Sulfate Utilization in Normal and Diabetic Female Rats[1] (35279)

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Numerous studies have indicated that the incorporation of sulfate into mucopolysaccharides is altered in diabetes (1–4), and it has been suggested that insulin has a regulatory action on sulfation (5). In a pervious communication, we reported that the enzymatic uptake of labeled sulfate into chondroitin sulfate was stimulated with low concentrations and noncompetitively inhibited with higher concentrations of soluble enzyme prepared from livers of diabetic male rats (6). It was subsequently demonstrated that the enzymatic formation of the active sulfate carrier phosphoadenosine-phosphosulfate (PAPS) was enhanced with low concentrations (<0.5 mg) and depressed with higher concentrations (>1.0 mg) of enzyme from pancreatectomized male rats (7). However, since sex differences in ester sulfate formation have been reported (8–10), indicating that sulfotransferase activity is greater in females than in males (11), the present experiments were performed to study the uptake of labeled sulfate into intermediate nucleotides and chondroitin sulfate in female rats, and to determine the effect of diabetes on these reactions in the female animal. Evidence is presented indicating that while the overall reaction is more efficient in normal female than in normal male rats, the uptake of sulfate-^{35}S into PAPS and chondroitin sulfate is still inhibited at higher concentrations of enzyme from diabetic female rats.

Materials and Methods. Enzyme preparation. A 95% pancreatectomy was performed on female white rats (Institute strain) weighing 80–120 g, and the development of diabetes was tested by blood sugar levels after 7 hr fasting. Operated animals were sacrificed when diabetes was well established (blood sugar 150–200 mg/100 ml), and were matched with control animals of the same age and sex. Soluble enzyme systems from livers of normal and diabetic rats were prepared as described by Hilz and Lipmann (12), through removal of the microsomes. The 30,000 rpm supernatant was used as enzyme source after measurement of protein content (13).

Incubations. The standard incubation mixture consisted of: 40 μm Tris–HCl, pH 7.5; 6 μm $MgCl_2$; 5 μm K_2ATP; 5 μm glucose; 5 μm cysteine–HCl; 50 μg chondroitin sulfate (Sigma Chem. Co.); 10 μCi (^{35}S)Na_2SO_4 (sp act 358 mCi/mmole); and 0.25–5.0 mg of enzyme protein as indicated in a total volume of 1.0 ml. Incubations were carried out for 2 hr at 37° using air as the gas phase. Following incubation, the samples were placed in boiling water for 1 min and then centrifuged at low speed to remove coagulated protein.

Separation of products. a. Labeled chondroitin sulfate. 1.0 ml each of 1% cetylpyridinium chloride (CPC) and 0.1% chondroitin sulfate (CS) were added to the low-speed supernatant, and the resultant precipitate was washed four times with 5 ml of 0.026 *M* Na_2SO_4. After the last wash, the final CPC-CS precipitate was dissolved in 1.0 ml of the following solution: *n*-propanol–glacial acetic acid–methanol–water (40:15:20:38.5, v/v) and 0.2-ml duplicate aliquots were pipetted directly into 5 ml of Bray's solution and counted in a Packard Tri-Carb scintillation counter. This procedure has been previously described (6).

b. Nucleotide intermediates. The low-speed supernatant was diluted with 3 vol of water, and the nucleotides were adsorbed by the addition of 1 ml of 5% suspension of charcoal. The charcoal precipitate was washed two times with 0.026 *M* Na_2SO_4 and the nucleotides were then eluted with pyridine. The samples were dried in an air stream and the

