SOLUBILIZATION OF RAT LIVER MICROSOMAL CHOLESTEROL 7 α-HYDROXYLASE

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1. Introduction

The 7 α-hydroxylation of cholesterol is considered to be the first and rate limiting step in the transformation of cholesterol to bile acids [1,2]. Cholesterol 7 α-hydroxylase (EC 1.14) is located in the microsomal fraction of rat liver and requires NADPH, molecular oxygen, and an electron transport system involving cytochrome *P*-450 and NADPH-cytochrome *c* oxido-reductase [3–7]. When the concentration of bile acids in the enterohepatic circulation is lowered, either by cannulation of the bile duct or by feeding rats a bile acid sequestrant, the specific content of cytochrome *P*-450 remains constant, but the specific activity of cholesterol 7α-hydroxylase is increased 3-fold [1,3]. Cholesterol 7α-hydroxylase appears to be more sensitive to detergents and salts than other cytochrome *P*-450 dependent reactions [8]. It is therefore likely that the bulk of the liver microsomal cytochrome *P*-450 is not associated with cholesterol 7α-hydroxylase activity. To study this enzyme in greater detail, it is first necessary to release it from the microsomal membrane. The mixed function oxidase system from rat liver microsomes has previously been solubilized and fractionated [9], but these methods rely on the detergency of sodium cholate or sodium deoxycholate. Since these detergents are both strong inhibitors of cholesterol 7α-hydroxylase it was necessary to explore other methods for the solubilization of this mixed function oxidase.

2. Materials and methods

Liver microsomes from rats fed a diet containing 4% w/w cholestyramine (a bile acid sequestering agent) were prepared in the usual way [10]. Microsomes from four livers (total 32 g wet weight) were resuspended in 0.154 M KCl to give a final volume of 7 ml. An aliguot of this suspension was added dropwise to 100 vol of stirred acetone cooled to approx. −30°C. The acetone was immediately filtered through a Buchner funnel, and the powder washed with diethyl ether then acetone, both cooled to −30°C. The powder was kept in vacuo at room temperature for one hour, then stored at −20°C. Cholesterol 7α-hydroxylase activity was retained for several weeks.

Cholesterol 7α-hydroxylase activity was assayed as described previously [10].

Cytochrome *P*-450 and cytochrome b_5 were measured according to the method of Omura and Sato [11].

NADPH-cytochrome *c* oxidoreductase activity was assayed by adding an aliquot of the sample to 2.8 ml 0.1 M potassium phosphate buffer pH 7.55, 1 mg cytochrome *c*, 100 μl NADPH generator (0.5 μmol $NADP^+$, 5 μmol glucose 6-phosphate and 0.5 IU glucose 6-phosphate dehydrogenase). The difference in absorbance between 551 nm and 540 nm was measured.

Protein was determined by the biuret method [12]. Cholesterol was measured by gas-liquid chromatography on a 1% SE30 column using pregnenolone acetate as the internal standard.

Nonidet P40 was obtained from BDH Chemicals Ltd., Poole, England. This surface active agent is an octyl-phenol-ethylene-oxide condensate, (average nine mol ethylene oxide). Other reagents used were

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the highest grade commerically avaible.

For solubilization, the acetone powder or microsomal suspension in 0.1 M phosphate buffer pH 7.6 was cooled to 0–4°C. After addition of Nonidet P42 detergent, the suspension was kept on ice for 20 min, stirring occasionally.

3. Results

An acetone powder was used to test the ability of enzymes and surface active agents to solubilize cholesterol 7α-hydroxylase. The results of these tests are summarized in table 1. Although most detergents

Table 1
Effect of solubilizing agents on the activity of cholesterol 7α-hydroxylase in rat liver microsomal acetone powder

