Isolation and Characterization of the K1 (L) Antigen of *Escherichia coli*¹

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ABSTRACT

BOLAÑOS, ROGER (Tulane University, New Orleans, La.), AND CHARLES W. DEWITT. Isolation and characterization of the K1 (L) antigen of *Escherichia coli*. J. Bacteriol. 91:987-996. 1966.—An acidic polysaccharide with the serological properties of the K1 (L) antigen has been isolated from *Escherichia coli* O2:K1:H4 by means of phenol-water extraction, fractionation with hexadecyltrimethylammonium bromide (Cetavlon), filtration through Sephadex G-200, and chromatography on anion-exchange cellulose columns. Nucleic acid and protein content were reduced to a nondetectable level. There is no contamination with O antigen. The active material appears in two different positions in the Cetavlon fractionation, each with a slightly different serological specificity, as followed by the inhibition of passive hemagglutination. By paper chromatography, the polysaccharide moiety of the O antigenic fraction is composed of glucose, galactose, hexosamine and rhamnose. The absence of colanic acid in either K1 fraction was not proven, although its participation in our assay system, as well as the participation of the common or cross-reacting antigen, was ruled out by serological means.

Escherichia coli has been recognized for some time as the causal agent of human infantile diarrhea, and is also known to cause various acute inflammatory infections, often fatal, in humans and animals. Attempts to find a correlation of virulence with the presence of cell wall lipopolysaccharide (24, 27), or sialic acid-containing compounds found in some *E. coli* strains (10), have not been successful. It has been shown, however, that there is a direct relation of mouse lethality to certain types of K antigens, the L (labile) type being most closely associated with virulence (16), and in particular K1, K5, and K44 apparently playing a very important role in the host-parasite relationship (10).

In the earlier experiments by Kauffmann (16) on the role of the L-type antigen, better protec-

tion was obtained in mice passively immunized with antiserum directed against the encapsulated strain than with antiserum against the smooth nonencapsulated strain. No protection could be demonstrated after active immunization, however. More recently Jenkin and Palmer (14) have shown that the rate of clearance of *E. coli* from the blood and peritoneal cavity of infected mice is directly proportional to the concentration of antibody directed against surface components of the bacteria, the effect being an enhancement of the phagocytic capabilities of the host defensive cells. Although this antibody is called "specific," it can be evoked by stimulation with apparently unrelated antigens (lipopolysaccharide prepared from different organisms), and it has not been determined whether it is directed against the somatic (O) antigen, the capsular (K) antigen, or the flagellar (H) antigen, all of which are external to the cell wall. In fact, there are almost no recent reports of studies on the role of K antigen and its homologous antibody in the host-parasite relationship of *E. coli*.

Envelope materials in the *Enterobacteriaceae* have been found, in general, to be acidic polysaccharides (34). Attempts to define chemically the envelope materials of *E. coli* have led to identification of several very interesting and related substances. Barry and Goebel (2), studying

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a colicinogenic strain of *E. coli* (K235-O1:K1), isolated colominic acid, a homopolymer of *N*-acetylneuraminic acid (1), from the supernatant fluid of the culture medium. This substance, or a very similar one, has been found in several other serotypes of *E. coli* as well as in *Salmonella djakarta* and *S. dahlum* (3). No serological role has been demonstrated for colominic acid (3), despite the fact that a substance containing neuraminic acid is responsible for the haptenic activity of *Neisseria meningitidis* group C polysaccharide (31). DeWitt and Rowe (4) and DeWitt and Zell (5) isolated *N*-acetylneuraminic acid and *N*,7-*O*-diacetylneuraminic acid from the cell surface of some serotypes (K1 and K5). Despite the fact that these compounds were shown to be associated with the envelope material, and their relationship with K antigen was suggested, no role in the determinant group was demonstrated. Recently, Goebel (11) obtained an acidic polysaccharide, colanic acid, from *E. coli* K235 L+O (m) responsible for the mucoid characteristics of the strain and present as a capsule which interferes with the serological activity of the O antigen. It proved to be antigenic in rabbits and serologically distinct from K1 antigen. Ørskov et al. (21) isolated A and B types of K antigens from *E. coli* strains O8:K42(A):NM and O141:K85ab → bc(B):H4. The polysaccharides were shown to contain K antigenic specificity and to be distinct from O antigen and from colanic acid by both chemical and serological criteria. The sugar composition was, in general, simple hexoses and methyl pentoses, amino sugars, and hexuronic acid.

