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THE CAPSULAR POLYSACCHARIDE OF A MUCOID VARIANT OF E. COLI K12

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The isolation and properties of a polysaccharide elaborated by a mucoid variant of the colicine K-producing bacillus *E. coli* K235 were described in a previous communication.[1] This substance, named colanic acid, encapsulates the bacillus and is antigenic in rabbits. There are many accounts in the literature of mucoid forms of *E. coli* and of other Enterobacteriaceae as well. In certain instances these variants have a special encapsulating antigen which has been termed the M antigen.[2] It has been suggested by Kauffmann that this substance, regardless of the microorganism which elaborates it, is in all instances the same or very nearly so. Many investigators have reiterated this concept, yet their evidence in support of it has for the most part been fragmentary.

During the course of an investigation on bacteriophage T6, it was observed that when cultures of *E. coli* K12W were plated with an excess of virus, resistant mucoid forms of the host cell developed. We became curious as to the nature of the substance which endowed these cells with their remarkable mucoid characteristics. From that which follows, it will be seen that this material is to all intents identical with the colanic acid elaborated by the colicinogenic bacillus *E. coli* K235(m).

Materials and Methods.—Bacterial strains: Two strains of *E. coli* were used in this study. One was the mucoid variant of the colicine K-producing bacillus *E. coli* K235 L+O,[1, 3] the other a mucoid variant of *E. coli* K12W 1485 picked from a plate which had been seeded with 5×10^7 bacilli and 2×10^8 T6 phage particles, then incubated for 24 hr.

Identification of sugar components of K12 polysaccharide: A sample of the K12 polysaccharide (25 mg) was hydrolyzed in 1 *N* H_2SO_4 at 100°C for 4 hr. The hydrolysate was freed of sulfate ions, and the solution concentrated *in vacuo* (40 mg/ml); 0.005 ml containing 200 γ of hydrolysate was then chromatographed on Whatman no. 1 paper. For purposes of comparison, a similar hydrolysate of colanic acid was prepared and spotted on the same paper, together with samples of glucose,

fucose, galactose, glucuronic acid, and galacturonic acid (15 γ each). The chromatogram was developed with pyridine:ethyl acetate:acetic acid:water (5:5:1:3) for 22 hr at 23°C, using the descending technique.[4] The paper was dipped in a dilute solution of silver nitrate and sprayed with 0.5 *N* NaOH in ethyl alcohol.[5] The chromatogram was finally fixed in sodium thiosulfate solution.

Quantitative determination of sugar components: A chromatogram of each hydrolysate was made by distributing a total of 5.44 mg of the hydrolyzed material in 17 separate spots across the line of origin on a piece of Whatman no. 1 paper, 7 inches wide.[6] The chromatograms were developed as described above. The position of the various sugars was ascertained by spraying the two outside strips which had been cut from each paper. The different sugar components were now cut from the unsprayed paper, eluted quantitatively, and then brought to a known volume. The amount of sugar in each was determined by means of the cysteine reaction.[7] In the case of the uronic acid, the determination was made by means of the carbazole reaction.[8]

Antisera: Antisera to *E. coli* K235 L+O(m) and to colanic acid were prepared as previously described.[1] Similarly, an antibacterial serum to *E. coli* K12W(m) was obtained by injecting rabbits with increasing doses of acetone-killed bacilli,[9] starting with an initial intravenous injection of 0.05 mg and ending with one of 0.5 mg. Antisera to the K12 polysaccharide itself were prepared by administering to rabbits intravenous injections of a sterile solution of the purified polysaccharide in doses of 0.1, 0.2, 0.5, 1.0, 2.0, and 2.0 mg at 3-day intervals. Absorption of the sera with colanic acid or with K12 polysaccharide to remove polysaccharide antibodies was performed as described earlier.[1]

Results.—Isolation of the K12 polysaccharide: A culture of the mucoid variant of *E. coli* K12W was grown at constant pH in a nutrient medium containing only dialyzable substances.[3] The bacilli were killed with chloroform and then removed by centrifugation. The centrifugate was rapidly concentrated *in vacuo* at 18°C, thoroughly dialyzed, and the crude nondialyzable material recovered by freeze-drying. The latter, predominantly polysaccharide, but still containing some bacterial nucleoprotein, was purified by precipitation at 0°C with ethanol. The polysaccharide was finally deproteinized by shaking with chloroform-octyl alcohol (3:1).[10] After dialysis and freeze-drying, 0.7 gm of the carbohydrate was recovered from 1 liter of bacterial culture. This material contained 0.3 per cent nitrogen. Attempts to purify it further by precipitation with cetavlon[11] failed to eliminate this small amount of nitrogenous contaminant.

