COLICINE K

V. The Somatic Antigen of a Non-Colicinogenic Variant of E. coli K235*

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Plate 9

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The isolation and characterization of colicines V and K was described in earlier communications from this laboratory (1, 2). These substances, both potent bactericidal agents, proved to be identical with the O antigenic complex of the respective colicinogenic bacilli from which they were derived. The antigens are toxic not only for bacteria, but for mammalian species as well, and they were shown to be constituted from polysaccharide, lipid, and protein. Despite concerted chemical effort it has not been possible to separate from these substances a component of low molecular weight having bactericidal activity. An enhancement of activity was achieved only by dissociating the O antigens into their component parts. When this was done, it was found that bactericidal activity remained associated with the protein component of the antigenic complex, whereas the lipocarbohydrate was the bearer of the endotoxin.

The antigenic mosaic of the colicine K–producing bacillus *E. coli* K235 L^+OC^+ is complex. In addition to its type-specific bactericidal O antigen, the bacillus elaborates a thermolabile L antigen (3), a serologically inert polysaccharide, colominic acid (4), and a serologically active capsular polysaccharide. The latter has been newly isolated in this laboratory and an account of it will appear forthwith.

During the course of our investigations a genetic variant of the colicinogenic bacillus was isolated which elaborated no colicine (5). This microorganism was designated as *E. coli* K235 L^-OC^-, and was found to have undergone considerable genetic change. Not only had it lost its capacity to synthesize a bactericidal O antigen or colicine (C^-), but it produced neither an L antigen (L^-), nor colominic acid, nor a capsular polysaccharide. Nevertheless, the original colicinogenic bacillus *E. coli* K235 L^+OC^+ and its non-colicinogenic variant are serologically related for the two cross-react, a fact which has led us to the conclusion that they must have very similar O antigens (5).

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It was of great interest therefore to isolate the somatic antigen of the non-colicinogenic bacillus (6) and to compare its properties with those of colicine K. By so doing it should be possible to learn whether the two might not exhibit chemical differences which could be correlated with bactericidal activity.

The following is an account of the isolation of the O antigen of *E. coli* K235 L^-OC^- and of a comparison of its properties with those of the antigen derived from the colicinogenic parent. It will be shown from evidence set out below, that the lipopolysaccharide components of the two O antigens are chemically and serologically indistinguishable. It will be seen, furthermore, that the bactericidal properties of colicine K do not reside in the constituent which endows it with serological specificity.

Materials and Methods

Bacterial Strains.—The two strains of *E. coli* K235 used in these studies are variants of the original colicine K–producing microorganism sent us nearly a decade ago from the University of Liège by Dr. Pierre Frédéricq. The colicinogenic bacillus has been designated by us as *E. coli* K235 L^+OC^+. It produces a thermolabile surface antigen (L^+), grows on nutrient agar as opaque (O) colonies, and elaborates colicine K (C^+). The non-colicinogenic variant *E. coli* K235 L^-OC^- was first isolated in this laboratory in 1958 (5). This microorganism fails to produce an L antigen (L^-), its colonies are opaque (O), and it produces no colicine K (C^-).

Culture Media.—The culture medium in which the non-colicinogenic bacillus was cultivated for the production of its antigen was the same as that used in our previous studies on colicine K (1, 7).

Antisera.—Antibacterial rabbit sera to *E. coli* K235 L^+OC^+ and L^-OC^- were obtained by injecting animals with living bacilli as previously described (5). Antisera to the purified somatic antigens of *E. coli* K235 L^+OC^+ and *E. coli* K235 L^-OC^- were prepared by immunizing rabbits with sterile solutions of the two antigens, using dosages similar to those employed in earlier studies (5). The precipitation reactions of the various antigens in these immune sera were carried out by the agar diffusion technique of Ouchterlony (8).

Analytical Methods.—Absorption spectra were measured in a Beckman recording spectrophotometer, model DK 2. Electrophoretic analyses were made in a Tiselius apparatus, using a 2 ml cell (9); ultracentrifugal determinations were performed in a Spinco analytical centrifuge, model E.

Nitrogen was determined colorimetrically by the technique of Koch and McMeekin (10) and phosphorus by the procedure of Allen (11). The determination of reducing sugars was made by the method of Shaffer and Somogyi (12), after hydrolyzing the materials for 6 hours in 1 N HCl at 100°C; the results presented in the Tables are expressed in terms of the reducing value of glucose. Lipid was determined gravimetrically by weighing the chloroform-soluble material liberated during hydrolysis for 6 hours in 1 N HCl. Protein analyses were performed by a modified procedure of Folin and Ciocalteau (13) using bovine serum albumin as standard. Hexosamine was determined by the technique of Sørensen (14) after 6 hours of hydrolysis of the substances in 6 N HCl at 100°C. The determination of volatile organic acid, calculated as acetyl, was made by the method of Elek and Harte (15).

EXPERIMENTAL

Isolation of the Somatic Antigen of E. coli K235 L^-OC^-.—The crude antigen of *E. coli* K235 L^-OC^- was obtained from the culture medium of the bacillus by a procedure similar to that used for the isolation of colicine K (1).

After concentrating the cell-free medium *in vacuo*, and dialyzing it free of electrolytes, the crude antigen was obtained by freeze-drying the residue. The antigen was purified by precipitating an aqueous solution with 3 volumes of ethanol at −10°C. The precipitated antigen was redissolved in water, dialyzed, and concentrated *in vacuo* to 200 ml. The requisite amount of ammonium sulfate was added to bring the solution to 50 per cent saturation; the precipitate was centrifuged off, redissolved, and reprecipitated twice more at 45 per cent saturation. The solution was finally dialyzed free of sulfate, concentrated *in vacuo*, and lyophilized. The yield of purified antigen was 2.1 gm.

The material was only slightly pigmented and is referred to as ammonium sulfate–precipitated C^- antigen. Further purification was achieved by passing a solution of the antigen through DEAE-cellulose as follows.

1.8 gm of the ammonium sulfate–precipitated C^- antigen was dissolved in 50 ml of 0.05 M tris buffer at pH 8.0. The solution was slowly passed through a column of 3.5 gm DEAE-cellulose (1.8 × 10 cm), which had been equilibrated with the same buffer. The column was washed with 250 ml of buffer, and after dialysis of the eluate, the solution was lyophilized. 1.7 gm of a white product was recovered. This material is referred to as purified C^- antigen. It is completely devoid of bactericidal activity.

A small amount of brown pigment is absorbed during the procedure by the DEAE-cellulose. The pigmented material can be partially eluted with 0.3 M tris–0.5 M NaCl buffer at pH 7.2 and contains a relatively high percentage of nitrogen.

During the purification of the C^+ antigen it was found that small amounts of an accompanying serologically active polysaccharide, unrelated to the O antigen, could be separated by precipitation with cetyl trimethylammonium bromide (cetavlon) (16). Although *E. coli* K235 L^-OC^- does not produce this substance and solutions of the purified C^- antigen do not precipitate with the reagent, we have nevertheless treated the antigen with cetavlon in order to study its effect.

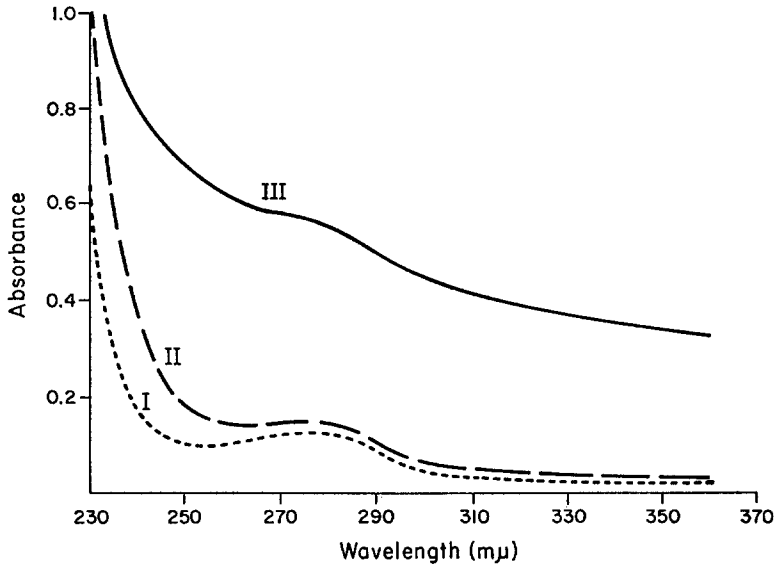
0.6 gm of purified C^- antigen was dissolved in 33 ml of 0.1 M borate at pH 9.2, and 16 ml of a 5 per cent solution of cetavlon was added. The mixture remained clear and was dialyzed for 20 hours to remove borate. The antigen was now repeatedly precipitated with ethanol (5 vol) to remove the cetavlon. The material was eventually freed of salts by dialysis, lyophilized, and 0.5 gm was recovered. The substance is referred to as cetavlon-treated C^- antigen.

Isolation of the Somatic Antigen of E. coli K235 L^+OC^+.—The initial isolation and purification of this material was carried out as previously described (1). A small amount of a serologically active capsular polysaccharide was separated from the partially purified O antigen by precipitation with cetavlon (16).