We present here evidence of the isolation of material containing the serological activity of one of the L type of K antigens of *E. coli* (O2:K1:H4) which differs chemically and serologically from the O antigen and from colanic acid.

MATERIALS AND METHODS

The *E. coli* strain (E-107; received from The Upjohn Co., Kalamazoo, Mich.) used in this study was isolated from a human systemic infection and has been found by us to have a mouse LD₅₀ of <10 organisms per mouse. It is serotype O2:K1:H4, but, as it has been propagated exclusively on solid media, flagellar antigen development is suppressed and no anti-H antibody is present in our antisera when tested against a motile culture. A smooth, translucent variant has been isolated which lacks the capsular antigen but retains the somatic antigen and is referred to as K(-). Two *E. coli* strains which produce colanic acid, K235 L+O(m) and K12(m), were kindly supplied by W. Goebel.

Anti-O and anti-OK sera were prepared in rabbits against infusion broth cultures and were standardized by bacterial agglutination according to Edwards and Ewing (7). Anti-K sera were prepared by adsorption

of the anti-OK sera with heated (100 C, 60 min) K-positive cells or live K-negative cells. Both gave identical results in the serological tests used. An anti-O 14 serum, kindly sent to us by E. Neter, was used in the assay of common or cross-reacting antigen (18, 33).

Passive hemagglutination (HA) was performed with sheep erythrocytes sensitized with untreated bacterial extracts in the case of K1 antigens or with extracts pretreated by exposure to sodium hydroxide (20) in the case of O antigenic material. (The term "antigen" will be used throughout because one fraction isolated early in the investigation was found to be antigenic in rabbits. Fractions obtained later have not been examined for antigenicity.) The optimal concentration of sensitizing K1 antigen was determined each time by using twofold dilutions of antigen and assaying for maximal titer against a standard anti-K1 serum. Optimal concentrations were remarkably constant, ranging from 5 to 10 µg per ml of a 2.5% suspension of erythrocytes. Sensitized erythrocytes (0.1 ml of a 1.0% suspension) were added to serial twofold dilutions of antiserum (0.4 ml), and the test was read after incubation for 1 hr at 37 C and then overnight at 5 C. Hemagglutination inhibition was read as the least amount of material which on preincubation at 37 C for 30 min with 0.2 ml of antiserum dilution completely inhibited 3 to 4 hemagglutination (HA) units.

The procedure used for extraction of the K1 antigen was modified from that of Ørskov et al. (21). Briefly, cells grown in chemically defined medium (22) were extracted with 45% aqueous phenol (32). The water-soluble portion was submitted to fractional precipitation with Cetavlon (hexadecyltrimethylammonium bromide) according to Scott (26). The entire procedure is presented in detail in Fig. 1.

As can be seen, the usual dialysis step was replaced by filtration through Sephadex G-50 (8) obtained from Pharmacia Fine Chemicals, Inc., Rochester, N.Y. Lyophilization was replaced by precipitation from cold ethyl alcohol. Both methods were introduced because of earlier loss of K1 activity during these steps. Purified materials have been found to be slightly less labile. To obtain dry fractions, the alcohol precipitates were washed twice in acetone, suspended in diethyl ether, centrifuged, and dried in air. Fractions were free from Cetavlon (<1 µg of quaternary ammonium ion) as measured by the reaction of Fogh et al. (9).

The number of fractions and yield of each obtained by the Cetavlon fractionation varied among the different experiments. In general, the more concentrated starting material yielded more fractions. However, those precipitates appearing at very close NaCl molarities were found to be similar in serological activity and chemical composition and were, therefore, pooled.

