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Metabolism of Exogenous Sialic Acid in the Rat.* (26258)

GARLAND A. JOHNSON, CHARLES R. KISTLER AND ROBERT H. MCCLUER
(Introduced by E. Ogden)
Department of Physiological Chemistry and Pharmacology, Ohio State University, and Columbus Psychiatric Institute and Hospital, Columbus

The ubiquitous occurrence of sialic acid in mammalian tissues as a component of glycoproteins, glycolipids and oligosaccharides(1) implicates a role for this acidic carbohydrate in a variety of basic physiological phenomena. Free, unbound, sialic acid has not been detected except in cerebrospinal fluid (2). The liberation of sialic acid by bacterial and viral neuraminidase(3) raises the question of the metabolic fate of this substance. This report is concerned with the metabolism of sialic acid following intra-arterial, intraperitoneal, subcutaneous and oral administration to the rat.

Materials and methods. Sialic acid was prepared from bovine submaxillary mucin by the method of Svennerholm(4). The lyophilized product obtained following chromatography on Dowex-1 was dissolved in 0.9% saline and neutralized in preparation for administration to the animals. Twice recrystallized material (m.p. 185-186°C), which was shown by paper chromatography(5,6) to contain N-acetylneuraminic acid and N-glycolylneuraminic acid in the approximate ratio of 9:1, was used for calculation of extinction coefficients with the thiobarbituric acid assay(7).

Adult male albino rats were anesthetized with chlorolose-urethane and heparinized. The abdominal aorta was cannulated with a T-cannula and sialic acid (approximately 100 mg/kg body wt, in 1.0 ml) was injected through the side arm followed by 1.0 ml of saline. Arterial blood samples were obtained at time intervals indicated and diluted plasma samples were analyzed for free sialic acid using the thiobarbituric acid assay of Warren(7). In several experiments the renal arteries were bilaterally ligated. Data from typical experiments are presented in Table I.

The assay of sialic acid in urine was complicated by the presence of a chromogen resembling that given by deoxyribose. How-

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ever, satisfactory results were not obtained with calculations based on the extinction coefficients of deoxyribose and sialic acid. Rat urine, chromatographed on Whatman No. 1 paper in n-butanol, acetic acid, water (4:1:5, v/v) and developed with the thiobarbituric acid spray reagent(8), showed a spot with a lower Rf than authentic deoxyribose. This chromogen was not identified, but interference was eliminated by use of the absorbancy ratio obtained with the thiobarbituric acid assay of control samples of rat urine. It was found that the ratio of optical densities ($O.D._{549}/O.D._{535}$) was equal to $0.71 \pm 0.06\sigma$. Since molecular extinction coefficients (ϵ) of sialic acid at 549 mμ and 535 mμ were found to be 55×10^3 and 26×10^3 respectively, and if it is assumed that the ϵ_{549} and ϵ_{535} of the urinary chromogen are 7×10^3 and 10×10^3 respectively, then equation (1) can be derived.

$$\mu\text{moles}_{S.A.} = 0.117\ O.D._{549} - 0.082\ O.D._{535} \quad (1)$$

It was shown that the recovery of various quantities (5 to 50 mg) of sialic acid added to 6 hour urine samples was $98.7\% \pm 1.2\sigma$ as calculated by equation (1). The measurement of free sialic acid excreted in the urine was carried out in a paraffinized metabolism cage. Six hour urine samples were collected and diluted to at least 100 ml for analysis. To determine the recovery of sialic acid added to the metabolism cage, 4.0 to 54.5 mg of sialic acid were pipetted into a cage with a rat. After 6 hours cage washings were analyzed with the thiobarbituric acid assay. Recovery of the added sialic acid was 95.4 to 99.0%. Antibiotic pretreatment of the gastrointestinal tracts of the rats was accomplished by adding phthalylsulfathiazole (sulfathalidine, Merck) to the rats' feed (10% by wt) and neomycin sulfate (Mycifradin sulfate, Upjohn) (0.25 g/100 ml) to the drinking water. This feeding was continued until the color of the feces turned from brown to gray or almost white. In experiments involving incubation of intestinal tissue with sialic acid, small strips (0.5-1.0 cm wide) were cut from the intestine and placed in 10 ml of Krebs-Ringer phosphate buffer(9) containing 0.4 mg of either glucose or sialic acid and incubated at 37°C under an atmosphere of 95% O_2/5% CO_2 in a Dubnoff metabolic incubator. Similarly, in experiments with intestinal contents, the cecum was washed out with buffer solution and 10.0 ml aliquots were incubated with known amounts of sialic acid in the Dubnoff incubator.