3.4 gm of C^+ antigen, purified to the point where the complex had been separated from colominic acid by precipitation with ammonium sulfate (1), was dissolved in 200 ml of 0.1 M sodium borate. 75 ml of a 5 per cent aqueous solution of cetavlon was added and the precipitate which formed was separated by centrifugation. The supernate, containing the somatic antigen, was dialyzed in the cold. The solution was then desiccated by freeze drying. The material was dissolved in a small volume of water and the cetavlon removed by repeated precipitation at 0° with 5 volumes of ethanol. The precipitate was finally dissolved in water, dialyzed, and dried from the frozen state. 2.5 gm of material was recovered.

A small amount of brown pigment could also be removed from this material by passing a solution through a small column of DEAE-cellulose as previously described. The final product is pure white and is highly bactericidal (1). This material is referred to as purified C^+ antigen.

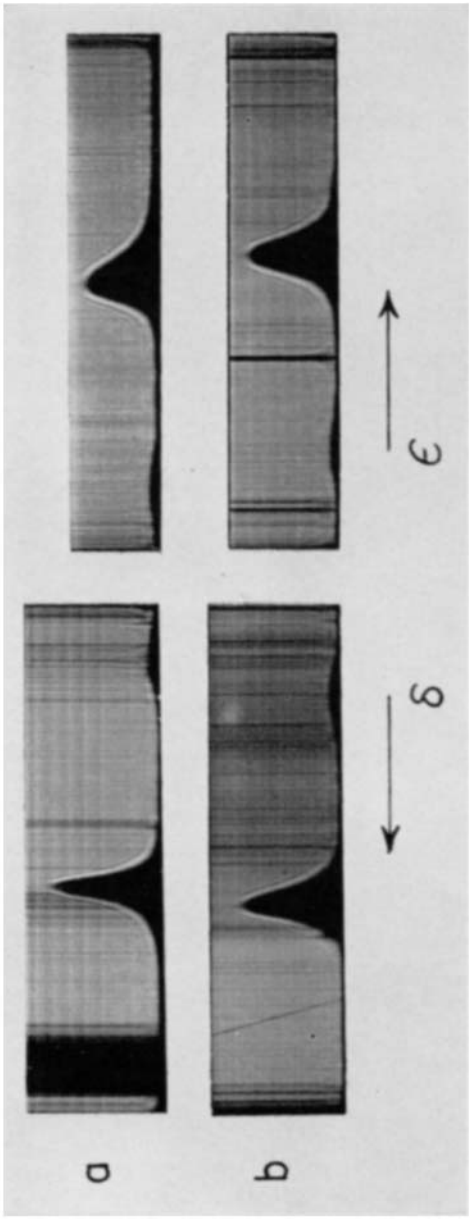
Properties of the Somatic Antigens of E. coli K235 L^+OC^+ and L^-OC^-.—Both the C^+ and C^- antigens are high molecular weight amorphous substances which are soluble in water. A solution of the purified C^- antigen is slightly opalescent,



TEXT-FIG. 1. Absorption spectra of the somatic antigens derived from *E. coli* K235 L^+OC^+ and L^-OC^-. *I*, purified C^+ antigen treated with cetavlon; *II*, purified C^- antigen treated with cetavlon; *III*, purified C^- antigen (observations made on 0.5 mg of substance per ml in 0.1 M phosphate buffer at pH 6.8).

whereas solutions of the cetavlon-treated product and the C^+ antigen are not. Treatment of the C^- antigen with cetavlon apparently brings about a change in its state of aggregation which in turn is reflected in the UV absorption spectrum (Text-fig. 1). An untreated and slightly opalescent solution of the purified C^- antigen shows a relatively high absorption over the whole UV range, due no doubt to light scattering. Cetavlon-treated C^+ and C^- antigens, however, have almost identical absorption curves, with maxima at 275 mμ, typical of proteins containing aromatic amino acids. The absence of peaks at 260 mμ indicates that both are free of nucleic acid.

Electrophoretic analyses of the C^+ and C^- antigens were made in 0.1 M borate at pH 9.2. The patterns are shown in Text-fig. 2. It will be seen that both



TEXT-FIG. 2. Electrophoretic patterns of the somatic antigens of *E. coli* K235 L^+OC^+ and L^-OC^- (0.1 M borate buffer, pH 9.2). *a*, purified C^+ antigen (13,000 seconds), $u = -3.7 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}. *b*, purified C^- antigen (11,000 seconds), $u = -4.6 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}.

substances form but a single boundary; in some preparations, however, the boundary was slightly asymmetric. There was no significant difference in the electrophoretic properties of the C^- antigen after cetavlon treatment.

It should be emphasized that the purified C^+ antigen is powerfully bactericidal (400 to 800 units of colicine K activity per mg) (1) whereas the antigen derived from the non-colicinogenic bacillus is completely devoid of such activity.

A summary of the chemical analyses of the C^- and C^+ antigen preparations is presented in Table I. It can be seen that both substances contain carbohydrate, lipid, and protein, and that they resemble the somatic antigens of other Gram-negative bacilli. Various preparations of the C^+ antigen invariably contained higher percentages of protein and nitrogen than did the C^- antigen, a

TABLE I

Chemical Properties of the Somatic Antigens Derived from E. coli K235 L^+OC^- and Its Non-Colicinogenic Variant

Analysis	C^+ antigen	C^- antigen
	per cent	*per cent*
Nitrogen	5.8 ± 0.2	4.3 ± 0.4
Phosphorus	1.3 ± 0.1	1.8 ± 0.1
Protein	18.0 ± 1.6	9.2 ± 2.7
Lipid	10.0 ± 0.4	13.8 ± 1.4
Reducing sugar	40.5 ± 1.7	41.4 ± 1.2
Hexosamine	17.5 ± 0.4	16.5 ± 0.5
Ash	0.9 ± 0.2	1.4 ± 0.4

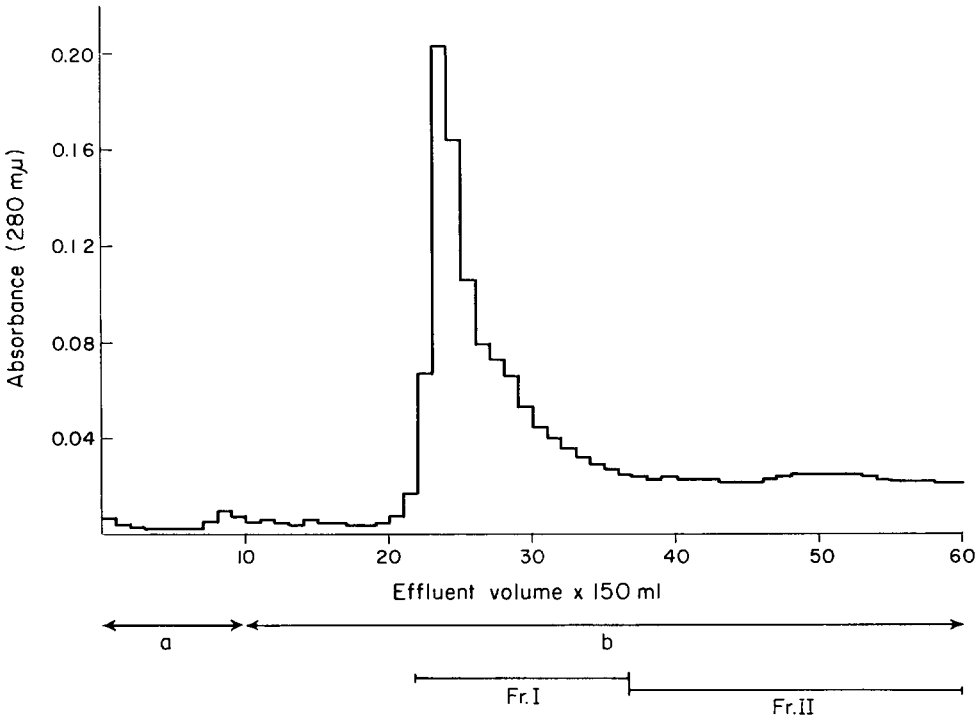
fact which might be related to the bactericidal activity of the former substance. The C^+ antigen, furthermore, contains less lipid and phosphorus, yet the reducing sugar and hexosamine content of both antigens is essentially the same. Analyses of the C^- antigen which were treated with cetavlon reveal that its chemical composition is indistinguishable from that of untreated material.

Chromatography of the C^- Antigen.—The C^- antigen was chromatographed on a DEAE-cellulose column as follows:

225 gm of DEAE-cellulose was equilibrated with 0.05 molar tris buffer at pH 8.0, and the slurry was packed in a column (48 × 6.2 cm). 750 mg of antigen was dissolved in the same buffer, placed on the column, and permitted to flow by gravity. Elution of the antigen was carried out (150 ml per hour), first with 1500 ml of 0.05 molar tris buffer at pH 8.0, and then with increasing concentrations of tris buffer. The concentration was gradually changed by allowing 0.3 M tris–0.5 M NaCl at pH 7.2 to flow into a mixing flask containing 3800 ml of 0.05 M tris at pH 8.0. Sixty 150 ml samples of effluent were collected and their absorbance was measured at 280 mμ.

The effluent curve is shown in Text-fig. 3. The samples of eluate in tubes 22 to 37 inclusive were combined, dialyzed, and lyophilized. The yield of substance was 384 mg (Fr. I). The material present in tubes 38 to 60 was also isolated; 66 mg was recovered (Fr. II).

