2-HYDROXYLATION OF PREGNENOLONE BY RAT LIVER MICROSOMES

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1. Introduction

The microsomal fraction of liver homogenate catalyzes hydroxylation of steroids in a number of different positions [1]. The positions hydroxylated often vary with the steroid and a given steroid is frequently hydroxylated only in a limited number of positions. Different steroids may be hydroxylated in the same position or positions. The question of the multiplicity of the enzyme system catalyzing hydroxylation of steroids has not been resolved nor are the factors governing the positional specificity of the enzyme system(s) known. In a recent report from this laboratory some aspects of the microsomal hydroxylation of cholesterol, pregnenolone* and dehydroepiandrosterone were studied with the aim of gathering information relevant to these questions [2]. Part of the investigation concerned the properties of the system(s) catalyzing 7α-hydroxylation of these substrates. Marked differences were observed between 7α-hydroxylation of cholesterol and 7α-hydroxylation of pregnenolone and dehydroepiandrosterone. The pattern of hydroxylations of pregnenolone and dehydroepiandrosterone also differed. Whereas the 7α-hydroxylated derivative was a major product of dehydroepiandrosterone, 7α-hydroxyprenenolone was a minor product of prenenolone. Instead, an unidentified compound or compounds with thin-layer chromatographic properties similar to those of 7β-hydroxypregnenolone was the major product. The present communication reports the identification of the unknown as a mixture of about 85% 2α-hydroxypregnenolone and about 15% of 2β-hydroxypregnenolone.

* Trivial names: pregnenolone, 3β-hydroxy-5-pregnen-20-one; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one.

2. Experimental

2.1. *Materials*

[4-^{14}C]Pregnenolone (Radiochemical Centre, Amersham, England) was purified by chromatography on a column of hydroxyalkoxypropyl-Sephadex with methanol–water–1,2-dichloroethane (7:3:1, v/v/v) as solvent [3]. The purified material was diluted with unlabelled pregnenolone and crystallized from an acetone–water mixture to yield material with a specific radioactivity of 3 μCi/mg.

2.2. *Methods*

Male rats of the Sprague-Dawley strain weighing 150–200 g were used. Liver homogenates (20%, w/v) were prepared in 0.25 M sucrose containing 0.001 M EDTA and the microsomal fraction was isolated as described previously [2]. The microsomal fraction was suspended in 0.1 M potassium phosphate buffer, pH 7.0, containing 0.028 M nicotinamide in a volume corresponding to the original volume of the 20,000 *g* supernatant fluid. Each incubation mixture consisted of 1 ml of microsomal fraction, 3 μmoles of NADPH and 2 ml of buffer. [4-^{14}C]Pregnenolone, 100 μg in 50 μl of acetone, was added and incubation was carried out for 20 min at 37°. The incubation was terminated by the addition of 20 vol of chloroform–methanol (2:1, v/v). The precipitate was filtered off and 0.2 vol of a 0.9% (w/v) sodium chloride solution were added. The residue of the chloroform phase was subjected to thin-layer chromatography with Kieselgel G (Merck, Darmstadt, Germany) as adsorbent and 2,2,4-trimethylpentane–isoamyl acetate–acetone (2:2:1, v/v/v) as solvent. The chromatoplate was run three times in the same solvent with drying of the plate between the runs.

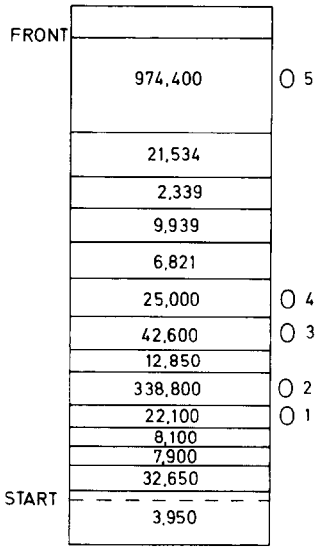

Fig. 1. Thin-layer chromatogram of extract of incubation of [4-^{14}C]pregnenolone with microsomal fraction of rat liver homogenate fortified with NADPH. Reference compounds were: 1) 7α-hydroxypregnenolone; 2) 7β-hydroxypregnenolone; 3) 16α-hydroxypregnenolone; 4) 7-ketopregnenolone.

