A reliable radiochromatographic assay technique for hepatic microsomal 16 α -hydroxylase activity towards oestrone 3-sulphate

Comparison between pigmented and non-pigmented mature guinea pigs

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A reliable procedure for the assay of liver microsomal 16 α -hydroxylation of oestrone 3-sulphate has been developed for the guinea pig. It is based on the rapid, quantitative separation of oestradiol and oestriol by Sephadex LH-20 columns after the chemical reduction and enzymic hydrolysis of the incubation products. Microsomal preparations and incubation conditions that optimized 16 α -hydroxylation of oestrone 3-sulphate were employed. Under these circumstances, reduction of the substrate at C-17 and hydrolysis of the sulphate were minimized. Conditions were established that yielded reaction linearity with respect to time and microsomal concentration. This hydroxylation had an absolute requirement for NADPH, which could not be satisfied by NADH. Apparent K_m values for oestrone 3-sulphate and NADPH, under the conditions used, were 14 μ M and 0.17 mM respectively. 16 α -Hydroxylase activity was present in the liver microsomal fraction from heavily pigmented, female English Shorthaired guinea pigs. Much lower activity was detected in mature pigmented males and albino females. No activity could be demonstrated in mature, albino males.

Conjugated steroids have, at various times in the past, been thought of as products solely destined for excretion. Although this may hold for the glucuronide forms, evidence from numerous sources suggests a role other than excretion for sulpho-conjugated steroids in some species (Gustafsson & Ingelman-Sundberg, 1974; Einarsson *et al.*, 1976). Sulphated steroids are conserved by many mammals. Oestrone 3-sulphate is quantitatively the most important circulating form of oestrogen in the human (Purdy *et al.*, 1961; Smith & Hagerman, 1965; Hawkins & Oakey, 1974).

Studies in our laboratory have shown that, in guinea pigs, oestrone is hydroxylated at the 16-position in the β -configuration, whereas oestrone 3-sulphate is 16 α -hydroxylated (Hobkirk *et al.*, 1975; Hobkirk & Nilsen, 1977; Harvey & Hobkirk, 1977). 16 α -Hydroxylation of oestrogens is a quantitatively important activity in the human (Beer & Gallagher, 1955; Zumoff *et al.*, 1968). Oestrone 3-sulphate has also been shown to be directly hydroxylated at various carbon atoms in human tissues (Fishman *et al.*, 1973; Ingelman-Sundberg *et al.*, 1975). Moreover, it has been suggested that oestrone 3-sulphate in the human may be the

primary substrate for oestrogen hydroxylation at C-16 (Fishman *et al.*, 1969). In view of the above and the yet undefined role of oestriol in certain carcinogenic states (Lemon, 1975), as well as its oestrogenic or anti-oestrogenic role in the uterus (Clark *et al.*, 1977), the study of the 16 α -hydroxylase becomes rather important.

Further evidence from our own laboratory, that 16 α -hydroxylation of phenolic steroids in guinea-pig liver appears to be uncomplicated by hydroxylation at other carbons (Hobkirk *et al.*, 1977), presents a strong case for the use of this species as a model for the detailed study of the 16 α -hydroxylase, an enzyme long known to exist, but about which remarkably little is known.

Evidence *in vivo* has been reported for the 16 α -hydroxylation of oestrogens in pigmented female guinea pigs (Hobkirk *et al.*, 1978). Recently, experiments *in vivo* from our own laboratory, on albino guinea pigs, have suggested a relationship between the ability to hydroxylate oestrogens and pigmentation of the animals. In the present paper we report a reliable method for the assay of the 16 α -hydroxylation of oestrone 3-sulphate. This was used to measure the activity of the enzyme in mature

male and mature non-pregnant female guinea pigs, with a view to confirming any relationship to pigmentation.

