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SIDE-CHAIN CLEAVAGE OF CHOLESTEROL SULFATE BY OVARIAN MITOCHONDRIA

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Summary—Mitochondria isolated from porcine corpora lutea and from the luteinized ovaries of gonadrotropin-treated immature rats were found to efficiently cleave the side-chain of cholesterol sulfate to produce 3β-hydroxy-5-pregnen-20-one sulfate (pregnenolone sulfate). When mitochondria were preincubated with cholesterol sulfate, the time-course for the side-chain cleavage of cholesterol sulfate was biphasic. With 200 μM cholesterol sulphate, the initial rate of the reaction was the same as that observed for 25-hydroxycholesterol. This rate was not increased when both cholesterol sulfate and 25-hydroxycholesterol were incubated together. The rate of side-chain cleavage by isolated mitochondria supplied with 75 μM cholesterol sulfate as substrate was inhibited by 97% by aminoglutethimide, a specific inhibitor of cytochrome P-450$_{scc}$. The slow phase of side-chain cleavage of cholesterol sulfate appeared to be limited by the rate of substrate movement to the mitochondrial site of the reaction. Cholesterol sulfate translocation rates were however up to 8 times greater than those observed for cholesterol when equivalent concentrations of the two substrates were added to the mitochondria. We conclude that cholesterol sulfate is a better substrate than cholesterol for side-chain cleavage by isolated mitochondria and that both reactions are catalysed by the same cytochrome P-450$_{scc}$ enzyme.

INTRODUCTION

Conversion of cholesterol into pregnenolone, termed cholesterol side-chain cleavage, occurs in the mitochondria of steroid producing tissues and is catalysed by cytochrome P-450$_{scc}$ [1–6]. There appears to be only one gene for cytochrome P-450$_{scc}$ which is expressed in the adrenal cortex, ovary, testis and placenta [4–6]. Mitochondria from the adrenal cortex can also catalyse the side-chain cleavage of cholesterol sulfate to yield pregnenolone sulfate [7–14]. Hochberg *et al.* [7] have reported that about 10% of the pregnenolone isolated from the bovine adrenal cortex is the sterol sulfate. There are conflicting reports in the literature regarding the role of cytochrome P-450$_{scc}$ in the conversion of cholesterol sulfate into pregnenolone sulfate. Greenfield *et al.* [11] and Greenfield and Parsons [12] have shown that their preparation of purified bovine adrenal cytochrome P-450$_{scc}$ can catalyse the conversion of cholesterol sulfate into pregnenolone sulfate. However, Lambeth *et al.* [13] did not detect side-chain cleavage of cholesterol sulfate by purified cytochrome P-450$_{scc}$ incorporated into phospholipid vesicles. Young and Hall [9], working with partially purified bovine adrenal cytochrome P-450$_{scc}$, concluded that cholesterol side-chain cleavage of cholesterol sulfate occurs via a different cytochrome P-450 isoenzyme than that which converts cholesterol into pregnenolone.

Lambeth *et al.* [13] and Xiang and Lambeth [14] have reported that in mitochondria from the rat adrenal gland, cholesterol sulfate is an inhibitor of cholesterol translocation to the inner-mitochondrial membrane site of the cholesterol side-chain cleavage reaction. They also found an inverse relationship between endogenous cholesterol sulfate concentrations in the mitochondria and the rate of cholesterol conversion into pregnenolone, suggesting a regulatory role for cholesterol sulfate in steroidogenesis.

Cholesterol sulfate is present in the rat ovary and in concentrations comparable to the adrenal gland [15]. In the present study we have measured the side-chain cleavage of exogenous cholesterol sulfate by mitochondria isolated from porcine corpora lutea and the ovaries of immature rats treated with gonadotropins. We have found that cholesterol sulfate is a better substrate than cholesterol for side-chain cleavage by isolated ovarian mitochondria and all evidence indicates the cleavage of both substrates occurs via the same enzyme.

EXPERIMENTAL

Materials

Antipregnenolone antibody was obtained from Radioassay Systems Laboratories (Carson, Calif.). Cholesterol sulfate, cholesterol, 25-hydroxycholesterol, pregnenolone, $NADP^+$ and DL-isocitrate (trisodium salt) were from Sigma, (St Louis, Mo.). Cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one) was a gift from the Sterling-Winthrop Research Institute (Rensselaer, N.Y.). [4-^{14}C]-Cholesterol (58 mCi/mmol) and [4,7-^{3}H]pregnenolone (11 Ci/mmol) were purchased from the Radiochemical Center, Amersham, England.

