SEPARATION, CHARACTERIZATION, AND BIOLOGICAL SIGNIFICANCE OF A COMMON ANTIGEN IN ENTEROBACTERIACEAE*

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An antigen common to members of the family Enterobacteriaceae detectable only by means of the hemagglutination test was recently described (1–3). It was discovered during the course of a study using this test concerned with serologic interrelationships among the almost 145 known *Escherichia coli* O antigen groups (2). Good correspondence was obtained between O antigen groups and homologous rabbit antisera, and a limited number of cross-reactions among various O groups was also observed. In addition, erythrocytes coated with crude preparations of any of the *E. coli* O groups were agglutinated by rabbit antiserum to *E. coli* O14 and to a lesser extent by antiserum to O 56, 124, and 144. Subsequent studies revealed that the same reaction occurred with these antisera when erythrocytes were coated with crude lipopolysaccharides from other Enterobacteriaceae including *Salmonella*, *Shigella*, *Proteus*, and *Aerobacter* species, but not with similar preparations from numerous other Gram-negative or positive bacterial species. It was postulated that the Enterobacteriaceae lipopolysaccharide possesses two types of antigenic determinants. One is the classic, specific O antigen; the other is common to all members of this family. *E. coli* O14 appears to differ from the others in its remarkable capacity to elicit antibody formation in rabbits to this common determinant.

Antibody to the common antigen is present in significant amounts in human and other mammalian sera (2, 4), and must be removed, either by absorption or by preincubation with excess crude heterologous O antigen preparations, to permit measurement of specific antibody directed against the O antigen. If this is not done, as has been the case in almost all previously published studies, the hemagglutination test measures the sum of antibody to both the specific and the common antigen.

The present report is concerned with the isolation of the common antigen by means of DEAE cellulose chromatography, with some of its physical, chem-

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ical, and biologic properties, with the relation of common antigen to the rough phenomenon, and with a reevaluation of the distribution of antibodies in human sera directed specifically against O antigens. A preliminary report of these studies has been presented (5).

Materials and Methods

Bacterial Strains and Antisera.—Prototype *E. coli* O groups, *Proteus* OX 19, and rough *E. coli*, *Salmonella*, and *Shigella* strains, together with corresponding antisera, were obtained from the Communicable Disease Center, Atlanta, Georgia.[1] Additional strains of *E. coli* were obtained from patients at this laboratory; a strain of *Shigella flexneri* was from the collection of the Department of Microbiology of the University of Virginia. Endotoxin preparations of *Salmonella typhosa* and various other organisms and group D salmonella antiserum were obtained from a commercial source (Difco Laboratories, Inc., Detroit).

Preparation of Crude Lipopolysaccharides.—Crude endotoxin was prepared by the phenol-water extraction method of Westphal (6). Cultures were incubated in 2-liter Erlenmeyer flasks containing 1 liter of the minimal liquid medium of Davis (7) supplemented with 0.5 per cent yeast extract and 1 per cent casamino acids (Difco Laboratories, Inc.) in a rotary shaker for 20 hours at room temperature. Cells were harvested by continuous flow centrifugation and lyophilized. They were then weighed, suspended in water, heated to 68°C, and mixed with an equal volume of 95 per cent phenol at the same temperature. Following 20 minutes' incubation with agitation at 68°C, the aqueous phase was removed by centrifugation, and the phenol phase was reextracted with an equal volume of water by the same procedure. Aqueous phases were pooled, cooled, and centrifuged to remove residual non-soluble material, and dialyzed for 48 hours against cold tap water. The non-dialyzable material was then concentrated to approximately 40 ml by evaporation at reduced atmospheric pressure at 40°C, and precipitated at 4°C with 10 volumes of absolute ethanol containing a trace of sodium acetate. The precipitate was washed with absolute ethanol, lyophilized, and stored at −20°C until subjected to further study. Recovery was 3 to 7 per cent of the initial dry weight of bacteria.

Serologic Studies.—Hemagglutination tests were conducted as described in detail elsewhere (8) employing plastic trays containing 96 cups.[2] Hanks' balanced salt solution (BSS) was employed as diluent. All reagents were added to the system in volumes of 0.25 ml; the mixture was then incubated at 37°C for 1 hour, and hemagglutination was read by pattern over a horizontally placed x-ray view box. Specific O antigens were assayed by determining the maximum dilution which, when preincubated with a standard dilution of specific rabbit antiserum (usually 1:50) for 30 minutes at 37°C, would block agglutination of erythrocytes coated with the specific antigen. Common antigen was similarly assayed employing a 1:50 dilution of a single lot of *E. coli* O14 rabbit antiserum obtained from the Communicable Disease Center and erythrocytes coated with a heterologous Enterobacteriaceae antigen. A hemagglutination-inhibition unit was defined as the maximum dilution at which antigen blocked agglutination by this standard dilution of serum. The specific O antigen of *E. coli* O14 was assayed by the hemagglutination method by first removing the common antibody either by absorbing 3 times for 30 minutes at 37°C with boiled, washed bodies of a rough *E. coli* strain, or by blocking the antibody by preincubation of a 1:50 dilution of O14 antiserum with a 10 per cent crude phenol extract of *E. coli* O1 at 37°C for 30 minutes. The *E. coli* O14 antiserum modified by either of these methods only agglutinated cells coated with O14 antigen.

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[1] I am indebted to Drs. John Winn and W. H. Ewing for providing these.

[2] Obtained from Limbro Manufacturing Company, New Haven, Connecticut, as disposo-trays.

Precipitin tests were performed by the method of Ouchterlony (9); bacterial agglutination tests were conducted according to the procedures of Edwards and Ewing (10).

Analytical Methods.—Absorption spectra were measured in a Beckman DU spectrophotometer; ultracentrifugal preparative studies were performed in Spinco model L centrifuge.

Sodium was determined by the flame photometric method, nitrogen by the micro Kjeldahl procedure (11), protein by the method of Lowry (12), and phosphorus by that of Fiske and Subbarow (13). Lipid was determined gravimetrically by weighing the chloroform-soluble material released during hydrolysis for 6 hours with 1 N HCl. Total reducing sugars were measured by the ferricyanide reduction method (14) after hydrolysis in 1 N HCl for 2 to 6 hours at 100°C; total hexoses were measured by the method of Dubois *et al.* (15), uronic acids by the method of Dische (16), and 6 deoxyhexoses by the method of Dische and Shettles (17).[3]

TABLE I

Assay of O and Common Antigens in Crude Lipopolysaccharide Preparations

Source	Reciprocal of maximum blocking dilution of 1 mg/ml	
	O antigen	Common antigen
E. coli O14*	256	640–2048
E. coli O1*	4096	128
E. coli rough*	—	256
E. coli O55‡	—	8
E. coli O127‡	128	16
*Sh. flexneri**	256	256
S. typhosa‡	2048	32
Proteus OX 19	—	Undiluted

Hemagglutination-inhibition tests were conducted against a 1:50 dilution of standard homologous rabbit antiserum.

