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Enzymatic Basis for Epimerization of Cardiotonic Steroids at Carbon 3 in Rat Liver*

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The conversion of 3β-hydroxysteroids to their 3α epimers, denoted as epimerization, has been studied using various fractions of liver homogenates from male or female rats. The greatest epimerization capacity was found to reside primarily in the clear supernatant and next in the microsomal fraction. The oxidation rates of a large set of 3-hydroxysteroids by the soluble fraction and DPN or TPN were determined using a spectrophotometric method. Both cardioactive and hormone-type steroids were effectively dehydrogenated to their 3-keto derivatives. Much parallelism between the two steroid types was found in the influence of structure variations on the oxidation velocities. The reduction of 3-ketosteroids of both types by DPNH or TPNH in the presence of the soluble fraction was also followed. The results suggest that the rat liver contains soluble as well as particulate 3β- and 3α-hydroxysteroid dehydrogenases which differ in their dependency upon DPN or TPN and in their relative affinity for DPNH or TPNH. On the assumption that DPN is the common hydrogen acceptor and TPNH is the major hydrogen donor for the interconversions of 3-hydroxy- and 3-ketosteroids in the liver cell, it has been concluded that the epimerization process involves an oxidation of the cardioactive steroids by the DPN-dependent 3β-hydroxysteroid dehydrogenase and an immediately subsequent reduction of the 3-keto derivatives produced by the TPN-dependent 3α-hydroxysteroid dehydrogenases. Both β- and α-enzymes showed certain sex differences in their activities. An explanation for the sex differences in the steric course of the 3-keto-(5α-H-)-steroid reduction has been offered. The biological significance of epimerization is discussed with regard to the mechanism of action of cardioactive steroids and to their detoxification in the animal body.

Cardiotonic steroids, probable intermediary products in the degradation of cardiac glycosides in the animal body (Repke, 1963), are themselves rapidly metabolized. The main locus of the biotransformation of genins is the hydroxy group at carbon 3. In the glycosides this group is involved in the binding of the sugar component to the steroid nucleus and hence becomes freely accessible only after glycoside fission. This hydroxy group is in the β position in nearly all natural representatives, but it is rapidly inverted to the α position in the animal body (Repke and Lauterbach, 1959). This inversion, denoted hereafter as epimerization, has been considered to be a major pathway for the detoxification of genins (Repke and Lauterbach, 1959) and therefore deserved further investigation. The present paper aims at a closer analysis of this reaction.

In addition to the genins, other 3β-hydroxysteroids like Δ^4-pregnen-3β-ol-20-one, Δ^4-androstene-3β,17β-diol (for review see Samuels, 1960), and cholesterol (Samuelsson, 1959) are metabolized by epimerization at C-3. For the sake of comparison, the study of this type of steroids is included in this paper.

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In the course of *in vitro* studies with tissue preparations from the rat, using digitoxigenin as substrate, it has been shown that the epimerization may be effected by isolated erythrocytes (Portius and Repke, 1960), pieces of diaphragm,[1] and slices from cardiac muscle[1] or liver (Lauterbach and Repke, 1960). It is probable that other tissues are also able to bring about the same reaction, but the liver seems to possess by far the highest activity (Repke, 1963). In addition to the rat tissue, liver slices of other animal species will invert the hydroxy group at C-3 from β to α position, the following order of increasing activities having been found: frog, pigeon, toad, guinea pig, cat, rat, mouse, and rabbit (Repke, 1960). Slices of human liver likewise have a distinct capacity for epimerization of digitoxigenin.[2] Because of accessibility and relatively high activity, rat liver was chosen as enzyme source in the present study. Cell fractions instead of slices have been used to analyze the enzymatic basis for epimerization at a subcellular level and to prevent side reactions like conjugation.

Experimental Procedure

Steroids.—Uzarigenin, digitoxigenin, strophanthidin, sarmentogenin, digoxigenin, gitoxigenin, bufalin, scillarenin (Tamm, 1956); 3-dehydrouzarigenin, 3-epiuzarigenin (Kuritzkes *et al.*, 1959); 17β-H-digitoxigenin

[1] F. Lauterbach and K. Repke, unpublished experiments.
[2] I. Herrmann and K. Repke, to be published.

(Frèrejacque, 1959); dihydrodigitoxigenin (Brown and Wright, 1961); xysmalogenin (Polonia *et al.*, 1959); diginatigenin (Tamm and Gubler, 1958); 3-dehydrobufalin, 3-epibufalin (Tamm, 1959); scillirosidin (von Wartburg and Renz, 1959) were gifts of various scientists.[3] 3-Dehydro-digitoxigenin (mp 198–200°), 3-epi-digitoxigenin (mp 275–279°), 3-dehydro-gitoxigenin (mp 205–211°), 3-epi-gitoxigenin (mp 205–215°), 3-dehydro-digoxigenin (mp 249–251°), and 3-epi-digoxigenin (mp 252–258°) were prepared according to known methods (Sigg *et al.*, 1953; Tamm and Gubler, 1958; Tamm and Gubler, 1959). The melting points (corrected) were determined on a Boetius-type hot stage. The cardioactive steroids were either paper chromatographically pure or contained no more than 5% of a single foreign steroid. The steroids of hormone type were chromatographically pure. All steroids were dissolved in pyridine (spectroscopic grade, Eastman) and made up to a concentration of 0.15 μmole/10 μl of solvent.

