SIALIC ACIDS (*N*,7-O-DIACETYLNEURAMINIC ACID AND *N*-ACETYLNEURAMINIC ACID) IN *ESCHERICHIA COLI*

I. Isolation and Identification

CHARLES W. DEWITT[1] AND JANET A. ROWE[2]

Research Laboratories, The Upjohn Co., Kalamazoo, Michigan

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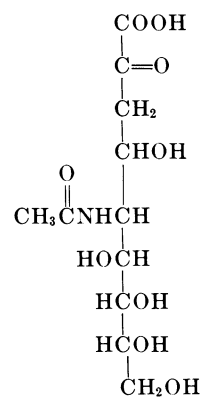
ABSTRACT

DeWitt, Charles W. (The Upjohn Co., Kalamazoo, Mich.) and Janet A. Rowe. Sialic acids (*N*,7-O-diacetylneuraminic acid and *N*-acetylneuraminic acid) in *Escherichia coli*. I. Isolation and identification. J. Bacteriol. **82:** 838–848. 1961.—Two sialic acids, *N*-acetylneuraminic acid and *N*, 7-O-diacetylneuraminic acid, were obtained in crude mixtures from whole cells of *Escherichia coli* and from its endotoxin by weak acid hydrolysis followed by anion exchange resin chromatography. Yields from whole cells were 0.1 to 0.2% (dry weight) with 50 to 60% purity. Identification of the sialic acids was by comparative paper chromatography and colorimetric assays using the acidic *p*-dimethylaminobenzaldehyde (direct Ehrlich), resorcinol and thiobarbituric acid reactions. The *N*-acetyl derivative was also shown to be susceptible to hydrolysis by clostridial *N*-acetylneuraminic aldolase and the end products identified, *N*-acetylamannosamine by paper chromatography and pyruvic acid by oxidation of DPNH with lactic acid dehydrogenase. The two sialic acids were separated on paper chromatograms, eluted, and assays for total and ester acyl groups showed the suspected *N*-acetyl derivative to contain 0.11 O-acyl and 1.16 *N*-acetyl groups per mole sialic acid and the diacetyl derivative to have 1.10 O-acyl and 0.93 *N*-acetyl groups per mole. The O-acyl group was identified as acetyl by preparation of the hydroxamate.

The sialic acids have been found to occur widely in mammalian tissue, chiefly in mucopolysaccharides and glycoproteins in association with D-galactose or galactosamine (Blix, 1950; Zilliken and Whitehouse, 1958; Castellani et al., 1960). The generic term "sialic acid" includes the entire series of substituted neuraminic acids, of which the *N*-acetyl-, *N*-glycolyl-, *N*,4-O-diacetyl-, *N*, 7-O-diacetyl-, and *N*-acetyl-O-diacetyl- derivatives have been isolated from nature (Blix, 1958). The presently accepted formulae for all these compounds are as derivatives of *N*-acetylneuraminic acid:



Little is known concerning their physiological role or the significance of their presence in biological materials with the exception of their participation in the attachment of the myxoviruses to mammalian cells where, in the bound form, they act as substrate for the viral enzyme, neuraminidase (Gottschalk, 1957).

The first report of the occurrence of a sialic acid in bacteria was by Barry and Goebel (1957), who described the elaboration of a sialic acid-like material, colominic acid, by a specific strain of *Escherichia coli*. Barry (1958) has since reported this to be a homopolymer of *N*-acetylneuraminic acid. *N*-acetylneuraminic acid has also been reported to be the main constituent of the group C specific hapten of *Neisseria meningitidis*

[1] Present address: Dept. of Bacteriology, University of Illinois, Urbana, Ill.

[2] Present address: Depts. of Surgery and Microbiology, Tulane University, School of Medicine, New Orleans, La.

(Watson, Marinetti, and Scherp, 1958). Materials yielding the characteristic color reactions of a sialic acid have recently been reported in other gram-negative bacteria (Aaronson and Lessie, 1960), but have not been further identified.

In a previous brief communication (DeWitt and Rowe, 1959), we reported the presence of *N*,7-O-diacetylneuraminic acid and *N*-acetylneuraminic acid in the endotoxins of several strains of *E. coli*. As the O-acetylated compound had not been found previously in other than mammalian tissues, we now wish to extend and amplify this original report.

MATERIALS AND METHODS

Crystalline *N*-acetylneuraminic acid (NANA) obtained by mild acid hydrolysis of *E. coli* O_1:K_1 and crystallized from glacial acetic acid was supplied through the courtesy of D. G. Comb, University of Michigan, and F. Zilliken, University of Pennsylvania. Both samples were the same in our hands and gave infrared adsorption curves identical to those published by Cornforth, Firth, and Gottschalk (1958).

Crystalline *N*,7-O-diacetylneuraminic acid (NODANA) was isolated from bovine submaxillary mucin and was kindly supplied by G. Blix, University of Uppsala.

