THE SQUALENE EPOXIDASE-CYCLASE ACTIVITY OF HUMAN TERM PLACENTA

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1. Introduction

The placental biosynthesis of lanosterol and cholesterol from labelled mevalonate was demonstrated by Zelewski and Villee [1] in 1966; in spite of a low yield, these two sterols have been identified by a recrystallization to constant specific radioactivity. In 1967, in a general survey of the placental lipids biosynthesis, Kleine [2] described the incorporation of labelled acetate into both free and esterified cholesterol. In 1968, Villee [3] observed that the placental microsomes were responsible for epoxidase-cyclase activity; in addition, this activity seemed to be inhibited by placental cytosol, but was increased by rat liver cytosol. The identification of the sterols in the last two publications may be in question; no data concerning the recrystallization to constant specific radioactivity was given.

The above results obtained with tissue slices have been recently re-examined by perfusion of whole placenta (Diczfalusy et al. [4]). No steroid biosynthesis could be observed from labelled acetate with midgestation placenta.

These contradictory observations, although obtained under slightly different conditions, have led us to study the epoxidase-cyclase activity of term placenta under conditions designed to improve the conversion: subcellular fractionation; high specific activity squalene instead of acetate or mevalonate (more remote precursors); addition of rat liver cytosol carrier protein (S.C.P.) according to Scallen et al. [5] and Ritter and Dempsey [6]. To this end, epoxidase-cyclase activity of three systems have been examined: placental microsomes + buffer; placental microsomes + cytosol; placental microsomes + S.C.P.

Our results demonstrate that human term placenta possesses an effective epoxidase-cyclase activity which leads under our experimental conditions, to lanosterol rather than cholesterol, that the sterols obtained are in part esterified and that the low basal epoxidase-cyclase activity of placental microsomes can be greatly enhanced by S.C.P, and therefore related more to the lack of placental cytoplasmic S.C.P. than to the presence of some inhibitor in placental cytosol (as envisaged by Villee [3]).

2. Material and methods

2.1. *Reagents*

The cholesterol, lanosterol, EDTA and glutathione were supplied by Merck (Darmstadt); the [11, 12-^{3}H]squalene (18 mCi/mM) was generously donated by Professor Biellmann (University Louis Pasteur, Strasbourg); NAD^+ and NADPH were obtained from Boehringer (Tubingen); the hexane (analytical grade) was purchased from Union Chimique Belge (U.C.B.). The phosphate buffer used (0.02 M, pH 7.0, 10^{-4} M EDTA) was prepared using double distilled water. The thin-layer chromatography (TLC) was performed on Kieselgel Merck F_{254} plates (thickness: 0.25 mm; support: aluminium sheet).

2.2. *Preparation of placental microsomes and rat liver cytosol*

All enzyme preparations were carried out at 4°C. The term placenta was processed within half an hour of delivery. Tissue (200 g) was homogenized (ultra-Turrax or Potter) in phosphate buffer (160 ml) for 2 to 3 min. The homogenate was centrifuged (13 000 *g* ×

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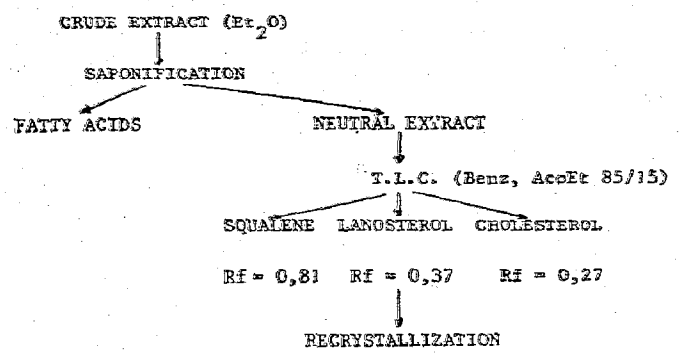

Fig. 1. Incubation 1, 2 and 3.

15 min) and the supernatant recentrifuged (140 000 $g \times 110$ min). The pellet (microsomes) was used directly for the incubations.

Rat livers (18.4 g) from 2 months-old animals were homogenized in phosphate buffer (20 ml, ultra-Turrax) for 3 min. The homogenate was centrifuged (13 000 $g \times 15$ min) and the supernatant recentrifuged (140 000 $g \times 135$ min); only the top 2/3 of the latter supernatant were taken.

