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Non-Esterified Cholesterol-Rich Adrenal Lipid Fractions

PREPARATION, PROPERTIES AND PREFERENTIAL UTILIZATION FOR CHOLESTEROL SIDE-CHAIN CLEAVAGE BY CORTICOTROPIN-STIMULATED ADRENAL MITOCHONDRIA

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Rat adrenal 105000*g* supernatant contains two lipid moieties, 'lipid-I' and 'lipid-II' which contain non-esterifed cholesterol and stimulate cholesterol side-chain cleavage in soluble or mitochondrial enzyme systems. Lipid-I contains relatively large low-density heat-stable particles, whereas lipid-II particles are smaller, more dense and heat-labile. Lipid-I and lipid-II can be separated from clear cytosol by ultracentrifugation and gel filtration respectively. Corticotropin plus cycloheximide treatment increases the non-esterified cholesterol concentrations in the lipid fractions, and stimulatory effects of lipids on cholesterol side-chain cleavage appear to correlate with non-esterified cholesterol concentrations therein.

On addition of saturating amounts of cholesterol-rich lipid, pregnenolone synthesis and cholesterol binding to cytochrome *P*-450 are stimulated more in mitochondria from corticotropin-stimulated adrenals than in mitochondria from control or corticotropin-plus cycloheximide-stimulated adrenals. These results support the contention that the corticotropin-induced increase in mitochondrial cholesterol side-chain cleavage involves an increase in cholesterol utilization as well as an increase in cholesterol availability.

Corticotropin ('ACTH') regulates steroidogenesis primarily by modulating the conversion of cholesterol into pregnenolone (3β-hydroxypregn-4-en-20-one) (Stone & Hechter, 1954; Karaboyas & Koritz, 1965; Billiar & Eik-Nes, 1965; Farese, 1971). The mechanism for such modulation is not clear. After corticotropin administration, adrenal cholesterol esterase activity increases (Davis & Garren, 1966; Boyd & Trzeciak, 1973), leading to increased non-esterified cholesterol in the lipid droplets (Garren *et al.*, 1971) and mitochondria (Mahaffee *et al.*, 1974). The increase in non-esterified cholesterol is associated with increased binding of cholesterol to cytochrome *P*-450 (Jefcoate *et al.*, 1970; Simpson *et al.*, 1972; Brownie *et al.*, 1973), an integral component of the cholesterol-side-chain-cleavage enzyme complex (Harding *et al.*, 1964). A labile protein is required for steroidogenesis (Ferguson, 1963; Farese, 1964; Garren *et al.*, 1965) and binding of cholesterol to cytochrome *P*-450 (Jefcoate *et al.*, 1970; Simpson *et al.*, 1972; Brownie *et al.*, 1973; Brownie & Paul, 1974), but it is unclear if corticotropin regulates this labile protein.

Although corticotropin increases the availability of intra-adrenal non-esterified cholesterol, it is unclear if this effect can account for enhanced binding of cholesterol to cytochrome *P*-450 and subsequent steroidogenesis, or if there is another corticotropin-induced factor that enhances cholesterol utilization. These possibilities could be tested experimentally by adding saturating amounts of non-esterified cholesterol to incubations of mitochondria from control and corticotropin-stimulated adrenals and observing the resultant effects on cholesterol–*P*-450 interaction and pregnenolone synthesis. Such an approach has been employed by Simpson *et al.* (1978) and Bell & Harding (1974), but the results were quite different; the former group observed that corticotropin pretreatment enhanced mitochondrial utilization of cholesterol added in acetone, but the latter group noted no effect of corticotropin with cholesterol added in a phospholipid suspension. In our approach to this problem, we turned to our previous findings (Farese & Prudente, 1977; Farese & Prudente, 1978*a*) that adrenal cytosol contains lipids that are enriched in non-esterified cholesterol by prior administration of corticotropin plus cycloheximide, and that appear to provide a relatively physiological substrate for the cholesterol-side-chain-cleavage reaction in a soluble enzyme system. In the present paper we describe the preparation and certain properties of two adrenal cytosolic lipid frac-

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tions, their utilization in soluble and mitochondrial cholesterol-side-chain-cleavage enzyme systems, and effects of corticotropin treatment on the latter. Our results support the contention that corticotropin enhances the utilization of non-esterified cholesterol, as well as its availability.