It can be seen from Text-fig. 3 that the C^- antigen forms but one major peak which trails considerably. When the effluent samples were assayed for carbohydrate, their sugar content was found to be proportional to their absorbance at 280 mμ, and no additional peak could be detected. The analytical properties of the C^- antigen before chromatography and those of fractions I and II were essentially identical. Furthermore fractions I and II were serologically indistinguishable when tested by the agar diffusion technique. It

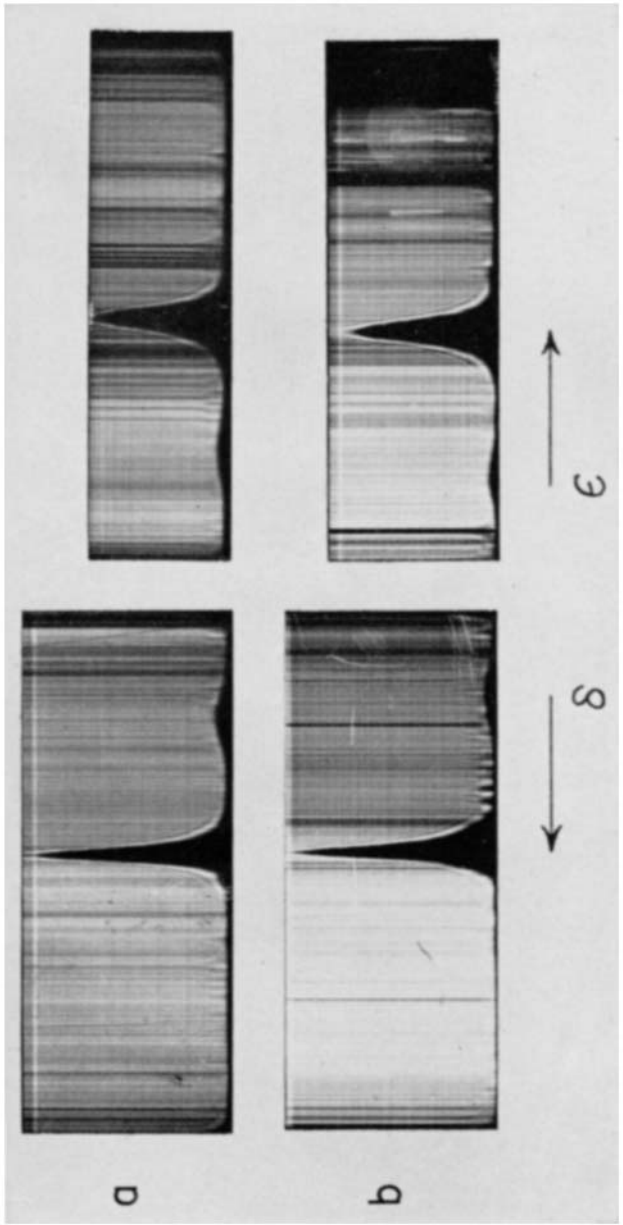


TEXT-FIG. 3. Chromatography of the purified antigen of *E. coli* K235 L^-OC^- (0.75 gm) on DEAE-cellulose (225 gm). Eluent: *a*, 0.05 M tris buffer, pH 8.0; *b*, gradient from 0.05 M tris buffer, pH 8.0, toward 0.3 M tris–0.5 M NaCl buffer, pH 7.2.

appears from this that the purified C^- antigen is relatively homogeneous and that no fractionation can be achieved under the conditions employed.

It should be pointed out, however, that the antigen is eluted as a single peak only when the ratio of antigen to DEAE-cellulose is 1:300 or lower. If this ratio is increased, part of the material is not absorbed and promptly appears in the effluent.

The Lipocarbohydrate Components of the C^+ and C^- Antigens.—The chemical composition of the lipocarbohydrate components of the two somatic antigens was next investigated in order to learn whether they differ.



TEXT-FIG. 4. Electrophoretic patterns of the lipocarbohydrates derived from the somatic antigens of *E. coli* K235 L^+OC^+ and L^+OC^- (0.1 M borate buffer, pH 9.2). *a*, C^+ lipocarbohydrate (7250 seconds), $u = -3.2 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}. *b*, C^- lipocarbohydrate (7500 seconds), $u = -4.7 \times 10^{-5}$ cm^2 volt^{-1} sec^{-1}.

1.4 gm of the C^- antigen was dissolved in 50 ml of redistilled 90 per cent phenol (17) in order to dissociate it into its protein and lipocarbohydrate components. The solution was maintained at room temperature for an hour, and then dialyzed at 4°C until free of phenol. The protein fraction, which precipitated as a granular substance, was separated by centrifugation and dried *in vacuo*. 250 mg was recovered. The supernate containing the lipocarbohydrate was concentrated and lyophilized. The substance was again dissolved in phenol, redialyzed, and lyophilized. 1.08 gm of the polysaccharide was finally obtained.

A sample of the C^+ antigen was treated in the same way. 500 mg of substance yielded 330 mg of the lipocarbohydrate component and 87 mg of the protein constituent.

The C^+ and C^- lipocarbohydrates are soluble in water and their solutions are slightly opalescent. When examined by electrophoresis in borate buffer at pH 9.2, the two substances were found to form but a single boundary, thus indicating the presence of only one major component (Text-fig. 4). The electrophoresis of the C^- lipocarbohydrate was also performed in 0.1 M veronal buffer at pH 8.2, and in 0.1 M acetate buffer at pH 4.2. Except for differences in the mobilities, the patterns observed were practically the same as those seen in borate.

When the C^- polysaccaride was examined in the ultracentrifuge[1] it exhibited a considerable degree of homogeneity (Text-fig. 5). The sedimentation constant in 0.1 M phosphate buffer was found to be approximately $41.0s_{20,w}$. When the lipopolysaccharide was examined in pyridine-water (2:8 by vol), however, the sedimentation constant was $7.6s_{20,w}$. From the figure it will be noted that the boundary in pyridine-water was much sharper than that in phosphate buffer. After recovering the substance from the pyridine-water solution and re-examining its sedimentation constant in phosphate buffer, it proved to be the same as that of the original material. Thus, the lipocarbohydrate seems to be highly aggregated in aqueous solutions, and less so in pyridine-water mixtures.

A comparison of the chemical properties of the C^+ and C^- lipocarbohydrates is given in Table II. It can be seen from the Table that the two substances have a very similar composition. Their reducing sugar and hexosamine content is essentially the same. Like the antigen from which it originates, the C^+ lipocarbohydrate has somewhat less lipid and more protein than does the C^- lipocarbohydrate.

Identification of the Monosaccharide Components of the Two Lipocarbohydrates.—

(*a*) *Identification of hexoses and of rhamnose:* In order to characterize the monosaccharide components of the two lipocarbohydrates, their acid hydrolysates were investigated by paper chromatographic techniques and by the cysteine color reaction (18).

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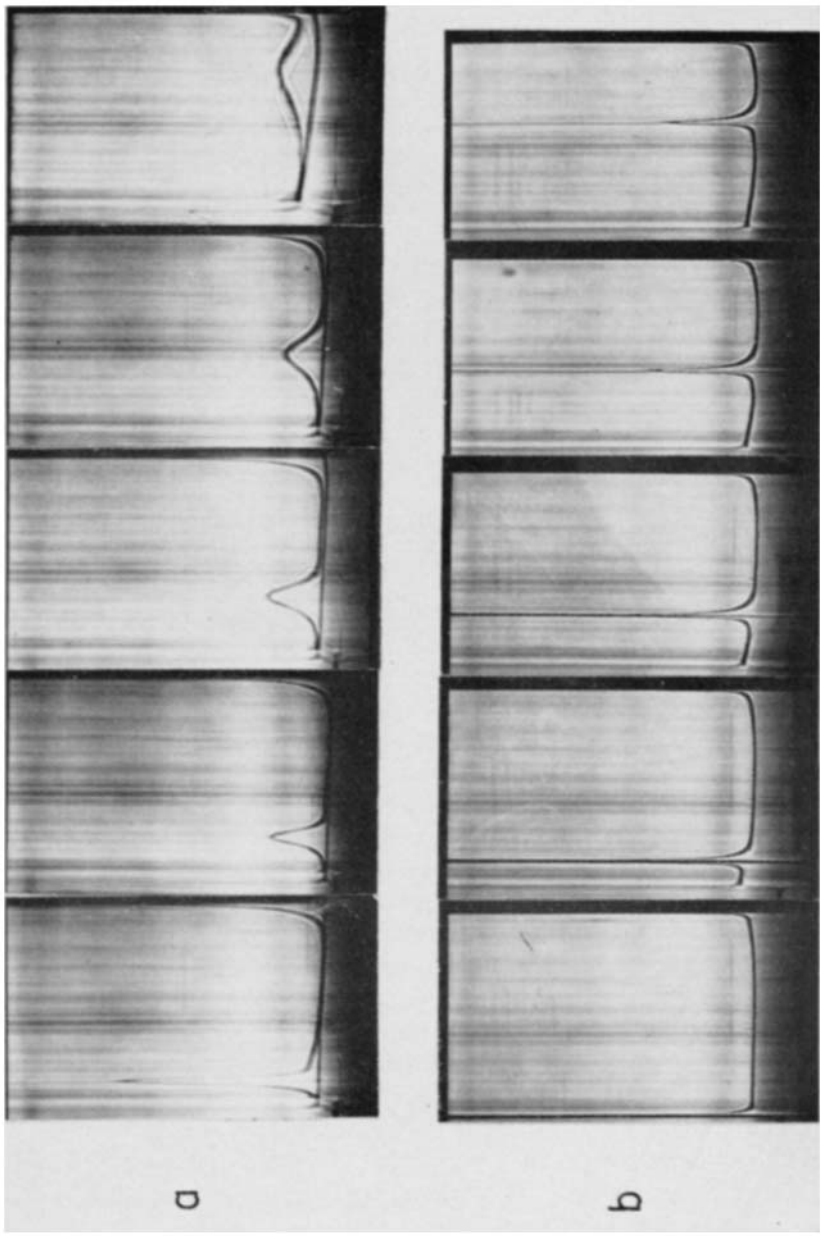
[1] We are greatly indebted to Dr. David A. Yphantis for his kindness in carrying out the ultracentrifugal analyses reported in this communication.