N-acetylmannosamine (NAM) was the gift of A. R. Hanze of this company and was prepared by the alkaline epimerization of *N*-acetylglucosamine (NAG) according to Roseman and Comb (1958) and partially purified by fractional crystallization. It contained residual NAG and traces of other components which did not interfere with visualization of NAM by paper chromatography, on borate treated paper, according to Cardini and Leloir (1957).

N-glycolylneuraminic acid (NGNA) was visualized in the paper chromatography system of Svennerholm and Svennerholm (1958) as a component (R_F = 0.35) of a crude mixture of bovine submaxillary sialic acids. Glycolic acid (i.e., NGNA) was determined according to Klenk and Uhlenbruck (1957).

Paper chromatography was by the descending front technique and compounds were detected by appropriate sprays. The direct Ehrlich reagent of Svennerholm and Svennerholm (1958) was made fresh daily with recrystallized *p*-dimethylaminobenzaldehyde. Resorcinol was used as a 3% solution in *n*-butanol containing 5% hydrochloric acid. Ninhydrin with S-collidine was prepared and used according to Moffat and Lytle (1959). The hexosamine spray of Partridge (1949) was modified in that the direct Ehrlich solution, cited above, was used in place of the *p*-dimethylaminobenzaldehyde solution given. The O-aminobiphenyl spray of Timell, Glaudemans, and Currie (1956) was used for detection of reducing sugars.

Total sialic acids were estimated by the resorcinol method of Svennerholm (1957). Free sialic acids were measured by the thiobarbituric acid (TBA) method of Warren (1959) or by removal of bound sialic acids from aqueous solution with benzoic acid-saturated chloroform (Schramm and Mohr, 1959), followed by resorcinol assay of the aqueous supernatant.

Ester acyl groups were estimated according to Hestrin (1949) and total acetyl by the micro steam distillation method of Ludowieg and Dorfman (1960).

The following method for extraction of bacterial sialic acids was used for the greater part of the results reported here and has been modified from that of Svennerholm (1958): Cells were harvested from 16 to 18 hr broth cultures (37 C) by centrifugation and washed with distilled water at 3 C. Any of the complete broth media are satisfactory except that brain heart infusion medium will result in considerably higher sialic acid yields. Cells were suspended in distilled water at 20 to 50 g (dry wt) per liter and conc H_2SO_4 added to 0.05 N. After heating at 80 C for 30 min, the cells were chilled to 3 C, and removed by centrifugation. The acidic clarified supernatant was then passed over a Dowex-50W, X-8, 50-100 mesh cation-exchange column (Dow Chemical Co., Midland, Mich.) in the hydrogen form.

The column was washed with water, the effluent adsorbed to a 9 mm × 140 cm Dowex-2, X-8, 100-200 mesh anion-exchange column in the acetate form, and this column well washed with distilled water. The column was then eluted gradiently with distilled water and 2 M sodium acetate-acetic acid buffer (pH 4.6) according to Bock and Ling (1954). The pH of the eluting buffer was later lowered to 3.5 to 3.8 on the basis of the stability curve for NODANA, as given by Blix (1958). NODANA yields were found to be higher at these increased acid concentrations whereas one run at pH 5.5 resulted in the recovery

of no NODANA. Three peaks of resorcinol reactive material were uniformly obtained.

The pooled eluates comprising peak I and appearing at 0.4 to 0.5 M were then passed over a Dowex-50 column in the hydrogen phase and this effluent dried from the frozen state to yield a white amorphous powder.

N-acetylneuraminic aldolase (NANAldolase) was prepared from a 16 hr old anaerobic broth culture of *Clostridium perfringens*. The cells were harvested and washed twice with 0.1 M phosphate buffer at pH 7.0. The culture medium was tryptone, 10 g; yeast extract, 10 g; K_2HPO_4, 5 g; glucose, 1.5 g; and cysteine, 80 mg; water to 1 liter; adjusted to pH 7. The cells were suspended in 0.05 M phosphate buffer (pH 7.0) and disrupted in a Raytheon 10 kc oscillator (Raytheon Co., Burlington, Mass.) for 30 min. The clarified supernatant contained 39 mg protein per ml and 0.2 ml, and was capable of completely degrading 1.3 μM of authentic NANA with the formation of the theoretical yield of pyruvic acid within 20 min. This was then partially purified by fractional precipitation with $(NH_4)_2SO_4$. The greater portion of the NANA degrading activity appeared in that material precipitating at 60 to 80% saturation. After dialysis, this fraction was resuspended in 0.1 M phosphate buffer (pH 7.0) and stored at −20 C.

The strain of *C. perfringens* used (Comb and Roseman, 1958) was supplied by Comb. Other cultures failed to produce detectable amounts of NANAldolase although large amounts of aldolase (as measured with hexose diphosphate) were readily produced.

After heating at 100 C for 2 min to destroy any DPNH oxidase present in the clostridial extract, the aldolase reaction mixtures were assayed enzymatically for pyruvate according to Kornberg (1955).