Experimental

Male Sprague–Dawley rats weighing approx. 200g were obtained from the Holtzmann Co. and kept in a light and temperature-controlled environment for at least 1 week before each experiment. For preparation of cholesterol-rich adrenal cytosolic lipid fractions, the rats were injected intraperitoneally with 10mg of cycloheximide (Nutritional Biochemical Co.) and 2 units of corticotropin-(1–24)-tetracosapeptide (Cortrosyn; kindly supplied by Organon Inc., West Orange, NJ, U.S.A.) in physiological saline (0.9% NaCl) 45 and 30min respectively before killing by decapitation. In experiments wherein mitochondria were prepared for incubation and measurement of pregnenolone-synthesizing activity, the cycloheximide and corticotropin (as above) were injected 20 and 10min respectively before killing.

Adrenals were rapidly removed from carcasses, chilled in ice-cold physiological saline, trimmed free of fat and connective tissue and homogenized in ST medium (250mM-sucrose and 50mM-Tris, pH7.5) or STMK medium (ST medium plus 5mM-$MgCl_2$ and 25mM-KCl) as follows. (*a*) For mitochondria, ST medium was employed and a 10% (w/v) homogenate was made by three gentle passes of a motor-driven (400rev./min) Teflon pestle in a Potter–Elvehjem homogenizer. (*b*) For soluble cholesterol-side-chain-cleavage enzyme complex, STMK medium was employed and a 10% (w/v) homogenate was made by ten vigorous passes of the homogenizer at 1600rev./min as described previously (Farese & Prudente, 1977, 1978*a*). (*c*) For lipid fractions, ST or STMK medium was employed and a 10–30% (w/v) homogenate was made by three or ten passes of the homogenizer (neither the buffer nor the homogenization procedure appreciably influenced the amount or activity of the purified adrenal lipid fractions).

Homogenates were centrifuged for 10min at 400*g* and 4°C to remove unbroken cells, nuclei and cell debris. The supernatant was centrifuged for 10min at 10000*g* and 4°C to obtain the mitochondrial pellet, which was washed once and resuspended in ST medium. As described previously (Farese & Prudente, 1977, 1978*a*), for preparation of cytosolic soluble cholesterol-side-chain-cleavage enzymes and lipid fractions, 2ml of 10000*g* supernatant was layered on 1ml of a 25% (w/v) sucrose solution, and then 2ml of 4 or 5% (w/v) sucrose solution (these solutions also contained Tris and electrolytes as in the 10000*g* supernatant) was placed on top. After centrifuging for 2h at 4°C and 300000*g* (SW-65 rotor), the lipid fraction, called 'lipid-I', which floated to the top, was obtained as quantitatively as possible by aspiration with the upper 1ml of the 'overlayed' 4 or 5% sucrose solution. A more dense sucrose solution was added to the lipid-I suspension so that the final sucrose concentration was 250mM. After discarding the lower 1ml of the overlayed 4 or 5% sucrose solution, 'clear cytosol' fraction was obtained, and this contained both soluble cholesterol-side-chain-cleavage enzyme complex and a second lipid fraction, 'lipid-II'. As described for plasma lipoproteins (Sata *et al.*, 1970; Quarfordt *et al.*, 1972), lipid-II was purified by chromatography on a Sephadex G-200, Sepharose 4B or Sepharose 2B column (1.6cm × 20cm) as follows: 0.1μCi [26-^{14}C]cholesterol (Amersham–Searle; sp. radioactivity 58Ci/mol) was added to clear cytosol to serve as a marker for cholesterol-rich lipids or lipoproteins; 1ml of radioactively labelled clear cytosol (containing the equivalent of 300mg of adrenal tissue) was applied to a column previously equilibrated at 8°C with ST or STMK medium; elution (4ml/h) was effected with the same buffers (elution profiles were identical in both buffers), and the three peak fractions containing [^{14}C]cholesterol were pooled for experiments utilizing purified lipid-II fraction.

Assays for cholesterol side-chain cleavage were conducted either in a soluble enzyme system as described previously (Farese & Prudente, 1977, 1978*a*), or in a mitochondrial system. In the former case, clear cytosol (approx. 0.3mg of protein) from 10mg of control adrenal tissue (prepared by more vigorous homogenization in STMK medium; see above) served as the enzyme source and was incubated for 20min at 37°C (Farese & Prudente, 1977, 1978*a*) in 250 or 375μl of a solution containing: 250mM-sucrose; 50mM-Tris (pH7.5); 5mM-$MgCl_2$; 25mM-KCl; 1mM-NADPH; 20μM-WIN-25540 (4α,5-epoxy-17β-hydroxy-5α-androstane-2α-carbonitrile; kindly supplied by Sterling–Winthrop Co.) to inhibit conversion of pregnenolone into progesterone (Farese & Prudente, 1977, 1978*a*), and other additions as described. The mitochondrial cholesterol-side-chain-cleavage assay system was identical with the soluble assay system except that mitochondria from 10mg of adrenal tissue were substituted for clear cytosol, and 5mM-DL-isocitrate (Sigma Chemical Co.) and 500μM-$CaCl_2$ (this and NADPH were required for demonstration of lipid-induced effects on pregnenolone synthesis in the mitochondrial cholesterol-side-chain-cleavage system) were also present in a final volume of 250μl; and incubation was for 10min at 37°C. After incubation, reaction mixtures were chilled, ice-cold

