STUDIES ON THE INCORPORATION OF S\textsuperscript{35}-SULFATE INTO CHARONINSULFURIC ACID BY CHARONIA LAMPAS

By SAKARU SUZUKI

(From the Department of General Education, Nagoya University, Nagoya)

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Recently Egami and coworkers (1) reinvestigated a new type of glucan polysulfate (charoninsulfuric acid) which was isolated from the mucus of Charonia lampas (Tritonium nodiferum), and showed that a part of the glucan has a cellulose structure and the other part an amylase structure. It was found in this experiment that a close relation exists between the sulfur content of charoninsulfuric acid and the constitution of the glucan, i.e. the less the sulfur content the less the cellulose structure. This parallelism implies that the biological transformation of amylase to cellulose may be preceded or accompanied by the sulfation, and hence the sulfation of charoninsulfuric acid is of particular interest.

The problems concerning the biological sulfation of polysaccharides in different mammalian tissues have been dealt with by several workers. Bostrom and Måansson (2) demonstrated that S\textsuperscript{35}-sulfate is retained in slices of cartilage of calf, and the major portion of the labeled sulfate is present in chondroitin sulfate. Sato and coworkers (3) demonstrated a similar uptake of sulfate in heparin by the liver of rats.

According to our study (4) on the incorporation of S\textsuperscript{35}-sulfate into different organs of Chalonia lampas, remarkably high concentrations of S\textsuperscript{35} are recognized in the mucous gland. It thus seems most reasonable to assume that the biological sulfation of charoninsulfuric acid occurs in the mucous gland. The present in vivo and in vitro investigations were undertaken to determine whether or not S\textsuperscript{35}-sulfate enters into charoninsulfuric acid of the mucous gland.

EXPERIMENTAL

In Vivo Experiments—Six groups of Charonia lampas, each group comprising four Charonia weighing 120 to 160 g. (without shell), were injected intramuscularly with S\textsuperscript{35}-sulfate in 0.2 ml. of distilled water (5×10\textsuperscript{6}...
c.p.m. per sq.cm. measured as benzidine sulfate at infinite thickness). After immersing the test animals in a sea of 20°, the respective groups were killed at different times; the first group after 3 hours, the last one after 69 hours. The mucous glands were removed and immediately placed in absolute methanol. After 4 days these were defatted with acetone and dried in vacuo. Charoninsulfuric acid was obtained from this preparation of each group as follows: about 10 g. of the dried mucous gland was ground to a powder and extracted with 150 ml. of 1.7 per cent HCl at 40° for 40 minutes. The clear extract was neutralized with KOH to salt out potassium charoninsulfate, which was then dissolved in 1 per cent HCl and reprecipitated with an equal volume of ethanol. After repetition of this salting out and precipitation procedures, the precipitate was dissolved in 50 ml. of distilled water and the last traces of S$_{35}$-sulfate were removed by electrodialysis. The dissolved charoninsulfuric acid was then precipitated with ethanol, washed with absolute ethanol and ether and dried in vacuo. About 200 mg. of each of the different preparations thus obtained were hydrolyzed with a mixture of conc. HNO$_3$ and HCl (1:3) in a boiling water bath, and after evaporation the sulfate was precipitated as the benzidine salt from aqueous solutions. The benzidine sulfate was washed with 50 per cent and 95 per cent aqueous acetone successively, suspended in 2 ml. of absolute ethanol and then transferred to a small porcelain dish of a diameter of 2.5 cm. After drying, the radioactivity was measured by means of a Geiger-Müller counter. Each sample had a thickness of 17 mg. per sq.cm., which corresponded approximately to infinite thickness. Under such conditions the number of counts is linearly proportional to the activity. The results are expressed as counts per sq. cm. The error in measuring the radioactivity of the sample amounted to 8 per cent.

The possibility that the samples of charoninsulfuric acid obtained under this experiment were contaminated with free S$_{35}$-sulfate need not be considered because of the following fact: 200 mg. of pure charoninsulfuric acid (S = 15 per cent) and S$_{35}$-sodium sulfate ($5 \times 10^6$ c.p.m.) dissolved in 150 ml. of 1.7 per cent HCl were treated as above; it was then found through the measurement of the radioactivity that the charoninsulfuric acid thus obtained was free from the labeled sulfate.

