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ENHANCEMENT OF  $3\beta$ -HYDROXYSTEROID  
DEHYDROGENASE-ISOMERASE IN THE HUMAN FETAL ADRENAL  
BY REMOVAL OF THE SOLUBLE CELL FRACTION

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SUMMARY

To assess whether there is some factor in the soluble cell fraction which might result in inhibition of the  $3\beta$ -hydroxysteroid dehydrogenase-isomerase enzyme system in the human fetal adrenal, separate homogenate and microsomal fractions of fetal adrenal tissue were incubated with isotopic pregnenolone and dehydroepiandrosterone. The amount of the  $\Delta^4$ -3-ketosteroid, androstenedione, found in the microsomal preparation was 22–27 times higher than that in the homogenate. No labeled  $C_{21}$ - $\Delta^4$ -3-ketosteroids were found.

The data are interpreted as suggesting that inhibition of the formation of  $\Delta^4$ -3-ketosteroids occurred in the homogenate, even though this enzyme capacity is present. The exact nature of this inhibition was not demonstrated. The data further imply a deficiency of a pregnenolone-specific  $3\beta$ -hydroxysteroid dehydrogenase-isomerase system and suggest substrate specificity for this enzyme function.

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INTRODUCTION

A number of studies have demonstrated the importance of the human fetal adrenal in steroid metabolism in pregnancy. Data from various *in vitro*<sup>1–13</sup> and *in vivo*<sup>14–18</sup> experiments have led to differing results regarding the ability of the fetal adrenal to convert  $\Delta^5$ - $3\beta$ -hydroxysteroids to their  $\Delta^4$ -3-ketosteroid analogues. Since the presence of  $3\beta$ -hydroxysteroid dehydrogenase-isomerase has been demonstrated in some studies, although its activity appears to be low, several inhibitory or regulatory mechanisms have been suggested. These have included inhibition of  $3\beta$ -hydroxysteroid dehydrogenase-isomerase by progesterone<sup>10</sup> and by  $17\beta$ -estradiol<sup>6</sup>.

The following trivial names and abbreviations have been used: pregnenolone,  $3\beta$ -hydroxy-pregn-5-en-20-one; pregnenolone sulfate, 20-oxo-5-pregnen- $3\beta$ -yl-sulfate; dehydroepiandrosterone,  $3\beta$ -hydroxy-5-androsten-17-one; dehydroepiandrosterone sulfate, 17-oxo-5-androsten- $3\beta$ -yl sulfate; progesterone, 4-pregnene-3,20-dione;  $17\alpha$ -hydroxyprogesterone,  $17\alpha$ -hydroxy-4-pregnene-3,20-dione;  $16\alpha$ -hydroxyprogesterone,  $16\alpha$ -hydroxy-4-pregnene-3,20-dione; androstenedione, 4-androstene-3,17-dione;  $11\beta$ -hydroxyandrostenedione,  $11\beta$ -hydroxy-4-androstene-3,17-dione; deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; hydrocortisone,  $17\alpha$ -21-hydroxy-4-pregnene-3,20-dione.

In addition, steroid sulfokinase activity in human fetal adrenals has been found and located in the 105000  $\times$  g subcellular fraction<sup>15-18</sup>, whereas sulfatase (a microsomal enzyme), at least in the second trimester of pregnancy, is virtually absent<sup>5,19</sup>.

To assess whether experimental removal of the soluble cell fraction results in changes in 3 $\beta$ -hydroxysteroid dehydrogenase-isomerase enzyme activity, separate homogenate and microsomal fractions from human fetal adrenal tissue were incubated with the  $\Delta^5$ -3 $\beta$ -hydroxysteroid substrates, pregnenolone and dehydroepiandrosterone. Using a microsomal preparation, a greater conversion of these steroids to the  $\Delta^4$ -3-ketosteroid, androstenedione, was demonstrable than in the tissue homogenate.

Nineteen adrenal glands (total weight 6.24 g) were obtained from 12 human fetuses at 15-20 weeks of gestation at the time of therapeutic termination of pregnancy, and frozen at -20° until analysis.