diethyl ether was added, and, after mixing, portions of the diethyl ether extracts were removed for pregnenolone determination by radioimmunoassay (Farese & Prudente, 1977, 1978*a*). Pregnenolone was undetectable in zero-time samples and added pregnenolone was not appreciably metabolized in either incubation system. Pregnenolone synthesis was linear or nearly linear during the incubation times employed, and the yield of pregnenolone was directly proportional to mitochondrial or cytosol protein concentrations.

In absorbance-spectrum studies, adrenal mitochondria were incubated at 22°C in STMK medium and the difference in absorbance between the wavelengths 390 and 420nm was continuously recorded in an Aminco–Chance DW-II spectrophotometer operating in the dual-wavelength mode (Jefcoate *et al.*, 1970; Simpson *et al.*, 1972; Brownie *et al.*, 1973; Farese & Prudente, 1978*a*). After attainment of a stable baseline, 10μg of pregnenolone in 10μl of propylene glycol was added, and a type-II spectral change, indicative of binding of cholesterol to cytochrome *P*-450, was measured as reported by Brownie and his co-workers (Brownie *et al.*, 1973; Brownie & Paul, 1974). Cytochrome *P*-450 was determined by the method of Omura & Sato (1964).

The efficacy of treatments *in vivo* was monitored in all experiments by measurements of serum corticosterone by the acid-fluorescence method (Silber *et al.*, 1958) (corticotropin increased serum corticosterone concentrations by 3–5-fold and cycloheximide blocked this effect of corticotropin). Proteins were determined by the method of Lowry *et al.* (1951) and cholesterol by the method of Schoenheimer and Sperry as outlined by Hawk *et al.* (1954).

Results

As shown in Fig. 1, when clear cytosol from corticotropin-plus cycloheximide-stimulated adrenals was equilibrated with [^{14}C]cholesterol and then chromatographed on Sepharose 4B, the [^{14}C]-cholesterol largely migrated with gel-excluded substances and eluted in the void volume. On addition of eluate fractions to a soluble cholesterol-side-chain-cleavage assay system, pregnenolone synthesis was stimulated only by fractions containing [^{14}C]cholesterol. Comparable results were obtained with Sephadex G-200 (see Farese & Prudente, 1978*a*) and Sepharose 2B chromatography (results not shown).

As shown in Fig. 2, the cholesterol-containing fractions that stimulated soluble cholesterol-side-chain-cleavage enzymes were devoid of intrinsic pregnenolone-synthesizing enzymes and were clearly separable from the latter by gel filtration. As shown in Table 1, pregnenolone-synthesizing activity of chromatographically purified (cholesterol-depleted) cholesterol-side-chain-cleavage enzyme preparations was fully dependent on the addition of either lipid-I or lipid-II fractions. These results

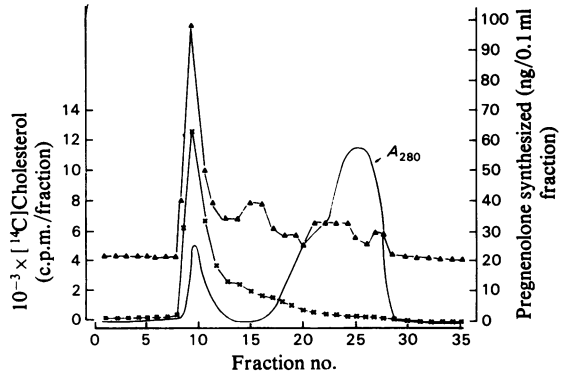

Fig. 1. *Sepharose 4B column chromatography of clear cytosol from corticotropin-plus-cycloheximide-stimulated adrenals*

ST medium was employed for sample application and elution. Fractions were assayed for [^{14}C]-cholesterol radioactivity (×) and ability to stimulate pregnenolone synthesis when added (0.1ml) to control clear cytosol and other components of the soluble cholesterol-side-chain-cleavage assay system (▲). (Incubation of fractions in the assay system without clear cytosol failed to yield any pregnenolone.) The solid line shows the A_{280} profile. Fractions (1ml) were collected. See the Experimental section for other details.

