FORM VARIATION IN *ESCHERICHIA COLI* K1: DETERMINED BY O-ACETYLATION OF THE CAPSULAR POLYSACCHARIDE*

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Barry and Goebel (1, 2) first described a sialic acid polymer from bacteria, which they called colominic acid, in an *Escherichia coli*, strain K235. Later, this sialic acid polymer was shown to be the K1 capsular polysaccharide of *E. coli* (2–9). There is much evidence that the K1 capsular polysaccharide confers invasiveness to *E. coli* (9–18). Among the serologically defined *E. coli* surface antigens, there are, to date, 164 O (lipopolysaccharide), 56 H (flagellar), and 103 K (envelope or capsular) antigens (8, 11, 19–21). In surveys of *E. coli* CSF isolates from newborns, ≅ 80% were K1 strains (10, 12, 13, 22). K1 strains comprised ≅ 50% of blood isolates from newborns without meningitis and 50% of upper urinary tract infections of infants (10, 12, 16, 17, 23). There were no associations with other *E. coli* K, O, or H antigens or biotypes among these disease isolates (10, 12, 13, 22, 24). Further evidence is derived from the protective effect of K1 antibodies in laboratory animals (9, 10, 13, 25). In an infant rat model, K1 strains, in contrast to *E. coli* with other capsular polysaccharides, induced bacteremia and meningitis after intestinal colonization (14). Passive administration of anti-K1 antibody protected against bacteremia and death but did not affect intestinal colonization in K1-fed infant rats (25).

Both the *E. coli* K1 and Group B meningococcal capsular polysaccharides consist of linear homopolymers of α 2-8-linked *N*-acetyl neuraminic acid (NANA)[1] (1-4, 26, 27). No antigenic differences were detected by double immunodiffusion of K1 and group B meningococcal polysaccharides with group B meningococcal and *E. coli* K1 antisera (10, 28, 29). In contrast to most encapsulated bacteria, K1 capsules are poor immunogens when injected intravenously as formalin-inactivated organisms in laboratory animals and as purified polysaccharides in most adult volunteers (8, 11, 20, 28, 29, 30). However, repeated intravenous injections of formalinized group B meningococcus organisms, strain B-11, into a horse (No. 46), yielded high-titered anticapsular antiserum used to identify both group B meningococci and *E. coli* K1 strains by precipitin formation (halos) in agar and by immunoelectrophoresis of whole organism extracts (10, 12, 14, 17).

It was observed that sera, produced at different times with living cultures of the test

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strain K1 (U5/41 = O1:K1:H−), varied in their ability to agglutinate that same strain (11, 21). It was further observed that K1 colonies could be divided into two types based upon their ability to give weak or strong agglutinates with rabbit typing antisera. Reculture of strongly agglutinating colonies, originally designated K1 positive, regularly gave rise to many strongly agglutinating colonies and a few weakly agglutinating colonies (K1 negative) with a ratio of ≅ 20–50/1. Upon reculture, the weakly agglutinating colonies (K1 negative) mostly gave rise at about the same ratio of 20–50/1. The K1-negative colonies were only weakly immunogenic when injected into rabbits. In contrast, the strongly agglutinating colonies induced K1 antibodies in a large proportion of rabbits. When this phenomenon was examined by the antiserum agar technique, using the horse 46 group B meningococcal antiserum, the K1-positive colonies gave only faint halos in contrast to the dense precipitin halos yielded by the K1-negative colonies. Both K1 types agglutinated with group B meningococcal antiserum although the K1-negative organisms gave the strongest agglutinates. The group B meningococcal antiserum agar was thus advantageous for study of the reversion phenomenon.

This frequent change in K1-capsular polysaccharide phenotypes, first described by Ørskov et al. (11, 32), has been designated form variation because of its similarity to antigenic variation in *Salmonella* O antigens (19, 31). Interest in K1-form variation was heightened by our studies of neonatal *Escherichia coli* K1-disease isolates because ≅ 90% were of the K1-negative colonial type (10, 12).

Materials and Methods

Bacterial Strains. To facilitate their description and to avoid confusion among other K antigens, the designation OAc+ (O-acetyl positive) and OAc− (O-acetyl negative) will be used for the form variants previously described as K1 positive and K1 negative, respectively. *E. coli* strains, C94 (O7:K1:H−), LH (O75:K1:H3), and EC3 (O1:K1:H−) were disease isolates (12, 14). Strains D698 OAc+ and D699 OAc− (originally designated K1$^+$ and K1$^-$, respectively), were derived from one parent strain (U5/41 = O1:K1:H7). The strain labeled O16 is the test strain for O antigen 16 (F11119/41 = O16:K1:H−) (20, 31). This strain is stable in the OAc− form. Strain C375 (O132:K1:H−), is a disease isolate from a newborn with meningitis (12). Dr. Walter Goebel, The Rockefeller University, N.Y., provided *E. coli* strain K235 (O1:K1:H−) (1, 2). The *E. coli* strain (O7:K1:H,−) studied by Grados and Ewing (28) and later by Kasper et al. (29), was kindly donated by Ms. Brenda Brandt, Walter Reed Army Institute of Research. The organisms were stored as freeze-dried 10% skimmed milk suspensions.

Analytical Methods. Sialic acid was determined by the methods of Svennerholm and/or Warren, using NANA (Sigma Chemical Co., St. Louis, Mo.), as a reference standard (36, 37). The effect of neuraminidase treatment was determined using influenza virus A2/Victoria (Parke Davis & Co., Detroit, MI, lot Rx9120, CCA content 15215 U/ml) and neuraminidase (purified enzyme type V) from *Clostridium perfringens* (Sigma Chemical Co.) (38). Protein was determined by the method of Lowry (39) using bovine serum albumin as a standard. Nucleic acid was determined by the absorption of a 1.0 mg/ml solution at 260 nm assuming an extinction coefficient of 20.0. O-acetyl was determined by the colorimeteric method of Hestrin (40). Moisture content was determined at 100° C by thermal gravimetric analysis and all values were expressed as dry weight (41). Lipolysaccharide (LPS or O antigen) was assayed by *Limulus polyphemus* amebocyte lysate gelation (LAL, Associates of Cape Cod, Woods Hole, Mass. and *E. coli* endotoxin lot EC2, Bureau of Biologics, Food and Drug Administration) (42).

