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SIALIDO-LACTOSE OF COW COLOSTRUM

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Sialic acid[1], also known as acetyl-neuraminic acid[2] or gynaminic acid[3], was first isolated from bovine submaxillary mucoid, and subsequently shown to be widely distributed in mammalian tissues[4]. Mammary gland extracts and colostrum also contain a substance similar to or identical with sialic acid[5,6,7]. A crystalline compound obtained by KUHN from cow colostrum seemed to differ in some respects from these, and was therefore named lactaminic acid. This laboratory in attempts to elucidate the mechanism of the receptor destroying enzyme[8], which catalyzes the hydrolysis of sialic acid in certain mucoids, explored the possibility of using cow colostrum as a source for less complex substrates than those encountered in submaxillary gland and urine. Upon finding dialyzable sialic acid in cow colostrum, an investigation of its chemistry was undertaken. Preliminary results of this study have been reported[9].

METHODS

Sialic acid was assayed by the Direct Ehrlich's and Bial's Orcinol tests as described by WERNER AND ODIN[10]. Its acid-labile COOH group was determined manometrically by TRACEY's method for glucuronic acid[11]. *Lactose* was analyzed by the anthrone method[12]. *Reducing values* were

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determined by a modification of the HAGEDORN-JENSEN method[13], as well as by hypoiodite reduction[14]. A modification of the Hestrin method for esters was used[15]. Nitrogen was determined by the micro-Kjeldahl method. Acetyl determinations* were made by saponification with *p*-toluenesulfonic acid, followed by steam distillation and titration of the liberated acetic acid.

Paper chromatography was carried out on Whatman No. 1 filter paper with descending irrigation by *n*-butanol, acetic acid and water (50:12:25). The dried papers were sprayed with a mixture of benzidine and trichloroacetic acid in aqueous ethanol, and then heated to 115° C for 10 min[16]. Sialic acid and sialic acid-containing compounds gave blue spots. All other reducing sugars gave brown spots, appearing after one minute at 115° C.

Paper electrophoresis was carried out in a Spinco Paper Electrophoresis Apparatus, Model R (Durrum Type). 30 μg of sialic acid or methyl sialate were applied on Whatman No. 3MM filter strips and run in the cold for 6 h in a supporting electrolyte of $0.1 M$ acetic acid with a gradient of 480 volts. The dried papers were sprayed with the benzidine trichloroacetic acid reagent.

Enzymic degradation of isolated sialic acid was carried out by incubation with a lyophilized filtrate of Cholerae vibrio (Behring Werke, Marburg, a/d Lahn, Germany). The filtrate was dialyzed against distilled water for 24 h, and then diluted in $0.1 M$ phosphate buffer, pH 7, so that each ml contained 2 mg of lyophilate. 200 μg of substrate were incubated at 37° C for three days in the presence of 1 mg of enzyme preparation, and the sample analyzed for sialic acid, hexosamine and pyruvic acid as described previously[17].

