The formation of adenosine-3′-phosphate-5′-phosphosulfate in extracts of chick embryo cartilage and its conversion into chondroitin sulfate*

During the last few years, work in this laboratory has clarified the mechanism of sulfate activation[1,2,3]. The biological sulfate carrier, "active sulfate", identified as adenosine-3′-phosphate-5′-phosphosulfate, PAPS, is enzymically formed in a two-step reaction:

$$ATP + S \rightleftarrows APS + PP \quad (1)$$

$$APS + ATP \rightarrow PAPS + ADP \quad (2)$$

PAPS acts as a sulfate donor in various sulfate acceptor systems. We wish to report here on the synthesis of PAPS in particle-free extracts of chick embryo cartilage and its conversion there to chondroitin sulfuric acid. In the whole animal and in slices, the incorporation of ^{35}S-sulfate into cartilage had previously been studied by DZIEWIATKOWSKI[4], LAYTON[5] and by BOSTRÖM[6] and his group[7].

Radioactive PAPS was prepared enzymically from radioactive sulfate as described by ROBBINS AND LIPMANN[2]. The condyles of the tibias and the femurs of 15-day old chick embryos, weighing approximately 100 mg per chick were mixed in a cold mortar with sand and homogenized with a pestle in calcium and magnesium-free saline-phosphate solution. Chondroitin sulfuric acid was isolated from the boiled centrifuged incubate by alcohol[8] or cetyl trimethyl ammonium bromide[9] precipitation and the residual ^{35}S removed by exhaustive washing.

First, a series of experiments was carried out with cartilage extracts and radioactive sulfate. Formation of PAP^{35}S together with small amounts of AP^{35}S was shown by radioautography of paper electrographs. The paper electrophoresis pattern shown in Fig. 1 was obtained with a pyridine eluate of a charcoal adsorbate from the incubate. The actual amounts of PAPS formed with the carrier-free sulfate were too small to be detected by U.V. quenching, but are identified by the position of the radioactive spots. An unidentified compound is indicated by the radioactive spot below the PAP^{35}S.

In further experiments, incorporation of $^{35}SO_4^{-2}$ into chondroitin sulfate was observed, but only in the presence of ATP and magnesium, as shown in Table I. Only a slight stimulation was observed if glutamine was added (*cf.*[7]). UTP in this system did not stimulate further but indeed rather showed an inhibitory effect, probably owing to excess of nucleotide. A *de novo* synthesis of chondroitin sulfuric acid from the component parts was further confirmed by incorporation of radioactive acetate into chondroitin sulfate with this extract. As shown in Table II, again only in the presence of ATP, a sizable incorporation of acetate was obtained. Omission of CoA significantly reduced the incorporation of acetate. In Table III, an experiment is shown in which, with the same extract, both incorporation of acetate and of sulfate into chondroitin sulfate were obtained. In these experiments, CoA was present in all samples.

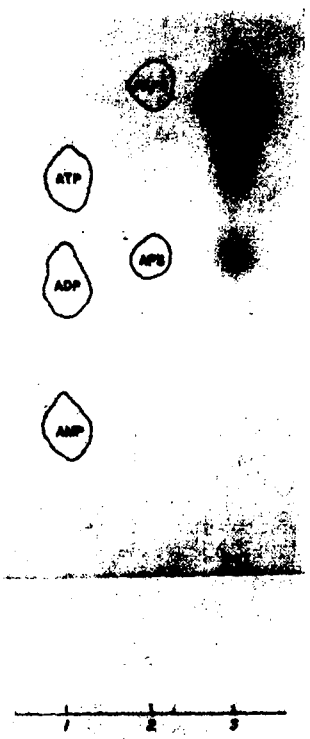

Fig. 1. Radioautogram of paper electrogram from $^{35}SO_4^{-2}$-incubated cartilage extract. Two embryos were homogenized in 3 ml saline phosphate to which 12 μmoles Mg^{++}, 10 μmoles Na-ATP, 1.5 μmoles UTP, 2 μmoles glutamine, 2.7 ml enzyme and 2 ml $^{35}SO_4^{-2}$ (100 μc, carrier-free) were added; total volume 5.3 ml. Incubated for 2 h at 37°. Markers electrographed on the same paper strip are identified in Nos. 1 and 2 by U.V. quenching, as indicated by encircling lines. No. 3 represents the radioautogram of electrogram in citrate buffer[1] of the pyridine eluate from a charcoal adsorbate of incubate.

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The following abbreviations are used: PAPS, adenosine-3′-phosphate-5′-phosphosulfate; APS, adenosine-5′-phosphosulfate; S, sulfate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; CoA, coenzyme A; UTP, uridine triphosphate; and PP, pyrophosphate.

TABLE I

INCORPORATION OF $^{35}SO_4^{-2}$ INTO CHONDROITIN SULFATE

No.	Incubation (37°) h	ATP 5 μmoles	Mg 6 μmoles	Glutamine 1 μmole	UTP 1 μmole	c.p.m.	%
1	0	–	–	–	–	60	
2	2	–	–	–	–	60	
3	2	+	–	–	–	405	
4	2	+	+	–	–	4,760	0.6
5	2	+	+	+	–	5,390	0.7
6	2	+	+	+	+	2,007	0.28

Condyles from tibias and femurs of 3 chick embryos broken up in deep-freeze cooled mortar with quartz sand with 6 ml saline, 0.01 *M* phosphate (pH 7.4). Centrifuged at 6,000 *g* (International centrifuge) for 10 min. The supernatant was mixed with 1.2 ml carrier-free $^{35}SO_4^{-2}$ (60 μc); 1 ml enzyme-^{35}S mixture in each sample; final volume, 1.35 ml.

