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Measurement of Progesterone and Pregnenolone 16α-Hydroxylase Activities by a Tritium-Exchange Method

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A new isotopic method, based upon the stereospecific replacement of a proton (^{3}H) by a hydroxyl group, has been developed for the measurement of progesterone and pregnenolone-16α-hydroxylase activity. The incubation medium consists of a phosphate buffer (pH 7; 150 mM), NADPH (1 mM), nicotinamide (10 mM) and magnesium chloride (4 mM). Tween-80 (1 mg/ml) is used to solubilize saturating concentrations of [16-^{3}H]progesterone (200 μM) or [16-^{3}H]-pregnenolone (50 μM). The microsomal fraction isolated from a male rat liver is used as the enzymic source.

The enzymically released tritium is recovered in the incubation medium as molecules of tritiated water which are distilled under reduced pressure. The amount of radioactivity present in the water exactly reflects the 16α-hydroxylase activity.

The method is easy to perform and is completely independent of any further metabolism of the 16α-hydroxylated products.

Although the hormonal steroids are already metabolized in the producing organs [1] as well as in target tissues [2], their main site of transformation and deactivation is the liver [3, 4]. The catabolic pathway of those molecules involves a limited number of enzymic reactions, *i.e.* 3α and 3β-hydroxy-steroid oxidoreduction, 5α and 5β reduction, ring and side chain hydroxylation, sulfate and glucurono-conjugation.

Specific positions (1β, 2α, 2β, 6α, 6β, 7α, 7β, 15α, 15β, 16α and 18; see [5] for review) of the steroid skeleton ring can be hydroxylated by the liver microsomal-bound monooxygenases. Those steroid hydroxylases share a number of biochemical properties in common with the (cytochrome *P*-450)-linked drug-metabolizing enzymes [6].

The 16α-hydroxylation is a common reaction occuring in the catabolism of estrogens, androgens and corticoids. In order to study the biochemical properties as well as the regulatory mechanism involved in the control of the enzymic activities, reliable assays are absolutely required.

The described methods are usually based on the incubation of ^{14}C-labelled substrates [7, 8] and the subsequent extraction and chromatographic separation of the metabolites. The main pitfalls of such techniques are a lack of resolution of the thin-layer chromatography procedures used to isolate the metabolites, variable losses of compounds during the various manipulations (usually corrected for by the use of tritiated carriers), and also the difficulties of applying those time-consuming methods in experiments needing the simultaneous assay of large numbers of samples.

In this report, a new sensitive and specific assay is described for the measurement of progesterone and pregnenolone 16α-hydroxylase activity. It is essentially based on the stereospecific exchange of the proton (^{3}H) located on the 16α-position by the hydroxyl group during the enzymic oxidation. The proton (^{3}H) is released in the incubation medium as a molecule of water which is distilled under reduced pressure and counted by liquid scintillation. A similar method has recently been set up in our laboratory for the

The previous papers of this series have appeared in this journal [9, 10].

Enzymes. Steroid, hydrogen-donor: oxygen oxidoreductase (17α-hydroxylating) or steroid 17α-hydroxylase (EC 1.14.99.9); glucose-6-phosphate dehydrogenase (EC 1.1.1.49).

Trivial Names. Progesterone, pregn-4-en-3,20 dione; pregnenolone, 3β-hydroxypregn-5-en-20-one.

measurement of cholesterol 7α-hydroxylase [9], progesterone and pregnenolone 17α-hydroxylase [10].

MATERIALS AND METHODS

Glucose 6-phosphate, NADP and glucose-6-phosphate dehydrogenase were supplied by Boehringer (Mannheim, Germany), Tween-80 (polyoxyethylene sorbitan monooleate) was purchased from Sigma Chemical Company (St Louis, U.S.A.). [4-^{14}C]Progesterone (49.5 Ci/mol), [4-^{14}C]pregnenolone (53 Ci/mol), [16-^{3}H]progesterone (26 Ci/mmol) (95% of the ^{3}H is located on the 16 position) and [16-^{3}H]pregnenolone (16 Ci/mmol) were obtained from I.R.E. (Fleurus, Belgium). The labelled steroids were always purified by thin-layer chromatography before use.