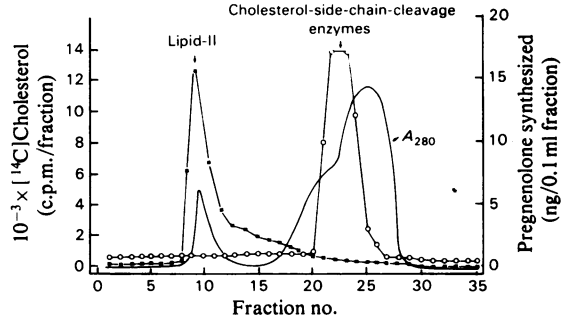

Fig. 2. *Separation of lipid-II and cholesterol-side-chain-cleavage enzymes by Sepharose 4B column chromatography*

Clear cytosol from corticotropin-plus-cycloheximide-stimulated adrenals was chromatographed as in Fig. 1 and assayed for [^{14}C]cholesterol content (×) and intrinsic cholesterol-side-chain-cleavage enzyme activity (○) by incubating 0.1ml of each fraction (1ml) plus 0.05ml of cholesterol-rich lipid-I (derived from 5mg of corticotropin-plus-cycloheximide-stimulated adrenal tissue) in the soluble cholesterol-side-chain-cleavage assay system (as described in the Experimental section, but without control clear cytosol as the cholesterol-side-chain-cleavage enzyme source).

Table 1. *Effects of cholesterol-rich lipids on substrate-poor chromatographically purified cholesterol-side-chain-cleavage enzymes*

After chromatography of adrenal clear cytosol on Sepharose 4B (see Fig. 3), fractions 21–25 were pooled ('substrate-poor soluble cholesterol-side-chain-cleavage enzymes') and 0.1 ml was employed in the incubation with 0.05 ml of lipid-I or 0.1 ml of lipid-II (derived from 5 or 10 mg of adrenal tissue respectively) as indicated, along with other components of the soluble cholesterol-side-chain-cleavage assay system. Results are means ± variation of duplicate values.

Incubation components	Pregnenolone synthesized (ng)
Soluble cholesterol-side-chain-cleavage enzymes	0 ± 0
Lipid-I	0 ± 0
Lipid-II	0 ± 0
Lipid-I + lipid-II	0 ± 0
Soluble cholesterol-side-chain-cleavage enzymes + lipid-I	22.8 ± 0
Soluble cholesterol-side-chain-cleavage enzymes + lipid-II	10.6 ± 0.3

Table 2. *Effects of corticotropin and cycloheximide treatment on non-esterified cholesterol concentrations in lipid-I and lipid-II*

Expt.	Treatment *in vivo*	Cholesterol (μg/100 mg of adrenal tissue)	
		Lipid-I	Lipid-II in clear cytosol
1	Control	12	8
	Corticotropin	12	8
	Corticotropin + cycloheximide	63	23
2	Control	12	5
	Corticotropin	12	5
	Corticotropin + cycloheximide	131	33
3	Control	22	8
	Corticotropin	14	8
	Corticotropin + cycloheximide	96	28

suggested that both lipids can provide substrates for the cholesterol-side-chain-cleavage reaction. In addition, it is noteworthy that, on a tissue-weight basis, lipid-I is approximately 4-fold more active than lipid-II, and this correlates well with the fact that the non-esterified cholesterol concentrations in lipid-I and lipid-II (derived from 100 mg of corticotropin-plus cycloheximide-stimulated adrenal tissue) were 100 ± 17 and 25 ± 3 μg (mean ± S.E.M. for 11 determinations) respectively.

With chromatography of clear cytosol from control adrenal tissue on Sephadex G-200 or Sepharose 4B, the elution profiles for [^{14}C]cholesterol and A_{280} were identical with those observed during chromatography of cytosol from corticotropin-plus cycloheximide-treated adrenals. However, on addition of control eluate fractions to a soluble cholesterol-side-chain-cleavage assay system, little or no stimulation of pregnenolone synthesis was observed (results not shown). The poor stimulatory effect of lipid-II fractions from control cytosol may be related to low non-esterified cholesterol concentrations therein (see Table 2 and Farese & Prudente, 1978*a*). These results are comparable with those obtained with lipid-I (see Tables 2 and 3 and Farese & Prudente, 1977, 1978*a*), where non-esterified cholesterol availability appeared to correlate with cholesterol-side-chain-cleavage-stimulatory properties.

