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ENZYMATIC ANDROGEN ASSAY: SOME PROPERTIES OF HUMAN PLACENTAL MICROSOMAL AROMATASE

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Summary—The androgen content of biological fluids can be determined after their conversion into estrogens using human placental microsomal aromatase (HPMA). The purpose of this paper is to report some physico-chemical properties of HPMA. Using an accurate, specific and sensitive assay for HPMA, K_m values for dehydroepiandrosterone (DHEA), androstenedione and testosterone were found to increase with increasing amount of the detergent (Triton X-100) added. Analysis at substrate concentrations 5–10 times above and below the K_m values did not indicate any anomalous kinetic behaviour. Triton X-100, used for enzyme solubilization, significantly decreased the rate of aromatization of the three substrates by increasing their K_m values. This effect was more important for testosterone than for androstenedione or DHEA. Using a new protocol for the determination of aromatase activity, kinetic properties of aromatase before and after solubilization are described.

INTRODUCTION

The availability of a sensitive and specific estradiol and estrone assay using 17β-estradiol dehydrogenase [1] prompted us to use this new method to determine androgens after their quantitative aromatization. Aromatization is achieved by the human placental microsomal aromatase (estrogen synthetase) [HPMA]. This enzymatic system is able to convert several androgens into estrogens quantitatively [2–4].

The determination of androgens using the aromatase system was previously proposed by Finkelstein *et al.*[5] and Horn *et al.*[6]. However, in that method the formed estrogens were measured by spectrofluorimetry.

We have studied the properties of the aromatase contained in the microsomal fraction of human placenta and have obtained a partial solubilization by using a non-ionic detergent, Triton X-100, which is subsequently removed by XAD-2 chromatography.

The purpose of this work was the determination of the optimal conditions of aromatase activity assay and the evaluation of the Michaelis constants of coenzyme and steroid substrates with a view to establishing an enzymatic assay for androgens [7].

EXPERIMENTAL

Steroids were purchased from Steraloids (Pawling, New York, U.S.A.) and purified by TLC. Nucleotides, D-glucose-6-phosphate and yeast glucose-6-phosphate dehydrogenase were purchased from Boehringer (Mannheim, West Germany). All other used reagents were from Merck, Darmstadt, W. Germany.

Enzyme preparation

We used human full term placenta, obtained less than 30 min after delivery. All further operations were carried out at 4°C. The placental tissue was minced and homogenized in a mixer with an equal volume of 0.03 M phosphate buffer, pH 7.2, 2 M glycerol. The homogenate was centrifuged at 1000 *g* for 15 min and the supernatant at 10,000 *g* for 30 min. The resulting supernatant was centrifuged at 105,000 *g* for 90 min and the microsomal pellet was collected. The microsomal fraction was washed several times with a 0.03 M phosphate buffer, pH 7.2, 2 M glycerol. The washed microsomal fraction was homogenized with a glass Potter-Elvehjem apparatus in an equal volume of 0.03 M phosphate buffer, pH 7.2, 2 M glycerol containing 0.4% Triton X-100. The mixture was centrifuged at 105,000 *g* for 90 min and the supernatant removed, made 5 M in glycerol and applied to an Amberlite XAD-2 column (2.5 × 10 cm) equilibrated at 4°C with 0.03 M phosphate buffer, pH 7.9 containing 5 M glycerol.

Protein determination

Protein determination was carried out according to the procedure of Lowry *et al.*[8] with bovine serum albumin as the standard. The protein concentration of the enzyme preparation was adjusted to 2.4 mg/ml.