2.3. *Incubations*

All incubations were carried out at 37°C, and were shaken under oxygen. A solution of Tween-80 (0.5 mg) in 20 µl of benzene was added to a solution of radioactive squalene (5 µl containing 1.85 µg = 160 dpm) in hexane. The solvents were removed in vacuum (60°C), buffer (2 ml) added and shaken to suspend the substrate. To the solution were added the following quantities of cofactors: NADPH 1.4 µmol; NAD 1.3 µmol; glutathione 20 µmol; Mg^{2+} 32 µmol (in 1.5 ml of water) and a suspension of the microsomal pellet in: 6.5 ml of placental cytosol (incubation 1); 6.5 ml of buffer (incubation 2); 6.5 ml of rat liver cytosol (incubations 3 and 4); 10 ml of liver cytosol and placental cytosol 1:1 (incubations 5 and 6); 10 ml of liver cytosol and buffer 1:1 (incubations 7 and 8). The incubation times were 140 min for incubations 1–3; 280 min for incubations 4–8.

2.4. *Isolation of incubation products*

2.4.1. Extraction

The incubation was stopped by adding ether (30 ml) to the cooled mixture which was then continuously extracted (travelling band, 5 hr) and centrifuged (3000 $g \times 15$ min). The ether solution was decanted, dried on sodium sulphate and concentrated. The weighed residue was dissolved in hexane (5 ml) and the radioactivity measured (50 µl aliquot).

2.4.2. Saponification

An aliquot of the hexane solution (3 ml) was concentrated and saponified by refluxing (1 hr) with 3 ml of 15% methanolic KOH. The methanolic solution was concentrated to 1 ml, diluted with water (10 ml) and the emulsion thus obtained extracted continuously (30 ml of ether; travelling band: 3 hr). The ether solution was decanted, dried and concentrated. The residue obtained (neutral extract) was dissolved in hexane after addition of 2 mg of non radioactive lanosterol.

2.4.3. Chromatography

The neutral extract was separated by TLC using benzene–ethylacetate (85/15) as the irrigant. The zones corresponding to cholesterol, lanosterol and squalene were determinated by using reference samples run on the edge of the plate. The strip carrying the standards was cut off and separately sprayed with a mixture of acetic acid–sulfuric acid–benzaldehyde (8/1/1) and heated at 120°C. The areas corresponding to these compounds are cut out: the silica gel obtained was inactivated by two drops of water, packed into a glass-column (5 × 150 mm). The eluates were concentrated and dissolved in 1 ml of benzene and the radioactivity was measured on an aliquot (0.1 ml).

2.4.4. Recrystallization

The radioactive lanosterol or cholesterol fraction was diluted with non radioactive lanosterol or cholesterol and recrystallized in methanol to constant specific radioactivity.

2.5. *Examination for esterified sterols in incubation 4*

2.5.1. Total sterols

Same operation work as above, on an aliquot of the crude extract.

2.5.2. Free sterols

To 7.8 mg of crude extract (27 000 dpm) were added 2 mg of non radioactive lanosterol, the mixture was submitted to TLC (no. 1) (Benzene–ethylacetate: 85/15) and revealed as in sect. 2.4.3). To locate the

Table 1
Fractionation steps and distribution of radioactivity after incubations 1–3.

		Incubation 1		Incubation 2		Incubation 3	
		Weight (mg)	Radio-activity (dpm)	Weight (mg)	Radio-activity (dpm)	Weight (mg)	Radio-activity (dpm)
Fractionation steps	Saponification	27.7	76 200	17.7	58 600	13.9	52 500
	Aliquot of neutral extract chromatographied	4.4	56 650	6.5	55 200	2.5	24 150
Distribution of radio-activity in:	Cholesterol zone		200		221		400
	Lanosterol zone	ND*	690	ND	890	ND	1 600
	Squalene zone		43 000		42 100		20 000

* ND = not determinated.

sterol esters, cholesterol oleate was added to usual standards.

2.5.3. Esterified sterols

The squalene and cholesterol ester zone was saponified by refluxing in 2.5 ml of 15% methanolic KOH (90 min). The neutral extract was submitted to TLC (no. 2) (hexane–ethylacetate: 95/5). The different zones (squalene R_f: 0.65; lanosterol R_f: 0.075 were eluted and recrystallized as described in sect. 2.4.

