The Influence of Thyrotropin on Harderian Gland Glycosaminoglycans of the Mouse[1] (35078)

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In experimental exophthalmos induced by injections of anterior pituitary extract containing thyrotropin, an increase in glycosaminoglycans content of the retrobulbar tissue has been shown by biochemical and radioautographic techniques (1, 2) but there is disagreement regarding the precise class of glycosaminoglycans. Whereas thyrotropin increased (nonsulfated) hyaluronic acid of the retrobulbar tissue of the guinea pig (3) and dog (4), similar investigations involving measurement of ^{35}S uptake were considered to reflect an effect of thyrotropin upon sulfated glycosaminoglycans (2, 5).

Many of the studies of experimental exophthalmos have used as a model the guinea pig harderian gland, since it constitutes most of the retro-ocular tissue, and its increase in size is a major factor in the proptosis induced by the administration of pituitary extract. Recently we reported that the harderian gland of the mouse is sensitive, in terms of ^{35}S-sulfate uptake, to thyrotropin (6), and the present study was undertaken to extend those observations by characterizing the fate of radiosulfate in the harderian gland under the influence or in the absence of thyrotropin.

Materials and Methods. Female albino mice[4] weighing 15–18 g were used in groups of four and the effect of thyrotropin on ^{35}S uptake by the harderian gland was measured as previously described (6). The mice were fed LID[5] (Remington type, General Biochemical) for 10 days, then 10 μg Na 1-thyroxine was injected in 0.1 ml 0.9% NaCl sc; thyroid USP was added to the diet to 0.06% concentration, and the animals were used 4 days later. Some experiments were done without giving thyroid hormone to the animals. To measure uptake of ^{35}S, the mice were injected with 40 μCi carrier-free Na ^{35}S-sulfate in 0.2 ml 0.9% NaCl solution ip and thyrotropin in 0.2 ml 1% HSA was given simultaneously; control mice received ^{35}S-sulfate and 1% HSA. The mice were killed by exsanguination under ether anesthesia 6, 12, or 24 hr after the injection of thyrotropin and ^{35}S. The harderian gland from each eye was removed and dissected free of adventitia; portions of salivary gland and pancreas were also obtained. In view of previous observations (6) most of the experiments were done when 10 mU thyrotropin was given with radiosulfate 6 hr before removal of the tissues.

To study the fate of radiosulfate in the harderian gland, both glands from each mouse were pooled and weighed and then twice extracted for 4 hr with acetone (5 ml/20 mg tissue) and dried *in vacuo* over P_2O_5. A portion, approximately 2 mg of the resulting powder, was twice extracted by suspension in

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[4] Mice were obtained from Carworth Inc., New City, N.Y. Sodium ^{35}S-sulfate was purchased from Charles E. Frosst & Co., Montreal, P.Q. Canada.

[5] Abbreviations used are: LID, low-iodine diet; HSA, human serum albumin; TCA, trichloracetic acid; ip, intraperitoneal.