* Phenol-water extract.

‡ Commercial material (Difco Laboratories, Inc.).

Hexosamines were assayed by the method of Neuhaus and Letzring (18). All assays are based on dry weight obtained after overnight incubation of aliquots at 105°C.

Chromatographic Studies.—These were performed with sephadex (Pharmacia, Upsala, Sweden) and DEAE cellulose. DEAE cellulose columns were equilibrated with 0.05 M tris buffer pH 8.0; antigen was dissolved in the same buffer and eluted from the column with this buffer followed by a gradient established between a reservoir containing 0.3 tris–0.5 M NaCl, pH 7.2, into a mixing flask containing 1000 ml of 0.05 M tris buffer, pH 8.0, according to the procedure described by Rüde and Goebel (19). Modifications in these procedures will be described as employed.

EXPERIMENTAL

Separation of Common and O Antigens by DEAE Cellulose Chromatography.—Rüde and Goebel (19) successfully applied chromatography on DEAE cellulose together with NaCl elution to the purification of *E. coli* O1 antigen

[3] I am grateful to Dr. Wesley Volk for advice in performing these procedures.

in the course of studies on colicines. Their method proved to be useful in separation of common and specific O antigens from crude lipopolysaccharide preparations from a series of different Enterobacteriaceae. Assays of common

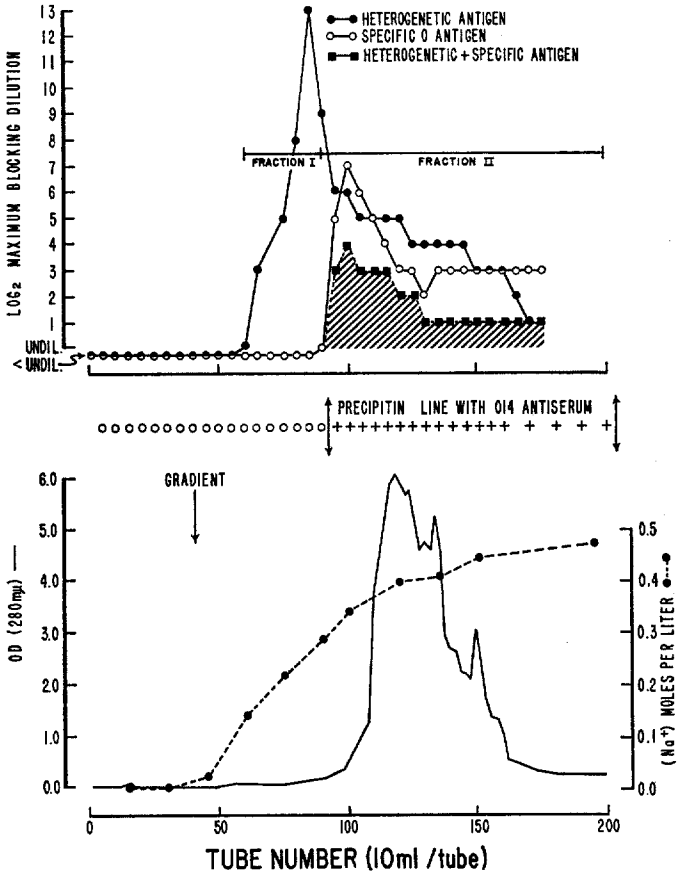

FIG. 1. Chromatography of crude phenol-water extract of *E. coli* O14 (500 mg) on DEAE cellulose column (26 × 2.5 cm).

and O antigens in crude preparations from various sources is presented in Table I. All preparations contained some common antigen, but *E. coli* O14 possessed far more activity per milligram than any of the others, including a rough strain of *E. coli*. The variation in relative amounts of specific O antigen, noted in this table, presumably reflects the relative purity of the materials studied.

Separations of the common and O antigens in a series of Enterobacteriaceae by chromatography are shown in Figs. 1 to 5. In each of these studies common

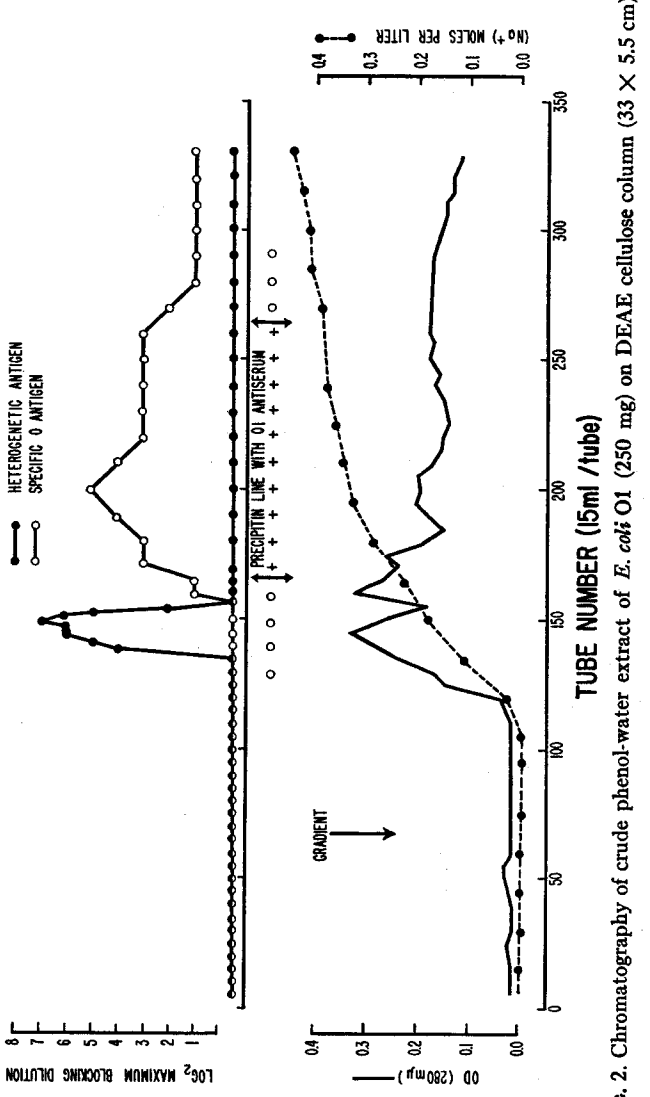

FIG. 2. Chromatography of crude phenol-water extract of *E. coli* O1 (250 mg) on DEAE cellulose column (33 × 5.5 cm).