Experimental

Materials

[6,7-³H]Oestrone 3-sulphate (sp. radioactivity 53 Ci/mmol), [6,7-³H]oestradiol (sp. radioactivity 54 Ci/mmol) and [4-¹⁴C]oestradiol (sp. radioactivity 55.7 Ci/mmol) were purchased from New England Nuclear (Canada), Dorval, Que., Canada. All labelled steroids were purified and stored for use as previously described (Hobkirk *et al.*, 1969a,b). Unlabelled oestrone 3-sulphate was prepared chemically (Fieser, 1948) from oestrone and purified by crystallization. All other unlabelled steroids used were obtained from Schwarz-Mann, Orangeburg, N.Y., U.S.A. NADPH, NADH and purified bovine serum albumin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-Sephadex (A25) and Sephadex LH-20 were purchased from Pharmacia (Canada) Ltd., Montreal, Que., Canada. Amberlite XAD-2 resin was a product of Rohm and Haas, Philadelphia, PA, U.S.A. Mylase P, containing a phenolsulphatase, was purchased from ICN Nutritional Biochemicals, Cleveland, OH, U.S.A. The scintillation fluid employed was xylene-based ACS and was purchased from Amersham, Oakville, Ont., Canada. The mini-vials, used for radioactivity counting, were from Canadian Scientific Products, London, Ont., Canada. Guinea pigs of the English Shorthaired variety were either bred in our own animal quarters or purchased from local breeders and housed for several weeks before use. Albinos had markedly pink eyes and bore no pigmentation. Pigmented animals possessed at least one colour (brown or black) and eye rims that were darkly pigmented. All other solvents, chemicals and reagents were purchased from Fisher Scientific Co., Toronto, Ont., Canada, and were of acceptable grade.

Microsomal preparation

Guinea pigs were killed by a blow to the head. Livers were rapidly excised and washed carefully with cold 0.9% NaCl. The livers were blotted, cut into small pieces and weighed. By using a motor-driven Teflon pestle in a glass homogenizing tube (clearance 0.1–0.15 mm), 6.0 g of liver were homogenized in 30 ml of ice-cold 20 mM-Tris/HCl, pH 7.4, unless otherwise stated. Each homogenate was centrifuged at 20 000 g for 20 min at 4°C in a Sorvall RC2-B refrigerated centrifuge with a Sorvall type SS-34 rotor. The resulting supernatant was centrifuged at 105 000 g for 60 min at 4°C in a Beckman model L3-50 ultracentrifuge with a type 50Ti rotor. The high-speed supernatant was discarded and the

microsomal pellet was re-suspended in a volume of ice-cold 20 mM-Tris/HCl, pH 7.4, equal in volume to the discarded supernatant. This was centrifuged again at 105 000 g for 60 min at 4°C. The supernatant was once again discarded and the resulting microsomal pellet was suspended in a volume of 20 mM-Tris/HCl, pH 7.4, equal to the wet weight of liver originally homogenized. Microsomal protein concentration was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Microsomal incubations

[6,7-³H]Oestrone 3-sulphate and unlabelled oestrone 3-sulphate in methanolic solution were measured into glass test tubes. The methanol was evaporated under N₂ and to the substrate, different buffers, with or without ions, were added to give a final incubation volume of 5 ml. Initially, concentrations of oestrone 3-sulphate, NADPH and microsomal protein corresponded to those used by Harvey & Hobkirk (1977). The final concentrations used in routine assays were 3 × 10⁴ d.p.m. of [6,7-³H]oestrone 3-sulphate, 54.4 μM-oestrone 3-sulphate, 1.2 mM-NADPH, 40 mM-MgCl₂, 2–4 mg of microsomal protein and 20 mM-Tris/HCl, pH 7.4, in a final volume of 1.0 ml. The reaction was started by the addition of microsomal protein. Incubations were carried out in a Dubnoff shaking incubator at 37°C for 30 min. The reaction was terminated by adding 20 ml of cold (–20°C) methanol to the test tube, which was then stored at –20°C overnight. Products from these incubations were initially analysed as described below.