Aromatase activity

The incubation tubes were prepared in duplicate with 10 μmol steroid substrates (testosterone, an-

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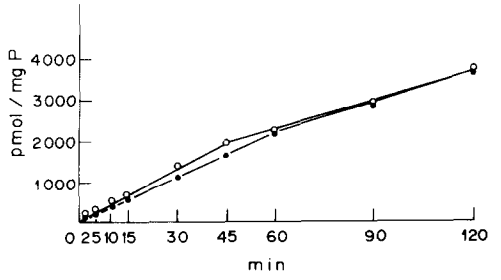

Fig. 1. Rate of aromatization for DHEA (●) and testosterone (○). In this and other Figures P denotes protein.

drostenedione or DHEA), 10 μmol $NADP^+$, 10 IU glucose-6-phosphate dehydrogenase, 100 μmol glucose-6-phosphate and 1 μmol dithioerythreitol in 0.02 M Tris–HCl buffer, pH 8. The aromatization reaction was then started by the addition of enzyme preparation to a final volume of 1 ml. We tested different protein concentrations of aromatase between 20 and 800 μg/ml. The reaction mixtures, including appropriate blanks, were incubated for various times (2–120 min) at 37°C. The formed estrogens were measured by the enzymatic method [1].

Estrogen determination

The enzymatic estrogen assay was described in details in 1979 by Nicolas *et al.*[1]. This method uses the transhydrogenase activity of the human placental estradiol dehydrogenase: the hydrogen of trace amounts of NADPH (10^{-6} M) is transferred by the enzyme to an excess of NAD^+ (10^{-3} M) via repeated cyclic interconversion of estrone and estradiol. The NADPH is continuously regenerated by the NADP regenerating system: glucose-6-phosphate (10^{-3} M) plus glucose-6-phosphate dehydrogenase. In the selected conditions, the rate limiting step of NAD^+ reduction is a linear function of estrone plus estradiol concentration in the medium. The sensitivity of the method is 8 pg of estrogens in the sample and the precision 5%.

RESULTS

All the experiments were done in triplicate and the average results are given below. The error is lower than 2%.

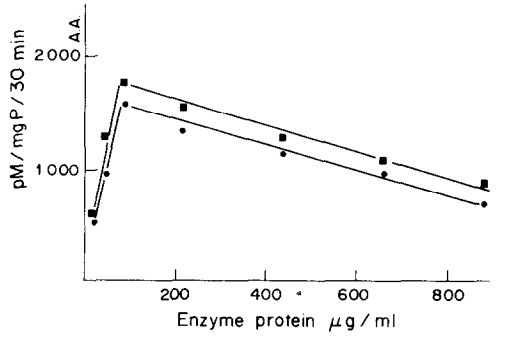

Fig. 2. Relationship between aromatase activity (A.A.) and enzyme protein concentration for DHEA (●) and testosterone (■).

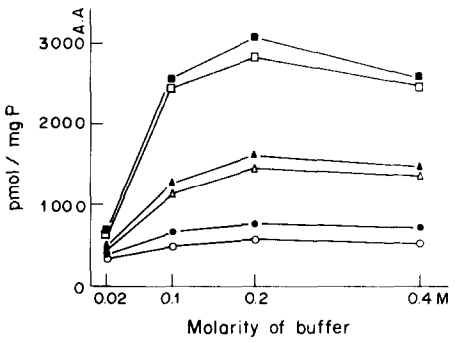

Fig. 3. Relationship between aromatase activity and the buffer molarity for DHEA during 15 min (○), 30 min (△), 45 min (□) and for androstenedione during 15 min (●), 30 min (▲), 45 min (■).

(a) Optimal measurement conditions

The rates of aromatization of DHEA and testosterone were compared (Fig. 1). For both substrates, aromatization was linear with time until 45 min. Beyond that, there is a variation in slope. Aromatization was also linear with protein concentration up to 100 μg/ml (Fig. 2).

Figure 3 shows that the formation of estrogens is controlled by the buffer molarity and the optimal value is 0.2 M.

The influence of the buffer pH is shown by the Fig. 4 with an optimal pH at 8.

(b) Kinetic analysis

NADPH and steroid Michaelis constant. Lineweaver–Burk plot (Fig. 5) indicates the same NADPH K_m value (10^{-6} M) with all three substrates.