RESULTS

Extraction of bacterial sialic acids from endotoxin. The first isolation of bacterial sialic acids in this laboratory was from *E. coli* endotoxin. These results are reported in detail sufficient only for comparison with those results obtained with living whole cells.

The endotoxin was a lipoprotein extracted by the hot 75% aqueous phenol method of Westphal, Lüderitz, and Bister (1952) from *E. coli* O_2:K_1. Nucleic acids were removed by precipitation in weak acetic acid and the resulting white amorphous powder fulfilled the biological and biochemical criteria for endotoxin (DeWitt, 1958). Hydrolysis and ion-exchange resin purification of the sialic acids followed the procedure given in Methods except that in these early experiments 0.03 N sulfuric acid was used in the hydrolytic step. Furthermore, the anion-exchange resin was Dowex-1, X-8, 100-200 mesh, in the formate form and the column was eluted gradiently with 2 M sodium formate and water. Elution curves of resorcinol reactive material resemble those given for whole cell hydrolyzates. Peaks appeared at slightly different salt concentrations but analysis by paper chromatography showed that similar compounds were obtained. One run yielded 165 mg in peak I which contained 23% total sialic acids. Paper chromatography in a two dimensional system using ethyl acetate-pyridine-acetic acid-water (5:5:1:3) showed two direct Ehrlich-positive spots which were shown later to correspond to NANA and NODANA. The *n*-butanol-*n*-propanol-0.1 N HCl (1:2:1) system

TABLE 1. *Release of bacterial sialic acids from Escherichia coli by heating[a] in various concentrations of sulfuric acid*

H_2SO_4	pH-Cell suspension[b]			Total sialic acids[c]		
	Before heating	After heating	After $Ba(OH)_2$	Supernatant of neutralized hydrolysate	Dowex-2 effluent[d]	Dowex-2 eluate Dowex-50 effluent[e]
N				*μg/ml*		
0	6.4	6.6	—	0	0	37
0.005	5.1	6.2	—	0	0	19
0.01	5.0	6.2	—	0	0	37
0.05	2.5	4.3	6.2	19	0	75
0.1	2.25	2.7	5.8	12	0	70
0.5	1.4	1.7	6.0	0	0	0

[a] At 80 C for 30 min.

[b] Samples at 0,0.005, and 0.01 N did not require $Ba(OH)_2$ neutralization. Note also that these pH changes apply only to these suspensions and that heavier or lighter bacterial suspensions will yield different pH changes.

[c] As NANA by resorcinol assay and adjusted to a common total volume.

[d] Material not adsorbing to Dowex-2.

[e] Material adsorbing to Dowex-2 and eluted from it with acetate ion but not adsorbing to Dowex-50W.

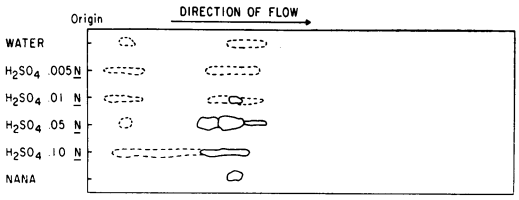


FIG. 1. *Paper chromatography of bacterial sialic acids obtained by hydrolysis in increasing acid concentrations at 80 C for 30 min. Whatman #1 paper, 16 hr at room temp, ethyl acetate-pyridine-acetic acid-water (5:5:1:3), direct Ehrlich spray. Solvent front off paper. NANA = N-acetylneuraminic acid.*

failed to show any spot attributable to NGNA and assay for glycolic acid showed less than 1% of total sialic acids as NGNA.

Determination of optimal conditions for the hydrolytic release of bacterial sialic acids. E. coli (O_2:K_1) was grown aerobically in Difco brain heart infusion broth and cells were suspended in distilled water at approximately 50 g/liter.

Preliminary experiments, using a final concentration of 0.1 N H_2SO_4 and a constant temperature of 80 C, showed that maximum yields of total sialic acids were obtained at 30 min. With a constant time of 30 min, 80 C resulted in a higher yield than did 100 C.

Sulfuric acid was then added to aliquots of an aqueous suspension of these same cells to obtain final concentrations ranging from 0.005 N to 0.5 N. The cell suspensions were heated at 80 C for 30 min with constant stirring, cooled to 3 C, centrifuged, and the supernatants, after neutralization with $Ba(OH)_2$ and removal of the $BaSO_4$, were assayed for total sialic acids according to the ion-exchange chromatography method. The Dowex-2 (CH_3COO^-) columns were eluted with 12 ml 1 M sodium acetate-acetic acid buffer at pH 4.6 and this eluate desalted by passage over Dowex-50 (H^+) columns.

The effluents were dried from the frozen state, taken up in minimal water and developed overnight on Whatman #1 paper in ethyl acetate-pyridine-acetic acid-water (5:5:1:3). Papers were then sprayed with direct Ehrlich reagent.