Methoxyamine hydrochloride, *N,O*-bis-trimethylsilyl acetamide, trimethylchlorosilane and dry pyridine to prepare *O*-methyloximetrimethylsilyl ethers for gas-liquid chromatographic analysis and the chromatoplates for thin-layer chromatography were supplied by Marcherey-Nagel (Düren, Germany).

Reference steroids were obtained from Steraloids (New York, U.S.A.) and from Ikapharm (Ramat-Gan, Israel). Scintillation cocktails (instagel, monophase, carbosorb, permafluor) were supplied by Packard Instrument (Groningen, The Netherlands). The other chemical compounds and organic solvents were analytical grade reagents provided by Merck (Darmstadt, Germany) and used without further purification.

Preparation of Subcellular Fractions

150-g male Sprague-Dawley rats (Centre des Oncins, Lyon, France) were sacrificed by cervical dislocation and carefully bled. The livers were removed and rapidly cooled in ice-cold isotonic KCl solution. $9000 \times g$ supernatant and microsomes were prepared as previously described [9].

Optimal Conditions for the 16α-Hydroxylase Assay

In the results section, we will describe the experiments which led to the selection of the following optimal conditions for the measurement of progesterone and pregnenolone 16α-hydroxylase.

1 ml of an acetonic solution containing 0.1 μmol (10^6 dis./min) of substrate ([16-^{3}H]progesterone or [16-^{3}H]pregnenolone) and 0.5 mg of Tween-80 was introduced into a glass tube and evaporated to dryness under a stream of nitrogen, as previously described [9, 10]. The substrate−Tween-80 residue was then dissolved in an incubation mixture composed of an NADPH-generating system (glucose-6-phosphate, 5 mM; NADP, 1 mM; glucose-6-phosphate dehydrogenase, 1 I.U. per tube), $MgCl_2$ (4 mM), and a phosphate buffer (150 mM; pH 7.4). An aliquot of $9000 \times g$ supernatant or of isolated microsomes equivalent to 50 mg of liver was added and the final volume of the incubation medium adjusted to 1 ml. After 20 min of incubation in a shaking water bath at 37 °C, the enzymic reaction was stopped by the addition of 1.5 ml of trichloracetic acid (20%, w/v in water). The tubes were centrifuged for 5 min at $2000 \times g$, and the supernatants were then distilled under reduced pressure. 1 ml of the distilled water was mixed with 10 ml Instagel and counted by liquid scintillation.

The total radioactivity released in the incubation water was divided by half of the specific activity of the substrate (as only 50% of the tritium is located on the 16α position; see Results section) to be converted into μmol of metabolized substrate.

Identification of the Metabolic Products

^{14}C-labelled substrate (progesterone or pregnenolone) was incubated under the optimal conditions described above. The incubation mixture was extracted twice with 10 ml of dichloromethane. The residue of the organic phase evaporation, dissolved in a few drops of chloroform, was chromatographed on a silicagel G plate (250 μ; solvent system: benzene/chloroform/methanol; 3/7/1; v/v). The compounds more polar than the substrate were recovered from the chromatoplate and chromatographed again in the same solvent system. The labelled metabolites localized by autoradiography were then scraped off and eluted from the silicagel by shaking it with ether. They were finally analyzed as methoxime trimethylsilyl ethers [11] by gas-liquid chromatography/mass spectrometry (LKB 9000 S). The gas-liquid chromatographic separation was realized on a glass column (2 m × 3 mm internal diameter) packed with a 1% OV-1 phase coated on gas-chrom P (AW-DMCS) 100−150 mesh. Flash heater temperature was 280 °C; separator and ion-source temperatures were respectively 270 and 290 °C. The helium flow rate was set at 30 ml/min to reach the maximum efficiency of the Ryhage jet separator. The column temperature was generally programmed at 2 °C/min, starting at 200 °C. Other experimental conditions were: trap current 60 μA, accelerating voltage 3500 V, electron energy 70 eV for mass spectrum recording.