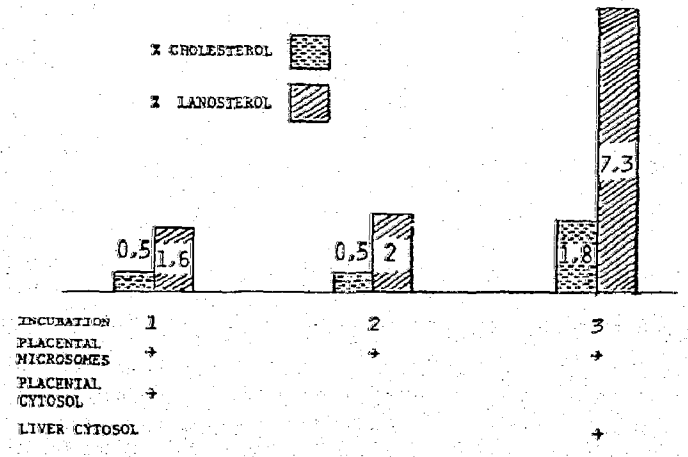

Fig. 2. The conversion of squalene into lanosterol and cholesterol (results expressed as a percentage of [^{3}H]squalene).

2.6. *Radioactivity measurement*

The radioactivity is measured by liquid scintillation counting (Packard 3320) in 15 ml of the following mixture: PPO, 3 g; POPOP, 0.1 g; toluene, 1 ℓ.

3. Results and discussion

The scheme describing the steps in the isolation of products from incubations 1, 2 and 3 is shown in fig. 1. Table 1 indicates the distribution of radioactivity

Table 2
Lanosterol recrystallizations in incubations 1–3.

Addition of non radioactive lanosterol	Incubation 1 15 mg (dpm/mg)	Incubation 2 20 mg (dpm/mg)	Incubation 3 20 mg (dpm/mg)
Recrystal-lization 1	25	25.5	69.5
Recrystal-lization 2	23.4	24.2	70.5
Recrystal-lization 3	25	25.6	67.5
Yield of radio-active lanoster-ol after recrys-tallization*	0.85%	1.15%	7%

* % of the total radioactivity eluted from TLC.

Table 3
Lanosterol recrystallization in incubation 4 (esterified + non-esterified).

Recrystallization	(dpm/mg)
1	168
2	161
3	155
Yield in recrystallized lanosterol*	10%

* % of the total radioactivity eluted from TLC.

in the different fractions separated by TLC. Fig. 2 compares the conversion of squalene into lanosterol in incubations 1, 2 and 3. The identification of lanosterol in incubations 1, 2 and 3 was carried out by successive recrystallizations of lanosterol to constant specific radioactivity (table 2). A comparison of the data in tables 1 and 2 shows that the radioactivities of the lanosterol zones before recrystallization (690 dpm/890 dpm = 0.77) and after recrystallization (0.85/1.15 = 0.74) is about the same for both incubation 1 and 2, suggesting that the lanosterol was adequately purified by TLC.

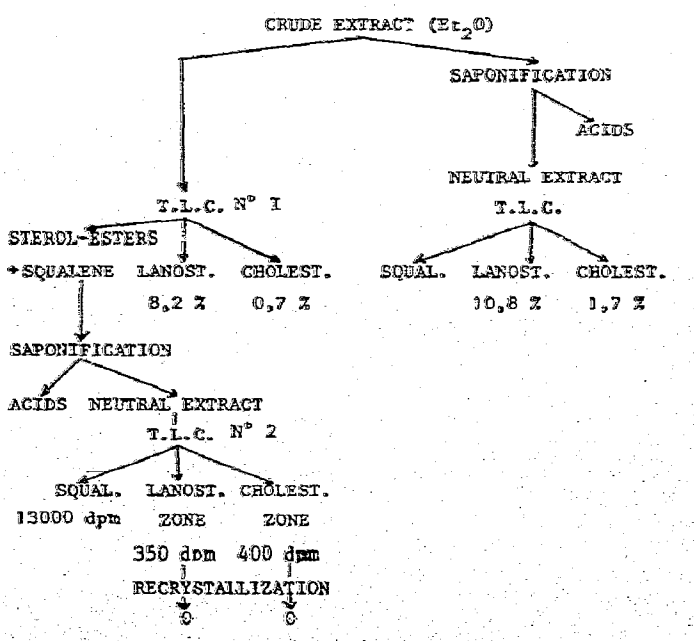

Fig. 3. Examination for sterol esters (incubation 4).

