COLOMINIC ACID, A POLYMER OF N-ACETYLNEURAMINIC ACID*

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Nitrogenous monosaccharides have frequently been found in the cell walls of microorganisms as well as in the capsular materials which surround them. The cell walls of enteric bacilli have been particularly well studied; they have been shown to contain, among other things, complex polysaccharides which are constituted from amino sugars and non-nitrogenous monosaccharides combined with lipide and protein (1). The chemical composition of the encapsulating substances of bacteria are as a rule less complex. For example, certain of the capsular polysaccharides of pneumococci are constituted from an amino sugar combined with other monosaccharides or with hexuronic acids (2). In general, it may be said that glucosamine and galactosamine as well as their *N*-acetyl derivatives are frequently encountered in polysaccharides of bacterial origin.

In recent years still other and hitherto unknown amino saccharides have been found in bacteria. Thus, in 1954, the isolation of an acidic hexosamine termed muramic acid from bacterial spores was reported, (3) and its detection in the cell walls of other bacterial species soon followed (4). Muramic acid was characterized provisionally by Strange as 3-O-α-carboxyethylhexosamine (5) and was later synthesized by Kent (6).

During the past several years chemical investigations of mammalian mucopolysaccharides have led to the discovery of a new group of acidic aminosaccharides known by various names such as gynaminic, lactaminic, neuraminic, and sialic acid. These substances are all related, and they are now termed "the sialic acids." (7). Until a recent communication from this laboratory (8) there was no evidence to indicate that the sialic acids were present in polysaccharides of bacterial origin. In this report a substance was described which was obtained from a strain of *Escherichia coli* known as $K_{235}L_{+}O$ and was termed colominic acid. Colominic acid was shown to be a macromolecular substance which appeared to be related to the sialic acids for it gave certain typical color tests (7).

From the work which is to be reported here it will be seen that colominic acid is related to the sialic acids, for it proves to be a polymer of *N*-acetylneuraminic acid, or O-sialic acid. Thus, a new and unique nitrogenous polysaccharide has for the first time been shown to be present in bacterial cells.

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Materials

Strains of Microorganisms: In the following experiments a strain of *Escherichia coli* known as K_{235} (10) was employed. Three variants of this microorganism have been obtained in this laboratory (11) of which two were used in the present study, *E. coli* K_{235} L_+O and *E. coli* K_{235} L_-O. These two strains differ in that the first (K_{235} L_+O) is not agglutinatable in homologous antiserum, whereas the second (K_{235} L_-O) is.

Culture Medium.—150 gm. of Difco (technical) casamino acids was dissolved in 1 liter of tap water. The solution was dialyzed in cellophane tubing against 15 liters of tap water for 3 days at 4°C. 18 gm. of Na_2HPO_4 and 7.5 gm. of KH_2PO_4 were added to the dialysate and the pH of the solution adjusted to 7.0 by the addition of 1 N NaOH (about 5 ml.). The solution was filtered and then autoclaved in a 5 gallon pyrex bottle for 1 hour at 10 pounds' pressure. Prior to seeding with the appropriate culture, 450 ml. of a sterile solution of 50 per cent glucose, and the filtered, sterile, dialysate of 75 gm. of Difco yeast extract were added.

Chemical and Physical Methods

Electrophoresis.—Electrophoretic analyses of colominic acid preparations were carried out in borate buffer at pH 9.2 and 0.1 ionic strength at 0.4°C. in the Tiselius apparatus, using the schlieren scanning method of Longsworth (12). A potential gradient of 7.0 v/cm.^{2} was employed. Mobilities were calculated from the descending patterns by the method of Tiselius and Kabat (13).

Analytical Methods

Nitrogen analyses were performed by the method of Koch and McMeekin (14), phosphorus was estimated by the procedure of Allen (15), and protein analyses were made by the modified procedure of Folin and Ciocalteu (16). Carbohydrate was determined by a modified anthrone method (17) and sialic acid by the modified Ehrlich procedure of Werner and Odin (9). A modified orcinol procedure (18) was used for the estimation of sialic acid in which samples were heated for 3 minutes at 100°C. Ammonia and amino acids were determined with ninhydrin using the method of Moore and Stein (19). Glucosamine was determined by the procedure of Sörensen (20) and reducing groups were determined by the ferricyanide method of Schales (21). Pentoses (22) and hexuronic acids (23) were ascertained by the methods of Dische.

Serology.—Antibacterial sera were obtained from rabbits which had been injected intradermally with 0.2 ml. of a slant washing of living *E. coli* K_{235} L_+O or L_-O microorganisms. One week later three intravenous injections of a saline suspension of living bacteria containing 10^8 B/ml. were made on consecutive days. After a rest of 1 week three more injections were given; at this time the concentration of bacilli was 10^9 B/ml. The animals were bled on the 7th day after the last injection.

