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The kinetics of 4-nitrophenol conjugation by perfused livers and hepatic microsomes from streptozocin-induced diabetic rats

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The formation of both glucuronide and sulphate conjugates of 4-nitrophenol is deficient in perfused livers from male diabetic rats. Experiments with 'native' hepatic microsomes demonstrated that the defect in glucuronidation is due to a decrease in the maximal velocity of the reaction. There is no alteration in the affinity of the glucuronyltransferase for 4-nitrophenol. Non-linear regression analysis of the 4-nitrophenol liver perfusate concentrations showed that the elimination follows saturable Michaelis-Menten kinetics. Clearance values in 'native' microsomal preparations and in perfused livers were calculated and found to be similar in both systems. This provides evidence that glucuronyltransferase is 'native' in the intact liver.

There have been reports recently of a decrease in glucuronyltransferase activity in freshly prepared untreated ('native') hepatic microsomes isolated from streptozocin-induced diabetic rats (Hawksworth & Morrison 1981, 1982; Morrison & Hawksworth 1982, 1984; Rouer et al 1981). This diabetes-induced defect in transferase activity was abolished by maximal detergent (Triton X-100) activation of the microsomes. In contrast, allosteric activation of the enzyme by either UDP *N*-acetyl glucosamine and/or magnesium ions did not significantly alter the defect (Morrison & Hawksworth 1982). Increased latency of the transferase enzyme in streptozocin-induced diabetes was thought to be responsible for the defect. The relevance of these results to metabolism in diabetes mellitus was emphasized when defects in hepatic microsomal conjugation and oxidative metabolism were also observed in spontaneously diabetic rats and mice (Rouer et al 1981; Warren et al 1983); although the results demonstrated that drug metabolism was altered in diabetic animals, they were confined to microsomal preparations.

The prediction of drug metabolism in the whole animal from in-vitro hepatic microsomal preparations is difficult where membrane-bound enzymes, such as glucuronyltransferase, are involved in the metabolism. The activity of glucuronyltransferase can be readily influenced by perturbations of the membrane environment, the presence of allosteric activators and inhibitors, and in-vitro sonication and storage of microsomal preparations. The extent to which the transferase is activated or latent in-vivo is not clear, and conflicting reports appear in the literature (Moldéus et al 1976; Otani et al 1976; Winsnes & Dutton 1973; Andersson et al 1978).

The present study was undertaken to investigate the activity of glucuronyltransferase in perfused livers isolated from streptozocin-induced diabetic rats and to compare glucuronidation rates in perfused livers with microsomal transferase activity. Metabolic studies with perfused livers have been reported to correlate well with those carried out in-vivo (von Bahr et al 1970; Bickel & Minder 1970). The substrate for glucuronidation in the present study was 4-nitrophenol, a substrate for the 3-methylcholanthrene-inducible form of the transferase. The glucuronidation of 4-nitrophenol has been previously shown to be deficient in streptozocin-treated rat liver microsomes (Morrison & Hawksworth 1984).

METHODS

Streptozocin treatment

Male, Sprague-Dawley rats (180–200 g) were starved for 24 h before receiving 60 mg kg^{-1} streptozocin, intravenously, in acetate buffer, pH 4·5, on day 1. Controls received an equivalent volume of the vehicle (0·1 ml/100 g). Thereafter, for the duration of the experiment, rats had free access to food and water. On day 6 after streptozocin administration the rats were divided into two groups. Livers were

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isolated and perfused or microsomes were prepared, and glucuronyltransferase activity measured in the first group. The second group received daily treatment with 16 iu insulin, subcutaneously, for 6 days before the hepatic glucuronyltransferase activity was measured on day 12.

Assessment of the induced diabetes

Blood and urinary glucose concentrations were determined by a specific enzymatic method using *o*-dianisidine as the chromogen according to Sigma Technical Bulletin No 310. On day 6 streptozocin-treated rats had a blood glucose concentration of 380 ± 26 mg/100 ml (n = 8) compared with 99 + 3 mg/100 ml in control rats. Between 4 and 6 days after treatment diabetic rats excreted approximately 16 g glucose/24 h in the urine. All the signs of streptozocin-induced diabetes were readily reversed within 48 h of initiating insulin treatment.

