Biochemical Pharmacology, Vol .20 ,pp. 1463–1470. Pergamon Press, 1971. Printed in Great Britain

SOME EFFECTS OF 3-METHYLCHOLANTHRENE ON URIDINE DIPHOSPHATE GLUCURONYLTRANSFERASE IN THE RAT AND GUINEA PIG*

RICHARD D. HOWLAND† and ALAN BURKHALTER

Department of Pharmacology, University of California, San Francisco, Calif. 94122, U.S.A.

(*Received* 20 *June* 1970; *accepted* 30 *September* 1970)

Abstract—Treatment of young rats and guinea pigs with 3-methylcholanthrene significantly increased liver microsomal glucuronyltransferase activity. The earliest detectable increase in rat glucuronyltransferase activity occurred between 6 and 12 hr following administration of the polycyclic hydrocarbon, and after 48 hr, glucuronyltransferase activity in treated animals was twice that in control animals. Liver glucuronyltransferase activity was not effected when rats were pretreated with nikethamide, chlorcyclizine or phenobarbital. Administration of *d,l*-ethionine or actinomycin D, inhibitors of protein synthesis, prevented the increase in glucuronyltransferase activity following 3-methylcholanthrene administration. Kinetic analysis of glucuronyltransferase activity indicated that the V_{max} was significantly increased in treated rats and guinea pigs. The K_m of the enzyme from 3-methylcholanthrene treated rats was significantly decreased, whereas, the K_m of the enzyme from treated guinea pigs was significantly increased. These changes in V_{max} and K_m suggest that induction of glucuronyltransferase by 3-methylcholanthrene may involve qualitative as well as quantitative changes in the enzyme.

URIDINE-5′-DIPHOSPHATE glucuronyltransferase (EC 2.4.1.17) catalyzes the transfer of glucuronic acid from UDP-glucuronic acid (UDPGA) to a variety of endogenous and exogenous acceptor substrates. Inscoe and Axelrod[1] reported that treatment of rats and guinea pigs with 3-methylcholanthrene (MC) caused an increase in hepatic glucuronyltransferase activity using *o*-aminophenol (OAP) as the acceptor. In mice, glucuronyltransferase activity toward OAP and bilirubin has been reported to increase following sodium barbital treatment.[2]

Recently, data have been presented indicating that inducers of microsomal enzymes have significant effects on the kinetic constants of certain of these enzymes. Guarino *et al.*[3] reported that treatment of rats with phenobarbital resulted in an increase in the Michaelis constant (K_m) as well as the maximal velocity (V_{max}) of aniline hydroxylase. Alvares, Schilling and Kuntzman[4] found that the K_m of rat liver benzpyrene hydroxylase was decreased and the V_{max} increased after MC administration. These hydroxylation reactions depend upon an intact and functioning microsomal electron transport system. A number of investigators[5–10] have demonstrated that the administration of MC to rats results in the appearance of a cytochrome P-450 which is different from that found in control animals or those treated with phenobarbital. Further-

* A preliminary report of this work was presented at the meeting of the Federation of American Societies for Experimental Biology in Atlantic City, 1969. This research was supported by NIH Training Grant GM-00475 and a research grant from the Academic Senate of the University of California, San Francisco.

† Present address: Roche Institute of Molecular Biology, Nutley, N.J. 07110.

more, Alvares *et al.*[11] have shown that the appearance of the new cytochrome P-450 after MC administration is dependent upon protein synthesis. These data indicate that MC administration results in the synthesis of at least one component of the microsomal electron transport system which differs qualitatively from control animals. It is possible that the changes in the K_m of benzpyrene hydroxylase following MC may reflect qualitative changes in the electron transport system and not qualitative changes in the enzyme protein itself.

We determined some of the effects of MC and other inducing agents on liver glucoronyltransferase, a microsomal enzyme which does not depend on cytochrome P-450 or the microsomal electron transport chain for its activity.

MATERIALS AND METHODS

Male Wistar rats (35–45 g) and randomly bred male abino guinea pigs (50–60 g) were allowed free access to food and water throughout the experiments. All drugs were injected intraperitoneally in a volume of 1·0 ml/100 g body weight. MC was dissolved in corn oil; all other drugs were dissolved in 0·9% saline. Control animals received the appropriate vehicle only. Unless otherwise noted, enzyme assays of MC-treated animals were performed 48 hr after a single injection. When other drugs were employed enzyme activity was determined 24 hr after the last injection. The animals were sacrificed between 8:00 and 9:00 a.m. and the livers rapidly removed and chilled on ice. All subsequent steps were carried out at 0–4°. The tissue was weighed, minced with scissors and then homogenized in 4 vol. of 1·15% KCl with a Potter–Elvejhem homogenizer (Teflon pestle).

