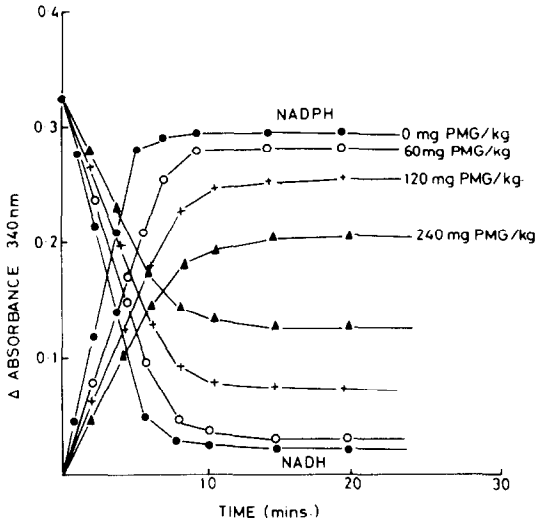

Fig. 2. Pattern of ATP-driven nicotinamide nucleotide transhydrogenase reaction in intact mitochondria fraction isolated from the livers of rats 5 hr after intraperitoneal dosing with *N*-(phosphonomethyl) glycine (PMG). Reaction medium same as described in text except that 0.3 mM ATP was added in place of 5 mM sodium succinate.

increased as the dose of the herbicide was raised to 240 mg PMG/kg which gave maximal inhibitions of 49 and 58% respectively for the ATP- and respiration-driven reactions. The levels of NADPH formed, 10 min after the start of the reactions decreased by at least 17.6% and 33.4% respectively at 120 mg PMG/kg and 240 mg PMG/kg dosage levels when compared to controls. These results suggest that like 2,4-dinitrophenol, a classical uncoupler, PMG induces a defect in the energy-linked nicotinamide nucleotide transhydrogenase reaction.

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The effect of spironolactone pretreatment on digitoxin metabolism in isolated hepatocytes from male and female rats

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In a previous paper [1], we reported a preliminary result for digitoxin (Dt3*) metabolism in isolated rat hepatocyte preparations. In order to establish the validity of this system for the study of Dt3 metabolism, the present study was designed to test the effect of spironolactone (SP) pretreatment on Dt3 metabolism in isolated rat hepatocyte preparations obtained from female and male rats.

[^{3}H] Dt3 (10.9 Ci/mmole, generally labeled) and [1α, 2α-^{3}H]cholesterol (57 Ci/mole) were purchased from New England Nuclear (Boston, MA). Unlabeled Dt3 and Dg3 were from Merck (Darmstadt, West Germany), Dt2, Dt1, Dt0, Dg2, Dg1 and Dg0 from Boehringer Mannheim (Mannheim, West Germany), collagenase (type I) from Sigma (St. Louis, MO). Thirteen-week-old Wistar derived rats of both sexes were used.

Aldactone A tablets (Dainihonseiyaku, Osaka, Japan) were ground into powder and suspended in distilled water. This suspension was given orally (10 mg/100 g body wt as a SP dose) twice daily for 4 consecutive days. About 18 to 20 hr after the last dose, rats were used for the preparation of isolated hepatocytes.

The isolation of rat hepatocytes was performed according to the method described previously [1]. After preparation of isolated hepatocytes, the cells were resuspended in Waymouth MB 752/1 medium containing HEPES, alanine and serine. A [^{3}H] Dt3 DMSO solution with various amounts of unlabeled Dt3 was added to the cell suspension. The range of the concentration of Dt3 in the medium was from

* Abbreviations used: Dt3, digitoxin; Dt2, digitoxigenin bis-digitoxoside; Dt1, digitoxigenin *mono*-digitoxiside; Dt0, digitoxigenin; Dg3, digoxin; Dg2, digoxigenin *bis*-digitoxoside; Dg1, digoxigenin *mono*-digitoxoside; Dg0, digoxigenin; DMSO, dimethyl sulfoxide; SP, spironolactone.