TABLE I. Blood Levels of Free Sialic Acid with and without Renal Ligation.

Time after inj.	mg %/kg body wt Rat 1,* without ligation	Rat 2,* with ligation
0 †	2.5	5.2
5 min.	160.4	74.2
30 "	19.4	43.0
1 hr	8.1	27.9
2 "	2.5	22.3
3 "	3.1	22.8
4 "	—	26.8
5 "	—	23.0
6 "	—	24.0

* Dosage of sialic acid—100 mg/kg body wt.
† Sample taken before inj.

Results. Table I presents levels of free sialic acid in the rat blood following intra-arterial injection, with and without renal ligation. Without ligation all detectable sialic acid disappeared from the blood after 2 hours. With renal ligation the sialic acid content in blood reached a plateau after 2 hours, and no significant decrease in values was observed during the next 4 hours, the duration of experiment.

A large percentage of intraperitoneally administered sialic acid was excreted in the urine in the 0-6 hour period (Table II). The per cent recovery varied from about 80% with a 4.9 mg dose to approximately 90% with a 37.4 mg dose. Subcutaneous administration delayed excretion so that detectable amounts of sialic acid appeared in the 6-12 hour samples; however, the per cent recovery did not appear to differ from that of the intraperitoneal administrations of corresponding dosages. Fecal analyses for sialic acid did not reveal any detectable amounts of sialic acid in either of the parenteral administrations.

Sialic acid was not detected in the urine up to 24 hours following oral administration. The 2 rats receiving the largest doses of sialic

TABLE II. Recovery of Administered Sialic Acid from Rat Urine.

Route of admin.	Sialic acid admin. (mg/kg body wt)	Sialic acid recovered	
		mg/kg body wt	%
Intraper.	9.0	7.3	80.4
	18.2	14.5	80.0
	18.3	14.8	81.3
	37.1	31.8	85.8
	41.1	34.5	83.8
	68.0	62.4	91.7
Subcut.	41.6	34.8	83.7
	52.5	45.5	86.6
Oral (without antibiotic)	36.9	.0	.0
	46.7	.0	.0
	54.4	.0	.0
	75.5	.0	.0
	90.8	.0	.0
	273	.0	.0
	315	.0	.0
Oral (with antibiotic pretreatment)	90.0	.0	.0
	98.0	.0	.0
	182	.0	.0
	205	.0	.0

acid (Table II) were sacrificed after 12 hours and the intestines excised and washed out with saline solution. These washings did not contain a detectable amount of free sialic acid. Following antibiotic pretreatment of the gastrointestinal tract, orally administered sialic acid was not detected in any of the urine samples, but the feces was found to contain free sialic acid.

Enzymatic degradation of sialic acid by intestinal tissue slices could not be demonstrated. In every experiment in which tissue was incubated *in vitro*, the thiobarbituric acid assay showed a marked increase in optical densities with an absorption maximum at 535 mμ. This phenomenon has been previously reported(10). When intestinal contents were incubated with sialic acid, sialic acid had disappeared in both control and experimental solutions after 18 hours. However, this destruction of sialic acid was completely inhibited by addition of neomycin sulfate (125 μg/ml) to the incubation solution.

Discussion. Following intra-arterial administration, sialic acid is rapidly cleared from the blood stream. If the renal arteries are tied off, equilibrium with the body fluids seems to be reached within 2 hours, since no detectable decrease in the free sialic acid in the blood is noticeable during the next 4 hours. Thus, utilization is probably not a major factor in clearance of the material from the blood.