Properties of K12 polysaccharide: Gross chemical analyses of this polysaccharide revealed that it was very similar to, if not identical with, colanic acid. Like the latter, the K12 carbohydrate is a white amorphous fibrous substance soluble in water to yield solutions which are even more viscous than those of the colanic acid derived from *E. coli* K235. The polysaccharide itself is somewhat toxic when administered intravenously to rabbits, perhaps because it is contaminated with small quantities of bacterial endotoxin. After acid hydrolysis the K12 polysaccharide yields approximately 60 per cent of reducing sugar, calculated as glucose. The polysaccharide contains 13 per cent of a uronic acid, calculated as glucuronic acid, when analyzed by the carbazole method of Dische.[8]

Proof that the K12 polysaccharide and colanic acid are identical was obtained by means of paper chromatography and by quantitative analyses. Thus, it may be seen from Figure 1 that the hydrolysates of both polysaccharides contain galactose, glucose, fucose, and a uronic acid which proved to be glucuronic acid. The slow-moving spot shown both by colanic acid and the K12 polysaccharide (*a* in Fig. 1) was believed to be an oligosaccharide. Its nature was elucidated in the following manner. The component was eluted from a similar chromatogram,

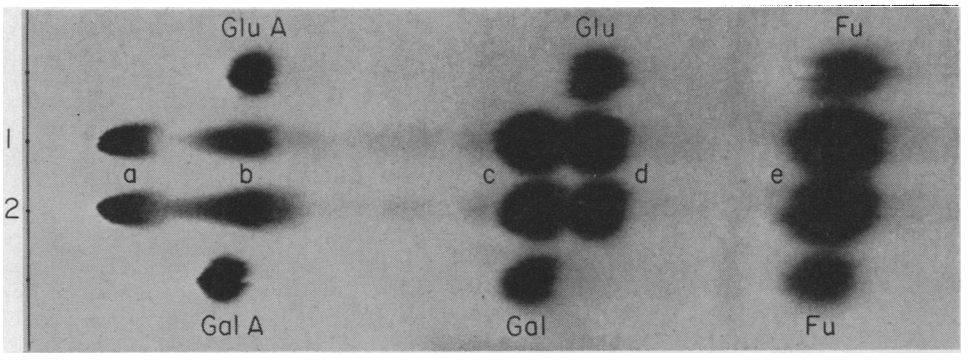

FIG. 1.—Chromatogram of sugars present in acid hydrolysates of colanic acid and polysaccharide from *E. coli* K12. (*1*) Colanic acid. (*2*) K12 polysaccharide. Glu, glucose; Gal, galactose; Fu, fucose; Glu A, glucuronic acid; Gal A, galacturonic acid.

