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Note

Pregnenolone production by adrenal mitochondria: a new high-performance liquid chromatographic analytical method for cholesterol side-chain cleavage

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Oxidative cleavage of the side-chain of cholesterol to form pregnenolone is the first step in adrenal steroid hormone biosynthesis. Side-chain cleavage occurs in the mitochondrion, catalyzed by cytochrome P-450$_{scc}$ (scc = side-chain cleavage [1]). Cholesterol in the mitochondrial inner membrane has been proposed to be the substrate pool for the inner membrane-located cytochrome, and movement of cholesterol to this membrane (e.g. from the outer membrane) may be the rate-limiting, corticotropin-regulated step in steroidogenesis [2–5].

Both direct and radiometric methods have been developed to measure pregnenolone production in isolated mitochondria and in the purified, reconstituted cytochrome P-450$_{scc}$ enzyme system. Direct assays of pregnenolone, such as gas–liquid chromatography and radioimmunoassay [6], measure pregnenolone mass and have been invaluable in defining the biphasic nature of pregnenolone output from activated mitochondria [4]. However, both methods entail cumbersome procedures involving multiple extraction and pipetting steps. In our experience, the radioimmunoassay can be highly condition-dependent, and multiple analyses are required to obtain statistically significant results.

Methods for the quantification of pregnenolone derived from labeled cholesterol require initial separation of pregnenolone from cholesterol. Separation has been achieved by both thin-layer chromatography [7] and by differential elution from Sephadex LH-20 mini-columns [8]. An alternative method employs a mixture of [7-^{3}H]- and [25-^{14}C]cholesterol [9]; side-chain cleavage yields [^{3}H]pregnenolone and [^{14}C]isocapraldehyde. A change in the isotopic ratio following volatilization of [^{14}C]isocapraldehyde provides a measure of the side-chain cleaved. Most of these radiometric methods require extensive handling of radio-

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active material which in the latter case involves volatile radioactive products and possible complications due to charring during the heating step.

Here, we report a new, convenient and rapid procedure involving isocratic high-performance liquid chromatographic (HPLC) separation of radiolabeled pregnenolone from cholesterol, with simultaneous quantification using a radioactive flow detector. By this approach, we have noted that the uptake of radiolabeled cholesterol added in ethanol and/or acetone can be rate-limiting in side-chain cleavage and have established conditions for "preloading" cholesterol into mitochondrial steroidogenic pools.

EXPERIMENTAL

Materials

Malate and $NADP^+$ were purchased from Sigma (St. Louis, MO, U.S.A.); [4-^{3}H]pregnenolone (5-pregnene-3β-ol-20-one), [1,2-^{3}H(N)]cholesterol, [4-^{14}C]cholesterol and Econofluor scintillation fluid from New England Nuclear (Boston, MA, U.S.A.); cyanoketone (2-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one) was a gift from Sterling Winthrop Institute (Rensselear, NY, U.S.A.). HPLC-grade isopropanol and acetonitrile were purchased from American Scientific Products (McGaw Park, IL, U.S.A.). Other chemicals were of reagent grade.