The homogenate was centrifuged at 9000 *g* (av.) for 20 min and the resulting supernatant was carefully transferred and centrifuged at 105,000 *g* (av.) for 60 min in a Beckman Model L ultracentrifuge. The supernatant was discarded and the microsomal pellet was gently resuspended in 1·15% KCl such that 1·0 ml contained microsomes derived from 200 mg wet weight of liver. Microsomal protein was determined by the method of Nayyar and Glick.[12] Bovine serum albumin was used as the standard.

Assay of glucuronyltransferase activity was performed using OAP as the substrate. In addition to microsomes, the final concentration of reactants in the incubation medium were: 50 mM tris–HCl buffer, pH 7·4, 15 mM $MgCl_2$, 2·0 mM UDPGA (Sigma Chemical Co.) when rat microsomes were assayed and 0·5 mM UDPGA when guinea pig microsomes were assayed. The final volume was 2·0 ml and the concentration of microsomal protein was between 1 and 2 mg/ml. OAP was sublimed before use and dissolved in 0·2% ascorbic acid just prior to addition to the reaction vessel. The reactants, including microsomes, were preincubated for 3 min and the reaction was started by addition of UDPGA. Incubation was at 37° for 15 min under air in a Dubnoff metabolic shaker. The reaction was stopped by addition of 2·0 ml of 0·625 N trichloroacetic acid (TCA) containing 1M KH_2PO_4, pH 2·2. Blanks contained no UDPGA and had the TCA-phosphate mixture added to them at the beginning of the incubation. OAP-glucuronide formed was measured by the method of Dutton and Storey,[13] using authentic OAP-glucuronide (Aldrich) as the standard.

Kinetic data were analyzed by regression analysis to fit a straight line to a Lineweaver–Burk plot. Statistical comparisons between control and treated groups were made by Student's *t*-test.

RESULTS

Preliminary experiments established that Mg^{2+} stimulated OAP-glucuronidation. When the final concentration of Mg^{2+} in the incubation media was 15 mM, glucuronyltransferase activity was three times greater than in the absence of the divalent cation. Rat glucuronyltransferase was found to require 1·0 mM OAP and 2·0 mM UDPGA, whereas the guinea pig enzyme required 0·1 mM OAP and 0·5 mM UDPGA. UDPGA was not present in saturating concentrations. However, under these conditions the enzyme activity was linear for at least 20 min and was proportional to microsomal protein concentrations between 0·5 and 3·0 mg/ml.

Treatment of rats or guinea pigs with MC resulted in a significant increase in glucuronyltransferase activity (Table 1). OAP conjugation of treated rats was two times greater than control whereas the activity of treated guinea pigs was only 1·5 times greater than control. The specific activity of the control guinea pig enzyme was three times the specific activity of the control rat. This result was in accord with the observation of Temple, Clement and Done.[14]

TABLE 1. EFFECT OF 3-METHYLCHOLANTHRENE ON RAT AND GUINEA PIG UDP-GLUCURONYLTRANSFERASE*

Species	Control (nmoles/mg prot./15 min†)	Treated (nmoles/mg prot./15 min†)
Rat	6·07±0·55 (4)	12·70±1·10‡ (4)
Guinea pig	18·52±0·60 (6)	25·87±1·90‡ (6)

* Rats and guinea pigs were injected with 40 mg/kg 3-methylcholanthrene and assayed 48 hr later.

† Figures in each column represent the mean ± S.E., and the number in parentheses is the number of determinations.

‡ $P < 0·01$ compared to the respective control.

The inducers of the phenobarbital type and inducers of the polycyclic hydrocarbon type are known to have different effects on the oxidative drug-metabolizing enzymes.[15] We were interested if there were also differences in their effects on glucuronyltransferase activity. Accordingly, we administered to rats either nikethamide (25 mg/kg for 3 days), chlorcyclizine (24 mg/kg for 3 days) or phenobarbital (35 mg/kg b.i.d. for 3 days). None of these treatments had any significant effect on OAP-glucuronidation.

When MC was administered to rats elevation of microsomal glucuronyltransferase activity was observed between 6 and 12 hr after the injection and continued to increase so that at 48 hr it was approximately twice that of control (Fig. 1). Experiments using the 9000 *g* supernatant indicated that glucuronyltransferase activity remained elevated for several days and then slowly declined.

The increase in OAP conjugation was not due to any direct effect on MC. When rat microsomes were preincubated with several concentrations of MC (10, 20 and 40 μg/ml) for 15 min prior to the addition of OAP and UDPGA no difference in enzyme activity was observed.

