J. steroid Biochem. Vol. 27, No. 4–6, pp. 781–789, 1987
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0022-4731/87 $3.00 + 0.00
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Proceedings of the VII International Congress on Hormonal Steroids (Madrid, Spain, 1986)

AROMATASE

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Summary—Aromatase catalyzes the conversion of androgens to estrogens through a series of monooxygenations to achieve the 19-desmolation and aromatization of the neutral steroid ring-A structure. We have separated two forms of aromatase, a major (P_{2a}) and a minor (P_3) form, from human term placenta through solubilization and chromatography. Partially purified aromatase in each form was immunoaffinity chromatographed to give a single band (SDS–PAGE) cytochrome P-450 of 55 kDa, utilizing a mouse monoclonal anti-human placental aromatase cytochrome P-450 IgG_i (MAb3–2C2) which is capable of suppressing placental aromatase activity. The purified cytochrome P-450 showed specific aromatase activity of 25–30 nmol/min per mg with K_m of 20–30 nM for androstenedione on reconstitution with NADPH-cyt P-450 reductase and dilauroyl L-α-phosphatidylcholine. This one step represents a higher than 100-fold purification with maintenance of the same K_m. The stability analysis showed a half-life of more than 5 yr for solubilized aromatase and 2 months for the aromatase cytochrome P-450 on storage at −90°C. Contrary to the recent claim that estrogen biosynthesis by reconstituted human placental cytochrome P-450 is by *trans*-diaxial 1α,2β-hydrogen elimination, all of our partially purified forms and reconstituted aromatase synthesized estrogens by *cis*-1β,2β-hydrogen elimination. Use of purified aromatase and [19-$^3H_3$, 4-^{14}C]androstenedione led us to discover a metabolic switching by aromatase to 2β-hydroxylation of androgen. Results of the MAb3–2C2 suppression of aromatase activity in different species and tissues including human, baboons, horses, cows, pigs and rats indicated the presence of various isozymes of aromatase.

INTRODUCTION

Biosynthesis of steroid hormones is catalyzed by hydroxylases and desmolases. The terminal stage of hormonal steroid biosynthesis, the conversion of C19- to C18-steroids, involves 19-desmolation. The androgen 19-desmolation is catalyzed by aromatase giving rise to estrogens through 19-hydroxylation/desmolation in the unsaturation mode and also by non-aromatizing 19-desmolase giving 19-norandrogens through 19-hydroxylation/desmolation in the saturation mode [1]. In this presentation we discuss our recent developments on aromatase which include (1) immunoaffinity purified aromatase of human placenta, (2) stereomechanism of aromatization by reconstituted system, (3) isotope effect and metabolic switching of aromatase, and (4) multiple forms and isozymes of aromatase.

IMMUNOAFFINITY PURIFIED AROMATASE OF HUMAN PLACENTA

We have previously described solubilization, separation and purification by stepwise chromatographies of two forms of human placental aromatase, a major P_2- and a minor P_3-form [2]. Solubilization with deoxycholate (0.5%) in glycerol–dithiothreitol–phosphate buffer and chromatography on DEAE-cellulose gives P_2-aromatase with 0.25 M Tris and P_3-aromatase with 0.5 M Tris buffer elution. P_2-Aromatase was further separated to a major P_{2a}- and a minor P_{2b}-form on a DEAE-cellulose column. Each form is obtained as a soluble 2×10^6 Da aggregate from the Bio-Gel A-15-m column without the presence of detergent and as a 135-kDa minimum unit of active enzyme in the presence of detergents such as Emulgen 913 plus sodium cholate or Triton N-101 plus deoxycholate. Following immunization of Balb/c mice with P_2-aromatase cyt P-450, we selected a clone of mouse hybridoma cells producing anti-human placental aromatase cyt P-450 antibodies and prepared a monoclonal antibody IgG_i, MAb3-2C2, which is capable of suppressing human placental aromatase activity [3]. The monoclonal IgG was coupled to CNBr-activated Sepharose (2 mG IgG/ml resin) and used for immunoaffinity purification of aromatase cyt P-450.

