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BILIRUBIN GLUCURONIDATION BY HEPATIC MICROSOMAL SUBFRACTIONS AND THE EFFECT OF 3-METHYLCHOLANTHRENE*

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Abstract—Rough-surfaced and smooth-surfaced microsomal fractions were prepared from the livers of adult male guinea pigs and Long–Evans rats. Glucuronyltransferase activity (EC 2.4.1.17), with bilirubin as substrate, was found predominantly in the rough subfraction of both species. Guinea pigs treated with a single dose of 3-methylcholanthrene had a higher enzyme activity in both the smooth and rough subfractions. The ratio of enzyme activity in the smooth-surfaced vs. rough-surfaced fractions was not significantly altered by 3-methylcholanthrene. Addition *in vitro* of 3-methylcholanthrene to unfractionated microsomal preparations had no significant effect on bilirubin glucuronyltransferase activity.

ALTHOUGH White[1] has reported that UDP-glucuronyltransferase (EC 2.4.1.17) with bilirubin as substrate is concentrated in the smooth microsomal subfraction, more recent investigations with substrates other than bilirubin have noted a preponderance of glucuronyltransferase activity in the rough subfraction.[2–4] Considering the agreement among the studies with nonbilirubin substrates and the relative paucity of information with bilirubin as substrate, the submicrosomal distribution of bilirubin glucuronyltransferase activity was evaluated in liver preparations from guinea pigs and rats. The effect of 3-methylcholanthrene (3-MC) on the enzyme activity of guinea pig submicrosomal fractions was also investigated.

METHODS

Animals and drug pretreatment. Adult male Long–Evans rats (200–250 g) and guinea pigs (300–500 g) were allowed access to food and water *ad lib.* Animals were maintained in local animal facilities for 3–5 days prior to use. For 3-MC studies, each set of three animals was matched for body weight. One animal of the group received an intraperitoneal injection of 3-MC (Sigma Chemical Co.) (40 mg/kg). Another animal, serving as a vehicle control, received a similar intraperitoneal injection of corn oil (5 ml/kg). The third animal in the group remained untreated for purposes explained in the results section.

Enzyme preparation. Drug-treated animals were sacrified 48 hr after the single dose of 3-MC. Rough-surfaced and smooth-surfaced microsomal fractions were prepared from liver homogenates by the method of Dallner[5] as described by Gram *et al.*[6]

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After the smooth-surfaced microsomes (Fig. 1a) were separated from the rough-surfaced pellet (Fig. 1b), this supernatant was sedimented at 105,000 *g* for 45 min in a Spinco model L preparative ultracentrifuge. The microsomal subfractions were resuspended for incubation by gentle homogenization with either isotonic alkaline KCl or hepatic soluble fraction. Microsomal protein concentrations were determined by the method of Lowry *et al.*[7] with bovine serum albumin (Sigma Chemical Co.) serving as the standard.

Enzyme assays. Incubation and assay conditions for bilirubin glucuronyltransferase have been previously described.[8] The final bilirubin concentration was 275 μM and the final uridine-5'-diphosphoglucuronic acid (UDPGA, ammonium salt, Sigma Chemical Co.) concentration was 10 mM. The amount of conjugation was determined by the difference between samples with and without UDPGA.

p-Nitrobenzoic acid reductase activity was also measured to serve as a marker enzyme for comparing the submicrosomal separations. The incubation procedure for both species was that established for the rat by Gram *et al.*[6] and the quantification of formed *p*-amino-benzoic acid was based on the method of Fouts and Brodie.[9]

Statistics. Data comparing the enzyme activities in the smooth-surfaced vs. rough-surfaced microsomes from the experimental series on nondrug-treated animals were analyzed by Student's *t*-test.[10] In the studies utilizing 3-MC the data for comparing the smooth-surfaced vs. rough-surfaced microsomal fractions were analyzed by an analysis of variance, partially nested design.[11] The ratios of smooth to rough enzyme activity were compared by an analysis of variance, completely random design, with the means being tested by Duncan's New Multiple Range test.[10] The acceptable significance level for all data was $P < 0.05$.