Pyridine Nucleotides.—DPN and TPN (Sigma purest grade) were dissolved in water and a small amount of 0.1 M NaOH added, sufficient to adjust the *p*H to 5.5. The final concentration was 1.50 μmoles of the nucleotide per 100 μl of solvent. DPNH and TPNH (Sigma purest grade), both enzymatically reduced, were dissolved in 1% solution of $NaHCO_3$ to give a final concentration of 0.25 μmole/100 μl of solution.

Buffer Solutions.—The glycine and Tris buffers (Repke and Samuels, 1964) contained 2 mM ethylenediaminetetraacetic acid.

Isolation of Particulate and Soluble Fractions of Liver Cells.—The cell components were isolated from the livers of adult rats of the Sprague-Dawley strain according to the method of Schneider, (1948), except that 40 mM nicotinamide was added to the isotonic sucrose. After separation of the microsomal fraction by centrifugation at 105,000 × *g* for 1 hour, the soluble fraction was prepared as described in the accompanying paper (Repke and Samuels, 1964). The protein content of the various preparations was estimated by a biuret procedure (Gornall *et al.*, 1949) and expressed as mg equivalent of bovine serum albumin.

Estimation of the Distribution of Epimerization Activities among the Liver Cell Fractions.—Aliquots of the whole homogenate or of the various cell fractions equivalent to 1 g liver were incubated for 1/2 and 1 hour with 0.1 and 0.5 μmole of steroids at 37.5° and *p*H 7.5 (phosphate or Tris buffer) in a total volume of 5 ml containing 200 μmoles of nicotinamide. When specified, the following substances were also added: 50 μmoles of glucose, 25 μmoles of fumarate, pyruvate, and glutamate, 5 μmoles of citrate, 0.5 and 10 μmoles of DPN or TPN. The epimerizing capacity of the various fractions was estimated by the amount of the epimeric metabolite produced during the incubation as estimated by paper chromatographic analysis.

Paper Chromatographic Analysis of Enzyme Reaction.—The reactions were stopped by extraction of the samples with chloroform. After evaporation of the solvent the extracted materials were chromatographed on paper. The solvent systems used were xylol/formamide[4] (Kaiser, 1955), benzene-chloroform (7:5)/formamide or xylol–methyl ethyl ketone (1:1)/formamide (Schmid *et al.*, 1959) for cardioactive steroids and hexane-benzene (1:1)/formamide or hexane/formamide (Neher, 1958) for hormone-type steroids. Parent compounds and metabolites were detected by treatment of the dried papers with trichloracetic acid and "chloramine" (Kaiser, 1955) (cardenolide type), with antimony trichloride (bufadienolide type and steroid alcohols), with I_2-HI solution (C_{19} and C_{21} steroid ketones) or with *m*-dinitrobenzene (3- and 17-ketosteroids) (Neher, 1958). A rough measurement of the quantities of metabolite and, in this way, an approximate estimation of enzyme activity, was obtained by comparing the size of spots and the strength of fluorescence or the depth of color produced by known amounts of the authentic compounds with those produced by the unknown amounts of the respective metabolites. Under suitable conditions (personal practice and appropriate substance range) this procedure proved to have an average error of about 15%.

Spectroscopic Determination of Enzymatic Activities in the Soluble and Microsomal Fractions.—Having established that the major reaction which occurred was reduction or oxidation at carbon-3, the interconversions of 3-hydroxy- and 3-ketosteroids were then followed by measuring at 340 mμ the amount of DPNH or TPNH which was formed or which disappeared in the course of the reactions. The dehydrogenation of 3-hydroxysteroids by DPN or TPN and soluble fraction was estimated as described in the previous paper (Repke and Samuels, 1964). The dehydrogenation of 3-hydroxysteroids by fresh microsomes and DPN or TPN was followed in the same manner except that 3.3 mM KCN was added (Hurlock and Talalay, 1959).

The reduction of 3-ketosteroids by DPNH or TPNH in the presence of supernatant was measured as follows:

After having noted any difference in optical density between standard and sample cells when both contained buffer solution and clear liver supernatant only, the reduced pyridine nucleotide was added to the sample cuvet and its absorbance was measured. By this procedure the solutions of reduced nucleotides could be checked for initial concentration routinely, assuming the absorbancy at 340 mμ to be 6.22×10^6 cm²/mole (Horecker and Kornberg, 1948). Then either 10 μl of steroid solution in pyridine or 10 μl of pyridine alone was mixed with the contents of the sample cells, and the absorbancy was measured every 30 seconds. The decrease in absorbancy during the second 30 seconds, after correcting for the pyridine blank, was taken as a measure of enzymatic activity.

Results

Survey of the Distribution of Epimerization Activities in the Liver Cell.—On incubation with unfractionated liver homogenate, the epimerization of digitoxigenin at physiological *p*H (7.5) proceeded considerably more slowly than with liver slices. The lowered activity after destruction of cells could be compensated to a considerable extent by addition of nicotinamide, glucose, and citrate, and probably was due to the degradation of pyridine nucleotides which was partially prevented by the presence of nicotinamide (Bassham *et al.*, 1959).