EXPERIMENTAL AND RESULTS

Isolation and characterization of lactose sialic acid

The dialyzable portion of 1 l of cow colostrum was defatted**, shaken with a small quantity of Dowex-50 (acid form) and then passed through a 1 ½" × 10" column of Dowex-1 (10% cross linked, formate form). The anion exchange column failed to retain sialic acid-containing material without pretreatment of the solution by Dowex-50. The Dowex-1 column was washed with 2 l of distilled water, and then eluted with increasing concentrations of formic acid. At $0.06 N$ formic acid, a sialic acid-containing fraction appeared. No other sialic acid fraction was encountered. The eluate was evaporated under reduced pressure, taken up in methanol and then precipitated by addition of acetone. The precipitate was redissolved in methanol an precipitated with ether. The dried white powder weighed 170 mg. On paper chromatography, this powder gave one blue spot (R_F 0.06) (sialic acid R_F 0.23). Upon hydrolysis with $0.5 N$ H_2SO_4 for 5 min at 100° C and subsequent neutralization with $Ba(OH)_2$, the material exhibited four spots. There was some unreacted material, R_F 0.06, another blue spot, R_F 0.22, and two brown spots, R_F 0.15 and R_F 0.27, respectively. When the acid hydrolysis was carried out for 30 min however, only one blue spot (R_F 0.22) and one brown spot (R_F 0.27) were found. The mobilities of these materials were compared with reference compounds, sialic acid (R_F 0.23), lactose (R_F 0.15), glucose and galactose (R_F 0.27 each). Having tentatively identified the compound as lactose sialic acid (neuramin lactose), analyses were carried out in order to establish the molecular composition of this material. The ratio of lactose to sialic acid was found to be unity. The analyses of lactose sialic acid are summarized in Table I. The ultraviolet spectrum of the compound exhibited a small maximum at 270 mμ. Upon heating for 30 min at 100° C in $0.05 N$ NaOH, a strong maximum at 261 mμ appeared, $E^{1\,vM}_{261\,m\mu} = 2.0$ (Beckman Spectrophotometer, Model DU, light path 1 cm).

The reducing value of the trisaccharide suggested that the reducing group of lactose was free. The nature of the linkage of sialic acid to lactose, however, could

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not be determined from the reducing value of the trisaccharide. When the latter was reacted with sodium borohydride, the reducing group of the compound disappeared, and the hexose analysis dropped to 24.8%. This indicated that the glucose moiety of lactose had been at the reducing end. At the same time, it was observed that sodium borohydride had failed to reduce sialic acid. Therefore, the reducing group of sialic acid must have been attached by a glycosidic linkage to lactose. The compound hence is sialido-lactose.

TABLE I

ANALYSIS OF SIALIDO-LACTOSE

Sialido-lactose (MW 633)	*Calculated*	*Found*
Sialic acid (Bial)	48.8	43.2
Lactose[12]	54.2	48.2
Reducing value[13]	28.4	27.1
Nitrogen	2.2	2.1
Acetyl	6.8§	6.4
$[\alpha]_D^{26°}$ =		+21° (H_2O)

§ Monoacetyl compound.

The isolation and characterization of methyl sialate

The dialysate of colostrum was heated for 30 min at 80–90° C with diluted H_2SO_4 at approximately pH 1. The hydrolysate was filtered on a large Buechner funnel and washed with three liters of distilled water. The filtrate was neutralized by addition of solid $Ba(OH)_2$ and refiltered. The solution was passed through a bed of Dowex-50 (acid form) in order to remove excess barium ions, and then passed through a 1½" × 10" column of Dowex-1 (10% cross linked, formate form) and washed and eluted as in the isolation of sialido-lactose. The sialic acid fraction eluted at 0.07*N* formic acid. After concentration of the eluate, and lyophilization, the material was dissolved in absolute methanol. Insoluble material was removed, and the clear liquid was placed in a desiccator containing concentrated sulfuric acid. Crystallization occurred upon slow evaporation of the solvent.

Methyl Sialate $C_{12}H_{21}NO_9$ (MW 323)	C	H	N
Calculated	44.56	6.56	4.33
Found	45.35	6.84	3.97

Yield: 250 mg — $[\alpha]_{26}^{D}$ = —38° Melting point: 171–174° C.

By paper chromatography this compound had the same R_F (0.48 and 0.58) as similarly prepared methyl sialate of bovine submaxillary mucoid. The analyses, including infrared spectra, of this material were also identical with methyl sialate obtained from the mucoid. The methyl ester was saponified on standing in 0.5*N* Na_2CO_3 for one hour at room temperature. After treatment with small amounts of Dowex-50 (acid form), sialic acid so obtained remained amorphous. A comparison of the characteristics of methyl sialate and sialic acid appears in Table II.