Microsomal preparation and incubation

Hepatic microsomal fractions were prepared by a modification of conventional ultracentrifugation methods (Eriksson et al 1978) involving the addition of 15% v/v glycerol to the buffer in which the liver is homogenized (Ichikawa & Yamano 1967). Incubations were carried out as described previously (Morrison & Hawksworth 1984) and concentrations of 4-nitrophenol between 0·1 and 1·0 mM were used to estimate the Michaelis-Menten constants, V_{max} and K_m.

Recovery of total liver glucuronyltransferase activity in microsomal preparations was estimated by comparing the activity of the optimally activated (Triton X-100) transferase in 0·25% (w/v) liver homogenate and in microsomal preparations. Homogenate formed 370·9 nmol glucuronide min^{-1} g^{-1} liver, compared with 133·2 nmol min^{-1} g^{-1} liver in microsomal fractions. This recovery of 36% was incorporated into the calculation of clearance values from microsomal preparations.

The perfusion system

Livers were perfused by a recirculating system essentially as described by Ross (1972). During perfusion the medium entered the liver vasculature via the portal vein, and flowed out from the liver through a cannula inserted into the vena cava. Humid 95% O_2/5% CO_2 was supplied to the system and the temperature was maintained at 37 °C.

The medium was based on Krebs-Henseleit buffer solution containing 2·5% (w/v) bovine serum albumin to maintain the osmotic pressure and provide the physiological binding protein. Expired human red blood cells, 3 to 5 weeks old, were obtained from Aberdeen Royal Infirmary Transfusion Service. They were washed twice with Krebs-Henseleit buffer, pH 7·4, to remove the citrate anticoagulant and added to the medium at a concentration of 12·5% (v/v). This gave a haematocrit reading of between 8 and 9 and a haemoglobin content of 2·5%. Pularin heparin, 10 iu ml^{-1}, was added to the medium to prevent blockage of the cannulae. Finally, the pH of the medium was adjusted to pH 7·4.

Operating procedure

Rats were anaesthetized by intraperitoneal injection of 1·5 g kg^{-1} ethyl carbamate. The bile duct was cannulated using polythene tubing (bore 0·58 mm, external diameter 0·96 mm), and the portal vein cannulated using polythene tubing of bore 1 mm, external diameter 2 mm. The inferior vena cava was cannulated above the diaphragm using polythene tubing of bore 1·57 mm, external diameter 2·08 mm.

The liver was dissected from the body, attached to the perfusion system, and the flow rate of the medium through the liver allowed to proceed at a maximum rate during the first 10–15 min. It was then adjusted to a physiological flow rate of 1 to 1·5 ml min^{-1} g^{-1} liver.

During perfusion the medium pH, flow rate and perfusion pressure remained constant. Bile flowed at between 30 and 50 μl h^{-1} g^{-1} liver for at least 2·5 h.

Measurement of 4-nitrophenol conjugation

Livers were left to equilibrate in the perfusion system for 30 min, and during this time a blank bile sample was collected. 4-Nitrophenol (0·5 mM) was added to the reservoir (total perfusate volume = 100 ml) to simulate intravenous dosing in-vivo. Samples (1 ml) of medium were removed for analysis of 4-nitrophenol and conjugates, and bile samples were collected at 30 min intervals throughout the experiment.

Samples were spiked with 1 mM phenolphthalein as internal standard, and centrifuged to remove blood cells. Methanol (2 vol) was added to the supernatant to precipitate proteins. Before analysing the samples by HPLC, it was necessary to treat them further to remove lipid contamination thought to originate largely from the albumin. After methanol precipitation, 1 ml of the supernatant was adjusted to pH 8–9 with 20 μl of 0·5 M NaOH, 3 ml hexane added, and the tubes shaken mechanically for 30 min. The hexane layer was removed and the aqueous

layer re-adjusted to pH 7 with 20 μl of 0·5 M HCl. Samples were filtered before HPLC analysis using a 1 cm Millipore filter of pore size 0·45 μm. Recovery of 4-nitrophenol, its glucuronide and sulphate conjugates, and the internal standard was over 90%. After appropriate dilution, the bile samples were treated as described for the perfusate samples.