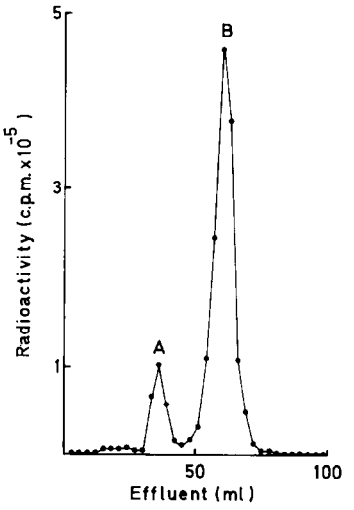

Fig. 2. Chromatogram of unknown on hydroxyalkoxypropyl-Sephadex. Column, 10 g; solvent, chloroform–hexane, 1:1 (v/v).

Radio-gas chromatography was performed with a Barber-Colman 5000 instrument equipped with a 1% QF-1 or 1% SE-30 column. Combined gas chromatography–mass spectrometry was performed with an LKB 9000 instrument equipped with a 1.5% SE-30 column.

Catalytic hydrogenation of the Δ^5 double bond was performed in ethanol with 10% palladium on charcoal as catalyst [4]. Acetonides were prepared as described by McCloskey and McClelland [5].

3. Results

A series of ten incubations of [4-^{14}C]pregnenolone was carried out with the microsomal fraction of rat liver homogenate fortified with NADPH. The extracts of the incubations were subjected to thin-layer chromatography (fig. 1). After autoradiography the labelled zones having about the mobility of reference 7β-hydroxypregnenolone were collected. The material was pooled and chromatographed on a column of hydroxyalkoxypropyl-Sephadex with chloroform–hexane (1:1, v/v) as solvent (fig. 2). Two peaks containing radioactivity (A, 33–43 ml of effluent, and B, 51–72 ml of effluent) were seen. Aliquots of both were converted to trimethylsilyl ethers and subjected individually to radio-gas chromatography and combined gas chromatography–mass spectrometry. Upon radio-gas chromatography each compound gave a single peak of mass and radioactivity. Upon combined gas chromatography–mass spectrometry again single peaks were observed. The t_R (retention time relative to cholestane) of compound A on a column of 1.5% SE-30 was 0.95 and that of compound B 1.31. The mass spectra of compounds A and B were practically identical (fig. 3 a and b). The base peak was at *m/e* 386 (M-90 (trimethylsilanol)). Peaks were seen also at *m/e* 461 (M-15). *m/e* 371 (M-(90+15)) and *m/e* (N-2 × 90). The mass spectra were similar to those of the trimethylsilyl ethers of 7α- and 7β-hydroxypregnenolone [6] and indicated that compounds A and B were dihydroxypregnenolones. Compounds A and B were hydrogenated catalytically. Radio-gas chromatography and combined gas chromatography–mass spectrometry of the trimethylsilyl ethers of hydrogenated compounds A and B showed predominant peaks with t_R on 1.5% SE-30 of 1.04 and 1.38,

