the intima is accelerated and intensified by epinephrine and that the catecholamine-phospholipid compounds seem to possess a particular affinity for arterial tissue. It seems possible that catecholamines injured the intima of the coronary arteries in the 2 test groups of monkeys and thus laid the foundation for subsequent lipid accumulation.

Summary. Two groups of squirrel monkeys (*Saimiri sciureus*) were subjected to intermittent stress of different types (restraint alone and restraint combined with conditioned avoidance) for a period of 25 months. The following statistically significant differences were observed between the test groups and a control group of monkeys confined to cages for the same period of time: (1) The mean serum cholesterol was higher following the periods of stress; (2) excretion of urinary 17-ketosteroids was increased; and (3) coronary artery atherosclerosis was more marked. Although no significant differences were observed in the *weight* of the adrenal glands, the adrenals from all except one of the test monkeys showed changes observable grossly and histologically. Electrocardiographic changes, present in 4 of the test monkeys, were not seen in any monkeys in the control group. The effects of stress-liberated catecholamines are discussed briefly.

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Urinary N-Sulfate Glycosaminoglycan Excretion in Children: Normal and Abnormal Values.*† (32361)

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Six seemingly distinct syndromes have been described in which there is an excessive urinary excretion of one or two glycosaminoglycans (GAG) (1). The quantitative determination of GAG in the urine has provided a use-

ful diagnostic procedure, and the widespread application of these determinations has become a necessity for delineation of the spectrum of mucopolysaccharidoses.

Heparan sulfate, one of the GAGs which occurs in excess in urine and tissues of patients with mucopolysaccharidoses, shares with heparin the distinction of containing N-sulfated hexosamine, so that the development of a specific assay for N-sulfate hexosamine(2) provides a simple and direct measure of these two GAGs. We report here the results of an assay for GAG N-sulfate hexosamine performed on urine samples from 1) a series of 56 children ranging in age from 3 months to 18 years without apparent stigmata of the mucopolysaccharidoses, and 2) a group of 6 patients with a clinical and/or autopsy diagnosis of mucopolysaccharidosis.

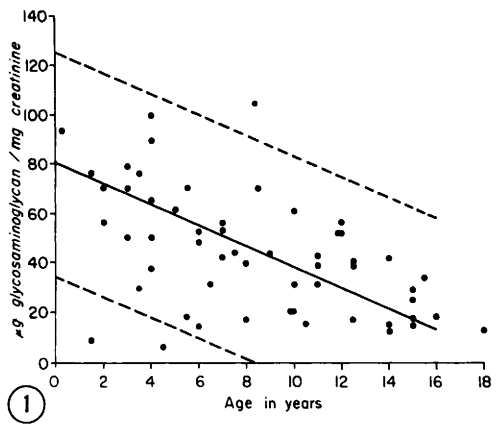
Methods. Urine samples were collected from 58 patients attending Pediatric Outpatient Clinic, University of Washington Hospital. Fifteen to 30 ml voided urine specimens were preserved with 1 ml of toluene and kept refrigerated until processed. Twenty-four hour urine specimens were collected in the presence of toluene from patients with clinically suspected mucopolysaccharidoses during hospitalization. Twenty-four hour specimens from parents and siblings were collected at home. Urine was obtained from the bladder at time of autopsy of 3 patients with mucopolysaccharidoses. Creatinine determinations were performed on all specimens but one; those containing less than 0.1 mg/ml creatinine were not included in the study. Two patients with diabetes insipidus were excluded by this criterion.

GAGs were precipitated from urine by the procedure of Teller *et al*(3), a modification of the method of diFerrante and Rich(4). The only change we made in the procedure was the substitution of cetylpyridinium chloride (Recip, Stockholm, Sweden) for cetyl trimethylammonium bromide.