The enzyme solution and addenda were incubated in stoppered test tubes in a 37° water bath, and after incubation dipped for 1 min into a boiling water bath. The heating caused only some cloudiness. To the cloudy solution 6 ml water and 0.5 ml 1% cetyl trimethyl ammonium bromide[9] were added. The precipitate that formed was washed 10 times with 5 ml 0.1 *M* Na_2SO_4. From samples 3 and 4, chondroitin sulfuric acid has been extracted with 0.9 *M* KCl, purified as described by SCOTT[9] and by KODICEK AND LOEWI[10] for paper electrophoresis, in 0.1 *M* phosphate, pH 6.8. For counting, the whole precipitate was transferred into planchets and counted in Tracerlab scaler.

TABLE II

SYNTHESIS OF CHONDROITIN SULFATE FROM ^{14}C-ACETATE

No.	Incubation (37°) h	ATP 2 μmoles	Mg^{++} 6 μmoles	l-Glutamine 2 μmoles	CoA 14 units	Cysteine 5 μmoles	c.p.m.
1	0	+	+	+	+	+	80
2	2	+	+	+	+	+	1,640
3	2	–	+	+	+	+	300
4	2	+	–	+	+	+	1,170
5	2	+	+	+	–	–	700
6	2	+	+	+	–	+	540

Condyles from tibias and femurs of four 16-day old chick embryos were extracted with 5 ml phosphate buffer, 0.1 *M*, pH 7.4, as described in Table I. Centrifuged for 10 min at 6,000 *g* in the International centrifuge and the supernatant recentrifuged in the Spinco for 45 min at 105,000 *g*. 6 ml of supernatant mixed with 0.6 ml 1-^{14}C-acetate (12 μmoles, 7 μc/μmole). 1 ml of enzyme-acetate mixture for each sample in final volume of 1.22 ml.

Chondroitin sulfuric acid was isolated[8] by precipitation with alcohol; 0.2 ml saturated solution of sodium acetate was added to each sample followed after heating by 3 volumes absolute alcohol. Precipitate was washed 12 times with 5 ml 80% alcohol.

A sample of chondroitin sulfuric acid was isolated from the experiments shown in Table III and electrographed. A radioautogram of the electrogram showed the radioactivity situated at the site of the chondroitin sulfuric acid as developed by staining with toluidine blue[10].

The transfer of sulfate from PAP^{35}S to chondroitin sulfuric acid is shown in Table IV. The addition of ATP stimulated greatly this incorporation into the polysaccharide. UTP gave a slightly higher incorporation than ATP. Addition of glutamine did not further stimulate, indicating that with the energy donor present in excess, this system appears to be saturated with glutamine.

Generally, in these experiments, extracts were used obtained by centrifugation in the International centrifuge. However, it was found that these incorporations are catalyzed by the supernatant fraction after centrifugation at 105,000 *g* in the Spinco. The extracts may also be frozen without appreciable loss of activity. It is concluded, therefore, that the chondroitin sulfate synthesis reported here is due to a soluble system without participation of particulate material. So far, only extracts of *embryonic* cartilage showed significant effects.

TABLE III

PARALLEL SYNTHESIS OF CHONDROITIN SULFATE FROM ^{14}C-ACETATE AND $^{35}SO_4^{-2}$

Incubation (37°) h	^{14}C-acetate (2 μmoles)	^{14}C-acetate	SO_4^{-2} (2 μmoles)	$^{35}SO_4^{-2}$	c.p.m.
0	+	-	+	+	1,350
2	-	-	+	-	6,100
2	-	-	+	+	5,400
0	-	+	+	-	125
2	-	+	+	-	1,890
2	-	+	-	-	1,890

Condyles from tibias and femurs of four 16-day old chick embryos were homogenized, as was described, in 7 ml phosphate buffer, pH 7.4. 3.5 ml enzyme mixed with 0.3 ml containing 6 μmoles $^{35}SO_4^{-2}$ (42 μc) and another 3.5 ml enzyme solution mixed with 0.3 ml containing 6 μmoles ^{14}C-acetate (42 μc). Each sample contained 1 ml enzyme-isotope mixture, 2 μmoles ATP, 6 μmoles Mg^{++}, 2 μmoles glutamine and 14 units CoA in a total of 1.32 ml and with $31 \cdot 10^6$ counts per sample. Chondroitin sulfuric acid was isolated as in Table II. In this experiment all samples were counted in gas flow counter (Nuclear).

TABLE IV

SULFATE TRANSFER FROM PAP^{35}S TO CHONDROITIN SULFATE

Incubation (37°) h	PAP^{35}S	ATP 1 μmole	UTP 1 μmole	c.p.m.	1	% incorporation
0	-	-	-	80		
2	+	-	-	340	260	1.7
2	+	+	-	600	520	3.4
2	-	-	+	670	590	4
2	+	+	-	550	470	3

Enzyme preparation from three chick embryos, as in the previous experiments. Extracted with 0.5 ml saline-phosphate solution and centrifuged. The supernatant was mixed with 0.25 ml PAP^{35}S and 1 ml immediately heated (zero-time). Each tube contained 1 ml enzyme in 1.35 ml total and 15,000 counts. Precipitation with Na acetate and 3 volumes of alcohol, 10 washings with 80% alcohol as in Table II.

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