TEXT FIG. 5. Sedimentation pattern of the lipocarbohydrate derived from the somatic antigen of *E. coli* K235 1–OC . *a*, In 0.1 M sodium phosphate buffer, pH 6.8. Photographs 1 to 4 taken at 4 minute intervals, frame 5 after 20 minutes at 31,410 RPM. Bar angles: frame 1 to 4, 70°; frame 5, 40°. *b*, In pyridine-water mixture (2:8 by volume). Photographs taken at 32 minute intervals and at 50,740 RPM. Bar angle, 70°.

10 mg of the C^+ or C^- lipocarbohydrate was hydrolyzed with 0.5 ml 1 N H_2SO_4 at 100°C for 6 hours. The brownish precipitate which formed, predominantly lipid, was filtered off and discarded. The filtrate was neutralized with $Ba(OH)_2$ to pH 4.0 and the $BaSO_4$ was filtered and washed. The combined filtrates were evaporated *in vacuo* to dryness and the residue dissolved in 0.25 ml of water. 8λ samples of the hydrolysate were spotted on sheets of Whatman paper No. 1 together with spots of known sugars (20 μg). The chromatograms were developed with pyridine-ethyl acetate-acetic acid-water (5:5:1:3) for 15 hours at room temperature, using the descending technique (19). They were then stained by spraying with aniline phosphate or with the Elson-Morgan reagent (20).

It can be seen from Text-fig. 6 that the chromatograms of the hydrolysates of the two lipocarbohydrates formed the same pattern of spots when stained with aniline phosphate. The R_f values of the predominant spots coincided with those of rhamnose, glucose, galactose, and glucosamine. It should be

TABLE II

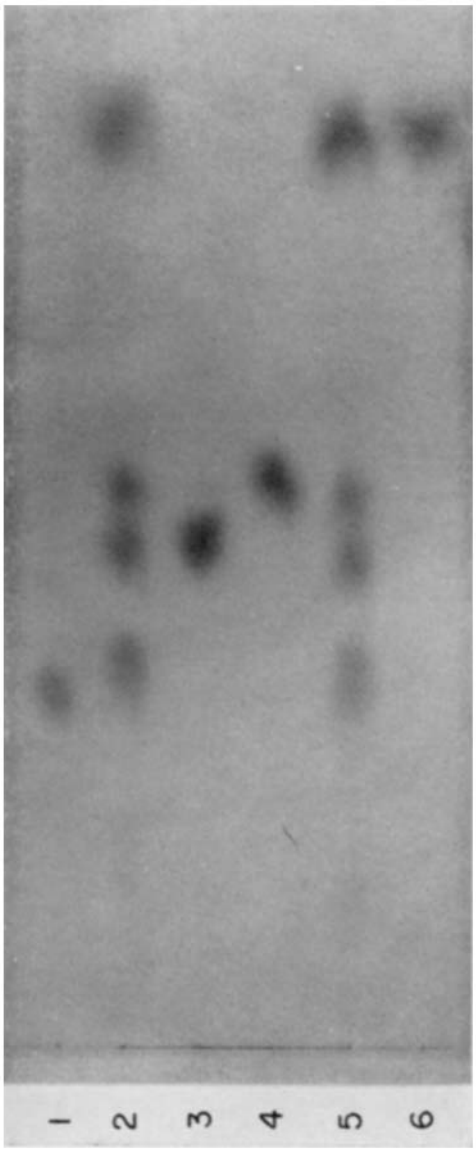
Chemical Properties of the Lipocarbohydrates Derived from the Somatic Antigens of E. coli K235 L^+OC^+ and Its Non-Colicinogenic Variant

Analysis	C^+ lipocarbohydrate	C^- lipocarbohydrate
	per cent	*per cent*
Nitrogen	3.0 ± 0.1	2.7 ± 0.1
Phosphorus	1.7 ± 0.1	1.9 ± 0.1
Protein	3.4 ± 1.2	1.5 ± 0.4
Lipid	10.1 ± 0.6	13.1 ± 0.9
Reducing sugar	49.6 ± 1.9	48.4 ± 0.6
Hexosamine	18.0 ± 1.0	19.0 ± 1.0
Acetyl	6.5	7.4
Ash	2.9 ± 0.3	3.7 ± 0.1

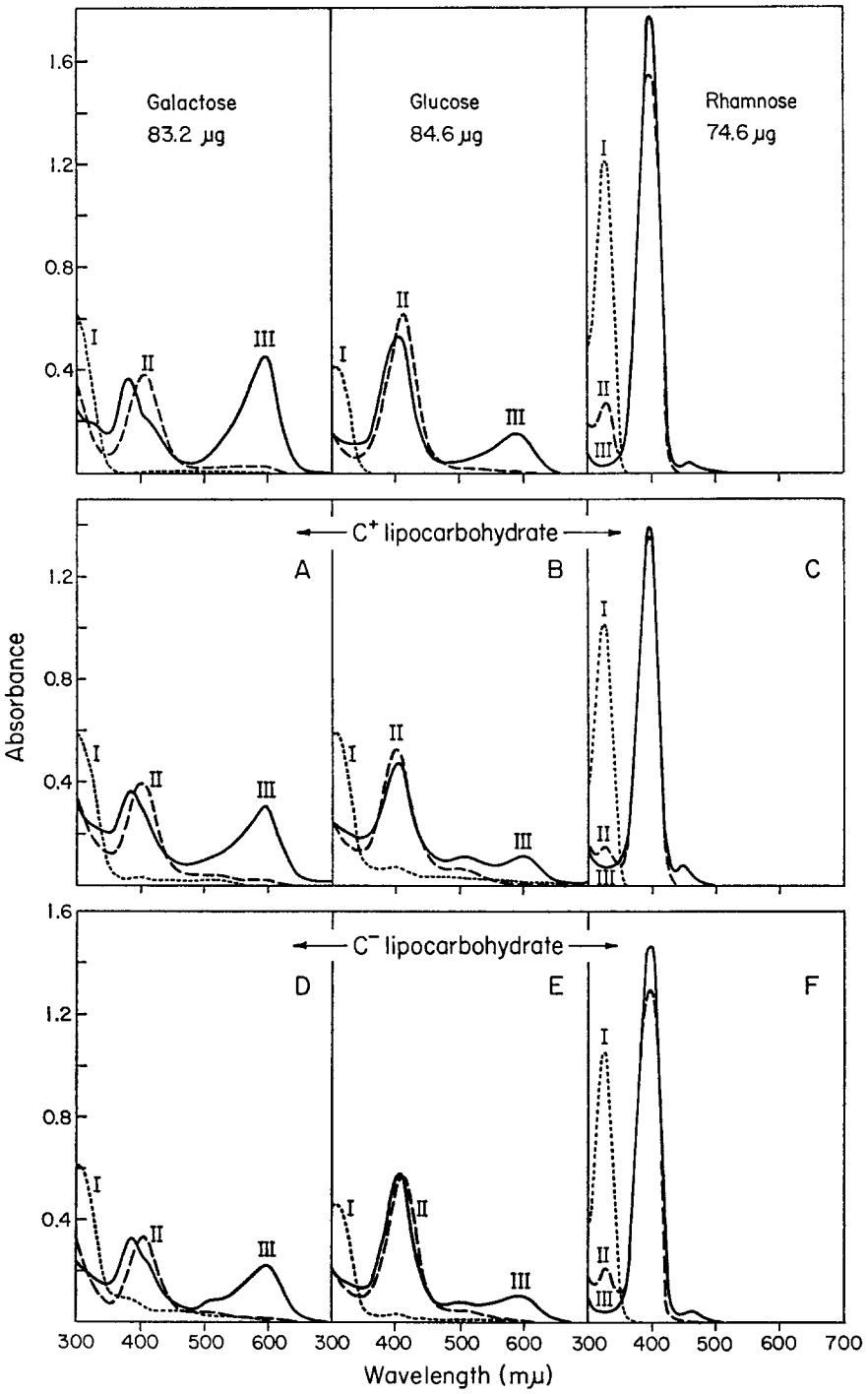
noted that small amounts of some slowly migrating components, the nature of which is unknown, are also to be seen. Upon spraying a second chromatogram with the Elson-Morgan reagent, only one component, which migrated at the same rate as glucosamine, appeared as an extended pink spot.

Further identification of the sugars present in the hydrolysates was achieved by first separating them on a paper chromatogram and then characterizing them by the cysteine reaction (18, 21).