On incubation of methyl sialate with filtrates of V. cholerae, splitting of sialic acid to N-acetylhexosamine and pyruvic acid was demonstrated. The rate of the enzymic degradation of sialic acid isolated from cow colostrum was identical with the rate observed with preparations obtained from bovine submaxillary gland.

TABLE II

COMPARISON OF THE PROPERTIES OF SIALIC ACID ISOLATED FROM BOVINE SUBMAXILLARY GLAND AND METHYL SIALATE ISOLATED FROM COW COLOSTRUM

	Sialic acid (Submaxillary)	*Methyl sialate (Colostrum)*
Direct Ehrlich's reaction	100%	100%
Bial's orcinol	100%	94%
Tracey CO_2	1 mole	1 mole
Ferricyanide reduction (glucose standard)	10%	10%
Hypoiodite reduction (glucose standard)	55%	55%
Decomposition point	151–160° C	171–174° C
Rotation $[\alpha]_D^{25}$	—32°	—32° – —38°
Ferric hydroxamate (glucurone standard)	0.29 mequiv.	2.67 mequiv.
Electrophoretic migration (0.1*M* acetic acid)	to anode	to cathode
Methoxyl group	none	1 mole
U.V. spectrum (maximum)	265 mμ	285 mμ
R_F butanol–acetic acid–water (50:12:25)	0.13 and 0.20	0.48 and 0.58

DISCUSSION

Hexose-linked sialic acid has been demonstrated in extracts of lactating rats' mammary glands[5,6], and cow colostrum[7,9]. In the more complex mucoids sialic acid apparently is linked to N-acetylhexosamine.

A comparison of the analyses of sialido-lactose of four different laboratories is shown in Table III. Deviations from theory occur in all four preparations but they are probably within the limits of the errors of the analytical methods.

TABLE III

A COMPARISON OF THE ANALYSES OF SIALIDO-LACTOSE OBTAINED IN FOUR LABORATORIES

	Sialic acid %	*Lactose %*	*Reducing value % glucose*	*Nitrogen*	*Rotation*	*Acetyl (moles)*	*pK*
Trucco and Caputto	43	47	28	2			
Bacon and Heyworth		~33	27–33	1.2–1.6	+6 to +22		2.2
Kuhn and Brossmer				2.3	+16	2	
Heimer and Meyer	43	48	27.1	2.1	+21	1	

Methyl sialate isolated from bovine colostrum and bovine submaxillary mucoid was found to be indistinguishable. Considerable doubt had existed concerning the identity of KUHN's lactaminic acid with sialic acid. KUHN's corrected analyses[18], KLENK's identification of neuraminic acid (deacetylated, methoxy sialic acid[19]) as a constituent of cow colostrum, and our analytical data, including the enzymic

References p. 494.

degradation by the sialic acid splitting enzyme, indicate the identity of sialic acid found in all tissues of cattle. It would seem that all naturally occurring substances reacting like sialic acid are 2-keto, 2-deoxy, 5-amino, 5-deoxy (2,6-pyranoside) D-(?)-glucononononic acids. Species-specific sialic acids, however, have been demonstrated by BLIX[4]. The substance isolated from bovine sources apparently contains a very labile O-acetyl group, located on carbon 7[4] which however was lost in the procedures used*.

SUMMARY

The sialic acid-lactose containing oligosaccharide of cow colostrum was isolated. On reduction with borohydride its hexose value decreased by one-half, while the sialic acid color was unaffected. Methyl-sialate was isolated from the complex in good yield and shown to be identical with that isolated from bovine submaxillary gland. From the properties of the complex, it is concluded that sialic acid is present in the complex in O-glycosidic bond and that the complex is sialido-lactose.

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* While this manuscript was in preparation, GOTTSCHALK in a short communication[20] proposed a structure for sialido-lactose. In the proposed structure sialic acid is linked in an O-glycosidic bond to D-galactose. No experimental evidence exists for this assumption.