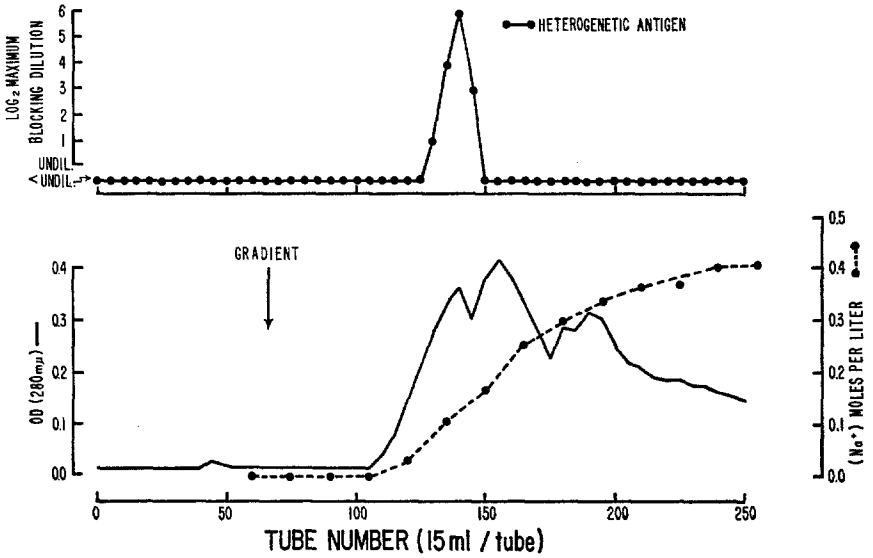

FIG. 3. Chromatography of crude phenol-water extract of *E. coli* rough:H10 (250 mg) on DEAE cellulose column (33 × 5.5 cm).

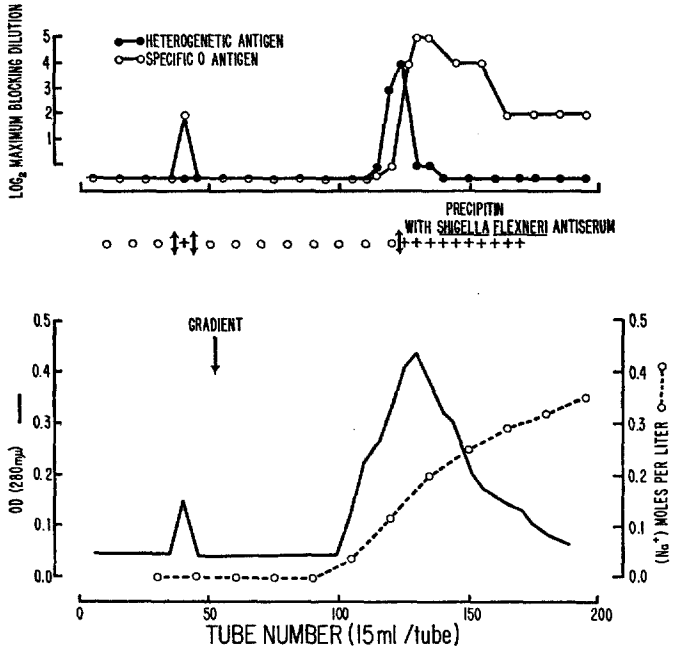

FIG. 4. Chromatography of crude phenol-water extract of *Shigella flexneri* (250 mg) on DEAE cellulose column (33 × 5.5 cm).

and O antigens were determined by the hemagglutination test, and, when possible, the presence of O antigens was substantiated by agar gel precipitin test as well. Good correlation was obtained between precipitin and hemagglutination tests for O antigens; the latter test, however, appeared to be more sensitive. Note that no precipitin line was found in any of these studies in zones where the common antigen appears alone, confirming previous observa-

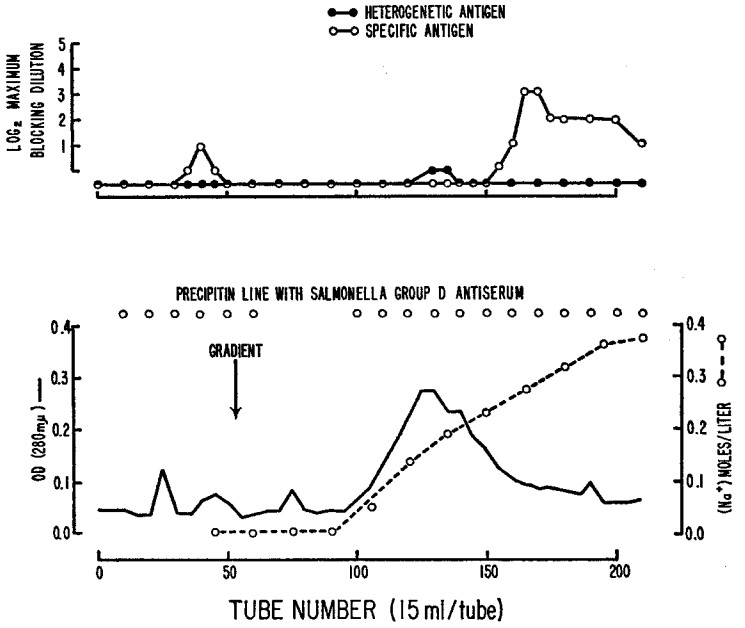

FIG. 5. Chromatography of *Salmonella typhosa* lipopolysaccharide (Difco Laboratories, Inc.) (250 mg) on DEAE cellulose column (33 × 5.5 cm).

tions that the common antigen is detectable only by means of the hemagglutination test.

A summary of a series of experiments in which determinations were made of the concentration of NaCl at which common and O antigens were eluted from a series of Enterobacteriaceae extracts employing various size chromatography columns is presented in Table II. In each of these experiments the common antigen was first eluted at molarities of NaCl varying from 0.08 to 0.16 (mean for all experiments 0.12); the peak elution of common antigen occurred at a mean NaCl molarity of 0.16. Specific O antigens, regardless of source, eluted at somewhat higher NaCl molarity; mean molarity at onset 0.21 and at peak 0.28. Rechromatography of purified common antigen on DEAE cellulose gave reproducible results. Recovery was 3.2 to 6.8 per cent common antigen

from the crude phenol-water extract of *E. coli* O14. Thus, a means was found which enabled purification of the common antigen; double chromatographed material was used in further studies of the properties of this substance. The most efficient procedure for separating O and common antigens from large

TABLE II

Concentration of NaCl at which O and Common Antigens Elute from DEAE Cellulose Columns