The 135-kDa aromatase preparation was further purified in the presence of androstenedione (2 μM). The major aromatase fraction was eluted from a hydroxylapatite column with 50 mM phosphate buffer containing 0.15% Emulgen 913. This was applied first to normal mouse IgG coupled Sepharose to remove some non-specific binding proteins, and then applied to the immunoaffinity resin. Non-aromatase cytochromes P-450 and NADPH-cyt P-450 reductase were washed away. The bound cyt P-450 was eluted stepwise with 0.5, 1, 2, and 3 M NaCl in phosphate buffer (pH 7.4) and glycine buffer (pH 2.8). The protein components analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) are given in Figs. 1(a) and (b). Contaminating proteins of similar sizes are con-

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tained in the MAb3-2C2 pass-through fraction (Lane 6, Fig. 1(a) and Lane 5, Fig. 1(b)) and this fraction shows a strong non-aromatase cyt P-450 spectrum. The initial "55-kDa" band was found to be a mixture of closely moving bands (55 and 56 kDa) overlapping each other as the results of this immunoaffinity separation. Aromatase active cyt P-450 is at the lower side of the mixture band and was chromatographed to apparent homogeneity on silver stained SDS-PAGE at 55 kDa as shown in Fig. 1(a), Lane 7 and 1(b), Lanes 9–12. The elution with 1, 2, and 3 M NaCl and glycine buffer gave the same protein band but showed varied heme content and aromatase activity. Both the aromatase specific activity and heme content increased with NaCl concentration with parallelism, which supports the concept that aromatization is indeed catalyzed by a cytochrome P-450. The acidic glycine buffer apparently elutes the same protein but loses its cyt P-450 and enzyme activity. The aromatase cyt P-450 in the 3 M NaCl eluant shows specific activity of 25–30 nmol estrone/min per mg on reconstitution with NADPH-cyt P-450 reductase and dilauroyl L-α-phosphatidylcholine.

Kinetic analysis of a pair of reconstituted P_{2a}- and P_3-aromatase is given in Figs. 2(a) and (b). The K_m obtained for P_{2a} and P_3 was 28 and 17 nM, respectively. These results show that the monoclonal antibody based immunoaffinity chromatography purified human placental aromatase cyt P-450 in one step without changing its K_m for the substrate to an apparent homogeneity judged by SDS-PAGE silver staining with a higher than 100-fold increase of the aromatase specific activity. They also suggest that even aromatase of extremely low content found in other tissues and species may be applicable to the immunoaffinity purification.

The instability of aromatase activity and

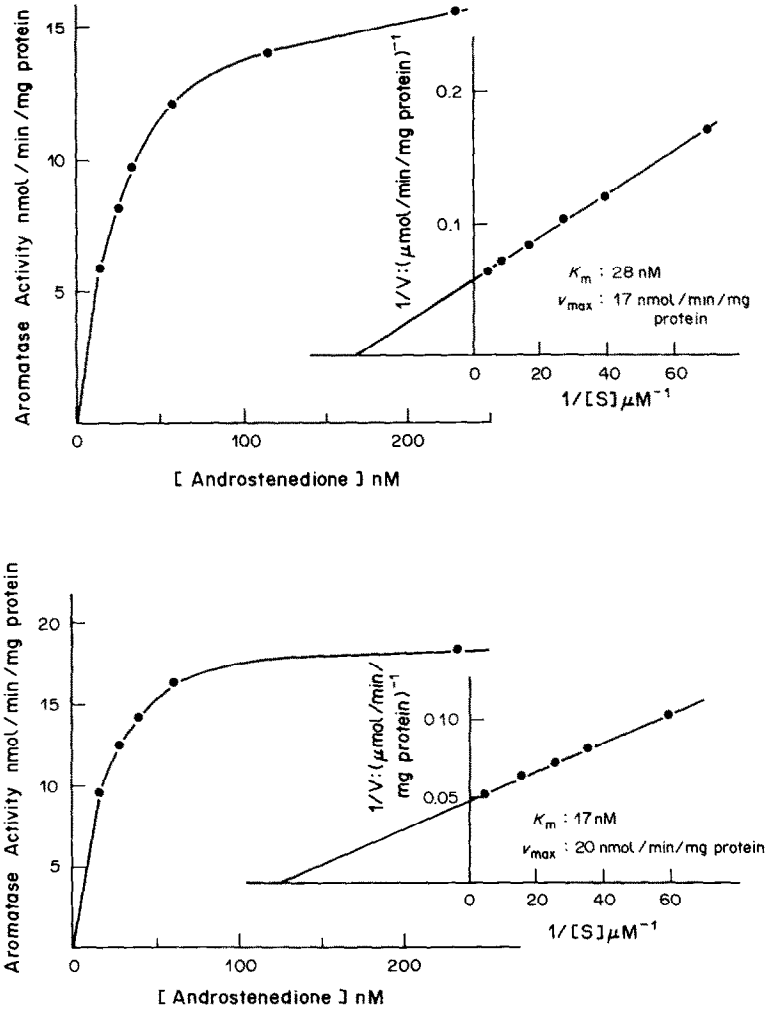

Fig. 2. Kinetic characterization of immunoaffinity purified reconstituted aromatase (a) P_2-aromatase, P_2 HA50 MAb3-2C2 3 M NaCl, (b) P_3-aromatase, P_3 HA50 MAb3-2C2 3 M NaCl. Enzyme activity was assayed by the [^{3}H]water method.