Analysis of incubation products

The investigation of the products was initially carried out as described by Harvey & Hobkirk (1977). The cold methanol extracts were filtered through Whatman no. 40 filter paper, and the precipitates were washed with cold methanol. The combined extracts were evaporated to dryness and taken up in water. The products were chromatographed on DEAE-Sephadex (A25) columns (Hobkirk *et al.*, 1969a). The monosulphate peak was concentrated by the use of Amberlite XAD-2 resin (Bradlow, 1968). The monosulphates were then chemically reduced overnight with NaBH₄ (Hobkirk *et al.*, 1969a). Acetic acid was then added dropwise until neutrality was reached. The steroids were once again recovered from the reaction mixture via Amberlite XAD-2 resin. The reduced monosulphates were incubated overnight at 37°C in 0.1 M-acetate buffer, pH 6.0, containing Mylase P (Emerman *et al.*, 1965). The reduced, free oestrogens so formed were extracted into ethyl acetate. The extract was evaporated to dryness and then chromatographed on Sephadex LH-20 with chloro-

Fig. 1. Routine assay of 16 α -hydroxylation of oestrone 3-sulphate

All the possible oestrogen metabolites from guinea-pig liver microsomal incubations are shown. They were subjected to reduction and hydrolysis as described in the Experimental section.

form/benzene/methanol (3:7:1, by vol.) as solvent (Carr *et al.*, 1971). The identities of the labelled metabolites were verified by crystallizing with the appropriate pure carriers (Hobkirk *et al.*, 1969a).

All radioactivity counting was performed on a Unilux IIA Nuclear-Chicago liquid-scintillation spectrometer with quench corrections by channels ratio. Sufficient counts were accumulated to ensure an error within, at the most, $\pm 5\%$ for all of the data presented.

Routine assay

The steroidal products from routine incubations, after being left overnight at -20°C in methanol, were subjected to chemical reduction and enzymic hydrolysis as described above and shown in Fig. 1. This resulted in the production of only oestradiol and oestriol from a number of possible incubation products, thus simplifying the assay system. The metabolites were then run on Sephadex LH-20 columns with toluene/methanol (9:1, v/v) as solvent as described below. Recovery of the incubated radioactivity after the manipulations shown in Fig. 1 was approx. 90%. Most of the loss occurred after the hydrolysis with Mylase P.

One unit of enzyme activity is the amount of enzyme required to hydroxylate 1 nmol of oestrone 3-sulphate at the 16-position in the α -configuration per h. Specific activity is defined as the number of units of enzyme activity per mg of protein.

Results and discussion

Conditions affecting 16 α -hydroxylase activity

Fig. 2. shows the chromatographic patterns on DEAE-Sephadex of incubation products formed by the liver microsomal fraction ('microsomes') prepared and incubated in various media. Conditions were sought that optimized the 16 α -hydroxylation of oestrone 3-sulphate. Recovery of the incubated radioactivity from the chromatographic step was better than 95%. All of the radioactivity was found in the monosulphate fractions. Absence of a free oestrogen fraction indicated the lack of sulphatase activity, which had been detected by Harvey & Hobkirk (1977), under their conditions.

There was no apparent difference in the elution pattern, when the microsomes were prepared with KCl or a modified Bucher buffer (Figs. 2e and 2f). The effect of preparing the microsomes in 20mM-

Fig. 2. Elution profile from a DEAE-Sephadex column (linear 0–0.8 M-NaCl gradient; 60 cm $\times$ 0.9 cm) of metabolites of oestrone 3-sulphate from microsomal incubations

Incubations were carried out as described previously. Each incubation contained oestrone 3-sulphate and NADPH unless otherwise stated. The methods of microsomal preparation and/or the incubation conditions were varied as follows. (a) Control with NADPH excluded from incubation; (b) microsomal fraction prepared in 20 mM-Tris/HCl, pH 7.4, and incubated in 20 mM-Tris/HCl, pH 7.4, plus 40 mM-MgCl₂; (c) microsomal fraction prepared and incubated in 20 mM-Tris/HCl, pH 7.4; (d) microsomal fraction prepared in 1.15% KCl and incubated in 20 mM-Tris/HCl, pH 7.4; (e) microsomal fraction prepared and incubated in a modified Bucher buffer without nicotinamide (Harvey & Hobkirk, 1977); (f) microsomal fraction prepared in 1.15% KCl and incubated in modified Bucher buffer without nicotinamide. The radioactivity in each peak is denoted as a percentage of the total radioactivity recovered from the column; 10 ml fractions were collected. Abbreviations used: $16\alpha\text{OHOE}_1\text{3S}$, 16α -hydroxyoestrone 3-sulphate; $\text{OE}_3\text{3S}$, oestriol 3-sulphate; $\text{OE}_2\text{3S}$, oestradiol 3-sulphate; $\text{OE}_1\text{3S}$, oestrone 3-sulphate.