Crude antigen		Column size	Na^+, moles per liter			
			Common antigen		O antigen	
			Onset	Peak	Onset	Peak
	mg	*cm*				
E. coli O14	100	2.5 × 20	0.11	0.16	—	—
	100	2.5 × 20	0.15	0.20	—	—
	500	2.5 × 20	0.15	0.28	0.30	0.33
Rechromatograph		2.5 × 20	0.16	0.18	—	—
	1500	5.5 × 33	0.12	0.25	0.28	0.34
Rechromatograph		2.5 × 20	0.08	0.14	0.21	0.21
	1000	5.5 × 33	0.12	0.15	0.21	0.21
Mean		—	0.13	0.19	0.25	0.27
E. coli O1	100	2.5 × 20	0.11	0.13	—	—
Rechromatograph		2.5 × 20	0.15	0.18	0.27	0.34
	250	5.5 × 33	0.13	0.18	0.23	0.33
Mean		—	0.13	0.16	0.25	0.33
E. coli rough: H10	250	5.5 × 33	0.08	0.13	—	—
S. typhosa	250	5.5 × 33	0.16	0.19	0.25	0.28
Sh. flexneri	250	5.5 × 33	0.09	0.15	0.11	0.20
Proteus OX 19	250	5.5 × 33	0.15	0.17	—	—
Mean, all sources		—	0.12	0.16	0.21	0.27

quantities (1 to 2 gm) of *E. coli* O14 phenol-water extract was achieved by employing 33 × 5.5 cm DEAE cellulose columns, applying crude material at a concentration of 10 mg per ml in 0.05 M tris buffer pH 8 to the column, followed by elution with 0.25 M NaCl in 0.3 M tris, pH 7.2, through a mixing flask containing 1 liter of 0.05 M tris, pH 8.

Physical and Chemical Studies of the Common Antigen.—The chemical constituents of DEAE cellulose purified common antigen determined in three preparations is shown in Table III. The material was slightly yellow in color, contained small amounts of hexose (calculated as glucose). Most of the reducing

sugar appears to be glucosamine.[4] Tests for 6 deoxyhexoses, uronic acid, and heptulose were negative. Nitrogen content of 6.5 per cent may be accounted for by the glucosamine, amino acids, and small amount of nucleic acid contaminant.

Common antigen recovered from *E. coli* O14 as shown in fraction I of Fig. 1, was found to absorb only slightly in the ultraviolet spectrum at 260 and 280 mμ, but preparations obtained in subsequent extractions were found to possess material absorbing at 260 mμ (Fig. 6).[5] It was therefore not clear as to whether nucleic acid might be responsible for some of the antigenic activity. This question was resolved by incubating 3 mg of common antigen, which possessed

TABLE III

Chemical Properties of Common Enterobacteriaceae Antigen Separated from E. coli O14 by DEAE Cellulose Chromatography

Analysis	Sample (dry weight)			
	I	II	III	Mean
	per cent	*per cent*	*per cent*	*per cent*
Nitrogen	5.4	7.4	6.8	6.5
Phosphorus	0.4	0.5	—	0.5
Protein	3.0	1.7	8.4	4.4
Lipid	—	—	—	Not demonstrable
Reducing sugar	18.5	12.5	20.0	17.0
Hexose*	—	—	2.3	2.3
Hexosamine	—	8.3	16.9	12.6

* As glucose.

considerable 260 mμ absorbance, and 16,284 blocking units per mg with 12 mg of activated charcoal in 3 ml of water at pH 2 for 30 minutes at 0°C. Following removal of the charcoal by centrifugation optical density in the ultravolet spectrum was almost entirely eliminated without effect on antigenic activity (as determined by hemagglutination inhibition tests). Furthermore, chromatography of the purified common antigen on sephadex G-100 (Fig. 7) resulted in 2 peaks, the first of which contained almost all of the antigenic activity while the second had almost all of the 260 mμ absorbance. Accordingly, nucleic acid can be considered to be a contaminant in some preparations.

Periodate Oxidation.—Three lipopolysaccharide preparations derived from

[4] Preliminary analysis of amino acid content employing chromatography by the method of Spackman *et al.* (20) reveals the hexosamine to be glucosamine. I am indebted to Dr. George Frimpter for this study which is still in progress.

[5] The only differences in procedures was that the flow rate in the first study was much slower than in later separations and that it was carried out at room temperature whereas all subsequent studies were performed at 4°C with a flow rate of 2 ml per minute.

S. typhosa, *E. coli* O127 (Difco Laboratories, Inc.) and phenol-water extracted *E. coli* O14 (25 mg in 25 ml) were incubated with an equal volume of 1/50 M potassium periodate at 4°C in the dark. Aliquots of 4 ml were removed at

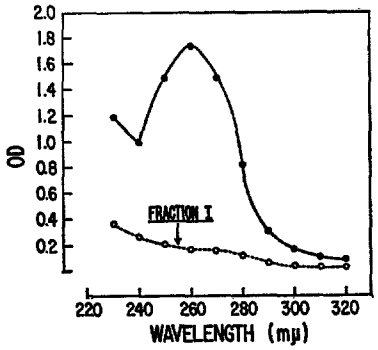

FIG. 6. Absorbance of 1 mg/ml of DEAE cellulose–purified common antigen prepared from *E. coli* O14. Fraction I corresponds to material recovered in the experiment shown in Fig. 1; the upper line is spectrum of preparations obtained in subsequent attempts to isolate the antigen by the same procedure.

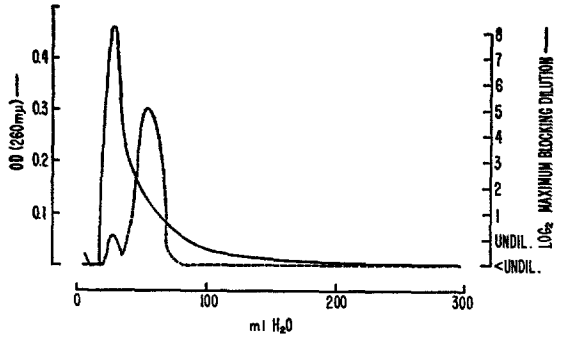

FIG. 7. Chromatography of DEAE cellulose–purified common antigen on sephadex G-100 (2.5 × 21 cm) demonstrating separation of antigenic activity from most of the 260 mμ absorbing material.

intervals and added to 1 ml of 10 per cent glucose and dialyzed overnight to remove reaction products. No reduction in hemagglutination-inhibition activity was observed with any of the antigens after incubations of 1, 3, 5, and 9 hours with periodate.