Reversed-phase ion-pair HPLC was used to quantify 4-nitrophenol and its conjugates (Karakaya & Carter 1980). Detection of the compounds was by their ultraviolet absorbance at 280 nm, which favoured the detection of the conjugates. Quantitation was by peak-height ratios, and standard curves were linear for all the eluents at the concentrations measured.

Equilibrium dialysis

Binding of 4-nitrophenol to microsomal protein in incubation mixtures, and to perfusion medium was quantified by equilibrium dialysis (Bridges & Wilson 1976) using MSE Dianorm equipment.

RESULTS AND DISCUSSION

Fig. 1 shows the formation of 4-nitrophenol glucuronide in 'native' microsomes from control and diabetic rats. There is a significant difference in the microsomal enzyme activity between control and diabetic rats throughout the concentration range of 4-nitrophenol studied.

The Michaelis-Menten parameters, K_m and V_{max} (Table 1), were calculated from these data by non-linear regression analysis using BMDP/PAR derivative-free non-linear regression analysis (Ralston 1981). In 'native' microsomes there is no significant difference in the affinity of the enzyme for 4-nitrophenol in control and diabetic rat microsomes. The deficient glucuronidation of 4-nitrophenol in the diabetic rat 'native' microsomes is due to a decrease in V_{max}.

Table 1. Values of K_m and V_{max} in microsomes.

	K_m	V_{max}
Control	0·253 ± 0·029 (4)	3·51 ± 0·32 (4)
Diabetic	0·253 ± 0·060 (5)	2·17 ± 0·19* (5)

K_m is mM and V_{max} is nmol glucuronide formed (mg microsomal protein)$^{-1}$ min^{-1}. Results are mean ± s.e. m., with the number of determinations given in parentheses. * $P < 0·02$, using a non-paired Student's *t*-test.

Fig. 2 shows the concentrations of 4-nitrophenol in the perfusates of livers isolated from control and streptozocin-treated rats. At all times after 10 min, 4-nitrophenol concentrations were higher in the perfusates of diabetic rat livers than in controls. This

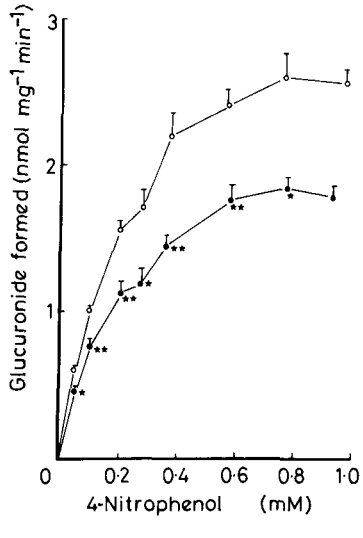

FIG. 1. 4-Nitrophenol glucuronidation in 'native' microsomes. Results are shown as the mean ± s.e.m. Control rats (○, n = 4); diabetic rats (●, n = 5). * $P < 0·05$; ** $P < 0·005$, using non-paired Student's *t*-test.

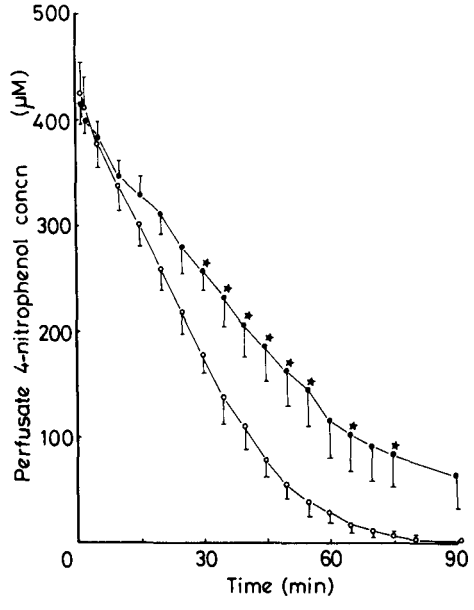

FIG. 2. Perfusate 4-nitrophenol concentrations in perfusions of control (○, n = 6) and diabetic (●, n = 8) rat livers. Results are shown as the mean ± s.e.m.* $P < 0·05$, using non-paired Student's *t*-test.

difference was significant ($P > 0{\cdot}05$) between 30 and 75 min after the introduction of substrate.