Mitochondrial preparation and incubation

Adrenals were removed from male Sprague–Dawley rats (200–250 g, King Animal Labs.) sacrificed by decapitation after 10 min of ether stress. Mitochondria were isolated as previously described [4] and were recentrifuged (5000 *g*, 10 min) and resuspended in 1 ml homogenization buffer [0.25 *M* sucrose, 1 m*M* ethylenediaminetetraacetic acid (EDTA), 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.0]. In some experiments, the mitochondria were then preloaded with [^{3}H]cholesterol. This was accomplished by incubation of the mitochondria with [^{3}H]cholesterol (0.3 μCi delivered in 15 μl ethanol or acetone) at 37°C in a shaking water bath for various periods. The radiolabeled mitochondria were reisolated by centrifugation (5000 *g*, 15 min) and resuspended in 0.5 ml side-chain cleavage reaction buffer (0.125 *M* potassium chloride, 25 m*M* HEPES, 5 m*M* magnesium chloride, 0.2 m*M* EDTA, pH 7.0). This mitochondrial preparation (100 μl) was added to reaction buffer (0.96 ml) at 37°C containing $NADP^+$ (0.7 m*M*), cyanoketone (4.0 μM), malate (5 m*M*, as a source of reducing equivalents) and, where indicated, [^{14}C]cholesterol (0.06 μCi delivered in 4 μl ethanol–acetone). At 0, 5, 15, and 30 min after initiation of side-chain cleavage, aliquots (150–200 μl) to be assayed for the presence of radiolabelled pregnenolone were vortexed in 1 ml dichloromethane. In the presence of mitochondria, greater than 99% of both pregnenolone and cholesterol were extracted into the organic phase.

Sample preparation

After separation of phases (approximately 1 h), the aqueous phase was removed by aspiration and the organic phase was dried under vacuum overnight. Acetonitrile–isopropanol (95:5, v/v, 60 μl) was added to each dry tube. These were then vortexed extensively to insure solubilization of both pregnenolone and cholesterol. Greater than 90% recovery was seen at this step for both steroids.

HPLC separation of pregnenolone from cholesterol

HPLC was carried out using a Beckman Model 332 gradient liquid chromatograph equipped with a Model 110A solvent delivery system and an Altex Model 100A injector with a 50-μl injector loop (Beckman Instruments, San Ramon, CA, U.S.A.). Separation was obtained under isocratic conditions on a 5-μm C_8 column (Altex Ultrasphere-Octyl, 25 cm × 4.6 mm) using a solvent system of acetonitrile and isopropanol. Flow-rate was maintained at 1.2 ml/min.

Quantitation of pregnenolone and cholesterol

A FLO-ONE Model HP radioactive flow detector (Radiomatic Instruments, Tampa, FL, U.S.A.) received eluant directly from the column. This was mixed in a 500-μl flow cell with liquid scintillant entering at a rate of 1.4 ml/min. Radioactivity was reported by digital output as ^3H, ^{14}C (net cpm per 0.5 min) or, when programmed for integration, as total cpm per peak. Data from incubations were expressed as the percentage conversion of cholesterol to pregnenolone (i.e., % conversion = cpm in the pregnenolone peak divided by the total cpm in both the pregnenolone and cholesterol peaks). Expression of data in this manner eliminates the need to correct for sample recovery or to normalize counts among different samples.

RESULTS

Chromatographic separation of pregnenolone and cholesterol

We sought isocratic conditions for rapid and complete separation of pregnenolone and cholesterol, as measured by the flow detector. These requirements were met using the chromatographic conditions described above and a mobile phase of acetonitrile–isopropanol (95:5, v/v) (see Fig. 1). With this solvent system, the retention times for pregnenolone and cholesterol were 4.3 and 7.3 min, respectively. Excessive retention of cholesterol with peak broadening was observed with 100% acetonitrile, and overlap of peaks occurred with mobile phases containing more than 5% isopropanol.

On the basis of cpm loaded onto the column, greater than 95% of the cholesterol and pregnenolone standards (typically 3.0–6.0 nCi) were recovered within 10 min, thus eliminating the need for column washing between analyses and allowing rapid processing of samples. From chromatograms in which ^{14}C was the only isotope, ^{14}C spillover into the ^3H channel was found to be between 22 and 24%; runs involving dual isotopes were corrected accordingly.

