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SHORT COMMUNICATION

SOLUBILISATION AND FRACTIONAL PRECIPITATION OF A STEROID ALPHA-KETOL OXIDASE

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Summary—An alpha-ketol oxidase that converts deoxycorticosterone to pregnenoic acid has been solubilised and fractionally precipitated with polyethylene glycol from rabbit liver microsomes. Maximal activity was obtained with the 12–14% fraction. Activity was linear with cytochrome P-450 concentrations up to 0.2 nmol and was inhibited with carbon monoxide.

INTRODUCTION

Cytochrome P-450 isozymes of rabbit liver microsomes have been shown to catalyse the hydroxylation of the progesterone ring system at C-6 and C-16 [1] and the acetyl side-chain at C-21 [2, 3]. 21-Hydroxylation of progesterone is of particular interest since it gives deoxycorticosterone which may undergo further oxidation *in vitro* to a 20-oxo-21-oic acid, termed pregnenoic acid,* and a C-20-etienic acid [4]. Although there was initial doubt as to whether a cytochrome P-450 system was involved in the side-chain oxidations, primarily because of a relative insensitivity to the presence of carbon monoxide [4], a more recent study found a variable inhibition of pregnenoic acid by rabbit liver microsomes and a highly significant inhibition of the same reaction catalysed by rabbit adrenal microsomes [5]. This led to a reconsideration of the possible involvement of a cytochrome P-450 system in the hepatic microsomes and as a preliminary to its isolation, the solubilisation, fractional precipitation, and reconstitution of the alpha-ketol oxidase system was investigated.

EXPERIMENTAL

Liver microsomes were prepared from outbred New Zealand White rabbits (3.5–4.0 kg; Ferme Cunicole, Claude Leonard, Quebec, Canada) of both sexes as previously described [5] with an additional wash of the microsomal pellets with 0.1 M sodium pyrophosphate buffer, pH 7.4 to remove hemoglobin [6]. Microsomal protein (1.5 g) was solubilised and precipitated with a 50% aqueous solution of polyethylene glycol (8000, Baker Chemical, Inc., U.S.A.) essentially as described by Coon and coworkers[6], except that PEG precipitates corresponding to 2% increments between 6 and 18% were collected. Each was dissolved in 10 mM Tris acetate buffer containing 20% glycerol and 1 mM EDTA, pH 7.4 (25 ml) and dialysed overnight against 2.5 l of the same buffer at 4°C. Protein [7], cytochrome P-450 [8] and cytochrome b_5 [9] were measured by published procedures. Cytochrome P-450 reductase was purified as described by French and Coon[10]. The reductase (0.1 U) and cytochrome P-450 fractions were reconstituted without addition of dilauroyl-L-α-lecithin. Steroid acid formation was assessed by incubation of [4-^{14}C]deoxycorticosterone (58.5 mCi/mmol; 0.05 μCi; New England Nuclear (Canada Ltd) with the reconstituted system. The reaction was started by the addition of NADPH (500 μM) to give a total reaction vol of 1 ml. Incubation conditions, thin layer chromatography and the detection of radiometabolites and standards was as previously described [5]. Pregnenoic acid was synthesised as described by Monder[11] and etienic acid was purchased from Steraloids Inc., U.S.A. Inhibition studies were carried out with mixtures of carbon monoxide–air prepared in an inverted separatory funnel submerged in a tank of water. The gas mixture was bubbled through a stainless steel needle inserted in the rubber cap of a glass test tube containing the incubation mixture. After 5 min NADPH was added, the needle withdrawn, and the tubes covered with alumina foil and incubated. Radiometabolites were quantitated by scraping silica gel zones into counting vials containing a PCS-Xylene scintillation fluid (10 ml; 50:50 v/v; Amersham–Searle, U.S.A.) and counting in a Beckman Liquid Scintillation Counter (Model 9000).

RESULTS AND DISCUSSION

Rabbit liver microsomes were solubilised with sodium cholate and the protein fractions were precipitated with increasing concentrations of polyethylene glycol under conditions which have been extensively used prior to the purification of cytochrome P-450 isozymes. One variation employed here was to examine the fractional precipitation of solubilised microsomal protein by increasing the PEG concentration by 2% increments. In addition the precipitation was carried on further than is usually employed to 18% PEG. Individual precipitates were incubated, after dialysis, with [^{14}C]DOC under conditions expected to reconstitute alpha-ketol oxidase activity, namely; PEG fraction + cytochrome P-450 reductase + air. The addition of a phospholipid did not influence acid formation. The solubilised microsomal liquor and the 0–6% PEG precipitate lacked detectable alpha-ketol oxidase activity. Figure 1 shows that activity was detected in all other fractions from 6–18% PEG precipitates with the highest levels being obtained with the precipitates above 12% PEG. Pregnenoic acid formation appeared to correlate with all three of the major PEG fraction constituents, namely; protein, cytochrome P-450 and cytochrome b_5. Though each fraction also contained a low level of cytochrome P-450 reductase, additional reductase (0.1 U) was routinely added to avoid it being a limiting factor. The cytochrome P-450 content of the PEG fractions is of importance to the alpha-ketol oxidase activity since the latter was inhibited by 49.7% by a 1:1 carbon monoxide–air mixture and 85.5% by a 9:1 ratio.