Polysaccharides. *E. coli* were cultivated in modified Frantz medium utilizing 5% sodium beta glycerol phosphate as a carbon source and 0.1% sterile-filtered yeast dialysate (43, 44). When the bacteria ceased to multiply logarithmically, an equal volume of 0.2% Cetavlon (Sigma Chemical Co.), was added to the culture and the resultant precipitate harvested by centrifugation. The polysaccharide was isolated by dissociation in 1.0 M CaCl$_2$, sequential precipitation

of nucleic acids and K1 polysaccharide with ethanol, removal of residual protein by cold phenol extraction and sedimentation of LPS by ultracentrifugation (43). The resultant supernate, containing K1 polysaccharide, was dialyzed extensively against deionized water at 4°C, lyophilized, and stored at −30°C. Group B meningococcal capsular polysaccharide, lot FRII, was generously donated by Dr. Emil C. Gotschlich, The Rockefeller University.

De-O-Acetylation. The K1 OAc⁺ variant D698 polysaccharide, 100 mg, was dissolved in 50 ml of 0.1 N NaOH. After heating at 37°C for 4 h, the material was neutralized with HCl, lyophilized, dialyzed against water at 4°C, and relyophilized to yield *ca* 60 mg.

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{13}C natural abundance NMR spectra were determined at 24.05 MHz (JEOL FX-100 spectrometer) and 67.89 MHz (homebuilt system) (33). Both spectrometers were equipped with quadrature phase detection. Samples were run at pH 7.0, ≅ 20°C at concentrations of ≅ 50 mg/ml. Spectral parameters were as follows: At 24.05 MHz, 12 μs (90°) pulse, 2.5 s pulse repetition time, 5 kHz spectral window with matched filter, 8 K data points with 8 points zero filling; at 67.89 MHz, 29 μs (90°) pulse, 2.5 s pulse repetition. 16 kHz spectral window with 20 kHz filter, 16 K data points with 16 K points zero filling. Before Fourier transformation, the free induction decay signals were exponentially multiplied to produce a 2.0 Hz additional line broadening. Broad band decoupling was employed in all cases, but gated to suppress the nuclear Overhauser effect enhancement for O-acetyl analysis (34, 35).

Gel Filtration. The molecular size of the K1 polysaccharides was estimated by calculation of the Kd (partition coefficient) after gel filtration through Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J., lot 3325) using 0.2 M ammonium acetate as eluent. The columns were calibrated by Blue Dextran (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc.) and [^{14}C]sodium acetate (Amersham Corp., Arlington Heights, Ill.) (41, 44).

Serological Methods. Antiserum agar was prepared with 10% horse 46 group B meningococcal antiserum in 1.0% agarose (Sigma Chemical Co.) and Davis Minimal Medium (Difco Laboratories, Detroit, Mich.) (14, 15, 45, 46). Halos were observed around the colonies after incubation at 37°C from 18 to 24 h and then for 4–8 h in a refrigerator. Immunodiffusion was performed in 0.9% agarose in phosphate-buffered saline (PBS), pH 7.4. Countercurrent immunoelectrophoresis (CIE), and qualitative and quantitative immunoelectrophoresis (IEP) were performed using horse 46 group B meningococcal and rabbit *E. coli* antisera (20, 47–51).

Quantitative Precipitin Analysis. Horse 46 serum was brought to 37% saturation with ammonium sulfate at 4°C and the resultant precipitate removed by centrifugation at 14,000 *g*, 4°C for 2 h. The supernate was brought to 55% saturation with ammonium sulfate and the precipitate collected by centrifugation at 14,000 *g*, 4°C for 2 h, dialyzed extensively against 0.15 M NaCl, sterile filtered through a 0.4 μm membrane (Nalge, Rochester, N. Y.) and stored at −20°C. Equal volumes (1.0 ml) of K1 polysaccharides from strains D698 (OAc⁺), D699 (OAc⁻), or O16 (OAc⁻) and the globulin were mixed and incubated at 37°C for 1 h and 3 d at 4°C. The precipitates were isolated by centrifugation and washed three times with saline at 4°C (52). The precipitates were dissolved in 5.0 ml of 0.8% sodium lauryl sulfate and the antibody content calculated by assuming an extinction coefficient of 14.0 at 280 nm. Antigen and antibody remaining in the supernates were detected by CIE (47).

Comparative Immunogenicity. Rabbits and two burros were injected intravenously with formalinized organisms taken from an exponentially growing culture (19, 21). Their preimmune sera showed no precipitins with either K1-variant polysaccharides. The animals were bled 4–6 d after the last intravenous injection and their sera separated under sterile conditions and stored at −20°C. Their K1-antibody content was estimated by CIE using an endpoint titration with dilutions of the antisera reacting with group B meningococcal (OAc⁻) or D698 (OAc⁺) polysaccharides.