0.2 ml of each hydrolysate was distributed on the base line of separate sheets of chromatographic paper. Spots of the same hydrolysates were also placed near the two edges of each sheet. After developing the chromatograms, the two side strips containing the separate spots, were stained in order to indicate the position of the various components. The center portion of the chromatogram was now cut parallel to the base line into strips containing the rhamnose, glucose, and galactose components. A blank strip of the same size served as control. Each strip was eluted with 25 ml of water and the eluates were evaporated *in vacuo* to dryness. The residues were dissolved in a measured quantity of water and the cysteine reaction (21) was



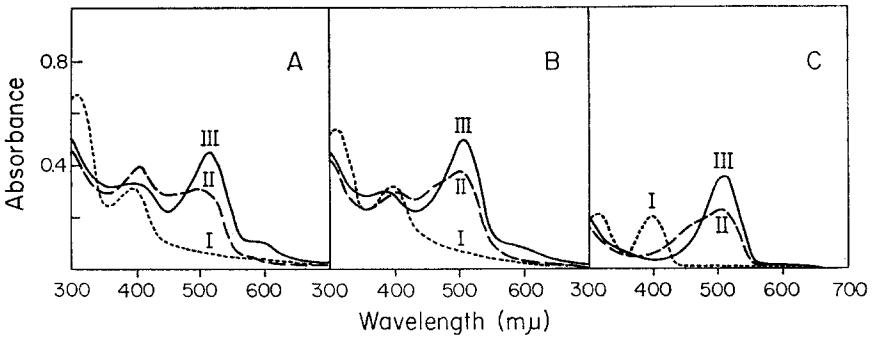
TEXT-FIG. 6. Chromatogram of the sugars present in the hydrolysates of C^+ and C^- lipocarbohydrates. *1*, Glucosamine; *2*, Hydrolysate of C^+ lipocarbohydrate; *3*, Galactose; *4*, Glucose; *5*, Hydrolysate of C^- lipocarbohydrate; *6*, Rhamnose.



TEXT-FIG. 7. Cysteine absorption spectra of sugars present in acid hydrolysates of C^+ and C^- lipocarbohydrates after separation on paper chromatograms. *A*, *B*, and *C*: fractions obtained from hydrolysate of C^+ lipocarbohydrate; *D*, *E*, and *F*: fractions obtained from hydrolysate of C^- lipocarbohydrate. Curve I, spectrum of control sample without cysteine. Curve II, spectrum of primary cysteine reaction. Curve III, spectrum of secondary cysteine reaction.

performed on 1 ml samples. The standards employed were solutions of the corresponding pure sugars having approximately the same concentration as those of the unknowns.

The cysteine absorption spectra of the sugars present in the eluates, and those of the known sugars, are shown in Text-fig. 7. It can be seen that fractions A, B, and C obtained from the C^+ lipocarbohydrate and fractions D, E, and F from the C^- lipocarbohydrate, exhibit absorption maxima corresponding to galactose, glucose, and rhamnose. The galactose (A and D) and glucose (B and E) fractions from both carbohydrates, however, show additional minor peaks at 400 mμ (curve I) and at 500 mμ (curves II and III). These peaks, not seen in the absorption curves of pure glucose and galactose, are characteristic of heptose (Text-fig. 8) and indicate that the hydrolysates of



TEXT-FIG. 8. Cysteine absorption spectra of the heptose component present in acid hydrolysates of C^+ and C^- lipocarbohydrates. *A*, heptose from C^+ lipocarbohydrate; *B*, heptose from C^- lipocarbohydrate; *C*, 100 μg D-*gala*-L-*manno*-heptose. Curve I, spectrum of control sample without cysteine. Curve II, spectrum of primary cysteine reaction. Curve III, spectrum of secondary cysteine reaction.

the two lipocarbohydrates contained a small amount of such a saccharide. The quantitative estimation of the sugars present in the hydrolysates will be described below.

(*b*) *Identification of heptose:* The separation of heptose from the other products of hydrolysis of the C^+ and C^- lipocarbohydrates was achieved by employing the procedure of Slein and Schnell (22). It will be recalled that heptoses frequently occur in lipocarbohydrates of Gram-negative bacilli as phosphate esters (22, 23) and that they can be separated as such by precipitation as their barium salts. The procedure is as follows:

40 mg of each of the lipocarbohydrates was hydrolyzed in 2 ml 1 N HCl at 100° for 3 hours. The hydrolysates were filtered and neutralized to pH 8.5 with NaOH and 50 mg of barium acetate was added. The solutions were first centrifuged and the barium salt of heptose phosphate was precipitated from the clear supernates by the addition of 3 volumes of ethanol.

After centrifuging, the sediment was dissolved in 2 ml of water and the barium ions precipitated with 3 ml of 2 N H_2SO_4. $BaSO_4$ was removed and the supernate, containing heptose phosphate and an excess of H_2SO_4, was heated to 100°C for 15 hours. The hydrolysate was finally neutralized with $Ba(OH)_2$, filtered, concentrated, and chromatographed on Whatman paper No. 1, using pyridine–*n*-butanol–water, 4:6:3. After spraying with a 3 per cent solution of *p*-anisidine hydrochloride in water-saturated *n*-butanol (24), and heating to 140°C, the heptose spots appeared as reddish brown areas, in contrast to the brown spots typical of hexoses. The unknown heptose migrates at about the same rate as D-*gala*-L-*manno*-heptose ($R_{glucose}$ = 0.93), a value which lies between that of glucose and galactose.

A portion of the two hydrolysates was now tested by the cysteine reaction, using D-*gala*-L-*manno*-heptose for purposes of comparison (21). The hydrolysates from both lipocarbohydrates gave absorption curves typical of heptose (Text-fig. 8).

(*c*) *Identification of hexosamines:* To determine whether hexosamines other than glucosamine are present in the C^+ and C^- lipocarbohydrates, hydrolysates were prepared and fractionated on Dowex 50 columns (25).

Samples of the two lipocarbohydrates were hydrolyzed with 3 ml of 6 N HCl, for 10 hours at 100°C. The pigmented solutions were filtered and evaporated *in vacuo*. In each instance the residue was dissolved in 1 ml of distilled water and applied to Dowex 50 columns (200 mesh, 0.9 × 15 cm, H^+ form). The columns were eluted first with distilled water (45 ml) and then with 150 ml of 0.33 N HCl. 3 ml samples were collected and assayed for their content of hexosamine, organically bound phosphate, ninhydrin-positive substances, and sugars. The solutions corresponding to individual peaks were combined, evaporated *in vacuo* to dryness, redissolved in water, and then tested on paper chromatograms. Two different solvent systems were used for development, pyridine–*n*-butanol–water (4:6:3) and *n*-butanol–water–acetic acid (12:5:3).

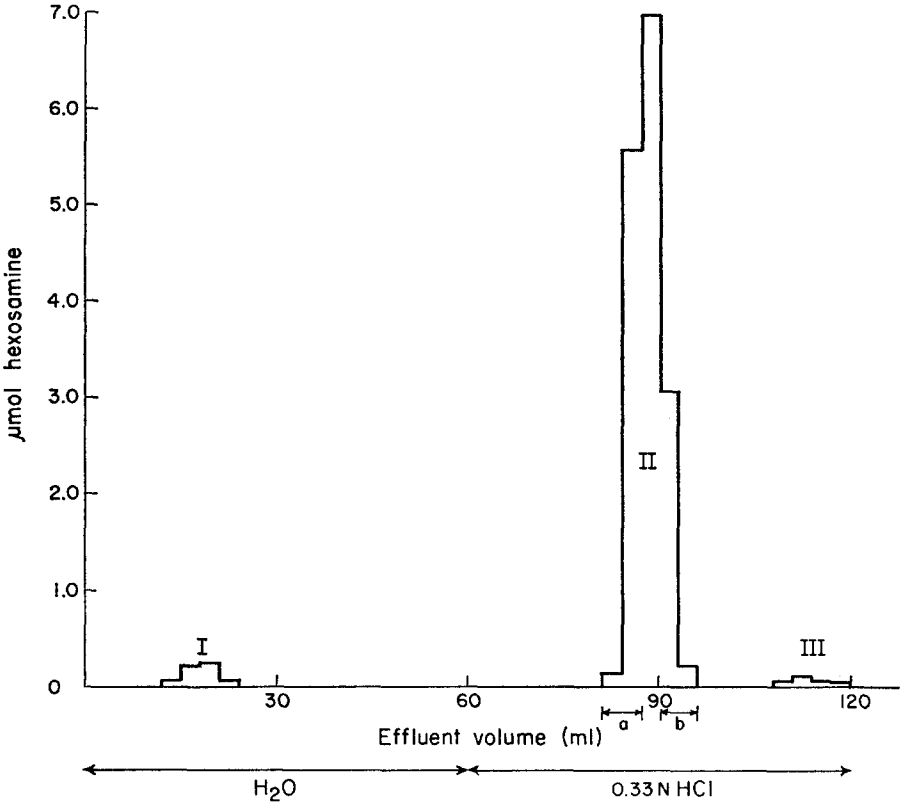
The effluent curve of the hydrolysate of the C^- polysaccharide is presented in Text-fig. 9, where the quantity of hexosamine in each sample is plotted as a function of the effluent volume. It can be seen that one major fraction (II) and 2 minor fractions (I and III) were eluted from the column. An identical curve, not shown in the figure, was obtained with the hydrolysate of the C^+ lipocarbohydrate.

The substance comprising fraction I appears to be glucosamine phosphate for it contains hexosamine and organically bound phosphate in approximately equimolar amounts. The rate of migration of the isolated component on paper chromatograms, and the position of its peak when eluted from the Dowex column, are identical with those of pure glucosamine-6-phosphate.[2] The nature of the substances in fraction III is not known.

Fraction II contains free hexosamine and represents at least 85 per cent of the amino sugars present in the lipocarbohydrates. The position of the peak coincides with that of pure glucosamine when the latter is chromatographed on the same column. On paper chromatograms the isolated material exhibits

[2] We are grateful to Mr. James W. Fristrom of the Rockefeller Institute for furnishing us with a sample of glucosamine-6-phosphate.