The pooled adrenals were homogenized in Krebs-Ringer-bicarbonate glucose buffer (pH 7.4). The ratio of tissue to buffer was 1:4 (w/v). Preliminary centrifugation was performed at 800  $\times$  g for 30 min. A quarter of this preparation, corresponding to 1.5 g of wet adrenal tissue, was incubated as the homogenate fraction. The 'microsomal pellet fraction' was obtained by differential centrifugation as described by SCHNEIDER AND HOGEBOOM<sup>20</sup> from a second quarter of the fetal adrenal pool and washed three times to minimize supernatant contamination. Both preparations were separately incubated using [4-<sup>14</sup>C]pregnenolone (1  $\mu$ C) and [7-<sup>3</sup>H]dehydroepiandrosterone (5.3  $\mu$ C) as substrates. The [4-<sup>14</sup>C]pregnenolone, specific activity 52.4 mC per mmole and [7-<sup>3</sup>H]dehydroepiandrosterone, specific activity 10.4 C/mmole were each purified by paper chromatography in Systems 1 and 3 (see below). Crystalline steroid standards were recrystallized prior to use and melting points were determined. The added cofactors were: ATP, 2  $\mu$ moles; NAD<sup>+</sup>, 0.005 M; NADP<sup>+</sup>, 0.005 M; MgCl<sub>2</sub>, 0.05 M; isocitric acid, 0.025 M; isocitrate dehydrogenase, 4  $\mu$ molar units. The two incubations were simultaneously performed for 3 h at 37°, with air as the gas phase. The reactions were terminated by the addition of dichloromethane and frozen immediately thereafter. The tissue was thawed 48 h later, and 300  $\mu$ g of each of the following nonradioactive steroids were added as carriers: in the homogenate flask, progesterone, androstenedione, 11 $\beta$ -hydroxyandrostenedione, deoxycorticosterone, and dehydroepiandrosterone sulfate; and in the microsomal flask, progesterone, androstenedione, pregnenolone sulfate and dehydroepiandrosterone sulfate.

The following chromatography systems were used and will be referred to in the text by number:

paper chromatography: 1, heptane-methanol-water (5:4:1, by vol); 2, ligroine-propylene glycol<sup>23</sup>; 3, heptane-benzene-methanol-water (33:17:40:10, by vol.); 4, isopropyl ether-*tert.*-butanol-NH<sub>4</sub>OH-water (4:4:1:9<sup>24</sup>, by vol.).

Column partition chromatography: 1, isoctane-*tert.*-butanol-1 M NH<sub>4</sub>OH (3:4:4, by vol.).

Approx. 89% of the starting radioactivity was recovered after the two incubations.

The distribution of radioactive material recovered after initial extraction and partition is presented in Table I.

The "free" steroid fractions from both the homogenate and microsomal incubations were submitted separately to paper chromatography System 1. In both, radioactivity was found in the same area in which carrier dehydroepiandrosterone

and androstenedione ran. When this material was submitted to paper chromatography System 2, two peaks of radioactivity were noted: one ( $R_t = 0.34$ ) similar in mobility to the dehydroepiandrosterone standard, and a second ( $R_t = 1$ ), similar to the mobility of the androstenedione standard. The first peak, mixed with additional authentic dehydroepiandrosterone was recrystallized to constant specific activity. The second peak, after acetylation, was resubmitted to paper chromatography System 2 in which its  $R_t$  was identical to that of authentic androstenedione standard. After mixing with additional crystalline androstenedione, it was recrystallized to constant specific activity.

No radioactive progesterone, deoxycorticosterone,  $11\beta$ -hydroxyandrostenedione,  $17\alpha$ -hydroxyprogesterone,  $16\alpha$ -hydroxyprogesterone or hydrocortisone were detected, although at least three chromatographic procedures were performed using crystalline standards in a search for each of these compounds. In Table II the amount of androstenedione found in the homogenate and microsomal incubations is expressed as the percent of the starting material. The amount of androstenedione found in the microsomal pellet incubation can be seen to be 22–27 times higher than that of the homogenate. When calculated on the basis of the radioactivity found in the "free" steroid fraction, the androstenedione isolated from the microsomal pellet was 9–12 times that from the homogenate.

The water-soluble fractions from both experiments were separately submitted to column partition chromatography in System 1. The homogenate fraction exhibited a major peak of radioactivity in the same hold-back volume as authentic dehydro-

TABLE I

DISTRIBUTION OF RADIOACTIVE MATERIAL RECOVERED AFTER INITIAL EXTRACTION AND PARTITION OF HOMOGENATE AND MICROSOMAL FRACTIONS OF HUMAN FETAL ADRENALS INCUBATED WITH [ $^{14}\text{C}$ ]PREGNENOLONE AND [ $^3\text{H}$ ]DEHYDROEPIANDROSTERONE

Data expressed as percent of recovered material.