Measurement of the $^{3}H/^{14}C$ Ratio in 16α-Hydroxylated Products

To study the stereochemical mechanism of the enzymic hydroxylation (see Results section), a mixture of (16-^{3}H)-labelled and (4-^{14}C)-labelled substrates was incubated under the conditions described above,

and the products of the reaction analyzed by gas-liquid chromatography after derivatization. Using a splitter device, (9:1) the chromatographic peaks corresponding to 16α-hydroxyprogesterone and 3β, 16α-dihydroxy-5α-pregnane-20-one were collected at −70 °C on glass-wool cartridges and burned in a Packard Tri-Carb 306 oxidizer. The radioactivity associated with ^3H_2O and $^{14}CO_2$ was then counted by liquid scintillation.

RESULTS

After incubation *in vitro* of (^{14}C)-labelled progesterone or pregnenolone in the presence of male rat liver microsomes, a number of metabolites were synthesized. In the case of pregnenolone, the metabolic pathway looks rather simple as only one major labelled compound could be identified: 3β,16α-dihydroxy-5-pregnane-20-one. Minor labelled spots also appeared on the autoradiography of the thin-layer chromatoplates, but it was nearly impossible to identify them by mass spectrometry (< 2 μg in the whole sample).

On the other hand, the incubation of progesterone under the same enzymic conditions produces a large number of metabolites which were not satisfactorily separated by thin-layer chromatography. About 10 spots, overlapping each other, were in fact observed on the autoradiography of the chromatoplates. As those spots could not be improved by using other solvent systems, the silicagel of the plates was scraped off in three separate sections and the compounds present in each fraction were identified by gas chromatography/mass-spectrometry as described in the Methods section (Table 1).

The major metabolite of progesterone, 3α,6β-dihydroxy-5α-pregnane-20-one was not separated from the 16α-hydroxylated metabolites which were present in lower amounts. Zone III also contained four isomers of 3,6,16-trihydroxy-5-pregnan-30-one. In conclusion, the complexity of progesterone metabolism in rat liver microsomes made it very difficult to determine accurately the 16α-hydroxylase activity simply by measuring the radioactivity of different 16α-hydroxylated metabolites.

Tritium Exchange Method

Steroid hydroxylase activities can be measured by a tritium exchange method based upon the following reaction: (16-^{3}H)-labelled steroid + NADPH + H^+ + O_2 → 16α-hydroxysteroid + ^{3}HOH + $NADP^+$. Three fundamental requirements must be fulfilled in order to develop such an assay. Firstly, the tritiated labelled substrate must be radiochemically stable under the assay conditions. ^{3}H release should only occur as a consequence of the enzymic hydroxylation. Secondly, the exact amount of tritium located on the position to be hydroxylated must be known. If tritium is located elsewhere, it should not be exchanged by either enzymic or non-enzymic reactions. Thirdly, the stereochemical mechanism of the enzymic reaction must be proven.