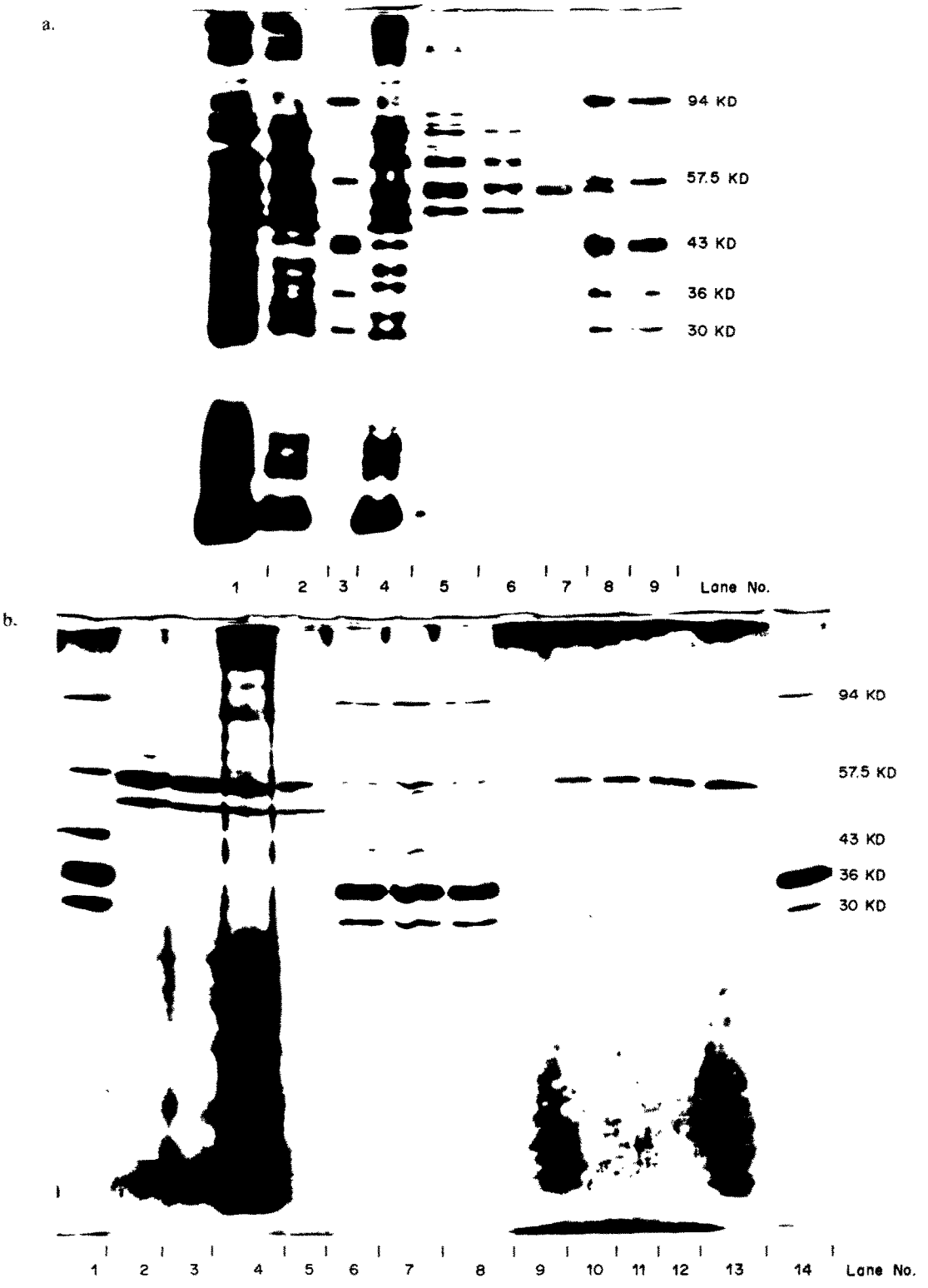

Fig. 1. SDS–PAGE of P_{2a} aromatase purified by immunoaffinity chromatography. Laemmli's procedure was used in a 10% separating gel and 3% stacking gel. (a) Lane 1—solubilized partially purified P_{2a}, lane 2—P_{2a} 15 m, lane 3—standard proteins, lane 4—P_{2a} 135 kDa G-25, lane 5—P_{2a} hydroxylapatite 50 mM fraction (P_{2a} HA50), lane 6—P_{2a} HA50 MAb pass, lane 7—P_{2a} HA50 MAb 3.0 M NaCl, lane 8—standard protein mixed with P_{2a} HA50 MAb 3.0 M NaCl, lane 9—standard protein. (b) Lane 1—standard proteins, lane 2—P_{2a} hydroxylapatite 50 mM fraction (P_{2a} HA50), lane 3—P_{2a} HA50 NM IgG pass, lane 4—P_{2a} HA50 NM IgG 0.2 M glycine, lane 5—P_{2a} HA50 MAb pass, lane 6—standard proteins, lane 7—standard protein mixed with P_{2a} HA50 MAb 3.0 M NaCl, lane 8—standard proteins, lane 9—P_{2a} HA50 MAb 0.5 M NaCl, lane 10—P_{2a} HA50 MAb 1.0 M NaCl, lane 11—P_{2a} HA50 MAb 2.0 M NaCl, lane 12—P_{2a} HA50 MAb 3.0 M NaCl, lane 13—P_{2a} HA50 MAb 0.2 M glycine, lane 14—standard proteins.

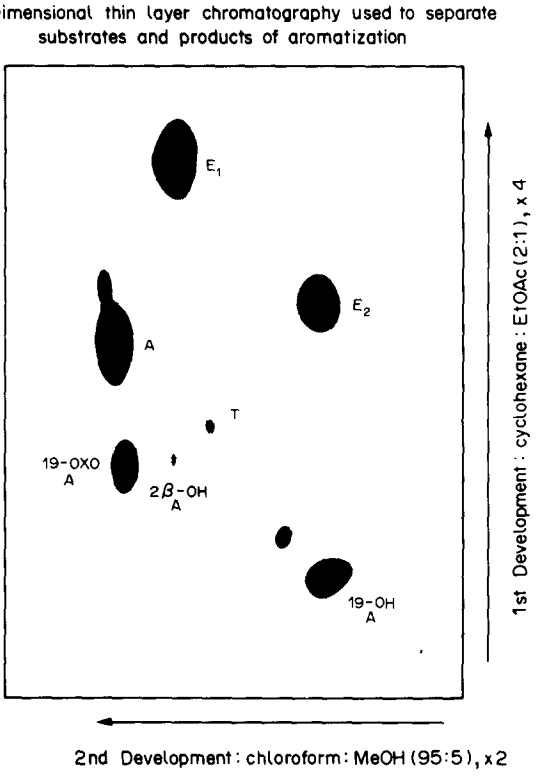

Fig. 5. Metabolites of androstenedione. Two-dimensional TLC pattern following exposure to radioautography of the metabolites of androstenedione by P_{2a}-aromatase.