The mitochondrial fraction supplemented with fumarate, pyruvate, glutamate, and glucose showed an activity considerably below that of the unfractionated homogenate. The very small activity with digitoxigenin as steroid substrate might have been caused by a contamination of this fraction with microsomes (Hulsmans, 1961). The turbid supernatant ob-

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[4] The solvent before the (/) is the moving phase while that after it is the stationary phase.

TABLE I

OXIDATION RATES OF 3β-HYDROXYSTEROIDS[a]

	Steroids	Differing Features	+ ΔA per 0.5 min	+ ΔA per 5.0 min	Relative Rates[b]
	Cardenolide Type (14β-OH)				
1.	Digitoxigenin	Basic type	0.021	0.092	1.0
2.	17β-H-Digitoxigenin	Butenolide residue α-positioned[c]	0.012	0.047	0.6
3.	Dihydrodigitoxigenin	Butenolide residue saturated	0.033	0.123	1.6
4.	Uzarigenin	A/B in *trans* junction[d]	0.002	0.012	0.1
5.	Xysmalogenin	Double bond between C-5 and C-6	0.000	0.001	≪0.1
6.	Strophanthidin	5β-OH, 19-oxo	0.000	0.000	0
7.	Sarmentogenin	11α-OH	0.000	0.000	0
8.	Digoxigenin	12β-OH	0.002	0.013	0.1
9.	Gitoxigenin	16β-OH	0.002	0.010	0.1
10.	Diginatigenin	12β-OH, 16β-OH	0.000	0.004	<0.1
	Bufadienolide Type (14β-OH)				
11.	Bufalin	Basic type	0.040	0.133	1.9
12.	Scillarenin	Double bond between C-4 and C-5	0.019	0.091	0.9
13.	Scillirosidin	$\Delta^{4,5}$, 6β-acetoxy, 8β-OH	0.001	0.007	<0.1
	Hormone Type (14α-H)				
14.	Etiocholan-3β-ol-17-one	Basic type	0.043	0.173	2.1
15.	5β-H-Pregnan-3β-ol-20-one[e]	Acetyl at C-17	0.057	0.227	2.7
16.	Androstan-3β-ol-17-one	A/B in *trans* junction[d]	0.006	0.038	0.3
			0.005[f]	0.033	

[a] On incubation with DPN and soluble fraction from liver homogenates at *p*H 10.2 and 37.5°, based on the increase in absorbancy (+ ΔA) due to formation of DPNH (mean values of double determinations: wavelength = 340 mμ, *d* = 1 cm). The final volume of 3 ml contained a mixture of the following components: 286 μmoles glycine–sodium hydroxide buffer, 2 mg protein, 1.50 μmoles DPN, and 0.15 μmole steroid. If not otherwise specified the soluble fraction was isolated from liver homogenates of male rats. [b] Based on the increase in absorbancy (+ ΔA) per 0.5 minute. [c] In all other representatives β-positioned. [d] *cis*-Junction in all other representatives whenever the double bonds $\Delta^{4,5}$ or $\Delta^{5,6}$ are lacking. [e] The 5α-H epimer was not sufficiently soluble. [f] Supernatant from liver homogenates of female rats.

TABLE II

OXIDATION VELOCITIES OF 3α-HYDROXYSTEROIDS[a]

	Steroid	Oxidation by DPN: + ΔA per 0.5 min	+ ΔA per 5.0 min	Relative Rates		Oxidation by TPN: + ΔA per 0.5 min	+ ΔA per 5.0 min	Relative Rates	
	Cardenolide Type (14β-OH)								
17.	3-epi-Digitoxigenin	0.004	0.026	1.0[b]	0.2[c]	0.158	0.268	1.0[b]	8[c]
18.	3-epi-Uzarigenin	0.005	0.031	1.3	2.5	0.128	0.263	0.8	64
19.	3-epi-Digoxigenin	0.000	0.000	0	<0.1	0.021	0.064	0.1	11
20.	3-epi-Gitoxigenin	0.004	0.026	1.0	2.0	0.151	0.255	1.0	76
	Hormone Type (14α-H)								
21.	5β-H-Pregnan-3α-ol-20-one[d]	0.008	0.047	2.0	0.1	0.104	0.260	0.7	2
22.	Androstan-3α-ol-17-one[e]	0.007	0.041	1.8	1.2	0.109	0.272	0.7	18
		0.015[f]	0.105			0.162[f]	0.308		