The bacterial suspensions used in the agglutination tests were obtained from an 18 hour broth culture of the microorganisms. The bacteria were collected by centrifugation, washed in saline, and resedimented. The cells were finally suspended in saline to the desired concentration. Agglutination tests were performed by mixing equal volumes of the serum dilutions and the bacterial suspension. The tests were incubated for 2 hours at 37°C. and after standing overnight at 4°C. readings were made.

EXPERIMENTAL

Preparation of Colominic Acid.—Fifteen liters of culture medium was seeded with 5×10^4 cells of *E. coli* K_{235} L_+O growing in the logarithmic phase. The medium was maintained at

37°C. and at pH 7.0 by means of an electronic pH control device (11). The culture was aerated at the rate of 4 liters per minute. Eighteen hours later, when growth of the organisms had ceased, the bottle was removed and 100 ml. of chloroform was added. The bacterial suspension was stirred for 5 minutes and then clarified in a Sharples centrifuge. The bacteria-free supernatant, containing colominic acid and the O antigen of the microorganism, was concentrated *in vacuo* (11 to 12 mm.) to 1 liter. The bath temperature was maintained at 75°C.

The concentrated medium was now dialyzed against distilled water for 24 hours at 4°C. The solution was then filtered, concentrated to 500 ml. *in vacuo*, and redialyzed. The solution was again concentrated *in vacuo*, redialyzed, and lyophilized. The residue was comprised primarily of the O antigen of the parent microorganism and some 25 per cent of colominic acid. Further purification was achieved by precipitating an aqueous solution of the crude substance with ethanol at 0°C.

A 3 per cent solution of the substance in 0.02 M sodium acetate was adjusted to pH 4.0 and absolute ethanol (−10°C.) was added to a concentration of 75 per cent. The solution was stored at −20°C. for 2 hours. The precipitate, containing a mixture of the O antigen and colominic acid, was collected by centrifugation at −10°C. The yellow pigmented supernate was discarded. The precipitate was dissolved in 100 ml. of distilled water and the solution dialyzed at 4°C. for 24 hours. After concentrating the solution *in vacuo*, the residue was recovered by lyophilization 5 gm. of material was obtained.

The separation of colominic acid from the O antigen of *E. coli* K_{235} $L_{+}O$ was accomplished by precipitating the latter with ammonium sulfate. Thus, a 3 per cent solution of the material was stirred at room temperature with sufficient solid ammonium sulfate to bring the concentration to 90 per cent saturation. After standing for 1 hour the solution was centrifuged and the supernate, containing colominic acid, was decanted. The precipitate of O antigen was redissolved in water and reprecipitated with ammonium sulfate. The combined supernates, containing the colominic acid, were now filtered and acidified with concentrated hydrochloric acid to incipient turbidity (0.5 ml.). The initial precipitate containing protein and colominic acid was collected by filtration (Fr. A.). Additional acid was added to the filtrate (2 ml.) until no more colominic acid precipitated and the solution was placed at 4°C. for 2 hours. The precipitated colominic acid is sticky and adheres to the walls of the flask. The solution was therefore decanted through a fine sintered glass filter and the precipitate then dissolved in 50 ml. distilled water. Two ml. of 1 N hydrochloric acid was added and the solution dialyzed until free of chloride and sulfate ions. Traces of salt were removed by electrodialysis. After concentrating the solution to 30 ml. *in vacuo*, the material was filtered and lyophilized. 1.0 gm. of pure colominic acid was recovered (Fr. B).

The acidified ammonium sulfate–containing supernate, from which the colominic acid had been removed, was also dialyzed against distilled water until free of sulfate and chloride ions. This solution was concentrated *in vacuo* and lyophilized (Fr. C).

The chemical analyses of the various fractions obtained are recorded in Table I where it can be seen that approximately half of the colominic acid originally present in the crude material could be recovered in purified form. It is of interest to note that Fr. A contained a considerable amount of protein as determined by the Folin-Ciocalteu reaction. On the other hand, Fr. C. contained a carbohydrate, other than colominic acid, as determined by the anthrone test. The fraction precipitated by 90 per cent saturated sulfate contained all of the O antigen and a portion of the colominic acid. The latter appears to be bound to the O antigen complex (17) because repeated reprecipitations with

ammonium sulfate fail to release additional colominic acid. The latter can be separated by employing the so called "lower phase" solvent technique (17). Further purification of the colominic acid obtained from this split material is

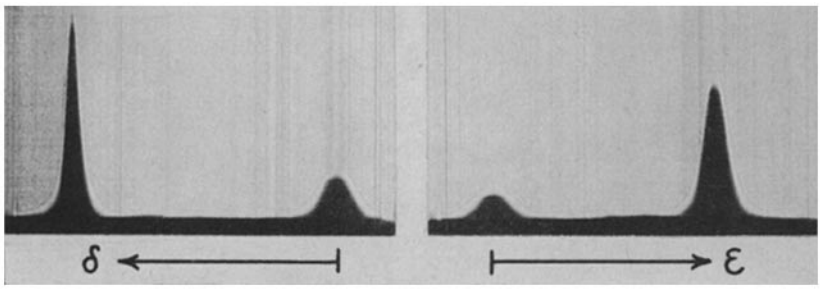

FIG. 1. Electrophoretic pattern of a 1.0 per cent solution of colominic acid in sodium borate buffer of pH 9.2 and 0.1 ionic strength. Patterns were recorded after 4800 seconds at a potential gradient of 7.0 volts/cm.