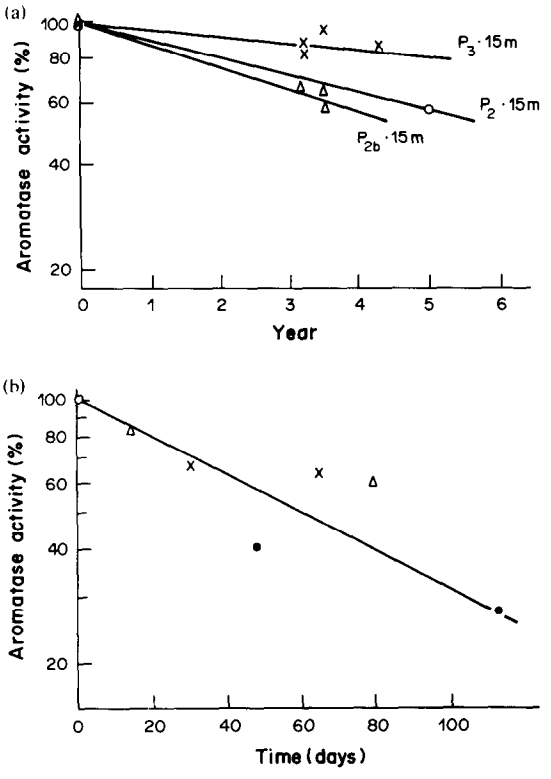

Fig. 3. (a) Stability of solubilized human-placental aromatase. Solubilized aromatase was separated by DE-52 column chromatography and stored at −90°C in glycerol (20%)–dithiothreitol (0.5 mM)–phosphate (100 mM, pH 7.4) (GPD buffer). Activity was assayed by the [^{3}H]water method. (b) Stability of immunoaffinity purified aromatase. Purified P_{2a}-aromatase cyt P-450 was stored at −90°C in GPD-Em 913 buffer and assayed upon reconstitution with P_{2a} NADPH-cyt P-450 reductase and dilauroyl phosphatidylcholine.

the heme of aromatase cyt P-450 has been previously noted. Nakajin *et al.* recently reported [4] that an aromatase cyt P-450 preparation showed a very high K_m of 30 μM for androstenedione and an activity half-life of only 2.5 days even when stored at −80°C. We have stored our partially purified aromatase and purified aromatase cyt P-450 in glycerol (20%)–dithiothreitol (0.5 mM)–phosphate (100 mM, pH 7.4) at −90°C and measured the stability. The results are shown in Figs 3(a) and (b). Solubilized and partially purified aromatase preparations showed a half-life of 5 yr or longer and P_{2a}-aromatase cyt P-450 showed a half-life of 2 months.