[a] On incubation with pyridine nucleotides and the soluble fraction from liver homogenates at *p*H 8.6 (DPN-dependent enzyme) or at *p*H 9.6 (TPN-dependent enzyme) and at 37.5°, based on the increase in absorbancy (+ ΔA) due to formation of DPNH or TPNH (wavelength = 340 mμ, *d* = 1 cm). The final volume of 3 ml contained a mixture of the following components: 286 μmoles glycine–sodium hydroxide buffer, 2 mg protein, 1.5 μmoles DPN or TPN, and 0.15 μmole steroid. Unless otherwise specified the soluble fraction was isolated from liver homogenates of male rats. [b] Based on the increase in absorbancy (+ ΔA) per 0.5 minute. [c] The increase in absorbancy per 0.5 minute observed with the corresponding 3β-hydroxysteroid, DPN and soluble fraction (see Table I) is taken as 1.0. [d] 5α-H-pregnan-3α-ol-20-one was not available. [e] Etiocholan-3α-ol-17-one was not sufficiently soluble in the test solution. [f] Supernatant from liver homogenates of female rats.

tained after centrifuging at 8500 × *g* for 10 minutes, containing the microsomal and soluble fractions, caused approximately the same amount of epimerization of digitoxigenin as the unfractionated homogenate, but the microsomal fraction separated from this fraction was much less active, even when fortified with DPN or TPN, DPN being superior to TPN.

These observations favored the conclusion that the soluble enzymes of the clear supernatant fraction were largely involved in the process of epimerization. This fraction, therefore, was examined further. Previously, one type of soluble DPN-dependent 3β-hydroxysteroid dehydrogenase and two types of DPN- or TPN-dependent 3α-hydroxysteroid dehydrogenases, based on differences in *p*H-activity curves, had been found (Repke and Samuels, 1964). Since the same differences in *p*H optima were found for either *cis* or *trans* C/D ring junction, the rates of dehydrogenation of various 3-hydroxysteroids on incubation with the soluble fraction and DPN or TPN could be directly compared (Tables I and II).

Oxidation of 3β-Hydroxysteroids at pH 10.2 by DPN and the Soluble Fraction.—As previously observed, the soluble fraction seemed to contain a single type of 3β-

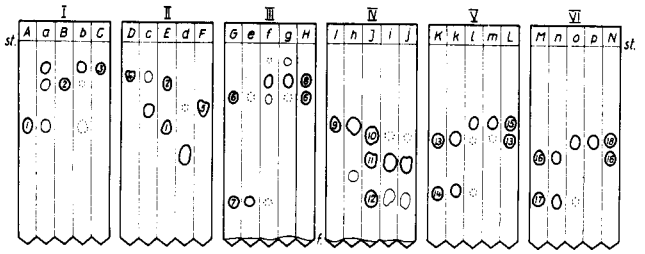

FIG. 1.—Examples of the identification by paper chromatography of the metabolites produced by incubating cardiac steroids with pyridine nucleotides and the soluble fraction from homogenates of male rat liver. On strips *A–N* are shown the mobilities of the following authentic compounds: (1) 3-dehydrodigitoxigenin, (2) digitoxigenin, (3) 3-epi-digitoxigenin, (4) 17β-H-digitoxigenin, (5) dihydrodigitoxigenin, (6) bufalin, (7) 3-dehydrobufalin, (8) 3-epi-bufalin, (9) xysmalogenin, (10) uzarigenin, (11) 3-epi-uzarigenin, (12) 3-dehydrouzarigenin, (13) digoxigenin, (14) 3-dehydro-digoxigenin, (15) 3-epi-digoxigenin, (16) gitoxigenin, (17) 3-dehydrogitoxigenin, (18) 3-epi-gitoxigenin. On strips *a-p* the migration of metabolites is documented. Incubation conditions determining the course of reactions: 3-dehydrodigitoxigenin and DPNH (*a*) or TPNH (*b*) at *p*H 7.5; 17β-H-digitoxigenin and DPN (*c*) or dihydrodigitoxigenin and DPN (*d*) at *p*H 10.2; bufalin and DPN at *p*H 10.2 (*e*); 3-dehydrobufalin and DPNH (*f*) or TPNH (*g*) at *p*H 7.5; xysmalogenin and DPN at *p*H 10.2 (*h*); 3-dehydro-uzarigenin and DPNH (*i*) or TPNH (*j*) at *p*H 7.5; digoxigenin and DPN at *p*H 10.2 (*k*); 3-dehydrodigoxigenin and DPNH (*l*) or TPNH (*m*) at *p*H 7.5; gitoxigenin and DPN at *p*H 10.2 (*n*); 3-dehydrogitoxigenin and DPNH (*o*) or TPNH (*p*) at *p*H 7.5. For further incubation details see explanatory captions of Tables I–III. The incubations were continued until the amount of metabolites formed seemed to be sufficient for paper chromatographic analysis. The enzymatic reactions were stopped by extraction of incubation mixtures with chloroform. The spots on these chromatograms are bounded by lines of different thickness or dotted lines indicating roughly the amount of substance found. St. = start, f. = front. Chromatographic systems: xylol/formamide, 12 hours (I and II) or 3.5 hours (III); benzene-chloroform (7:5)/formamide, 4 hours (IV); xylol–methyl ethyl ketone (1 : 1)/formamide, 18 hours (V) or 12.5 hours (VI). The location of cardenolide or bufadienolide derivatives was identified by their fluorescence reactions with trichloracetic acid and "chloramine" (Kaiser, 1955) or with antimony trichloride (Neher, 1958). Further examples of the paper chromatographic separation of cardenolide derivatives are illustrated in the accompanying paper (Repke and Samuels, 1964).