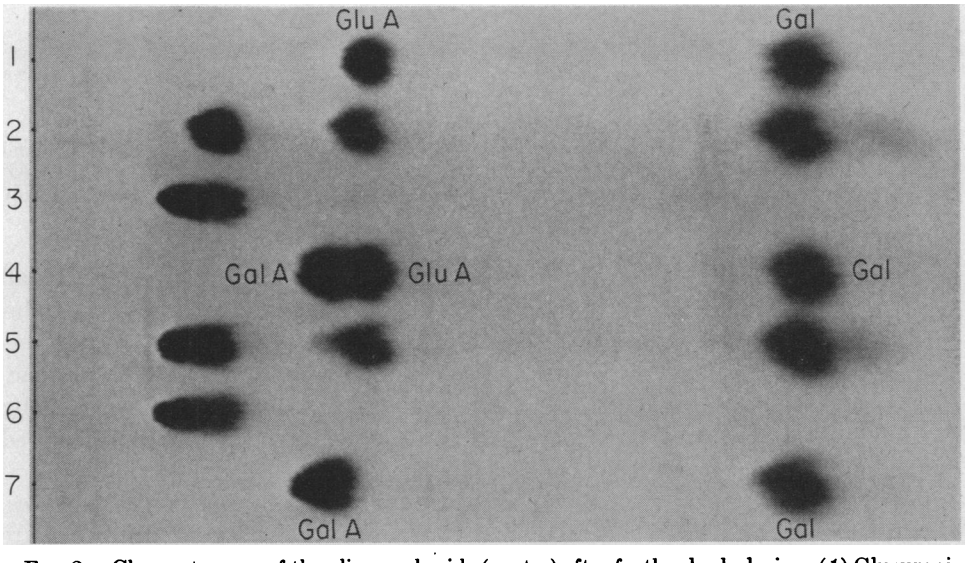

FIG. 2.—Chromatogram of the oligosaccharide (spot *a*) after further hydrolysis. (*1*) Glucuronic acid, galactose (15 γ each). (*2*) Spot *a* from colanic acid, rehydrolyzed (200 γ). (*3*) Spot *a* from colanic acid (100 γ). (*4*) Galacturonic acid, glucuronic acid, galactose (15 γ each). (*5*) Spot *a* from K12 polysaccharide, rehydrolyzed (200 γ). (*6*) Spot *a* from K12 polysaccharide (100 γ). (*7*) Galacturonic acid, galactose (15 γ each).

hydrolyzed for 4 hr at 100°C in 1 *N* H_2SO_4, and the hydrolysate rechromatographed. Figure 2 reveals that the material obtained from spot *a* now yields two sugars which migrate at the same rate as galactose and glucuronic acid. It should be noted, however, that unhydrolyzed material remains, for there is still a slow-moving spot.

In chromatograms which had been developed for only 22 hr, the uronic acids were not fully separated (*b* in Fig. 1). The uronic acid component (spot *b*) of each polysaccharide was therefore eluted from a paper chromatogram, and rechromatographed for 67 hr under the same conditions. It can be seen from Figure 3 that now the uronic acids are well separated and those present in the acid hydrolysates of the two polysaccharides are clearly identified as glucuronic acid. It should be

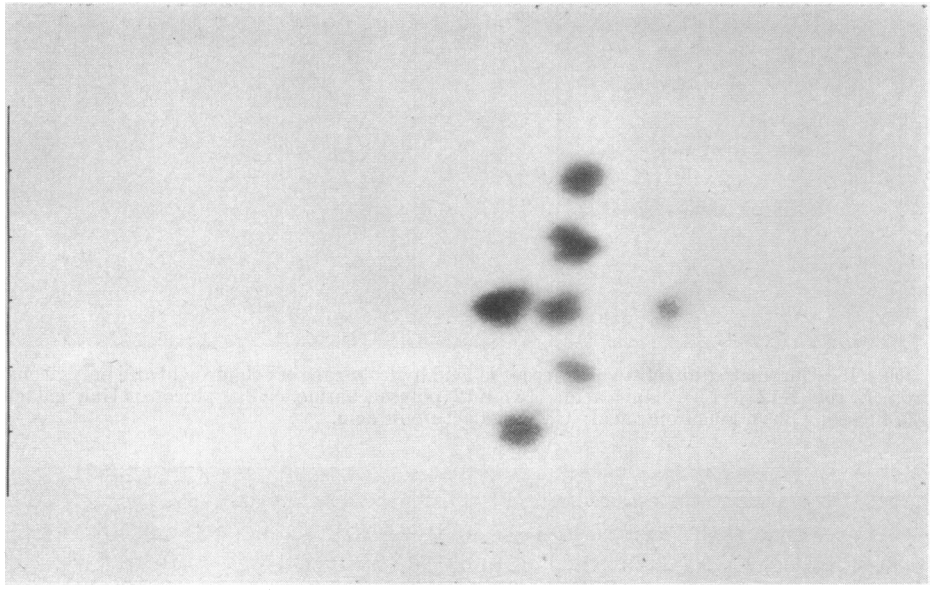

FIG. 3.—Chromatogram of the uronic acid (spot *b*) present in acid hydrolysates of colanic acid and K12 polysaccharide. (*1*) Glucuronic acid (10 γ). (*2*) Spot *b* from colanic acid (10 γ). (*3*) Galacturonic, glucuronic, and mannuronic acids (10 γ each). (*4*) Spot *b* from K12 polysaccharide (10 γ). (*5*) Galacturonic acid (10 γ).

noted, however, that the material from the K12 polysaccharide contained a small amount of an additional component having an Rf value greater than that of glucuronic acid. The nature of this substance is unknown. In regard to the percentages of the various sugars present in acid hydrolysates of the K12 polysaccharide and in colanic acid, it was stated earlier that these were determined by means of the cysteine and carbazole reactions of Dische. These values, calculated on the basis of 100 per cent recovery, are listed in Table 1.