Total carbohydrate was determined by the anthrone reaction (13) with glucose as standard, and was considered to represent polysaccharide content. Nucleic acid was estimated by differential adsorption at 260 to 280 mµ, and was confirmed by the orcinol reaction of Nejbaum (25). Uronic acids were estimated with the carbazole reaction of Dische (6) with glucuronic acid standards, and protein was determined with the Lowry modification of

FIG. 1. Fractionation scheme for preparation of K1 (L) antigen. All Cetavlon precipitates were washed twice with NaCl solutions of the same molarity as the precipitating solution, dissolved in 1 M NaCl, and precipitated three times from ethyl alcohol to remove Cetavlon. The occurrence of the fraction insoluble in 0.5 M NaCl is not constant.

the phenol reaction (15) with crystalline ovalbumin as a standard. Total sialic acids were measured by the resorcinol reaction (28), and free sialic acids with the thiobarbituric acid assay of Warren (30).

From optical density readings obtained with crystalline *N*-acetylneuraminic acid (NANA), and bacterial polysaccharide free from sialic acid, simultaneous equations were derived which yielded: micromoles of sialic acid equals absorbancy at 580 $m\mu$ (0.618) minus absorbancy at 450 $m\mu$ (0.114) for use with the resorcinol assay. From density readings with NANA and

crystalline 2-deoxyribose was obtained: micromoles of sialic acid equals absorbancy at 549 $m\mu$ (0.102) minus absorbancy at 532 $m\mu$ (0.06) for use with the thiobarbituric acid assay.

Acid hydrolysis was in 2 N H_2SO_4 at 100 C for 90 min; the solution was neutralized with $BaCO_3$, and the resulting $BaSO_4$ was removed by centrifugation.

Paper chromatography was by the descending front technique with the use of Whatman no. 1 paper and pyridine-ethyl acetate-acetic acid-water (5:5:1:3) or *n*-butanol-pyridine-water (6:4:3) as solvents. Papers

were dried and developed with the *O*-aminobiphenyl reagent of Timell et al. (29).

ECTEOLA-cellulose columns were prepared according to Ringertz and Reichard (23) with elution by stepwise increments of NaCl concentration from 0.1 to 1.0 M.

RESULTS

The preferred assay of K antigen and thus control of antigen isolation procedures would have been bacterial agglutination inhibition with K(+) cells and anti-K antiserum. Crude K antigen, obtained by extraction of O2:K1 cells with aqueous phenol (Fig. 1), was shown by rabbit immunization to contain both O and K antigens. As was expected, anti-K titers of the standard antisera were found to be as much as seven twofold dilutions higher by passive hemagglutination (PHA) with the crude antigen as compared with bacterial agglutination (BA), with a corresponding increase in sensitivity of the inhibition assay of antigen. Although a drastic reduction in the bacterial cell concentration (optical density at 660 m μ , 0.3; 0.05 ml per 0.5 ml of total volume) resulted in an increase in BA serum titer so that it equaled the PHA titer, the method was not sufficiently reproducible. Use of inhibition of PHA required a more careful analysis of the specificities of the anti-K serum; the results of these tests are presented in Tables 1 and 2.

In Table 1 it is shown that the anti-O activity of the parent anti-O2:K1 serum is removed by adsorption with O2 bacterial cells to yield an antiserum with good anti-K1 activity when assayed by either BA or PHA. Both of these antisera, as

well as anti-O2 serum, fail to agglutinate cells of either of two colanic acid-producing strains, K12(m) or K235 L + O(m), assuring us that anticolanic acid antibody is not interfering. Further, although the anti-O14 serum shows a minor cross reaction with O2-sensitized erythrocytes, it is completely negative against K1-sensitized erythrocytes, thus indicating that the common or Kunin antigen-antibody system is not interfering.

In Table 2, these results are confirmed by the complete adsorption of anti-K1 antibody, as measured either by BA or PHA, by living O2:K1 cells or erythrocytes sensitized with K1 antigen, but not by O2 cells or K12(m) cells.