Table 1 shows that although sialic acids could be detected in the unfractionated hydrolyzates of only the 0.05 or 0.1 N acid-treated samples, they were actually released by all methods, as is seen in the last column. The optimum acid concentration for release of total sialic acids is 0.05 N to 0.10 N. Figure 1 shows, however, that the optimum acid concentration for release of free NANA is 0.05 N and that the other samples contain large amounts of other resorcinol and direct Ehrlich positive compounds. The small area of higher mobility appearing only in the 0.05 N treated sample was later shown to be NODANA.

Extraction of bacterial sialic acids from whole living cells. Numerous strains of the K_1 serotype of *E. coli* have been grown in various media in amounts ranging from 500 ml to 250 liters and used for extraction of bacterial sialic acids, according to the procedure given in Methods, with almost identical results.

The yields obtained on one occasion with *E. coli* O_1:K_1 are representative. Washed living cells (190 g dry wt/1500 ml) upon hydrolysis yielded 22.8 g of released soluble material (at 8000 × *g* for 40 min) containing 245.7 mg total sialic acids. Passage of this material over the cation exchange resin resulted in loss of about 3% of the total solids and recovery of 242 mg total sialic acids. Of this, 220 mg sialic acids was passed over the anion-exchange column with complete adsorption. On elution, peak I consisted of 149 mg solids containing 52.5 mg total sialic acids. Peaks II and III also contained resorcinol-positive materials but yielded amber syrups in vacuo and dry weights were not obtained.

The anion-exchange resin elution pattern of sialic acids as measured in the standard resorcinol reaction ($OD_{585m\mu} - OD_{450m\mu}$), and of material adsorbing at 260 mμ as obtained in another experiment, are shown in Fig. 2. The eluate at 0.4 to 0.7 M (peak I) contained 60% total sialic acids on this occasion. Descending chromatography of these resin eluates on either Whatman #1 or Gryksbo #3 paper in ethyl acetate-pyridine-acetic acid-water (5:5:1:3) revealed only two direct Ehrlich-positive materials (Fig. 3) which later proved to be NANA as the major component and NODANA as the faster-moving minor component. The ultraviolet-adsorbing components were separated from both of these. A minor, very slow-moving component which was acidic but nonreducing, nonultraviolet-adsorbing and direct Ehrlich negative was also present.

The eluate appearing at 1 M buffer concentration (peak II) contained most of the components

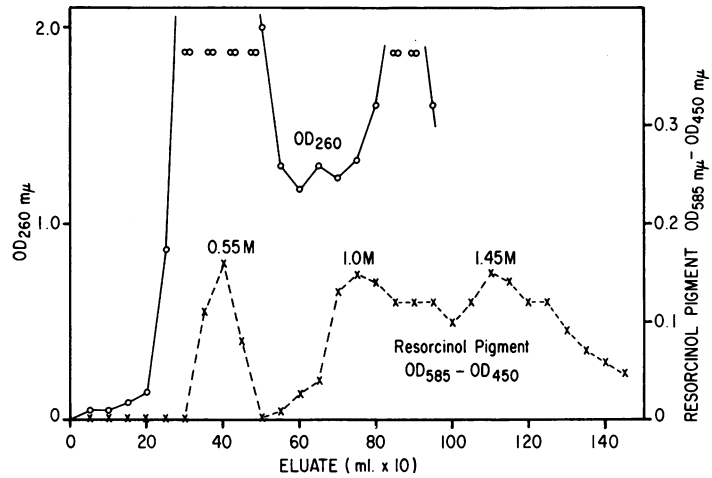


FIG. 2. *The elution of resorcinol-positive ($OD_{585\ m\mu} - OD_{450\ m\mu}$) and ultraviolet-adsorbing material from Dowex-2-acetate anion exchange resin loaded with bacterial hydrolyzates. Eluting buffer concentrations are indicated for each peak.*