Treatment with Trypsin.—*E. coli* O55 lipopolysaccharide (Difco Laboratories, Inc.) was treated with trypsin by incubating 200 mg of lipopolysaccharide with 250 mg of trypsin (Difco Laboratories, Inc.) in 125 ml of water adjusted to pH 8.2 with 1 N NaOH. Five ml of toluene was added to inhibit bacterial

growth. The mixture was incubated at 37°C for 20 hours, then concentrated by evaporation, dialyzed overnight, and precipitated with absolute ethanol at final volume of 68 per cent and washed with absolute ethanol. The dried product was dissolved in 0.85 per cent NaCl at a concentration of 1 mg per ml, boiled 2 hours, and coated on fresh erythrocytes. Sensitized cells were strongly agglutinated by *E. coli* O14 serum. Thus, trypsinization failed to remove the common antigen from *E. coli* O55 lipopolysaccharide.

Molecular Size.—DEAE cellulose purified common antigen was not dialyzable

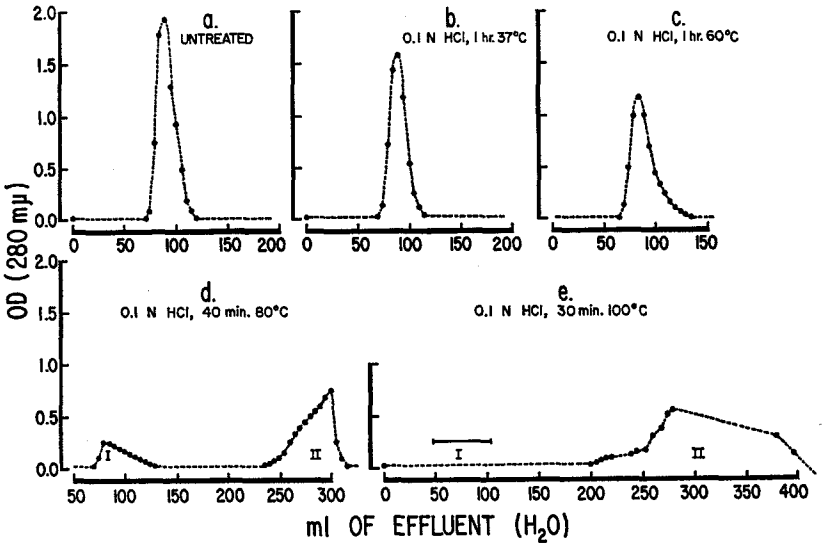

FIG. 8. Chromatography of products of hydrolysis of *S. typhosa* lipopolysaccharide on sephadex G-100 (bed volume, 30 × 4 cm).

through cellophane membranes and was excluded from sephadex G-100, indicating a molecular weight well in excess of 40,000. The common antigen was found to migrate with the O antigen when crude lipopolysaccharides of *E. coli* O14 and O55 and *S. typhosa* were chromatographed on sephadex G-100 and G-200 suggesting that they are bound together or are of very similar molecular size. An example of the migration in sephadex G-100 of the 2 antigenic components in crude *S. typhosa* lipopolysaccharide is shown in Fig. 8, obtained during a study of the relative resistance of O and common antigens to acid hydrolysis.

Resistance to Acid Hydrolysis.—Ten mg quantities of *S. typhosa* lipopolysaccharide were dissolved in 0.1 N HCl and heated at various temperatures, allowed to cool, and chromatographed in turn on a single 30 × 4 cm G-100 sephadex column; elution was carried out with water.

Both antigens were present in the single peak found with untreated *S. typhosa* lipopolysaccharide (Fig. 8 *a*): heating for 1 hour at 37°C in 0.1 N HCl slightly decreased the amount of optically dense material recovered (Fig. 8 *b*), but the single peak obtained contained both antigens; heating at 60°C for 1 hour removed the *S. typhosa* O antigen, but both coating and blocking activity of the common antigen were retained (Fig. 8 *c*); heating at 80°C for 40 minutes produced 2 peaks (Fig. 8 *d*), but only the first (I), at the elution volume point of the untreated material, still possessed some of the common antigen, detectable only by hemagglutination-inhibition tests. Finally, boiling for 30 minutes in 0.1 N HCl (Fig. 8 *e*) entirely removed optically dense material, from position I, but a slight amount of common antigen remained at the original elution

TABLE IV

Ethanol Fractionation of Crude Lipopolysaccharides of E. coli

	Lipopolysaccharide					
	O55		O127		O14	
	mg	*per cent*	*mg*	*per cent*	*mg*	*per cent*
Crude extract	260	100.0	200	100.0	700	100.0
Ethanol fraction I	165	63.5	151	75.5	486	69.4
II	11	4.2	42	21.0	48	6.9
III	14	5.3	3	1.5	85	12.1
Total recovered	190	73.0	196	98.0	619	88.4

volume of the preparation (I). Products of hydrolysis (Fig. 8 *e*, II) failed to block hemagglutination by antibody to either the common or specific antigens. Thus, the common antigen appeared to be more stable than the O antigen under these experimental conditions.

Failure to Separate Common and O Antigens by Westphal Methods—

(*a*) *Ethanol fractionation:* It had been previously shown that Boivin preparations of *E. coli* O14 and commercially prepared endotoxins (Difco Laboratories, Inc.) contain the common Enterobacteriaceae antigen as well as the specific O antigen. Accordingly, an attempt was made to further fractionate these preparations by the ethanol method described by Westphal (6) in the hope that the two antigenic activities could be separated by this means.

E. coli O55 and 127 lipopolysaccharide (Difco Laboratories, Inc.) and phenol-extracted *E. coli* O14 were dissolved in water. An equal volume of absolute ethanol was added to each, yielding precipitate I. The supernates were concentrated to 20 to 30 ml by flash evaporation at 40°C and an equal volume of ethanol added yielding precipitate II. The remaining supernate was adjusted

to 6 volumes ethanol for each of water and precipitate III obtained. Precipitates were dried, weighed, and tested for antigenic activity *in vitro*. Recovery of ethanol fractions, by weight, is summarized in Table IV. Most of the starting material was precipitated during the first step in the procedure. Only small amounts (2 to 12 per cent) were recovered during the third step which, according to Westphal, should contain most of the O antigen contaminated with small amounts of nucleic acid. Results of antigenic activity determinations obtained with fractions of *E. coli* O127 are shown in Table V; similar results were obtained with the other lipopolysaccharides. Most of the activity, by weight, of *both* antigens was recovered in precipitate III. The absence of detectable common antigen in precipitates I and II in the presence of small amounts

TABLE V

Antigenic Activity of Ethanol Fraction of E. coli O127 Lipopolysaccharide as Determined by Hemagglutination Tests*

	Ethanol fraction			
	Untreated	I	II	III
O127 antigen	128	8	16	640
Common antigen	16	0	0	40
Sensitizes RBC's to O127 serum	+	0	0	+
Sensitizes RBC's to O14 serum	+	0	0	+

* Activity expressed as reciprocal of maximum dilution of 1 mg/ml of antigen capable of blocking agglutination of erythrocytes coated with O127 antigen with a 1:50 dilution of O127 or O14 (common) rabbit antiserum.

of O antigen does not necessarily indicate that the O antigens had been purified, since these fractions contained large amounts of inactive material. These studies indicated that the bulk of the crude lipopolysaccharide possessed little antigenic activity, and that ethanol fractionation did not clearly dissociate the two antigenic activities.