When crude antigen (Fig. 1) was fractionally precipitated as Cetavlon complexes from solutions of decreasing electrolyte concentration, numerous fractions were collected, the number of which varied among different experiments. Early experiments yielded only one fraction (that precipitating at 0.19 M NaCl) positive for K antigen on the basis of PHA inhibition with the crude K antigen-anti-K1 serum system. Two additional fractions (0.28 and 0.075 M) were capable of sensitizing erythrocytes without pretreatment by heat or alkali. Erythrocytes so sensitized were agglutinated only in anti-K1 serum, and each hemagglutination reaction was inhibited by either fraction.

The results of two such experiments, one reporting inhibitory levels as dry weight, the other as total carbohydrate, are given in Table 3. Inhibitory levels of the 0.28 and 0.075 M fractions were 0.12 and 0.016 μ g (as carbohydrate), when tested in their homologous PHA systems, 0.50

TABLE 1. Bacterial agglutination and hemagglutination titers of various antisera

Antiserum	Bacterial agglutination					Hemagglutination*		
	Reciprocal of serum dilution vs. <i>Escherichia coli</i> strains					Isolated K1 antigen	Lipo-polysaccharide	None
	O2: K1(+)	O2: K1(-)	O2: K1 (+) (100 C, 60 min)	K12(m)	K235 L + O (m)			
Anti-O2:K1	160	5,120	5,120	0†	0	800-3,200	16,000	0
Anti-K1	80	0	0	0	0	800-3,200	0	0
Anti-O2	0	10,240	5,120	0	0	0‡	32,000	0
Anti-O14						0	160	0
Antigen revealed	K1	O2		Colanic acid	Colanic acid	K1	O2	—

* Hemagglutination was determined with the indicated sensitizing fractions. Titers with isolated K1 antigen depend on whether the crude antigen or one of the Cetavlon fractions (Fig. 1) was used for erythrocyte sensitization. Lipopolysaccharide was prepared by centrifugation (30,000 $\times$ g for 4 hr) of a Westphal-type lipopolysaccharide (15) followed by treatment with NaOH according to Neter et al. (20).

† Zero indicates <1:10.

‡ Cells sensitized with crude antigen yield moderate titers here due to residual O antigen. Cells sensitized with purified fractions are uniformly negative.

and 0.06 μg in the reciprocal heterologous systems, and $> 50 \mu\text{g}$ when crude antigen was used for red cell sensitization. In each case, the erythrocytes sensitized with the 0.075 M fraction were more susceptible to inhibition than were the cells sensitized with the 0.28 M fraction.

These results were confirmed by experiments in which anti-K1 serum was adsorbed with erythrocytes optimally sensitized with either the 0.28 or the 0.075 M fraction and the residual homologous and heterologous titers were determined (Table 4).

Later, more careful fractionation of crude antigen by Cetavlon precipitation showed that, although precipitates were obtained at about 0.19 M NaCl, they no longer displayed any serological activity. Further, an artificial mixture of the 0.28 and 0.075 M fractions was capable of inhibiting the erythrocyte-crude antigen versus anti-K1 serum system, suggesting that the 0.19 M fraction obtained earlier was the result of incomplete separation of the 0.28 and 0.075 M activities. The occurrence of the 0.075 M fraction and its serological specificity remained constant. The 0.28 M fraction could be separated into several smaller fractions, appearing at closely related NaCl concentrations but showing the same serological specificity.

The results of such an experiment are given in Table 5, and include the chemical and serological analyses of the fractions. Those precipitates which usually appear at NaCl concentrations above 0.4 M are insoluble and contain a brown pigment. Those fractions precipitating at about 0.3 and 0.075 M NaCl show K1 specificity, the O specificity appearing in the supernatant fluid of the 0.075 M NaCl precipitation. It was not possible to relate directly or simply the serological specificity to any one of the major biochemical components (car-

bohydrate, protein, or nucleic acid), or to sialic acid. Fractions 5, 6, and 7 have K1 activity and are characterized by a high nucleic acid content; fractions 3 and 4 are also mainly nucleic acid yet have no K1 activity. Also, fractions 5 and 6, which contain the main portion of the K1 antigen precipitating in this region, show an increased carbohydrate content. This relationship is not supported by fractions 9, 10, and 11, however. Although all fractions were negative for uronic acids, their absence cannot be assumed because of the high interference of simple sugars in the carbazole reaction (6).