Tissue preparation

Female, Wistar albino rats, aged 20 days, were injected subcutaneously with 50 IU of pregnant mare's serum gonadotropin and 54 h later with 25 IU of HCG [16]. Rats were killed 6 days after the first injection and ovaries, comprising mainly corpora lutea, were pooled from 10–20 animals for each experiment. Porcine ovaries were collected from the abattoirs and corpora lutea from at least 10 ovaries were dissected out [17]. Mitochondria were prepared from rat ovaries and porcine corpora lutea as described before [18].

Synthesis of radiolabeled cholesterol sulfate

[4-^{14}C]Cholesterol (2 μCi; 3.0 mg) was converted to cholesterol sulfate as described by Lambeth *et al.* [13] and was purified by thin-layer chromatography on silica gel G plates with the solvent chloroform:methanol:acetone:acetic acid:water (8:2:4:2:1). [^{3}H]Pregnenolone sulfate was also synthesized and purified by this procedure.

Mitochondrial incubations to determine side-chain cleavage activity

Mitochondria (0.5–1 mg protein) were incubated in 2 ml of buffer, pH 7.3, comprising 0.25 M sucrose, 25 mM Tris–HCl, 20 mM KCl, 10 mM KH_2PO_4, 5 mM $MgSO_4$, 0.2 mM EDTA, 1 mg/ml bovine serum albumin (fatty acid free), 5 mM isocitrate, 0.5 mM $NADP^+$ and 8 μM cyanoketone. The cyanoketone was added to prevent conversion of pregnenolone into progesterone [19]. Cholesterol sulfate was added to the incubation medium in 20 μl dimethylsulfoxide while cholesterol or 25-hydroxycholesterol were added in 10 μl of ethanol. Control incubations (no exogenous substrates present) contained the solvent vehicle. Unless otherwise stated, preincubation was carried out at 37°C for 10 min with all components except isocitrate and $NADP^+$ which were used to start the reaction. Where non-radiolabeled substrates were used, 50 μl aliquots of the incubation medium were removed at various times (see Results section) and were added to 0.4 ml ice-cold methanol. These samples were stored at −20°C.

Determination of total (free and sulfated) pregnenolone content of samples

To hydrolyse any pregnenolone sulfate present in the above samples, solvent was removed under N_2, 200 μl of 1 M methanolic HCl was added and the samples incubated for 16 h at 37°C [15]. Samples were neutralized by adding methanolic ammonia solution, diluted with methanol and triplicate aliquots taken for the determination of their pregnenolone content by radioimmunoassay [20]. The anti-pregnenolone antiserum used (serum 5-32-7, from Radioassay Systems Laboratories) showed a cross-reactivity with pregnenolone sulfate of 71% compared to molar equivalents of pregnenolone, similar to that observed by other workers using this antiserum [21].

Measurement of side-chain cleavage of tracer [4-^{14}C]cholesterol

Mitochondrial incubations were carried out as described above using 50 nCi [4-^{14}C]cholesterol as substrate excepting that $NADP^+$ and isocitrate were present in the preincubation and the reaction was started by adding the radiolabeled substrate. Reactions were terminated by the addition of 1 ml ice-cold methanol containing 15 μg of each of cholesterol, pregnenolone and progesterone. Measurement of the radioactivity associated with each of these steroids following thin-layer chromatography was determined as described elsewhere [22, 23]. No radioactivity was detected in the progesterone band, confirming that the cyanoketone present in the incubation efficiently inhibited conversion of pregnenolone into progesterone.

Analysis of products of [4-^{14}C]cholesterol sulfate metabolism by isolated mitochondria

Mitochondria were incubated with 45 nCi [4-^{14}C]cholesterol sulfate (75 μM) as described above. After incubation for 30 min at 37°C the reaction was stopped by the addition of 2 ml cold butanol containing 15 μg of each of