hydroxysteroid dehydrogenase which functions with DPN only (Repke and Samuels, 1964). This enzyme system used a wide variety of 3β-hydroxysteroids as substrates, but they were distinguished by their relative rates of dehydrogenation (Table I). The type of substitution at C-14 and C-17 did not have an important influence. 14β-Hydroxysteroids (e.g., nos. 1, 3, 11 in Table I) were dehydrogenated in the same range of rates as 14α-H-steroids (e.g., nos. 14, 15 in Table I). The β-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* was reported to oxidize a 14α-hydroxy derivative much more slowly than its 14α-H-congener (Talalay, 1957), but no such compound was available to us. Substitution of 17β-H by a butenolide ring (no. 1), a cumaline ring (no. 11), an oxygen (no. 14), or an acetyl side chain (no. 15) appeared to be of minor importance. The inversion of the butenolide ring from β to α position (no. 1 → 2) halved the oxidation velocity of 3β-OH, while the saturation of the double bond in this ring (no. 1 → 3) or the enlargement from a five-membered to a six-membered ring (no. 1 → 11) caused a distinct increase in rate under the conditions of these experiments.

In both the cardenolide and hormonal series A/B *cis*-steroids (nos. 1, 3, 11 and 14, 15) were dehydrogenated more rapidly than A/B *trans* isomers (nos. 4 and 16). The introduction of a double bond between C-5 and C-6 (nos. 1 → 5) reduced the oxidation velocity of 3β-OH even more than the presence of the A/B *trans* configuration. The introduction of unsaturation between C-4 and C-5 (no. 11 vs. no. 12) did not have as much effect. Additional hydroxy groups in positions 8β (no. 13), 12β (no. 8), 16β (no. 9), or 12β and 16β (no. 10) resulted in a marked drop in oxidation of 3β-OH, while hydroxy groups in positions 5β (no. 6) or 11α (no. 7) prevented any oxidation. A similar detrimental influence of additional hydroxy groups had been observed with the β-hydroxysteroid dehydrogenase from *Pseudomonas*, a 6β-OH being the only exception to this rule (Talalay, 1957).

The results of the spectroscopic examinations (Table I) were checked by paper chromatographic analyses of the reaction products (for examples see Fig. 1). When there was an increase in the absorbancy at 340 mμ, the expected 3-ketosteroid was found. The 3-dehydroderivatives of 17β-H- and dihydrodigitoxigenin have not been described. However their identity seems probable on the basis of the origin and the chromatographic behavior of the metabolites. Using the same solvent system on a single paper sheet, the migration distance of 3-dehydrodigitoxigenin and that of the metabolites of 17β-H-digitoxigenin and dihydrodigitoxigenin were found to be increased in respect to the corresponding genins by nearly identical factors: 2.12, 2.02, and 2.04, respectively. The metabolite of xysmalogenin which was found only after prolonged incubation may be identical with anhydroperiplogenone (double bond in Δ^4 position) and not with xysmalogenone (double bond in Δ^5 position) because there is a highly active Δ^5-3-ketosteroid isomerase in the soluble fraction of rat liver (Talalay and Wang, 1955). This conclusion is substantiated by comparing the $R_{\text{xysmalogenin}}$ values of the respective compounds in the same solvent system. For the isolated metabolite this value amounts to 1.60 which is nearly the same as that described for anhydroperiplogenone (1.76), but quite different from that of xysmalogenone (2.02) (Polonia *et al.*, 1959).

*Oxidation of 3α-Hydroxysteroids at p*H *8.6 or 9.6 by DPN or TPN and the Soluble Fraction.*—There were apparently two types of 3α-hydroxysteroid dehydrogenases in this fraction, both DPN- and TPN-dependent enzymes (Repke and Samuels, 1964), but for the sake of comparison the structure-activity relations with the different coenzymes will be discussed together (Table II). The type of substitution at C-14 and C-17 (compare nos. 17 and 21) again did not have a major influence on the oxidation velocities. In contrast with the 3β-dehydrogenase (Table I), the two α enzymes from liver showed no great differences in oxidation velocity between A/B *cis*-steroids (nos. 17, 21) and A/B *trans*-steroids (nos. 18, 22). Depending on their position, the influence of additional hydroxy groups on the oxidation of 3α-OH differed markedly, 12β-OH (no. 19) being again very detrimental but 16β-OH (no. 20) showing no influence.

The TPN-dependent 3α-hydroxysteroid dehydrogenase proved to be fifteen to forty times more active than the DPN-dependent enzyme if compared at their *p*H optima; i..e, *p*H 9.6 and 8.6, respectively. Compared with the DPN-dependent 3β-hydroxysteroid dehydrogenase (optimum at *p*H 10.2), the TPN-dependent 3α-enzyme was always more active with the different substrates (Table II, right-hand column under the caption Relative Rates).