Finally, a comparison was made of the infrared spectra of the two polysaccharides. It will be seen from Figure 4 that these are essentially indistinguishable. We are greatly indebted to Dr. Herbert Jaffe of this Institute who kindly made these measurements for us.

Immunological studies: Like colanic acid, the K12 polysaccharide is serologically active and will incite a definite immune response in rabbits. Here again our immunological experiments supported our chemical evidence that the two substances are identical. Thus, in an agar diffusion test employing an antibacterial serum to *E. coli* K235(m), or one to colanic acid itself, each polysaccharide forms but one band and these do not cross (Fig. 5). Furthermore, each polysaccharide gives a

TABLE 1

MONOSACCHARIDE COMPONENTS OF COLANIC ACID AND *E. coli* K12 POLYSACCHARIDE

Monosaccharide	Colanic acid,* %	K12 polysaccharide,* %
Glucuronic acid	17.1–19.9	16.7–18.0
Galactose	32.9–34.5	31.9–32.7
Glucose	16.2–16.9	15.5–16.8
Fucose	30.4–32.2	33.8–34.6

* The total sugars recovered by elution of the chromatograms of colanic acid and K12 polysaccharide were 82–94% and 74–75%, respectively. The figures in the columns are calculated on the basis of 100% recovery.

strong precipitin reaction in homologous antiserum and in the heterologous immune serum as well (Table 2). In both instances the antibodies can be completely removed by absorption with either the homologous or the heterologous polysaccharide, a fact which lends proof that the two are identical.

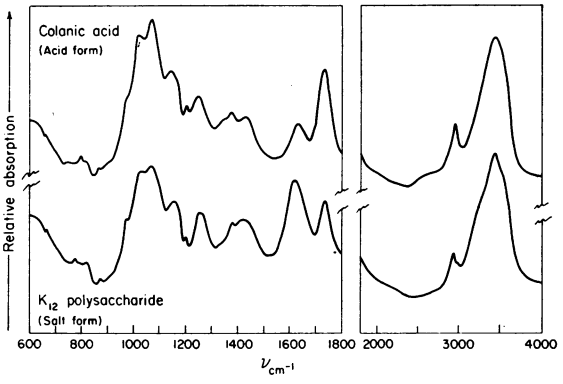

FIG. 4.—The infrared spectra of colanic acid and of K12 polysaccharide in potassium bromide at a concentration of 2 mg % by weight.

Finally, it should be pointed out that young cultures of *E. coli* K235(m) and K12(m), grown in Todd-Hewitt broth for a short interval to ensure maximum capsule formation, are both agglutinated in antibacterial sera to the two mucoid variants, as well as in antisera elicited by the polysaccharides themselves (Table 3). This agglutination cannot be attributed to the interaction of a common O antigenic component, for it is well known that such antigens are masked in encapsulated Enterobacteriaceae. Furthermore, the O antigens of *E. coli* K235(m) and K12(m) are unrelated serologically.[12]

Discussion.—The isolation of a polysaccharide from a mucoid strain of *E. coli* was first described by Dorothea Smith in 1927.[13] She showed this substance to be serologically active and attempted to relate its presence to the virulence of the *coli* strain from which it was obtained. In addition, she partially characterized two of the sugar components, glucose and a uronic acid. Some 10 years later Kauffmann described a similar polysaccharide, the so-called slime substance, from mucoid variants of *S. paratyphi* B.[14] This new antigenic material he named the M antigen, and in his book *Enterobacteriaceae*, he states that "the M antigen of Salmonella bacteria appears to be identical in all types."[2] Chemical studies revealed that the M antigen of *S. paratyphi* B was a nitrogen-free polysaccharide.[15]

The mucoid variants of a number of different *E. coli* strains were investigated by Henriksen.[16] By serological means he showed that all these strains produced the same encapsulating M antigen and that the latter was identical with the M antigen elaborated by *S. paratyphi* B. In 1957, Beiser and Davis[17] isolated several genetically different mucoid variants of a strain of *E. coli*, designated as W. They obtained the encapsulating polysaccharides of these strains and found them to be

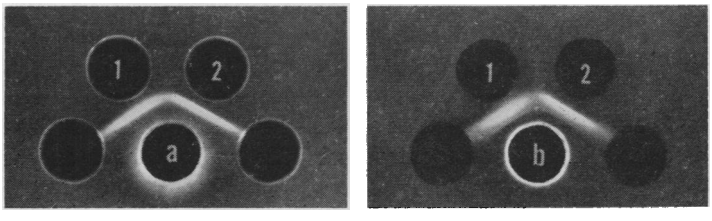

FIG. 5.—Gel precipitation reactions of colanic acid and K12 polysaccharide. (*1*) Colanic acid (0.125 mg/ml). (*2*) K12 polysaccharide (0.25 mg/ml). (*a*) Antiserum to *E. coli* K235 (mucoid). (*b*) Antiserum to colanic acid.