TABLE I

Analyses of Purified Colominic Acid and of Fractions Obtained during Its Purification

Substance analyzed	Fraction wt.	N	P	Absorbance/mg.			Total absorbance in Ehrlich test. Fraction wt. × absorbance/mg.	Total colominic acid recovered
				650 mμ Folin-Ciocalteu	620 mμ Anthrone	530 mμ Ehrlich		
	gm.	*per cent*	*per cent*					*per cent*
Crude residue	6.5	—	—	—	—	0.321	2,140	100.0
Ppt. 75 per cent ethanol	5.1	5.0	1.10	0.492	2.79	0.412	2,100	98.0
Ppt. 90 per cent ammonium sulfate	3.5	5.3	1.80	0.700	3.55	0.208	730	34.0
Fr. A-1st acid ppt.	0.06	8.1	0.36	0.288	0.512	0.692	41	1.9
Fr. B-2nd acid ppt.	1.30	4.65	0.07	0.038	0.102	0.870	1,130	53.0
Fr. C-supernate	0.07	5.65	0.93	0.289	3.045	0.395	27	1.3
Fr. B-electrodialyzed	1.10	4.35	0.00	0.021	0.078	0.980	1,002	47.0

difficult, however, because of the presence of large amounts of carbohydrate derived from the O antigen.

Electrophoretic Analysis of Colominic Acid.—A 1 per cent solution of colominic acid in 0.1 M sodium borate buffer at pH 9.2, when subjected to electrophoretic analysis, showed but a single peak (Fig. 1). The mobility of the acid was calculated to be -11.1×10^{-5} cm.^{2}/volt sec.

Properties of Colominic Acid.—Purified colominic acid is a white amorphous powder which is soluble in water. Aqueous solutions are not viscous but they are

markedly acidic (pH 3.7). Such solutions do not show a characteristic absorption band in the ultraviolet, but upon boiling in 0.1 N alkali, an absorption band appears which has a maximum at 260 mμ. Humin is readily formed when colominic acid is heated with dilute mineral acids. Purified colominic acid gives only feeble anthrone and Folin-Ciocalteu tests. Tests for hexosamines, pentoses, and hexuronic acids are entirely negative. When heated with Erhlich's reagent, colominic acid gives a red color which has an absorption maximum at 530 mμ. A reddish purple color is produced when the substance is heated with Bial's orcinol reagent, with an absorption maximum at 540 mμ. Colominic acid is neither toxic nor antigenic when injected into rabbits nor does it precipitate in the sera of rabbits which have been immunized with *E. coli* $K_{236}L_{+}O$.

Chemical Analysis of Colominic Acid.—Analyses for carbon, hydrogen, nitrogen, and acetyl were performed on several preparations of purified colominic acid dried to constant weight over phosphorous pentoxide at various temperatures. It was observed that as the drying temperature was raised there was a slight increase in the amount of water lost. Thus, a sample which had been dried at 25°C. lost 5.69 per cent of its weight, and upon drying at 100°C. a loss of 7.58 per cent was incurred. In addition, there was some discolorization of the material. Drying at 78°C. appeared best, for at this temperature no discoloration occurred. The following is a typical analysis of colominic acid:—

	C	H	N	CH_3CO
Colominic acid found	46.08	5.87	4.35	14.82
$C_{12}H_{19}NO_9$ calculated	44.86	5.92	4.36	13.39
$C_{11}H_{19}NO_9$ calculated	42.72	6.19	4.53	13.92

From the analytical date it appears that the formula $C_{12}H_{19}NO_9$ represents most closely the monomer unit of colominic acid. The monomer is believed to contain one acetyl group. Alkalimetric titrations of colominic acid gave an equivalent weight of 324. The calculated value for $C_{11}H_{18}O_7$ COOH is 321. From this it would appear that the monomer of colominic acid contains one carboxyl group. Methoxyl groups, sulphur, and phosphorus are absent.

The optical rotation of a 5 per cent aqueous solution of colominic acid, when measured in a 2 decimeter tube, gave the value $[\alpha]_D^{20^\circ} = -50.5^\circ \pm 2^\circ$. An infrared spectrum of colominic acid was taken in a potassium bromide pellet using 1 mg. of the acid mixed with 250 mg. of the salt. The spectrum is recorded in Fig. 4 A.