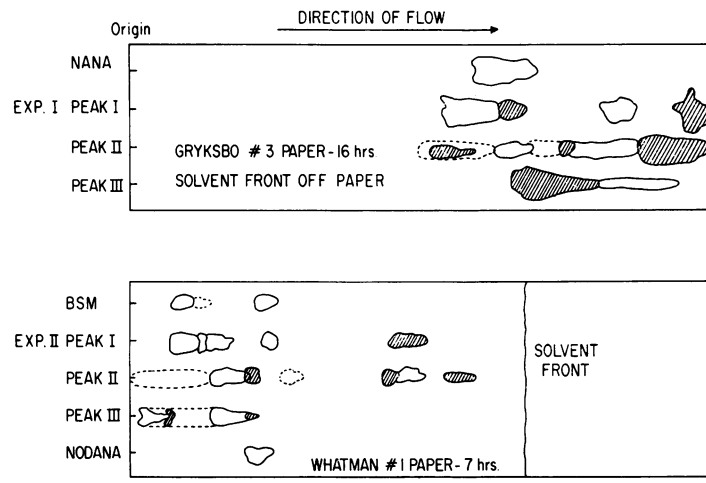


FIG. 3. *Paper chromatography of resorcinol-reactive eluates obtained from Dowex-2-acetate resin loaded with bacterial hydrolyzates. System is ethyl acetate-pyridine-acetic acid-water (5:5:1:3), sprayed with direct Ehrlich reagent. Crosshatched areas are ultraviolet fluorescing. NANA = N-acetylneuraminic acid (cryst); NODANA = N, 7-O-diacetylneuraminic acid (cryst); BSM = crude mixture of N-acetyl-, N-glycolyl- and N, O-diacetylneuraminic acids from bovine submaxillary mucin.*

seen in the earlier peak except that the area corresponding to NODANA was considerably smaller. It also contained at least three additional direct Ehrlich-negative components which are unidentified. Total resorcinol-positive concentration (35% as NANA) was higher than paper chromatography revealed. Again, no component was seen which was both direct Ehrlich-positive and ultraviolet-adsorbing.

The eluate appearing at 1.3 to 1.5 M (Peak III) gave less well defined spots and individual components were not identified. Here, also, total resorcinol-positive concentrations (45% as NANA) were considerably higher than was expected from paper chromatographic results and no correlation of sialic acids and ultraviolet adsorption was seen.

Paper chromatographic behavior and color re-

actions of bacterial sialic acids. The resorcinol and direct Ehrlich-positive materials isolated from either endotoxin or whole cells of *E. coli* and comprising "peak I" were separated by descending paper chromatography in several developing systems into two main components. The slower moving component A migrates with NANA and the faster component B with *N*,7-ODANA. Two-dimensional chromatography yielded no further separation and the separated components could be eluted and redeveloped on paper in any combination of two systems with no change in R_{NANA} or R_{NODANA} of either component. Relative mobilities of these components are given in Table 2, together with the mobilities of NANA and NODANA for comparison. Numerous other determinations have been made in which the solvent front was allowed to leave the paper in order to enhance minor differences in mobility with no separation of experimental and authentic crystalline material. Figure 4 gives the results of a series of such experiments in four different solvent systems. Other unidentified resorcinol and direct Ehrlich-positive materials (therefore presumably neuraminic acid derivatives or complexes) result from weak acid hydrolysis of *E. coli* K_1, but they do not appear in this chromatographic fraction.

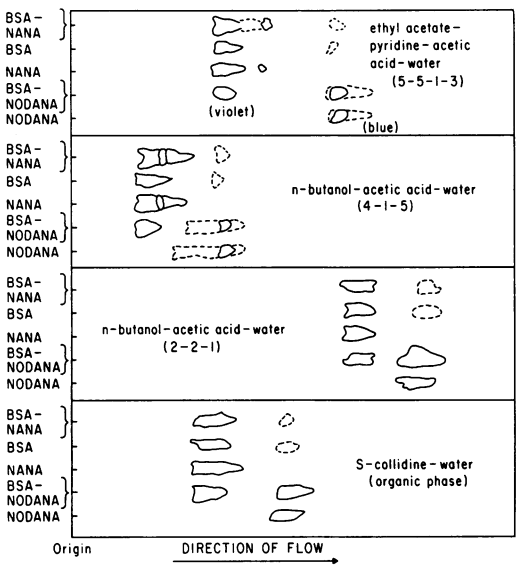


FIG. 4. *Paper chromatographic comparison of bacterial sialic acids* (BSA) *with N-acetylneuraminic acid (NANA) and N,7-O-diacetylneuraminic acid (NODANA). Whatman #1 paper, 16–20 hr, direct Ehrlich spray.*

In Table 2 it can also be seen that no component appeared in the *n*-butanol-*n*-propanol-0.1 N HCl system which migrated with *N*-glycolylneuraminic acid. Furthermore, assays for glycolyl end groups were consistently negative.

Component A is, in addition to the above, ninhydrin negative, a weak reducing sugar and reacts as an α-keto acid. The adsorption spectra of its resorcinol and *p*-dimethylaminobenzaldehyde pigments are identical to those formed with authentic NANA. Material eluted from paper bar chromatograms gives negative tests for hexosamines, 3-0-substituted hexosamines, pentoses, 5-methylpentoses and hexuronic acids. The faster moving component B reacts as a sialic acid in the color reactions cited above

TABLE 2. *Relative mobilities of two components of bacterial sialic acids*

Solvent system	R_F			
	NANA	Component A	NODANA	Component B
n-Butanol-pyridine-water (6:4:3) (5 determinations)	0.12–0.15	0.12–0.16	0.23–0.25	0.22–0.28
n-Butanol-acetic acid-water (4:1:5) (3 determinations)	0.13–0.17	0.13–0.17	0.23–0.25	0.22–0.25
Ethyl acetate-pyridine-acetic acid-water (5:5:1:3) (9 determinations)	0.29–0.32	0.29–0.33	0.56–0.58	0.54–0.65
n-Butanol-n-propanol-0.1 N HCl (1:2:1)	0.44	0.44	0.44	0.44
	(*N*-Glycolylneuraminic acid = 0.35)			
n-Butanol-acetic acid-water (2:2:1)	0.32	0.33	0.44	0.40

except that its *p*-dimethylaminobenzaldehyde pigment is blue rather than violet and the adsorption curve agrees with that formed by NODANA rather than NANA.