Table 3. *Effects of corticotropin and cycloheximide treatment on cholesterol-side-chain-cleavage-stimulating properties of lipid-I*

Lipid-I from 10 mg of adrenal tissue was incubated in soluble cholesterol-side-chain-cleavage assay system with control clear cytosol. Results are means ± S.E.M. (n = 4).

Addition	Pregnenolone synthesized (ng)
None	18.9 ± 0.8
Control (lipid-I)	36.0 ± 1.1
Corticotropin + cycloheximide (lipid-I)	90.8 ± 2.9

A number of results distinguished lipid-II from lipid-I. First, more prolonged centrifugation (16 h versus the routine 2 h) failed to diminish the yield of lipid-II and clearly the latter does not simply represent lipid-I, which is not removed by a 2 h centrifugation. Secondly, whereas lipid-I was previously found to be heat-stable (Farese & Prudente, 1978*a*), lipid-II lost most cholesterol-side-chain-cleavage-stimulating activity after heating for 4 min at 60°C. Thirdly, as shown in Figs. 3 and 4, in sucrose-density-gradient analyses, lipid-I remained in the

upper fractions (density < 1.030 g/ml), whereas lipid-II migrated to the lower fractions yielding several peaks in the density range of 1.030 to >1.110 g/ml.

Both lipid-I and lipid-II were visualized by electron microscopy by using negative staining (phosphotungstic acid) techniques (performed by Ms. Joann Gaudsmith). Larger forms (up to 1–2 μm diameter) were more prevalent in the lipid-I preparations, but lipid-I and lipid-II were otherwise similar. Most particles in the lipid-II preparations were approx. 0.2 μm in diameter.

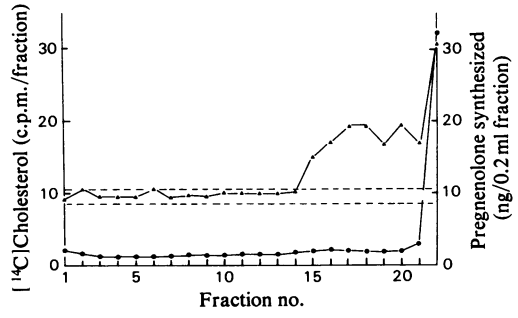

Fig. 3. *Sucrose-gradient analysis of lipid-I*
Lipid-I (0.5 ml in 4% sucrose/Tris) derived from 150 mg of corticotropin-plus-cycloheximide-stimulated adrenal tissue was layered on a 12 ml 5–25% (w/v) sucrose gradient (containing 50 mM-Tris, pH 7.5) and centrifuged at 35000 rev./min and 8°C in an SW-41 rotor. Fractions (0.5 ml) were obtained after piercing the bottom of the tube with a needle, and assayed for [14C]cholesterol content (●) and stimulation of pregnenolone synthesis (▲) when added (0.2 ml) to the soluble cholesterol-side-chain-cleavage assay system. The horizontal broken lines show the range of replicate assays conducted without addition of gradient fraction. The assay of 5–25% (w/v) sucrose/Tris did not affect the assay system. The top of the gradient is to the right.

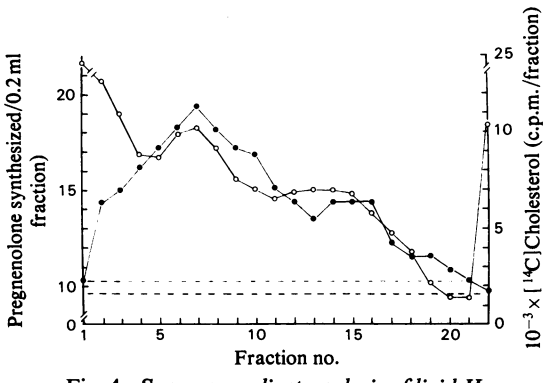

Fig. 4. *Sucrose-gradient analysis of lipid-II*
This experiment was identical with that of Fig. 3, except that 0.5 ml of chromatographically purified lipid-II in 4% (w/v) sucrose/Tris was applied to the gradient. The horizontal broken lines show the range of replicate assays conducted without addition of gradient fraction. Symbols: ●, pregnenolone synthesized; ○, [14C]cholesterol content.