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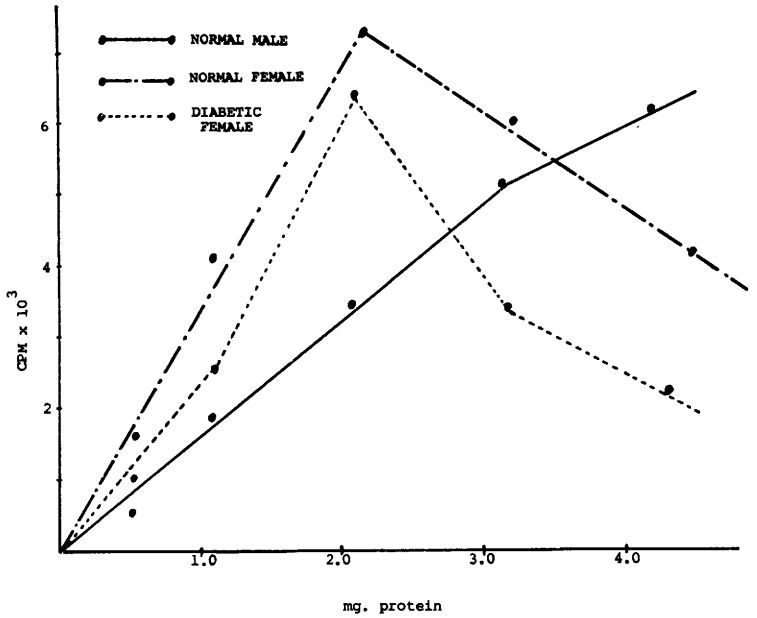

FIG. 1. Uptake of ^{35}S into chondroitin sulfate by normal and diabetic rat liver preparations. Conditions: standard reaction mixture as described in text, with 10 μCi of ^{35}S Na_2SO_4, incubated for 2 hr at 37°. Each value is the mean of 6 observations.

residue was taken up in 0.2 ml of water and subsequently applied as a streak to Whatman 3MM chromatography paper. All chromatography paper had been previously prepared by washing with 10% Na_2CO_3, 2 *N* acetic acid, 10^{-3} *M* EDTA, and distilled water (14). Samples were chromatographed overnight in a solvent consisting of 7.5 vol of ethanol, 3 vol of ammonium acetate buffer, pH 3.8, and 10^{-3} *M* in EDTA buffer, pH 7.4 (7, 15). Since appropriate spots were not always detectable with UV light due to low concentrations, the entire paper was cut at 1 cm intervals from the origin to the solvent front. One half of these 1 cm strips were placed into 7 ml of Bray's solution for determination of radioactivity, and the other half was eluted with 2 ml of water for determination of nucleotide content (absorption at 260 mμ). This chromatographic separation of nucleotide products after incubation with hepatic enzyme has been described (7).

Results. Figure 1 shows the results of incubations using increasing concentrations of enzyme prepared from normal and diabetic female rats; results with enzyme from normal male rats are given for comparison. Reaction rate was linearly related to enzyme concentration up to 2 mg of protein and then decreased with increasing concentrations of protein using preparations from both normal and diabetic female rats. Compared to values obtained using normal male preparations, velocity was significantly increased at 2 mg of enzyme protein from both normal and diabetic females, and significantly decreased at 4 mg of diabetic female protein.

Formation of $PAP^{35}S$ was linearly related to protein concentration up to 2 mg and then declined with increasing protein concentration using enzyme from normal female animals. In contrast, formation of $PAP^{35}S$ was enhanced with low concentrations (0.5 mg) but abruptly inhibited with higher concentrations (>1.0 mg) of enzyme protein from diabetic females. These findings were confirmed by calculation of the specific activities of $PAP^{35}S$ formed at the various enzyme concentrations employed (Table I).

Formation of adenosine phosphosulfate (APS), the nonactive sulfate intermediate, linearly increased with up to 2.0 mg of enzyme protein from normal or diabetic females, and was then moderately decreased

TABLE I. $PAP^{35}S$ Formation in Incubations with Normal and Diabetic Hepatic Enzyme.[a]

Preparation	Enzyme protein (mg)	cpm	Sp act (cpm/μg of nucleotide)
Normal	0.25	1010	20
	0.50	1550	107
	1.00	2710	195
	2.00	3400	171
	3.00	1100	102
	4.00	900	70
Diabetic	0.25	810	105
	0.50	4080	309
	1.00	1920	155
	2.00	1250	62
	3.00	700	85
	4.00	560	24

[a] Conditions: Standard incubation mixture as described in text; 10 μCi of ^{35}S-Na_2SO_4; incubated for 2 hr. Each value is the mean of 4 observations.

with higher protein concentrations. Specific activity of the $AP^{35}S$ formed at each protein concentration of diabetic enzyme was not significantly different from that of normals (Table II).