Conditions	Suspension	Supernatant	Pellet
Sodium cholate 1.5 mg/mg protein	70% inhibition	70% inhibition	80% inhibition
Sodium deoxycholate 1.5 mg/mg protein	inactive	inactive	inactive
Phospholipase A (from *Crotalus adamanteus* venom, 4 μU/mg acetone powder)	–	inactive	some loss of activity
3 M Urea	inactive	inactive	inactive
8% Butanol	inactive	inactive	inactive
Pancreatin 5 mg/60 mg acetone powder	–	inactive	–
Naja naja venom; 5 μg and 1 mg/40 mg acetone powder	active	inactive	active
0.1% and 1% Lubrol WX	90% inhibition	inactive	inactive
0.1% Lubrol W	90% inhibition	inactive	inactive
0.1% Triton X-100	inactive	–	–
1% Digitonin	inactive	inactive	inactive
0.1% and 2% cetyl trimethylammonium bromide	inactive	–	–
0.1% and 2% Saponin	inactive	–	–
0.1% Tween 80	inhibition	inhibition	inactive
0.1% Nonidet P40	active	active	loss of activity
0.1% Nonidet P42	active	active	loss of activity

Acetone powder (approx. 50 mg.) was suspended in 12 ml 0.1 M potassium phoshate buffer pH 7.7 and treated with the solubilizing agents shown in the table. Cholesterol 7α-hydroxylase activity was measured as described previouslv [10].

solubilized cytochrome *P*-450, all but Nonidet P42 led to substantial inhibition of cholesterol 7α-hydroxylase. The surfactant Nonidet P42 (a 27% solution of Nonidet P40 in water) solubilized the components of the microsomal mixed function oxidase, and the resultant supernatant after centrifugation at 100 000 *g* for 1 hr still retained cholesterol 7α-hydroxylase activity.

3.1. *Effect of concentration of Nonidet P42 on the solubilization of acetone powder*

To determine the optimal conditions for solubilization of cholesterol 7α-hydroxylase, an acetone powder was suspended in 0.1 M phosphate buffer pH 7.6 at 0°C and increasing amounts of Nonidet P42 added. The percentage solubilization of protein, cytochrome *P*-450, cytochrome b_5, NADPH-cytochrome *c* reductase, and the activity of cholesterol 7α-hydroxylase is plotted against Nonidet P42 concentration in figs.1 and 2. From these graphs it

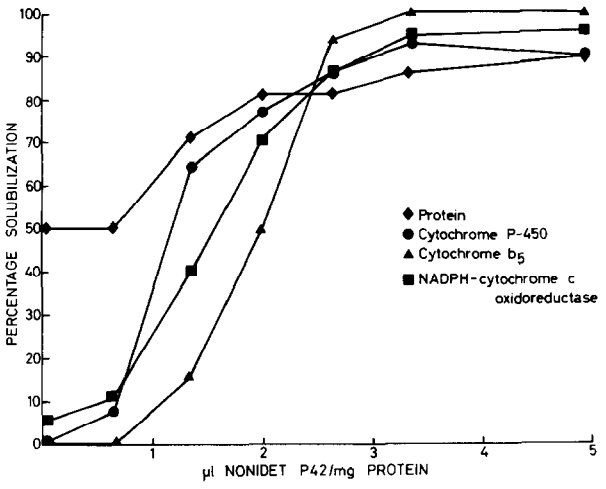

Fig.1. 30 mg liver microsomal acetone powder (15 mg protein) were suspended in 10 ml of 0.1 M phosphate buffer, 1 mM EDTA pH 7.55, and solubilized, as described in the text, with increasing quantities of Nonidet P42. After centrifugation at 105 000 *g* for 1 hr, the pellets were resuspended in 10 ml of buffer and the suspensions and supernatants assayed for protein, cytochrome *P*-450, cytochrome b_5, and NADPH cytochrome *c* oxidoreductase. Percentage solubilization is the ratio

$$\frac{\text{supernatant assay}}{\text{supernatant assay + pellet assay}} \times 100$$

