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NOVEL METHOD OF EVALUATING BIOLOGICAL 19-HYDROXYLATION AND AROMATIZATION OF ANDROGENS

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[19C^{3}H]Androstenedione of high specific activity has been prepared. In liver incubation the isotope was shown to be stable to biological processes other than 19-hydroxylation. Incubation of the new substrate with human placental microsomes yielded ^3H_2O, ^3HCOOH and estrogens devoid of radioactivity. The formation of ^3H_2O and ^3HCOOH was close to the expected 2:1 ratio indicating that the material can be used to discriminate between 19-hydroxylation which yields ^3H_2O and aromatization which results in ^3HCOOH. Comparison of the formation of ^3H_2O from [1β,2β^{3}H]androstenedione and of ^3HCOOH from [19C$^3H_3$]androstenedione in placental microsomal incubation showed that the aromatization of the former was 3.2 times faster indicating an isotope effect of that magnitude for the aromatization of [19C^{3}H] vs [19CH_3]androstenediones. The new substrate will be an effective probe and discriminant of both 19-hydroxylation and aromatization of androgens *in vivo* and *in vitro*, reactions which have been reported to be dissociated in specific tissues.

The biosynthesis of the C-18 estrogens proceeds by the aromatization of C-19 androgens and requires the elimination of the C-19 methyl group and one hydrogen each from C-1 and C-2. The process involves three sequential enzymatic hydroxylations the first two of which take place at the C-19 methyl group to generate an aldehyde function, while the third appears to be at the 2β position leading to an unstable intermediate which collapses rapidly and nonenzymatically to estrogen (1). In the course of the transformation of androgens to estrogens the 1β and 2β hydrogens are stereospecifically transferred to water (2, 3) while the C-19 fragment is eliminated as formic acid (4). The aromatization process is currently assessed by two different analytical procedures. One method involves the use of androgen precursors isotopically labeled in biologically stable positions and depends on the isolation and quantitation of the labeled estrogen products (5). The other procedure relies on 1β^{3}H-labeled androgen substrates with the formation of the ^{3}H water serving as a measure of the aromatization process (6, 7). Since the 1β

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or 2β hydrogens are transferred into water only in the final steps of the transformation both of these methods monitor only the last stage of the aromatization sequence and provide no information on the preceding hydroxylations at the C-19 position. The rapid and sensitive radiometric method also has to contend with oxidative reactions at the C-1 and C-2 sites which are unrelated to aromatization (8) and which can lead to spurious results particularly in in vivo studies or in in vitro experiments with enzymatically active tissues such as the liver. A substrate which would provide a measure of both 19-hydroxylation and aromatization and would exhibit greater radiometric specificity would be a [19C^{3}H] androgen. Tritium transfered from this substance into water would reflect hydroxylation at C-19 while the appearance of ^{3}HCOOH would be a measure of aromatization (4). To be useful such a substrate must have high specific activity to allow the measurement of aromatization in tissues in which it is a low yield but physiologically important process such as the brain (9) or adipose tissue (10). The stereospecificity of the C-19 hydroxylation (11) and the intervention of isotope effects (12) also requires that the tritium labeling of the 19-methyl group be homogenous. In this communication we report on the preparation of such a substrate and demonstrate its utility in the evaluation of the sequential enzymatic processes participating in aromatization.

MATERIALS AND METHODS

[19^{3}H$_3$]Testosterone (60 Ci/mmole) was prepared from estrone employing a modification of a described synthetic pathway (13). The evenly labeled methyl group was introduced by means of a Grignard reaction between [C^{3}H$_3$]methylmagnesium bromide and 17β-acetoxy-5α,10α-epoxyestrane-3-cycloethylene ketal. The product of the reaction, [19C^{3}H$_3$]17β-acetoxy-5α-hydroxyandrostane-3-cycloethylene ketal, was further processed (13) without isolation to give the labeled testosterone, which by reverse isotope dilution and HPLC employing a flow-cell type radiation detector (Packard Trace 7140) was found to be of greater than 90% radiohomogeneity. This material was converted to [19C^{3}H$_3$]androstenedione in good yield by oxidation with pyridinium dichromate (14). The radiochemical purity of the purified [19C^{3}H$_3$]androstenedione product as determined by reverse isotope dilution was better than 97%.

The [19C^{3}H]androstenedione, 60 Ci/mmole, diluted to 4 nM concentration with inert androstenedione in 50 µl of ethanol was incubated with 5 ml of human placental microsomes (1 mg/ml of 0.05 M Tris HCl (pH 7.2)) in the presence 5 mg of NADPH, 1 mg of glucose-6-phosphate and 3.1 units of glucose-6-phosphate dehydrogenase. The incubations were carried out at 37°C in air for the specified time.

For assay purposes 1 ml of the incubation mixture was mixed with an equal volume of 1 M phosphoric acid, flash frozen and lyophilized. To the distilled fraction (1.2 ml), which was a mixture of formic acid and water, 0.3 ml of 1N sodium hydroxide was added and the mixture was then flash frozen and lyophilized; 0.5 ml aliquots were then counted. The amount of ^{3}H water and ^{3}H formic acid in 0.5 ml of the original distillate was calculated.

