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BIOSYNTHESIS OF HEPARIN

EVIDENCE FOR THE TRANSFER OF RADIOACTIVE SULFATE TO SMALL-MOLECULAR-WEIGHT ACCEPTORS

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SUMMARY

The incorporation of [^{35}S]sulfate into the heparin fraction was studied in a high-speed supernatant from the Furth mast-cell tumor. The rate of incorporation was found to be linear over a 45-min period. Preincubation prior to the addition of carrier-free [^{35}S]-sulfate caused a marked decrease in labeling of the heparin fraction.

These results were interpreted to indicate that a dialyzable sulfate acceptor system was depleted during the preincubation period. The data support the hypothesis that sulfate is incorporated primarily into small-molecular-weight heparin precursors which are then polymerized to form heparin.

INTRODUCTION

The incorporation of sulfate into heparin by sub-cellular preparations of mouse mast-cell tumors has been shown to involve transfer from PAPS[1-4]. The nature of the carbohydrate acceptor is not known, but three general types may be considered. (a) Sulfate may be transferred to a hexose derivative which in turn is built into a polymerizing oligosaccharide. This possibility is supported by the identification of uridine diphosphate *N*-acetylgalactosamine sulfate in polysaccharide-producing hen oviducts[5] and by the demonstrated sulfation of small-molecular-weight hexose derivatives[6]. (b) Sulfate may be transferred to a polymer chain after each addition of unsulfated residue[7]. And finally, (c) sulfate may be transferred to a fully formed mucopolysaccharide molecule. This latter possiblity is suggested by the work of a number of investigators[2,8-14]. This communication presents evidence that the first of these mechanisms is responsible for a significant degree of sulfate incorporation into heparin in a soluble enzyme system derived from the Furth mast-cell tumor.

Abbreviation: PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

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EXPERIMENTAL

Procedures for preparation of a high-speed supernatant fraction from the Furth mast-cell tumor, incubation of the supernatant fluid with [^{35}S]sulfate, and isolation of two sulfate-containing fractions from the incubated samples have been described in an earlier communication[15]. The principal component of Fraction I was heparin. Fraction II included the heparin fraction and one or more dialyzable substances not characterized as yet.

The experimental conditions are shown in Fig. 1 and in the tables. In some of the samples, carrier-free [^{35}S]sulfate was added at the beginning of the experimental period, or "zero" time. Other samples were brought to 37° at "zero" time, and incubated for 15 or 30 min without added sulfate; the carrier-free [^{35}S]sulfate was then added at the end of this "preincubation period". The time between the addition of radioactive sulfate and termination of the experiment will be referred to as the "incubation period". The concentrations of enzymes, substrates and various adjuvants were identical in all cases, regardless of the time at which the [^{35}S]sulfate was added.

RESULTS

The time course of [^{35}S]sulfate incorporation into Fractions I and II of samples incubated for periods of from zero to 120 min is shown in Fig. 1. There was a linear increase in radioactivity in Fraction I during the first 45 min; the rate of incorporation was somewhat reduced during the remainder of the incubation. Fraction II was labeled to a much greater degree; the incorporation reached a peak in about 30 min and then declined gradually during the subsequent 90 min.

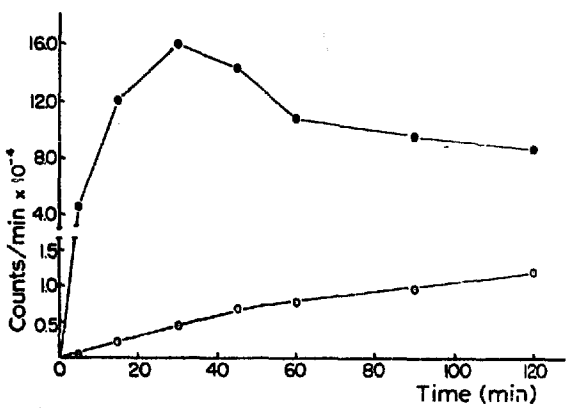

Fig. 1. Time course of [^{35}S]sulfate incorporation into Fractions I (⊙—⊙) and II (●—●) in incubations of a high-speed supernatant derived from mouse mast-cell tumor. Each incubation sample contained in a total volume of 0.5 ml; high-speed supernatant from 50% Furth-tumor homogenate, 0.13 ml; [^{35}S]sulfate, carrier-free, $4.5 \cdot 10^6$ counts/min; Tyrode's solution (pH 7.4), 125 μl; and the following substances in μmoles: Tris buffer (pH 7.4), 26; phosphate buffer (pH 7.4), 4.0; ATP, 1.0; $MgCl_2$, 0.81; cysteine, 0.41; niacinamide, 0.5; L-glutamine, 0.5; and DPN, 0.025. The incubation was carried out at 37° in a Dubnoff metabolic shaker.

Preincubation for 15 min prior to [^{35}S]sulfate addition caused a substantial reduction in isotope incorporation into Fraction I during subsequent incubations of 15 and 45 min (Table I). There was also a significant reduction in the amount of label incorporated into Fraction II of the same samples. A 30-min preincubation resulted in even lower uptake of [^{35}S]sulfate during subsequent incubation periods.

Since treatment of preincubated samples was identical to that of the samples incubated from zero time with radioactive sulfate, except that during preincubation only endogenous sulfate was present, it is relevant to compare the uptake of [^{35}S]sulfate into the heparin fraction in these samples during corresponding time intervals (Table II). For example, by subtracting the radioactivity incorporated during a 15-min incubation from that incorporated during 30 min (samples incubated with labeled sulfate from zero time, Table I), it can be determined that 2350 counts/min were incorporated during the 15- to 30-min interval. In contrast, the sample which had been preincubated for 15 min incorporated only 537 counts/min during the corresponding time, *i.e.*, the

TABLE I

INCORPORATION OF [^{35}S]SULFATE INTO FRACTIONS I AND II DURING VARIOUS INCUBATION PERIODS. EFFECT OF PREINCUBATION

Each sample was treated as described in Fig. 1.