The K_m values of steroid substrates obtained with a microsomal preparation before solubilization are shown in Fig. 6. The Lineweaver–Burk plots for these experiments indicate that K_m values for an-

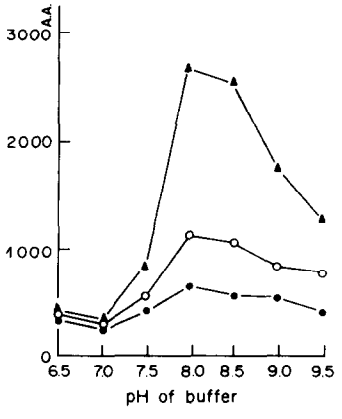

Fig. 4. Relationship between aromatase activity and the pH of the buffer for androstenedione during 15 min (●), 30 min (○) and 45 min (▲).

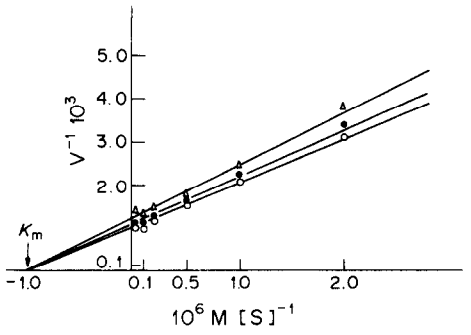

Fig. 5. Lineweaver–Burk plot for K_m NADPH for DHEA (○), androstenedione (●) and testosterone (△).

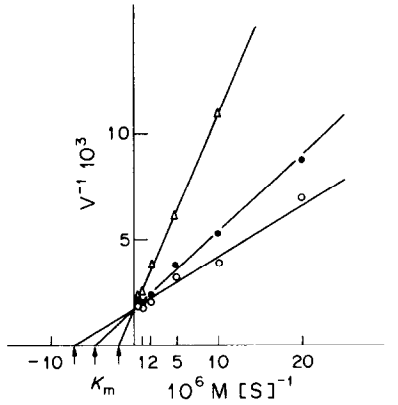

Fig. 6. Lineweaver–Burk plot for K_m of substrates: DHEA (○), androstenedione (●) and testosterone (△) with non-solubilized enzyme.

drostenedione and testosterone were 200 and 500 nM respectively. These values are consistent with those reported by Bellino and Osawa[9], Reed and Ohno[10], and Canick and Ryan[11] (Table 1).

According to Bellino and Osawa[9], the values must be accepted very cautiously, since they represent the net effect of several enzymatic reactions, as a crude microsomal preparation was the source of enzyme activity.

Our values are lower than those reported by Thompson and Siiteri[12] and Brodie *et al.*[13] (Table 1). This may result from the low sensitivity of the analytical methods ued by these authors.

More recently, Gibb and Lavoie[14] (Table 1) reported K_m values for androstenedione and testosterone ten times lower than our values. In spite of these differences, our results show that androstenedione is utilized more efficiently than testosterone by the aromatase.

In the microsomal fraction, there is a great amount of 3β-hydroxysteroid dehydrogenase-5-ene-isomerase (3β-HSDH), and it is noteworthy that DHEA is converted as efficiently as androstenedione. The apparent Michaelis constant which results in fact from two enzymatic reaction (oxidation and aromatization) is of the same order of that of androstenedione. This suggests that the 3β-HSDH and the aromatase are closely located in microsomes.

Kinetic constants with solubilized enzyme. Table 2 shows K_m values obtained with solubilized preparation at various concentrations of Triton X-100 (0.01–0.04%) for all the three steroid substrates. These experiments were performed to compare and contrast the K_m values of the crude microsomal aromatase and Triton X-100 solubilized aromatase, and then to determine whether or not the detergent influences the substrate affinity. K_m values were calculated by Lineweaver–Burk plotting (Fig. 7). Estimates of the K_m for androstenedione or testosterone aromatization are lower than or similar to the most recently reported values (Table 1).