Tris/HCl, pH 7.4, as opposed to KCl, is evident, however, in Figs. 2(c) and 2(d). Enzymic reduction of oestrone 3-sulphate at C-17 is decreased somewhat when 20mM-Tris/HCl, pH 7.4, is used for microsomal preparation. The result of incubating in Tris/HCl is shown by comparing Figs. 2(d) and 2(f). The apparent suppression of enzymic activity at C-17 when using Tris/HCl to prepare the microsomes and as the incubating buffer is magnified when 40mM-MgCl₂ is included in the incubation medium (Fig. 2b). The inclusion of nicotinamide (Harvey & Hobkirk, 1977) in any combination had no effect.

To ensure that hydroxylation was not also being inhibited, the peak containing the presumptive hydroxylated products was further analysed. After chemical reduction and hydrolysis as described above, the metabolites were chromatographed on Sephadex LH-20 with the elution system described by Carr *et al.* (1971). Fig. 3(b) is typical of the elution pattern obtained. The peaks in Figs. 2(a) and 2(b) were re-chromatographed on Sephadex LH-20 after the manipulations described above. Fig. 3(a)

Fig. 3. Elution profile from a Sephadex LH-20 column (20cm × 0.6cm) of two of the monosulphate fractions from a DEAE-Sephadex column

The solvent system used was chloroform/benzene/methanol (3:7:1, by vol.); 2ml fractions were collected. The monosulphate fractions were reduced and hydrolysed before being chromatographed as described in the text. (a) Control [corresponds to Fig. 2(a)]; (b) +NADPH [corresponds to Fig. 2(b)]. Abbreviations used: OE₂, oestradiol; OE₃, oestrinol.

shows that incubating oestrone 3-sulphate in the absence of NADPH results in no hydroxylation. The oestrinol peak in Fig. 3(b) represents the 16 α -hydroxylation of oestrone 3-sulphate under conditions that allowed little enzymic reduction at C-17. It is possible that reduction was inhibited under the experimental conditions or that the hydroxylase was activated, resulting in much less reduction of oestrone 3-sulphate.

The optimum conditions employed routinely consisted of [³H]oestrone 3-sulphate, NADPH, 40mM-MgCl₂ and microsomes prepared and washed with Tris/HCl. This was incubated in 20mM-Tris/HCl, pH 7.4.

Quantitative separation of oestradiol from oestrinol by using Sephadex LH-20

A rapid and reliable system was needed for the routine separation of oestradiol and oestrinol as shown in Fig. 1. The system used previously seemed inadequate in our hands for routine use. A smaller elution volume and faster flow rate were required. The system developed was as simple as possible, i.e. toluene/methanol (9:1, v/v). The columns were 10cm in length with an inner diameter of 0.6cm and were fitted with Teflon stopcocks. They appeared to be reusable indefinitely. An elution profile of standard oestradiol and oestrinol is shown in Fig. 4. A routine incubation sample, after chemical reduction

Fig. 4. Elution profile from a Sephadex LH-20 column (10cm × 0.6cm) of standard oestradiol and oestrinol

The solvent system used was toluene/methanol (9:1, v/v); 2ml fractions were collected. The flow rate was 0.5ml/min. Oestradiol was eluted with the first 24ml of solvent. Oestrinol was eluted with the next 30ml of the solvent system. Abbreviations used: OE₂, oestradiol; OE₃, oestrinol.

and hydrolysis, was taken up in 1.0 ml of the solvent system and applied to the column. The test tube containing the sample was then washed twice with 1 ml of the solvent system, and the washings were transferred to the column. Two fractions were collected, one containing oestradiol and the other oestriol. Both fractions were evaporated to dryness and a known volume of methanol was added. A portion was taken for counting of radioactivity. The percentage 16 α -hydroxylation was calculated as follows:

Percentage 16 α -hydroxylation =

$$\left(\frac{\text{d.p.m. in fraction 2}}{\text{d.p.m. in fraction 1} + \text{d.p.m. in fraction 2}} \right) \times 100$$

Incubations with boiled microsomal protein, or in the absence of NADPH, gave values of 2–4% apparent hydroxylation. These were corrected for in all subsequent experiments. Identification of oestradiol and oestriol, arising from the analysis of routine incubations, were made by crystallization with unlabelled carriers to radiochemical homogeneity (Hobkirk *et al.*, 1969a).