In Vitro Experiments—The mucous gland of newly killed Charonia was sliced to a thickness of approximately 0.5 mm. with a safety razor blade. About 35 g. each of the slices, which contain large amounts
of charoninsulfuric acid, were quickly transferred to three vessels containing 100 ml. of sea water as the basic medium. The vessels were shaken in a thermostat at 30°. One of the samples (No. I) was taken out and boiled for 20 minutes, cooled, and returned to the thermostat. When the temperature of this sample was 30°, S\textsuperscript{35}-sodium sulfate (3 × 10\textsuperscript{6} c.p.m. per sq. cm.) in 1 ml. of distilled water were added to each sample and the reaction was allowed to proceed at 30°. Samples II and III were removed after 4 and 8 hours respectively, and boiled for 20 minutes. Each suspension was acidified with 5 ml. of conc. HCl, and charoninsulfuric acid was extracted as described in the preceding experiment. About 200 mg. each of the charoninsulfuric acid samples I to III were hydrolyzed with a mixture of conc. HNO\textsubscript{3} and HCl, and the sulfate was precipitated as the benzidinie salt for the determination of the radioactivity.

RESULTS

The results of the sulfur and nitrogen analyses are given in Table I. The sulfur values, being relatively high, indicate that it must be

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<th>Sample No.</th>
<th>Per cent of dry substance</th>
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<td>III</td>
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excluded in this paper to make discussions on the charoninsulfuric acid with a low sulfur content. The nitrogen values indicate that small amounts of protein are present in all samples; but this contamination need not be taken into consideration, since the amounts of sulfur-con-
taining amino-acids are negligible.

In Fig. 1 the radioactivities measured in the in vivo experiments are plotted against time in hours after injection. As can be seen, the curve shows an increase in the radioactivity from 50 c.p.m. per sq. cm. after 3 hours to 825 c.p.m. after 41 hours and then a slow increase to 857 c.p.m. after 69 hours. A decrease in the radioactivity, i.e. elimination of labeled sulfur from the charoninsulfuric acid, could not be observed, since Charonia could not be kept alive beyond 3 days after injection.

![Figure 1](image)

**Fig. 1.** Uptake of radioactive sulfate in charoninsulfuric acid of mucous gland.

Counts per minute per sq. cm. as found in measuring radioactivity plotted against time in hours.

The results of radioactivity measurements in the experiment performed with slices are shown in Table II. Even when no substrates have been added, slight uptake of $S^{35}$-sulfate was observed after 8 hours, while there was no significant uptake of $S^{35}$ if the slice was boiled prior to the addition of the isotope. As the amounts of $S^{35}$ fixed within 8 hours
were small, a clear-cut result regarding the effect of several inhibitors has not been obtained. It was impossible to incubate the reaction mixtures more than 8 hours owing to the putrefaction of the slices. Additions of 0.02 M glucose, 0.03 M phosphate or 0.5 per cent charoninsulfuric acids with different sulfur contents (S=2,5 and 15 per cent) did not accelerate the reaction.

**DISCUSSION**

From the consideration of these results and those obtained with autoradiography (4), it seems most reasonable to conclude that biological sulfation of charoninsulfuric acid occurs in the mucous gland, and that the sulfate ion in sea water is a natural source of the sulfate group of charoninsulfuric acid.

In the studies on the conjugation of sulfate with phenols, it has been demonstrated by several workers (5-6) that the preliminary step of the conjugation is an enzymatic activation of inorganic sulfate by ATP. Recently Hilz and Lipmann (7) have suggested that the active sulfate corresponds to a mixed anhydride between sulfate and phosphate, the phosphate being most likely linked to adenosine. Their suggestion must be useful for the studies on the biological sulfation of polysaccharides.

**SUMMARY**

1. The uptake of S$^{35}$-sulfate into the charoninsulfuric acid of the mucous gland of Charonia lampas has been studied by in vivo and in vitro experiments.

2. An increase in the radioactivity of charoninsulfuric acid was
demonstrated during 69 hours after intramuscular injection of S\textsuperscript{35}-sulfate.

3. Sodium sulfate labeled with S\textsuperscript{35} has been found to be taken up by the slice of the mucous gland and built into charoninsulfuric acid. In the boiled mucous gland no similar uptake occurs.

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REFERENCES

(2) Boström, H., and Månsson, B., *J. Biol. Chem.*, 196, 483 (1952)