Results

Characterization of K1-Form Variation. Several serologically defined *E. coli* K1 strains yielded a proportion of colonies weakly agglutinated with World Health Organization (WHO) rabbit typing antisera. This lesser agglutinability of daughter colonies ranged from 1/20 to 50 colonies and was constant for a given strain. Cultivation of these weakly agglutinating colonies revealed a similar low reversion to a stronger aggluti-

nation reaction. When grown on group B meningococcal antiserum agar, strains weakly agglutinating with rabbit K1-typing antisera yielded a majority of colonies with dense halos. When a faintly haloed colony was subcultured, it was found that most of the daughter colonies yielded the faint halo type and were strongly agglutinable in the WHO rabbit K1 typing antisera. Similar results for changes in colony phenotype were obtained for the weakly agglutinated densely haloed colonies. The WHO rabbit K1 typing antisera were produced with *E. coli* D698, a strain mostly in the OAc+ form. It was not possible to illustrate this difference in halo intensity between the variants by photographs, accordingly an artist's touch up of the originals is shown in Fig. 1. Strain D699, derived from the same *E. coli* parent as D698 yielded mostly colonies with dense halos (Table I). The strain O16 yielded only dense halos even after repeated dilution and transfer of single colonies. Upon initial culture, strains C94, LH, and EC3 yielded mostly densely haloed colonies. These three K1 strains behaved similarly to D698/D699 as subcultures of single variant colonies produced cultures with a predominance of one halo type. These variants had the same O and H antigens as their parent. The reversion rates of these form variant pairs is shown in Table I. The WHO O16 test strain, O16:K1:H- (F11119/41) and strain K235 (O1:K1:H−) yielded only densely haloed colonies. Grados and Ewing strain O7:K1:H−, used for preparation of K1 capsular polysaccharide vaccine in human volunteer studies, yielded mostly densely haloed colonies (OAc−). Strain C375 yielded only faintly haloed colonies (OAc+).

Polysaccharides. Table II shows the chemical composition and characteristics of K1 polysaccharides from variant pairs derived from the same parent. All K1 preparations were composed of sialic acid with only traces of protein and nucleic acid. The molecular sizing characteristics, as determined by Kd. values were similar. The endotoxin content of the polysaccharides, used as an estimate of O-antigen contamination, ranged from 2.5% for D698 and 0.25% for D699 to <0.1% for the other K1 and group B meningococcal polysaccharides. The only detectable difference among these preparations was their O-acetyl content. K1 polysaccharide, derived from strongly agglutinable faint-haloed colonies, had O-acetyl values ranging from 2.5 to 4.3 μmol/mg as determined by the Hestrin method and from 0.2 to 0.85 O-acetyl eq/NANA as determined by ^{13}C NMR. K1 polysaccharide extracted from strain C375 that yielded only faint haloes had an O-acetyl content of 2.5 μM/mg by the Hestrin method and 0.22 O-acetyl equivalents/NANA residue. K1 polysaccharides derived from poorly agglutinated densely haloed colonial variants had little or no detectable O-acetyl. The K1 preparation from the O16 strain, stable in the OAc− form, and group B meningococcal polysaccharide had no detectable O-acetyl (20, 27). K1 polysaccharides extracted from *E. coli* strains K235 and O7:K1:NM (26, 27) had no detectable O-acetyl.

[13]*C Nuclear Magnetic Resonance Spectroscopy.* The natural abundance ^{13}C NMR spectrum of the K1 OAc− variant polysaccharide is identical to that of the group B meningococcal polysaccharide (Fig. 2). Its composition and linkage is, therefore, confirmed as an α-2-8 homopolymer of sialic acid (3, 4, 26, 27, 53). The ^{13}C-NMR spectrum on the K1 OAc+ variant exhibited a relatively more complex spectrum, similar to the naturally occurring pseudo-randomly O-acetylated group C meningococcal polysaccharides (26, 54). A resonance attributable to the $OC(O)CH_3$ carbon in the K1-positive variant was observed at $\cong$1.5 ppm to higher field of the $NHC(O)CH_3$
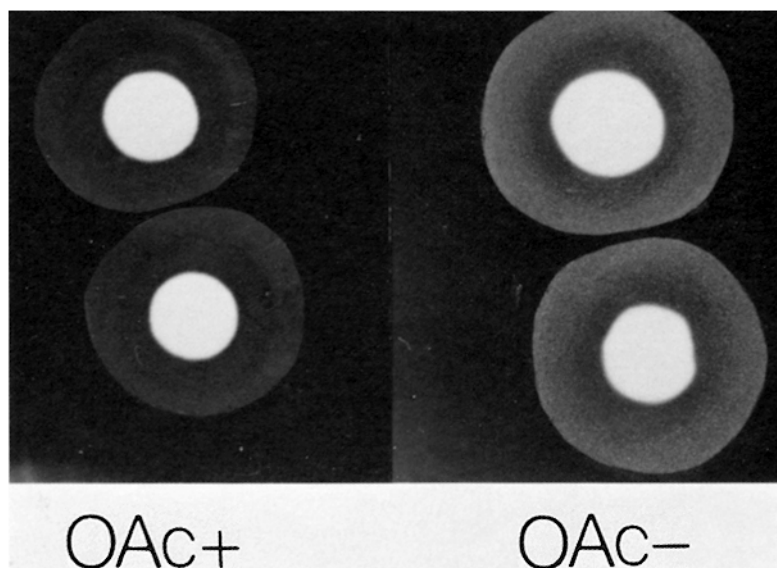
OAc+   OAc−

Fig. 1.   Halo precipitin formation of *E. coli* K1 strains cultivated on horse 46 group B meningococcal antiserum agar. *E. coli* strain D698 (OAc+) on the left and D699 (OAc−) on the right derived from the same parent, (U5/41 = O1:K1:H7), were grown for 18 h at 37°C and incubated at 8 h in a refrigerator. Representative colonies indicating the majority faint haloed from variant D698 (OAc+) and dense haloed D699 (OAc−) are shown. The halos are touched up by an artist because it was not possible to illustrate this difference with direct photographs.