Further attempts to purify the 0.28 M (pool of fractions 5, 6, 7, and 8 of Table 5) and 0.075 M (fraction 12, Table 5) fractions were made by use of gel filtration and ion-exchange chromatography. Elution patterns from G-75, G-100, and G-200 Sephadex were similar in that the K1 activity of both fractions appeared in the excluded volume, indicating a molecular size in excess of

TABLE 2. *Specificity of the standard anti-K1 serum as shown by antibody adsorption*

Adsorbing material	Reciprocal of serum dilution	
	Bacterial agglutination (O2:K1 cells)	Hemagglutination (crude antigen)
None.....	80	800
O2:K1 cells.....	0*	0
O2 cells (100 C, 60 min)....	80	800
K12(m) cells.....	—	800
Sheep erythrocytes sensitized with crude antigen...	0	0

* Zero indicates $< 1:10$.

TABLE 3. *Serological specificity of K antigenic fractions by hemagglutination inhibition*

Antiserum	Erythrocyte-sensitizing fraction	Fraction used for inhibition						
		Crude antigen /		0.28 M		0.075 M		Supernatant fluid
		Dry wt (μg)	CHO (μg)	Dry wt (μg)	CHO (μg)	Dry wt (μg)	CHO (μg)	CHO (μg)
Anti-K1	Crude antigen	1.56*	0.15	>100	>50 †	>100	>50 †	>50
	0.28 M		0.30	2.0	0.12	12.5	0.5	>50
	0.075 M	0.30	0.10	0.5	0.06	0.05	0.016	>50
Anti-O2	Lipopolysaccharide ‡		0.06		>50	>100	>50	0.05

* Minimal inhibitory amounts vs. 3 to 4 hemagglutination units of antiserum.

† A mixture of 2 μg of 0.28 M fraction and 0.16 μg of 0.075 M fraction (as total carbohydrate) inhibited the standard anti-K1 antiserum and crude antigen sensitized erythrocyte hemagglutination system. No end point was determined.

‡ See Table 1.

200,000. Typical experiments with the use of Sephadex G-200 with each K1 antigenic fraction are presented in Fig. 2. It should be noted that the scale for the two experiments is different, as about four times as much material was recovered from the 0.28 M fraction as from the 0.075 M fraction. There was considerable increase in specific K1 activity of both fractions. Most of the nucleic acid and about 50% of the protein was separated from the K1 activity of both fractions by this method, with a resultant increase in specific serological activity.

Sialic acid behaved differently in the two fractions. In the 0.28 M fraction, the K1 activity was not separated from this compound. Further, a constant proportion of resorcinol and thiobarbituric acid-positive material (15% free sialic acid) was obtained. In the 0.075 M fraction, however, most of the resorcinol-positive material was separated from the K1 activity and that not separated showed a variable percentage of free sialic acid (8 to 77%). The polysaccharide peak is quite homogeneous in both fractions, appearing mainly in the excluded or large molecule effluent. The results of these experiments indicate a very similar gross composition for the two K1 antigenic fractions, i.e., a high carbohydrate content with low amounts of associated protein, nucleic acid, and sialic acid. It is possible that a better separation of these components, particularly of the sialic acid from the 0.075 M fraction, could be obtained by the use of longer columns (8).

Preliminary experiments have suggested that

ion-exchange column chromatography may be useful for further purification. Cation-exchange resins (Dowex-50) do not bind the K1 activity, but are excellent for removal of traces of detergent after the Cetavlon fractionation. Anion exchangers in the chloride or acetate form effectively retain K1 activity, but elution methods tried with Dowex-2 (NaCl, sodium acetate, pH 5.5 acetate buffer) have either been unsuccessful or have resulted in K1 inactivation. ECTEOLA-cellulose in the chloride form retained the 0.075 M fraction, and upon elution with increasing chloride ion concentration gave a complete separation of the carbohydrate and K1 activity (0.4 M NaCl) from the nucleic acid (0.8 M NaCl).