TABLE III
REDUCTION VELOCITIES OF 3-KETOSTEROIDS[a]

	Steroids	Reduction by DPNH: − ΔA per 0.5 min	Reduction by DPNH: − ΔA per 5.0 min	Reduction by DPNH: Relative Rates	Reduction by TPNH: − ΔA per 0.5 min	Reduction by TPNH: − ΔA per 5.0 min	Reduction by TPNH: Relative Rates	
	Cardioactive Type (14β-OH)							
23.	3-Dehydrodigitoxigenin	0.025	0.161	1.0[b]	0.104	0.292	1.0[b]	4.2[c]
24.	3-Dehydrouzarigenin	0.016	0.114	0.6	0.027	0.200	0.3	1.7
25.	3-Dehydrobufalin	0.022	0.150	0.9	0.093	0.294	0.9	4.2
	Hormone Type (14α-H)[d]							
26.	5β-Pregnane-3,20-dione	0.053	0.248	2.1	0.061	0.286	0.6	1.2
27.	5β-Pregnan-3α-ol-20-one	0	0	0				
28.	Etiocholan-17β-ol-3-one	0.051	0.236	2.0	0.094	0.272	0.9	1.9
29.	Androstan-17β-ol-3-one	0.011	0.093	0.4	0.024	0.176	0.2	2.2

[a] On incubation with DPNH or TPNH and the soluble fraction from liver homogenates of male rats at *p*H 7.5 and 37.5°, based on the decrease in absorbancy (− ΔA) due to disappearance of reduced pyridine nucleotide (mean value of double determinations; wavelength = 340 mμ, *d* = 1 cm). The final volume of 3 ml contained a mixture of the following components: 135 μmoles Tris buffer, 10 mg protein, 0.25 μmole DPNH or TPNH, and 0.15 μmole steroid. [b] Based on the decrease in absorbancy (− ΔA) per 0.5 minute. [c] The decrease in absorbancy (− ΔA) per 0.5 minute observed with the corresponding 3-ketosteroid, DPNH, and soluble fraction is taken as 1.0. [d] 5α-H-Pregnane-3-20-dione, cholestan-3-one, and coprostan-3-one were not sufficiently soluble in the test solution.

Reduction of 3-Ketosteroids at pH 7.5 by DPNH or TPNH and the Soluble Fraction.—Since the soluble fraction contains both a 3α- and a 3β-hydroxysteroid dehydrogenase requiring DPN as coenzyme, the products of ketosteroid reduction by DPNH could be both 3α- and 3β-hydroxysteroids. Surprisingly enough, paper chromatographic analysis revealed that the 3-ketosteroids of cardioactive and hormone types yielded 3α-hydroxymetabolites almost exclusively (for examples see Fig. 1). Hence, it must be assumed that at physiological *p*H DPNH combines with the 3α-enzyme much more readily than with the 3β rival. The reduction rates of 3-ketosteroids by DPNH and the soluble fraction (Table III), therefore, mainly reflect the activity of the 3α-hydroxysteroid dehydrogenase. A/B *cis*-steroids (nos. 23, 28) were reduced more rapidy than their A/B *trans* isomers (nos. 24, 29). Similar structure-activity relations had been found earlier using a partially purified enzyme preparation (Tomkins, 1956). An isooctyl side chain at C-17 prevented the reduction of 3-ketosteroids in the presence of the enzyme preparation, while a six-membered lactone ring at the same position (no. 25) did not disturb the reaction. A keto group at C-20 (no. 27) was not reduced by DPNH and the soluble fraction.

With TPNH, as with DPNH, the enzymes of the soluble fraction reduced A/B *cis*-ketosteroids (nos. 23 and 28 in Table III) more actively than their A/B *trans* isomers (nos. 24 and 29). The relative effectiveness of the coenzymes, however, was quite different if the reduction rates of cardioactive and hormone-type steroids are compared. The reduction of 3-ketosteroids by TPNH should yield their 3α derivatives exclusively because in the soluble fraction there is only the TPN-dependent type of 3α-enzyme (Repke and Samuels, 1964). The paper chromatographic analysis of the reduction products from 3-ketosteroids of cardioactive and hormone types, however, proved that, in addition to overwhelming amounts of 3α-hydroxysteroids, in some cases traces of 3β analogs were also formed (Fig. 1). This fact may tentatively be explained by assuming that the high-speed supernatant used was contaminated by the microsomal fraction which in subsequent experiments was shown to contain a TPN-dependent 3β-hydroxysteroid dehydrogenase. The enzymatic reduction of 3-dehydrobufalin by DPNH, and especially by TPNH, yielded an additional metabolite which is unidentified as yet ($R_{Bufalin}$ = 0.45).

Preliminary Experiments on the Oxidation of 3-Hydroxysteroids by the Microsomal Fraction and DPN or TPN at pH 9.6.—The spectrophotometric determinations were done in the presence of KCN (Hurlock and Talalay, 1959) to prevent the reoxidation of DPNH or TPNH formed in the course of the reactions. The possibility cannot be excluded, however, that cyanide may also inhibit the DPN- or TPN-dependent hydroxysteroid dehydrogenases (cf. Meyerhof and Kaplan, 1952). Thus the quantitative significance of the rates in Table IV should be regarded with some reservation, particularly as the *p*H optima of the enzymes may not be identical and perhaps are far from the somewhat arbitrary *p*H used in these studies. Yet the results suggest that the microsomal fraction from livers of rats contains both DPN- and TPN-dependent dehydrogenases for 3β- as well as 3α-hydroxysteroids.