pregnenolone sulfate, pregnenolone, progesterone and cholesterol. The butanol layer was removed and the aqueous phase re-extracted twice with 2 ml aliquots of butanol. The butanol extracts were combined and the butanol was removed under nitrogen at room temperature. The remaining residue was dissolved in a small volume of chloroform:methanol (1:1) and applied to a silica gel G thin-layer chromatography plate. Plates were developed 3 times in diisopropyl ether:hexane:acetic acid (70:30:2). This gave separation of cholesterol, progesterone and pregnenolone from the sulfated steroids which remained at the origin. The location of radiolabeled steroids on the plate was detected using a Varian Berthold scanner. Silica gel on the plate corresponding to cholesterol, progesterone and pregnenolone standards was removed and the associated radioactivity was determined by scintillation counting following elution of the steroids with two 4 ml aliquots of chloroform:methanol (1:1). The sulfated steroids at the origin were similarly eluted from the silica gel and the solvent removed under nitrogen. The sulfate esters were hydrolysed by adding 1 ml of 1.0 M methanolic HCl and incubating for 20 h at 37°C [15]. One millilitre of Na_2CO_3 saturated methanol, 1.5 ml 0.88% KCl and 4 ml chloroform were added. The lower phase was retained and the solvent removed under nitrogen. The residue was dissolved in chloroform:methanol (1:1) and subjected to thin-layer chromatography as before. The radioactivity associated with the cholesterol, pregnenolone and progesterone bands was also determined as described above. The average recovery of radioactivity was 82%. Results were calculated from the percentage of radioactivity corresponding to each product relative to the total recovered radioactivity.

Other procedures

The concentration of cytochrome $P\text{-}450_{scc}$ in isolated mitochondria was determined by CO-reduced minus-reduced difference spectroscopy using an extinction difference of 91 $mM^{-1}\,cm^{-1}$ for the absorption difference between 450 and 490 nm [24]. Protein was measured by the Ponceau S method [25].

RESULTS

Products of cholesterol sulfate metabolism by ovarian mitochondria

Incubation of mitochondria (0.87 mg protein) prepared from rat ovaries for 30 min with 75 μM [4-^{14}C]cholesterol sulfate resulted in 15.8% of the cholesterol sulfate being converted into pregnenolone sulfate. The only other products detected were cholesterol (0.9%) and pregnenolone (0.3%). For mitochondria prepared from the porcine corpus luteum, a similar incubation (0.71 mg protein) resulted in 7.9% of the cholesterol sulfate being converted to pregnenolone sulfate. No free cholesterol or pregnenolone was detected. Pregnenolone sulfate has been found to be the major product of cholesterol sulfate metabolism by rat and bovine adrenal mitochondria also [8, 9, 13, 14].

Table 1. Comparison of side-chain cleavage rates by mitochondria using endogenous cholesterol, endogenous cholesterol plus cholesterol sulfate, and [4-^{14}C]cholesterol sulfate as substrates

Substrate	Total pregnenolone formation (nmol/30 min/mg protein	
	Rat ovary	Pig corpus luteum
Endogenous cholesterol	9.5	5.1
[4-^{14}C]Cholesterol sulfate	27.8	15.2
Endogenous cholesterol plus cholesterol sulfate	33.0	22.5

Total (free plus sulfated) pregnenolone formation by isolated mitochondria during a 30 min incubation at 37°C, was measured using endogenous cholesterol, 75 μM [4-^{14}C]cholesterol sulfate or 75 μM cholesterol sulfate plus endogenous cholesterol as substrates. For unlabeled substrates, product formation was measured by radioimmunossay. For [4-^{14}C]cholesterol sulfate, radiolabeled pregnenolone and pregnenolone sulfate were measured following their separation by thin-layer chromatography (see Experimental section).

Total (free and sulfated) pregnenolone production from isolated mitochondria incubated with unlabelled cholesterol sulfate was in reasonable agreement with pregnenolone production determined by adding the values for total pregnenolone production from [4-^{14}C]cholesterol sulfate and endogenous cholesterol, measured separately (Table 1). It appears that there is little competition of side-chain cleavage between endogenous cholesterol and 75 μM cholesterol sulfate.

Competition between cholesterol sulfate and cholesterol for side-chain cleavage

When an electron donor such as isocitrate is added to isolated mitochondria prior to addition of tracer [4-^{14}C]cholesterol, then the rate of [4-^{14}C]cholesterol conversion to pregnenolone is limited by the rate of [4-^{14}C]cholesterol movement to the inner-mitochondrial membrane [26]. Under these conditions we found that 30 μM cholesterol sulfate had little effect on the rate of [4-^{14}C]cholesterol conversion while 200 μM cholesterol sulfate caused the rate of conversion to decrease by approximately 50% (Fig. 1). The inhibition by cholesterol sulfate could be due to