TABLE 2

PRECIPITIN REACTIONS OF COLANIC ACID AND K12 POLYSACCHARIDE

Antiserum	Antigen tested	Final Dilution of Test Antigen 1:10,000	1:50,000	1:250,000	1:1,250,000
Colanic acid	CA	3	3	2	1
	K12	3½	3½	3	1
K12 polysaccharide	CA	½	2	2	1
	K12	½	2½	2½	1½

CA, colanic acid; K12, K12 polysaccharide; 3, heavy disk-like precipitate, clear supernate; 2, 1, partial precipitation; ½, trace reaction.

TABLE 3

AGGLUTINATION OF *E. coli* K235 L+O(m) AND OF *E. coli* K12W(m) IN ANTIBACTERIAL SERUM AND IN POLYSACCHARIDE ANTISERUM

Antiserum	Strain of *E. coli* tested	Final Dilution of Antiserum 1:10	1:20	1:40	1:80	1:160	1:320
E. coli K235 L+O(m)	K235 L+O(m)	3	3	2½	2	1	0
	K12W(m)	4	2	1	0	0	0
E. coli K12W(m)	K235 L+O(m)	3	2½	2	1½	1	0
	K12 W(m)	4	3	2	1½	1	½
Colanic acid	K235 L+O(m)	2	2½	2	1	½	...
	K12W(m)	3	2	2	1½	½	...
K12 polysaccharide	K235 L+O(m)	2½	2	2	1	0	...
	K12W(m)	2	2	1	½	½	...

4, complete agglutination; 3, 2, 1, partial agglutination; ½, trace agglutination.

identical. Their chemical characterization of the sugar components, though incomplete, revealed the presence of fucose and galactose in acid hydrolysates of the polysaccharides, as well as an additional unidentified reducing component.

More recently, Anderson[18] has investigated the slime-wall formation of Salmonellae and other Enterobacteriaceae. From a number of strains he obtained serologically active polysaccharides which, to all intents, were identical. He showed them to contain glucose, galactose, fucose, and a uronic acid-containing saccharide which he believed to be an aldobiuronide.

Within the past months a communication has appeared by Markovitz[19] concerning the capsular polysaccharides elaborated by a number of mucoid mutants of *E. coli* K12W. He investigated the regulatory mechanisms for the synthesis of these substances and characterized in part their chemical make-up. Here again, the numerous mutants which he studied appeared to elaborate the same polysaccharide, the synthesis of which is controlled by one regulator gene R_1 and possibly by a second.

This brief summary is presented largely to focus attention on the fact that the accumulation of evidence regarding the similarities in the encapsulating polysaccharides of Salmonellae and *E. coli*, cursory though it be, points to the identity of these substances. In contrast, it should be recalled that different strains of another genus in the same family of Enterobacteriaceae, the Klebsiellae, synthesize encapsulating polysaccharides which are different in chemical composition[20] and hence in serological specificity.[21]

In the work presented here we have shown that the encapsulating polysaccharide of a phage-resistant mucoid variant of *E. coli* K12W, strain 1485, is identical with that elaborated by the colicine K-producing bacillus, *E. coli* K235. This polysaccharide, which we have named colanic acid, is constituted from galactose, fucose, glucose, and glucuronic acid in the molar ratio 2:2:1:1, respectively. It is anti-

genic in experimental animals and confers upon the bacterial cell certain of its serological properties. Colanic acid must not be confused with the K antigens described by Kauffmann[22] and recently characterized by Ørskov and her coworkers.[23] These, too, contribute to the serological specificity of the bacilli which elaborate them, but, unlike the M antigen, differ from strain to strain and exhibit diverse immunological specificities.

In the light of our investigations and those of others, it is our belief that it is none other than colanic acid which constitutes the M antigen of mucoid variants of *E. coli* and the Salmonellae. It must be remembered, however, that the validity of our suggestion remains to be substantiated by yet more extensive and painstaking work.

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