Table 1. *Identification of the different metabolites obtained after progesterone incubation*
The different zones of the thin-layer chromatogram were analyzed as methoxime trimethylsilyl ethers by gas chromatography/mass spectrometry in LKB 9000 S equipment. For each compound, the relative retention time (methylene units) and the mass spectrometric data are given

Thin-layer chromatography zone	Relative retention time	M^+	Main characteristic fragments	Name
I	28.55	372	357, 341, 311, 286, 273, 220, 100	4-Pregnene-3,20-dione
II	28.17	417	402, 386, 372, 327, 312, 296, 288, 239, 129, 100	3α-hydroxy-4-pregnene-20-one
	28.31	419	404, 388, 328, 314, 298, 241, 129, 100	3β-hydroxy-5α-pregnan-20-one
III	28.2	507	476, 417, 402, 386, 331, 327, 317, 312, 296, 100	3α,6β-dihydroxy-5β-pregnan-20-one
	28.4	507	492, 476, 435, 417, 402, 386, 317, 246, 255, 215, 188, 129	3β,16α-dihydroxy-5α-pregnan-20-one
	29.1	507	492, 476, 462, 417, 402, 386, 331, 328, 296, 241, 129, 100	3β,6β-dihydroxy-5α-pregnan-20-one
	30.2	460	455, 429, 399, 388, 370, 355, 339, 188	16α-hydroxy-4-pregnene-3,20-dione
	30.4	548	533, 517, 458, 443, 427	6β,16α-dihydroxy-4-pregnene-20-dione
	29.2	595		
	29.7	595	580, 564, 523, 505, 474, 384, 369, 294, 188, 129, 117	3(α or β), 6β,16α-trihydroxy-5-(α or β)-pregnan-20-one
	30.0	595		
	30.3	595		

Stability of the Substrate

[16-^{3}H]Progesterone and [16-^{3}H]pregnenolone are radiochemically stable: when incubated in the absence of an enzyme, no significant amount of tritium should be released into the medium. The same was true if the substrates were incubated in the presence of an active enzymic preparation, but under anaerobic conditions or in the absence of cofactors, or if the incubation was run in the presence of a sub-cellular preparation isolated from muscle or adipose tissues.

Specificity of the Labelling

The location of the tritium on the substrate has been partly determined in the study of the stereochemistry of the enzymic reaction (see below). Close to 50% of the radioactivity was in fact located on the 16α-position of both substrates.

According to the chemical method used for the synthesis of [16-^{3}H]progesterone [12], more than 95% of the tritium is situated on carbon 16 and is more likely equally distributed between the α and the β positions, those positions possessing the unique feature of being sterically equivalent on the carbon 16.

Among other positions which have the highest possibility of also being labelled, the most likely is carbon 15. [16-^{3}H]Progesterone was incubated for 130 h at room temperature in the presence of *Fusarium lini*, a microorganism which specifically hydroxylates the 15α position of various steroids [16]. Under these conditions, no more than 2% of the substrate was hydroxylated on the 15α position (R. Cantineau and P. Kremers, unpublished results). Less than 15% of the total incubated radioactivity was found in the water of the incubation medium.

As our metabolic study demonstrated that no detectable amount of 16β- or 15-hydroxylated steroids was synthesized under our incubation consitions, the tritium located on those positions would not be released and consequently, would not interfere in the assay.

Stereochemistry of the Reaction

To assess the chemical mechanism of the enzymic substitution, a mixture of [4-^{14}C]progesterone and [16-^{3}H]progesterone was incubated in the presence of isolated rat liver microsomes. The ^{3}H/^{14}C ratio was then measured in three metabolites and compared to the ratio in the incubated substrate (Table 2). It was estimated that respectively 52% (3β,16α-dihydroxy-5α-pregnane-20-one) (measuring in the decreasing part of its gas-liquid chromatography peak) and 51% for 16α-hydroxyprogesterone of the tritium had been lost by the 16α-hydroxylated metabolites, while virtually no tritium was released from the 6β-hydroxypregnane-20-one (measuring in the rising part of its chromatographic peak).