TABLE I

Rate of Form Variation among Escherichia coli K1 Strains

E. coli strain	Reversion rate	No. of colonies examined
D699 K1 OAc−	1/30	5,500
D698 K1 OAc+	1/30	5,500
C94 K1 OAc−	1/40	2,847
C94 K1 OAc+	1/50	4,886
LH K1 OAc−	1/40	413
LH K1 OAc+	1/40	596
O16 K1 OAc−	None detected	5,000
C375 K1 OAc+	None detected	5,000

Single colonies were identified for their variant status by halo formation on horse 46 group B meningococcal antiserum agar. These colonies were suspended in saline, replated on antiserum agar, and halo formation examined after 24 h at 37°C. 10 colonies from each variant of the three strains and of C375 and O16 were transferred from 7 to 10 times.

resonance. After mild base hydrolysis of the K1-OAc+ polysaccharide, the ^{13}C-NMR spectrum was indistinguishable from that of the K1-OAc− polysaccharide, establishing the presence of O-acetylation as a difference between the two. The degree of O-acetylation (given as equivalents of O-acetyl per sialic acid residue) is readily established by comparative integration of the NHC(O)CH_3 and OC(O)CH_3 signals. These values are given in Table II. The polysaccharide appears equally acetylated at the C9 and C7 position, as approximately one-half of each of the C9 and C7 resonances are shifted downfield by $\cong 3$ ppm and the C8 resonance is almost entirely
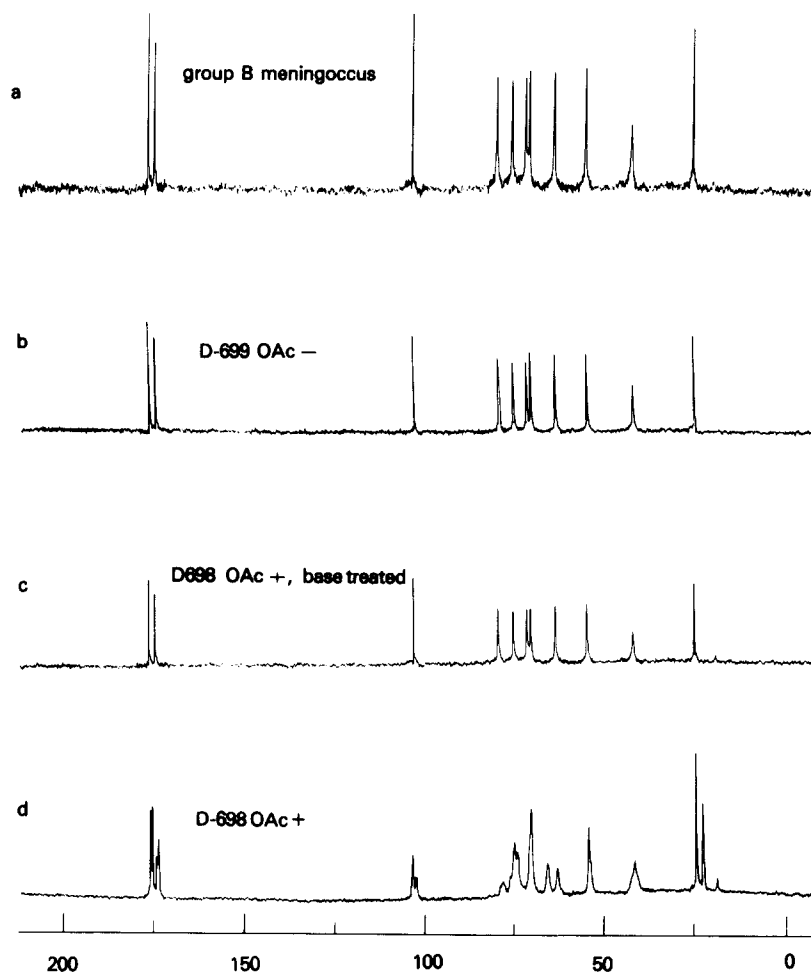
Fig. 2. High resolution ^{13}C (67.89 MHz) NMR spectra of *E. coli* K1-variant polysaccharides. A. Group B meningococal polysaccharide. B. D699 (OAc−). C. De-O-acetylated D698. D. D698 (OAc+). The spectra for the D699 (OAc−), De-O-acetylated D698 (OAc+), and group B meningococcal polysaccharides are identical. The D698 (OAc+) spectrum differs from the latter three by the presence of O-acetyl and the various spectral shifts induced by this moiety are described in Results.

shifted upfield by $\cong 3$ ppm. The downfield shifts on C9 and C7 are results of direct substitution and the upfield shift on C8 is a result of proximal substitution. The effect of C9 and C7 O-acetylation is also seen in the splitting of the anomeric carbon signal (C9 105 ppm). ^{13}C-NMR spectra of group B meningococcus and the *E. coli* K1-form variant pair D698 (OAc+) and D699 (OAc−) are given in Fig. 2. The line broadening of the K1-OAc+ polysaccharide resonances, in addition to the above mentioned resonance shifts, is best explained by heterogeneity of the distribution of the O-acetyl within the NANA polymer. A comparable heterogeneity of O-acetyl moieties in group C meningococcal polysaccharide, an α-2-9-NANA homopolymer, have been observed (26, 54).

Serologic Properties. It was not possible to prepare a homogeneous OAc+ polysac-

TABLE II

Physicochemical Characteristics of Escherichia coli K1 OAc+ and OAc— Form Variant Capsular Polysaccharides

Strain	Serotype	Variant status	Sialic acid	O-acetyl		Protein	Nucleic acid	Molecule size
				Hestrin	^{13}C-NMR (O-acetyl/ NANA)			
			% wt/wt	μM/mg		% wt/wt	% wt/wt	Kd
D698	O1:K1:H—	OAc+	80.2	2.7	0.85	0.1	0.5	0.44
D699	O1:K1:H—	OAc—	86.4	0.6	0.0	0.2	0.4	0.44
C94	O7:K1:H—	OAc+	94.0	4.3	0.31	0.3	0.4	0.39
C94	O7:K1:H—	OAc—	82.0	ND*	0.0	0.3	0.2	0.41
LH	O75:K1:H3	OAc+	90.0	3.4	0.67	0.1	0.6	0.36
LH	(prep. 2)	OAc+	85.0	2.7	0.70	0.1	ND	0.31
LH	O75:K1:H3	OAc—	76.0	ND	0.00	0.4	0.8	0.43
C375	O132:K1:H—	OAc+	92.2	2.5	0.22	0.1	0.3	0.43
O16	O16:K1:H—	OAc—	94.1	ND	0.00	0.1	0.3	0.43

E. coli K1-form variants, derived from the same parent, were selected on the basis of halo formation on antiserum agar. The O-acetyl content, determined by ^{13}C NMR, is expressed as O-acetyl equivalents per residue of NANA.