Table 4
Results of lanosterol recrystallization in incubations 5–8.

Incubations	5	6	7	8
Placental cytosol	+	+	–	–
Lanosterol*	4.0%	4.3%	4.6%	4.0%

* % of the total radioactivity eluted from TLC.

Tables 1 and 2, as well as fig. 2 show that the placental microsomes are able to convert squalene into sterol even when incubated without rat liver cytosol. After recrystallization to constant specific radioactivity, more than 1% of the radioactivity obtained is present in the lanosterol. We observed (incubation 3: placental microsomes + rat liver cytosol) the yield of lanosterol to be much higher than in incubations 1 and 2. This increase can be explained by the results of Dempsey and Ritter [6] and of Scallen et al. [5] which show that the liver cytosol contains a squalene and sterol carrier protein (S.C.P.). Thus, the addition of liver cytosol (and therefore presumably S.C.P.) increases the epoxidase-cyclase activity of placental microsomes 3 to 5 times. Incubation 4 was carried out for a longer period of time in order to increase the yield of radioactive sterols (fig. 3, table 3) and thus make the separation of the free sterols from the esterified ones easier. The lower yield obtained in incubations 5–8 can be explained by a difference in experimental conditions (Potter homogenization).

Our results disagree with Villee's hypothesis [3]: the placental cytosol does not seem to inhibit the conversion of squalene into sterols, since the placental microsomes, incubated in the presence of buffer or placental cytosol, have similar epoxidase-cyclase activities (fig. 2). Moreover, the epoxidase cyclase activities of placental microsomes stimulated by rat liver S.C.P. is the same (table 4) in the presence (incubations 5, 6) or in the absence (incubations 7, 8) of placental cytosol. Thus, neither the basal, nor the S.C.P. activated epoxidase-cyclase activity of placental microsomes are inhibited by placental cytosol.

Fig. 3 shows the scheme for the separation of the products in incubation 4 and the distribution of radioactivity in the cholesterol and lanosterol zones for the free and esterified sterol fractions. We obtained a radioactive product which co-crystallized with lano-

sterol (table 3). After TLC no. 1, some radioactivity was present in the sterol esters zone (fig. 3). The ratio between the radioactivities incorporated in the esterified and in the total sterols is 3.6/12.5 = 28% (fig. 3). This value can be compared with the ratios of esterified and total cholesterol of the placental tissue (29% determined by Nelson et al. [7], 32% according to Eberhagen [8]) and is in agreement with the enzymatic studies of Robertson et al. [9] who demonstrated the capacity of the placenta to esterify sterols.

In the case of incubation 4, neither the lanosterol, nor the cholesterol constitute the main component of the esterified sterols since the radioactivity was eliminated during the recrystallization (fig. 3). In addition, no digitonin precipitable material could be obtained from the mother liquors of the recrystallizations. With the solvent system used, the respective R_f's of the squalene (0.65) and the nearest sterol (0.075) are such that a contamination by the radioactive precursor is unlikely. We surmise that the radioactivity lost during these recrystallizations is associated with metabolites (e.g. methylsterols) implicated in the biosynthesis pathway between lanosterol and cholesterol. Such intermediates have been identified by Ramsey et al. [10] from the brain of young rats incubated with labelled mevalonate, squalene or oxidosqualene. The brains of young rats (Shah [11]) are rich in these metabolites especially in the esterified fraction.

There are other similarities between placental and cerebral microsomes: we have found that S.C.P. increases enzymic activity and the synthesis of lanosterol is greater than cholesterol.

Our results show that placental microsomes can oxidize and cyclise squalene and these reactions are enhanced by the addition of rat liver cytosol S.C.P. Though the latter is able to bind most of the cholesterol precursors, lanosterol accumulates. Thus we can observe a partial blockade of cholesterol biosynthesis at the stage of demethylation at C-4. We intend to study the role of the carrier protein in the regulation of the steroidogenesis; the low basal epoxidase cyclase activities cannot be related to the presence of an inhibitor.

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