Table 1. Rates of increase of concentrations of digitoxin metabolites in isolated hepatocyte preparations

Rat group		Dt3	Dt2	Dt1 (pmoles/min/10^6 cells)	Dt0	Dg3	Dg2	Origin
Control female	(5)*	−0.70 ± 0.17	0.32 ± 0.05	0.01 ± 0.02	−0.02 ± 0.02	0.36 ± 0.19	0.04 ± 0.04	0.01 ± 0.01
Spironolactone treated female	(7)	−7.06 ± 2.71	6.15 ± 2.47	0.16 ± 0.15	0.00 ± 0.01	0.48 ± 0.16	0.27 ± 0.23	0.08 ± 0.05
Control male	(6)	−6.25 ± 1.47	5.22 ± 1.29	0.20 ± 0.09	0.02 ± 0.03	0.40 ± 0.14	0.18 ± 0.14	0.09 ± 0.08
Spironolactone treated male	(6)	−41.22 ± 6.85	38.48 ± 6.75	2.50 ± 1.00	0.15 ± 0.19	0.46 ± 0.18	0.31 ± 0.32	0.41 ± 0.28

* Number in parentheses indicates the number of rats studied.
The initial concentration of Dt3 was 2.5 μM. Values were calculated from the initial changes in metabolite concn, which could be regarded to be linear with time. The incubation periods used for calculation were 60 min for control and SP-pretreated female rat studies, 30 min for control male rat studies and 15 min for SP-pretreated male rat studies.
All data are expressed as mean ±S.D.

0.6–125 μM. The incubation was carried out at 37° in an atmosphere of 95% O_2 and 5% CO_2 with constant shaking.

Immediately after the addition of [^{3}H] Dt3 in the medium, duplicate samples (each 0.5 ml) were withdrawn as 0-min samples. Duplicate samples were successively withdrawn from the medium with appropriate time intervals and were analysed for Dt3 metabolites by the method previously reported [1].

The velocity of Dt3 degradation was calculated from the initial part of the disappearance curve of Dt3 radioactivity in the medium which could be regarded as linear with time. From the velocity thus obtained a kinetic analysis was performed to obtain parameters (K_m, V_{max}) for Dt3 degradation by using the non-linear least square method described by Wilkinson [2], with minor modifications for a PANAFACOM U-300 computor. We then selected the best fit to the Michaelis–Menten equation.

Statistical significance was analysed by the Student's unpaired *t*-test. Cleland's method [3] was used for the comparison of apparent K_m and V_{max} values between different groups.

Rates of increase in the concentrations of Dt3 metabolites in studies with an initial Dt3 concentration of 2.5 μM are shown in Table 1. Table 2 shows the kinetic parameters for Dt3 degradation calculated using a computer. In control female rats, the V_{max} value for Dt3 degradation was lowest but it was increased nearly 5 times by SP pretreatment. The V_{max} value for control male rats was more than 10 times higher than the control female value, and this was further increased 2.3 times by SP pretreatment. Interestingly, apparent K_m values of both female and male rats decreased with SP pretreatment resulting in a very small difference between SP-pretreatment male and female values ($P > 0.5$), although in control rats the male value was 2 times higher than the female value ($P < 0.005$).

The results of the present study revealed that isolated rat hepatocyte preparation can efficiently metabolize Dt3 and convert it to a variety of metabolites. The predominant metabolite found in most of the studies was Dt2 which occupied about 70–90% of the total metabolites found in incubation preparations from control male and SP treated female and male rats (Table 1). Only in preparations obtained from control female rats did the concentration of Dg3 slightly exceed that of Dt2. These observations are in good agreement with previous *in vitro* studies reporting that the major metabolite of Dt3 by rat liver preparations was Dt2 [1, 4, 5] as well as results of *in vivo* studies that the major metabolite found in the chloroform soluble fraction in the bile of the male rats was Dt2 [6, 7].

In preparations obtained from control female rats, the proportion of Dg3 related metabolites (i.e. Dg3, Dg2) was relatively large compared with the other three rat groups which agrees with *in vivo* observations for female rat bile [8] and feces [9].

