# THE CONVERSION OF CHOLESTEROL-$^{3}$H-SULFATE-$^{35}$S INTO PREGNENOLONE-$^{3}$H-SULFATE-$^{35}$S BY SONICATED BOVINE ADRENAL MITOCHONDRIA

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This paper describes the results of in vitro experiments which prove that bovine adrenal mitochondrial preparations that are disrupted by sonication can efficiently convert cholesterol sulfate into pregnenolone sulfate. Intact mitochondria fail to effect this conversion.

Cholesterol sulfate, which occurs in a variety of mammalian tissues (Drayer et al., 1964, 1965, 1967 and Moser et al., 1966), has been shown in an in vivo experiment to serve as a precursor, without separation of the sulfate radical, of several sulfated urinary metabolites of adrenal secretory products. When cholesterol sulfate labeled with $^{3}$H and $^{35}$S was injected by catheter into the blood supplying an adrenal carcinoma, it was possible to isolate from the urine of the patient sulfates of various 3β-hydroxy-$\Delta^{5}$ steroid metabolites which bore a $^{3}$H/$^{35}$S ratio similar to that of the administered sterol conjugate (Roberts et al., 1964). By this in vivo experiment, the previous demonstration that steroid sulfates could serve as biosynthetic intermediates (Calvin et al., 1963; Calvin and Lieberman, 1964) was extended to include cholesterol

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sulfate. However, subsequent attempts in this and in other laboratories to study the sulfate pathway using in vitro techniques with cholesterol sulfate as a reactant have been unsuccessful. When, in this laboratory, the doubly-labeled sterol conjugate was incubated with hog adrenal mince, with whole homogenates of human adrenal tumors, or with a mitochondrial suspension made from normal human adrenal tissue, no identifiable $C_{19}$ or $C_{21}$ product could be isolated although, from one experiment, 7-ketocholesterol sulfate was characterized and 7α-hydroxycholesterol sulfate was tentatively identified. Raggatt and Whitehouse (1966) observed that the isooctyl side-chain of cholesterol sulfate was cleaved by mitochondria from bovine adrenals yielding isocaproic acid. The steroid fragment was not characterized although it probably was pregnenolone sulfate since the preparation was devoid of sulfatase.

Several other in vivo experiments have been reported and each has revealed that circulating cholesterol sulfate is not an important precursor for the synthesis of adrenal secretory products. Gurpide et al. (1966), by injecting radioactive cholesterol sulfate intravenously, observed that little or none of the circulating conjugate was converted into the major secretory products of the adrenals, cortisol or dehydroisoandrosterone sulfate. LeBeau and Baulieu (1966) observed that cholesterol, administered intravenously, is a more efficient precursor of urinary steroid metabolites than is its sulfate. Moreover, even a mid-term human fetus, which elaborates huge quantities of sulfurylated steroids, is unable to utilize circulating cholesterol sulfate when it is presented to the fetus by perfusion (Solomon et al., 1967).

As was pointed out by Gurpide et al. (1966), the apparent discrepancy between the findings of Roberts et al. (1964) and those of others might be explained by assuming that circulating cholesterol sulfate is not easily transported into adrenal cells and that only intracellular cholesterol sulfate serves as an intermediate for the biogenesis of de-

hydroisoandrosterone sulfate. The success of Roberts et al. (1964) in demonstrating the conversion of cholesterol sulfate into dehydroisoandrosterone sulfate by the in situ perfusion of an adrenal tumor may have been due to the well-recognized "leakiness" exhibited by some cancer cells. To test this possibility, the capacity of mitochondria whose membranes were ruptured by sonication to effect the conversion of cholesterol sulfate into pregnenolone sulfate was investigated.

Experiment 1. The adrenal cortices from twelve bovine glands were chopped finely in a Waring blender and then homogenized in 0.25 M sucrose solution in a glass homogenizer. The method used for the isolation and sonication of the mitochondria has been reported by Cooper et al. (1965) and by Simpson and Boyd (1966). The incubation medium, containing 0.1 M phosphate buffer, pH 7.4, magnesium ion and a TPNH-generating system, was identical with that described by Simpson and Boyd (1966). Following centrifugation of the sonicate at 105,000 x g for 30 minutes, the resultant supernatant was divided into two portions. To the first half (A) was added 110 µg of doubly-labeled cholesterol sulfate ($2 \times 10^6$cpm $^3$H, $0.6 \times 10^6$cpm $^{35}$S). To the second portion (B) was added 0.7 µg of cholesterol 4-$^{14}$C ($1.4 \times 10^6$cpm $^{14}$C). Both portions were incubated at 37°C in a Dubnoff shaker during which time air was bubbled constantly through each. After 70 minutes, the contents of each flask were poured into 3 volumes of ethanol. The precipitate was removed by filtration and discarded. The solvents were evaporated to dryness and each residue was chromatographed on a reversed-phase chromatographic system using 50 gm of Celite as support (System 1: methanol 4: n-propanol 1: water 1.3: toluene 2: isooctane 2). This column served the dual purpose of removing lipid as well as the unused labeled cholesterol in B. Chromatographic analysis of the residue obtained from A on System 1 led to the elution of the sterol sulfate and the more polar metabolites in the early fractions. To remove the unmetabolized substrate, the residue

