Age-dependent changes in the multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver

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The soluble NADP-dependent 17 β -hydroxysteroid dehydrogenase activity of female rabbit liver increases with the age of the animal, the specific activity of the enzyme in the 56-day-old rabbit being 3 times that of the 28-day-old animal. The increase in activity is accompanied by a change in the molecular heterogeneity of the enzyme. Three forms (enzymes I, II and III) were identified in the liver cytosol of the 56-day-old female rabbit, whereas only one major form (enzyme II) was present in the 28-day-old animal. Peptide maps of the four purified enzymes showed that there were minor differences in structure. The enzyme present in the liver of the 28-day-old rabbit was distinct from the three enzymes of the 56-day-old animal. All of the enzymes exhibited bifunctional activity, having 17 β -hydroxysteroid dehydrogenase activity towards androgen and oestrogen substrates and 3 α -hydroxysteroid dehydrogenase activity towards androgens of the 5 β -androstane series. The differences in substrate specificity of the enzymes paralleled their differences in structure. The data suggest that one enzyme (enzyme III) may have a special role in steroid metabolism during development in the female rabbit.

17 β -Hydroxysteroid dehydrogenase activity has been shown to be age-dependent in a number of mammalian tissues. Inano *et al.* (1967) reported that the 17 β -hydroxysteroid dehydrogenase activity of rat testis increased with age, reaching a maximum in 60-day-old animals. Furubayashi *et al.* (1982) observed that the 17 β -hydroxysteroid dehydrogenase activity in the testis of the golden hamster was approx. 10-fold higher in adult than in immature animals. Ghraf *et al.* (1975b) carried out an extensive examination of the 17 β -hydroxysteroid dehydrogenase in the cytosol and microsomal fractions from a variety of tissues, including the kidney, liver, adrenal and gonads, of 15–120-day-old male and female rats. The enzyme activity in at least one of the subcellular fractions of each tissue displayed an age-dependent development, maximal activities being attained in 60-day-old animals.

Bongiovanni & Marino (1972) measured the 17 β -hydroxysteroid dehydrogenase in the liver homogenates of male rabbits during different stages of development. No enzyme activity was

detected in foetal liver homogenates, but the enzyme was present in liver homogenates of the young (20-day-old) and mature (17–19-month-old) animal. In the preceding paper (Antoun *et al.*, 1985) we described the purification of multiple forms of a soluble NADP-dependent 17 β -hydroxysteroid dehydrogenase from the liver of the female rabbit (8–12 weeks old). The enzyme forms had distinct properties and substrate specificities. The possibility that these enzyme forms undergo age-dependent changes that result in the differences in enzyme activity observed by Bongiovanni & Marino (1972) was therefore investigated. The present paper compares the structures and substrate specificities of forms of the 17 β -hydroxysteroid dehydrogenase purified from the liver cytosols of 28- and 56-day-old female rabbits. The paper also presents further evidence for the distinct nature of the three enzyme forms present in the 56-day-old animal.

Experimental

Materials

[1-¹⁴C]Acetic anhydride (sp. radioactivity 115 mCi/mmol) was purchased from Amersham Corp., Oakville, Ont., Canada. [1,2-³H]-

Trivial names used: androsterone, 3 α -hydroxy-5 α -androstane-17-one; aetiocholanolone, 3 α -hydroxy-5 β -androstane-17-one.

Androsterone (sp. radioactivity 41 Ci/mmol), [1,2-³H]aetiocholanolone (sp. radioactivity 45 Ci/mmol), [6,7-³H]oestradiol-17 β (sp. radioactivity 40 Ci/mmol), [6,7-³H]oestrone (sp. radioactivity 44 Ci/mmol) and [7-³H]testosterone (sp. radioactivity 25 Ci/mmol) were obtained from New England Nuclear, Lachine, Que., Canada. The purity of all radioactive steroids was verified by t.l.c. on Silica Gel N (Macherey Nagel und Co., Düren, Germany) in the solvent system benzene/acetone (4:1, v/v). Non-radioactive steroids were purchased from Research Plus Steroid Laboratories, Bayonne, NJ, U.S.A., or Sigma Chemical Co., St. Louis, MO, U.S.A. LKB carrier ampholyte, pH5-7 (40%, w/v), was obtained from Fisher Scientific Co., Ottawa, Ont., Canada. Polybuffer 74 and Polybuffer Exchanger PBE 94 were purchased from Pharmacia, Dorval, Que., Canada. Agarose-immobilized Procion Red HE3B (Matrex Gel Red A) was obtained from Amicon, Oakville, Ont., Canada. All chemical reagents used were of analytical grade.