A large percentage (80-90%) of the sialic acid administered by intraperitoneal or subcutaneous injection is excreted in the urine during the first 6 hours after injection. The per cent recovery increases as dosage is increased, perhaps indicating saturation of the systems degrading sialic acid. Sialic acid was not detected in the urine or feces following oral administration of relatively large quantities. The presence of sialic acid in feces of rats whose gastrointestinal tracts had undergone extensive antibiotic treatment preceding oral administration indicated that intestinal bacteria are the principal cause of destruction of sialic acid. Furthermore, no evidence was obtained which indicates that intestinal tissue is capable of degrading sialic acid, and the disappearance of sialic acid when incubated in the presence of intestinal juice is inhibited by antibiotic. The fact that sialic acid did not appear in the urine of rats receiving antibiotics after oral administration may indicate that there is little or no absorption of sialic acid across the intestinal wall.

Summary. Sialic acid, prepared from beef submaxillary mucin and assayed by the thiobarbituric acid assay, was administered to rats intra-arterially, intraperitoneally, subcutaneously, and also orally with and without antibiotic pretreatment of the gastrointestinal tract. Cannulation experiments demonstrated the rapid removal of free sialic acid from the vascular system following intra-arterial administration. Analyses of 6 hour samples of rat urine following parenteral administrations showed that 80-92% of free sialic acid was recovered. Amount recovered increased as dose was increased. Intestinal bacteria destroyed the orally administered sialic acid which was not absorbed to any appreciable extent.

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Response of Starved Rats and Polycythemic Rats to Graded Doses of Erythropoietin.* (26259)

NEIL I. GALLAGHER, DUANE Q. HAGAN, JOHN M. MCCARTHY AND ROBERT D. LANGE
(Introduced by R. Wegria)
Departments of Medicine, Schools of Medicine, St. Louis University and Washington University; Veterans Administration Hospital, St. Louis, Mo.

Present studies of the humoral regulation of erythropoiesis are based on diverse *in vivo* bio-assay technics, described by Gordon(1). Gordon also noted that methodology influenced results. Few specific attempts have been made to compare results obtained by different assay procedures(2,3).

This report details results of assays carried out using starved rats or rats rendered polycythemic by hypertransfusion. Reticulocyte counts and red cell Fe^{59} incorporation were measured; erythropoietic response of both types of assay animal to human and rabbit erythropoietin was assessed. Serially graded doses of erythropoietin were assayed and the relationship between response in red cell iron incorporation or reticulocyte production and dose of erythropoietin was determined.

Methods and materials. Erythropoietin was prepared from rabbit plasma and human urine: a) Hemolytic anemia was produced in albino rabbits by daily subcutaneous injections of 1 ml of 2.5% phenylhydrazine solution. When the hematocrits were 15% or less the rabbits were exsanguinated into heparinized flasks. The separated plasma was pooled, and extracted by the method of Borsook(4). This extract was concentrated 10-fold, dialyzed for 12 hours against distilled water at 4°C and stored frozen. The erythropoietic stimulating activity of such extracts had been previously demonstrated(4,5). b) Urine was obtained from a patient with severe anemia due to erythroid failure. His hematocrit ranged between 17 and 20%; there was persistent, absolute reticulocytopenia, and marrow was devoid of red cell precursors. Urine was dialyzed against running tap water for 18 hours, concentrated by flash evaporation to one-twentieth of the original volume and stored at −18°C.

Two types of bio-assay were used. On the first day of a 4-day assay period, female Sprague-Dawley rats weighing 180 to 200 g were started on starvation(6) or rendered polycythemic by a single transfusion of homologous packed red cells equivalent to 50% of the animals' original predicted red cell mass(7). Starvation was continued for the entire 96 hour assay period; the polycythemic animals were fed a regular rat chow diet. The remainder of the assay technic was standard. Plasma extract or urine concentrate was injected intravenously 30 and 54 hours after transfusion or institution of starvation. Although the dose of plasma extract or urine concentrate varied, volume of injection was kept constant by dilution with saline.

Eighteen hours prior to completion of the assay, each rat was given an intravenous injection of approximately 1 μc of Fe^{59} citrate in saline. An appropriate standard was also

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