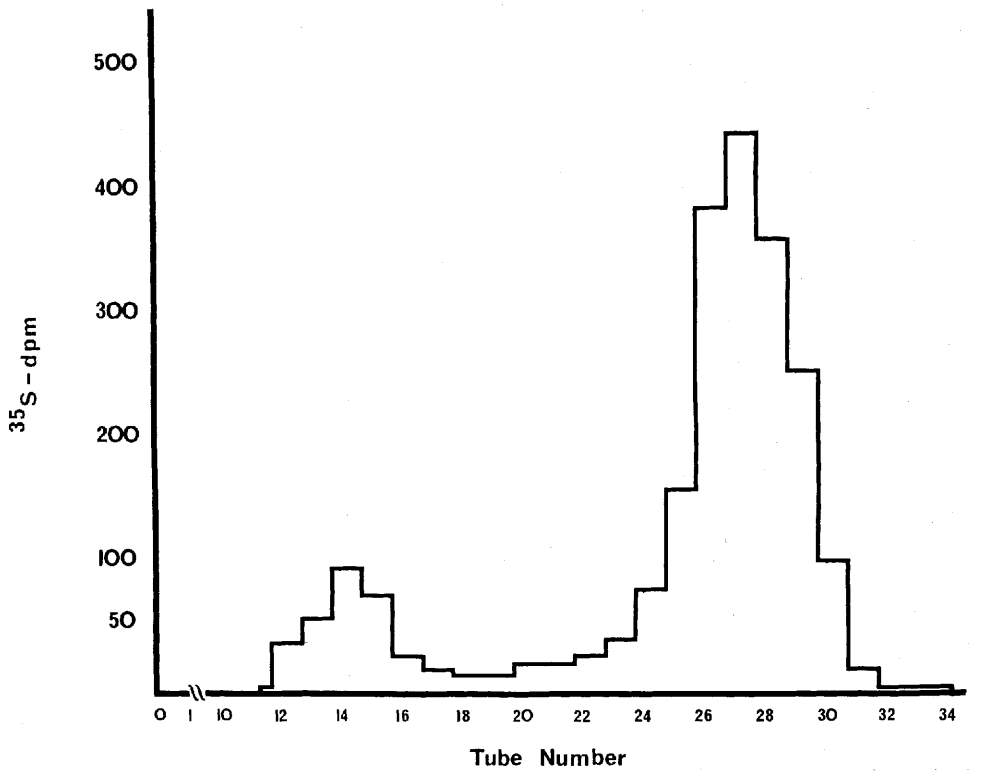

FIG. 1. Chromatography on Sephadex G-25 (1 × 15 cm) of NaOH extract of pooled harderian glands from a mouse. ^{35}S was injected ip 6 hr before the glands were removed. An aliquot (0.2 ml) of NaOH extract was applied to the column and eluted at room temperature with distilled water. Effluent was collected in 0.5-ml fractions and radioactivity was determined in a liquid scintillaton counter as described in the text.

0.5 *N* NaOH in the cold with occasional mixing for about 12 hr. An aliquot of the supernatant fluid was filtered on Sephadex G-25, thus separating inorganic ^{35}S-sulfate from that associated with larger molecules.

To study the effect of thyrotropin on the incorporation of radiosulfate into glycosaminoglycans 40 μCi Na ^{35}S-sulfate with or without 10 mU thyrotropin was given ip 6 hr before removal of the harderian glands. Pooled glands from four mice were cut into 0.2-mm pieces with a McIlwain chopper before being weighed. The tissue was defatted with several changes of acetone over 48 hr, dried to a constant weight over P_2O_5 *in vacuo,* and then digested with papain for 16 hr at 65° (7). The digests were cooled to 4°, TCA was added to give a final concentration of 5% and after 10 min the insoluble material was washed twice with 5% TCA. Glycosaminoglycans were recovered as insoluble potassium salts after addition to the pooled supernatant solutions of 3 vol of 5% potassium acetate in ethanol, and storage overnight at 4°. The precipitate was washed with ethanol, ethanol–ether, ether, and dried *in vacuo.* The residue was dissolved in 0.1 ml 0.075 *M* NaCl; an aliquot was used for uronic acid determination (8) and another aliquot of the dissolved glycosaminoglycans was applied to a cellulose column for fractionation by a modified method of Svejcar (9) [as described previously (6)] wherein there is stepwise elution of various categories of glycosaminoglycans.

Results. By Sephadex G-25 chromatography of a NaOH extract of harderian gland, ^{35}S was in two eluted fractions. The results of a representative experiment are shown in Fig. 1; one fraction (varying from 70–90%