Hydrolysis of Colominic Acid.—Colominic acid has weak reducing properties when tested with alkaline ferricyanide (21). This may be due to the presence of a free-reducing group on one end of the polymer chain. Different preparations of colominic showed variations in this respect, a fact which suggests some

heterogenicity in molecular size. Upon heating colominic acid in aqueous buffered solutions between pH 1.0 and 5.0, hydrolysis occurs and the amount may be estimated by measuring the increase in reducing value. The formation of reducing groups occurs at a temperature as low as 37°C., but hydrolysis is accelerated at higher temperatures. When colominic acid is heated in buffered solutions at pH 6 or higher there is no release of reducing groups. In order to ascertain the optimum conditions for the hydrolysis of colominic acid a study of this reaction was made at various temperatures and pH values. It was observed that optimum results were obtained by heating aqueous solutions of colominic acid at 100°C. for 90 minutes. In Fig. 2 is shown a hydrolysis curve of the acid under these conditions. Here it may be seen that a rapid initial liberation of reducing groups occurs which soon levels off.

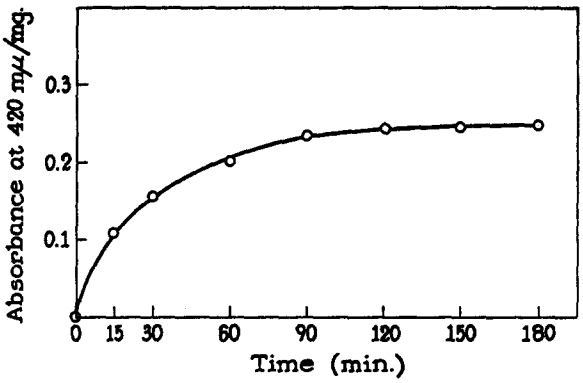

FIG. 2. The increase in ferricyanide consumption of a 1 per cent solution of colominic acid heated to 100°C.

A standard curve of ferricyanide consumption *versus* glucose concentration was constructed and this curve was employed to determine the degree of hydrolysis of colominic acid. The value obtained from the standard curve was then multiplied by the ratio of the molecular weight of the monomer unit of colominic acid to that of glucose. It should be realized that this calculation is empirical and that the values obtained are probably minimal. Thus it was found that some 57 per cent of the reducing groups are liberated after heating an aqueous solution of colominic acid for 1½ hours at 100°C., and that some 63 per cent are liberated after 3 hours. There is no further increase in reduction following prolonged hydrolysis because of the formation of humin.

Products of Hydrolysis of Colominic Acid.—In view of the fact that a satisfactory procedure for the hydrolysis of colominic acid had been devised, a study was undertaken to identify the products of hydrolysis. The latter were separated by means of ion exchange chromatography (24).

A solution of 812 mg. of purified colominic acid in 20 ml. of distilled water was heated for 1½ hours in a boiling water bath. The hydrolysate was dialyzed at 4°C. against two portions of distilled water (200 ml.) for 24 hours, combined, and concentrated *in vacuo* to 10 ml. The solution was decolorized with charcoal (darco-60), filtered, and lyophilized. 595 mg. of an amorphous white powder was obtained. The non-dialyzable material was also isolated by freeze-drying. 7.1 mg. of a gray material was recovered. Thus a total of 602 mg. of substance was recovered which represented 75 per cent of the starting material.

The dialyzable hydrolysate was now fractionated on a Dowex 1-×8 column. The resin, which was purchased in the chloride form, was first converted to the formate (25). A column 3.5 × 14.5 cm. was prepared and 500 mg. of the lyophilized hydrolysate, dissolved in 5 ml. of water, was placed upon it. After the sample had been adsorbed it was washed with 100 ml. of water. The washings were collected in 5 ml. portions by means of a Technicon fraction collector. The column was now eluted with dilute formic acid by the gradient elution technique. A 500 ml. Erlenmeyer flask containing water served as the mixing vessel. To this was gradually added 0.8 N formic acid and again 5 ml. fractions of the eluate were collected. In all some 500 fractions were obtained and analyses were made upon samples selected throughout the series for carbohydrate, amino acids, and ammonia. In addition, analyses were performed for sialic acid, using both the Ehrlich and orcinol reagents. Acidity titrations were also made with standard sodium hydroxide solution.

The analyses of a typical chromatographic separation are plotted in Fig. 3. Here it will be noted that three distinct fractions emerged from the column. The first appeared at the beginning of the experiment and contained ammonia, which could be detected by boiling an aliquot of the peak fraction (tube 12) with alkali. When the contents of this tube were made alkaline with 1 N NaOH and evaporated to dryness *in vacuo* the residue gave no ninhydrin test for amino acids. The curve of the second fraction to emerge from the column was symmetrical in shape and gave intense Ehrlich and Bial color reactions. It should be noted that the curves obtained by these two analytical procedures were superimposable, a fact which suggested that only a single substance was present. The third band to emerge could also be detected by the Ehrlich and orcinol reactions. It is of interest that no substances were eluted which reacted solely with the anthrone reagent.