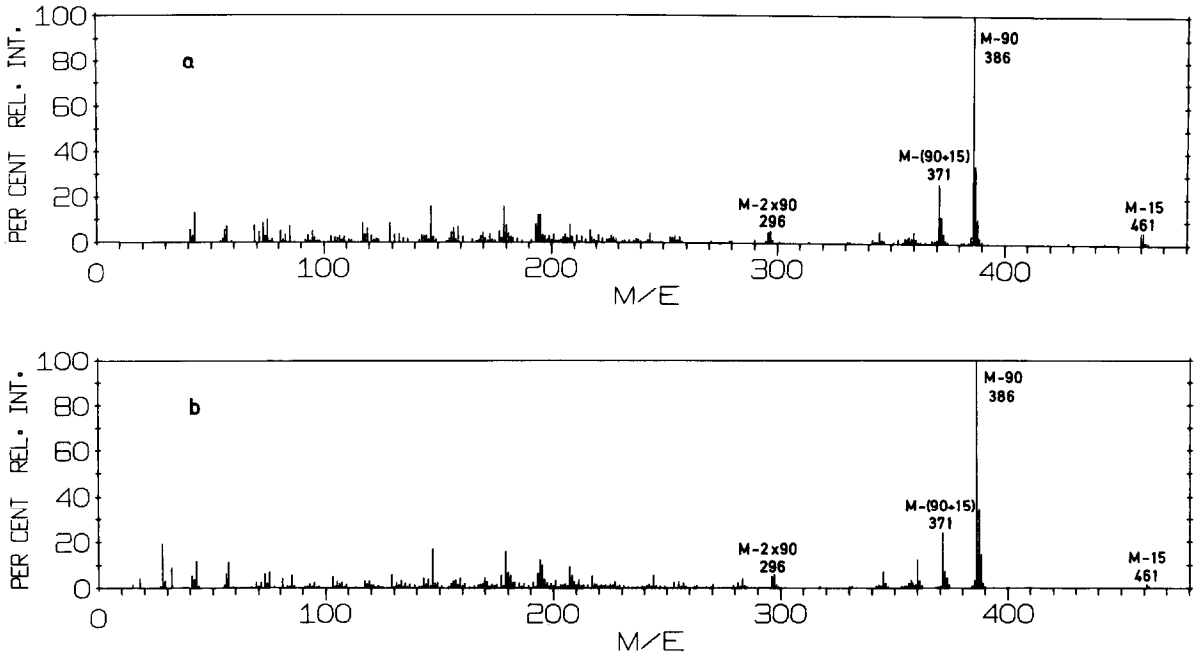

Fig. 3. Mass spectra of trimethylsilyl ethers of compound A (a) and compound B (b).

respectively. Hydrogenated compounds A and B gave almost the same mass spectra (fig. 4 a and b). The spectra were practically identical with that of the trimethylsilyl ether of authetic 2α,3β-dihydroxy-5α--pregnan-20-one (fig. 4 c). The molecular weight was 478 and the base peak was at *m/e* 299 (M-(89 + 90, trimethylsiloxy group and trimethylsilanol)). Prominent peaks were seen at *m/e* 463 (M-15), *m/e* 338 (M-90) and *m/e* 143, 142 and 129. Peaks at *m/e* 143, 142 and 129 are seen in 2,3-*bis*-trimethylsiloxy steroids [7, 8]. These results indicated that compounds A and B were the epimeric 2-hydroxypregnenolones. The relative retention times indicated that compound A was the 2β-epimer and that compound B was the 2α-epimer. The t_R of the trimethylsilyl ether of hydrogenated compound B was found to be 1.38 and the trimethylsilyl ether of 2α,3β-dihydroxy-5α-pregnan-20-one has been reported to have a t_R of 1.34 under similar chromatographic conditions [7]. The question of the configuration at C-2 of compounds A and B was established by preparation of acetonides. Hydrogenated compounds A and B were treated with acetone and dry copper sulfate. Compound B remained unchanged after this treatment showing that the hydroxyl groups in compound B are oriented *trans* to each other. Compound A was transformed into a derivative which showed the mass spectrum expected of an acetonide of 2β,3β-dihydroxy-5α-pregnan-20-one (fig. 4 d). The base peak was at *m/e* 359 (M-15) and a prominent peak was seen at *m/e* 299 (M-75). This fragmentation pattern is typical of acetonides of steroids with the vicinal hydroxyl groups in the steroid nucleus ([9], cf. also [5 and 10]).

4. Discussion

In the previous study of the metabolism of pregnenolone in the presence of microsomal fraction of rat liver homogenate fortified with NADPH [2], 7α-, 7β- and 16α-hydroxypregnenolone and 7-ketopregnenolone were identified as metabolites. The major metabolite could not be identified. The mass spectrum of the trimethylsilyl ether of the unknown indicated the structure of a hydroxylated pregnenolone. On biological grounds it appeared very likely that the unknown had retained the Δ^5-3β-hydroxy structure