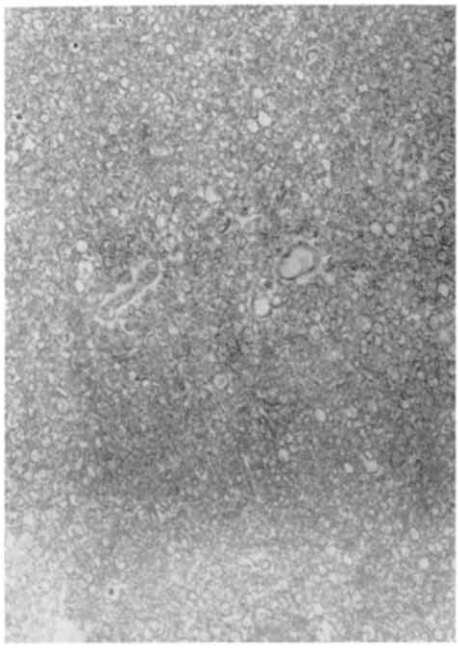
RESULTS

Enzyme activities for the guinea pig. As seen in Table 1, the proportion of microsomal protein was greater in the rough-surfaced subfraction. Bilirubin glucuronyltransferase activity was predominantly found in the rough-surfaced microsomal fraction, whereas

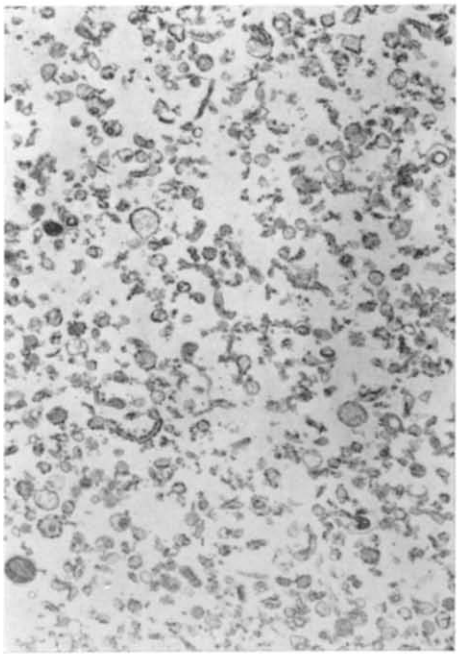
TABLE 1. HEPATIC SMOOTH-SURFACED AND ROUGH-SURFACED MICROSOME ENZYME ACTIVITIES FOR GUINEA PIG AND LONG–EVANS RAT*

Animals	Smooth	Rough	S/R	P†
Guinea pig				
Microsomal protein (mg/g liver)	6·2 ± 0·4	8·9 ± 0·4	0·70	< 0·001
Bilirubin glucuronyltransferase‡	3·2 ± 0·3	5·5 ± 0·2	0·58	< 0·001
p-Nitrobenzoic acid reductase§	82·4 ± 6·7	36·1 ± 2·8	2·3	< 0·001
Long-Evans rat				
Microsomal protein (mg/g liver)	6·1 ± 0·4	10·0 ± 0·4	0·61	< 0·001
Bilirubin glucuronyltransferase‡	3·4 ± 0·3	7·0 ± 0·3	0·49	< 0·001
p-Nitrobenzoic acid reductase§	52·9 ± 4·5	14·7 ± 1·3	3·6	< 0·001

* Data are expressed as mean ± S.E. for 11 guinea pigs or 10 rats.
† As determined by Student's *t*-test, two-tailed.
‡ Bilirubin conjugated (mμmoles) per milligram of microsomal protein per half-hour.
§ *p*-Aminobenzoic acid formed (mμmoles) per milligram of microsomal protein per hour.



(a)



(b)

FIG. 1. Electron micrographs of representative sections from a pellet of guinea pig liver smooth-surfaced microsomes (a) and rough-surfaced microsomes (b) prepared by the method of Dallner.[5] Magnification of 24,000 ×.

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more than twice as much *p*-nitrobenzoic acid reductase activity occurred in the smooth-surfaced microsomal fraction as compared to the rough-surfaced subfraction.

Enzyme activities for the Long–Evans rat. Similar patterns were obtained for the rat (Table 1). Again, microsomal protein content and bilirubin glucuronyltransferase activity were mainly concentrated in the rough-surfaced microsomal subfraction while *p*-nitrobenzoic acid reductase activity was predominantly in the smooth-surfaced microsomal fraction.