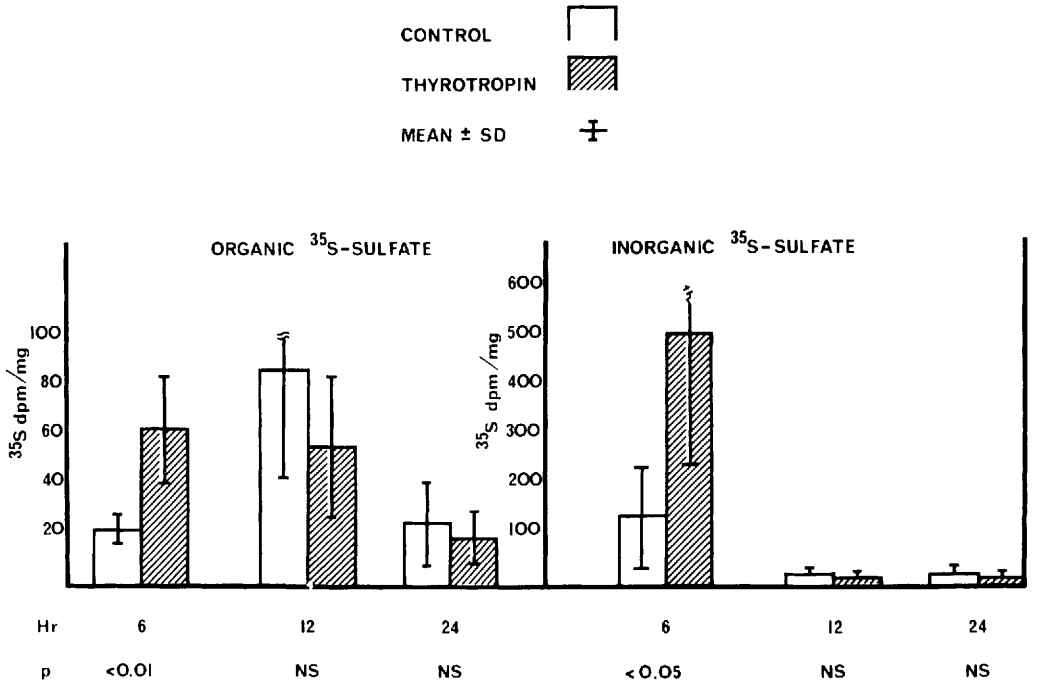

FIG. 2. ^{35}S–sulfate in the mouse harderian gland at various time intervals after simultaneous administration of radiosulfate and 10 mU thyrotropin ip. Fractionation of NaOH extracts was as described in the legend to Fig. 1. Mean and 2 SD of observations with four mice are shown in each instance. Diet: LID + thyroid (see text).

of the total ^{35}S) had an elution volume corresponding to inorganic sulfate and the other, presumed to be organically bound radiosulfate, was eluted at the void volume; when the excluded (*i.e.*, presumed organic) fraction was dialyzed against 1% HSA for 24 hr, 91.1 ± 12.6% of the radioactivity was retained inside the dialysis bag. Similar patterns were obtained with NaOH extracts of pancreas and salivary gland.

Figure 2 shows that a single injection of 10 mU thyrotropin produced a statistically significant increase in ^{35}S content of both organic ($p < .01$) and of inorganic ($p < .05$) sulfate. The thyrotropin response was evident only at 6 hr after the injection; when there was a greater interval before removing the gland no response to thyrotropin occurred. Approximately a 2-fold increase in radioactivity in both inorganic and organically incorporated ^{35}S was obtained with 10 mU thyrotropin and there was no further increase with higher doses (Fig. 3). Salivary gland and pancreas, taken from the mice given 10 mU thyrotropin, that provided the data shown in Fig. 2, were less responsive in terms of accumulating and incorporating ^{35}S-sulfate (Table I). The mean value for both inorganic and organic ^{35}S was increased in both tissues but the spread of values was such that only with organic radioactivity in salivary gland was there statistical significance.

By pooling eight harderian glands from four mice at a time, reproducible estimates of glycosaminoglycans as uronic acid were possible. In four experiments with pooled glands uronic acid (4.5–9.7 μg) was 0.064–0.077% of the weight of dried defatted tissue that ranged from 7.0–12.5 mg. Thyrotropin (10 mU) increased significantly the concentration of glycosaminoglycans; uronic acid values (μg/mg dry weight, mean ± SD) were 0.8 ± 0.07 for control and 1.1 ± 0.06 for thyrotropin-injected ($p < .01$). These results were obtained only with animals given thyroid hormone (see Methods) although thy-

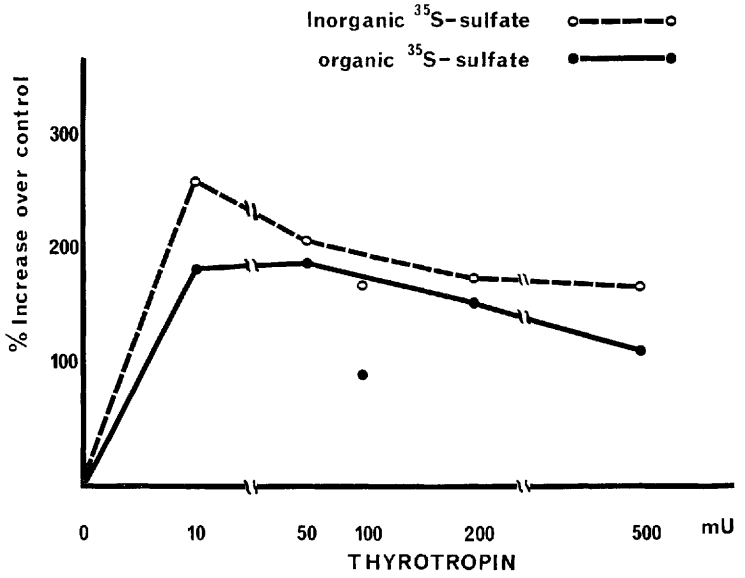

FIG. 3. Effect of various doses of thyrotropin on ^{35}S-sulfate distribution in harderian gland of mice. Thyrotropin was injected simultaneous with radiosulfate ip 6 hr before the glands were removed. Fractionation of NaOH extract was as described in the legend to Fig. 1. Mean value of observations with four mice is shown in each instance. Diet: LID + thyroid (see text).