Enzyme purification and assay

Liver tissue was obtained from 28- and 56-day-old virgin female New Zealand White rabbits, and the soluble NADP-dependent 17 β -hydroxysteroid dehydrogenase was purified from these tissues as described by Antoun *et al.* (1985). Hydroxysteroid dehydrogenase activity was measured as described by Lau *et al.* (1982a). Steroid substrates were adjusted to a specific radioactivity of 4.5 μ Ci/ μ mol and incubated at a final concentration of 1.0 μ M. Incubations were carried out at 37°C for 30 min in 67 mM-glycine/NaOH buffer, pH9.5, containing 167 μ M-NADP⁺. The incubation volume was 3.0 ml. The amount of enzyme assayed was adjusted so that substrate oxidation did not exceed 25%. Protein was measured by the method of Bradford (1976), with bovine serum albumin as the standard. Enzyme activity is expressed as units (μ mol of substrate oxidized/min).

Electrophoresis in polyacrylamide gels

Purity of the enzymes was assessed by polyacrylamide-gel electrophoresis at pH8.3 (Davis, 1964). The M_r values of the enzymes were determined by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate as described by Weber *et al.* (1972). The following protein standards were used: ribonuclease, chymotrypsinogen A, fructose biphosphosphate aldolase, ovalbumin and bovine serum albumin.

Isoelectric focusing in polyacrylamide gels over the pH range 5-7 was carried out as detailed by Antoun *et al.* (1985). The gels were stained for 17 β -hydroxysteroid dehydrogenase activity by the

procedure of Lau *et al.* (1982a) with the substrate testosterone.

Peptide mapping

In order to obtain peptide maps of the small quantities of purified enzymes obtained, the proteins were first acetylated with [1-¹⁴C]acetic anhydride, and the peptides generated by proteolytic digestion and separated by electrophoresis were detected by autoradiography. This procedure has been employed in our laboratory to compare the multiple forms of the 17 α -hydroxysteroid dehydrogenase of female rabbit liver and kidney (Lau *et al.*, 1982b). Protein acetylation was carried out by the method described by Kaplan *et al.* (1971). Each freeze-dried enzyme sample (400 μ g) was dissolved in 1 ml of 8 M-urea previously acidified to destroy any cyanate formed. The mixture was transferred to a reaction vessel, and sodium borate was added as a buffer. The pH was adjusted to 9.5 with 1 M-KOH. Acetonitrile (200 μ l) containing 0.2 mCi of [1-¹⁴C]acetic anhydride was added to the reaction mixture in two equal portions at 10 min intervals. The reaction mixture was stirred throughout the procedure, and a pH of 9.0 was maintained by using a pH-stat with 5 M-KOH as titrant. Unlabelled acetic anhydride (20 μ l) was then added to the reaction mixture in four equal portions at 10 min intervals. After acetylation, each sample was acidified with conc. HCl, and 1 mg of bovine serum albumin, acetylated with unlabelled acetic anhydride by the above procedure, was added to limit procedure losses during subsequent steps. The acetylated samples were dialysed against several changes of distilled water until no radioactivity was detected in the diffusate. Each sample was freeze-dried, dissolved in 1 ml of 0.5% NH₄HCO₃ and digested for 6 h at 37°C with papain in the presence of 2-mercaptoethanol (50 μ l). An amount of papain equivalent to one-tenth of the protein sample was used for digestion. The digested samples were freeze-dried and subjected to high-voltage electrophoresis at pH6.5 (acetic acid/pyridine/water, 3:100:900, by vol.) in the first dimension and at pH2.1 (acetic acid/formic acid/water, 4:1:45, by vol.) in the second dimension. Electrophoresis of peptides neutral at pH6.5 was also performed at pH3.5 (water/acetic acid/pyridine, 190:10:1, by vol.). Peptides were located by autoradiography.

Results

Table 1 compares the 17 β -hydroxysteroid dehydrogenase activity in the liver cytosols of female rabbits 28-56 days of age. There is a progressive increase in the enzyme activity, so that the specific

activity of the enzyme in the 56-day-old animal is 3-fold higher than that of the 28-day-old animal.

