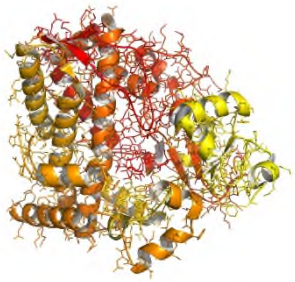

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RECONSTITUTION OF PURIFIED HUMAN LIVER P450 ENZYMES

A protocol is given for reconstituting purified liver P450 enzymes with P450 reductase (Fp) and cytochrome b_5 (b_5) using phospholipids. Fp is required to pass electrons from the cofactor NADPH to the P450 enzyme while inclusion of b_5 in the reconstituted system often results in metabolic properties (e.g., K_M) that more closely resemble those of intact liver microsomes. The phospholipid used is dilauroylphosphatidylcholine (DLPC), since reconstitution with this lipid gives optimal catalytic activity with all human P450s examined (except for CYP3A4; see note below).

In the following experiment, two human P450s (CYP2A6 and CYP2C9) are being tested for their ability to metabolize a substrate. Each reconstituted system is prepared in a quantity sufficient to assess substrate metabolism by both enzymes ± b_5 in triplicate. Amounts of CYP2A6 and CYP2C9 used are such that rates of product formation are proportional to the amount of P450 added. The P450:Fp, P450:b_5, and P450:DLPC ratios given have already been optimized.

SET-UP FOR RECONSTITUTED SYSTEMS (RCS)

Enzyme	Concentration
Human CYP2A6	3.25 nmol/ml
Human CYP2C9	8.45 nmol/ml
Human Fp	15.7 nmol (55 kU)/ml
Human b_5	25.8 nmol/ml
DLPC (Serdary/Avanti)	1 mg/ml in H_2O (see ☆ below)

ORDER OF ADDITION TO 10 x 75 mm GLASS TUBES KEPT @ ROOM TEMP:
DLPC ➔ Fp ➔ P450 ➔ b_5 - Vortex After Each Addition

	DLPC	Fp	b_5	P450	λ/assay
RCS-**A**	48 λ	32λ	25 λ	--	33 (n=3)
RCS-**B**	48 λ	32 λ	--	49λ **2A6** Prep#5	40 (n=3)
RCS-**C**	48 λ	32 λ	25 λ	49λ **2A6** Prep#5	48 (n=3)
RCS-**D**	48 λ	32 λ	--	19λ **2C9** Prep#7	31 (n=3)
RCS-**E**	48 λ	32 λ	25 λ	19λ **2C9** Prep#7	39 (n=3)

RCS-A contains **NO P450**,150 pmol (500 units) Fp, 15 µg DLPC and 200 pmol b_5. RCS-B ➔ RCS-E contain 50 pmol P450 enzyme, 150 pmol Fp, 15 µg DLPC **±** 200 pmol b_5.

NOTE: The amounts of DLPC, Fp, and P450 enzyme used to form each reconstituted system are slightly greater than that required for triplicate assay tubes. For example, while only 45λ (45 µg) DLPC, 30λ (450 pmol) Fp and 46λ (150 pmol) 2A6 are needed to run triplicate tubes with RCS-B, 48λ DLPC, 32λ Fp and 49λ 2A6 are actually added so that 40λ of the mix can be readily retrieved for each of the 3 tubes.

Once All The Above Components Are Added:

1. Incubate tubes containing RCS @ 37°C for 5 min, then place back onto ice.

2. Add the appropriate amount of each RCS to the tubes in which the metabolism reactions will be performed. These tubes should at least contain assay buffer (e.g, 100 mM KPO_4).

3. Add the remainder of the reaction components.

4. Start reactions with NADPH, and terminate after 5-10 min according to substrate protocol.

☆Prepared fresh by adding 100λ of DLPC stock solution [10 mg/ml in $CHCl_3$] to a 13 x 100 mm glass tube, evaporating off **ALL** the $CHCl_3$ with a gentle stream of nitrogen, adding 1.0 ml of **H_2O**, and resuspending using 4 x 15 sec bursts of a probe-type sonicator set @ 100 watts (**NOTE:** cool solution for 30 seconds on ice between bursts).

IMPORTANT NOTE: The phospholipid used here for optimal reconstitution of CYP2A6 and CYP2C19 catalysis is DLPC. This same phospholipid has also proved best for reconstituting the catalytic activity of all other P450 enzymes with the singular exception of CYP3A4. The latter P450 exhibits a different phospholipid requirement for successful reconstitution, which is comprised of a mixture of DLPC plus dioleoylphosphatidylcholine (DOPC) and dilauroylphosphatidylserine (DLPS)(1:1:1 ratio). The addition of CHAPS (100 µg/ml) and/or glutathione (3 mM) may enhance CYP3A4-mediated metabolism in the reconstituted system, depending upon the particular substrate.