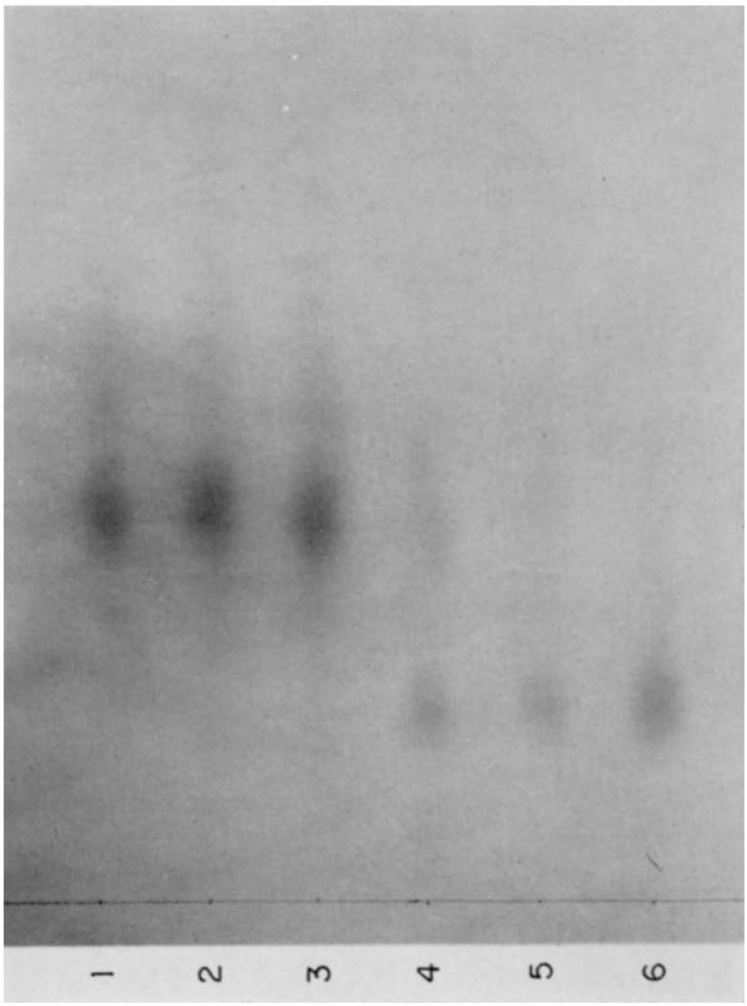
one extended spot with an R_f value identical with that of glucosamine. It has been reported, however, that mannosamine is eluted from columns of zeokarb 225 resins (comparable to Dowex 50) very close to glucosamine and that the R_f values of the two sugars on paper chromatograms are also very similar (26). If mannosamine is indeed present in the hydrolysate, it can therefore



TEXT-FIG. 9. Fractionation of hexosamines present in the acid hydrolysate (6 N HCl, 10 hours, 100°C) of C^- lipocarbohydrate on a Dowex 50 column (0.9 × 15 cm).

not be detected by the methods employed. It is possible, however, to differentiate the two hexosamines clearly by chromatographing their *N*-acetyl derivatives on borate-treated paper (27, 28). The substance present in the first two and last two tubes comprising fraction II was isolated and the two samples were *N*-acetylated (26, 29). The *N*-acetyl derivatives were now chromatographed on borate paper using authentic samples of *N*-acetylglucosamine and mannosamine for comparison.[3] It will be seen from Text-fig. 10, that the

[3] We wish to express our thanks to Dr. Saul Roseman of the University of Michigan, Ann Arbor, for a sample of *N*-acetylmannosamine.



TEXT-FIG. 10. Chromatogram of hexosamines from acid hydrolysates of C^+ and C^- lipocarbohydrates after *N*-acetylation. *1*. *N*-acetylglucosamine; *2*. Acetylated hexosamine from hydrolysate of C^- lipocarbohydrate (Fr. *a*, peak II, Text-fig. 9); *3*. Acetylated hexosamine from the corresponding fraction of C^+ lipocarbohydrate; *4*. Acetylated hexosamine from hydrolysate of C^- lipocarbohydrate (Fr. *b*, peak II, Text-fig. 9); *5*. Acetylated hexosamine from the corresponding fraction of C^+ lipocarbohydrate; *6*. *N*-acetylmannosamine.

last two samples comprising Fr. II do indeed contain mannosamine together with some glucosamine, whereas the first two samples contain only glucosamine. The amount of mannosamine is small, however, and it is therefore questionable whether its presence in the two hydrolysates might not represent an artifact. It has been reported that the epimerization of *N*-acetylglucosamine and mannosamine takes place in alkaline solutions (28, 30). Under conditions of acid hydrolysis, however, no similar rearrangement has, to our knowledge, been reported. When pure glucosamine was heated with 6 N HCl for 10 hours and the solution treated in exactly the same way as were the carbohydrate hydrolysates, no mannosamine could be detected.[4] This sugar must therefore be considered a minor constituent of both lipocarbohydrates.

When the latter were hydrolyzed under milder conditions (3 N HCl for 4 hours), the hydrolysates yielded, in addition to the three fractions described above, a fourth fraction which emerged from the column before the free hexosamine. This fraction contains both rhamnose and hexosamine which are probably in chemical combination as oligosaccharides.

Quantitative Determination of Monosaccharides.—The quantities of monosaccharides present in the acid hydrolysates of the C^+ and C^- lipocarbohydrates were determined by means of the cysteine reaction after separating the various sugars on paper chromatograms as previously described. The absorption curves of the fractions so obtained are shown in Text-fig. 7. The absorbance at wavelengths characteristic for the sugar in question was compared with that of a known quantity of the appropriate monosaccharide standard, and the amount of sugar was calculated.

It was found that the hydrolysate of the C^+ lipocarbohydrate contained 20.3 per cent rhamnose, 5.0 per cent glucose, and 3.7 per cent galactose, whereas that of the C^- lipocarbohydrate contained 21.0 per cent rhamnose, 4.5 per cent glucose, and 3.9 per cent galactose. These values, however, account for only that portion of the monosaccharides which is liberated after 6 hours of hydrolysis in 1 N H_2SO_4. Under these conditions, part of the rhamnose is probably still bound as an oligosaccharide and part of the heptose still occurs as heptose phosphate.

The total amount of these two sugars was therefore estimated by carrying

[4] Our observations regarding the presence of a small amount of mannosamine in acid hydrolysates of the two lipocarbohydrates have been confirmed in the laboratory of Dr. Stanford Moore. He observed that hydrolysates of the C^- and C^+ somatic antigens (6 N HCl, 20 hours, 110°C) when chromatographed, under conditions for amino acid analysis (31), showed the presence of mannosamine corresponding to 3 per cent of the total glucosamine present in the hydrolysate. When pure glucosamine was heated in 6 N HCl for 10 hours, and then chromatographed under the same conditions, the hexosamine did not undergo rearrangement. The solution did not show the presence of any traces of mannosamine. We are greatly indebted to Dr. Moore for his kind assistance in this matter, and we wish to express our thanks to him.

out the cysteine reaction directly on the lipocarbohydrates, without prior hydrolysis. The cysteine absorption curves of the two substances are presented in Text-fig. 11. They reveal that both polysaccharides contain a large amount of rhamnose. Except for the maxima typical of this sugar, only the minor peaks due to the presence of heptose, curve I (400 mμ) and curves II and III (500 mμ), can be seen. The absorption maxima caused by the hexoses are completely hidden.

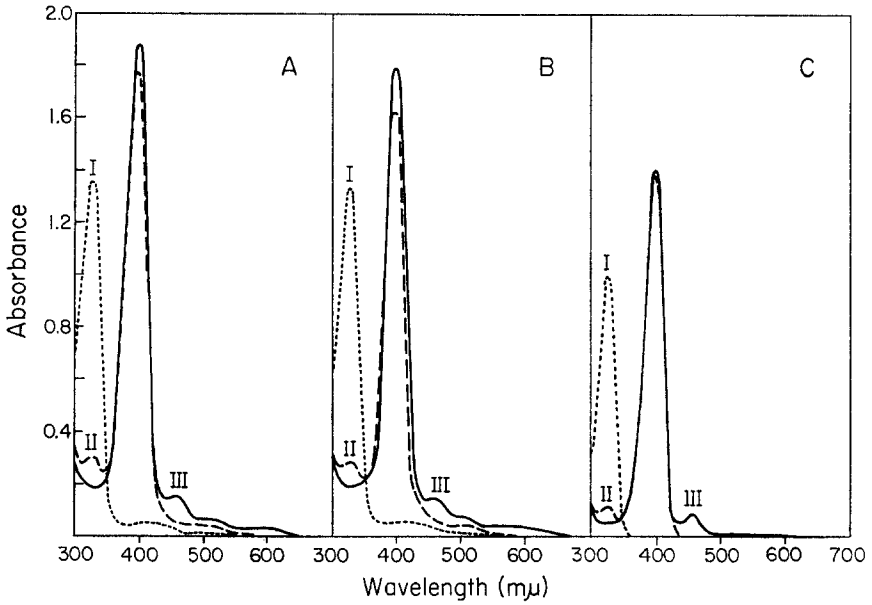
It has been reported by Dische that 6-deoxyhexoses can be determined in the presence of hexoses by employing the primary cysteine reaction (32). Thus, the content of rhamnose in the lipopolysaccharides was estimated by comparing the difference in their absorbances at wave lengths 396 mμ and 427 mμ with that of an authentic sample of rhamnose. The amounts proved to be 33.9 per cent and 33.8 per cent for the C^+ and C^- lipocarbohydrates respectively. Because of their high rhamnose content, the amount of hexoses present in the unhydrolyzed polysaccharides could not be estimated by this method; one must rely upon the values obtained after chromatographic separation of the saccharides present in acid hydrolysates.