The concentrations of 4-nitrophenol glucuronide and sulphate in the perfusates from control and diabetic rat livers are shown in Figs 3 and 4. At this concentration of substrate (0·5 mM), 4-nitrophenol is preferentially converted to the glucuronide conjugate; glucuronide concentrations in the perfusate are twice those of sulphate. There is significantly less glucuronide and sulphate conjugation of 4-nitrophenol in diabetic rat liver perfusions compared with controls. When the diabetic rats were treated with insulin the hepatic elimination of 4-nitrophenol was not significantly different from controls (data not shown).

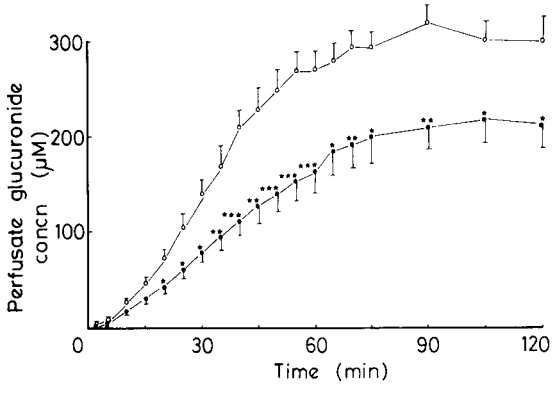

FIG. 3. Perfusate glucuronide concentrations in perfusions of control (○, n = 6) and diabetic (●, n = 8) rat livers. * $P < 0{\cdot}05$; ** $P < 0{\cdot}001$; *** $P < 0{\cdot}005$, using non-paired Student's *t*-test. Error bars represent s.e.m.

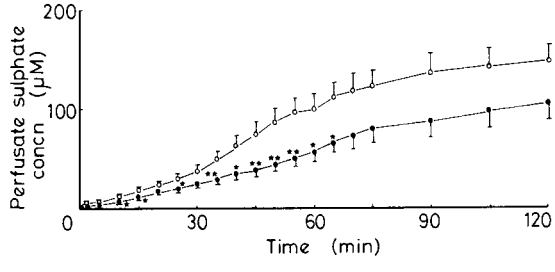

FIG. 4. Perfusate sulphate concentrations in perfusions of control (○, n = 6) and diabetic (●, n = 8) rat livers. * $P < 0{\cdot}05$; ** $P < 0{\cdot}02$, using non-paired Student's *t*-test. Error bars represent s.e.m.

In control rat livers, 10·5 ± 2·8% of the 4-nitrophenol was excreted in the bile as the glucuronide, and this was not significantly different in the diabetic livers. Only trace amounts of the sulphate conjugate were detected in the bile, and these were too low to be quantified accurately.

Fig. 5 shows the $\log_{10}$ concentration versus time plot of the mean 4-nitrophenol perfusate concentrations for control and diabetic rat livers. The data obtained from individual control and diabetic rats did not fit a linear $\log_{10}$ concentration versus time plot, indicating that the elimination of 4-nitrophenol is not an exponential process. This is confirmed by the computer fit of the data to Model 2 rather than to Model 1 below. This type of non-linear kinetics has been observed previously during the in-vivo elimination of diphenylhydantoin (Arnold & Gerber 1970), where drug concentrations were higher than the K_m and the elimination process was saturated. In such cases the time course of drug levels in the body shows Michaelis-Menten kinetics (Gibaldi & Perrier 1975).

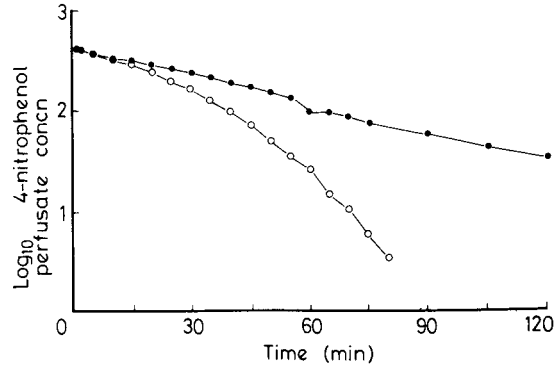

FIG. 5. Log_{10} of the mean 4-nitrophenol perfusate concentrations for control (○, n = 6) and diabetic (●, n = 8) rat livers.