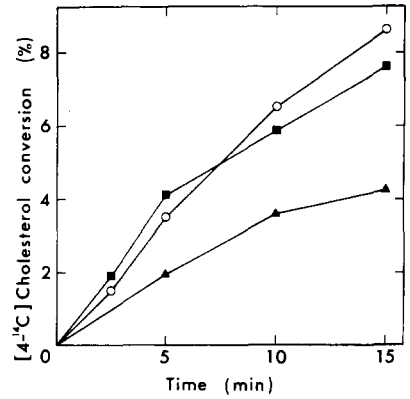

Fig. 1. The effect of cholesterol sulfate on the side-chain cleavage of [4-^{14}C]cholesterol. Mitochondria were prepared from the luteinized ovaries of immature rats treated with gonadotropins and were preincubated with cholesterol sulfate, $NADP^+$ and isocitrate before the reaction was started by the addition of the [4-^{14}C]cholesterol. The conversion of [4-^{14}C]cholesterol into [4-^{14}C]pregnenolone by the mitochondria was measured in the presence of 30 μM cholesterol sulfate (squares), 200 μM cholesterol sulfate (triangles) or the dimethylsulfoxide vehicle (circles).

competition for the active site of cytochrome P-450$_{scc}$ or due to competition between cholesterol sulfate and cholesterol for translocation to the site of reaction.

Time-courses for side-chain cleavage of cholesterol and cholesterol sulfate by mitochondria from rat ovaries

Following preincubation of isolated mitochondria for 10 min with 75 μM cholesterol sulfate and initiation of the reaction with isocitrate, the time-course of the side-chain cleavage reaction was biphasic (Fig. 2). There was an initial non-linear rapid phase followed by a linear slow phase. Addition of the cholesterol sulfate to mitochondria 16 min after the initiation of the reaction by the addition of isocitrate, resulted in a linear rate of side-chain cleavage equal to the slow phase observed when the mitochondria were preincubated with the cholesterol sulfate. It would appear that during preincubation, cholesterol sulfate and cholesterol are transported to the inner-membrane site of the reaction and the initial rate of side-chain cleavage reflects conversion of the accumulated substrates. After this is used up the rate of the reaction becomes limited primarily by the rate of cholesterol sulfate transport to the site of the reaction.

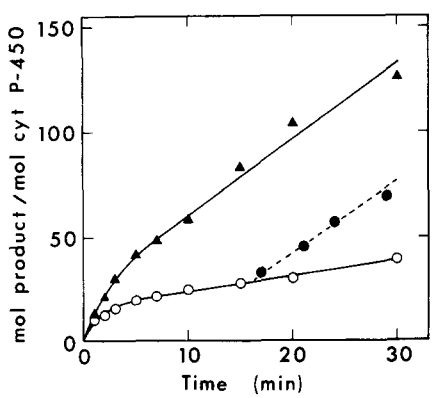

Fig. 2. Side-chain cleavage of cholesterol and cholesterol sulfate by mitochondria from rat ovaries. Product (pregnenolone plus pregnenolone sulfate) formation from endogenous cholesterol (open circles), 75 μM cholesterol sulfate added prior to preincubation (closed triangles) and 75 μM cholesterol sulfate added after incubation of mitochondria for 16 min (closed circles) was determined as described under Experimental. Cyt P-450, cytochrome P-450$_{scc}$.

Figure 3 shows that the addition of 200 μM cholesterol to isolated mitochondria from rat ovaries caused a doubling of the transport-limited slow phase of pregnenolone synthesis. Cholesterol sulfate translocation to the site of the side-chain cleavage reaction (Figs 2 and 4) therefore occurs much more rapidly than the transport of cholesterol to the cytochrome.

The effect of cholesterol sulfate concentration on the rate of side-chain cleavage by mitochondria from rat ovaries

Both the fast and slow phases of the side-chain cleavage reaction by mitochondria from rat ovaries increased with increasing concentrations of cholesterol sulfate (Fig. 4). With 200 μM cholesterol sulfate the fast phase of the reaction was linear for 7 min and gave a substrate turnover rate of 12.2 mol steroid/min/mol cytochrome P-450$_{scc}$. This rate was the same as that observed with 60 μM 25-hydroxycholesterol indicating that it is the maximum turnover rate

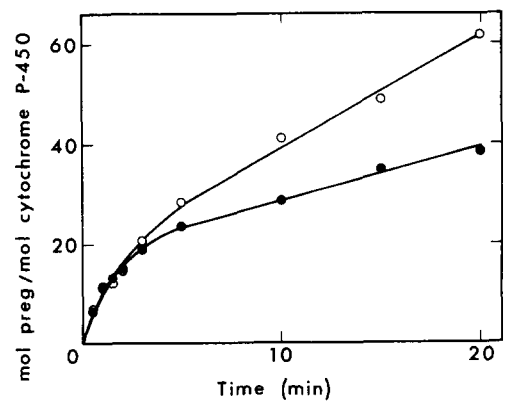

Fig. 3. The effect of exogenous cholesterol on the rate of side-chain cleavage by mitochondria from rat ovaries. Pregnenolone (preg) production was measured using mitochondria to which 200 μM cholesterol (open circles) or the ethanol vehicle (closed circles) had been added.