(*b*) *Ultracentrifugation:* Westphal (21) has also shown that relatively pure lipopolysaccharide endotoxins, free of nucleic acid, can be prepared by repeated high speed centrifugation of crude phenol extracts of Gram-negative bacteria. Accordingly, a 10 per cent aqueous solution (270 mg in 27 ml) of phenol-water extract of *E. coli* O14 was centrifuged at 136,000 × *G* for 1 hour. The sediment was washed 3 more times under the same conditions then dried at room temperature in a desicator and weighed. Most of the initial material was recovered in the first supernate (196 mg or 72.6 per cent). The final sediment produced an opalescent solution in water and contained 22 mg or 8 per cent of the starting material. Both of these fractions possessed O14 antigenic activity as determined by ability to coat erythrocytes and react with O14

antiserum, but the final sediment was most intensively studied since it corresponded most closely to a Westphal preparation of O antigen. This material was highly lethal to rabbits; 7/10 animals injected intravenously with 25 micrograms died within 24 hours. The surviving animals were bled at 1 week and pre- and postimmunization sera tested for ability to agglutinate erythrocytes coated with extracts of *E. coli* O14 and O127, *S. typhosa*, and a rough strain of *Shigella*. A rise in antibody to all antigens from less than undiluted to up to 1:16 was obtained indicating that this preparation was capable of inducing rabbits to form antibody to the common antigen. A solution of 1 mg/ml of the final precipitate tested in an agar gel diffusion system against hyperimmune *E. coli* O14 rabbit antiserum produced 3 well-defined precipitin lines within 24 hours' incubation at 4°C, indicating the presence of specific O antigen as well. Thus, in this experiment, ultracentrifugation failed to separate the common antigen from specific O antigen.

Measurement of Antibody to Specific O Antigens in Human Sera and Colostrum.—A study of the distribution in human serum and colostrum and antibodies directed against eleven non-enteropathic O groups of *E. coli* (O1, 2, 4, 6, 7, 20, 35, 58, 74, 75, and 110), previously reported (8), did not take into account the presence of antibody to the common Enterobacteriaceae antigen. It was reported that antibodies to all O groups were demonstrable in serum of almost all subjects over the age of 2 years, and that the geometric mean titers gradually leveled off above this age. In addition, human colostrum was found to be rich in antibody to *E. coli*, but transplacental passage and nursing were relatively ineffective in transferring antibody to the human neonate. Subsequent studies (2), employing crude extracts of *E. coli* O14 to block the common antigen in human serum, produced generally a two-fold reduction in titer when tested against erythrocytes coated with any of the 136 available O antigens, but rarely removed all reactivity. It appeared that part, but not all, of the antibody measured in human sera by the hemagglutination test was directed towards the common antigen.

To reassess the distribution of antibody to the specific O antigens in human serum, blood from 69 individuals of various ages, with and without a history of urinary tract infection, were tested against erythrocytes coated with *E. coli* O1. Diluted serum was preincubated for 30 minutes at 37°C with an equal volume of the common antigen as purified 2 times on DEAE cellulose. The amount of antigen used was 16 times more than necessary to completely block a 1:50 dilution of *E. coli* O14 rabbit antiserum from reacting with *E. coli* O1 antigen even when the serum was diluted 4 times further.

Pooled adult human sera tested against *E. coli* O1 gave a titer of 1:32; this was reduced to 1:16 when preincubated either with a crude extract of *E. coli* O14 or with DEAE cellulose purified common antigen. The geometric mean titers of antibody specifically directed against *E. coli* O1 (common antigen

having been blocked out) in sera from individuals of various ages, sex, and with or without a history of urinary tract infection is shown in Fig. 9. Note the low titer of antibody in cord blood, the absence of detectable antibody in children

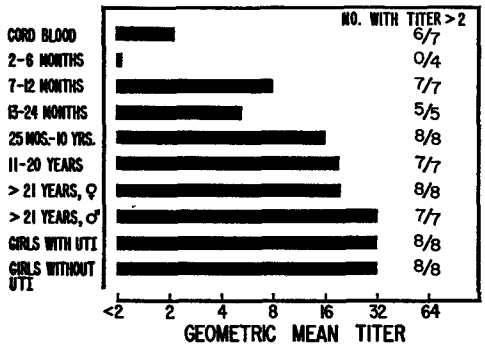

FIG. 9. Geometric mean titer of antibody to *E. coli* O1 according to age, sex, and past history of urinary tract infection in 69 human serums in which antibody to the common antigen was blocked by preincubation with excess purified common Enterobacteriaceae antigen.

TABLE VI

Effect of Preincubating Human Serum and Colostrum with Crude Extracts of E. coli O14 on Antibody Titer to E. coli O2

Subject preincubated with	Reciprocal of maximum hemagglutination dilution					
	Mother		Colostrum		Cord	
	O14	BSS	O14	BSS	O14	BSS
Cl.	40	40	80	160	Undiluted	2
Du.	80	160	1280	2560	4	8
Fi.	80	80	320	1280	—	—
Wa.	160	160	>2560	>2560	—	—
Th.	20	40	>2560	>2560	—	—

aged 2 to 6 months, and the rise above this age with a tendency to level off above the age of 2 years. Girls with active urinary tract infection possessed about the same amount of antibody to this antigen as age-matched girls with no known history of infection. Titers of antibody to *E. coli* O1 in colostrum were also found to be high; *e.g.*, colostrum titers were 1:320 and 1:640 compared to respective maternal titers of 1:8 and 1:32, when specimens obtained within 2 days of delivery were studied.

The relationship between titer of antibody to *E. coli* O2 in mother's serum, colostrum, and cord blood, tested with and without blocking out antibody to

the common antigen by use of crude *E. coli* O14 antigen, is shown in Table VI. A twofold reduction in titer was obtained in most instances but the previously shown relationships between these fluids remained intact; *e.g.*, very much higher concentrations of antibody in colostrum and poor transplacental transfer. In extensive studies of antibodies directed toward a great variety of *E. coli* O groups found in the urine of subjects with urinary tract infection, to be reported elsewhere, a twofold reduction of antibody titer was generally observed following preincubation of serum with *E. coli* O14 (22).