No.	*Preincubation period (min)*	*Incubation period* (min)*	*Incorporation of radioactive sulfate (total counts/min)*	
			Fraction I	*Fraction II*
1	0	15	2200	120 000
2	15	15	537	58 900
3	0	45	6970	143 000
4	15	45	2020	73 700
5	0	30	4550	160 000
6	30	30	620	12 000
7	0	90	9730	95 300
8	30	90	765	9 770

* [^{35}S]Sulfate was added at the beginning of this period.

TABLE II

INCORPORATION OF [^{35}S]SULFATE INTO FRACTION I (HEPARIN FRACTION) DURING CERTAIN TIME INTERVALS OF THE EXPERIMENTAL PERIOD. EFFECT OF PREINCUBATION

Each sample was treated as described in Fig. 1.

Time interval (min)*	*Net radioactivity incorporated into Fraction I during time interval** (total counts/min)*		
	No preincubation	*15 min preincubation*	*30 min preincubation*
15–30	2350	537	—
15–60	5690	2020	—
30–60	3340	1480	620
30–120	7650	—	765

* Refers to interval of the overall experimental period. Samples were brought to 37° at zero time.

** [^{35}S]Sulfate was added at start of incubation period either at zero time (no preincubation) or after the indicated preincubation period.

following 15-min period. Similarly, a large decrease in sulfate incorporation into the heparin fraction occurred in samples preincubated for 30 min.

In order to determine whether one of the agents in the cofactor mixture might be depleted during the early part of the incubation, a second dose of the mixture was added to some of the preincubated samples at the time of addition of [^{35}S]sulfate. The results are shown in Table III. The addition of cofactors produced only a negligible stimulation of incorporation of radioactivity into the heparin fraction in the samples preincubated for 15 min. After a 30-min preincubation a somewhat greater effect was observed, particularly when the period of incubation with [^{35}S]sulfate was increased to 90 min. On the other hand, the uptake of radioactive sulfate into Fraction II was markedly stimulated by a second cofactor addition. In samples which had been preincubated for 15 min, additional cofactors almost doubled [^{35}S]sulfate incorporation during subsequent incubations for 15 or 45 min; this uptake was of the same order as that observed in the samples which were incubated with the label for 15 or 45 min from zero time (Table I, Nos. 1 and 3, and Table III). Samples preincubated for 30 min showed an even greater response to the cofactor mixture, although the level of incorporation reached was not so high as in samples which had not been preincubated.

TABLE III

EFFECT OF A SECOND ADDITION OF COFACTORS ON THE INCORPORATION OF [^{35}S]SULFATE INTO FRACTIONS I AND II

Each sample was treated as described in Fig. 1, except that, where indicated, a second addition of the following cofactor mixture (CF) was made: ATP, 1.0 μmole; L-glutamine, 0.5 μmole; niacinamide, 0.5 μmole.

Preincubation time (min)	*Incubation time (min)*	*Incorporation of radioactive sulfate (total counts/min)*			
		Fraction I		*Fraction II*	
		— CF	*+ CF*	*— CF*	*+ CF*
15	15	537	640	58 900	103 000
15	45	2020	2310	73 700	134 000
30	30	620	951	12 000	88 300
30	90	765	2720	9 770	58 900

DISCUSSION

The data presented above were interpreted to indicate that in the experimental system used here, only a minor portion, if any, of ^{35}S-labeled heparin formation is accomplished through direct transfer of radioactive sulfate to a fully formed mucopolysaccharide molecule. This interpretation arises from the following considerations.

The time study shows that in samples not preincubated, formation of ^{35}S-labeled heparin fraction proceeded linearly for close to 60 min. On the other hand, a short preincubation prior to [^{35}S]sulfate addition resulted in a substantial decrease in incorporation of radioactivity into the heparin fraction. This reduction might be explained on the basis of one of the following processes (occurring during preincubation): (1) accumulation of unlabeled active sulfate (PAPS), resulting in a subsequent dilution of [^{35}S]PAPS; (2) depletion of sulfate-activating system; or (3) depletion of sulfate acceptor system.

It is considered highly unlikely that the first process played a significant role, because addition of a second dose of cofactors, including ATP, at the time of [^{35}S]-sulfate addition markedly increased the uptake of label into Fraction II but not into Fraction I (heparin fraction). If accumulation of unlabeled active sulfate during preincubation had resulted in a subsequent dilution of [^{35}S]PAPS, then additional cofactors should have modified incorporation of the label to the same extent in both Fractions I and II.

The possibility that the observed effects of preincubation (in particular, the decreased [^{35}S]sulfate incorporation into Fraction I, Table II) might be due to depletion of the sulfate-activating system is ruled out by the fact that treatment of the preincubated samples was identical to that of the samples incubated from zero time with [^{35}S]sulfate, except that during preincubation only endogenous sulfate was present. Thus, any changes in the sulfate-activating system must have occurred to the same extent in both the preincubated samples and in those incubated with radioactive sulfate from zero time.

It would appear, therefore, that it is the sulfate acceptor system which is influenced (by being depleted) during preincubation. The data suggest the early incorporation of sulfate into dialyzable molecules which are subsequently polymerized to form heparin. The effect of preincubation is interpreted to indicate that a limiting factor in the formation of ^{35}S-labeled heparin is the sulfate acceptor system, probably involving relatively small molecules.

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