Susceptibility of bacterial sialic acids to N-acetylneuraminic acid aldolase. Bacterial sialic acid (BSA) was also examined for its susceptibility to attack by NANAldolase as reported by Comb and Roseman (1958; 1960) and was shown to be cleaved in part by this enzyme with the production of pyruvic acid and *N*-acetylmannosamine.

When 300 μg of crude BSA (0.28 μmoles total sialic acids calculated as NANA and showing NODANA as a minor component) was compared with 0.97 μmoles authentic NANA by incubation with 0.2 ml NANAldolase as described in Methods, the results in Table 3 were obtained. Presence of the *N*,O-diacetyl compound or of NANA in any combined form would serve to explain the less than complete loss of total sialic acids in the bacterial extract (Comb and Roseman, 1958).

When a similar reaction mixture was examined by paper chromatography, a new material was seen which migrated with *N*-acetylmannosamine and there was an apparent loss of NANA (Fig. 5 and 6). Crude BSA (1.75 mg containing 1.65 μmoles total sialic acids) was incubated at 37 C for 60 min with 0.05 ml NANAldolase in a total volume of 0.15 ml. NANA and NODANA were differentiated in the ethyl acetate-pyridine-acetic acid-water (5:5:1:3) system and the *N*-acetylhexosamines were visualized in the borate treated-paper system of Cardini and Leloir (1957). Figure 5 depicts the loss of NANA but not

TABLE 3. *Degradation of bacterial sialic acids* (SA) *by NANAldolase*[a]

NANAldolase	NANA	Residual NANA[b]	NANA degraded
ml	*μg*	*μg*	*%*
0.2	300 (crystalline)	0	100
—	300 (crystalline)	275	8
0.2	87 [in 300 μg crude bacterial SA]	27	58
—	87 [in 300 μg crude bacterial SA]	87	0
0.2	—	0	—

[a] Enzyme 7.8 mg; phosphate buffer (pH 7.1) 30 μmoles; total volume 2.0 ml; 37 C; 60 min.

[b] Total sialic acids as measured by resorcinol reaction.

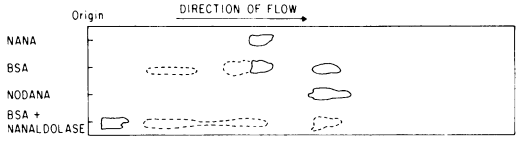


FIG. 5. *Paper chromatography of bacterial sialic acids (BSA) before and after treatment with NANAldolase. Whatman #1 paper, ethyl acetate-pyridine-acetic acid-water (5:5:1:3), 18 hr, direct Ehrlich spray. Solvent front off paper.*

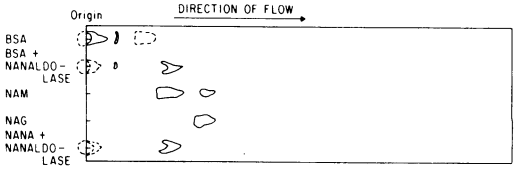


FIG. 6. *Paper chromatography of bacterial sialic acids (BSA) before and after treatment with NANAldolase. Whatman #1 paper treated with borate, n-butanol-pyridine-water (6:4:3), for 18 hr, indirect Ehrlich spray. NAM = N-acetylmannosamine; NAG = N-acetylglucosamine. Solvent front off paper.*

NODANA following incubation with NANAldolase. The new component of low mobility is unidentified. Figure 6 depicts the formation of an *N*-acylamino sugar which is obviously not *N*-acetylglucosamine and is presumably *N*-acetylmannosamine.

When a more highly purified sample of bacterial sialic acids was exposed to NANAldolase action, pyruvic acid was identified as an end product.

Crude BSA (190 mg containing 76 mg total sialic acids calculated as NANA but containing NODANA) obtained by the usual hydrolysis of *E. coli* K_1 followed by ion-exchange chromatography was further purified by passage over an ECTEOLA-cellulose (Brown Co., Berlin, N. H.) column (9 × 175 mm-0.01 M phosphate buffer at pH 7.0) prepared according to Bendich, et al., (1958). Sixty milligrams of sialic acids (100% as NANA) were obtained in the first peak of resorcinol-reactive material and shown by paper chromatography to consist of NANA and a small amount of unidentified material which was neither NODANA nor NGNA. Assay for glycolic acid following hydrolysis with N H_2SO_4 at 100 C for 60 min indicated 1.2% NGNA, which we do not consider significant.