On the other hand, the extremely small proportions of conjugated metabolites found in incubation preparations obtained from all the four rat groups (less than 2% of total Dt3 metabolites), agree well with our previous study with isolated hepatocyte preparations from Wistar male rats [1] and with Castle's work on both isolated hepatocyte preparations and microsomal preparations enriched with a NADPH generating system [4] but are in marked contrast with what has been reported for Dt3 metabolites in the rat bile in *in vivo* studies [6–8, 10].

The percentage of conjugated metabolites in the bile was reported to be near or over 50% and it was further increased by SP pretreatment [6, 10]. The reason why isolated rat hepatocytes make so few conjugates of Dt3 metabolites compared to *in vivo* liver remains unknown.

Another finding with regard to the change in metabolite production in the present study was a more than 10-fold increase in the appearance of Dt3 related metabolites (i.e. Dt2, Dt1, Dt0) but only a 1.4 to 2-fold (insignificant)

Table 2. Apparent kinetic parameters for digitoxin degradation in isolated hepatocyte preparation

Rat group		V_{max} (pmoles/min/10^6 cells)	K_m(μM)
Control female	(7)*	9.7 ± 0.7	29.9 ± 2.8
Spironolactone treated female	(7)	48.8 ± 2.1	14.1 ± 1.4
Control male	(11)	142.0 ± 8.2	61.9 ± 4.9
Spironolactone treated male	(5)	321.0 ± 12.0	15.9 ± 0.8

* Number in parentheses indicates the number of rats studied.
Values are expressed as mean ± S.E. (calculated by nonlinear regression).

increase in Dg3 related metabolites with SP pretreatment in both female and male rats (Table 1). This is in accord with Schmoldt's observations using liver microsomal preparations from male rats [11]. However, our results do not support the claim by Vöhringer *et al.* [10] that SP pretreatment reduced the production of Dg3 related metabolites in female rats.

Schmoldt has shown that both sugar moiety cleavage and 12β-hydroxylation are mediated by the hepatic cytochrome P-450 system [11, 12]. It is well known that unlike phenobarbital, SP has different effects on the hepatic microsomal P-450 system in rats depending on sex [13]. Most of the drug metabolizing enzyme activities dependent on P-450 were decreased in male rat liver except for ethylmorphine demethylation, which increased 1.4 times in male rats while most of the activities studied increased in female rat liver [13]. The marked inducing effect of SP pretreatment on Dt3 metabolism in particular for hydrolysis of sugar chain in both female and male rat livers appears to be rather an exceptional case among P-450 dependent enzyme activities as SP effect in rats.

The relative relationship of V_{max} values found in different rat groups in the present study is comparable with that of percent recovery of the i.v. injected Dt3 activity in the bile in corresponding rat groups studied by ourselves [14]. This may suggest that, at least quantitatively, the biotransformation velocity for Dt3 in isolated hepatocytes reflects the hepatic Dt3 metabolism *in vivo* and thus provides more detailed information in terms of kinetic parameters.

Large differences in apparent K_m values between different rat groups suggest that the hepatic clearance of Dt3 from plasma is determined not only by the change in V_{max} value but by the changes in apparent K_m values which were shown to occur by SP pretreatment as the hepatic clearance of a drug like Dt3 is determined mainly by the change in a free intrinsic clearance which is a function of not only V_{max} but apparent K_m value (V_{max}/K_m) [15]. This also explains our observation on Dt3 metabolism rates in control male and SP treated female rat groups. Values with lower substrate concentration (Table 1) were very close to each other, while V_{max} for control males was 3 times as high as that of treated females (Table 2).

In conclusion, by using rat hepatocyte preparations, the kinetic study of Dt3 biotransformation appears to be possible. Quantitatively the data thus obtained agrees reasonably with *in vivo* data previously obtained. However, the reason for the much smaller proportion of conjugated metabolites found in isolated hepatocyte preparations compared with previously reported *in vivo* data remains to be elucidated.

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