TABLE I. Fractionation on Sephadex G-25 of NaOH Extract of Harderian Gland, Salivary Gland, and Pancreas of Mice After Administration of ^{35}S-sulfate and 10 mU Thyrotropin.

	^{35}S dpm/mg (mean ± SD)					
	Harderian gland		Salivary gland		Pancreas	
Sephadex fraction	Organic	Inorganic	Organic	Inorganic	Organic	Inorganic
Control	22 ± 6[a]	139 ± 108[b]	28 ± 8[e]	185 ± 122	62 ± 44	138 ± 66
Thyrotropin	63 ± 20[d]	508 ± 207[e]	108 ± 59[f]	426 ± 291	98 ± 50	357 ± 430

Radiosulfate and 10 mU thyrotropin were injected simultaneously ip 6 hr before the tissues were removed. Fractionation of NaOH extract of each tissue was carried out on Sephadex G-25 and radioactivity of each fraction was expressed as dpm per mg of wet weight. Each result is of analysis from four mice.

p. [a] vs [d] $<.01$; [b] vs [e] and [e] vs [f] $<.05$ by Student's t test.

roid had no significant effect *per se* on the concentration of uronic acid. With mice not given thyroid hormone, the effect of thyrotropin on the distribution of ^{35}S within fractions of glycosaminoglycans was to enhance labeling of keratan sulfate only, but, as reported previously (6), when mice were given thyroid hormone, thyrotropin enhanced ^{35}S incorporation into heparatan sulfate and dermatan sulfate fractions also.

Discussion. Anterior pituitary extract containing thyrotropin stimulates ^{35}S-sulfate uptake by the harderian gland of the mouse (6)[6] and guinea pig (11). As reported here most of the ^{35}S taken up is inorganic sulfate but some radiosulfate is incorporated organically. A single injection of thyrotropin in-

[6] As a further check on the specificity of this effect of thyrotropin we recently tested the influence of placing the tube containing the solution of thyrotropin in boiling water for 5 min. This resulted in complete loss of thyroid-stimulating activity (10) and also of the effect of ^{35}S-sulfate uptake by mouse harderian gland.

creased ^{35}S content of both fractions and according to uronic acid and ^{35}S assays, the organic sulfate was at least partly in glycosaminoglycans. We previously reported (6)that thyrotropin enhanced the blood concentration of ^{35}S in a similar experimental preparation but this is probably not the sole basis for enhanced labeling of glycosaminoglycans since the concentration of uronic acid also increased.

Recently Sisson and Miles reported (3) that in the guinea pig harderian gland thyrotropin enhanced hyaluronic acid and had little effect on other glycosaminoglycans. Our experiments involved ^{35}S assay of glycosaminoglycans fractions and so would not reflect alterations in nonsulfated glycosaminoglycans like hyaluronic acid and heparin. Therefore, the increase we found in uronic acid, that represents sulfated as well as nonsulfated glycosaminoglycans, may reflect an influence on both moieties.

A generalized effect of thyrotropin on connective tissue was suggested 20 years ago (12) and our observations that there were some effects on salivary glands and pancreas similar to those on harderian glands would be in line with this possibility. The biological significance of these effects, particularly in relation to exophthalmos, remains obscure.

Summary. The nature of ^{35}S sulfate accumulated by the harderian gland of untreated and thyrotropin-treated mice was characterized by Sephadex G-25 and cellulose-column chromatography. Most of the ^{35}S remained inorganic but some was organically incorporated; a single injection of 10 mU thyrotropin increased the radiosulfate content of both moieties and higher doses of thyrotropin had no greater effect. Salivary gland and pancreas showed similar trends of response to thyrotropin. In mice pretreated with thyroid hormone, thyrotropin (as reported previously) increased ^{35}S incorporation into sulfated glycosaminoglycans, especially keratan sulfate and dermatan sulfate, in harderian gland; under these conditions there was also an increase in total uronic acid content.

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