When 2.43 μmoles of the above sample of

bacterial sialic acid and 1.3 μmoles of crystalline NANA were incubated with NANAldolase for 60 min and followed by assay for pyruvic acid, the results in Table 4 were obtained. The discrepancy between the amount of NANA degraded as shown by resorcinol assay and the amount of pyruvate formed is unexplained. The evident possibility of removal of pyruvate in some manner during the long incubation period by the relatively crude NANAldolase preparation was not substantiated by an assay in which pyruvate was added. In the absence of further data, we can conclude only that pyruvate is released from bacterial sialic acid and have no knowledge of the stoichiometry involved.

Molar ratios of N-acetyl and O-acetyl substituents in the two main components of bacterial sialic acid. The behavior of component B in various color reactions and paper chromatographic systems and its insusceptibility to NANAldolase action suggest strongly that it is the 7-O-acetyl derivative of NANA. Other derivatives could conceivably react in a similar manner, however, and we therefore sought to prove that this component is indeed an O-acetylated neuraminic acid.

TABLE 4. *Formation of pyruvate by the action of NANAldolase on bacterial sialic acid*[a] *(BSA)*

NANAldolase	NANA	Residual NANA[b]	NANA degraded[b]	Pyruvate formed[c]	NANA degraded[c]
ml	*μmoles*	*μmoles*	*%*	*μmoles*	*%*
—	2.43 (BSA)[b]	2.43	0	0	0
0.03	—	0	—	0	—
0.03	2.43 (BSA)	1.21	50	0.4	13
0.03	1.3 (crystalline)	0.2	84	0.6	46
—	1.3 (crystalline)	1.3	0	0	0

[a] Phosphate buffer (pH 7.1) 30 μmoles; total volume 2.0 ml; 37 C; 60 min.

[b] As measured by resorcinol reaction.

[c] As measured with lactic acid dehydrogenase and reduced DPN (Kornberg, 1955).

Accordingly, 18 mg of impure bacterial sialic acids (containing 4.1 mg total sialic acids by resorcinol assay and showing paper chromatographic components A and B) were streaked on Whatman #1 paper (46 × 57 cm) and developed for 16 hr in ethyl acetate-pyridine-acetic acid-water (5:5:1:3). After drying, the two strips containing these separated components were located, cut out, and eluted with distilled water. Yields were 845 μg of component A and 1360 μg of component B for 54% recovery. Redevelopment of these two components on paper in an *n*-butanol-acetic acid-water system (4:1:5) showed no separation from NANA in the case of component A nor from NODANA in the case of component B.

Table 5 gives the results obtained when these two components were examined for total sialic acids and total acetyl and ester acyl groups. Amide acyl was obtained by difference. The mole ratios of 0.11 O-acyl and 1.16 *N*- acyl per sialic acid for component A, and 1.1 O-acyl and 0.93 *N*-acyl for component B, substantiate the structures previously assigned on the basis of paper chromatographic mobilites and masking. It should be noted that crystalline *N*-acetylglucosamine, colominic acid and crude NANA obtained by hydrolysis of colominic acid all showed 0.18 to 0.20 moles O-acyl per mole and the results of Table 5 have been corrected accordingly.

The ester-acyl group of component B was identified as acetyl by reaction of this same crude bacterial sialic acid mixture with alkaline hydroxylamine according to Stadtman and Barker (1950). The alcohol soluble derivatives were recovered, developed on paper in *n*-butanol-water (organic phase), and visualized with ferric chloride. Mobilities of R_f = 0.44 were identical

TABLE 5. *Ester and amide linked acyl groups in bacterial sialic acids (SA)*

Component	Total[a] SA	Total Acyl	Ester Acyl	Amide[b] Acyl	O-Acyl / SA	N-Acyl / SA	Designation
	μmoles	*μmoles*	*μmoles*	*μmoles*	*mole/mole*	*mole/mole*	
A	0.55	0.7	0.06	0.64	0.11	1.16	*N*-acetylneuraminic acid
B	0.79	1.6	0.87	0.73	1.1	0.93	*N*,O-diacetylneuraminic acid

[a] By resorcinol assay.

[b] Total acyl minus ester acyl.

with that shown by authentic acethydroxamate obtained in a like manner from acetyl phosphate.

DISCUSSION

The results reported here establish the presence of both *N*,7-O-diacetylneuraminic acid (NODANA) and *N*-acetylneuraminic acid (NANA) in certain strains of *E. coli*. *N*-glycolylneuraminic acid (NGNA) is either absent or present in amounts too small to permit detection by our methods. Although the *N*-acetyl derivative is by far the main component isolated, it has not been proven that it exists as such on the cell. The lability of the O-acetyl moiety is so great as to preclude quantitative isolation of NODANA by present methods. The recovery of these two compounds from isolated endotoxin suggests that they occur in the surface lipoprotein but this depends, of course, on the homogeneity of the extracted antigen. This is discussed further in the following paper (DeWitt and Zell, 1961). Although the ester-acetyl group is considerably more labile to acid than the amide acetyl, it is evidently sufficiently stable to permit release of an appreciable quantity of NODANA by standard hydrolytic techniques.