* ND, not detectable.

charide because of the observations cited above. A similar problem in obtaining homogeneous anti-K1 antibodies was encountered. Considering the 7-yr immunization with formalinized organisms and the wide prevalence of *E. coli* K1 strains, it is likely that horse 46 antisera is not an exclusive OAc— reagent (12, 19, 55). There was, however, no detectable O-acetyl in the O16 K1 and group B meningococcal polysaccharides.

The effect of increasing K1 polysaccharides D698 (OAc+), D699 (OAc—) and the stable O16 (OAc—) concentrations upon the formation of antibody precipitate from horse 46 group B meningococcal globulin is shown in Fig. 3. Maximum precipitation occurred at lower concentrations of OAc— polysaccharides. However, the D698 OAc+ polysaccharide, at about fivefold higher concentrations, precipitated a comparable amount of antibody as the two OAc— polysaccharides. At the equivalence zones for each antigen, no antibody was detectable in the supernates using both variant polysaccharides as antigens.

In a representative double-immunodiffusion experiment, horse 46 globulin was reacted with D698 (OAc+), D699 (OAc—) and group B meningococcal polysaccharides (Fig. 4). A single identity line was observed with the D699 and group B meningococcal polysaccharides. In contrast, two precipitin lines, showing identity with the OAc— polysaccharides, were observed with D698 (OAc+). Not shown are the comparable results obtained in double-immunodiffusion experiments with rabbit and the burro antisera. Both burros yielded single precipitin lines with OAc— and double precipitin lines with OAc+ polysaccharides. All lines showed an identity reaction. Some rabbits, injected with OAc+ organisms, produced antiserum reactive only with OAc+ polysaccharides. Other rabbits injected with OAc+ organisms and the rabbits injected with OAc— organisms showed the same reactivity as the two burros and horse 46 (Table III). IEP showed that the single OAc— and double OAc+ precipitin lines had identical rapid anodal mobility characteristic of K acidic capsular polysaccharides (11, 20, 48). A finer characterization of horse 46 K1 antibodies was revealed by tandem-crossed IEP (Fig. 5). A single identical precipitin line was formed
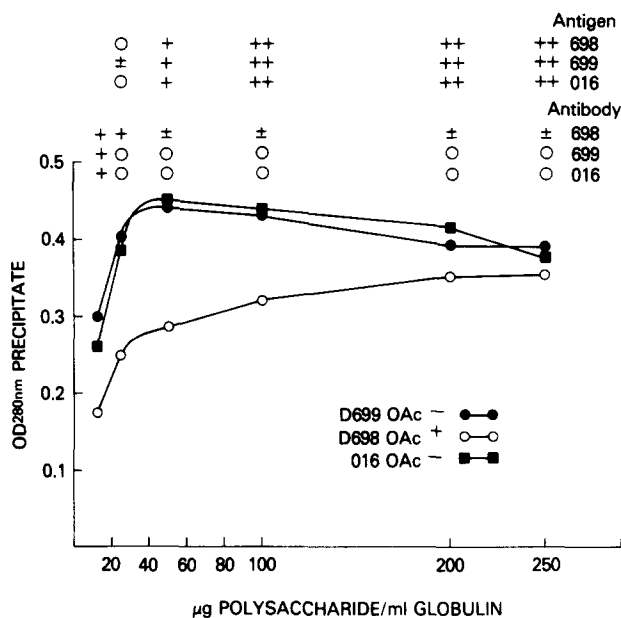
Fig. 3.   Quantitative precipitin analysis using equal volumes (2 ml) horse 46 group B meningococcal globulin and K1-variant polysaccharides of increasing concentration. The reactants were mixed, incubated at 37°C for 1 h and 3 d at 4°C. The precipitates were removed by centrifugation and the washed precipitates analysed for their antibody content (Materials and Methods). Excess antigen and antibody in the supernates were detected by CIE using horse 46 group B meningococcal globulin for the K1 antigens and the K1 antigens for excess antibody.
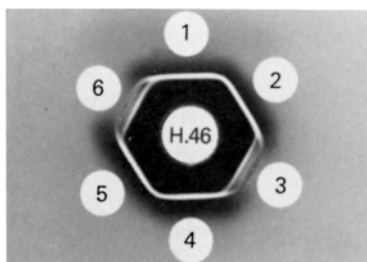

Fig. 4.   Double immunodiffusion with horse 46 group B meningococcal globulin (center well) and 0.5 mg/ml polysaccharides. Wells 1 and 4 contain group B meningococcal polysaccharide (FRII), wells 2 and 5 contain E. coli K1 D699 (OAc−), and wells 3 and 6 contain E. coli K1 D698 (OAc+).

with O16 (OAc−) and D698 (OAc+) polysaccharides. A precipitin spur extends from the O16 (OAc−) line (arrow 1) and another fine, difficult to see spur extends from the O16 (OAc−) to the D698 (OAc+) (arrow 2). Consistent with the results obtained by double immunodiffusion and IEP, an additional precipitin line was formed between horse 46 antiserum and D698 (OAc+) polysaccharide. Not shown were in situ absorptions using tandem-crossed IEP with both variant polysaccharides. All precipitin lines could be elevated and ultimately removed from the horse 46 globulin with either polysaccharide.