The amount of heptose present in the unhydrolyzed polysaccharides was calculated from the maxima observed at 400 mμ (curve I) and at 500 mμ (curve II), using D-*gala*-L-*manno*-heptose as a standard (Text-fig. 11). The C^+ lipocarbohydrate proved to contain 9.9 per cent of heptose, the C^- lipocarbohydrate 10.5 per cent. These values must be regarded as tentative, however, for it is not yet known which heptose is present in the polysaccharides. Finally, it should be stated that the hexosamine content of the polysaccharides was also determined on acid hydrolysates and the values obtained have already been presented in Table II.

The results of our chemical investigation are summarized in Table III where it is seen that the two lipocarbohydrates are constituted from identical sugars and that their proportions are essentially the same. From that which follows, it will be shown that this chemical similarity is reflected in the serological properties of the two lipocarbohydrates, and in the somatic antigens from which they are derived.

The Serological Properties of the C^+ and C^- Antigens and their Lipocarbohydrate Components.—The various fractions obtained during the purification of the C^- antigen were tested serologically by the agar diffusion technique (8), employing an antiserum to the purified C^- antigen. Samples of the latter, which had been freshly precipitated with ammonium sulfate, were found to be serologically homogeneous, for they showed but one precipitation band (Fig. 1 *A*, well *1*). The antigen was unstable, however, because further manipulation, such as purification with DEAE-cellulose or even prolonged storage at room temperature, brought about a change in the agar diffusion pattern (Fig. 1 *A*, well *2*). A new weak band now appeared close to the serum well. This band

was also observed if the antigen was subjected to electrodialysis, or treated at room temperature with 0.1 N acetic acid, or treated with sodium hydroxide (Fig. 1 *B*). If the antigen was permitted to stand in alkali for a week, it underwent even further serological change. The original band close to the antigen well became very weak, whereas the intensity of the band near the serum well was greatly enhanced (Fig. 1 *B*, well *4*).



TEXT-FIG. 11. Cysteine absorption spectra of unhydrolyzed C^+ and C^- lipocarbohydrates. *A*, C^+ lipocarbohydrate (200 μg); *B*, C^- lipocarbohydrate (200 μg); *C*, rhamnose (56 μg). Curve I, spectrum of the control sample without cysteine. Curve II, spectrum of primary cysteine reaction. Curve III, spectrum of secondary cysteine reaction.

The C^- lipocarbohydrate, obtained by dissociating the antigen with phenol, also forms two precipitation bands when tested against an antiserum to the purified C^+ or C^- antigen. In this instance, the band close to the serum well was also the more pronounced (Fig. 1 *C* and *D*, wells *2*). It can be seen that both bands of the lipocarbohydrate merged with those of the purified C^- antigen (wells *1* and *2*). These observations indicate clearly that treatment of the C^- antigen with various agents changes its serological properties. In all likelihood, some chemical degradation takes place, but the nature of the degradation products is not yet known. It is conceivable that some labile groups are split off, resulting in a change in the serological properties of the substances in question. In this connection it should be pointed out that the capsular polysac-

charide of Type I pneumococcus was shown to have a very labile acetyl group which could be split off in faintly alkaline solution (33). More recently, Baker has shown that the Vi antigen of *Paracolobactrum ballerup* was also sensitive to dilute alkali. Treatment of the polysaccharide with alkali brought about degradation which resulted in a change in its serological characteristics (34).

When the purified C^+ antigen was tested by the agar diffusion technique, using either an antiserum to the C^+ or to the C^- antigen, it too formed two precipitation bands (Fig. 1 *C* and *D*, wells *3*). This was not surprising because the antigen had also been fractionated on DEAE-cellulose. Similarly, the C^+ lipocarbohydrate showed two bands. As in the case of the C^- antigen and its

TABLE III

Monosaccharide Composition of the Lipocarbohydrates Derived from the Somatic Antigens of E. coli K235 L^+OC^+ and its Non-Colicinogenic Variant

Sugar	C^+ lipocarbohydrate	C^- lipocarbohydrate
	per cent	*per cent*
Rhamnose*	33.9	33.8
Heptose‡	9.9	10.5
Glucose§	5.0	4.5
Galactose§	3.7	3.9
Hexosamine	18.0	19.0

* Determined by cysteine reaction on the unhydrolyzed lipocarbohydrates.

‡ Determined by cysteine reaction on the unhydrolyzed lipocarbohydrates, using D-*gala*-L-*manno*-heptose as standard.

§ Determined after paper chromatographic separation of sugars present in acid hydrolysates.

lipocarbohydrate, the bands formed by the C^+ antigen merged with those of the C^+ lipocarbohydrate (Fig. 1 *C* and *D*, wells *3* and *4*).

A comparison was finally made between the C^+ and C^- antigens themselves and their lipocarbohydrate components. Sera to the two purified antigens were employed in these tests. The diffusion patterns, shown in Fig. 1 *E* and *F*, reveal that the bands formed by the two antigens merge (wells *1* and *2*) as do those of their lipocarbohydrates (wells *3* and *4*). It can be seen, furthermore, that the precipitation patterns of the various substances are the same whether one employs an antiserum elicited by colicine K, or one evoked by the C^- antigen. From these experiments it can be concluded that the antigen derived from the colicinogenic bacillus *E. coli* K235 L^+OC^+ and that of its non-colicinogenic variant are serologically indistinguishable. Their lipocarbohydrate components, so closely related chemically, are also indistinguishable by serological test.

Precipitation Reactions.—In order to learn if there is any serological difference between the two antigens, their antisera were absorbed with the hetero-

logous substance. In each instance the precipitins were removed by the addition of a slight excess of heterologous antigen. The sera were incubated at 37° for 2 hours, and after standing in the cold room for 24 hours, they were centrifuged. The two sera were now tested for the presence of precipitins reactive with the homologous antigens. At the same time, control tests of the unabsorbed sera were made.

A protocol of this experiment is presented in Table IV. It can be seen from the data that in each instance the precipitins were completely removed by ab-

TABLE IV

Precipitation Reactions of the Somatic Antigens Derived from E. coli K235 L^+OC^+ and Its Non-Colicinogenic Variant

Antiserum No.	Antigen tested	Final dilution of antigen			
		1:2000	1:10,000	1:50,000	1:250,000
47 unabsorbed	C^+	3	2½	2½	2½
	C^-	3	2½	2½	3
10 unabsorbed	C^+	2	3	3	2½
	C^-	2½	3	3	3
47 absorbed with C^- antigen	C^+	0	0	0	0
	C^-	0	0	0	0
10 absorbed with C^+ antigen	C^+	0	0	0	0
	C^-	0	0	0	0

No. 47: serum of a rabbit immunized with the purified somatic antigen of the colicinogenic bacillus (C^+ antigen).

No 10: serum of a rabbit immunized with the purified somatic antigen of the non-colicinogenic bacillus (C^- antigen).

3 = heavy disk-like precipitate, clear supernate.

2 = moderately heavy precipitate.

0 = no precipitate.

sorption with the heterologous antigen. Neither absorbed antiserum showed any trace of a reaction when tested with the homologous substance. This experiment substantiates the results of the agar diffusion experiments which also demonstrated that the C^+ and C^- antigens are serologically indistinguishable.

Colicine K Neutralization Tests of C^+ and C^- Antisera.—In a previous report it was shown that anstisera elicited by colicine K contained antibodies which neutralized the bactericidal activity of the colicine (5). A similar test was now performed, using the antisera of rabbits which had been immunized with the C^- antigen.

0.3 ml of colicine K (C^+) antiserum and 0.3 ml of a C^- antigen immune serum was added to each of two tubes containing 2.5 ml of molten 0.7 per cent nutrient agar at 50°C. To each tube was then added 0.05 ml of a culture of the test organism *E. coli* B containing 5×10^7 bacilli. The soft agar was then poured upon two nutrient agar (1.5 per cent) plates. After 10 minutes, each plate was spotted with 0.02 ml of serial dilutions of a solution of colicine K, beginning with 10 μg/ml and ending with 0.3 μg/ml. The plates were incubated for 6 hours, and the inhibition of growth of *E. coli* B was observed.

The plate containing the colicine K antiserum showed complete inhibition of colicine activity, indicating that the bactericidal activity of the colicine has been neutralized by the immune serum. The second plate, containing the immune serum to the C^- antigen, showed no neutralization of colicine K activity. The colicine gave complete inhibition of growth of *E. coli* B, even at a concentration of 1.25 μg/ml. Thus, the antiserum elicited by the C^- antigen is entirely devoid of colicine K–neutralizing antibodies.

DISCUSSION

The objective of the study here presented was to learn whether or not the type-specific antigens of the colicine K–producing bacillus *E. coli* K235 L^+OC^+ and its non-colicinogenic variant exhibit differences which can be correlated with bactericidal activity. Our efforts have been directed primarily toward the isolation and purification of the O antigen of the non-colicinogenic bacillus of *E. coli* K235 and an investigation of its lipopolysaccharide component. It is the latter which determines the serological specificity of O antigens and of the microorganism from which they are derived (35).