The eluent fractions numbered 218 to 252 (inclusive) were combined and concentrated *in vacuo* to 10 ml. The solution was filtered and lyophilized. 262.4 mg. of substance (Fr. 1) was recovered which represented a 52.5 per cent yield. The material present in fractions 410 to 458 (inclusive) was also isolated. The weight of this fraction (Fr. 2) was 9.7 mg., which represented a yield of 1.9 per cent. Thus, 55 per cent of the material originally placed upon the column was recovered by the gradient elution technique.

Crystallization of Fr. 1.—170 mg. of Fr. 1 was dissolved in 0.6 ml. of water and to this was added 9 ml. of 95 per cent ethanol. Ethyl ether was then added until a faint turbidity developed (approximately 9 ml.). After standing for 24 hours at 0°C., an amorphous precipitate separated which was collected by filtration. Petroleum ether was now added until the solution became turbid (2.6 ml.). After again standing at 0°C. for 5 hours, the clear supernate was removed and 6.0 ml. more of petroleum ether was added. After standing at 0°C. overnight, the heavy precipitate which formed was collected on a scintered glass filter and

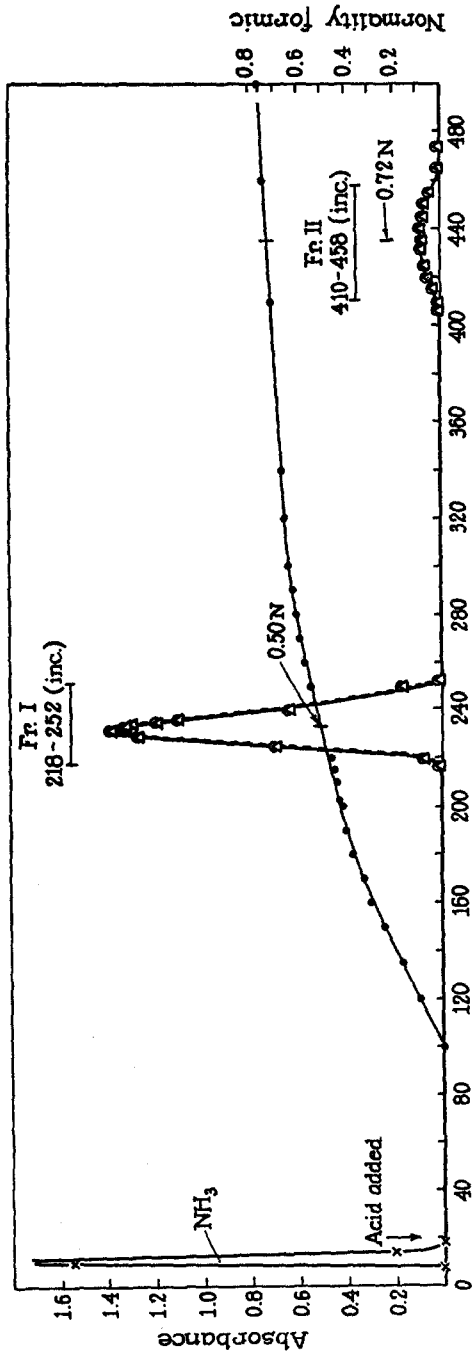

FIG. 3. Elution curve of adsorbed colominic acid hydrolysate from Dowex 1-X8 resin column with formic acid concentration, ●; Ninhydrin, X; Ehrlich at 530 mμ O; orcinol at 540 mμ, Δ.

washed with 5 ml. of petroleum ether. 45 mg. of substance was recovered. This material, which crystallized in the form of small needles, represented a recovery of 26.5 per cent.

Properties of crystalline Fr. 1.—Crystalline Fr. 1 is freely soluble in water and gives acidic solutions. Humin is readily formed when Fr. 1 is heated in dilute mineral acid. When heated with Ehrlich's reagent Fr. 1 gives a red color with an absorption maximum at 530 mμ. Upon heating with Bial's orcinol reagent a reddish purple color is formed, with an absorption maximum at 540 mμ.

Fr. 1 was dried to constant weight at 78°C. and the following is a typical analysis.

	C	H	N	CH_3CO
Fr. 1 found	42.55	6.31	4.45	14.00
$C_{11}H_{19}NO_9$ calculated	42.74	6.19	4.53	13.92
$C_{12}H_{19}NO_9$ calculated	44.86	5.92	4.36	13.39

From the data it can be seen that the formula $C_{11}H_{19}NO_9$ represents most closely the composition of the crystalline material.