Fig. 6 shows the difference in precipitin patterns when K1 polysaccharides of different O-acetylation are reacted with group B meningococcal serum. D698 (OAc+), with 85% O-acetyl equivalents/NANA had two precipitin lines that were

TABLE III

Specificity and Antibody Activity of Rabbit and Burros Injected with Escherichia coli K1 Variants Derived from the Same Parent Strain

Animal number	Immunogen	K1 capsular polysaccharide			
		Group B Mening. (OAc−)		E. coli K1 D698 (OAc+)	
		Titer*	No. lines	Titer	No. lines
Rabbits					
5626	EC3 OAc−	0	—	0	—
5627	" "	10	1	5	1
5628	" "	0	—	0	—
5629	" "	1	1	0	—
5630	Ec3 OAc+	0	—	0	—
5631	" "	0	—	20	2
5632	" "	0	—	1.4	2
5633	" "	0	—	0	—
5634	C94 OAc−	10	1	3.3	2
5635	" "	5	1	0	−
5636	" "	3.3	1	1	1
5637	" "	0	—	0	−
5638	C94 OAc+	0	—	5	1
5639	" "	0	—	5	2
5640	" "	0	—	5	2
5641	" "	0	—	5	1
5642	D698 OAc+	0	—	10	1
5643	" "	0	—	0	—
5644	" "	0	—	20	1
5645	" "	0	—	0	—
5647	D699 OAc−	0	—	0	—
5648	" "	2	1	1	1
5649	" "	0	—	0	—
Burros					
B279	D698 OAc+	1	1	3.3	1
B283	D699 OAc−	10	1	3.3	1

* Reciprocal dilution yielding detectable precipitation with 0.1 mg polysaccharide/ml in CIE. Antisera were diluted in saline and assayed immediately for precipitins by CIE as described in Methods. The number of distinct lines formed by the reaction mixtures is listed.

widest apart. The two precipitin lines were closest with C94 (OAc+) with 31% O-acetyl eq/NANA and the lines were of intermediate distance with LH (OAc+) (70% O-acetyl/NANA).

Neuraminidase Sensitivity. After incubation with influenza A2 virion, rapid degradation into sialic acid monomers was observed with two K1 OAc− preparations. In contrast, the K1 OAc+ preparation showed only slight degradation with this enzyme. The degree of hydrolysis of the OAc− preparation by the influenza virion was low, owing to the inhibitory effect of the reaction product, NANA. The OAc+ preparations were also resistant to *C. perfringens* neuraminidase (Fig. 7). It was not possible to demonstrate directly NANA hydrolysis of *C. perfringens* neuraminidase, because this enzyme preparation contained 4-6% NANA aldolase activity (38). The NANA released by the *C. perfringens* enzyme was a substrate for this contaminant, and therefore, could not be detected by the colorimetric reactions. The comparative susceptibility of the K1 variants to *C. perfringens* neuraminidase was measured by two
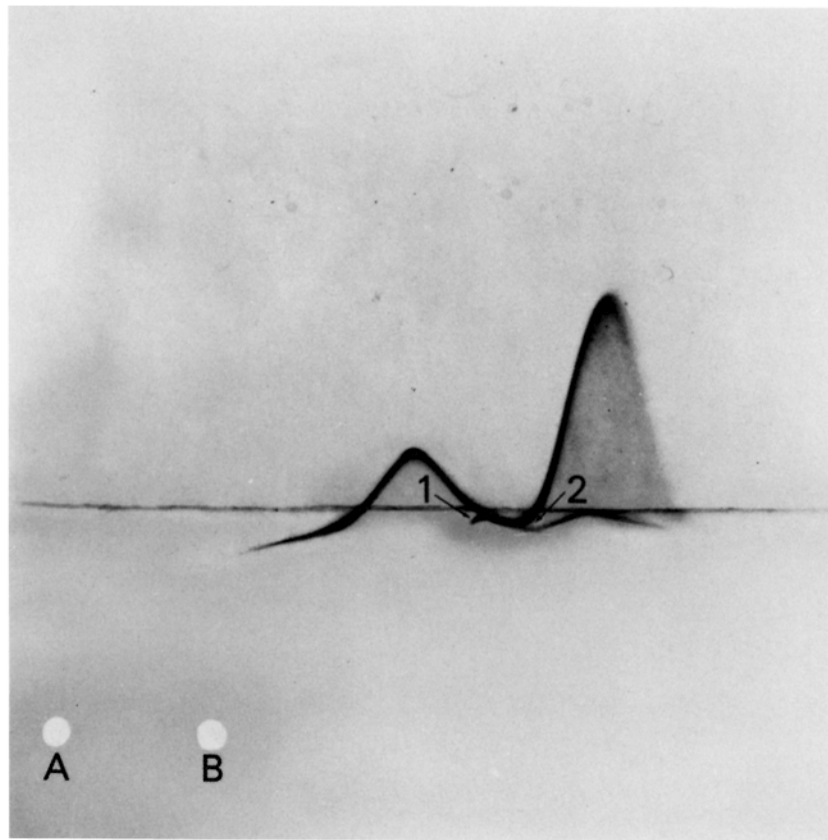
Fig. 5. Tandem-crossed immunoelectrophoresis of *E. coli* K1 capsular polysaccharides, O16 (OAc−) in well A and D698 (OAc+) in well B, were reacted against horse 46 group B meningococcal globulin. The anode is to the right in the first dimension electrophoresis and at the top in the second dimension electrophoresis.

methods. First, the unreleased or polymeric NANA was measured using acid hydrolysis of the reaction mixture (36). Fig. 7 shows that only 12% of the NANA from the OAc+ preparations were released. In contrast, 88% of the OAc− NANA were released after incubation with *C. perfringens* neuraminidase. Second, the degradation of the K1 polysaccharides was characterized by gel filtration through Sepharose 4B which revealed that only OAc− preparations were depolymerized by *C. perfringens* neuraminidase.