STEREOMECHANISM OF AROMATIZATION BY RECONSTITUTED SYSTEM

The stereospecific 1β,2β-hydrogen elimination in placental androgen aromatization has been established using crude preparations of human term placentas [5–9]. A stereospecific 1α,2β-*trans* diaxial elimination mechanism was postulated for human ovarian aromatization [10, 11]. However, this was later refuted and determined to be the same stereospecific 1β,2β-hydrogen elimination as for the placenta by use of stereoselectively labeled [1α,2α-$^3H_2$, 4-^{14}C]testosterone as the substrate [12]. Recently, Muto and Tan [13, 14] reported that human placental aromatase cyt P-450, when purified and reconstituted with NADPH-cyt P-450 reductase and phospholipid, aromatized [1β,2β-$^3H_2$]androstenedione by the 1α,2β-*trans* diaxial hydrogen elimination mechanism. The reconstituted aromatase was reported to have a K_m of 240 nM for androstendione and a specific cyt P-450 content of 4.1–4.2 nmol/mg. The stereomechanism was deduced from the measurement of [^{3}H]water released rather than [^{3}H]estrogen formed.

Contrary to these reports, we have never observed a switch or change of the stereomechanism of the 1,2-hydrogen elimination through the stages of purification or reconstitution. We had no reason to suspect a switching of the stereomechanism but since we extensively use the [^{3}H]water method for the aromatase assay based on the 1β-^{3}H elimination, it was important to determine if the extent of [^{3}H]-removal from 1β is constant among aromatase preparations. One series of results obtained by incubation of [1β-^{3}H,4-^{14}C]substrates (75% at 1β and 25% at 1α) and the determination of the change of ^{3}H/^{14}C ratio in the isolated [^{3}H, ^{14}C]estrogens are given in Fig. 4. The ^{3}H-distribution was as previously determined [9] and estrogens were isolated by two-dimensional TLC on silica gel. The theoretical ^{3}H% in the estrogen is 25% of the androgen, if it is by the stereospecific 1β-elimination. Our observed retention was 21–24% through the crude, partially purified, and reconstituted aromatase preparations. Similarly, when [1α,2α-$^3H_2$, 4-^{14}C]substrates (87% at 1α + 2α) [9] were incubated, estrogens retained 81–86% of the initial ^{3}H and [1β,2β-$^3H_2$, 4-^{14}C]androstenedione gave estrogens retaining 20–26% of the initial ^{3}H (data not shown). These results show that solubilized and partially purified aroma-

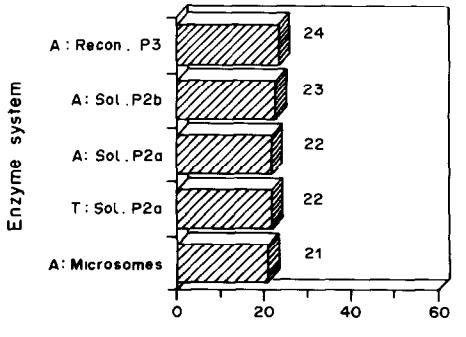

Fig. 4. Stereomechanism of 1,2-hydrogen elimination. Incubation of [1β-^{3}H, 4-^{14}C]substrate (75% at 1β and 25% at 1α) with various forms of aromatase. The graph shows the percentage of the original tritium label retained in the isolated [^{3}H, ^{14}C]estrogens.

tase as well as reconstituted aromatase produce estrogens by the stereospecific 1β,2β-hydrogen elimination mechanism and that the [^{3}H]water assay based on the 1β-^{3}H elimination is valid.

ISOTOPE EFFECT AND METABOLIC SWITCHING OF AROMATASE

The progress in aromatase purification and use of [19-$^3H_3$, 4-^{14}C]androgens have led us to find the metabolic switching of aromatase to androgen 2β-hydroxylation due to the isotope effect caused by the 19-tritium substitution. Although androgen 2β-hydroxylation by human placenta has been suspected, it has never been established. The activity is far lower than other metabolic activities and the products have escaped positive identification. When we synthesized isotope labeled 2β-hydroxytestosterone we found that it was hardly aromatized by human placental microsomes [15], and therefore we considered the pathway to be insignificant. The mechanism of aromatization proposed by Fishman's group [16–18] includes the 2β-hydroxylation of 19-oxoandrogen as the third step but claims specifically that the 2β-hydroxylation occurs only to the 19-aldehyde and not to the 19-alcohol or the initial 19-methyl substrate.