A better fit was sought for the data obtained for 4-nitrophenol elimination through a saturable elimination model (Model 2) using non-linear regression analysis. This fit was compared with that through the single exponential model (Model 1).

Model 1: a one compartment, single exponential model

$$\dot{c} = -kc$$

Model 2: Michaelis-Menten kinetics, i.e. saturable drug elimination

$$\dot{c} = \frac{-V_{max}\, c}{K_m + c}$$

$\dot{c}$ is the change in substrate concentration (c) with time, V_{max} the maximal velocity, k the elimination rate constant, and K_m is the dissociation constant for the substrate-enzyme complex.

The fit of the data to the models was compared using the sum of the squared deviations (SS) and the

distribution of residuals (observed minus calculated 4-nitrophenol concentrations predicted by each model). Fig. 6 shows that Model 2 gave a more even distribution of residuals for the control rat C1 than did Model 1. For the same animal the SS with Model 1 was 19822 while for Model 2 it was 1772. The values of SS and distribution of residuals indicated that Model 2 was a better fit for all the data, and the application of Michaelis-Menten kinetics was appropriate. Addition of a second saturable elimination pathway into Model 2 did not improve the fit of the data.

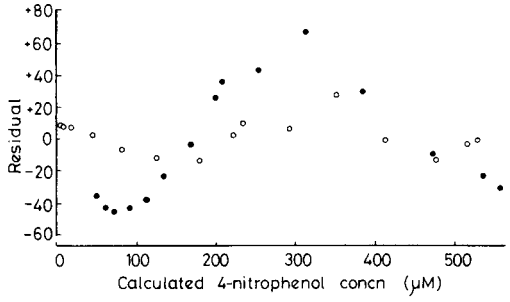

FIG. 6. Spread of residuals obtained during fitting of 4-nitrophenol elimination data of Model 1 (●) and Model 2 (○). Data from Rat C1.

At low substrate concentrations, where the $\log_{10}$ substrate concentration versus time curve is linear, exponential elimination kinetics can be considered. The elimination rate constant, k, and the half-life of the elimination, $t_{\frac{1}{2}}$, were calculated by linear regression analysis using the exponential terminal part of the semilogarithmic plot (Table 2). The time point after which elimination was exponential was different for each rat, and occurred later in diabetic rats (50–60 min) than in controls (20–30 min). The values of $t_{\frac{1}{2}}$ vary considerably in the diabetic group, and due partly to this, $t_{\frac{1}{2}}$ is not significantly different between control and diabetic rats. The values of k were significantly lower in diabetic rats than in controls probably due to the increased variance obtained in the inverse relationship between k and $t_{\frac{1}{2}}$.

The 4-nitrophenol elimination data from each control and diabetic rat were then analysed using Model 2 to calculate K_m and V_{max}. Initial estimates were required so that the final parameter estimates converged to the true values (Ralston et al 1979) and these were provided from the data by the method of Marquardt (1963). Table 3 shows the final parameter values obtained. V_{max} is slower in diabetic animals than in controls. The V_{max} in controls was 99·23 ± 18·63 nmol min^{-1} (g liver)$^{-1}$ compared with 75·30 ± 11·20 nmol min^{-1} (g liver)$^{-1}$ in diabetic rats. These values are not significantly different at the 5% level. The estimates for the apparent K_m show large deviations, with the result that any difference in K_m between the diabetic and control rats could not be detected.

The diabetic rats D4, D5 and D6 which had the slowest elimination of 4-nitrophenol also had the

Table 2. Pharmacokinetics of 4-nitrophenol elimination from control and diabetic perfused livers.

Control Rat	$t_{\frac{1}{2}}$ (min)	k(min^{-1})
C1	7·6	0·091
C2	8·8	0·079
C3	7·0	0 099
C4	11·9	0 058
C5	11·2	0·062
C6	12·2	0·057
Mean ± s.e.m.	9·8 ± 0·9	0·074 ± 0·007
Diabetic rat	$t_{\frac{1}{2}}$ (min)	k(min^{-1})
D1	16·1	0·043
D2	11·2	0·062
D3	11·5	0·060
D4	77·5	0·009
D5	35·4	0·020
D6	—	—
D7	8·7	0·080
D8	10·4	0·067
Mean ± s.e.m.	24·4 ± 9·5	0·049* ± 0·010

* $P < 0·05$, using non-paired Student's t-test. Significance value refers to differences between control and diabetic rats. The hepatic elimination of D6 was so slow that during the experiment the exponential phase was not reached. $t_{\frac{1}{2}}$ = half life and k = elimination rate constant.