The substance does not have a sharp melting point for it chars at 182 to 186°C. Titration of the substance with standard alkali gave a neutral equivalent of 307; this value is in agreement with the formula $C_{11}H_{19}NO_9$. The optical rotation of the substance, when measured at a concentration of 1.0 per cent in a 1 decimeter tube, gave the value $[\alpha]_D^{24°} = -35.0° \pm 2.0°$.

Partition chromatography of Fr. 1 on paper was performed as follows.

A 100 μg. of the material was placed upon a washed Whatman No. 1 filter paper and the chromatogram was developed at 37°C. for 16 hours by the descending technique using the solvent system secondary butanol-water-acetic acid (4:5:1). The chromatogram was now dried, sprayed with Ehrlich's reagent, and after drying a second time it was heated at 105°C. for 5 minutes. A pinkish spot appeared which revealed the position of the substance; its R_f value was calculated to be 0.65 at 27°C.

A second method was employed for the detection of the substance on the paper. In this case the chromatogram was first sprayed with 50 per cent hydrochloric acid and then dried. The paper was now exposed to the vapors of hydrochloric acid for 3 hours at 60°C. A brown spot appeared at the location of the material due to its decomposition and the formation of humin.

The analysis of crystalline Fr. 1 is recorded in Table II and its infrared spectrum, taken in potassium bromide, is given in Fig. 4 A. The measurements were made by Dr. H. Jaffe to whom the author is indebted.

From the analytical data it is apparent that Fr. 1 is identical with *N*-acetylneuraminic acid. A small amount of the latter was kindly given us by Dr. Gunnar Blix, Kungliga Universitetet I Uppsala, Sweden, and a comparison was made of the properties of the two materials. The decomposition temperature of an authentic sample of *N*-acetylneuraminic acid and its R_f value in the sol-

vent system described were determined. The results are recorded in Table II. In addition the infrared spectrum of *N*-acetylneuraminic acid was taken in a potassium bromide pellet (Fig. 4 B).

From the values recorded in Table II it is evident that the crystalline material obtained from the hydrolysate of colominic acid is identical in all respects with *N*-acetylneuraminic acid (26). The slight discrepancy in the specific optical rotation is within the error of measurement. The identity of the two materials is further substantiated by the fact that their infrared absorption spectra (Fig. 4 C and 4 B) are also identical.

From the data which has been presented it seems justifiable to conclude that colominic acid must be regarded as being constituted, for the most part at least, of units of *N*-acetylneuraminic acid. This concept is substantiated by the

TABLE II

Comparison of the Properties of Fraction 1 and of N-Acetylneuraminic acid

	N-Acetylneuraminic acid	Crystalline Fr. 1
C, *per cent*	42.72*	42.55
H, *per cent*	6.19*	6.34
N, *per cent*	4.54*	4.45
CH_3CO, *per cent*	13.92*	14.00
$[\alpha]_D^{24°C}$	31 ± 2°*	35 ± 2°
Decomposition temperature	184–7°C.	182–6°C.
Neutral equivalent	309*	307
$R_f^{27°C}$ Sec. butanol:water:acetic acid (4:5:1)	0.65	0.65

* These values were taken from a communication of Blix (26).

fact that upon hydrolysis of colominic acid some 40 per cent of *N*-acetylneuraminic acid is obtained, a value which is probably low because of the instability of the end product. Finally, it should be pointed out that repeated analyses of purified colominic acid indicate that its carbon content is somewhat higher than would be expected were this substance poly-*N*-acetylneuraminic acid. At the moment this fact cannot be satisfactorily explained.

Properties of Fr. II.—The amount of Fr. II which was obtained upon elution of the column was small and the yield depended upon the conditions employed for the hydrolysis of colominic acid. Thus, it was found that the more drastic the conditions, the larger was the recovery of this fraction. In one experiment, in which sulfuric acid was employed for the hydrolysis, the amount of Fr. II obtained was even greater than that of Fr. I. It is believed, therefore, that Fr. II represents a degradation product of Fr. I. Although Fr. II gives tests for sialic acid, its true chemical nature has not been extensively investigated. However, its infrared spectrum, taken in a potassium bromide pellet and recorded in Fig. 4 D, indicates that it differs markedly from *N*-acetylneuraminic acid.