Ion-exchange resin chromatography of these hydrolysates consistently yielded mixtures of NANA and NODANA which were separable only by paper chromatography. Chromatography on various other ion exchange resins, carbon, cellulose or modified cellulose failed to yield separation. As amounts of NODANA obtained by paper chromatography were too small to permit crystallization, we have depended here on other methods of identification.

N-acetylneuraminic acid was identified by comparative paper chromatography, various organic color reactions, and by its susceptibility to *N*-acetylneuraminic acid aldolase. The demonstration of *N*-acetylmannosamine and pyruvic acid as products of the latter reaction substantiates the identification.

The primary evidence in support of the identification of *N*,7-O-diacetylneuraminic acid was obtained with material eluted from paper bar chromatograms. Quantitative estimation of total and ester-acyl substituents showed this secondary component of bacterial sialic acids to contain one amide-acyl and one ester-acyl per molecule. The ester-acyl was identified as an acetyl group by formation of the hydroxamic acid derivative and, on the basis of comparative paper chromatography, shown to be at carbon seven. The same experiments confirmed the identification of the slower moving component as NANA (i.e., one amide acetyl group per sialic acid molecule).

It is apparent from the results of O-acyl estimations that whole cells contain approximately three times as many such groups as are required to establish all of the sialic acid as the diacetyl compound. A high proportion of O-acyl groups in crude sialic acid preparations from these enteric bacteria is also a common finding. It is possible, of course, that bacterial sialic acid exists on the cell as an *N*-acetyl-O-triacetyl derivative, but there is no evidence supporting such an assumption. The 6-O-acetyl glucose of Duff and Farmer (1958) could be present or this could be evidence of the existence of yet other O-acetylated sugars. Also, it must be noted in opposition to the proposal for a tri-O-acetyl compound that the cytidine-monophosphoneuraminic acid isolated by Comb, Shimizu, and Roseman, (1959) under neutral conditions yielded only the *N*-acetyl compound upon very mild hydrolysis. This could be interpreted as support for the thesis that NANA as well as NODANA is present in the surface lipoprotein, if it is assumed that the nucleotide is part of a transglycosidation mechanism. It must be further assumed, however, that O-acetylation does not occur after formation of the sialyl glycoside.

The demonstration of O-acetylated sialic acids in various strains of the K_1 serotype of *E. coli* does not in any way conflict with the existence of colominic acid (poly-*N*-acetylneuraminic acid). This compound has been well characterized by Barry (1958 and Barry, Tsai, and Chen, 1960) as containing one amide acetyl group per neuraminic acid molecule. It should be noted, however, that colominic acid was extracted from the culture medium, not from the bacteria themselves (Barry, 1958). Use of the term "colominic acid" to designate sialic acids extracted from cells or surface antigens should be discouraged unless proof of structure is presented. This would seem highly desirable in view of the relative biological inactivity of pure colominic acid as evidenced by its serological inactivity (Barry, 1958) and its insusceptibility to neuraminidase (Gottschalk, 1960). Colominic acid may well be an end product resulting from the breakdown of the surface mucopolysac-

charides or a compound formed by shunting of sialic acid before incorporation into surface polymers. As mentioned above, the O-acetyl groups may not be added until after formation of the O-glycosidic bonds composing the surface heteropolymer.

The crude mixtures of bacterial sialic acids obtained from ion-exchange resin chromatography contained considerable amounts of ultraviolet adsorbing materials and the elution patterns led us at first to believe that one or more of these might prove to be a nucleotide linked sialic acid, such as those described by O'Brien and Zilliken (1959). It was immediately apparent, however, that the sialic acids and ultraviolet adsorbing components were not identical but merely similar in their elution characteristics. To date, no component has been obtained by us from *E. coli* which upon paper chromatography is ultraviolet-adsorbing and also gives the standard color reactions for a sialic acid. The acid hydrolysis step and low pH of our eluting buffer in the fractionation procedure would, however, certainly destroy any acid labile nucleotide such as the cytidine monophosphate compound described by Comb, et al., (1959).

The discovery of sialic acids in the bacteria stimulates speculation on the role of this material in host-parasite relationships. The wide occurrence of these acids in animal mucoid secretions and ground substance leads us to suggest, for example, that one or more of the bacterial enzymes involved in the metabolism of the sialic acids might be capable of action against mammalian substrates and thus be involved in virulence of these organisms. Although the somatic lipopolysaccharide is undoubtedly responsible for the toxicity of enteric bacteria, there has been as yet no definition of the factor(s) which permit survival and growth in vivo of a small inoculum to the critical toxic level.

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