	<i>Homogenate</i>		<i>Microsomal pellets</i>	
	$^{14}\text{C}$ -labeled material (%)	$^3\text{H}$ -labeled material (%)	$^{14}\text{C}$ -labeled material (%)	$^3\text{H}$ -labeled material (%)
'Free' fraction ( $\text{MeCl}_2$ -soluble)	51.4	28.8	91.4	90.0
'Conjugated' fraction (water-soluble)	48.6	71.2	8.6	10.0

TABLE II

RADIOACTIVE ANDROSTENEDIONE FOUND IN HOMOGENATE AND MICROSOMAL FRACTIONS FOLLOWING INCUBATION OF HUMAN FETAL ADRENALS WITH [ $^{14}\text{C}$ ]PREGNENOLONE AND [ $^3\text{H}$ ]DEHYDROEPIANDROSTERONE

Data expressed as percent of starting material.

	<i>Androstenedione</i>	
	$^{14}\text{C}$	$^3\text{H}$
Microsomal	7.4	8.9
Homogenate	0.3	0.3
Ratio microsome:homogenate	22:1	27:1

epiandrosterone sulfate. This material was submitted to paper chromatography System 4 in which it ran with an  $R_F = 0.50$ , similar to standard dehydroepiandrosterone sulfate. It was mixed with additional dehydroepiandrosterone sulfate and recrystallized to constant specific activity. The aqueous fraction from the microsomal incubation, representing 8.6% and 10.0% of the  $^{14}\text{C}$  and  $^3\text{H}$ , respectively, completely separated from the dehydroepiandrosterone sulfate and pregnenolone sulfate carriers in System 1, 90% of the counts appearing in the first hold-back volume.

The lack of sulfokinase activity in the microsomal preparation is demonstrated by the minimal sulfoconjugation found. Since, after column partition chromatography, the radioactivity present in the aqueous fraction of the microsomal preparation was much less polar than the sulfoconjugated carrier steroids added, it can be assumed that those counts represent principally material from the "free" fraction not separated during the partition procedure.

The enzymatic capacity of the human fetal adrenal to convert  $\Delta^5$ -3 $\beta$ -hydroxysteroids to the  $\Delta^4$ -3-ketosteroid analogues is demonstrated by the isolation, in both experiments, of radioactive androstenedione. As shown in Table II the extent of this conversion appears to be markedly higher in the microsomal fraction. Therefore, in the human fetal adrenal, it seems possible that some factor present in the soluble fraction might preclude the formation of  $\Delta^4$ -3-ketosteroids, even if this latter enzyme capacity is present. Whether this factor is a biologic enzyme inhibitor, due to the presence of a relatively large concentration of steroid sulfokinases and sulfotransferases or some other factor remains to be elucidated.

The finding of androstenedione derived from both substrates, and the lack of  $\text{C}_{21}$ - $\Delta^4$ -3-ketosteroids, suggests a deficiency of the pregnenolone-specific 3 $\beta$ -hydroxysteroid dehydrogenase-isomerase system. Further support for this suggestion is furnished by the  $^3\text{H}:^{14}\text{C}$  ratios of dehydroepiandrosterone (5.8:1) and androstenedione (6.7:1) in the microsomal fraction and the corresponding ratios of 8.5:1 and 7.6:1 in the homogenate preparation. These ratios suggest the pathway of conversion of pregnenolone to androstenedione *via* dehydroepiandrosterone. Other studies have also demonstrated the existence of more than one substrate-specific isomerase<sup>30</sup>. In addition, studies in neoplastic adrenal tissue have shown a deficiency of a pregnenolone-specific 3 $\beta$ -hydroxysteroid dehydrogenase-isomerase system<sup>31,32</sup>. Thus, a similar pattern of steroid metabolism might be inferred in both the neoplastic and fetal adrenal gland, an observation also noted in our previous studies<sup>33</sup>.

Persistently elevated plasma levels of  $\Delta^5$ -3 $\beta$ -hydroxysteroids in some infants with congenital adrenal hyperplasia have been attributed to a relative deficiency of 3 $\beta$ -hydroxysteroid dehydrogenase-isomerase<sup>34-36</sup>. In light of our results, the possibility exists that these high levels of  $\Delta^5$ -3 $\beta$ -hydroxysteroids may be due, in part, to the persistence of an inhibitory factor present in the soluble cell fraction resulting in relative inactivity of the 3 $\beta$ -hydroxysteroid dehydrogenase-isomerase system.

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