These studies support the previously reported conclusions concerning the distribution of *E. coli* antibody in human body fluids, but indicate that titers to the specific O antigens are slightly lower than originally obtained because of the presence of antibody to the common antigen.

Relation between the Rough Phenomenon and the Common Antigen.—Rough strains of *E. coli* and other Enterobacteriaceae are characterized by their capacity to agglutinate in the presence of antiserum prepared against many different O antigen groups. Westphal and coworkers have proposed that the rough antigen forms the basal portion of the polysaccharide upon which the smooth O antigen determinants are built (23). Since both the rough and common antigen, described here, are widely distributed in Enterobacteriaceae the possibility that they may be related was considered.

Ten pools of rabbit *E. coli* antisera prepared against 8 to 15 different O antigens each, were incubated at a dilution of 1:50 with an equal volume of crude O14 antigen, at 37°C for 1 hour. Three rough strains of *E. coli* when tested against these treated sera were agglutinated to the same extent as by untreated serum. *E. coli* O14 cells were grown on trypticase soy agar in quart milk bottles, washed from the surface, boiled 2 hours, and washed with saline. *E. coli* O1, O6, and O75 rabbit antisera (1 ml undiluted serum plus 4 ml of formalinized saline) were incubated with these cells for 6 hours at 37°C, then overnight at 4°C. Bacteria were removed by centrifugation and the sera tested for ability to agglutinate a rough strain of *E. coli*. Absorbed sera agglutinated the rough strain as well as homologous unabsorbed sera.

Crude boiled antigens from 6 rough strains and *E. coli* O14 were tested in an agar gel diffusion plate against undiluted hyperimmune *E. coli* O14 serum. Precipitin lines were observed only between *E. coli* O14 antiserum and homologous antigen. Furthermore, serum from a rabbit immunized with a formalinized broth-grown culture of a rough strain by the intravenous route (0.5, 1, 2, 3, and 4 ml given at 3 day intervals) failed to form precipitin lines with *E. coli* O14 antigen. Thus, no *E. coli* O14 O antigen could be detected in rough strains, nor did rough strains induce antibody formation to this antigen.

Twelve rough strains of *E. coli* were tested, by the hemagglutination method, against rabbit antisera to each of the 145 odd O antigen groups of *E. coli*. Sensitized cells were agglutinated only by antiserum to *E. coli* O14, 124, and

144 (each of which will detect the common antigen). This observation was not surprising since as shown in Table I and Fig. 3, rough strains contained amounts of common antigen comparable to those observed in other Enterobacteriaceae, but less than found in *E. coli* O14.

Crude, boiled O antigen preparations of numerous rough strains of Enterobacteriaceae were prepared by the method of Neter (24) and coated on human group O erythrocytes. These strains included 3 *Salmonella* and 2 *Shigella*. The sensitized erythrocytes were agglutinated by pooled human serum and gamma globulin at dilutions of 1:8 to 1:32. Crude O14 antigen and DEAE cellulose–purified common antigen when preincubated with serum and gamma globulin in most instances reduced the titer observed against the rough strains coated on erythrocytes twofold and occasionally abolished the reactivity of the serum to rough antigens entirely. It was not entirely clear from these experiments whether the rough strains reacted with human serum and gamma globulin because the bacteria had retained a small amount of O antigen or because the sera contained antibodies against some component of the rough antigen. In any event, the common antigen did not appear to be entirely responsible for reactivity of rough strains in the hemagglutination test.

These experiments indicate that rough strains behave very much like other Enterobacteriaceae in that they possess the common antigen and coat erythrocytes. There does not appear to be any other special relation between *E. coli* O14 and the rough strains. Erythrocytes coated with antigens from some rough strains may provide an excellent reagent for measuring common antibody in serum directly, if it can be shown that agglutination of erythrocytes, coated with a particular strain, can be completely inhibited by preincubation of serum with purified common antigen.

Other Properties of the Common Antigen.—

(*a*) *Ability to coat erythrocytes:* DEAE cellulose–purified common antigen boiled for 2 hours in saline failed to sensitize erythrocytes to agglutinate in the presence of *E. coli* O14 serum, despite good hemagglutination inhibition activity (20,240 units/ml), nor could this be accomplished by addition of heterologous lipopolysaccharides derived from *Serratia marcescens* or *Brucella abortus*. Thus, attachment of common antigen to erythrocytes appeared to require direct association with its native lipopolysaccharide.

(*b*) *Ability to coat latex particles:* Whang and Neter (3) reported that latex particles coated with crude extracts from Enterobacteriaceae can be agglutinated by homologous antisera, but that the heterologous reaction with *E. coli* O14 was not observed. We were able to confirm these observations, and have been able to demonstrate that common antigen in crude O antigen preparations is also adsorbed to latex. Boiled *E. coli* O55 lipopolysaccharide (5 ml at 1 mg/ml) was incubated with 1 ml of 0.81 micron latex particles (Difco Labora-

tories, Inc.) in saline for 30 minutes at 37°C. Latex particles were removed by centrifugation and fresh latex was added for 2 more cycles of incubation. Assay of residual O and common antigens following this treatment revealed that one half of the initial amount of *both* antigens were removed. The particles prepared in this manner agglutinated only in the presence of O55 antiserum.

(*c*) *Ability to stimulate antibody production:* One-half mg of purified common antigen was injected intravenously into each of 6 rabbits. One week later, 3 animals received another injection of 0.5 ml and 3 received 1.0 ml by the same route. Serum obtained from animals bled 2 and 3 weeks after the initial injection failed to agglutinate erythrocytes sensitized to *E. coli* O1, O14, or a rough strain. Under these conditions, therefore, purified common antigen was not

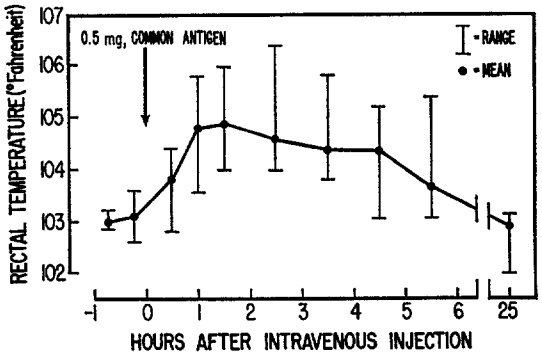

FIG. 10. Pyrogenic responses of 6 adult rabbits to intravenous injection of DEAE cellulose–purified common Enterobacteriaceae antigen.

antigenic even though it possessed 5,120 to 16,284 blocking units per mg. This was in striking contrast to previous observations that smaller amounts of common antigen found in Boivin and Westphal preparations or in whole bacteria of *E. coli* O14 readily stimulate antibody to the common antigen when injected under the same conditions in rabbits.