Several findings suggested that lipid-II was not a non-specific artefact arising from preparative techniques (e.g. contamination by serum lipoproteins, 'extraction' from other subcellular organelles). First, with chromatography of rat serum lipoproteins on Sepharose 4B, most [14C]cholesterol migrated behind lipid-II (i.e. on the front shoulder of the major protein peak) and sucrose-gradient analysis of the small fraction migrating in the position of lipid-II revealed only low-density (<1.030 g/ml) [14C]-cholesterol-containing lipoproteins. Secondly, with Sepharose 4B chromatography of clear cytosol from rat liver, [14C]cholesterol migrated behind adrenal lipid-II, producing a nearly symmetrical peak (at fraction 15; cf. Fig. 1) on the front shoulder of the major protein peak [this corresponds to the approximate expected chromatographic migration of β-lipoproteins, or low-density lipoproteins, in human plasma (Sata *et al.*, 1970; Quarfordt *et al.*, 1972)]. Thirdly, 'extraction' of adrenal mitochondria and purified lipid-I fraction by vigorous homogenization yielded only minor peaks of [14C]cholesterol migrating in the lipid-II area during Sepharose 4B chromatography. Sucrose-gradient analyses of these fractions revealed only low-density (<1.030 g/ml) [14C]cholesterol-containing lipoproteins.

As shown in Fig. 5, cholesterol-rich lipid-I or lipid-I + lipid-II (the results were indistinguishable) enhanced pregnenolone synthesis in mitochondria from

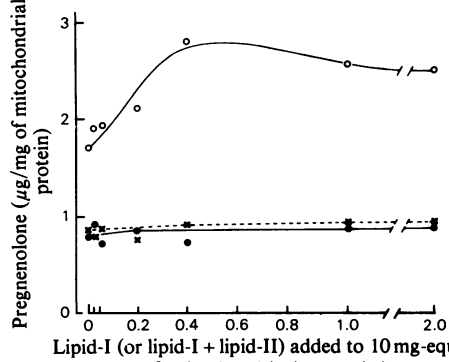

Fig. 5. *Stimulation of pregnenolone synthesis by addition of cholesterol-rich lipid-I (or lipid-I plus lipid-II) from corticotropin-plus cycloheximide-stimulated adrenals to mitochondria from control* (●), *corticotropin-plus-cycloheximide-stimulated* (×) *or corticotropin-stimulated adrenals* (○)
The mg-equiv. unit for the abscissa refers to the wet weight of tissue from which the subcellular fraction was derived. Concentrations of mitochondrial protein and cytochrome P-450 were nearly identical in all groups.

Table 4. *Effects of cholesterol-rich lipid on pregnenolone synthesis in mitochondria from control, corticotropin-stimulated or corticotropin- + cycloheximide-treated rats*

Cholesterol-rich lipid-I (or I + II) was prepared from corticotropin-plus cycloheximide-stimulated rat adrenals, and lipid derived from 0.4 mg of adrenal tissue was incubated for 10 min at 37°C with mitochondria derived from 10 mg of adrenal tissue, and other constituents of the mitochondrial cholesterol-side-chain-cleavage assay system. Results of ten experiments are shown and values are means ± S.E.M.

Treatment *in vivo*	Lipid addition	Pregnenolone synthesized (μg/mg of mitochondrial protein)	Change on addition of lipids (%)
None (control)	−	1.02 ± 0.28	
	+	1.28 ± 0.26	+38 ± 9 ($P < 0.01$)†
Corticotropin	−	2.10 ± 0.42 ($P < 0.05$)*	
	+	3.24 ± 0.65 ($P < 0.025$)*	+64 ± 15 ($P < 0.01$)†
Corticotropin + cycloheximide	+	1.15 ± 0.25	
	+	1.42 ± 0.03	+32 ± 9 ($P < 0.025$)†

* Comparison of mean with control mean value by standard *t* testing.

† Comparison of results obtained with and without addition of lipid (paired data analysis) and *t*-test evaluation of the mean difference.

Table 5. *Effects of high-Ca^{2+} concentrations (22 mM) on stimulatory properties of cholesterol-rich lipid-I + lipid II in control and corticotropin- plus cycloheximide-treated mitochondria*

In this experiment, the mitochondria were obtained from control rats or rats treated for 30 and 15 min with cycloheximide and corticotropin respectively, to produce high mitochondrial non-esterified cholesterol concentrations in the latter group. The mitochondrial incubation system did not include the 500 μM-$CaCl_2$ usually present. Results are mean values (± S.E.M.) for four incubations. Comparable results were obtained in repeat experiments.