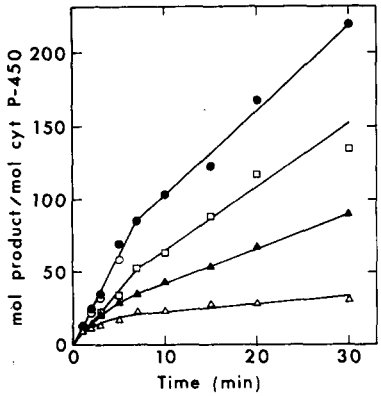

Fig. 4. The effect of cholesterol sulfate concentration on the rate of side-chain cleavage by mitochondria from rat ovaries. Product (pregnenolone plus pregnenolone sulfate) formation was measured from endogenous cholesterol (open triangles), 30 μM cholesterol sulfate (closed triangles) 75 μM cholesterol sulfate (open squares), 200 μM cholesterol sulfate (closed circles or 60 μM 25-hydroxycholesterol (open circles). Cyt P-450, cytochrome P-450$_{scc}$.

of substrate by the cytochrome [18]. There was no evidence for an enzyme other than cytochrome P-450$_{scc}$ catalysing the side-chain cleavage of cholesterol sulfate, as the initial rate was not increased when both cholesterol sulfate and 25-hydroxycholesterol were added together (not shown). Further evidence that cholesterol-sulfate conversion into pregnenolone sulfate is catalysed by cytochrome P-450$_{scc}$ was obtained by including the cytochrome P-450$_{scc}$ inhibitor, aminoglutethimide (200 μg/ml), in the incubation [27]. This resulted in a 97% inhibition of total (free plus sulfated) pregnenolone synthesis by mitochondria incubated with 75 μM cholesterol sulfate.

A good fit to a hyperbolic curve was obtained when the substrate turnover rates for the slow phase of side-chain cleavage of cholesterol sulfate (Fig. 4) were plotted against the cholesterol sulfate concentrations (not shown). The K_m determined for this transport-limited side-chain cleavage of cholesterol sulfate was 67 μM and the maximum velocity was 8.0 mol steroid/min/mol cytochrome P-450$_{scc}$. The rate of transport of cholesterol sulfate into the mitochondria is therefore less than the maximum substrate turnover rate of the cytochrome (12.2 mol steroid/min/mol cytochrome P-450$_{scc}$ for the preparation used in Fig. 4) even at concentrations of cholesterol sulfate saturating for its transport.

Time-courses for side-chain cleavage of cholesterol and cholesterol sulfate by mitochondria from porcine corpora lutea

Time-courses for total pregnenolone synthesis by mitochondria from porcine corpora lutea were similar to those for the rat ovary (Fig. 5). We have previously reported that the addition of 200 μM cholesterol to unwashed porcine luteal mitochondria did not alter the rate of pregnenolone synthesis [17]. The mitochondria used for Fig. 5 were washed twice and 200 μM cholesterol caused a 70% increase in rate of the slow phase of pregnenolone synthesis. In contrast, 200 μM cholesterol sulfate increased the rate of the slow phase 14-fold over that observed with endogenous cholesterol. The initial rate of side-chain cleavage after preincubation of the mitochondria with 200 μM cholesterol sulfate for 10 min was 11.5 mol steroid/min/mol cytochrome P-450$_{scc}$, which is close to the maximum substrate turnover rate we have observed for cytochrome P-450$_{scc}$ in mitochondria from porcine ovaries [17].

DISCUSSION

The present study demonstrates that cholesterol sulfate is a better substrate than cholesterol for side-chain cleavage by isolated ovarian mitochondria. This comes from the more rapid movement of cholesterol sulfate to the mitochondrial site of the cholesterol side-chain cleavage reaction, where it is subsequently converted into pregnenolone sulfate. Under transport-limiting conditions, 200 μM cholesterol sulfate increased the rate of side-chain cleavage more than 10-fold over that observed for endogenous cholesterol, while 200 μM exogenous cholesterol caused only a doubling of the rate.