The monosaccharide constituents of the O antigens of many *E. coli* strains have been extensively studied during the past years. Thus, Westphal and his coworkers have examined some twenty-nine strains, classified serologically according to the nomenclature of Kauffman and White (36). These investigators divided their coli strains into fourteen groups, each having the same qualitative monosaccharide composition (37). These groups, termed "chemotypes," proved to be identical with those used to designate a variety of *Salmonella* strains (23). Nearly all of the fourteen coli chemotypes were found to contain glucosamine, glucose, galactose, and heptose. In certain instances galactosamine and mannose were also detected. Some strains also contained a deoxyhexose, either fucose or rhamnose, and in certain instances a dideoxy sugar, colitose (3,6-dideoxy-L-xylo-hexose).

The coli strain used in our investigations, *E. coli* K235 L^+OC^+, has been classified serologically and belongs to O group 1 (38). Because this microorganism is serologically indistinguishable from the non-colicinogenic *E. coli* K235 L^-OC^- variant, the latter must also belong to O group 1. Our chemical investigation has revealed that the lipocarbohydrate component of the somatic antigen of this bacillus is essentially identical with that of the colicinogenic

parent. These two lipocarbohydrates, comprising some 75 per cent of their respective bacterial antigens, were found to be not only serologically indistinguishable but to be constituted from the same sugars. Both lipocarbohydrates contain glucosamine, galactose, glucose, heptose, rhamnose, and mannosamine in the same relative proportions.[5] To our knowledge mannosamine has never been found as a constituent of the somatic antigens of Gram-negative bacilli. This may be because its R_f value is very similar to that of glucosamine. The presence of small amounts of mannosamine in the hydrolysates of the two lipopolysaccharides does not appear to be an artifact; it must therefore be regarded tentatively as a true constituent of both lipocarbohydrates. Because of their composition, the two antigens may be classified as chemotype VII.

Both colicine K and the antigen derived from the non-colicinogenic bacillus are homogeneous when examined by electrophoresis. Freshly prepared materials show but one precipitation band when tested in agar gels against immune serum. Such preparations, however, after chemical manipulation exhibit a second component as manifested by the appearance of a new precipitation band. Presumably some labile grouping is removed, leaving a small amount of substance with slightly altered chemical composition and hence with altered serological specificity. Speculation regarding this phenomenon is fruitless, however, without further study. Despite the lability of these substances, our studies have indicated that colicine K and the O antigen derived from the non-colicinogenic bacillus *E. coli* K235 L^-OC^- are serologically indistinguishable as are their lipocarbohydrate components.

Our studies, however, have revealed one significant immunological difference between the two antigens: Colicine K elicits neutralizing antibodies, whereas the antigen of the non-colicinogenic variant does not (5). Furthermore, we have now found that there is a difference in chemical composition between the two antigens which appears to be of some significance. The antigen derived from the colicinogenic bacillus invariably has a higher nitrogen and protein content than does the antigen of the non-colicinogenic variant. To our mind, this is additional evidence that bactericidal activity is not related to the lipopolysaccharide moiety of the antigenic complex, but resides, in all probability, in its protein component.

In sum, our studies have shown that the two lipopolysaccharides derived from the O antigens of the colicine K–producing microorganism and its non-colicinogenic variant are serologically and chemically indistinguishable. That the two antigens must differ, however, is self-evident for the one is powerfully bacterici-

[5] In an earlier communication (1) fucose was reported to be a constituent of the lipocarbohydrate component of purified colicine K. It has subsequently been found that the presence of fucose was due to a contamination of the somatic antigen with a small amount of a capsular polysaccharide which is also elaborated by this bacillus. An account of this immunologically active carbohydrate will appear in a separate communication.

dal whereas the other is not. In whatever this difference may consist, it must be directly related to the bactericidal property of the colicine. It is our belief that a study of the protein components of the two antigens should reveal the nature of this difference.

SUMMARY

The somatic antigen of the non-colicinogenic bacillus *E. coli* K235 L^-OC^- has been isolated, and its chemical and serological properties have been compared with those of colicine K. The antigen of the non-colicinogenic bacillus has a protein content significantly lower than that of the C^+ antigen, a difference which might be related to the antibacterial activity of the latter.

The lipocarbohydrate components of the two antigens are chemically very similar; both contain the same proportions of galactose, glucose, heptose, rhamnose, glucosamine, and mannosamine.

When tested by agar diffusion, the two antigens are indistinguishable, as are their lipocarbohydrate components.

Our studies indicate that the bactericidal activity of colicine K does not reside in its lipocarbohydrate but in its protein component.

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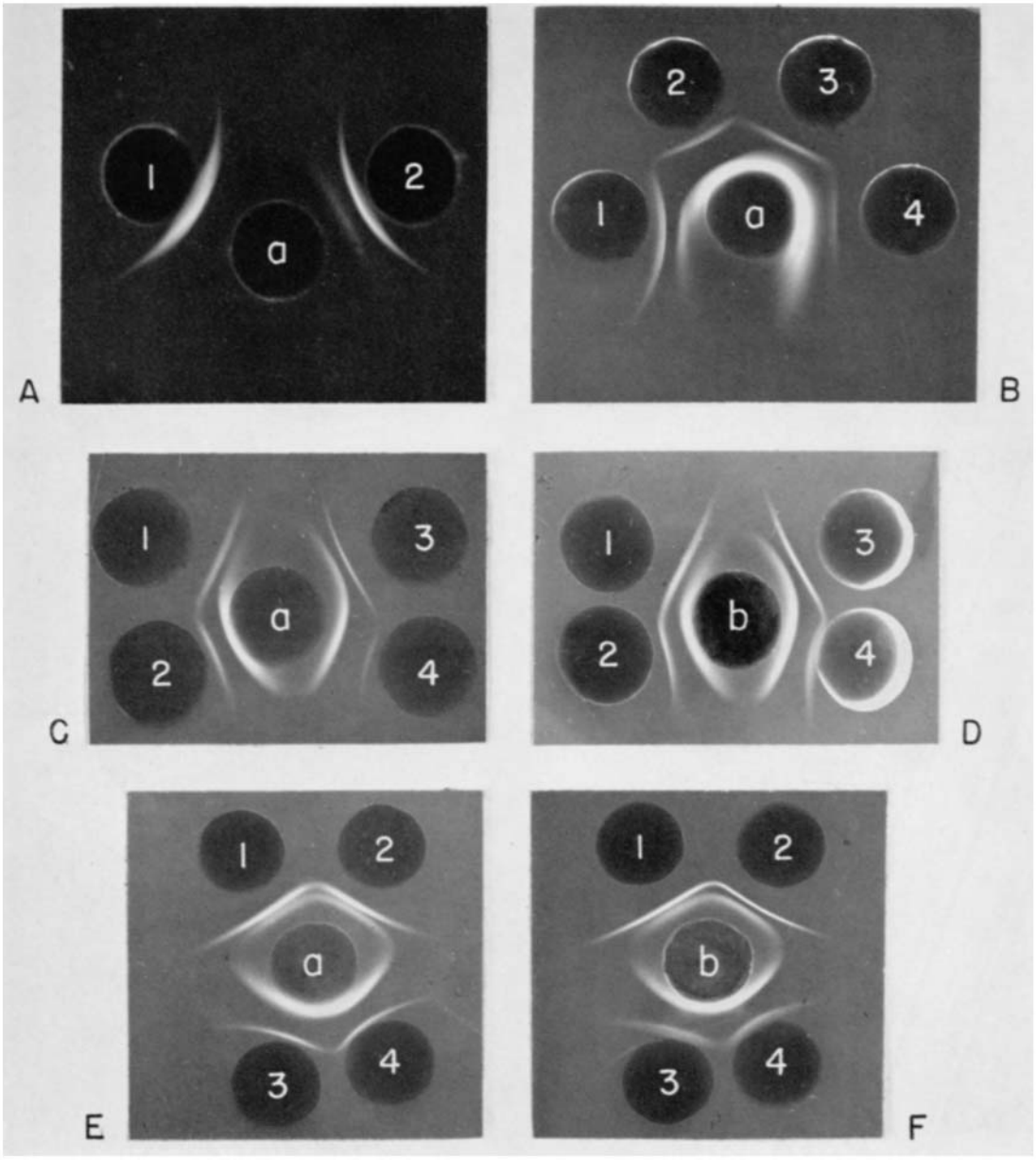
EXPLANATION OF PLATE 9

FIG. 1. Gel precipitation reactions of the somatic antigens derived from *E. coli* K235 L^+OC^+ and L^-OC^- and their lipocarbohydrate components. (*A*) *a*, C^- antigen immune serum; *1*, C^- antigen freshly precipitated with $(NH_4)_2SO_4$; *2*, C^- antigen purified with DEAE-cellulose. (*B*) *a*, C^- antigen immune serum; *1*, C^- antigen purified with DEAE-cellulose; *2*, C^- antigen, electrodialyzed; *3*, C^- antigen treated with 0.1 N NaOH for 1 day; *4*, C^- antigen treated with 0.1 N NaOH for 1 week. (*C*) and (*D*) *a*, C^- antigen immune serum; *b*, C^+ antigen immune serum; *1*, Purified C^- antigen; *2*, C^- lipocarbohydrate; *3*, Purified C^+ antigen; *4*, C^+ lipocarbohydrate. (*E*) and (*F*) *a*, C^- antigen immune serum; *b*, C^+ antigen immune serum; *1*, Purified C^- antigen; *2*, Purified C^+ antigen; *3*, C^- lipocarbohydrate; *4*, C^+ lipocarbohydrate.



(Rüde and Goebel: Colicine K)