To ensure that the isotope was present only at the C-19 position a mixture of [19C$^{3}H_3$] and [4-^{14}C]androstenedione was incubated as described above. The incubation mixture (3.5 ml) was diluted with saturated sodium chloride (3.5 ml), and extracted 3 times with chloroform containing estrone (30.5 mg) and estradiol (30.4 mg). The organic extract was dried over anhydrous sodium sulfate, and evaporated. The residue was submitted to preparative TLC using n-hexane-ethyl acetate (1:1) as a developing solvent and the estrone and estradiol were isolated and acetylated. Following purification of the acetates by preparative TLC using n-hexane-ethyl acetate (2:1) they were successively recrystallized from methanol. Aliquots of each crystallization were counted to determine the $^{3}H/^{14}C$ ratios.

To evaluate the biological stability of the label to processes other than aromatization [19C^{3}H]androstenedione was incubated with rat liver homogenate. For comparison [1β,2β-^{3}H]androstenedione was similarly incubated with the liver tissue and the $^{3}H_2O$ formed was lyophilized. The [1,2-^{3}H]- and [19C$^{3}H_3$]androstenediones in 40 µl of ethanol were incubated separately with 2.4 ml of liver homogenate (10 mg/ml of 0.05 M Tris HCl pH 7.4) in the presence 3 mg of NADPH, 5 mg of glucose-6-phosphate and 5 units of glucose-6-phosphate dehydrogenase. The incubations were carried out at 37°C in air for 1 hr. Subsequent assay procedures were the same as in the placental incubation for the [19C^{3}H]androstenedione. In the case of [1,2-^{3}H]androstenedione, 1 ml of the incubation mixture was mixed with equal volume of 1% charcoal suspension, alllowed to stand at 0°C for 15 min, and centrifuged. The supernatant was flash frozen and lyophilized and 0.5 ml of the distilled water was counted.

RESULTS

The $^{3}H/^{14}C$ ratios in the estrone and estradiol isolated from the incubation of a mixture of [19C$^{3}H_3$]- and [^{14}C]androstenediones with placental microsomes. The $^{3}H/^{14}C$ were found to be 0.005 for estrone and 0.004 for estradiol indicating that a maximum of 0.5% of the ^{3}H label in [19C$^{3}H_3$]androstenedione was located at positions other than C-19. The results of the rat liver incubations reveal also that less than 0.05% of the ^{3}H was released from the [19CH$_3$]androstenedione substrate while over 4% of the ^{3}H in the [1,2-^{3}H]androstenedione was transferred into $^{3}H_2O$.

The time course of tritium release in the form of $^{3}H_2O$ or $^{3}HCOOH$ from a 4 nM incubation of [19C$^{3}H_3$]androstenedione with placental microsomes is shown in Fig. 1. A simultaneous incubation with [1,2-^{3}H]androstenedione (1β 44%, 2β 32%, 1α 12%, 2α 12%) at the same 4 nM substrate concentration and assayed by $^{3}H_2O$ formation is also presented in Fig. 1. The values of $^{3}H_2O$ formed have

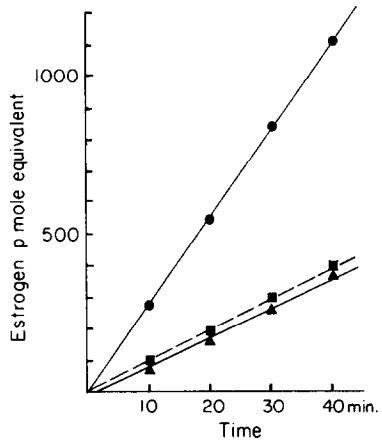

Fig. 1. Time course of aromatization as measured by radiometric procedures. ▲ $^{3}HCOOH$ and ■ $^{3}H_2O$ formation from 4 nM [19C^{3}H]-androstenedione. ● $^{3}H_2O$ formation from 4 nM [1,2^{3}H]androstenedione.

been normalized for isotope content and stereochemistry in the substrates to allow expression of the results in terms of mole estrogen equivalents. The formation of $^{3}H_2O$ from [19C$^{3}H_3$]androstenedione was linear for the 40 min studied. Formation of $^{3}HCOOH$ was also linear and was essentially identical with that of $^{3}H_2O$ in mole equivalents. The $^{3}H_2O$ and $^{3}HCOOH$ formed at the early times of the incubation expressed in dpm rather than mole equivalents are shown in Table 1. The ratio of $^{3}H_2O$ to $^{3}HCOOH$ decreased rapidly from 8.2 at 1 min to 2.4 at 15 min, remaining relatively constant thereafter. When the incubation was carried out at subsaturating substrate concentrations the ratio of $^{3}H_2O$ to $^{3}HCOOH$ formed was 2 or lower.

