# THE BINDING OF SQUALENE BY HUMAN PLACENTAL CYTOSOL: ROLE IN CONVERSION OF SQUALENE TO LANOSTEROL

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## 1. Introduction

Squalene epoxidation and cyclisation are steps of cholesterol biosynthesis which result in conversion of squalene to lanosterol. We could demonstrate [1] that human placenta possesses an effective epoxidase cyclase activity which leads under our experimental conditions to lanosterol rather than to cholesterol; however this epoxidase cyclase has a low basal level. More recently we located the rate limiting step in the epoxidation process [2].

The placental epoxidase activity is highly increased [1] by addition of liver squalene carrier protein (SCP). Thus, the low basal epoxidase activity of placental microsomes could be related to a lack of SCP or to an alteration of the squalene binding capacity of the placental cytosol proteins. The aim of this work is to look for these two possibilities.

The squalene binding capacities of native and heated human placental 105 000 *g* supernatant (S 105), of native and heated rat liver S 105, bovine serum albumin and human plasma proteins were studied by Sephadex gel filtration. The binding capacity of these fractions was compared to their ability to activate microsomal squalene epoxidase. It is shown that native placental S 105 has an important squalene binding capacity but is not very efficient in activating liver or placental epoxidase. Thus, at least in placenta, the squalene binding and the epoxidase activation should be related to different factors.

## 2. Materials and methods

### 2.1. *Microsomes and S 105 of rat liver and human placenta*

Fresh human placentas are hashed (meat grinder) and washed with ice-cold 0.02 M phosphate buffer, pH 7.35, 0.001 M EDTA (buffer A), to remove blood prior to homogenization. After centrifugation (13 000 *g*, 20 min), the pellet is homogenized (Ultra Turrax) in buffer A and microsomes and S 105 are separated by differential ultracentrifugation as previously described [1], except that we carried out an additional washing of microsomes with buffer A. Protein concentration was determined by the spectrophotometric method of Kalckar [3] or the colorimetric method of Lowry [4].

### 2.2. *Conversion of squalene to lanosterol*

In a final volume of 10 ml (buffer A) 40 mg of microsomal proteins were incubated during 210 min at 37°C under oxygen, with 25 mg of placental S 105 proteins or the same amount of bovine serum albumin or rat liver S 105 proteins. Cofactors and squalene were present in the following concentrations: NAD, NADPH (Boehringer): 0.4 mM; reduced glutathione (Boehringer): 5 mM; $Mg^{2+}$: $3 \times 10^{-2}$ M; [11,12 $^3$H]squalene (18 mCi/mM): $0.45 \times 10^{-6}$ M (total radioactivity: 180 000 dpm). Other conditions and the procedure used to dissolve squalene have been described in detail [1]. The isolation of reaction products was slightly modified as described earlier [2]. The results are expressed as percent of conversion of squalene to lanosterol [1].



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### 2.3. *Studies on squalene binding*

#### 2.3.1. Gel filtration of G25 Sephadex

30 μl of a squalene solution in hexane (180 000 dpm) are evaporated to dryness in the incubation vial and dissolved in 20 μl of dioxane–glycol (2/1 : v/v). This solution is diluted in 2.5 ml of S 105 and the radioactivity of two 0.1 ml aliquots is determined by liquid scintillation counting in 15 ml of scintillator 1 (toluene/PPO/POPOP: 1 liter/1 g/0.1 g). Two ml of squalene solution in S 105 are incubated (37°C, 15 min) and applied to a G 25 Sephadex column (2 × 30 cm). The elution is processed at 4°C with buffer A (at a rate of 1 ml/min). The protein bound radioactivity is determined by liquid scintillation counting of 0.2 ml aliquots in 3 ml ethanol + 12 ml of scintillator 1.

Controls are performed in the same conditions with buffer A instead of S 105.

It must be pointed out that dioxane should be freshly distilled because peroxidized dioxane abolishes the binding of both placental and liver S 105, probably by protein inactivation. The non protein bound squalene (free squalene) is adsorbed on the G25 Sephadex column, and accumulates as chromatographies are repeated; control experiments have shown that this column should not be used more than twice.

#### 2.3.2. Gel filtration on G 200 Sephadex

Placental S 105 protein (20 mg) are filtered on a G 200 Sephadex column (2 × 65 cm). Fractions containing proteins of molecular weight lower than 100 000 are concentrated (Carbowax), incubated (37°C, 15 min) with [11,12-$^3$H] squalene ($1.1 \times 10^{-6}$ M) and filtered again on the same column.