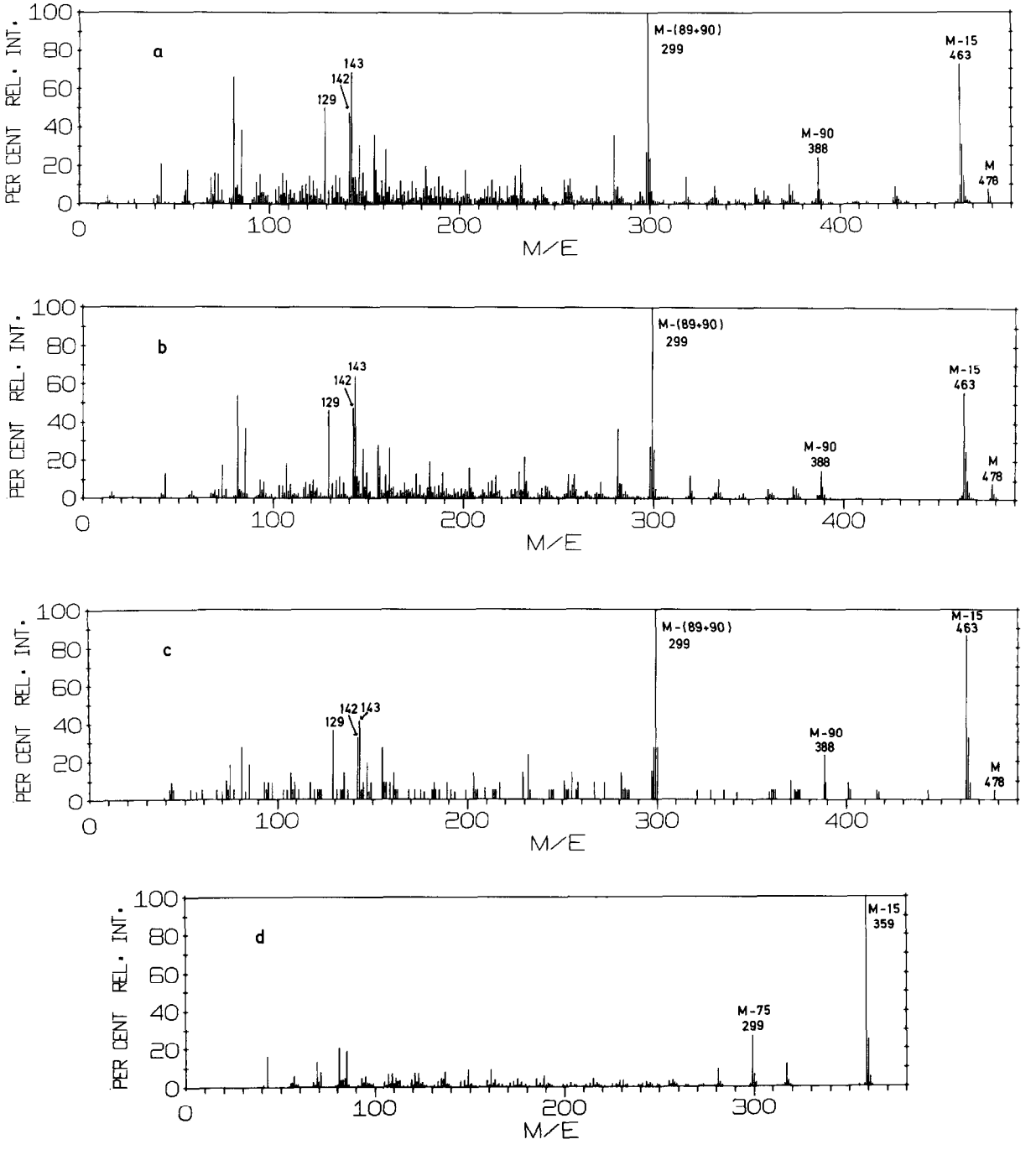

Fig. 4. Mass spectra of trimethylsilyl ethers of hydrogenated compound A (a), hydrogenated compound B (b) and authenic 2α, 3β-dihydroxy-5α-pregnan-20-one (c) and mass spectrum of acetonide of hydrogenated compound A (d). The 2α,3β-dihydroxy-5α-pregnan-20-one was a generous gift of Dr. J.-A. Gustafsson.

of pregnenolone. The mass spectrum of the trimethylsilyl ether of the unknown showed a very prominent base peak at M-90. Such a peak is found in mass spectra of Δ^5-3β-hydroxysteroids having a hydroxyl group at C-7 of C-18 [6, 9, 11]. Since C-7 could be excluded it was suggested that the unknown could be 18-hydroxypregnenolone [2]. However, comparison of chromatographic and mass spectrometric data of an authentic sample of 5-pregnene-3β,18,20β-triol with those of a sample of the unknown after reduction with sodium borohydride showed that the compounds were not identical. At this stage of the investigation the structure of 2-hydroxypregnenolone was considered less probable. The mass spectrum of the trimethylsilyl ether of the unknown did not show any prominent peaks at *m/e* 143, 142 and 129. Such peaks are found in the spectra of steroids with a 2,3-*bis*-trimethylsilyloxy structure [7, 8]. However, the comparison spectra available were of compounds without a Δ^5 double bond. The possibility that the presence of Δ^5 double bond could influence the fragmentation pattern had to be taken into account. A series of studies to identify the unknown metabolite was therefore initiated. As described in the Results section the unknown has now been found to be a mixture of about 85% 2α-hydroxypregnenolone and about 15% 2β-hydroxypregnenolone. These compounds have not been previously found in animal tissues. 2α-Hydroxylation of progesterone by rat liver microsomes has been reported [12] and 2α-hydroxylated pregnane derivatives have been isolated from feces of rats [7] and have been shown to be formed from progesterone in the isolated perfused rat liver [8].

The results of the present and the previous investigations [2] show the pronounced influence of the side chain on the pattern of hydroxylation of Δ^5-3β-hydroxysteroids by rat liver microsomes. The number of positions hydroxylated in dehydroepiandrosterone and pregnenolone contrasts sharply with that in cholesterol which in essence is hydroxylated only in the 7α-position.

Acknowledgements

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