Table 2. *^{3}H/^{14}C ratios of different metabolites*
^{3}H/^{14}C ratios were measured on purified metabolites trapped after separation in a gas chromatographic column of the metabolites extracted from an incubation of [16-^{3}H]progesterone and [4-^{14}C]-progesterone (experiment 1) and [16-^{3}H]pregnenolone and [4-^{14}C]-pregnenolone (experiment 2) with rat liver microsomes

Experiment	Compound	^{3}H/^{14}C
I	Progesterone	9.2
	16α-Hydroxy-5β-pregnan-3,20-dione	4.4
	16α-Hydroxyprogesterone	4.5
	6β-Hydroxy-5β-pregnan-3,20-dione	9.1
II	Pregnenolone	2.9
	16α-Hydroxypregnenolone	1.5

A similar experiment was realized using a mixture of [4-^{14}C]pregnenolone and [16-^{3}H]pregnenolone (^{3}H/^{14}C: 2.9) as a substrate. A ^{3}H/^{14}C ratio of 1.5 was found in the purified 16α-hydroxypregnenolone, corresponding to a loss of 48% of the tritium.

These results are in complete agreement with the hypothesis that 50% of the tritium is located on the 16α position. It is also clearly established that no tritium is released into the incubation mixture by the 6β-hydroxylase which is a very active enzyme in the rat liver microsomes.

As no 16β-hydroxylated or 16-keto products could be detected in the metabolic study (see above), the ^{3}H located on the 16β position cannot interfere in the 16α-hydroxylase assay.

OPTIMIZATION OF THE ENZYMIC ASSAY

Substrates

By using Tween-80 as a solubilizing agent, concentrations of progesterone and pregnenolone, in sufficient quantities to saturate the enzymic active site, were introduced into the incubation medium (Fig. 1). An apparent K_m value was estimated for the two substrates: 100 μM for progesterone and 8 μM for pregnenolone. Routinely, 200 μM progesterone and 50 μM pregnenolone were used for the measurement of their respective 16α-hydroxylase activities.

Co-factors

The enzymic reaction requires the presence of NADPH in the incubation medium, 1 mM being necessary to obtain the optimal activity. NADH or NAD alone was unable to support the hydroxylation (Table 3) reaction, but displayed a synergistic effect when added to sub-optimal concentrations of NADPH, the latter, in the form of a generating sys-

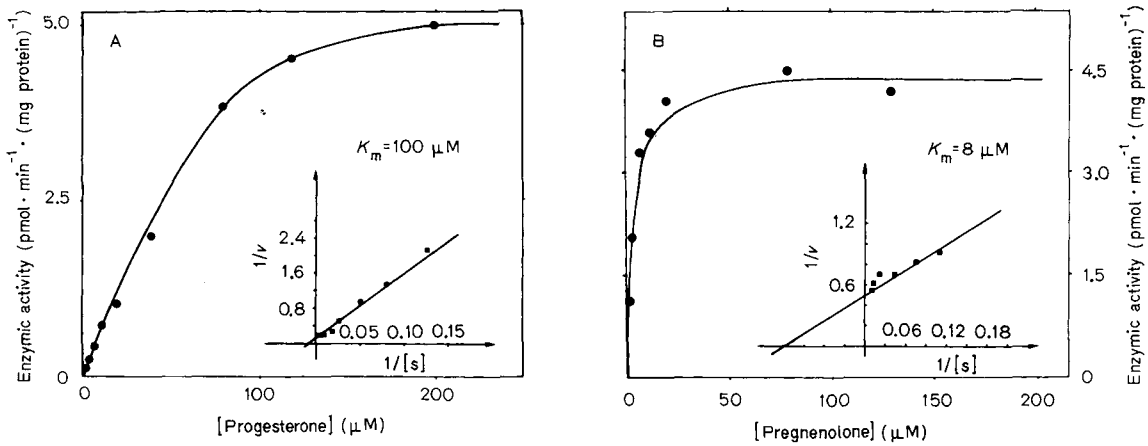

Fig. 1. *Progesterone (A) and pregnenolone (B) 16α-hydroxylase activity as a function of substrate concentration*