TABLE IV
OXIDATION VELOCITIES OF 3-HYDROXYSTEROIDS[a]

Steroids	Oxidation by DPN: + ΔA per 5.0 min M	Oxidation by DPN: + ΔA per 5.0 min F	Oxidation by TPN: + ΔA per 5.0 min M	Oxidation by TPN: + ΔA per 5.0 min F
Androstan-3β-ol-17-one	0.014	0.005	0.031	0.000
Androstan-3α-ol-17-one	0.054	0.018	0.026	0.056

[a] Incubated with DPN or TPN and fresh microsomal fraction at *p*H 9.6, based on the increase in absorbancy (+ ΔA) due to formation of DPNH or TPNH (wavelength = 340 mμ, *d* = 1 cm). The final volume of 3 ml contained a mixture of the following components: 286 μmoles glycine–sodium hydroxide buffer, 10 μmoles KCN, 2 mg protein, 1.5 μmoles DPN or TPN, and 0.15 μmole steroid. The microsomal fraction was isolated from liver homogenates of male or female rats, denoted as M or F in the table.

The particulate β-enzymes apparently were more active in the livers of males than in the livers of females. As far as the DPN-dependent enzyme is concerned, this result confirms an earlier statement of Rubin and Strecker (1961). The 3α-enzymes, too, showed sex differences in their activities. Using androstan-3α-ol-17-one as substrate, the DPN-dependent dehydrogenase apparently was more active in

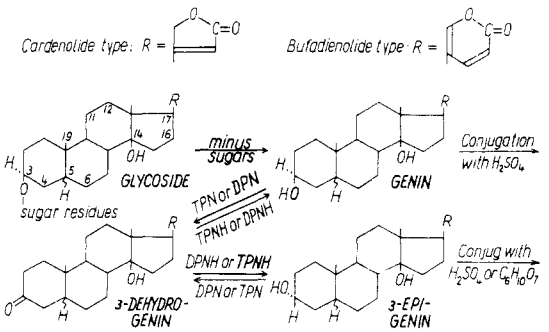

FIG. 2.—Schematic representation of epimerization and conjugation of genins occurring after glycoside fission in the liver of the rat.

males while the TPN-dependent enzyme seemed to prevail in females.

DISCUSSION

The oxidation-reduction reactions at C-3 of the steroids studied in this paper are catalyzed by DPN- or TPN-dependent 3-hydroxysteroid dehydrogenases. At physiological *p*H the kinetics of such reactions are determined by the amounts of oxidized and reduced pyridine nucleotides bound to the enzyme protein, while the thermodynamics are dependent on their free concentrations (Holzer *et al.*, 1956). Only the over-all levels of nucleotides in rat liver cells are known. According to recent determinations the DPNH/DPN$^+$ ratio amounts to about 0.3 (Bassham *et al.*, 1959; Slater and Sawyer, 1962) and the TPNH/TPN$^+$ ratio lies in the range between 2 (Bassham *et al.*, 1959) and 8 (Slater and Sawyer, 1962). In liver homogenates which had been incubated for 30 minutes in the presence of 0.1 M nicotinamide at 38° with air as gas phase, the first ratio was found to be reduced distinctly to a still lower value, while the second one was increased quite considerably to a much higher value (Bassham *et al.*, 1959). Hence in the following discussion the oxidation of 3-hydroxysteroids in the intact liver cell or with liver homogenates will be assumed to be effected predominantly with DPN as cofactor. On the other hand the reduction of 3-ketosteroids will be considered to be associated with TPNH as cofactor under the same conditions. These assumptions also rely on the suggestion (Kaplan *et al.*, 1956; Foster and Bloom, 1961) that DPNH serves primarily in the cell as a source of energy production and that TPNH is the primary source of hydrogen for reductive processes.

Sex Differences.—3-Ketosteroids of the A/B *trans* (5α-H) type, added as substrates or arising in the course of biotransformation, are reduced to 3α- and 3β-hydroxy compounds in characteristically differing amounts by homogenates of livers from female and male rats. With homogenates from *females* the 3α-derivatives have been found to prevail by a factor of about 5 (Rubin, 1957; Kupfer *et al.*, 1960) or to be the only products (Taylor, 1954; Bell *et al.*, 1962). These facts may readily be explained by comparing the relative rates of the three types of TPN-dependent enzymes in female livers (Tables II and IV). The 3β-hydroxysteroid dehydrogenase of the microsomal fraction appears to be absent or to have an exceptionally low activity, while the activity of the 3α-enzymes in the particulate and soluble fractions appears to be twice as high as that of the corresponding enzymes in male livers.