Effect of 3-methylcholanthrene pretreatment. Experiments were performed on guinea pigs 48 hr after a single dose of 3-MC (40 mg/kg). In addition to the corn oil control group, an additional untreated control group was added since Boyd *et al.*[12] have recently reported that corn oil *per se* can elicit toxic effects in high doses.

The effect of 3-MC on the protein content of hepatic microsomal fractions is shown

TABLE 2. EFFECT OF 3-METHYLCHOLANTHRENE (3-MC) PRETREATMENT ON GUINEA PIG HEPATIC MICROSOMAL PROTEIN CONTENT AND *p*-NITROBENZOIC ACID REDUCTASE ACTIVITY

Pretreatment	Smooth	Rough	S/R
Microsomal protein content*			
Nontreated	5·0 ± 0·5	8·8 ± 0·4 †	0·57 ± 0·05
Corn oil treated	4·8 ± 0·5	8·7 ± 0·3 †	0·55 ± 0·06
3-MC treated	6·9 ± 0·3	9·8 ± 0·3†	0·71 ± 0·02
p-Nitrobenzoic acid reductase activity*			
Nontreated	85·1 ± 9·8	41·4 ± 3·8 †	2·1 ± 0·2
Corn oil treated	83·5 ± 10·1	42·7 ± 7·0 †	2·0 ± 0·3
3-MC treated	88·0 ± 12·0	58·5 ± 5·5 †	1·5 ± 0·2

* Data are for six animals per mean (± S.E.). Protein content in milligram per g wet weight of liver. Enzyme activity in millimicromoles of *p*-aminobenzoic acid formed per milligram of microsomal protein per hr. Animals were killed 48 hr after intraperitoneal injection of 3-MC (40 mg/kg).

† Smooth vs. rough (horizontal comparisons) significantly different at $P \leq 0{\cdot}05$. Treatment means (vertical comparisons) connected by the same line are not significantly different at the 5 per cent level.

in Table 2. The rough-surfaced microsomal fraction contained the highest protein content for all three groups of animals. 3-MC produced a significant increase in protein content for both subfractions as compared to nontreated and corn oil control groups. Pretreatment with the hydrocarbon also caused a greater increase of protein in the smooth fraction rather than in the rough fraction, thereby increasing the smooth:rough ratio of protein.

p-Nitroreductase activity after 3-MC is presented in Table 2. Preparations from all three groups of animals exhibited almost twice as much enzyme activity in the smooth-surfaced microsomal fraction as compared to the rough subfraction. 3-MC did not significantly alter the reductase activity of either subfraction.

Bilirubin glucuronyltransferase activity was significantly increased by 3-MC (Table 3). Glucuronyltransferase activity was higher in the rough submicrosomal fraction for all three treatments. Although 3-MC pretreatment produced greater

TABLE 3. EFFECT OF 3-METHYLCHOLANTHRENE (3-MC) PRETREATMENT ON GUINEA PIG HEPATIC BILIRUBIN GLUCURONYLTRANSFERASE*

Pretreatment	Smooth	Rough	S/R
Nontreated	3·7 ± 0·3	4·5 ± 0·3 †	0·82 ± 0·04
Corn oil	3·8 ± 0·3	5·1 ± 0·3 †	0·76 ± 0·07
3-MC	8·8 ± 0·3	10·6 ± 0·4†	0·83 ± 0·02

* Enzyme activity expressed for six animals per mean (± S.E.) as millimicromoles of bilirubin conjugated per milligram of microsomal protein per half-hour. Animals were used 48 hr after intraperitoneal injection of 3-MC (40 mg/kg).

† Smooth vs. rough (horizontal comparisons) significantly different at $P \leq 0{\cdot}05$. Treatment means (vertical comparisons) connected by the same line are not significantly different at the 5 per cent level.

activity in both the smooth and rough subfractions compared to nontreated or corn oil controls, the ratio of smooth:rough microsomal enzyme activity was not significantly different.

Effect of 3-methylcholanthrene in vitro. 3-MC in 5 μl of acetone was added to incubates of unfractionated microsomal preparations from three guinea pigs at concentrations of 0, 0·5, 1·0, 2·0, 5·0 and 10·0 μM. The control values averaged 4·3 ± 0·1 mμmoles bilirubin conjugated per mg microsomal protein per half-hour. The conjugation values for the 3-MC concentrations used ranged from 4·0 ± 0·1 to 4·3 ± 0·3 with all values not significantly different from controls.