Comparative Immunogenicity. 23 Rabbits and 2 burros were immunized with OA+ and OA− variants derived from the same parents (Table III). The bacterial suspensions used for injection contained a small amount of the other variant due to the low, but constant, rate of reversion between the OAc+ and OAc− forms. Rabbits immunized with OAc− organisms produced antibodies reactive with OAc− polysaccharides (6/11). Four of these animals injected with OAc− organisms also produced anti-K1 OAc+ antibodies. Injection of OAc+ strains induced OAc+ antibodies in 8/12 rabbits. None of the OAc+-injected rabbits produced detectable precipitins to OAc− polysaccharides. The geometric mean titer of OAc+ antisera, raised by OAc+ organisms, was 6.58 compared to 3.86 for anti-OAc− antibodies raised by OAc−
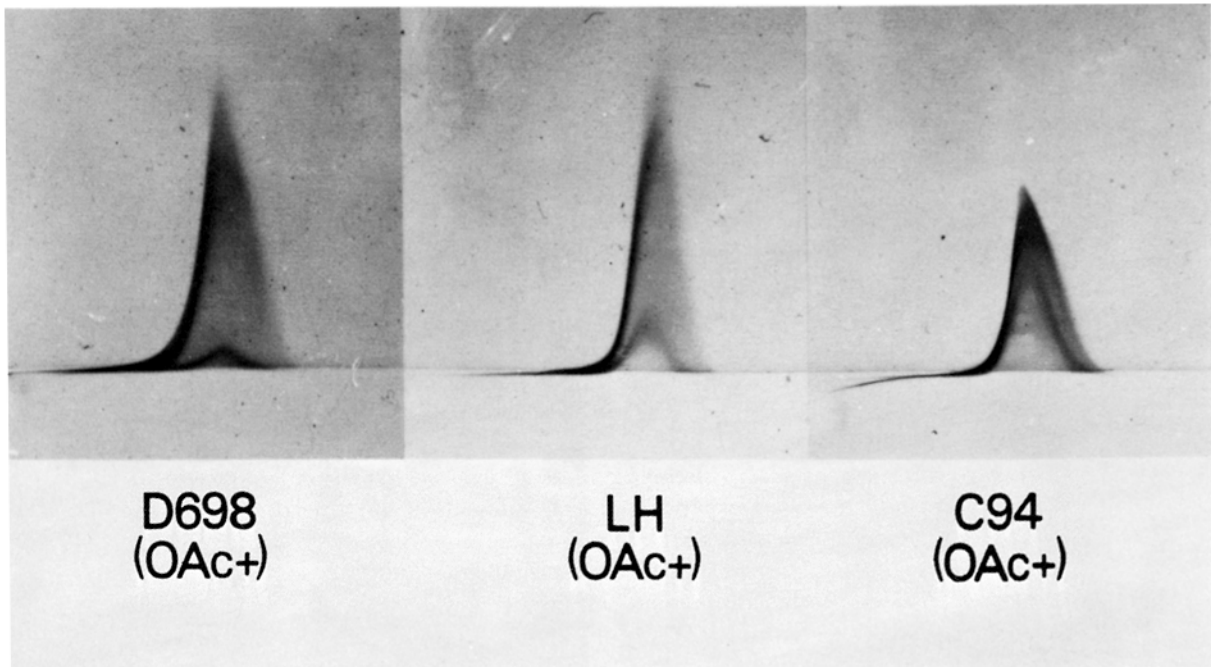
D698
(OAc+)

LH
(OAc+)

C94
(OAc+)

Fig. 6. OAc+ K1 polysaccharides prepared from various *E. coli* form variant pairs of different O-acetyl content were reacted against horse 46 group B meningococcal globulin by crossed immunoelectrophoresis. All the OAc+ K1 polysaccharides showed two precipitation lines. The distance between the two lines was related to their O-acetyl as measured by ^{13}C NMR. D698 (OAc+), with 85% O-acetyl/NANA/residue was widest apart, C94 (OAc+) with 31% was closest and LH with 70% was intermediate.
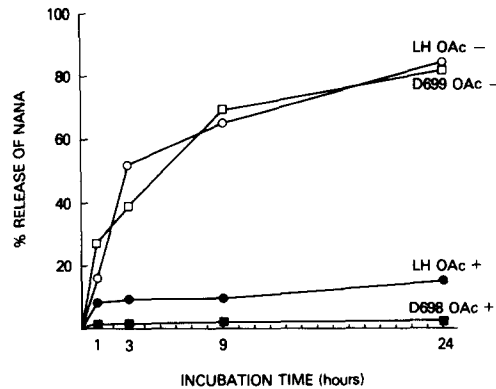

Fig. 7. Hydrolysis of *Escherichia coli* K1 form variant polysaccharides by *C. perfringens* neuraminidase. OAc+ and OAc— K1 polysaccharides were reacted with *C. perfringens* neuraminidase as described in Methods. The unreleased NANA was measured by the method of Warren (37) after acid-catalyzed hydrolysis.

organisms. Both burros made low levels of antibodies reactive with both K1-variant polysaccharides.

Discussion

Variation among bacterial structures, not mediated by usual genetic mechanisms such as mutation or the loss of chromosomal or extrachromosomal DNA, has been

described in Enterobacteriaceae (56–58). Form variation of Salmonella O antigens was first described by Kauffman as an oscillating, serologically detectable, change between a positive state in which a certain determinant is present, and a negative state in which it is absent (31). One such form variation is illustrated by antigen 12_2 of group B Salmonella (58). The term, form variation, was chosen for $E.$ $coli$ K1 polysaccharide because of its similarity to that observed in some somatic (O) antigens of Salmonella (20, 21, 31). The antigenic variation of the K1 polysaccharide is a result of random O-acetylation of C7 and C9 as determined by chemical assay, ^{13}C-NMR spectroscopy and antigenic analysis. The mechanism for this phenotypic change has not been identified but the theory that an unstable part of the DNA loop regulating biosynthesis or activity of a membrane transacetylase is related to this variation seems plausible and is subject to experimental verification (59). An antigenic specificity may be conferred by O-acetyl as has been shown for abequose of antigen 5 of group B Salmonella (56, 58).