from the early fractions was rechromatographed on a Celite partition system designed for the isolation of cholesterol sulfate (System 2: isooctane 4: ethyl acetate 1: tertiary butanol 2: methanol 2: 1 M $NH_4OH$ 3), which is eluted in the fourth to fifth hold-back volume. The radioactive material remaining on the column was eluted with methanol and was rechromatographed on a Celite partition column using System 3 (isooctane 8: ethyl acetate 12: tertiary butanol 6: methanol 6: 1 M $NH_4OH$ 9) in which pregnenolone sulfate is isolated in the fifth hold-back volume. The material in this fraction contained 209,000 cpm $^3H$ and 46,000 cpm $^{35}S$ which, uncorrected for procedural losses, represents a yield of 10%.

A one-quarter aliquot of this, doubly-labeled product, was diluted with 10 mg of carrier ammonium pregnenolone sulfate and the mixture was rechromatographed in System 3. One peak of radioactive material was eluted in the fifth hold-back volume. The curves obtained by plotting the fraction number against the counts of $^3H$, the counts of $^{35}S$ and the weight as evidenced by the methylene-blue reaction, were superimposable. The radioactive material in the peak was further diluted with carrier (15 mg) and the mixture recrystallized twice from methanol:acetone. The crystallization data are recorded below. The specific activities of the

CRYSTALLIZATION DATA* OF AMMONIUM PREGNENOLONE SULFATE

| | Experiment 1 | | | Experiment 2 | | |
|---|---|---|---|---|---|---|
| | $^3H$ | $^{35}S$ | $^3H/^{35}S$† | $^3H$ | $^{35}S$ | $^3H/^{35}S$† |
| 1st Crystallization | 1863 | 401 | 4.66 | 2820 | 636 | 4.43 |
| Mother Liquor | 1851 | 398 | 4.65 | 2854 | 640 | 4.45 |
| 2nd Crystallization | 1894 | 404 | 4.68 | 2762 | 614 | 4.49 |
| Mother Liquor | 1960 | 426 | 4.60 | 2836 | 638 | 4.44 |

* Specific activities in cpm/mg.
† These ratios were identical with the ratio of the incubated sterol sulfate when the latter was corrected for isotopic decay of the $^{35}S$ which occurred during the course of these experiments.

products indicated that the only radioactive metabolite isolated from the chromatogram using System 3 was pregnenolone sulfate.

The extract from (B), in which cholesterol 4-$^{14}$C was the substrate, was chromatographed on System 1. The early fractions, containing the more polar metabolites, were combined, diluted with carrier pregnenolone and rechromatographed on System 4 (isooctane:90% methanol). Both the radioactivity (341,000 cpm $^{14}$C) and the carrier were eluted in the sixth hold-back volume. This represents a yield of 25%, uncorrected for procedural losses. Radiochemical homogeneity was established by recrystallization to constant specific activity.

In the absence of knowledge of the concentrations of endogenous cholesterol and cholesterol sulfate, a comparison of the yield in this experiment with that obtained in A (10%) is of undetermined significance. It is clear that, with sonicated mitochondria, both substrates were converted into their respective $C_{21}$ products efficiently.

Experiment 2 was carried out to compare the results obtained from intact mitochondria with those from sonicated mitochondria when doubly-labeled cholesterol sulfate was the substrate. When sonicated mitochondria were incubated for only 15 minutes, doubly-labeled pregnenolone sulfate was isolated, using procedures described above, in 2.3% yield (see Table for crystallization data). In accord with our previous experience, no radioactive pregnenolone sulfate was isolated when intact mitochondria were employed.

These experiments suggest that cholesterol sulfate approaching intact mitochondria from the outside cannot penetrate into the sites where the enzymes necessary to convert the sterol sulfate into pregnenolone sulfate reside.

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