Attempts to Isolate Colominic Acid from E. coli K_{235} L_O.—It will be recalled that colominic acid is elaborated into the culture medium during the growth of *E. coli* K_{235} L_+O. It was of interest to determine whether the mutant, *E. coli* K_{235} L_O, derived from the L_+ strain also elaborates colominic acid. These two organisms can be distinguished by their agglutination reactions, for the viable L_+ strain does not agglutinate in homologous antisera, whereas the L_- variant does. In order to observe agglutination of the former, the microorganisms must first be heated at 100°C. for 1 hour a procedure which apparently destroys an

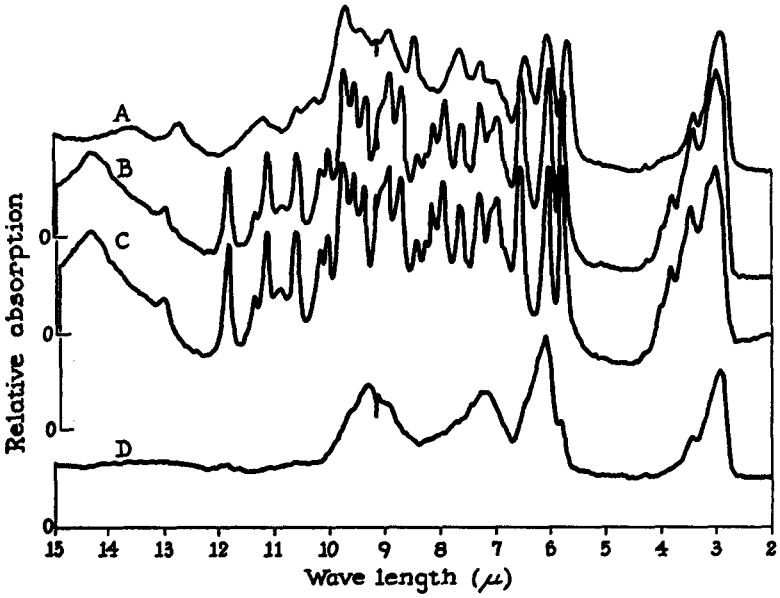

FIG. 4. Infrared spectrograms taken on 1 mg. of substance in potassium bromide pellet Curve A, colominic acid; Curve B, *N*-acetylneuraminic acid; Curve C, crystalline Fr. I; Curve D, amorphous Fr. II.

interfering substance (27). In contrast to this the L_O strain is deficient in this substance, and hence, by virtue of its O antigen, agglutinates spontaneously in homologous antiserum. Since both strains appear to have the same O antigen, their only difference resides in the presence or absence of the thermolabile interfering substance.

15 liters of culture medium was seeded with *E. coli* K_{235} L_O and the microorganisms were grown under the same conditions as previously described. The bacteria were separated from the supernatant medium and the soluble bacterial products isolated as previously described. This material was now precipitated with 75 per cent alcohol and then fractionated with ammonium sulfate. Two fractions were collected, one at 0.35 per cent, the other at 0.60 per cent saturation with ammonium sulfate. Unlike the material from the L_+O organism, there was no fraction which precipitated at 0.90 saturation. Acidification of the final am-

monium sulfate supernate with concentrated hydrochloric acid also failed to result in the precipitation of material. The two ammonium sulfate precipitates were now dissolved in water and dialyzed until free of sulfate ions. The acidified ammonium sulfate supernatant was also dialyzed. The various fractions were filtered and lyophilized. Their analyses are recorded in Table III.

It is apparent from the results presented in Table III that only a very small amount of colominic acid is elaborated into the culture medium during growth of the organism *E. coli* K_{235} L_O. If a comparison is made of the amount of colominic acid present in the various alcoholic precipitates (*cf.* Table I and III)

TABLE III

Analyses of Fractions Obtained during Fractionation of Material Elaborated by E. coli $K_{235}L\_O$

Substance analyzed	Fraction wt.	N	P	Absorbance/mg.			Total absorbance in Ehrlich test Fraction wt. × absorbance/mg.
				650 mμ Folin-Ciocalteu	620 mμ Anthrone	530 mμ Ehrlich	
	gm.	*per cent*	*per cent*				
Crude residue	4.52	—	—	—	—	0.014	63.0
Ppt. 75 per cent ethanol	2.28	5.95	2.12	1.022	4.030	0.026	59.3
Ppt. 35 per cent ammonium sulfate	0.35	6.85	1.58	1.400	3.650	0.000	0.0
Ppt. 60 per cent ammonium sulfate	1.34	4.60	1.91	0.705	4.400	0.000	0.0
Sol. 90 per cent ammonium sulfate	0.090	8.30	2.13	1.470	3.323	0.071	6.4

derived from the two strains, it is apparent that the L_+ strain elaborates some 40 times as much as does the L_ strain.

Release of Colominic Acid from E. coli K_{235} L_+O upon Heating.—It was of interest to determine whether heating L_+O bacteria would result in the release of colominic acid.

Thus the bacilli from 1 liter of culture of *E. coli* K_{235} L_+O were collected by centrifugation. The bacteria were washed and resuspended in 6 ml. of isotonic saline. Three ml. of the suspension was heated in a boiling water bath for 1 hour, the remainder was kept at room temperature to serve as a control. Both suspensions were now centrifuged, and the supernatants drawn off and analyzed for colominic acid by the Ehrlich reaction. It was found that the colominic acid present in the supernate obtained from the heated cells gave an absorbance of 0.197, whereas that from the unheated cells gave an absorbance of 0.050.