Addition	Pregnenolone synthesized (μg/mg of mitochondrial protein): Control mitochondria	Pregnenolone synthesized (μg/mg of mitochondrial protein): Corticotropin- + cycloheximide-treated mitochondria
None	0.55 ± 3	0.49 ± 6
Lipid	0.71 ± 9	0.58 ± 18
22 mM-$CaCl_2$	1.32 ± 15	5.37 ± 113
Lipid + 22 mM-$CaCl_2$	2.60 ± 10	2.70 ± 19

corticotropin-stimulated adrenals. In the absence of added lipid, mitochondria from corticotropin-stimulated adrenals were twice as active as mitochondria from control or corticotropin plus cycloheximide-stimulated adrenals. Moreover, the latter two mitochondrial types responded poorly, if at all, to addition of lipid fraction, and, consequently, the induced effect of corticotropin on mitochondrial pregnenolone synthesis was intensified by addition of saturating amounts of cholesterol-rich lipid fraction. This was also evident in a larger number of experiments (Table 4) in which saturating amounts of cholesterol-rich lipids were added in incubations of mitochondria from control, corticotropin-stimulated and corticotropin-plus cycloheximide-stimulated adrenals. Improved responsiveness of control mitochondria to lipid was observed by co-addition of large amounts of Ca^{2+} (22 mM) (Table 5). Presumably, this effect is due to mitochondrial swelling (oxidative phosphorylation is uncoupled by high Ca^{2+} concentrations) and improved interaction of non-esterified cholesterol of the lipid fraction with cytochrome *P*-450. Corticotropin-plus cycloheximide-stimulated mitochondria, on the other hand, were not stimulated by lipid in these conditions, conceivably because these mitochondria already possessed high non-esterified cholesterol concentrations (Mahaffee *et al.*, 1974).

As shown in Fig. 6, on addition of cholesterol-rich lipid fraction to mitochondria from control and corticotropin-stimulated adrenals, pregnenolone-induced type-II spectral changes were enhanced, particularly in mitochondria from corticotropin-stimulated adrenals. These results are in keeping with the possibility that lipid fraction supplies substrate for binding to mitochondrial cytochrome *P*-450 [comparable lipid-induced absorbance changes were observed in a soluble cholesterol-side-chain-cleavage system (Farese & Prudente, 1978*a*)].

Discussion

It seems clear that the rat adrenal yields two lipid fractions, which have distinctive physical properties. Lipid-I appears to be the classical low-density lipid-droplet fraction obtained by simple ultracentrifugation. Lipid-II, on the other hand, has a higher density and cannot be readily separated from clear cytosol by ultracentrifugation. The relatively large size of the lipid-II particles, however, allows ready separation from other cytosolic components by gel filtration (particularly Sepharose 2B or 4B). It is noteworthy that lipid-II, with its relatively high density and large size, is quite unusual when compared with serum lipoproteins (Sata *et al.*, 1970; Quarfordt *et al.*, 1972).

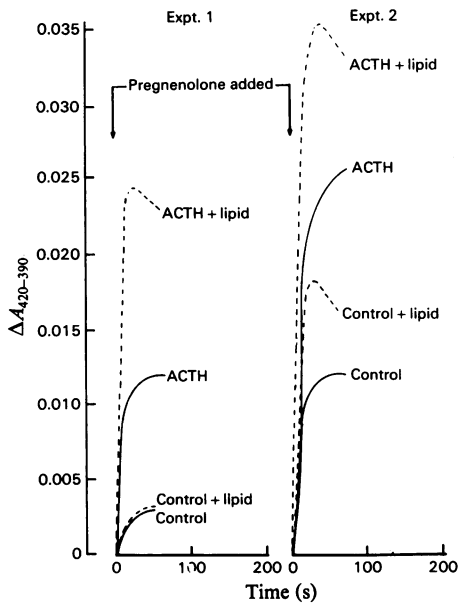

Fig. 6. *Pregnenolone-induced type-II spectral changes in mitochondria from control or corticotropin-stimulated adrenal tissues incubated with* (----) *or without* (——) *cholesterol-rich lipid-I (derived from corticotropin-plus-cycloheximide-treated adrenals)*

Mitochondria (0.5 ml derived from 50 mg of adrenal tissue) and 0.05 ml of lipid-I (derived from 5 mg of adrenal tissue) were incubated for 5 min at 22°C to attain a stable baseline, and pregnenolone was then added. The mitochondria from control and stimulated adrenals contained nearly identical concentrations of protein and cytochrome *P*-450. Addition of lipid-I to an assay system lacking mitochondria failed to affect the basal or pregnenolone-induced $\Delta A_{420-390}$. Abbreviation: ACTH, corticotropin.