Abbreviations: PEG, Polyethylene glycol; pregnenoic acid, 4-pregnen-3,20-dione-21-oic acid; DOC, 21-hydroxy-4-pregnen-3,20-dione.

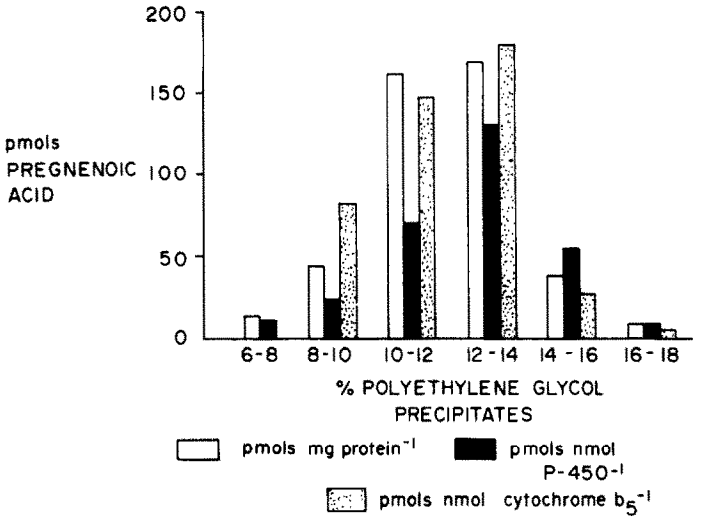

Fig. 1. Correlation of pregnenoic acid formation with the distribution of protein, cytochrome P-450 and cytochrome b_5 between PEG fractions. Incubates contained the PEG fraction equivalent to 0.22 nmol P-450, reductase (0.1 U) and NADPH (500 μM).

Table 1. Distribution of cytochrome P-450 and the formation of pregnenoic acid by the polyethylene glycol precipitates

% PEG fraction	Total P-450/PEG fraction (nmol)		Total pregnenoic acid/ PEG fraction (pmol × 10^{-3})		
	1	2	1	2	Mean
6–8	194	165	9.2	1.7	5.5
8–0	735	448	29.4	9.6	19.5
10–12	552	334	39.0	23.3	31.1
12–14	310	143	47.0	28.6	37.8
14–16	109	51	15.0	26.8	20.9
16–18	52	49	0.3	0.4	0.4

Results are given for 2 rabbits. Incubations were carried out for 30 min with 0.2 nmol cytochrome P.450 and [^{14}C]pregnenoic acid was isolated by TLC.

This is in contrast to the microsomal preparations which have exhibited variable but relatively low sensitivity to carbon monoxide [4, 5]. Comparison of the fractional precipitation of cytochrome P-450 and alpha-ketol oxidase activity (Table 1) shows that the bulk of the microsomal cytochrome P-450 is precipitated in the 8–12% PEG fractions, whereas the oxidase activity is highest in the 12% plus PEG fractions. The metabolism of DOC to pregnenoic acid is often accompanied by variable amounts of etienic acid. Figure 2 shows that only the formation of pregnenoic acid was linear with cytochrome P-450 up to 0.2 nmols. The low level of alpha-ketol oxidase activity in rabbit liver microsomes, which gives pmolar amounts of pregnenoic acid, presents problems for its isolation and purification. The appropriate selection of PEG precipitates and of rabbits with relatively high levels of hepatic alpha-ketol oxidase activity may facilitate the purification of this enzyme.

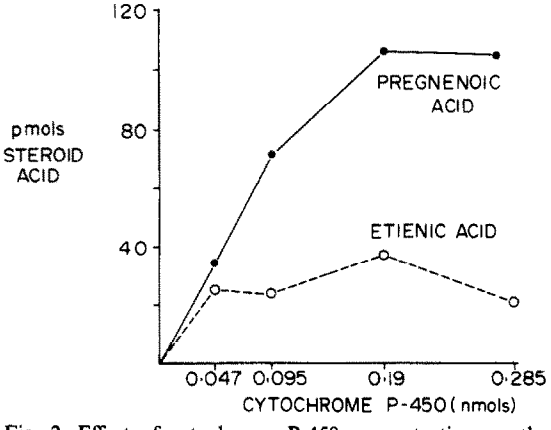

Fig. 2. Effect of cytochrome P-450 concentration on the formation of pregnenoic and etienic acid by the 12–14% PEG fraction.

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