DISCUSSION

The symposium presentation by White[1] reported bilirubin glucuronyltransferase activity in rat liver smooth and rough microsomal subfractions with the smooth fraction being 15 times as active as homogenates, and the rough fraction being only six times as active. Another literature citation supporting this preponderance of bilirubin glucuronyltransferase activity in the smooth fraction refers to unpublished observations[13] and a third makes no reference to experimental data.[14] White[1] also found a smooth: rough ratio of greater than 3:1 when *p*-nitrophenol was used as substrate. More recent studies with nonbilirubin substrates have found greater glucuronyltransferase activity in the rough fraction.[2–4] Gram *et al.*[2] reported almost a 2:1 ratio of rabbit liver rough to smooth enzyme activity with *p*-nitrophenol and *o*-aminophenol as glucuronide acceptors but a 1:1 ratio with phenolphthalein. Mulder[3] found the transferase concentrated in the rough fraction of rat microsomal preparations with *p*-nitrophenol and Schroeder *et al.*[4] also noted a greater activity in the rough fraction using monkey, guinea pig and mouse liver preparations with the same substrate.

The experiments presented here have shown that bilirubin glucuronyltransferase activity is greater in the rough-membrane fraction of the rat and guinea pig. Electron micrographs of the guinea pig liver microsomal subfractions (Fig. 1) showed the rough-microsomal fraction with ribosomes attached to the vesicles, and the smooth-microsomal fraction with vesicles of varying size but lacking bound ribosomes.

Various possibilities need to be considered in contrasting the data of White[1] with those of other investigators.[2–4] Species differences do not provide an adequate basis since rats were also used by Mulder[3] and in the experiments reported herein. Although White[1] used the microsomal subfractionation methods originated by Rothschild[15] as used by Peters,[16] whereas this investigation and the others referred to[2–4] used the methods of Dallner,[5] both the Rothschild and Dallner methods depend on sucrose gradient separations. It is therefore difficult to state whether the subfractionation procedures could explain the differences. It is doubtful that the analytical techniques for *p*-nitrophenol assay could readily explain the difference between White[1] and others[2–4] but the differences between White's bilirubin data and the findings reported herein may reflect analytical problems associated with using bilirubin as substrate. In particular, spurious results with the method of Lathe and Walker[17] used by White[1] have been reported from this laboratory[18] and Lathe commented on the lack of specificity for the method following White's presentation.[19]

A substantial increase in the unfractionated microsomal conjugation of bilirubin can be produced by treatment of guinea pigs with 3-MC.[8] We therefore investigated whether the administration of 3-MC could preferentially cause a stimulation in the amount of bilirubin conjugated by the rough-surfaced microsomes. 3-MC significantly changed the enzyme activity for bilirubin conjugation but not for the reduction of *p*-nitrophenol. Although increased glucuronyltransferase activity was observed in the rough-surfaced fraction, a nearly equal increase was present in the smooth-surfaced fraction (Table 3).

The nontreated and corn oil groups of animals were not significantly different in any of the parameters studied. Corn oil is used by many as a vehicle control and would not be expected to affect the enzyme activities, although higher concentrations might have detrimental effects.[12]

The concentrations *in vitro* of 3-MC used in the current study were inactive although they were reasonably comparable to the levels found by Bresnick *et al.*[20] in hepatic tissue 44 hr after the administration of 3-MC in rats. Enzyme activation from residual drug or metabolites therefore does not appear to explain the action of 3-MC on glucuronyltransferase activity. Polycyclic hydrocarbons such as 3-MC have been shown to cause little or no increase in the amount of microsomal protein per gram of liver, but can stimulate liver growth and the synthesis of total liver protein.[21,22] In a recent publication, 3,4-benzpyrene significantly increased microsomal protein in rats 72 hr after treatment.[23] The possibility then exists that hydrocarbons can selectively result in increased amounts of some tissue proteins without influencing the amounts of others. The increased protein content and increased enzyme specific activity noted in both subfractionated (Table 2) and unfractionated[8] microsomal preparations are therefore more consistent with 3-MC causing an increase in the net amount of bilirubin glucuronyltransferase than its causing some form of activation phenomenon.

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