Total glycosaminoglycans were estimated from hexuronic acid determination by Bitter and Muir's modification(5) of Dische's carbazole reaction. The standard was glucuronolactone. Values obtained were converted to glycosaminoglycan using a factor of 4.4 mg sodium chondroitin-4-sulfate per mg of hexuronic acid, determined on a commercially available product (Sigma Chemical Co., St. Louis.)

N-sulfate hexosamine was determined by the procedure of Lagunoff and Warren(2). Aliquots of a solution of GAG in .05 N NaOH were assayed in duplicate. Two-tenths ml of sample and the same volume of an appropriate concentration of standard were each mixed with 0.2 ml of 5% sodium nitrite (prepared weekly and stored at 4°C in a brown bottle) and 0.2 ml of 33% acetic acid. The solution was left for 80 minutes at room temperature and two 0.1 ml aliquots of 12.5% ammonium sulfamate were added to each solution, with vigorous shaking, allowing a 15-minute interval between additions. One ml of 5% concentrated hydrochloric acid and 0.1 ml of a fresh 1% solution of indole (Eastman) in absolute ethanol were next added to each solution. After 10 minutes at room temperature, the tubes were heated on a boiling water bath for 5 minutes, then cooled. One ml of absolute ethanol was added, and the difference between the absorbance at 492 mμ and 520 mμ measured. Porcine heparin (generously provided by Dr. F. McIntyre of Abbott Laboratories, Chicago—c.f. ref. 2) was used as a standard. Since samples of heparan sulfate vary in their N-sulfate/N-acetyl ratios, all values have been calculated as heparin equivalents.

Results. The values for total GAG per mg of creatinine fell along an age dependent regression line (Fig. 1) reasonably close to those previously determined by Rich, diFerrante and Archibald(6) and by Teller *et al* (3). A similar age dependence for GAG N-sulfate hexosamine excretion exists (Fig. 2). Low values for N-sulfate GAG in Fig. 2, particularly when associated with low creatinine levels, represent concentrations in the limiting range of sensitivity of the assay, so that the reliability of such values is low. The ratio of N-sulfate GAG (heparin equivalents) to total GAG in this series varied between 0 and 0.26 with a mean of .076 and a standard deviation of .060. The relative contribution of heparan sulfate and heparin to the total N-sulfated GAG found in the urine of



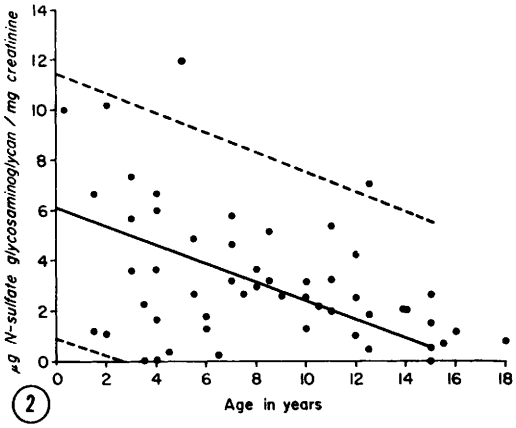


FIG. 1. Glycosaminoglycan excretion in pediatric patients without mucopolysaccharidosis. Concentrations were calculated from uronic acid determinations. The equation for the regression line is [GAG] = −4.4 [Age] + 81. Dotted lines include ± 2 standard deviations (the square root of the error variance estimate) about the regression line, as calculated from all the deviations from the regression line. Coefficient of correlation is −.67.

FIG. 2. N-sulfate glycosaminoglycan excretion in pediatric patients without mucopolysaccharidosis. Concentrations are determined as heparin equivalents. The equation for the regression line is [N-Sulfate GAG] = −.37 [Age] + 6.2. Dotted lines include ± 2 standard deviations (the square root of the error variance estimate) about the regression line, calculated as for Fig. 1. Coefficient of correlation is −.51.

normal children is not known(cf. 7).