The NADP-dependent 17 β -hydroxysteroid dehydrogenases were purified from the liver cytosols of 28-day-old and 56-day-old female rabbits by affinity chromatography on agarose-immobilized Procion Red HE3B and chromatofocusing as described by Antoun *et al.* (1985). The enzyme profiles obtained on chromatofocusing are shown in Fig. 1. As previously shown (Antoun *et al.*, 1985), three enzyme activities (enzymes I, II and III) were present in the 56-day-old rabbit. Three enzyme activities were also present in the 28-day-old animal (Fig. 1); however, in this case there was only one major enzyme activity (enzyme IIIY). The enzymes I, II, III and IIIY were each subjected to a second chromatofocusing under the conditions described in Fig. 1. By this procedure the enzymes were purified to homogeneity as established by polyacrylamide-gel electrophoresis. M_r values of 37000, 40000, 39000 and 40500 were calculated for enzymes I, II, III and IIIY respectively by comparing the mobilities of these enzymes with those of proteins of known M_r values after sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (results not shown).

The enzymes III and IIIY were eluted at similar positions on the pH gradients during chromatofocusing, indicating that these enzymes have similar isoelectric points (Fig. 1). The enzymes were therefore compared by isoelectric focusing-polyacrylamide-gel electrophoresis, and the results are shown in Fig. 2. The enzymes III and IIIY have different isoelectric points. Similar results were obtained when enzyme IIIY was compared with enzymes I and II. Enzyme IIIY has the most acidic isoelectric point of the four purified enzymes.

The enzymes were acetylated with [14 C]acetic anhydride by the method of Kaplan *et al.* (1971), and the primary structures of the acetylated proteins were compared by peptide mapping. Fig. 3 shows autoradiograms of the peptide maps

obtained after papain digestion of the enzymes and electrophoresis at pH6.5 and 2.1. The peptide maps demonstrate that there is extensive homology in the primary structures of the enzymes. No distinctive differences are observed between I and II. Enzyme III has at least four peptides that are not present in enzymes I and II. Although the peptide map of enzyme IIIY is most similar to those of enzymes I and II, this enzyme does have

Fig. 1. Chromatofocusing of the soluble NADP-dependent 17 β -hydroxysteroid dehydrogenases from the liver of 28- and 56-day-old female rabbits

The NADP-dependent 17 β -hydroxysteroid dehydrogenase activity was isolated from the liver of 28- and 56-day-old female rabbits by affinity chromatography on agarose-immobilized Procion Red HE3B as described in the text. Each enzyme fraction was applied to a column of Polybuffer Exchanger PBE 94 that had been equilibrated with 10mM-imidazole/HCl buffer, pH6.15, containing 0.5mM-dithiothreitol. Protein was eluted with a diluted Polybuffer 74 solution, pH5.15. Fractions (1.5ml) were collected and assayed for 17 β -hydroxysteroid dehydrogenase activity with the substrate testosterone. $\blacktriangle$, Activity in the 28-day-old rabbit; $\bullet$, activity in the 56-day-old rabbit; -----, pH gradient.

Table 1. 17 β -Hydroxysteroid dehydrogenase activity of the liver cytosol from female rabbits at different ages. Enzyme activity was measured at pH9.5 with testosterone (1 μ M) and NADP $^{+}$ (167 μ M). Experimental details are given in the text. The data show the average values ($\pm$ range) for four animals 28 or 56 days old and for two animals 35, 42 or 49 days old.

Age (days)	Specific activity (μ units/mg of protein)
28	220 $\pm$ 30
35	240 $\pm$ 20
42	440 $\pm$ 40
49	490 $\pm$ 50
56	640 $\pm$ 60

Table 2. *Substrate specificities of the multiple forms of the soluble 17 β -hydroxysteroid dehydrogenase of female rabbit liver*
The enzymes are those identified in Fig. 1. Experimental details are given in the text. The specific activities of the enzymes for each substrate are reported as the percentages of their specific activities with oestradiol-17 β (4.9, 8.4, 1.1 and 0.27 munits/mg of protein for enzymes, I, II, III and IIY respectively).

Substrate	Enzyme ...	Specific activity (% of activity with oestradiol-17 β)			
		I	II	III	IIY
Oestradiol-17 β		100	100	100	100
Testosterone		2570	1100	4760	3150
Androsterone		80	18	471	274
Aetiocholanolone		673	436	3140	750

Fig. 2. *Isoelectric focusing-polyacrylamide-gel electrophoresis of enzymes IIY and III from the liver of 28- and 56-day-old female rabbits*

Isoelectric focusing and substrate staining of the enzyme fractions were carried out as described in the text. The enzymes IIY and III (approx. 2000 μ units of activity towards testosterone) were applied to the gels, and isoelectric focusing was carried out initially at 400 V for 0.5 h and then at 500 V for 3.5 h. Gel a, enzyme III; gel b, enzyme IIY; gel c, enzyme III plus enzyme IIY.

phoresis at pH 2.1, no distinct peptides were observed (Fig. 3). These peptide bands were therefore subjected to electrophoresis in the second dimension at pH 3.5. The peptide maps obtained for the four enzymes are shown in Fig. 4. Although the peptide maps again show that there is considerable structural homology among the enzymes, peptides unique to each of the enzymes can be distinguished.