(*d*) *Pyrogenicity and toxicity:* All 6 rabbits given 0.5 mg of purified common antigen intravenously developed fever within 1 to 2 hours (Fig. 10). None, however, appeared to be ill after this dose and all tolerated well a second injection of 0.5 or 1.0 ml given 1 week later. This is in marked contrast to the lethality previously observed in the same rabbit stock given 25 micrograms of purified Westphal type antigen. It could not be ascertained whether or not the pyrogenic reaction observed in the rabbits given purified common antigen was due to slight contamination with *E. coli* O14 endotoxin from which it was separated.

(*e*) *Inhibition tests with monosaccharides:* In a previous study it was found that antibody to the common antigen was not blocked by preincubation with D-glucose, D-galactose, D-mannose, L-rhamnose, lactose, L-arabinose, D-levulose,

and D-glucosamine. Studies with these sugars were repeated employing a 1:50 dilution of standard *E. coli* O14 rabbit antiserum and erythrocytes coated with *E. coli* O1. The following sugars as 1 per cent solutions were tested in the same system: D-arabinose, *N*-acetylgalactosamine, *N*-acetylglucosamine, L-fucose, galactosamine, L- and D-xylose. None inhibited agglutination.

DISCUSSION

Initial experiments conducted following the identification of a common Enterobacteriaceae antigen strongly suggested that it was closely associated with, if not an integral part, of the large lipopolysaccharide complex which possesses both O antigen and endotoxin activity (1). Thus, common antigen was found in Boivin and both alcohol and ultracentrifuged preparations of phenol-water extracted antigen of the Westphal types; it was found to coat latex particles together with O antigen and migrated with O antigen on sephadex G-100 and G-200 columns. Furthermore, the initial observation depended upon the fact that common antigen coated erythrocytes along with the O antigens. Despite these findings, chromatography on DEAE cellulose revealed the common antigen to be separable from the O antigen. However, in the process of purification it lost some of its properties. The purified common antigen was incapable of coating erythrocytes, and was not antigenic when injected in relatively large amounts into rabbits. It was also much less toxic than endotoxin. The observation that it is non-dialyzable and excluded from sephadex G-100 and chemical studies indicates that it is located on a macromolecule made up of polysaccharide (most of which is glucosamine) and polypeptide.

The common antigen superficially resembles O antigen in chemical composition since it contains hexose, glucosamine, and protein, but it is not clear from the present experiments which of these components is responsible for antigenicity. The small amount of hexose present, the failure to inhibit antiserum with a large variety of monosaccharides, as well as failure of periodate treatment to inactivate the antigen, suggest, but by no means establish, that polysaccharide is not responsible for antigenicity. Nucleic acids appear as contaminants in some preparations; they can be removed by adsorption with charcoal or by sephadex chromatography without interfering with activity. Studies on the composition of the polypeptide fractions are now in progress and are of some interest since the glucosamine may be part of the basal structure of the gram negative bacterial cell wall which contains *N*-acetylglucosamine, alanine, glutamic acid, and meso-diaminopimelic acid (25).

The major significance of the common antigen-antibody system, thus far determined, appears to relate to its interference with the otherwise highly sensitive and specific hemagglutination reaction commonly employed to measure antibodies to Enterobacteriaceae. As shown in the present and an

earlier study by the author (8), and by Whang and Neter (4), some of the activity of human serum against erythrocytes coated with various Enterobacteriaceae lipopolysaccharides is due to antibody to the common antigen. This observation compelled us to repeat our observations on the distribution of antibody to *E. coli* in human serum after blocking the common antibody. Previous observations (8) that *E. coli* antibody passed the placenta very poorly, was gradually acquired with age, was present in high titer in human colostrum and was present in serum from all humans over the age of 2 that were studied, were confirmed. Nevertheless, titers of antibody directed against the O antigen in serum were less than earlier studies had indicated. These observations demonstrate that antibody to the common antigen must be removed from serum prior to testing for specific O antigens with sensitized erythrocytes. This may be readily accomplished by preincubating the serum with crude preparations of heterologous Enterobacteriaceae antigens and such procedure is now routinely performed in our laboratory.

The biologic significance of the common antigen has not been fully determined. Previous studies (2) failed to demonstrate that *E. coli* O14 rabbit antiserum, which possesses the largest amounts of common antigen, possesses bactericidal activity *in vitro*, in the presence of complement, against heterologous strains. The antigen may be of some taxonomic significance in characterizing Enterobacteriaceae, in which case *S. marcescens* would be excluded from this family since the strains of this species, thus far tested, do not possess common antigen. The peculiar ability of *E. coli* O14 to induce antibody to the common antigens appears most likely to be due to the relatively large amount of common antigen in this group. The common antigen present in smaller amounts in other *E. coli* and Enterobacteriaceae appears qualitatively similar to that in *E. coli* O14 as shown by their elution from DEAE cellulose at about the same NaCl concentration. One may speculate that the wide distribution of antibody to common antigen in mammalian sera is due to continuous antigenic exposure throughout life to small amounts of this substance from the large variety of Enterobacteriaceae resident in the gastrointestinal tract.

Finally, the rough phenomenon does not appear to be related to common antigen since sera absorbed with *E. coli* O14 bacterial cells remain capable of agglutinating rough strains and rough strains do not appear to possess more common antigen activity than do some other Enterobacteriaceae in which the O antigen may be characterized.

SUMMARY

An antigen common to Enterobacteriaceae and closely associated with endotoxin fractions has been separated by chromatography on DEAE cellulose employing elution with a NaCl gradient. The purified common antigen fails to coat erythrocytes, is poorly, if at all antigenic, it is non-dialyzable and

excluded from sephadex G-100 gel. It is composed of polysaccharide and polypeptide. The most important property of this antigen thus far determined appears to be its interference with the specificity of the hemagglutination test commonly employed to measure antibody to O antigen of Enterobacteriaceae. It may also have taxonomic significance in classification of this family of bacteria.

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Note Added in Proof.—A 5.2 mg sample of common *Enterobacteriaceae* antigen was hydrolyzed for 20 hours at 110°C under nitrogen and analyzed by Dr. George Frimpter of Cornell University Medical College according to the method of Spackman *et al.* (20). The product contained the following constituents (in micromoles): NH_3, 60.1; glutamic acid, 14.8; alanine, 14.3; glycine, 5.8; aspartic acid, 2.4; and 4.6 micromoles of a ninhydrin-positive material (calculated as diaminopimelic acid) which appears but has not definitely been shown to be diaminopimelic acid. In addition, small amounts (1 to 2 micromoles) of the following amino acids were also identified: threonine, serine, isoleucine, and leucine. These data suggest that the common antigen is of cell wall origin. See Discussion above.

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