#### 2.3.3. Electrophoresis

Electrophoresis on cellulose acetate was done with 'Cellogel' Chemetron, in the conditions described by the manufacturer. For liquid scintillation counting, the strips are cut and counted after elution of squalene in a mixture Triton X 100 and scintillator 1 (1/2:v/v).

Polyacrylamide gel electrophoresis was performed with S 105 preincubated (or not) with radioactive squalene (incubation conditions of 2.3.1.), according to the method of Weber [5]. The gel were cut and the fragments digested in NCS (Amersham) (1 ml, 15 min, 60°C) before counting.

#### 2.3.4. Dextran coated charcoal technique

Conditions are similar to those used by Mercier [6] for the study of steroid binding by plasma proteins: S 105 proteins preincubated in the conditions described above (2.3.1) with [11,12-$^3$H] squalene ($1.8 \times 10^{-6}$ M) were stirred with an equal volume of a dextran coated charcoal suspension in buffer A (Norit Prolabo: 0.5 g/100 ml; Dextran T 70 Pharmacia: 0.05 g/100 ml). Aliquots are taken at 0, 15, 40, 60 min and centrifuged (4000 rpm × 20 min). The radioactivity of the supernatant is determined by liquid scintillation counting as in 2.3.1.

## 3. Results and discussion

### 3.1. *Effect of placental 105 000 g supernatant on microsomal squalene to lanosterol conversion*

Table 1 shows the results of a serie of incubations. The conversion of squalene to lanosterol by washed placental microsomes is activated (2 or 3 times) by native placental S 105, but not by heated placental S 105 or bovine serum albumin. However, a higher activation is observed when placental microsomes are incubated in the presence of rat liver S 105.

A placental lack in SCP could explain the different abilities of placental S 105 and of hepatic S 105 to activate microsomal conversion of squalene into lanosterol. Thus we looked for the presence of a squalene binding protein in placental S 105.

### 3.2. *Squalene binding capacity of placental S 105*

Since equilibrium dialysis was inadequate to study this binding (in the conditions used, squalene is not dia-

Table 1
Squalene to lanosterol conversion by washed placental microsomes

| Incubation medium | Conversion (%) |
|---|---|
| Buffer A | 0.5 (2) |
| Native placental S 105 | 1.4–1.9 (3) |
| Heated placental S 105 | 0.4 (1) |
| Native rat liver S 105 | 3.6–5.3 (2) |
| Bovine serum albumin | 0.9 (1) |

The number of experiments is indicated in brackets.

Table 2
Squalene binding by native placental 105 000 *g* supernatant studied by gel filtration on G 25 Sephadex column

| Sample applied to the column | | | Characteristcs of $V_0$* effluent | | Protein bound Squalene |
|---|---|---|---|---|---|
| | Proteins (mg) | Radioactivity (dmp X $10^{-3}$) | Radioactivity (dpm X $10^{-3}$) | Radioactivity/mg of proteins (dpm X $10^{-3}$/mg) | (ng/mg protein) |
| Control: Buffer A | 0 | 120 | 0 | 0 | 0 |
| Fresh native placental S 105 | 12 | 136 | 115 | 9 | 90 |
| (Sample 1) | 6 | – | 111 | 19 | 180 |
| | 3 | 170 | 122 | 40 | 380 |
| | 1.2 | 113 | 48 | 40 | 380 |
| | 0.3 | 48 | 11 | 39 | 370 |
| | 0.15 | 25 | 5 | 39 | 370 |
| Sample 1 after two months storage at −20°C | 1.2 | 58 | 8 | 7 | 70 |
| | 0.3 | 42 | 3 | 11 | 106 |
| Fresh native placental S105(Sample 2) | 9.5 | 145 | 89 | 9 | 88 |
| | 1.9 | 96 | 66 | 34 | 324 |
| | 0.95 | 97 | 27 | 29 | 280 |
| | 0.475 | 80 | 13 | 29 | 280 |

* $V_0$ = Exclusion volume, determined by blue dextran filtration.

lysable), we used the method of gel filtration of G 25 Sephadex columns.

Table 2 shows that after incubation with native placental S 105, radioactive squalene is partly eluted with the proteins in the exclusion volume, free squalene being completely adsorbed on the column, as shown by the results obtained with buffer alone.