Determinations on urine obtained from the bladder at autopsy were performed on 3 patients with diagnosed mucopolysaccharidosis. One 24-hour specimen from a patient with Type II mucopolysaccharidosis was also studied. All of these patients had total GAG concentrations at least 10 times the average normal age corrected value (Table I). N-sulfate GAG concentrations were also greatly elevated in the 3 patients and the N-sulfate GAG/total GAG ratio did not differ significantly among the several cases (Table I).

Two sisters clinically diagnosed as Scheie's syndrome (Type V mucopolysaccharidosis) provided several 24-hour specimens (Table II). Although a 3-fold variation in total GAG excretion per 24 hours was observed, all the values were more than 2 standard deviations greater than the mean age-corrected normal value. N-sulfate GAG concentrations were also consistently elevated (Table II). Both parents of the patients with Scheie's syndrome and 2 siblings had normal urinary levels of GAG and N-sulfate GAG (Table II).

Discussion. The identification and quantitation of heparan sulfate in urine has previously depended on either 1) measurements

TABLE I. Total and N-Sulfate Glycosaminoglycan in Urine of Patients with Mucopolysaccharidoses. The clinical type refers to McKusick *et al*'s classification(1).

Patient	Sex	Age	Mucopoly-saccharidosis, clinical type	Urine sample	Total glycosaminoglycan μg/ml	Total glycosaminoglycan μg/mg creatinine	N-sulfate glycosaminoglycan (heparin equivalents) μg/ml	N-sulfate glycosaminoglycan (heparin equivalents) μg/mg creatinine	2 (N-sulfate GAG)/total GAG*
Du	♀	3	I (Hurler)	Bladder†	2100		357		.34
Hu	♂	11	I (Hurler)	" †	865	3400	136	522	.31
LeM	♂	11	II (Hunter)	24 hr	246	532	40	87	.32
McG	♂	22	II (Hunter)	Bladder†	664	664	150	150	.45

* The N-sulfate GAG/total GAG ratio is multiplied by 2 to give an approximation of heparan sulfate/total GAG(2,13).

† Post mortem.

TABLE II. Total and N-Sulfate Glycosaminoglycan in 2 Patients with Type V (McKusick *et al*)(1) and Unaffected Family Members. Specimens analyzed were 24 hr collections.

Patient	Sex	Age	Date	Total glycosaminoglycan		N-sulfate glycosaminoglycan (heparin equivalents)		2 (N-sulfate GAG)/total GAG*
				μg/ml	μg/mg creatinine	μg/ml	μg/mg creatinine	
JDe	♀	10	2/13	173	154	19.5	17.4	.23
			2/15	108	106	20.5	20.1	.38
			2/16	152	332	24.8	53.9	.33
MDe	♀	11	2/13	301	304	38.7	39.0	.26
			2/15	386	263	58.5	39.0	.30
			2/16	218	179	33.4	27.4	.31
Parents								
De	♂	51		21.1	15.8	1.9	1.4	.18
De	♀	49		20.1	24.6	1.9	4.6	.38
Siblings								
DiDe	♀	7½		14.1	30.8	.9	2.0	.12
DeDe	♀	7½		38.3	53.2	1.7	2.4	.08

* The N-sulfate GAG/total GAG ratio is multiplied by 2 to give an approximation of heparan sulfate/total GAG(2,13).

of carbazole/orincol or carbazole/naphthoresorcinol ratios(8), 2) partial separation of GAGs by alcohol precipitation(9) or chromatography(10,11), or 3) determination of glucosamine/galactosamine ratios(12). The orcinol and naphthoresorcinol tests are not specific for N-sulfate GAG, but because of the greater absorbance resulting from the reaction with dermatan sulfate than with heparan sulfate, low ratios indicate the presence of heparan sulfate. The separation procedures are relatively laborious and not entirely satisfactory because of heterogeneity of GAGs with respect to sulfation, molecular weight and covalently-bound peptides. The recently reported electrophoretic separation (13) of dermatan sulfate and heparan sulfate may prove useful but has not yet been thoroughly tested.