Table 2 and Fig. 8 show that the K_m values for androstenedione and the apparent value for DHEA are very similar, and different from those for testosterone, and that the solubilization of the enzyme (followed or not by removal of the Triton X-100) affects the K_m of the three steroids in the same ratio. The similarity between DHEA and androstenedione and the deviation of testosterone are maintained.

The best substrate seems to be androstenedione which is actually the physiological substrate. The apparent affinity constant of DHEA is similar to the affinity constant of androstenedione. This can be explained by the fact that the 3β-HSDH and the aromatase are associated in the same complex even after solubilization.

DISCUSSION

For this study, we have used an enzymatic method for the determination of estrogens since we intend to

Table 1. K_m values of androstenedione and testosterone for aromatase according to different authors

	K_m (μmol)			
	Androstenedione		Testosterone	
Bellino and Osawa[9]	0.53		0.1	
Reed and Ohno[10]	0.1		0.4	
Canick and Ryan[11]	0.1			
Thompson and Siiteri[12]	5	(7)		
Brodie *et al.*[13]	20			
Gibb and Lavoie[14]	0.014		0.041	
Kelly *et al.*[16]	0.045			
Present paper	0.2	(0.058)	0.5	(0.1)

Values between brackets are from solubilized enzyme.

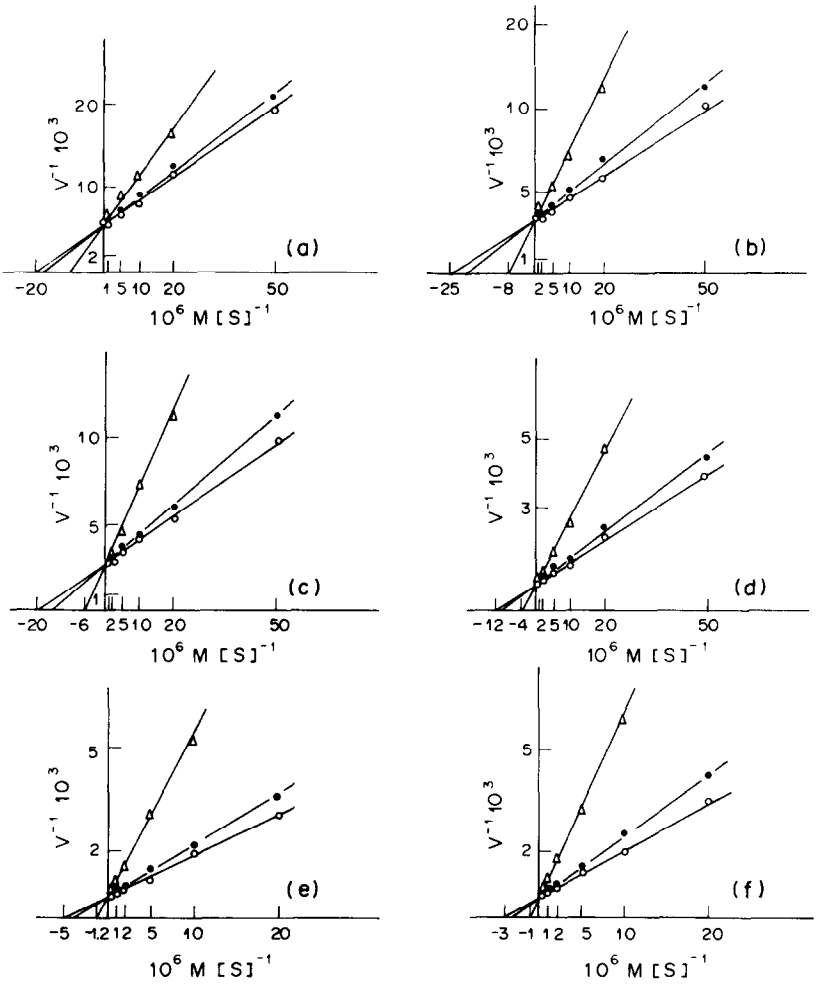

Fig. 7. Lineweaver–Burk plots for K_m of substrates DHEA (○), androstenedione (●) and testosterone (△) with solubilized enzyme at different concentrations of Triton X-100; 0% (a), 0.01% (b), 0.05% (c), 0.1% (d), 0.2% (e) and 0.4% (f).