In contrast to the numerous metabolites detectable in the placental microsomal incubate, partially purified and reconstituted aromatase preparations give much cleaner and fewer metabolites thus making identification easier. A two-dimensional thin layer chromatogram pattern of androstenedione metabolites by P_{2a}-aromatase is shown in Fig. 5. When we incubated [19-$^3H_3$, 4-^{14}C]androstenedione with purified aromatase preparations and positively identified 2β-hydroxyandrostenedione by use of authentic standard, we found that the ^{3}H/^{14}C ratio of the product is 4–28 times higher than that of the substrate. The results are given in Figs 6 and 7. The formation of [19-$^3H_3$]-2β-hydroxyandrostenedione reached 8–10% of the initial [19-$^3H_3$]androstenedione, whereas that of [4-^{14}C]-2β-hydroxyandrostenedione remained less than 0.5%. This tremendous kinetic isotope effect cannot be explained by the usual inverse secondary isotope effect (1.3 or less). Since aromatase has a large kinetic isotope effect (3.2) on the first 19-hydroxylation [19] the tritium-labeled substrate is selectively backed-up and thus would be more subjected to 2β-hydroxylation by metabolic switching. The fact that the effective van der Waal's radius is smaller for the [$^3H_3$]methyl than the [$^1H_3$]methyl group may facilitate 2β-hydroxylation through a relief of 1,3-diaxial steric hindrance. Supporting that the metabolic switching occurs at the active-site of aromatase, MAb3-2C2, monoclonal antibody to aromatase cyt P-450, was found to equally suppress the formation of 2β-hydroxyandrostenedione, 19-hydroxyandrostenedione, 19 - oxoandrostenedione and estrogen. These results also suggest that the

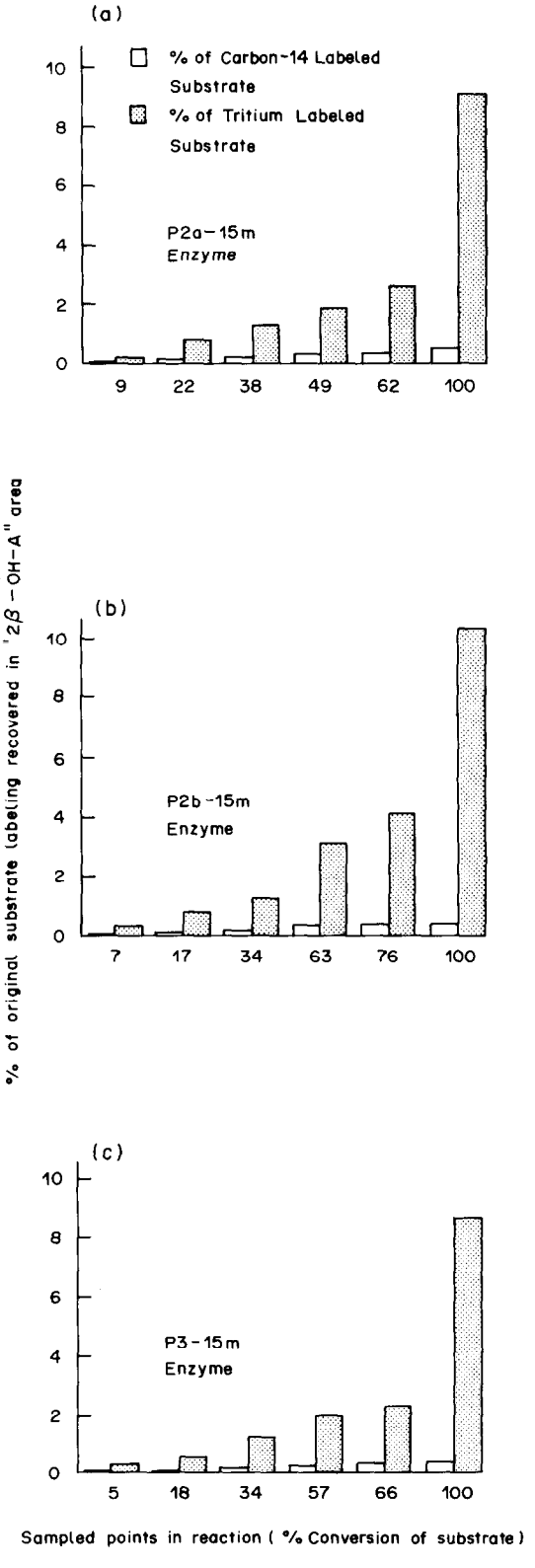

Fig. 6. Radiometric analysis of 2β-hydroxyandrostenedione by TLC after incubation of [19-$^3H_3$, 4-^{14}C]-androstenedione with purified aromatase preparations. The variation was observed as a function of percentage of conversion of substrate to products showing apparent kinetic isotope effect of 2β-hydroxylation.