The activities of the four enzymes towards oestrogen and androgen substrates are compared in Table 2. The absolute specific activities of the enzymes differ. Therefore in Table 2 the activity of each enzyme towards the different steroid substrates is expressed relative to its activity towards oestradiol-17 β in order that the activity profiles of the four enzymes can be more readily compared. Activity towards testosterone is much higher than towards oestradiol-17 β with all of the enzyme forms, ranging from 11-fold higher with enzyme II to 48-fold higher with enzyme III. The enzymes all exhibit 3 α -hydroxysteroid dehydrogenase activity with a preference for aetiocholanolone, an androgen of the 5 β -androstane series. In general, the substrate-specificity profile of enzyme IIY is most similar to that of enzyme I. However, enzyme IIY exhibits a higher 3 α -dehydrogenase activity towards the 5 α -androstane androsterone than does enzyme I. Enzyme III is the most distinct in its substrate specificity. It has the highest relative 17 β -dehydrogenase activity towards testosterone. Of even more significance is the high 3 α -dehydrogenase activity of this enzyme towards aetiocholanolone when compared with the other enzymes.

Discussion

In the preceding paper (Antoun *et al.*, 1985) we described the purification of three forms of a soluble 17 β -hydroxysteroid dehydrogenase from the liver of the 8–12-week-old female rabbit. The enzymes exhibited differences in their relative activities towards a number of androgen and

one peptide identical in mobility with a peptide found in enzyme III but not in enzymes I and II.

When the peptides that were neutral on electrophoresis at pH 6.5 were subjected to electro-

Fig. 3. Peptide maps of the papain digests of the ^{14}C -acetylated female rabbit liver enzymes I, II, III and IIIY. High-voltage paper electrophoresis (3000 V, 1 h) was carried out at pH 6.5 in the first dimension and at pH 2.1 in the second dimension. The peptides were located by autoradiography. Peptides not identified in all peptide maps are indicated by arrows.

oestrogen substrates. The results of the present paper support the conclusion that the three enzymes are distinct and differ in their primary structure. Of the three enzymes, enzyme III is the most distinct in terms of primary structure, as indicated by peptide mapping (Figs. 3 and 4). This enzyme also has a different *N*-terminus (aspartic acid or asparagine) from those of enzymes I and II (serine) (Kaplan *et al.*, 1983). The extent to which the structures of the enzymes differ is consistent with their differences in substrate specificity (Antoun *et al.*, 1985). The molecular heterogeneity of the 17 β -hydroxysteroid dehydrogenase could

result either from genetic variants or post-synthetic modification of the enzyme. Possible mechanisms for post-synthetic modification would include deamination of asparagine and glutamine residues to produce a series of charge isomers or limited proteolytic cleavage of *N*- or *C*-terminal sequences from one enzyme to generate additional forms. The different *N*-terminus and lower M_r value of enzyme III support the latter possibility.

The increase in the NADP-dependent 17 β -hydroxysteroid dehydrogenase activity in the liver of female rabbits from 28 to 56 days of age (Table 1) appears to be related to changes in the multiple

Fig. 4. Peptide maps of the neutral peptides from the papain digests of the ^{14}C -acetylated female rabbit liver enzymes I, II, III and IIIV

The peptides that were neutral at pH 6.5 were subjected to high-voltage paper electrophoresis (3000 V, 1 h) at pH 2.1 in the first dimension (Fig. 3) and at pH 3.5 in the second dimension. Peptides were located by autoradiography.

forms of the enzyme. One enzyme form is responsible for most of the enzyme activity in the 28-day-old rabbit, whereas a second major form is present in the 56-day-old animal (Fig. 1). We have examined the heterogeneity of this enzyme activity in female rabbits between the ages of 28 and 56 days and in animals older than 56 days. The enzyme profiles obtained with 49-, 70- and 84-day-old rabbits were the same as those observed with the 56-day-old animal (G. R. Antoun & D. G.

Williamson, unpublished work). This enzyme profile was also obtained with a 42-month-old rabbit. Therefore the 17β -hydroxysteroid dehydrogenase forms in the liver of the mature female rabbit are established in the animals by the age of 7 weeks. The enzyme profiles of 35- and 42-day-old rabbits differ from each other and from the profiles obtained with 28- and 56-day-old animals, suggesting that during this stage of development a relatively complex change in the heterogeneity of

the 17 β -hydroxysteroid dehydrogenase is taking place. We have not examined animals younger than 28 days of age to determine whether different forms of the enzyme are present before this stage of development.