On using homogenates of *male* livers 3β-hydroxysteroid metabolites have been reported either to predominate by a factor of about 2 (Rubin, 1957), to be formed in almost equal amounts (Bell *et al.*, 1962), or, with added excess TPNH, to constitute about one-third of the 3α isomer (Wettstein *et al.*, 1959). The particles of liver homogenates sedimenting between 7000 and 26,000 $\times$ *g*, on incubation with androstane-3,17-dione and DPNH, produced two to five times as much androstan-3β-ol-17-one when obtained from male rats as when obtained from female rats (Rubin and Strecker, 1961). Actually, however, TPNH should be the major hydrogen supplier. The present studies (Table IV) indicate that TPN-dependent types of 3β-hydroxysteroid dehydrogenase exist in the microsomal fraction of liver homogenates from males, but the activity does not seem to be greater than that of the TPN-dependent α-enzyme of the same fraction, judged from the oxidation velocities of their substrates. The reduction of 3-ketosteroids by this fraction and TPNH, however, yielded almost exclusively 3β-hydroxy compounds (McGuire and Tomkins, 1959). TPNH accordingly seems to combine preferentially with the β-enzyme of the microsomal fraction from male livers.

3-Ketosteroids of hormone type with A/B *cis*-ring juncture (5β-H-) were reduced only to 3α-hydroxy metabolites by liver homogenates from both female and male rats (Rubin, 1957; Bell *et al.*, 1962; Wettstein *et al.*, 1959). In accord with this fact, it had been observed[5] that the 5β-H-cardenolide genins were epimerized by slices from livers of both sexes. The TPN-dependent 3α-hydroxysteroid dehydrogenases have been reported to be distributed in all cell fractions; i.e., the nuclear, mitochondrial, microsomal, and supernatant fractions of male liver homogenates fortified with a TPNH-regenerating system in all cases yielded only 3α-hydroxy metabolites from cortisol (DeVenuto and Westphal, 1961). As far as the soluble fraction is concerned, this statement agrees with our observations made on 3-ketosteroids of hormone and cardioactive type.

Enzymatic Mechanism of Epimerization.—Two hypotheses have been proposed to explain the epimerization of the hydroxy group at carbon 3 occurring during the conversion of cholesterol to bile acids (Rosenfeld and Hellman, 1961): replacement of the original hydroxy group at C-3 with inversion by attack of a second OH from the opposite side, or oxidation of 3β-OH to a ketone followed by reduction to an alcohol with the opposite configuration. In the case of cholesterol the second alternative was found to be true (Rosenfeld and Hellman, 1961). The two possibilities must also be taken into account for the epimerization of cardioactive steroids in the animal body. In studies on the metabolic transformation of some genins by rat liver, carried out *in vivo* (Repke, 1962) and *in vitro* with liver slices (Lauterbach and Repke, 1960) or homogenates (this paper), their 3-keto derivatives were never found. This fact might speak in favor of the first possibility. The failure to detect the ketones as intermediary products, however, can readily be explained. With all substrates studied, the activity of the DPN-dependent 3β-hydroxysteroid dehydrogenase was always considerably weaker than that of the TPN-dependent 3α-enzyme (Tables I and II). Thus, the 3-ketone formed by dehydrogenation with the DPN-dependent enzyme would immediately be reduced by the TPNH-dependent enzyme under physiological conditions. However, during incubations of genins with cell preparations and oxidized pyridine nucleotides at an elevated *p*H, the 3-dehydro deriva-

[5] K. Repke, to be published.

tives were easily detected. Thus, the scheme of Figure 2 apparently correctly depicts the steps of epimerization in the intact liver cell which occur after splitting of the genins from the glycosides.

The genins of the cardiac glycosides usually possess an A/B *cis*-ring configuration (Tamm, 1956), but there are some representatives like uzarigenin with an A/B *trans* structure. As already pointed out, the A/B *trans*-steroids are handled differently from their A/B *cis* isomers by the liver enzymes of male rats. Liver slices from male rats metabolized uzarigenin more slowly than digitoxigenin,[6] but both were transformed into the 3α-OH derivatives. It seems justified, therefore, to leave the A/B-ring junction in the chemical formulas of Figure 2 unspecified.

Biological Significance of Epimerization.—The enzyme system from liver, which in the presence of hormone type steroids may function as a transhydrogenase (Hurlock and Talalay, 1958), likewise uses cardioactive steroids for transferring hydrogen from TPNH to DPN (Repke and Lauterbach, 1960). This ability to use the genins may possibly be connected with the oxidation-reduction processes at carbon-3 which occur in epimerization (Fig. 2). There is no positive correlation between their effectiveness in hydrogen transfer, however, and their biological activity (Repke and Lauterbach, 1960). As previously mentioned, the enzymes of epimerization can barely be traced in the heart muscle. Hence the genin effect on transhydrogenation seems not to be related to the metabolic effects or to the mechanism of action of the cardioactive steroids.

The epimerization of the genins, rather, can be regarded as a process of detoxification because the 3-dehydro- and 3-epimetabolites exert biological effects which are much weaker or almost negligible in comparison with the parent compounds (Repke, 1961). The detoxification of the epi- derivatives is then completed by formation of their glucuronides (Lauterbach and Repke, 1960) or sulfates (Herrmann and Repke, 1962). On the whole, the short lifetime of aglycones in the body (Repke, 1962) apparently is due to the rapid processes of epimerization and conjugation as pictured in Figure 2. Hence, these detoxification processes may explain the extremely transistory nature of biological effects which distinguish genins afrom glycosides.

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[6] K. Repke, to be published.