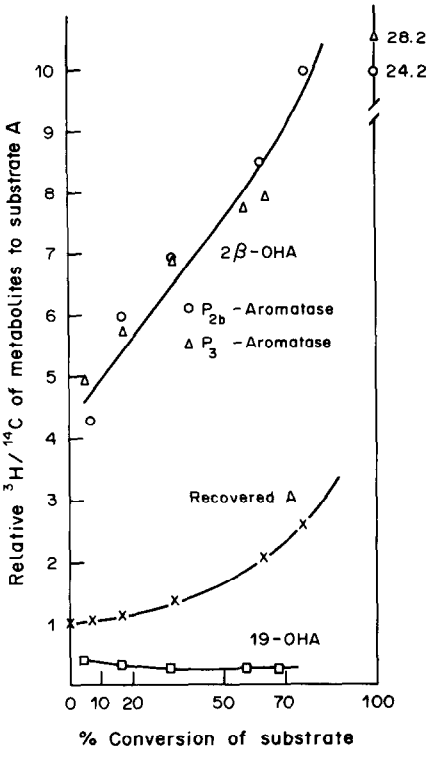

Fig. 7. Time-course measurement of $^3H/^{14}C$ ratio of 2β-hydroxyandrostenedione during incubation of [19-$^3H_3$, 4-^{14}C]androstenedione with purified aromatase preparations. The $^3H/^{14}C$ ratio of the isolated [19-$^3H_3$, 4-^{14}C]-2β-hydroxyandrostenedione is 4–28 times higher than that of the initial androstenedione.

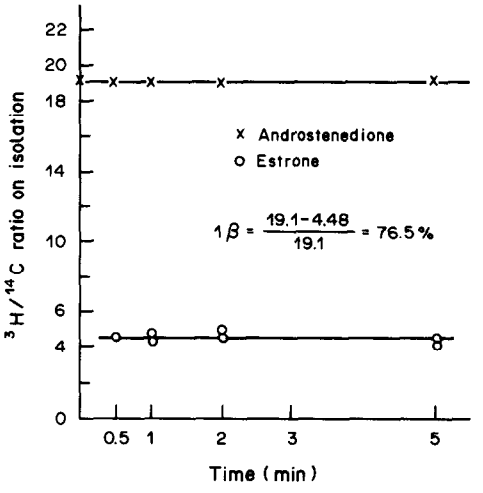

Fig. 8. Time-course measurement of $^3H/^{14}C$ ratio in aromatization of [1-^3H, 4-^{14}C]androstenedione with human placental microsomes. The $^3H/^{14}C$ ratio of the recovered substrate and product (E_1) were found to be constant throughout the time course, using the product isolation method.

heme bound oxygen of the active-site situates at a place close to both the 19-methyl and 2β-hydrogen.

The [^3H]water method for the aromatase assay gained popularity because it is simple and rapid. [19-$^3H_3$]Androgen releases 2 mol of [^3H]water and 1 mol of [^3H]formic acid on aromatization and thus could be considered a good substrate for the assay. The results shown in Figs. 6 and 7, however, indicate that the tritium labels do not represent well the behavior of [^{14}C]androgen and thus the natural substrate. A large portion of the tritium becomes unavailable for aromatization and the $^3H/^{14}C$ ratio of the remaining substrate changes non-linearly during aromatization. In contrast, [1-^3H]androgen, which gives a constant ratio of $^3H/^{14}C$ for estrogen and an unchanged ratio of the substrate on aromatization, as shown in Fig. 8, is an appropriate substrate when one corrects for the β% (75%).