Table 1

Ratio of $^{3}H_2O$ and $^{3}HCOOH$ formed in the incubation of [19C^{3}H]androstenedione with placental microsomes

Incubation period (min)	$^{3}H_2O/^{3}HCOOH$
1	8.2
2	3.6
4	2.7
8	2.7
15	2.4
25	2.4
35	2.3
40	2.2

[19C^{3}H]androstenedione (4 nM) was incubated with placental microsomes. Aliquots were removed at specified times and analyzed for $^{3}H_2O$ and $^{3}HCOOH$ content.

The formation of $^{3}H_2O$ from [1,2^{3}H]androstendione was also linear during the time course of the study but in terms of mole equivalents it was at all times much greater than that of either $^{3}H_2O$ or ^{3}HCOOH derived from [19C^{3}H]androstenedione with the average ratio being 3.2. This value is precisely matched by the results of the double label study where the yield of ^{4}C labeled estrogens from [^{14}C]androstenedione exceeds ^{3}HCOOH formation from [19C^{3}H]androstenedione by the same factor of 3.2.

DISCUSSION

With the exception of specific tissues, such as the placenta, the detection and measurement of estrogen formation from androgen precursors is complicated by the multistep nature of the process and the low yields of the reaction. The isolation and quantitation of estrogen products from stably labeled precursors is tedious and time consuming and high specific activity ^{3}H labeled substrates must be used in tissues with low enzymatic activity such as the brain. The radiometric method employing [1,2^{3}H] androgen precursors is experimentally superior but suffers from nonspecific contributions and requires precise knowledge of the stereochemical distribution of the isotope. More importantly, both procedures monitor only the final step of estrogen biosynthesis and provide no information about the initial 19-hydroxylative sequences. The recent report of the *in vivo* formation of 19-hydroxy androgens (15) and their unusual pharmacological properties (16) emphasizes the importance of assessing both those 19-hydroxylations which do and those which do not proceed to further aromatization. The preparation of the high specific activity [19C^{3}H]androstenedione provides a material which can be used not only to discriminate between the initial 19-hydroxylations and the final aromatization but which also can serve as a superior substrate for the radiometric measurement of estrogen biosynthesis. In rat liver incubations, an enzymatically active tissue which however is deficient in aromatization activity, only minimal quantities of ^{3}H were transfered to the medium indicating the resilience of the label at the C-19 methyl site to reactions other than 19-hydroxylation, a significant advantage over the alternate radiometric sub-

strate labeled at C-1,2 which released more than 80 times as much ^{3}H under the same conditions. This biological stability to nonspecific reactions makes the [19C^{3}H]androstenedione a particularly suitable substrate for in vivo radiometric measurement of aromatization.

With placental microsomes [19C$^{3}H_3$]androstenedione yields $^{3}H_2O$ and ^{3}HCOOH close to the expected 2 to 1 ratio except in the early stages of the reaction when $^{3}H_2O$ predominates. The latter provides evidence of the sequential nature of the aromatization process with the 19-hydroxylations which yield $^{3}H_2O$ preceding the third hydroxylation at 2β which results in aromatization and the expulsion of ^{3}HCOOH (1). The small excess of $^{3}H_2O$ over ^{3}HCOOH during the entire time course of the incubation can be interpreted as showing that the 19-hydroxylation is a more rapid reaction than the terminal hydroxylation step, a conclusion already previously supported by the isolation of the 19-hydroxy intermediates in studies with stably labeled substrates (17, 18).

The transfer of ^{3}H from [1,2^{3}H]androstenedione to water and the formation of ^{3}HCOOH from [19C$^{3}H_3$]androstenedione both represent the final step in aromatization and can therefore be used as indices of estrogen biosynthesis from these substrates. Comparison of the kinetics of [1,2^{3}H]- and [19C^{3}H]androstenedione aromatization by placental microsomes reveals that the former is approximately 3.2 times faster than the latter. Since the loss of the [1,2^{3}H] in aromatization is not accompanied by an isotope effect (3, 7) this rate difference must reflect the different rates of aromatization of [19C^{3}H]- vs [19CH_3]androstenedione. Based on the reported 3.2 isotope effect for the corresponding deuterium labeled material (12) the ^{3}H isotope effect observed by us is lower than expected. Methodological differences in obtaining the deuterium and tritium isotope effect values may be responsible for this discrepancy. The isotope effect must be taken into consideration when computing aromatization from the $^{3}H_2O$ on ^{3}HCOOH formed from this substrate. Under saturating maximum velocity conditions which are almost universally encountered in studies of aromatization the 3.2 isotope factor would provide the

appropriate correction. The existence of the isotope effect can be an advantage since it can be a valuable probe of the enzymatic mechanisms of 19-hydroxylation of androgens.

The use of the [19C^{3}H]androstenedione reveals that in placental microsomes there appears to be little 19-hydroxylation of androgens which does not result in aromatization. It will be important to employ this method to examine whether this applies also to other aromatization sites, and such studies are now in progress.

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