TABLE 4. Serological specificity of K1 antigenic fractions by adsorption of hemagglutinins*

Fraction on erythrocytes for hemagglutination	Fraction used for adsorption		
	None	0.075 M	0.28 M
0.075 M	800	100	<100
0.28 M	1,600	800	200

* A 1-ml amount of a 1:50 dilution of anti-K1 serum was adsorbed once at 37 C for 30 min and a second time at 4 C for 18 hr with 0.2 ml of packed erythrocytes optimally sensitized with the indicated fraction. Adsorbed serum was then assayed in doubling dilution in the appropriate hemagglutination system.

TABLE 5. Characterization of Cetavlon precipitates of aqueous phenol extracts of O2:K1 cells^a

Fraction no.	NaCl concn	Protein (mg)	Ribonucleic acid (mg)	Sialic acid (mg)	Carbohydrate (mg)	Serological activity ^b
1 ^c		7.5	70	10	107	O2:K1
2	0.5 M ^d					
3	0.4 M	0.5	10	0.4	1.7	—
4	0.36 M	0.15	6.0	0.23	1.2	—
5	0.33 M	0.4	30	0.63	3	K1
6	0.32 M	0.14	4.0	0.33	2	K1
7	0.30 M	0.13	2.3	0.15	0.4	K1
8	0.28 M	0.04	0.13	0.6	0.06	K1
9	0.24 M	0.05	0.03	0.28	0.04	—
10	0.20 M	0.01	0.02	0.35	0.05	—
11	0.15 M	0.14	0.1	0.37	1.0	—
12	0.075 M	0.13	2.0	1.13	1.0	K1
13	Supernatant fluid	3.0	16	4.2	90	O2

^a The extract used was 1.0 g (dry wt) or 250 mg (carbohydrate) of aqueous-phenol extract from 7.0 g (dry wt) of acetone-dried cells.

^b Hemagglutination inhibition. Erythrocytes sensitized with NaOH-treated lipopolysaccharide for assay of O antigen and with either 0.075 or 0.28 M fraction for assay of K antigen. Both the latter tests gave similar results.

^c Starting material (crude antigen).

^d Insoluble in water or organic solvents.

Samples of the 0.075 M supernatant fluid (O antigen), 0.20 to 0.19 M precipitate (colanic acid), and the 0.075 and 0.28 M precipitates were submitted to acid hydrolysis and paper chromatography. The identification of components is tentative, and is based on coincident mobilities of known and unknown in two solvent systems. The results (Table 6) show identity of our 0.19 to 0.20 M fraction with colanic acid (11). The O antigen appears identical with chemotype VIII of Kauffmann et al. (17), and is thus compatible with serotype O2. No reliable results were obtained from the examination of K1 active fractions, due mainly to the low yield, and possibly also to the interference of high levels of sialic acid in the recovery of other sugars after hydrolysis (12). Chromatographic patterns varied in different experiments, with the exception of the consistent absence of rhamnose and uronic acids, which distinguishes the material from O antigen and colanic acid, respectively. Large-scale preparations for further fractionation are in progress.

Crude K1 antigen was exposed to different temperatures and hydrogen ion concentrations, and the residual activity was measured by hemagglutination inhibition. It showed no decrease in

hemagglutination inhibitory activity after 2 weeks in aqueous solution at either 5 C or room temperature (22 to 26 C). At temperatures above this, however, there was a gradual loss of activity, the speed of inactivation being proportional to the temperature (Fig. 3).

When held in phosphate buffers of different pH at a constant temperature of 5 C, crude K1 antigen was stable after 4 days in the pH range of 5 to 8. At hydrogen ion concentrations below or above this range, however, there was a rapid loss in serological activity (Fig. 4). This lability to temperature and pH extremes is in agreement with the known behavior of L-type antigens when studied in their natural state as part of the *E. coli* capsule.

DISCUSSION

The results presented in this paper show that it is possible to partially purify a material containing the serological characteristics of the K1 (L) anti-

TABLE 6. Component sugars of O antigen and colanic acid

Sugar	0.075 M supernatant fluid	O2 antigen	0.19-0.20 M ppt	Colanic acid
Glucose	+	+	+	+
Galactose	+	+	+	+
Fucose			+	+
Rhamnose	+	+		
Glucosamine	+	+		
Galactosamine	+	+		
Glucuronic acid			+	+

FIG. 2. Fractionation of the two K1 active Cetavlon preparations by Sephadex G-200. Columns prepared with a height-diameter ratio of 10:1.