The binding of squalene to S 105 placental proteins can also be shown by the incubation in the presence of dextran coated charcoal (fig. 1) as described in 2.3.4.: after a 60 min dextran charcoal treatment, more than 85 per cent of [11,12-$^3$H] squalene remains in the supernatant, bound to the placental S 105 proteins, whereas in the absence of placental S 105, 86 per cent is adsorbed on charcoal.

As the squalene concentration could be neither increased (because of its low solubility) nor decreased (because of its low specific activity), the saturation of the protein binding could be studied only by varying the protein concentration and an eventual high affinity and specific binding (Kd $\leqslant 10^{-7}$ M) could not be tested. Table 2 shows that when $1.8 \times 10^{-6}$ M squalene is incubated in the presence of decreasing amounts of native placental S 105, a 'saturation' is observed with the binding of about 1 nmole of squalene per mg protein. The binding capacity of placental S 105 is decreased after a 2 months storage at −20°C.

Table 3 shows that the 'saturation' of the S 105 is not observed with heated placental S 105. Similarly, in

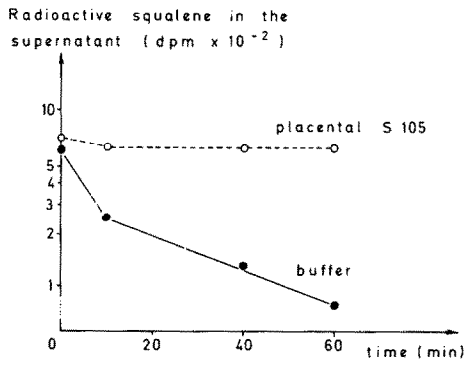


Fig. 1. Squalene binding to proteins of native placental 105 000 *g* supernatant. After preincubation (37°C, 15 min) of [11,12-$^3$H] squalene with native placental 105 000 *g* supernatant, free squalene is adsorbed by dextran charcoal treatment, separated by centrifugation and the radioactivity of the supernatant (protein bound squalene) determined by liquid scintillation counting. For experimental details and references see text (2.3.1. and 2.3.4.).

Table 3
Squalene binding by heated placental 105 000 *g* supernatant studied by gel filtration on G 25 Sephadex column

| Sample applied to the column | | | Characteristics of $V_0$* effluent | | Protein bound Squalene (ng/mg protein) |
|---|---|---|---|---|---|
| | Proteins (mg) | Radioactivity (DPM × $10^{-3}$) | Radioactivity (DPM × $10^{-3}$) | Radioactivity/mg of proteins (DPM × $10^{-3}$/mg) | |
| Sample 1 | 1.2 | 142 | 110 | 91 | 870 |
| | 0.6 | 141 | 106 | 177 | 1670 |
| | 0.3 | 130 | 99 | 330 | 3137 |
| | 0.12 | 142 | 86 | 719 | 6838 |
| | 0.06 | 108 | 76 | 1270 | 12065 |
| | 0.03 | 93 | 39 | 1300 | 12354 |
| | 0.015 | 54 | 29 | 1900 | 18050 |
| Sample 1 | 0.12 | 76 | 56 | 474 | 4500 |
| after two months | 0.06 | 75 | 43 | 717 | 6905 |
| storage at −20°C | 0.024 | 75 | 30 | 1270 | 12000 |

* $V_0$ = Exclusion volume, determined by blue dextran filtration.

Table 4
Squalene binding by native rat liver 105 000 *g* supernatant studied by gel filtration on G 25 Sephadex column

| Sample applied to the column | | | Characteristics of $V_0$* effluent | | Protein bound Squalene |
|---|---|---|---|---|---|
| | Proteins (mg) | Radioactivity (DPM × $10^{-3}$) | Radioactivity (DPM × $10^{-3}$) | Radioactivity/mg of proteins (DPM × $10^{-3}$/mg) | (ng/mg protein) |
| Fresh native | | | | | |
| rat liver S 105 | 30 | 120 | 88 | 2935 | 28 |
| | 12 | 109 | 64 | 5330 | 51 |
| | 6 | 111 | 55 | 9188 | 87 |
| | 3 | 95 | 47 | 15690 | 149 |
| | 0.6 | 94 | 12 | 20700 | 196 |
| | 0.3 | 78 | 9 | 31840 | 302 |
| | 0.2 | 106 | 67 | 336250 | 3194 |
| | 0.15 | 100 | 75 | 502060 | 4769 |

* $V_0$ = Exclusion volume, determined by blue dextran filtration.

the same conditions, human plasma proteins, native and heated bovine serum albumin (BSA), native and heated rat liver S 105, also bind squalene but the binding does not seem to be limited. Table 4 shows as an example the results obtained with native rat liver S 105.