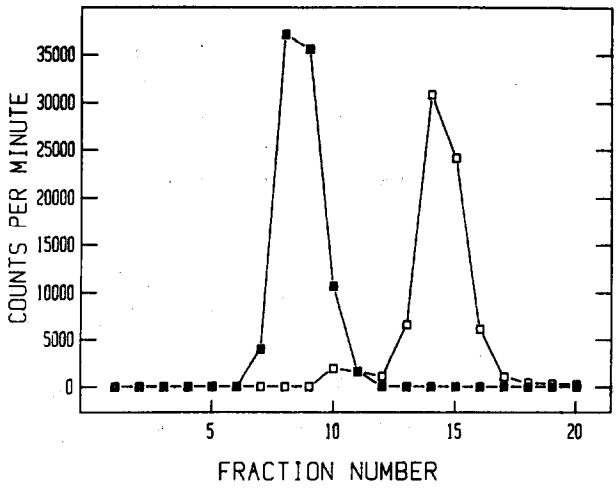

Fig. 1. Separation of [^{14}C]pregnenolone and [^{3}H]cholesterol by HPLC. A standard mixture containing approximately 4 nCi of [^{14}C]pregnenolone and [^{3}H]cholesterol in 60 μl acetonitrile was injected onto a 5-μm reversed-phase C_8 column pre-equilibrated with acetonitrile–isopropanol (95:5, v/v) as described in Experimental. Counts were measured by a radioactive flow detector. Filled squares are ^{14}C (pregnenolone) counts and open squares are ^{3}H (cholesterol) counts. The flow-rate was 1.2 ml/min and the fractions were collected every 0.5 min.

Mitochondrial preloading

Preloading was accomplished by adding cholesterol (0.3 μCi) in ethanol–acetone (1:1) to mitochondrial suspensions and incubating for various periods, up to 1 h. The mitochondria were then reisolated by centrifugation, and mitochondrial-associated label quantified. As shown in Fig. 2, the extent of cholesterol incorporation was dependent on the time of preincubation. Complete uptake of cholesterol was not seen even after 60 min, and maximal incorporation required 20 min or more. Thus, it is clear that added cholesterol does not become instantaneously associated with the mitochondrion. It is likely that under some circumstances this cholesterol association may limit side-chain cleavage activity.

Analysis of side-chain cleavage activity in isolated adrenal mitochondria

When tritium-labeled cholesterol was added immediately prior to initiation of side-chain cleavage (malate addition) typically less than 20% conversion was seen at 30 min (not shown). Fig. 3A shows that with preloaded mitochondria greater than 60% conversion was achieved in this time. Thus, under cholesterol preincubation conditions, a much greater fraction of the substrate is available for conversion.

Fig. 3B and C also demonstrates that [^{14}C]cholesterol added in a minimal volume (0.5%, v/v) of acetone becomes only slowly available for side-chain cleavage. In panel B, [^{14}C]cholesterol is added to the same preparation of [^{3}H]cholesterol-preloaded mitochondria simultaneously with malate initiation of side-chain cleavage. While the conversion of the preloaded cholesterol remains efficient, the same quantity of acetone-added cholesterol is only poorly converted.

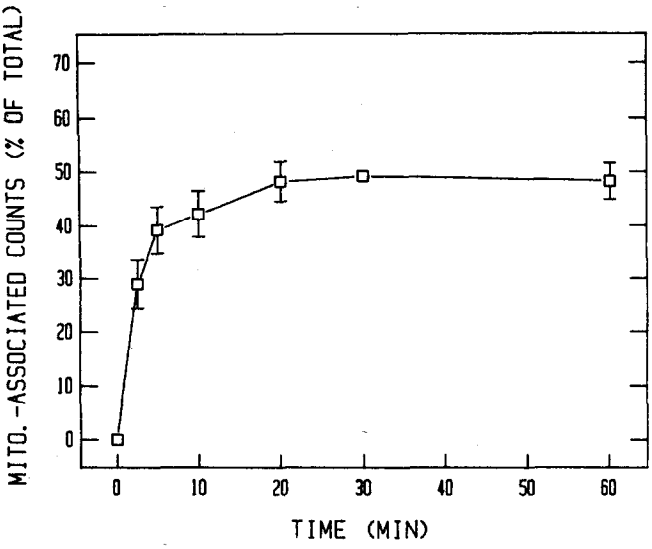

Fig. 2. Time dependence of cholesterol incorporation into isolated adrenal mitochondria. Mitochondria (4 mg protein per ml) were incubated at 37°C in homogenization buffer containing 0.3 μCi [^{3}H]cholesterol. The mitochondria were reisolated by centrifugation (5000 *g*, 15 min) and resuspended in 0.5 ml reaction buffer. Aliquots (100 μl) were assessed for extent of radiolabel incorporation by counting in a Beckman Model LS-7000 scintillation counter.