These results clearly indicate that colominic acid is released from the bacterial cells by boiling.

Immunological Properties of Colominic Acid.—In view of the fact that colominic acid appears to be involved in the agglutination reaction of *E. coli* K_{235}

L_+O it was of some interest to ascertain whether it would elicit antibodies when injected into rabbits. It was found, however, that the injection of colominic acid, either intravenously or intradermally failed to elicit any detectable immune response. Furthermore, intramuscular injections of colominic acid mixed with Freund's adjuvant were also without effect. It should also be noted that injections of as much as 10 mg. gave no gross toxic or inflammatory reactions. Finally, it should be stated that colominic acid itself does not precipitate in the antisera of rabbits injected with *E. coli* K_{235} L_+O.

Virulence of E. coli K_{235} L_+O and E. coli K_{235} L_-O in Mice.—It was of interest to determine whether the two bacterial strains *E. coli* K_{235} L_+O and L_-O showed any differences in virulence. In this regard it should be recalled that Kauffmann (27) has demonstrated that some *E. coli* strains which possess capsular substances are more virulent than those which are unencapsulated.

Mice were inoculated intraperitoneally with a 5 per cent mucin solution containing varying amounts of viable *E. coli* K_{235} L_+O or with *E. coli* K_{235} L_-O bacilli. The concentration of bacilli ranged from 10^6 organisms to 0. At the termination of the experiment the animals were observed for a 3 week interval. All animals had survived. They were then sacrificed and attempts were made to cultivate the microorganism from various organs, but without success.

DISCUSSION

Of the few nitrogen-containing homopolysaccharides which have been found in nature, the best known is chitin, a substance which is constituted solely of molecules of *N*-acetylglucosamine. There are also several heteropolysaccharides which have been extensively studied; among these are hyaluronic acid, chondroitin, and heparin. These substances contain two monosaccharides,—a hexosamine and a hexuronic acid. From the results which have been presented in this communication it appears that the enteric microorganism *E. coli* K_{235} L_+O elaborates considerable amounts of a nitrogenous polysaccharide which has been named colominic acid. This is a new and hitherto undescribed polysaccharide constituted primarily, if not solely, of units of *N*-acetylneuraminic acid. Thus, colominic acid differs markedly in its chemical composition from the serologically active carbohydrate which forms part of the O antigenic complex of *E. coli* K_{235} L_+O, for the latter is built up from units of glucose, galactose, rhamnose, fucose, and hexosamine (17).

The presence of a heat-labile anti-agglutination factor in certain strains of *E. coli* has been well substantiated (27). This factor is believed to be a substance which surrounds the microorganism, and is thought to have immunological activity. From the work which has been presented here there is no immunological evidence which would link colominic acid with the heat-labile anti-agglutination factor present in *E. coli* K_{235} L_+O. It may be coincidence that the removal of colominic acid and the appearance of O agglutinability of *E.*

coli K_{235} L_+O occur simultaneously upon heating the cells. The fact remains, however, that colominic acid is destroyed under these conditions. Furthermore, the L_-O variant contains no anti-agglutinating factor and it elaborates only minimal amounts of colominic acid. These observations strongly suggest that it is indeed the colominic acid which renders the L_+ strain inagglutinable.

In regard to the distribution of the sialic acids among bacterial species, this is at present unknown. It would seem highly unlikely, however, that *E. coli* K_{235} L_+O is the only microorganism capable of synthesizing this substance in view of the fact that the sialic acids are so widely distributed in mammalian tissue.

The chemical investigation of colominic acid and its hydrolytic products has revealed that this substance is composed largely of polymerized units of *N*-acetylneuraminic acid. No attempt has been made in the present study, however, to determine the arrangement of the *N*-acetylneuraminic acid units in the colominic acid macromolecule. Although the yield of *N*-acetylneuraminic acid is by no means quantitative, it must not be forgotten that this acid is extremely labile. The high absorbance values of colominic acid per unit of weight as determined by the Ehrlich reaction indicates strongly that it is indeed constituted solely of sialic acid-like substances.

SUMMARY

An acidic carbohydrate has been isolated from the culture medium of the enteric microorganism *E. coli* K_{235} L_+O. This substance has been named colominic acid.

N-acetylneuraminic acid, which has been isolated from the hydrolysate of colominic acid, is believed to be the monomer unit from which colominic acid is constituted.

Colominic acid is not antigenic in rabbits. Tests have failed to reveal a relationship between the ability of *E. coli* K_{235} to elaborate colominic acid and its virulence in mice.

Addendum.—After the completion of this manuscript the author was informed through a personal communication that his results have been substantiated in the laboratories of Dr. S. Roseman of the University of Michigan who employed a culture of the same microorganism *E. coli* $K_{235}L_+O$.

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