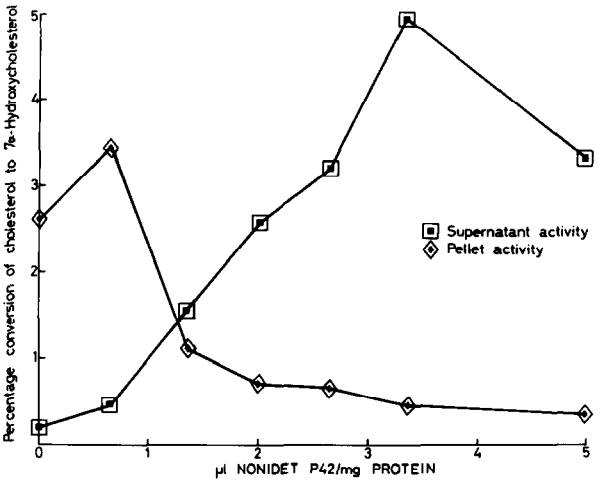

Fig.2. Experimental details were as in fig.1. Cholesterol 7α-hydroxylase activity was determined in the supernatants and resuspended pellets and expressed as the percentage conversion of [4-^{14}C]cholesterol to [4-^{14}C] 7α-hydroxycholesterol.

may be seen that the gradual release of cholesterol 7α-hydroxylase into solution follows the progress curve of solubilization of the mixed function oxidase components. Nonidet P42 solubilizes effectively over the concentration range 2–6 μl Nonidet P42/mg protein, but there is a narrow concentration range where substantial activation of cholesterol 7α-hydroxylase occurs.

3.2. *Criterion of solubility*

A solubilized rat liver microsomal acetone powder was centrifuged at 100 000 *g* for 7 hr, and after this period, results showed that approximately 75% of the enzyme activity still remained in the supernatant fraction. Since the enzyme may have lipoprotein characteristics, the specific gravity of the supernatant was determined and found to be 1.014.

3.3. *Effect of Nonidet P42 on native microsomes*

The applicability of the method used on a microsomal acetone powder to solubilization of native microsomes was studied using rat liver microsomes. A gradual release of mono-oxygenase components into solution on increasing the concentration of Nonidet P42 was again observed, and at a concentration of 4 μl Nonidet P42 X (mg protein)$^{-1}$ over 90% of

these components were solubilized. Cholesterol 7α-hydroxylase was measured as a function of Nonidet P42 concentration and results showed an activation of the enzyme at low concentration 0.3 μl Nonidet P42 × (mg protein)$^{-1}$.

Increasing detergent concentration caused a loss of activity in the pellet and a concomitant increase in cholesterol 7α-hydroxylase activity in the supernatant after centrifugation. Maximum cholesterol 7α-hydroxylase activity was observed in the supernatant fraction when 4 μl Nonidet P42 × (mg protein)$^{-1}$ was used for solubilization.

4. Discussion

Microsomes are a heterogeneous and complex mixture and study of the components necessitates solubilization. Cytochrome *P*-450 has proved to be difficult to resolve and where enzymic activity is to be studied, gentle solubilization procedures with minimal loss of activity are usually necessary prerequisites. Sodium deoxycholate holds the most prominent position in solubilization of microsomes as evidenced by the successful solubilization and reconstitution of several cytochrome *P*-450 dependent reactions, based on the method originally devised by Lu et al. [13]. Non-ionic detergents have been used by Miyake, [14] and more recently by Sato [15], Imai and Sato [16] and Ichihara et al. [17]. Because of the considerable inhibition of cholesterol 7α-hydroxylase by sodium deoxycholate, trial and error use of other surface active agents was employed, and it was found that although many detergents were capable of solubilizing microsomes, most inhibited or inactivated cholesterol 7α-hydroxylase even when the cytochrome *P*-450 content was not altered. Nonidet P42 solubilized both microsomal acetone powder and native microsomes with retention of cholesterol 7α-hydroxylase activity. The apparent activity of cholesterol 7α-hydroxylase solubilized from a liver microsomal acetone powder is at least as great as the control microsomal acetone powder with no added detergent and in most cases, at a concentration of approx. 3.5 μl Nonidet P42/mg protein, there is a very substantial activation. The reason for this activation is not understood.

With this preparation, resolution of the soluble cholesterol 7α-hydroxylase is now in progress in this laboratory.

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