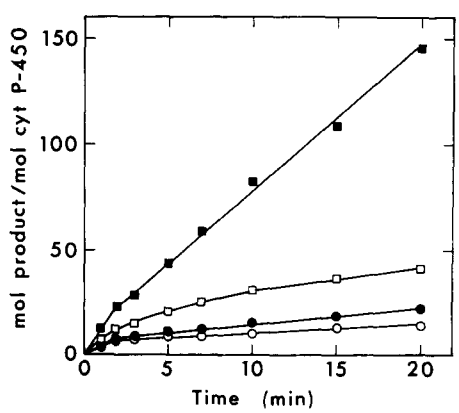

Fig. 5. Side-chain cleavage of cholesterol and cholesterol sulfate by porcine luteal mitochondria. Product (pregnenolone plus pregnenolone sulfate) formation was measured following incubation of mitochondria with 200 μM cholesterol (closed circles), 30 μM cholesterol sulfate (open squares), 200 μm cholesterol sulfate (closed squares) or with the solvent vehicle (open circles). Cyt P-450, cytochrome P-450$_{scc}$.

There are conflicting reports in the literature (see Introduction section) concerning whether cholesterol sulfate is converted into pregnenolone sulfate in adrenal mitochondria, by the same enzyme that converts cholesterol into pregnenolone [9, 11–14]. Our data for the ovary indicates that side-chain cleavage of cholesterol sulfate, like cholesterol, is catalysed by cytochrome $P\text{-}450_{scc}$. Evidence for this is that the substrate turnover rates for cytochrome $P\text{-}450_{scc}$ are the same following preincubation of rat ovarian mitochondria with either 60 μM 25-hydroxycholesterol or 200 μM cholesterol sulfate. We have previously shown that 25-hydroxycholesterol enables the maximum velocity of cholesterol side-chain cleavage by mitochondria from rat ovaries to be determined [18]. It is improbable that another enzyme system would yield an identical substrate turnover rate to this, with cholesterol sulfate as the substrate. If this was the case, then the addition of cholesterol sulfate and 25-hydroxycholesterol together would be expected to increase the maximum rate of side-chain cleavage. This was not observed. The inhibition of the side-chain cleavage of cholesterol sulfate which occurred following the addition of aminoglutethimide, provides further evidence for the involvement of cytochrome $P\text{-}450_{scc}$ in cholesterol sulfate metabolism.

We observed competition for side-chain cleavage between free and sulfated cholesterol when the rate of [4-^{14}C]cholesterol conversion into [4-^{14}C]pregnenolone was measured in the presence of cholesterol sulfate. A high concentration of cholesterol sulfate (200 μM) was required to inhibit [4-^{14}C]cholesterol conversion by 50%. While data is consistent with the competition occurring at the level of substrate binding to the cytochrome, we cannot exclude the possibility of competition also occurring at the level of translocation of the substrates to the inner-mitochondrial membrane site of the reaction. Lambeth *et al.* [13] and Xiang and Lambeth [14] have reported that cholesterol sulfate inhibits the translocation of cholesterol in mitochondria from the rat adrenal cortex. However, even if cholesterol sulfate does inhibit cholesterol translocation into mitochondria from the ovary, the overall rate of cholesterol side-chain cleavage is stimulated by cholesterol sulfate, due to its rapid rate of conversion into pregnenolone sulfate. We also did experiments which revealed that this was the case for the adrenal cortex too. When we incubated rat adrenal mitochondria with cholesterol sulfate, similar time-courses for side-chain cleavage to those shown in Fig. 4 for the ovary were observed.

We observed little hydrolysis of pregnenolone sulfate by mitochondria isolated from rat ovaries and no detectable hydrolysis of pregnenolone sulfate by mitochondria from the porcine corpus luteum. We also tested the rate of pregnenolone sulfate hydrolysis by the post-mitochondrial supernatant fraction of the rat ovary and observed a rate of 0.29 nmol pregnenolone/30 min/mg protein using 75 μM pregnenolone sulfate as substrate. This activity may be sufficient to ensure that any endogenous cholesterol sulfate in the ovary [15] that undergoes side-chain cleavage, can be converted into progesterone, the major steroid secreted by the luteinized rat ovary. An alternative fate of the pregnenolone sulfate might be conversion into dehydroepiandrosterone sulfate as occurs in the fetal adrenal cortex [21].

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