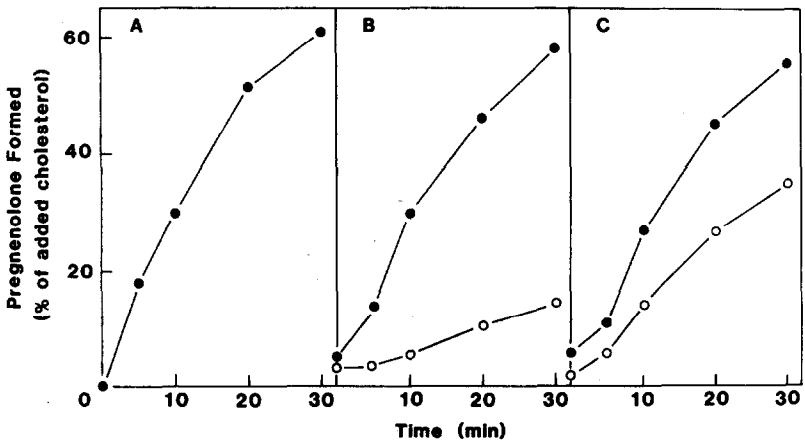

Fig. 3. Time course of pregnenolone formation from added cholesterol. Mitochondria (0.2 mg protein per ml) were incubated in reaction mixture containing NADP$^+$ and cyanoketone. Side-chain cleavage was initiated by addition of malate. Aliquots (150–200 μl) were removed at indicated times of analysis of radiolabeled pregnenolone and cholesterol content by HPLC. (A) Mitochondria were preloaded with [^{3}H]cholesterol for 60 min. (B) Mitochondria preloaded with [^{3}H]cholesterol were added to reaction mixture containing [^{14}C]cholesterol and malate. (C) Preloaded mitochondria were incubated for 30 min in reaction mixture containing [^{14}C]cholesterol prior to initation of side-chain cleavage with malate. (●) [^{3}H]Pregnenolone; (○) [^{14}C]pregnenolone.

However, if the acetone-added cholesterol is allowed to preincubate for 30 min with the preloaded mitochondria, the percentage conversion is increased several-fold, without significantly affecting the metabolism of the preloaded cholesterol. Thus, cholesterol added in organic solvents is initially poorly available for side-chain cleavage and requires slow equilibration into the metabolically active pool. Preloading overcomes this problem and allows efficient utilization of substrate.

DISCUSSION

The procedures outlined herein accomplish efficient extraction and HPLC separation with simultaneous quantification of pregnenolone and cholesterol. The principal advantage of this method is its speed and accuracy and the minimization of artifacts due to sample handling. Further, the chromatographic recovery is virtually complete, eliminating the need for column recycling between analyses. The major disadvantage is the inability to measure total pregnenolone production. Isolated adrenal mitochondria contain 20–40 nmol cholesterol per mg protein. Unlike direct assays, radiometric methods monitor the conversion of exogenously supplied radiolabeled substrate rather than the mass of pregnenolone produced from all substrate sources (i.e., endogenous and exogenous). Caution must therefore be applied when interpreting data since rates may not reflect substrate conversion from all pools. Nonetheless, with careful consideration of the experimentally appropriate conditions, the method described herein provides a simple assay for pregnenolone, amenable to a variety of manipulations. In particular, the method is likely to be useful for testing the effects of various putative activators and inhibitors and for other situations where mass measurements are not required.

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