Table 3. *Effect of NADPH, NAD and nicotinamide on progesterone 16-hydroxylase activity*
Results are expressed in specific activity and in percentage of the control (NADPH 1 mM). NADPH (generating system), NAD and nicotinamide concentrations are expressed in terms of molarity of the final incubation medium

NADPH	NADH	Nicotinamide	Progesterone-16α-hydroxylase	
mM			pmol × min^{-1} × mg^{-1} protein	% of control
0.05	–	–	15	6
0.1	–	–	52	21
0.05	–	–	196	78
1	–	–	250	100
–	1	–	37	15
0.05	1	–	176	70
–	–	10	12	5
0.05	–	10	235	94

tem. As a similar result was obtained with nicotinamide (Table 3), we concluded that the synergistic effect observed was linked to a protection of the NADPH towards the action of agents such as the lysosomal pyrophosphatase.

Further Requirements

Atmospheric oxygen is another absolute requirement of the steroid 16α-hydroxylase. The enzymic activity obtained after an incubation run in an atmosphere composed of pure oxygen was about twice as high as the corresponding activity measured in the presence of air. On the other hand, the enzymic activity was completely abolished when the incubation was realized in an atmosphere composed of carbon monoxide and reduced to 30% of its volume in an atmosphere made of pure nitrogen.

No strict dependence on the presence of any cations could be demonstrated. Magnesium slightly favored the enzymic activity (10–20%) and was routinely added into the incubation mixture. Heavy cations (0.05 mM) inhibited the progesterone and pregnenolone 16α-hydroxylase activity as it was demonstrated for cholesterol 7α-hydroxylase [9]. The inhibition was almost complete in the presence of mM concentrations of Hg^{2+}, Cu^{2+} or Ag^{+} ions.

Time of Incubation and Enzyme Concentration

Under the optimal conditions (summarized in the Methods section), the enzymic reaction proceeds linearly as a function of the time of incubation (up to 20 min) and the concentration of the enzyme concentration of the enzyme preparation (up to the equivalent of 50 mg of liver/ml incubation).

DISCUSSION

Incubated *in vitro* in the presence of the microsomal membranes isolated from male rat liver, pregnenolone is actively metabolized into one major product, *i.e.* 16α-hydroxypregnenolone. In contrast, progesterone is transformed into a large number of metabolites. In the introduction, we have detailed the main reasons which render the use of conventional methods (^{14}C-labelled substrates) difficult in measuring steroid hydroxylase activities. Obviously, in the case of progesterone-16α-hydroxylase, the problems are particularly critical as it is almost impossible to separate the 16α-hydroxylated metabolites from the other ^{14}C-labelled products.

The new method described in this paper, is based on a specific exchange of the tritium located on carbon 16 during the enzymic hydroxylation. No product ex-

traction and purification are required, the measurement of the radioactivity released in the incubation medium allows a precise determination of the progesterone or pregnenolone 16α-hydroxylase activity.

Providing that no hydroxylation occurs on the 16β position, the new method can be applied to the measurement of steroid 16α-hydroxylase activities in other tissues or subcellular preparations, such as cells in culture, human biopsy, tumors.

The sensitivity of the assay is very high; using a substrate with a specific activity of 8 Ci/mol, it is possible to estimate an enzymic activity corresponding to 0.1 nmol of metabolized progesterone per hour in the incubation medium.

The enzymic hydroxylation of steroid nucleus carbons proceeds in a direct and stereospecific manner in the case of cholesterol 7α-hydroxylase [9] and of progesterone and pregnenolone 17α-hydroxylase [10]. Indirect evidence supports the hypothesis of a similar mechanism for the 16α-hydroxylase of progesterone [13] and androgens [14] and for the 17α-hydroxylase of pregnenolone [15]. Even though a direct demonstration of the exact location of the tritium on the two substrates was not provided in our work, it is quite reasonable to consider that under our experimental conditions, the tritium is stereospecifically released from the 16α position by the microsomal monooxygenase.

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