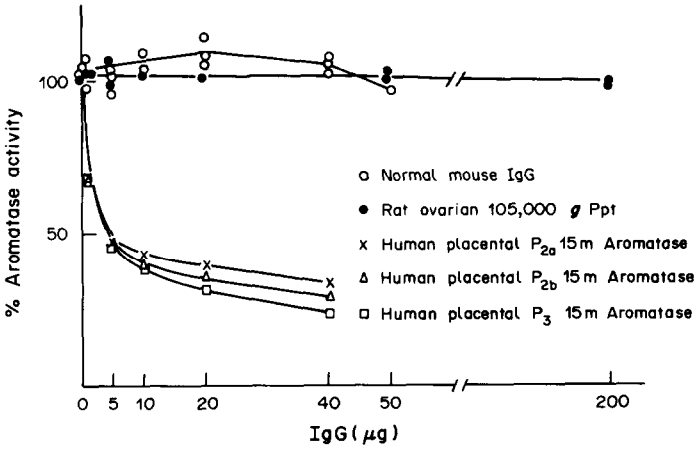

Fig. 9. Effect of monoclonal antibody (MAb3-2C2) on human placental and rat ovarian aromatases. Suppression of the aromatase activity was measured by the [^3H]water method. The effective suppression of P_{2a} 15 m (×, 28 μg protein), P_{2b} 15 m (Δ, 36 μg protein) and P_3 15 m (□, 78 μg protein) by the MAb3–2C2 was contrasted to the non-suppression of rat ovarian 105,000 g ppt (●, 670 μg protein) aromatase.

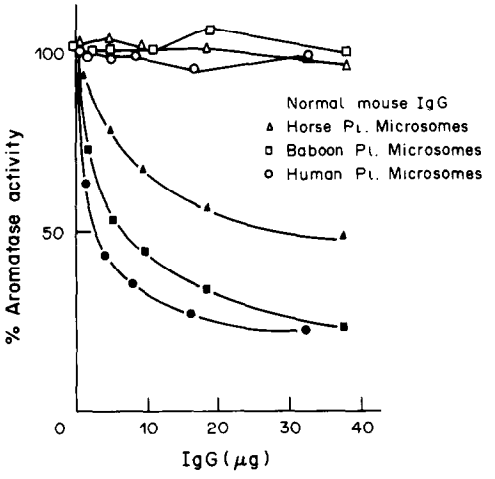

Fig. 10. Effect of MAb3-2C2 on placental aromatase of different species. Suppression by the monoclonal antibody was shown by incubations with horse (▲, 145 μg protein), baboon (■, 296 μg protein) and human (●, 5 μg protein) placental microsomes.

MULTIPLE FORMS AND ISOZYMES OF AROMATASE

Although the multiplicity and isozymes of hepatic cyt P-450 are well known, there has been no definitive evidence for isozymes of aromatase cyt P-450. Since a monoclonal antibody is made against a single antigenic structure the specificity of the interaction is expected to be very high. We have used MAb3-2C2, a mouse monoclonal antibody to human placental P_2-aromatase cyt P-450, to evaluate aromatase preparations of various species and tissues by its suppressibility of aromatase activity. MAb3-2C2 equally suppressed P_{2a}, P_{2b} and P_3 forms of human placental aromatase but did not affect the rat ovarian aromatase activity at all, as shown in Fig. 9. When placentas from horse, baboon and human were compared, the suppression curve of the baboon

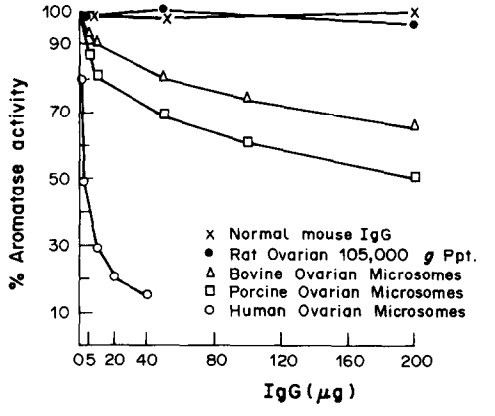

Fig. 11. Species specificity of ovarian aromatase activity. Suppression of aromatase activity by the monoclonal antibody in incubations of bovine (△, 3.4 mg protein), porcine (□, 5.0 mg protein) and human (○, 0.2 mg protein) ovarian microsomes was contrasted to the non-suppression of rat ovarian 105,000 *g* ppt (●, 0.67 mg protein).

placental microsomes was much closer to that of human than was that of horse placental microsomes, as shown in Fig. 10. In order to ascertain if the inability of MAb3-2C2 to suppress rat ovarian aromatase is due to a tissue specificity or a species difference, ovarian microsomal aromatases from rat, cow, pig and human were compared. The results are given in Fig. 11. The human ovarian aromatase activity was as equally suppressed as the placental activity. Porcine and bovine ovarian aromatase responded with an intermediate degree between the human and the rat. The results indicate the presence of aromatase isozymes among the different species. Tissue specificity requires more extensive evaluation. It is suggested that monoclonal antibodies would be useful for identification of the aromatase isozymes.

Acknowledgements—This work was supported by Grants HD-04945, AM07368 and RR-05716 from the National Institutes of Health of the United States Public Health Service and by Sidney H. Stilling Fellowship Fund. The authors gratefully acknowledge Dr N. Washida and Mrs G. Koscinski for preparation of the monoclonal antibody and Drs B. Tochigi and M. Tochigi for the study of human ovarian microsomes.

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