The absence of saturation observed both with native and heated rat liver S 105 can be related to the presence of several proteins able to bind this molecule: SCP [7,8] serum albumin, lipoproteins, the two latter being able to give 'non-specific', non-saturable squalene binding. However, in placental S 105, the squalene binding cannot be related to the presence of serum albumin or lipoproteins, since the hemoglobin determinations have shown this contamination to be less than 1–2%. Furthermore, on cellogel electrophoresis, there is no squalene binding protein in the albumin zone.

### 3.3. *Squalene induced aggregation of the placental 'SCP like' proteins*

We looked for such an aggregation since Scallen [8,9] and Ritter [10] reported that rat liver SCP has a low molecular weight (< 100 000) and aggregates in the pre-

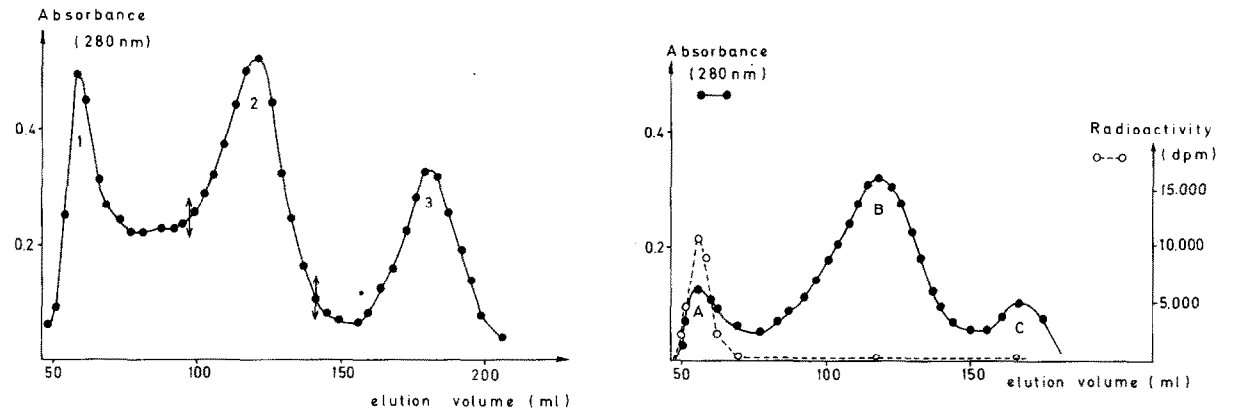


Fig. 2a: G 200 Sephadex filtration of native placental 105 000 *g* supernatant. Fig. 2b: G 200 Sephadex filtration of proteins of peak 2 (from fig. 2a) after preincubation with tritiated squalene.

sence of squalene.

When the placental S 105 is filtered on a G 200 Sephadex column, three peaks of proteins are eluted contain the SCP (mol. wt. < 100 000)) after concentracontain the SCP (mol. wt. < 100 000) after concentration and incubation in the presence of labelled squalene, are filtered on the same Sephadex G 200 column. The fig. 2b shows that a squelene binding protein appears in the exclusion volume (speak A, mol. wt. ⩾ 200 000). This new fraction accounts for only a small part of the proteins of peak 2; the main part (peak B) being eluted in the same volume as formerly. If proteins of peak 2 are not incubated with squalene, they are eluted in their former elution volume, showing that aggregation is not related to the concentration procedure.

This aggregation is also confirmed by polyacrylamide gel disc electrophoresis performed on placental or rat liver S 105 preincubated in the presence of labelled squalene. In both cases, the squalene binding proteins are excluded from the gel (7% acrylamide concentration).

## Conclusions

Our results show that placental S 105 has a true squalene binding capacity, but is not very efficient in activating liver or placental squalene epoxidase [2]. This latter observation could be explained by the limited binding capacity of placental S 105 with regards to the unlimited binding capacity of rat liver S 105. But we show that heated rat liver S 105 or native serum albumin, which have also an unlimited squalene binding capacity, does not activate squalene epoxidase. Thus there is no correlation between total squalene binding capacity of S 105 as measured till now by ourselves or others, and squalene epoxidase activation. Our results do not rule out the presence of a true SCP in the human placental S 105 but, as Bloch suggested for the liver [11], in the placenta, the binding capacity and the epoxidase activation should be related to different protein factors or two different functions of a same protein.

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