Despite the physical differences of density, size and heat lability, it is not clear that lipid-I and lipid-II differ functionally. Both lipid moieties have increased non-esterified cholesterol concentrations after corticotropin plus cycloheximide stimulation, and both stimulate the cholesterol-side-chain-cleavage reaction. As suggested previously (Farese & Prudente, 1978*a*) and confirmed in the present paper, lipid-I is considerably more active than lipid-II in stimulating cholesterol side-chain cleavage, and this correlates with the greater non-esterified cholesterol concentrations (3–4-fold) in lipid-I.

Although not certain, it seems likely that the major stimulatory effect of adrenal lipid fraction on pregnenolone synthesis is due to provision of non-esterified cholesterol as substrate for the cholesterol-side-chain-cleavage reaction. In support of this, the following may be noted: (*a*) cholesterol-rich lipid fractions from corticotropin-plus cycloheximide-stimulated adrenals stimulated the cholesterol-side-chain-cleavage reaction considerably better than cholesterol-poor lipid fractions from control adrenals; and (*b*) under conditions of Ca^{2+}-induced mitochondrial swelling, cholesterol-rich lipid enhanced pregnenolone synthesis in cholesterol-poor control mitochondria, but not in cholesterol-rich mitochondria from corticotropin-plus cycloheximide-stimulated adrenals.

The addition of cholesterol-rich lipid fraction to adrenal mitochondria allowed us to test several hypotheses concerning corticotropin action. First, the greater stimulation of pregnenolone synthesis in mitochondria from corticotropin-stimulated adrenals, as opposed to mitochondria from control or corticotropin-plus cycloheximide-stimulated adrenals, suggests that corticotropin enhances cholesterol side-chain cleavage by a mechanism involving increased utilization of cholesterol [this conclusion is also supported by the finding (R. V. Farese, unpublished work) of enhanced formation of [^{14}C]isocaproic acid (4-methylpentanoic acid) from [^{14}C]cholesterol in corticotropin-stimulated mitochondria]. Secondly, this effect of corticotropin occurs in the presence of relatively large amounts (500 μM) of Ca^{2+}, and although Ca^{2+} alone could not explain the corticotropin-induced effect on mitochondrial cholesterol side-chain cleavage (Simpson *et al.*, 1978; Farese & Prudente, 1978*b*), it seems clear that the combination of increased Ca^{2+} and increased non-esterified cholesterol [both are reportedly stimulated by corticotropin (Mahaffee *et al.*, 1974; Leier & Jungman, 1973)] does not account for the major effect on corticotropin on cholesterol side-chain cleavage.

The present finding, suggesting that corticotropin stimulates steroidogenesis primarily by increasing the utilization of cholesterol, is in keeping with several other recent reports. Simpson and co-workers (1978) have reported that effects of corticotropin on mitochondrial pregnenolone synthesis are maintained or increased on addition of non-esterified cholesterol in acetone to adrenal mitochondria. Similarly, we have recently reported (Farese & Prudente, 1978*c*) that adrenals rich in non-esterified cholesterol due to previous aminoglutethimide blockade of cholesterol side-chain cleavage do not manifest increased steroidogenesis after aminoglutethimide washout, unless other corticotropin-induced factors are present (in which case the steroidogenic effects of corticotropin are potentiated by the elevated non-esterified cholesterol concentrations).

The present absorbance-spectrum findings add further insight into the mechanism whereby corticotropin increases cholesterol binding to cytochrome *P*-450 in adrenal mitochondria (Jefcoate *et al.*, 1970; Simpson *et al.*, 1972; Brownie *et al.*, 1973, 1974). Although previously observed differences in absorbance spectra could be explained by a simple cortico-

tropin-induced increase in non-esterified cholesterol, the persistence or intensification of such effects of corticotropin after addition of cholesterol-rich lipid seems incompatible with such an explanation. Another corticotropin-induced factor appears to enhance cholesterol binding to cytochrome *P*-450 and subsequent utilization, but the identity of the factor is presently unclear. Although many investigators, including ourselves, have postulated that the putative labile protein is the steroidogenic factor, we have recently found that corticotropin rapidly increases a polyphosphorylated glycerolipid, which in turn enhances mitochondrial pregnenolone synthesis (R. V. Farese & A. M. Sabir, unpublished work).

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