develop this technique for androgen determination. Moreover, the enzymatic method allows the direct determination of estrone and estradiol without any separation step. It does not require radioactive material and the results are given in picomole of estrogens without any conversion or calculation (specific activity, yield of the separation step or percent of isotopic distribution).

The aromatase is required for the quantitative conversion of androgens into estrogens. In order to establish the best conditions of aromatization, we have studied the effect of pH, ionic strength and solubilization. The solubilization of the aromatase system using Triton X-100 which is subsequently removed has made it possible to obtain the optimal enzymatic affinity. The persistence of a detergent content higher than or equal to 0.1% in the enzymatic preparation results in the same K_m values as those of the crude preparation.

These results suggest that 3β-HSDH and aromatase systems are closely associated in the crude microsomal preparation. This hypothesis is supported by an aromatase activity with DHEA which is higher than, or equivalent to, that with androstenedione. Thus, the solubilization simplifies the dissociation of the two enzymatic systems as reported

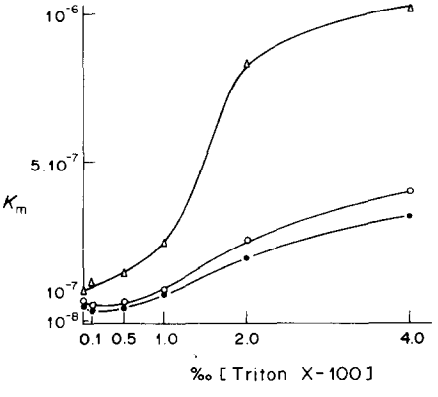

Fig. 8. Relationship between K_m of substrates DHEA (●), androstenedione (○), testosterone (△) and the Triton X-100 concentration.

Table 2. K_m values obtained with solubilized preparations at various concentrations of Triton X-100 for the three steroid substrates

Substrates	Non solubilized microsomes	Solubilized microsomes – different concentrations Triton X-100					
		0%	0.01%	0.05%	0.1%	0.2%	0.4%
DHEA	$1.3 \cdot 10^{-7}$	$5 \cdot 10^{-8}$	$4 \cdot 10^{-8}$	$5 \cdot 10^{-8}$	$8 \cdot 10^{-8}$	$2 \cdot 10^{-7}$	$3.3 \cdot 10^{-7}$
Androstenedione	$2 \cdot 10^{-7}$	$5.8 \cdot 10^{-8}$	$5 \cdot 10^{-8}$	$6 \cdot 10^{-8}$	10^{-7}	$2.5 \cdot 10^{-7}$	$4 \cdot 10^{-7}$
Testosterone	$5 \cdot 10^{-7}$	10^{-7}	$1.25 \cdot 10^{-7}$	$1.6 \cdot 10^{-7}$	$2.5 \cdot 10^{-7}$	$8 \cdot 10^{-7}$	10^{-6}

for other multienzymatic complexes [15]. The observed persistent difference between androstenedione and testosterone lends additional support to the proposition that the former is the favoured substrate for aromatization. However, we have no evidence that the aromatase has two different sites for androstenedione and testosterone aromatization, as suggested by Bellino and Osawa[9].

The results give information on the conditions required for a quantitative conversion of androgens into estrogens and allow the use of a small amount of microsomal preparation.

The assay of various androgens (testosterone or androstenedione plus testosterone) will be described in forthcoming papers.

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