Table. 3. Pharmacokinetic parameters for 4-nitrophenol hepatic elimination calculated by Model 2.

Rat	C_o (μM)	Vmax (nmol min^{-1} (g liver)$^{-1}$)	K_m (μM)
Control rats			
C1	540·8 ± 7·3	107·4 ± 7·8	66·7 ± 21·0
C2	414·6 ± 7·2	85·1 ± 6·7	12·7 ± 12·3
C3	327·8 ± 2·6	86·8 ± 4·0	39·4 ± 8·0
C4	430·0 ± 6·5	69·0 ± 5·6	33·3 ± 16·9
C5	489·5 ± 4·5	60·7 ± 13·2	147·8 ± 26·7
C6	395·9 ± 3·8	186·4 ± 21·2	268·0 ± 51·3
Mean ± s.e.m.		99·2 ± 18·6	94·6 ± 39·6
Diabetic rats			
D1	499·8 ± 8·4	58·5 ± 6·2	14·7 ± 25·5
D2	472·8 ± 8·0	87·3 ± 8·0	42·2 ± 21·4
D3	368·2 ± 5·4	119·2 ± 14·2	102·4 ± 31·9
D4	382·7 ± 3·7	44·9 ± 14·2	176·2 ± 141·5
D5	—	—	—
D6	—	—	—
D7	393·8 ± 3·4	85·9 ± 4·3	42·8 ± 10·1
D8	435·4 ± 3·3	56·0 ± 2·9	68·5 ± 14·0
Mean ± s.e.m (n = 6)		75·3 ± 11·2	74·5 ± 23·6

C_o is substrate concentration at time zero, and V_{max} and K_m are the Michaelis-Menten constants. The individual values for C_o, V_{max} and K_m are shown with the standard deviations of the estimates. Data from rats D5 and D6 did not fit Model 2.

lowest concentrations of both glucuronide and sulphate in the perfusate. This suggested that some inhibitory process may be operating to a greater extent in these animals. Elimination from these animals did not fit a model incorporating a term for substrate or product inhibition (data not shown).

A more likely explanation for the slower elimination of 4-nitrophenol is that, at the concentration of substrate used in these experiments, glucuronidation is deficient in the diabetic rat perfused livers, as observed in 'native' but not activated hepatic microsomal preparations (Morrison & Hawksworth 1982). If the transferase enzyme is in a fully activated form in-vivo, diabetes would, therefore, not be expected to decrease glucuronide formation in the perfused liver. The data obtained in the diabetic rat perfused livers provide further evidence that the transferase is latent in-vivo. It is thought to be capable of activation by endogenous activators such as UDP *N*-acetylglucosamine and divalent metals ions (Zakim & Vessey 1976).

In order to obtain more information on the form of glucuronyl transferase present in the intact liver, the clearance values obtained from the microsomal preparations were compared with those obtained from the perfused livers. Clearance (Cl_i) of total drug (free and bound 4-nitrophenol) was measured by the method of Rowland (1972), after correcting for protein binding and the recovery of liver glucuronyltransferase in microsomal fractions. Binding of substrate to microsomal protein was 4·1% (n = 36) and 57·9% to perfusion medium (n = 24), and 36% of the total glucuronyltransferase activity was recovered in microsomal fractions.

Table 4 compares the Cl_i of 4-nitrophenol from microsomal preparations and perfused livers. After correcting the elimination of 4-nitrophenol for the extent of drug binding in each system (α is the fraction of drug which is free i.e. not protein bound in each system), and the recovery of liver glucuronyltransferase in microsomal fractions, the clearance values from the perfused livers compare well with those from the 'native' microsomal preparations. This confirms that in the intact liver glucuronyltransferase is present in the 'native' form.