FIG. 3. Effect of temperature on K1 as measured by hemagglutination inhibition. Crude antigen; constant pH 7.2.

FIG. 4. Effect of pH on K1 activity as measured by hemagglutination inhibition. Crude antigen; constant temperature of 5 C.

gen of *E. coli*. Two different fractions were obtained, each one precipitating at a characteristic sodium chloride concentration in the presence of hexadecyltrimethylammonium bromide and each one apparently displaying a slightly different serological specificity as measured by hemagglutination inhibition. This difference may be real and K1 antigen may be composed of two specificities, or it may be an artifact of the isolation procedure, in which partial degradation of one or both fractions has occurred. It is well known that hyperimmune sera may contain a gamut of antibodies, all directed against the main determinant group, yet showing small differences in the size and specificity of the combining site (15). It is possible that such differences are being measured in the two K1 fractions presented here. Furthermore, the fact that neither of the isolated fractions, nor the crude antigen containing both, is able to inhibit completely bacterial agglutination by the adsorbed anti-K1 antiserum, supports this latter possibility. We cannot, at any rate, conclude that we have isolated the K1 antigen of *E. coli* as it appears in the capsule; however, we have prepared material with significant specific activity, containing far less nucleic and sialic acids and protein than our crude extract.

The polysaccharide moiety of the partially purified material is acidic in that it forms salts with quaternary ammonium compounds such as Cetavlon and anion-exchange resins. Both K1 active fractions were obtained free from O antigen as measured by hemagglutination inhibition

with lipopolysaccharide-sensitized erythrocytes, this test being capable of measuring as little as 0.02 μ g (20). Isolation of K1-specific material free from O antigen now allows us to study the role of this product in virulence, and such studies are in progress.

The serological analysis of the adsorbed anti-K1 serum assures us that we are not measuring colanic acid, rather than K1 hapten; however, we cannot disregard the possible presence of the former as a contaminant of our isolates, and must assume temporarily that part of our polysaccharide is colanic acid. Not only does colanic acid precipitate with Cetavlon at the approximate NaCl molarity at which we obtain one of the K1 fractions (11, 21), but also even those *E. coli* strains not obviously mucoid do produce small quantities of this substance (11, 19).

Two biochemical entities apparently remain associated with the K1 activity: carbohydrate and a small amount of protein. The continual direct association of the polysaccharide peak and the peak of K1 activity suggests that it contains the K1 hapten. Protein is probably not involved directly in K1 activity. We have on occasion obtained fully active fractions with no detectable protein, and further there is no direct relationship between protein concentration and K1 inhibitory activity.

We believe that sialic acid deserves further consideration, despite the fact that neither colominic acid (3) nor sialyl compounds released from the cell surface (5) show any serological activity in the K1 system. When the results of Sephadex filtration of the 0.075 M fraction (Fig. 2) are carefully analyzed, it can be seen that the major portion of the sialic acid is delayed in elution from the column, being separated from K1 activity, and that the ratio of free to total sialic acid varies throughout the elution series. It is possible that the slow-moving portion of sialic acid corresponds to colominic acid, which is reported to dialyze slowly (2), and thus would be of a molecular size small enough to diffuse into G-200 Sephadex. That portion of the sialyl compound that cannot be separated from K1 activity may be linked electrostatically to the active material as a result of the extraction procedure, and thus be a contaminant rather than an integral part of K1 antigen. This is supported by the high ratio of thiobarbituric acid positive material in the early eluates of this experiment. However, it is also possible that sialic acid constitutes a small but integral part of the serologically active molecule, and thus might play a role in the haptenic group.

The lability of the K (L) antigen in nature when submitted to high temperatures and extremes in pH (7, 16) agrees with the results found

for the fractions isolated here. This suggests that an extraction technique which uses elevated temperature or acid hydrolysis, or both, is not the most suitable.

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