Properties of the 16 α -hydroxylase

16 α -Hydroxylation activity toward oestrone 3-sulphate was examined as a function of time and protein concentration. Enzyme activity as a function of protein concentration proved to be linear up to 5 mg of microsomal protein. The reaction rate was linear for 1 h. All routine assays were performed within the specifications, which preserved linearity.

The pH-activity curve for the 16 α -hydroxylase was obtained by using various buffers for different pH ranges. Incubations performed in Tris/HCl gave higher activity than any other buffers used over similar pH ranges. The pH optimum occurs at approx. 7.4.

Substitution of NADH for NADPH in the system resulted in no measurable hydroxylation compared with blanks. To determine the saturating conditions for both NADPH and oestrone 3-sulphate, incubations were performed in the presence of a constant non-limiting concentration of NADPH while the concentration of oestrone 3-sulphate was varied, or vice versa.

Curves for each of the velocity versus oestrone 3-sulphate concentration and velocity versus NADPH concentration showed typical Michaelis-Menten curves. The apparent K_m values derived from double reciprocal plots are 14 μ M for oestrone 3-sulphate and 0.17 mM for NADPH. The V_{max} values for oestrone 3-sulphate and NADPH are 7.0 units of specific activity and 19 units of specific activity respectively. Based on these data the concentrations of oestrone 3-sulphate and NADPH

Table 1. 16 α -Hydroxylation of oestrone 3-sulphate by liver microsomes from albino and pigmented guinea pigs

The data below represent the specific activities found in eight pigmented females and four pigmented males, four non-pigmented females and four non-pigmented males. Liver microsomes, from each guinea pig, were subjected to the routine assay procedure specified in the text. Each value represents the mean $\pm$ s.d. for four incubations.

Specific activity (units/mg)			
Mature pigmented (non-pregnant)		Mature albino (non-pregnant)	
Female	Male	Female	Male
4.58 $\pm$ 0.28	0.67 $\pm$ 0.09	0.46 $\pm$ 0.04	N.D.
4.19 $\pm$ 0.26	0.79 $\pm$ 0.08	0.38 $\pm$ 0.02	N.D.
5.83 $\pm$ 0.24	N.D.*	0.32 $\pm$ 0.05	N.D.
5.03 $\pm$ 0.30	0.90 $\pm$ 0.1	0.21 $\pm$ 0.04	N.D.
6.17 $\pm$ 0.43			
3.45 $\pm$ 0.25			
9.25 $\pm$ 0.56			
8.66 $\pm$ 0.58			
5.89 $\pm$ 2.08†	0.59 $\pm$ 0.21	0.34 $\pm$ 0.10	N.D.

* Not detectable.

† Mean $\pm$ s.d.

used in studying 16 α -hydroxylation in albino and pigmented guinea pigs were not limiting or inhibitory.

16 α -Hydroxylation of oestrone 3-sulphate in albino versus pigmented guinea pigs

Table 1 shows the specific activities of the liver microsomal enzyme in albino and pigmented, mature guinea pigs, and indicates an apparent difference related to the presence or absence of pigmentation. This is most marked when comparing females. There seems to be a complete lack of 16 α -hydroxylase activity in non-pigmented males and very low activities in non-pigmented females. The activities in pigmented males are slightly lower than, but comparable with, those of non-pigmented females. When comparing males with females, either pigmented or albino, the activities in females are always higher. This is particularly evident among the pigmented animals. A sex-specific hydroxylase system has been reported in rat liver (Gustafsson & Ingelman-Sundberg, 1974). Each guinea pig in the present study was sexually mature, but differed in age. The variability within a group could possibly be explained by age differences but more likely, the values represent individuality among guinea pigs.

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