Discussion. Chondroitin sulfate is among a wide variety of sulfated compounds for which a common metabolic pathway exists (16–18), involving displacement by sulfate of pyrophosphate in adenosine triphosphate (ATP) to form adenosine phosphosulfate (APS), followed by phosphorylation of APS by the terminal phosphate of another ATP to form phosphoadensine-phosphosulfate (PAPS), and transfer to sulfate from PAPS to the acceptor molecule (19, 20).

The data presented here indicate that the uptake of labeled sulfate into chondroitin sulfate is more efficient in female than in male animals. Since specific activity of the active sulfate carrier $PAP^{35}S$ formed in incubations with female hepatic enzyme approximated that previously seen using male preparations (7), it would appear that it is the transfer of radioactive sulfate from $PAP^{35}S$ to chondroitin sulfate that is enhanced in female animals. This is in agreement with other investigations which have demonstrated that sulfotransferase activity is greater in livers of female than male animals (8, 10, 11).

Although the stimulation of radioactive sulfate uptake into chondroitin sulfate which had been previously observed at low concentrations of soluble enzyme prepared from diabetic male rats was not seen in the current experiments using diabetic female rats, enhancement of $PAP^{35}S$ formation at low concentrations was again demonstrated in the diabetic state. Presumably, the already efficient sulfotransferase system in female animals, whether normal or diabetic, was not further stimulated by the increased $PAP^{35}S$ substrate formed in incubations with low concentrations of diabetic female protein.

The inhibition of $PAP^{35}S$ and chondroitin sulfate-^{35}S formation seen with higher protein concentrations of diabetic female enzyme corresponds to that which was previously demonstrated in diabetic male preparations, further confirming the existence of an alteration in APS-phosphokinase activity in diabetes.

The enhancement of sulfate incorporation at low concentrations of diabetic enzyme protein suggests a source of stimulation which is unmasked in the absence of insulin. Growth hormone profoundly influences sulfate metabolism (21, 22), and numerous complex interrelationships *in vivo* between growth hormone and insulin are now recog-

TABLE II. $AP^{35}S$ Formation in Incubations with Normal and Diabetic Hepatic Enzyme.[a]

Preparation	Enzyme protein (mg)	cpm	Sp act (cpm/μg of nucleotide)
Normal	0.25	270	10
	0.50	1530	48
	1.00	5300	97
	2.00	4900	296
	3.00	3320	283
	4.00	3320	181
Diabetic	0.25	550	15
	0.50	2380	44
	1.00	3760	155
	2.00	4600	330
	3.00	3970	305
	4.00	4040	150

[a] Conditions: Standard incubation as described in text; 10 μCi of ^{35}S-Na_2SO_4; incubated for 2 hr. Each value is the mean of 4 observations.

nized (23, 24). The control of sulfation may be another of these interactions. Previous work has indicated that in the absence of either insulin or growth hormone, the incorporation of sulfate-^{35}S into aortic mucopolysaccharides is increased 4 hr after an intraperitoneal injection of radioactive sulfate; whereas in the hypophysectomized-pancreatectomized animal, sulfate-^{35}S incorporation returns to a regular pattern of steady increase over a 6-hr period (5). While diabetes results in an overall depression of sulfation, the role of insulin in this process may be regulatory in nature, and other factors may stimulate this same metabolic site, perhaps in a competitive manner. Further work is in progress to determine the nature of the stimulatory and inhibitory factors involved in these reactions.

Summary. The formation of the nucleotide intermediates adenosine-phosphosulfate (APS) and phosphoadenosine-phosphosulfate (PAPS), and the uptake of radioactive sulfate into chondroitin sulfate was examined using hepatic enzyme preparations from normal and pancreatectomized female rats. It was found that the uptake of ^{35}S into PAPS and chondroitin sulfate is inhibited at high concentrations of enzyme from diabetic female rats, while PAP^{35}S formation is stimulated at lower concentrations of diabetic enzyme. Chondroitin sulfate-^{35}S formation is enhanced in female animals, probably due to greater sulfotransferase activity in the female than in the male.

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