The K1-form variants have different antigenic, immunogenic, and biochemical properties. We have no clear idea of the selective force(s) regulating this variation. The OAc+ polysaccharide resists hydrolysis by neuraminidases, hence, this variant may favor survival of K1 organisms in the intestinal tract (60). On the other hand, the OAc+ K1 polysaccharide is more immunogenic, accordingly host immune mechanisms may favor survival of OAc− organisms (16). The finding of lytic bacteriophage specific for the K1 polysaccharide provides another variable to study the relation of O-acetylation and $E.$ $coli$ physiology (64). The relation of the O-acetyl moiety to the virulence of $E.$ $coli$ K1 organisms has not been identified. The observation that most K1 isolates from CSF of newborns were of the OAc− form may be related to the practice of isolating K1 colonies from the group B meningococcal antiserum agar. The OAc− form variant yields a much more distinct and intense halo of precipitation. This property may have favored their selection for further serologic analysis. Studies involving several laboratories to study the frequency of OAc+ and OAc− variants among $E.$ $coli$ K1 isolates from healthy and diseased individuals are under way.

Serological studies show two major antigenic determinants in the K1-polysaccharide variants. One is a result of the OAc− polysaccharide. The other is probably a result of O-acetylation modifying the OAc− determinants. K1 polysaccharides derived from OAc+ strains contain at least two populations of molecules: one with O-acetylated and non-O-acetylated determinants and another, in lesser amounts, that is only OAc−. The O-acetyl itself is not an antigen. The observation that either K1 variant can remove all OAc− antibodies from horse 46 and rabbit OAc− antisera indicates that O-acetyl groups are only modifying OAc− molecules. The minor antigenic determinants, revealed by the fine spurs of precipitation in the tandem-crossed iep, may be explained by the random O-acetylation at C7 and C9. The O-acetyl moiety, does however, confer immunogenic specificity to the K1 antigens when whole bacteria are injected. Rabbits injected with K1-OAc+ strains produced mostly OAc+ antibodies. In contrast, OAc− strains induced antibodies in some rabbits reactive with both variants. The level of these OAc− antibodies was lower than the OAc+ injected rabbits. No conclusions regarding species reactivity can yet be drawn because the two burros produced cross-reactive antibodies. Studies in volunteers, using purified K1-variant polysaccharides, are planned to characterize their comparative immunogenicity and specificity to $E.$ $coli$ K1 and group B meningococci.

Three explanations for the different O-acetyl contents in the OAc+ preparations from the four *E. coli* strains can be forwarded. The first, is that the reversion rates from OAc+ to OAc— are related to their O-acetyl content. The low O-acetyl content (0.22 μM/mg) of C375 K1 polysaccharide, derived from a nonreverting OAc+ strain, rules against this possibility. The second, is that the extent of acetylation is unique for each strain. Our preliminary evidence favors this possibility. Third, is that the culture conditions, as has been shown for group C meningococcal polysaccharide (26), may influence the extent of O-acetylation for each strain. Presently, we are not able to define the extent to which each variable operates within a given strain. O-acetylation of polysaccharides, including group C meningococcal polysaccharide, another sialic homopolymer, occurs after biosynthesis of the polymeric NANA (62, 63). Further studies of this process are in progress to characterize more accurately these aspects of K1-form variation.

Form variation may have been the cause of problems in identifying *E. coli* disease isolates (22, 65, 66, 68, 69). Several authors have commented about the different serologic reactivity of strains designated K1 by conventional typing antisera. Examination of *E. coli* sent to the Central Public Health Laboratories of the United Kingdom at Colindale revealed that $\cong$50% of CSF isolates from newborns and young infants were K1-containing strains, as adjuged by halo formation on meningococcal group B antiserum agar. With rabbit *E. coli* typing antisera, an additional 22 isolates were identified as K1. The authors concluded that halo reaction with horse group B meningococcal antiserum agar was a more reliable K1-typing assay than agglutination with rabbit *E. coli* typing antisera (22). Further investigation of *E. coli* isolates using this new information regarding the K1-form variation is indicated.

The neuraminidase susceptibility of both the *E. coli* K1 and group B meningococcal capsular polysaccharides may be an important clue to their unusual immunologic behavior. These two polysaccharides, confering virulence to two bacterial species, are unusual compared to other bacterial capsules. As purified polysaccharides, both fail to induce detectable serum antibodies in most adult humans (29, 30). Even when injected as whole encapsulated bacteria, the capsules of both *E. coli* K1 and group B meningococcus are poor immunogens (20, 28, 29, 47). One obvious difference between *E. coli* K1 and meningococcus group B polysaccharide compared to other bacterial capsular polysaccharides is their susceptibility to rapid enzymatic depolymerization by neuraminidase (27). This enzyme is present as an extracellular product of several intestinal bacteria and in mammalian cells (60). Thus, the discovery of the neuraminidase-resistant K1 capsular polysaccharide may provide an effective vaccine for these two organisms. Studies using whole encapsulated bacteria suggest that the OAc+ variant may be more immunogenic. The presence of O-acetyl moieties has been related to the comparative immunogencity of other bacterial capsular polysaccharides. For pneumococcal type 1, O-acetyl is directly related to immunogenicity (67). In contrast, the O-acetyl negative group C meningococcal polysaccharide variant is more immunogenic in young adults than the more prevalent O-acetyl positive capsule (66, 68). These findings suggest that the O-acetyl moiety, per se, is not directly related to immunogenicity. Rather, the differences in overall polysaccharide structure are involved.

Summary

The chemical basis for the alternating antigenic change called form variation noted for the *Escherichia coli* K1-capsular polysaccharide has been shown by ^{13}C nuclear

magnetic resonance to be a result of random O-acetylation of C7 and C9 carbons of the α-2-8-linked sialic acid homopolymer. A serologic method (antiserum agar) was developed to identify and isolate the form variants. The O-acetyl positive and O-acetyl negative K1 polysaccharides had unique biochemical and immunologic properties. The O-acetyl-positive variants resisted neuraminidase hydrolysis in contrast to the susceptibility of the O-acetyl negative variant to this enzyme. In addition, O-acetylation altered the antigenicity of the O-acetyl polysaccharides. When injected as whole organisms, O-acetyl positive organisms produced anti-K1 antibodies in rabbits specific for this polysaccharide variant. O-acetyl negative organisms were comparatively less immunogenic; however, antibodies induced by these organisms reacted with both K1 polysaccharide variants. Burros, injected with either variant, produced antibodies reactive with both K1 polysaccharides.

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