The difference in the heterogeneity of the 17 β -hydroxysteroid dehydrogenase between the 28- and 56-day-old female rabbit is not only quantitative. On the basis of the isoelectric points and peptide maps of the four enzymes purified in this study we have concluded that the major enzyme form (enzyme IIIY) present in the 28-day-old rabbit is not present in the 56-day-old animal. The results do indicate, however, that the structural differences among the enzymes are minor. Although enzyme IIIY has the same *N*-terminal aspartic acid (or asparagine) as enzyme III (Kaplan *et al.*, 1983), the peptide map of enzyme IIIY is more similar to those of enzyme I and II. The substrate specificity of enzyme IIIY is also more similar to those of enzymes I and II than to that of enzyme III (Table 2). The distinct differences of enzyme III in substrate specificity suggest that *in vivo* this enzyme could have a specialized role in maturation of the rabbit. A detailed kinetic analysis of the enzyme must be carried out in order to substantiate this possibility, particularly with regard to the apparently high activity of this enzyme towards 3 α -hydroxysteroids of the 5 β -androstane series.

It is possible that enzyme IIIY of the 28-day-old rabbit is the precursor of one or more of the enzymes of the 56-day-old animal. Post-synthetic modification of enzyme IIIY could occur through an age-dependent factor or component of rabbit liver to produce the enzyme profile observed in the older animal. Kahn *et al.* (1977) have proposed such a mechanism to explain the appearance of a heat-labile glucose-6-phosphate dehydrogenase in senescent cultured cells of human liver. When the forms I, II and IIIY of the 17 β -hydroxysteroid dehydrogenase are compared, the similarities of their substrate specificities and peptide maps, the higher *M_r* value of enzyme IIIY and the distinct *N*-terminus of enzyme IIIY are all consistent with the hypothesis that enzyme I or II is formed by proteolytic cleavage of an *N*-terminal peptide from enzyme IIIY. It is less likely that such a modification of enzyme IIIY would produce enzyme III. There are more differences in their substrate specificities and peptide maps and they have the same *N*-terminus. It is also unlikely that the enzyme forms present in the 56-day-old rabbit are produced *in vivo* by deamidation of the enzyme of the 28-day-old animal, since enzyme IIIY is the most acidic of the four enzymes.

The age-related changes in the liver 17 β -hydroxysteroid dehydrogenase could be hormone-

dependent, resulting from an alteration in steroid secretion during maturation of the gonads. Hormonal regulation of 17 β -hydroxysteroid dehydrogenase activity has been described. Macartney & Thomas (1969) reported an age-dependent increase in the conversion of oestrone into oestradiol-17 β in rabbit uterine preparations. Breuer *et al.* (1979) have observed that oestrogens can suppress the 17 β -hydroxysteroid dehydrogenase activity of rat liver. This suppression was due to an oestrogen-dependent decrease in one of two enzyme activities that they identified in rat liver cytosol. Kochakian *et al.* (1973) showed that the 17 β -hydroxysteroid dehydrogenase activity of male guinea-pig kidney cytosol was decreased by castration and restored by testosterone administration. In subsequent studies Shen & Kochakian (1978) showed that this enzyme activity was low in the cytosol of female guinea-pig kidney but that it could be increased to the values found in the male animal by testosterone treatment. In contrast, the 17 β -hydroxysteroid dehydrogenase of liver did not respond to testosterone treatment, suggesting that in the female guinea pig the liver 17 β -dehydrogenase is under different regulation from that for the kidney 17 β -dehydrogenase. A role for hypophyseal hormones in the regulation of 17 β -hydroxysteroid dehydrogenases has been proposed by a number of investigators (Musto *et al.*, 1972; Lax *et al.*, 1974; Ghraf *et al.*, 1975a; Muroso & Payne, 1979; Kondo *et al.*, 1980). Hormones shown to affect 17 β -dehydroxysteroid dehydrogenase activity include prolactin, luteinizing hormone (lutropin) and follicle-stimulating hormone (follicitropin). To date, the measurement of circulating concentrations of steroid or hypophyseal hormones in the rabbit during development has not been carried out. This information is necessary to establish whether the age-related changes in the multiple forms of the soluble NADP-dependent 17 β -hydroxysteroid dehydrogenase of female rabbit liver are hormone-dependent.

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