Table 4. Clearance of 4-nitrophenol from microsomal preparations and perfused livers.

	Control	Diabetic
'Native' microsomal ($\alpha = 0{\cdot}96$)	$1{\cdot}00 \pm 0{\cdot}03$ (4)	$0{\cdot}78 \pm 0{\cdot}11$ (5)
Perfusion ($\alpha = 0{\cdot}42$)	$0{\cdot}96 \pm 0{\cdot}39$ (6)	$0{\cdot}70 \pm 0{\cdot}19$ (7)

Clearance is ml min^{-1} g $liver^{-1}$. α is the fraction of the drug which is free (i.e. not protein bound) in each system. Values are mean ± s.e.m., with the number of animals given in parentheses.

Although the diabetes-induced defect in microsomal glucuronyltransferase activity has been attributed to increased membrane constraint (Morrison & Hawksworth 1982), other factors, such as decreased availability of cofactor, may contribute to the effect seen in the perfused rat liver. The glucuronidation of 4-nitrophenol in perfused rat liver is extremely sensitive to carbohydrate reserves and to the availability of UDP-glucuronic acid (Reinke et al 1981; Thurman et al 1981). The formation of UDP-glucuronic acid depends on an adequate supply of NAD, and in conditions where this supply is limited glucuronidation has been shown to be deficient (Moldéus et al 1976, 1978). NAD levels are known to be decreased in the livers of streptozocin-induced diabetic animals (Gunnarsson et al 1974).

Reinke et al (1981) have shown that inclusion of 10 mM glucose in their liver perfusion medium increased glucuronide formation in fasted, phenobarbitone-treated rats. This effect is probably due to fresh synthesis of UDP-glucuronic acid by NAD-dependent UDP-glucose dehydrogenase. In the present experiments the perfusion medium contained 11·1 mM glucose, so the potential supply of UDP-glucuronic acid should be in excess of the concentration of 4-nitrophenol present (maximum 0·5 mM). However, in alloxan-induced diabetic rats the activity of hepatic UDP-glucose dehydrogenase, and the UDP-glucuronic acid content of the liver have been previously reported to be decreased (Müller-Oerlinghausen et al 1967; Wong & Sourkes 1967). Thus, despite the relatively high glucose concentration in the perfusion medium, diabetic rat livers may not be able to synthesize sufficient UDP-glucuronic acid to produce the concentration of 4-nitrophenol glucuronide observed in control animals.

At 0·5 mM 4-nitrophenol, the contribution of sulphation to hepatic elimination is much less than that of glucuronidation. Therefore, although the concentrations of both glucuronide and sulphate are decreased by 50% in perfusions of diabetic rat livers, the decrease in sulphation has less effect on the overall elimination of 4-nitrophenol. Sulphation of phenolic substrates seems less sensitive to the effects of enzyme inducers and fasting than glucuronidation (Suolinna & Mantyla 1980; Reinke et al 1981; Thurman et al 1981; Mulder 1981).

The interpretation of results from the present study contrast with those proposed by Eacho et al (1981a,b) to explain the effect of streptozocin-induced diabetes on glucuronidation. They observed an increase in the conjugation of 4-nitrophenol in hepatocytes isolated from male streptozocin-induced diabetic rats and attributed this to an increased availability of UDP-glucuronic acid in diabetic rat liver, despite the fact that sulphation was predominant in their system at the concentrations of 4-nitrophenol used, up to 100 μM. They substantiated this conclusion with evidence that the V_{max} of 4-nitrophenol glucuronidation was not altered in microsomes from diabetic rat livers. However, they investigated enzyme activity only in microsomes which had been maximally activated by Triton X-100, and, therefore, no difference in activity would have been expected between the diabetic and control livers. Hepatocytes from diabetic rats, isolated by collagenase perfusion, may exhibit altered permeability, resulting from changes in the membrane composition.

In conclusion, both the glucuronidation and sulphation of 4-nitrophenol are deficient in livers from male diabetic rats. In 'native' hepatic microsomes the defect in glucuronidation is due to a decrease in the maximal velocity of the reaction. Comparison of 4-nitrophenol clearance from perfused livers and 'native' microsomes provides evidence that glucuronyltransferase is in the 'native' form in the intact liver.

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