Since only heparin and heparan sulfate of the GAGs contain glucosamine, measurement of the glucosamine/galactosamine ratio provides yet another method of measuring heparan sulfate. The procedure requires hydrolysis of the GAGs and subsequent chromatography on columns, paper or thin layer materials. Hydrolysis of N-sulfated GAG is notoriously difficult and both large losses of hexosamine and incomplete hydrolysis occur.

The test described here for N-sulfate GAG is probably simpler than the other measures of heparan sulfate and is suitable for routine diagnostic laboratories. One drawback is the known variability of N-sulfate content of heparan sulfate(14) making it impossible to determine the absolute heparan sulfate concentration on the basis of N-sulfate GAG measurement. We have adopted the expedient of expressing heparan sulfate in terms of heparin equivalents. Heparin itself has not been found in excess in the urine in any of the mucopolysacchardioses.

It is generally agreed that in Types I and II mucopolysaccharidosis both dermatan and heparan sulfate are excreted in excess, while dermatan sulfate is excreted exclusively in Type VI and heparan sulfate in Type III. Terry and Linker(9) have reported data indicating that the excretion patterns of dermatan sulfate and heparan sulfate in Type I and Type II mucopolysaccharidosis differ quantitatively; Type I patients, according to their findings, excrete roughly 4 times as much dermatan sulfate as heparan sulfate, whereas Type II patients excrete approximately equal quantities of the two. The results reported here are not consistent with such a difference; however, it is conceivable that the discrepancy in results could arise from variation in the degree of N-sulfation of heparan sulfate in the two syndromes, since Terry and Linker measured glucos-

amine/galactosamine ratios, and we have measured N-sulfate hexosamine.

Three patients with Scheie's syndrome have been reported to excrete an excess of dermatan sulfate only in their urine(1), but determinations in another laboratory(9) on 2 of these patients have indicated the presence of heparan sulfate as well as dermatan sulfate. The two sisters in the present series excrete excessive quantities of both dermatan sulfate and heparan sulfate.

Summary. An assay for N-sulfate hexosamine has been adapted to the determination of the urinary excretion of N-sulfated glycosaminoglycans. Values were obtained for N-sulfate glycosaminoglycan concentrations in the urine of 56 pediatric outpatients without clinical signs of mucopolysaccharidosis. Abnormal levels of N-sulfate glycosaminoglycan were found in 6 cases of mucopolysaccharidosis.

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In vitro Porphyrin Synthesis by Iron Deficient Erythrocytes.* (32362)

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The synthesis of heme-containing compounds, hemoglobin, myoglobin and a variety of enzymes, is restricted in the iron deficient state(1). This is not solely the result of a lack of iron for incorporation into protoporphyrin to form heme. The associated anemia is characterized by erythrocytes with a low hemoglobin content. Despite an excess of free erythrocyte protoporphyrin in iron deficiency (2), when the hemoglobin deficit is considered, the total amount of porphyrin produced per erythrocyte is reduced. Adequate concentration of iron appears to be necessary for optimal heme biosynthesis(3,4). Erythrocytes from humans with clinical iron deficiency are as efficient *in vitro* as normal cells in augmenting the conversion of δ aminolevulinic acid to porphobilinogen. This implies normal activity of δ aminolevulenic acid dehydrase. Iron deficient cells, however, were found to be less adequate than normal as a source of necessary enzymes for the synthesis of uro and coproporphyrinogens from δ aminolevulinic acid(5).

In the experiments to be reported here the addition of iron to the *in vitro* incubation mixtures did not augment porphyrin synthesis by iron deficient erythrocytes. However, administration of iron salts to iron deficient patients greatly increased porphyrin synthesis from δ aminolevulinic acid by their erythrocytes *in vitro*.

Materials and methods. Using δ aminolevulinic acid as substrate, porphyrin production was measured *in vitro* by methods previously described(5). Coproporphyrin and protoporphyrin were determined by a modifi-

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