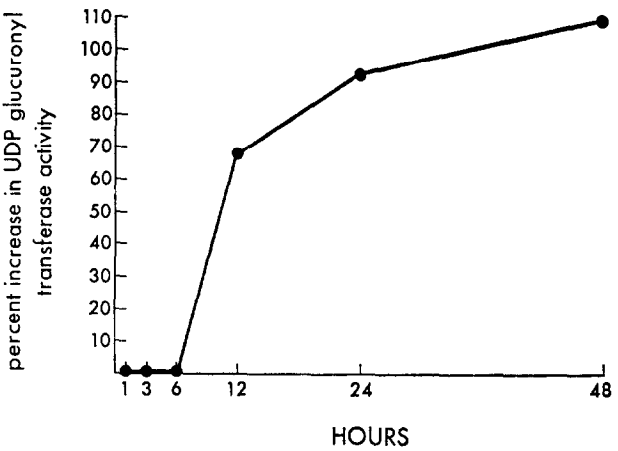

FIG. 1. Time course of 3-methylcholanthrene effect on rat UDP-glucuronyltransferase. Animals were injected at zero time with 40 mg/kg MC and sacrificed at the time noted. Enzyme assays were performed on the microsomal fraction. Each point represents the mean per cent increase of three animals as compared to the control value of 7·19±0·30 nmoles/mg prot./15 min (mean ±S.E., $n = 21$).

The increase in enzyme activity was found to be related to protein synthesis. Treatment of rats with either *d,l*-ethionine or actinomycin D prior to the administration of MC prevented the increase in enzyme activity observed 24 hr after the polycyclic hydrocarbon (Table 2).

Kinetic analysis of microsomal glucuronyltransferase activity after MC administration revealed marked changes in the V_{max} and apparent K_m of the reaction (Table 3).

TABLE 2. EFFECT OF ETHIONINE AND ACTINOMYCIN D ON INCREASED UDP-GLUCURONYLTRANSFERASE ACTIVITY FOLLOWING 3-METHYLCHOLANTHRENE ADMINISTRATION

Treatment*	nmoles/mg prot./15 min†
Control	7·77 ± 0·86
3-Methylcholanthrene	14·36 ± 1·32
3-Methylcholanthrene + ethionine	7·23 ± 0·85‡
Control	6·95 ± 0·65
3-Methylcholanthrene	13·62 ± 1·21
3-Methylcholanthrene + actinomycin D	5·69 ± 0·35‡

* Twenty-four rats were divided into six groups of four each. Ethionine was administered in a dose of 1 g/kg divided into two equal closes given 30 min apart. 3-Methylcholanthrene, 40 mg/kg, was administered 30 min after the last dose of ethionine. Actinomycin D was dissolved in 10% ethanol in saline (v/v) and administered in a dose of 0·7 mg/kg. One hr later 40 mg/kg of 3-methylcholanthrene was given. Control groups received the appropriate vehicles only. All animals were sacrificed 24 hr after the administration of 3-methylcholanthrene.

† The values are the mean ± S.E.

‡ $P < 0·05$ compared to the group treated with 3-methylcholanthrene only.

TABLE 3. EFFECT OF 3-METHYLCHOLANTHRENE ON THE KINETIC CONSTANTS OF RAT AND GUINEAPIG LIVER GLUCURONYLTRANSFERASE*

Species	$K_m \times 10^{-5}$ M		V_{max}	
	Control	Treated	Control (nmoles/mg prot./min)	Treated (nmoles/mg prot./min)
Rat	25·8 ± 6·2 (5)	7·9 ± 1·1† (5)	0·47 ± 0·03 (5)	0·70 ± 0·05† (5)
Guinea pig	2·6 ± 0·5 (4)	8·8 ± 2·0† (4)	1·5 ± 0·3 (4)	4·2 ± 0·8† (4)

* Rats and guinea pigs were injected with 40 mg/kg 3-MC. Enzyme assays were performed 48 hr later. The figures represent the mean ± S.E. and the number in parentheses is the number of determinations. When rats were assayed, the livers from two animals were pooled. Concentrations of OAP ranged between 0·1 to 1·0 mM in the rat and 0·01 to 0·1 mM in the guinea pig.

† $P < 0.05$ as compared to respective control.

The V_{max} was significantly greater ($P < 0.05$) in MC-treated rats and guinea pigs than in their controls.

The enzyme of control rats and guinea pigs exhibited markedly different Michaelis constants; the K_m of the rat enzyme being an order of magnitude greater than the K_m of the guineapig enzyme. This result is in agreement with our finding that the guinea-pig enzyme was saturated at a lower concentration of OAP than the rat enzyme.

The effect of MC on the K_m of the enzyme was different for each of the species. In the rat, pretreatment with MC resulted in a significantly lower K_m ($P < 0.05$), suggesting that the relative affinity of the enzyme for OAP was increased. In contrast, the

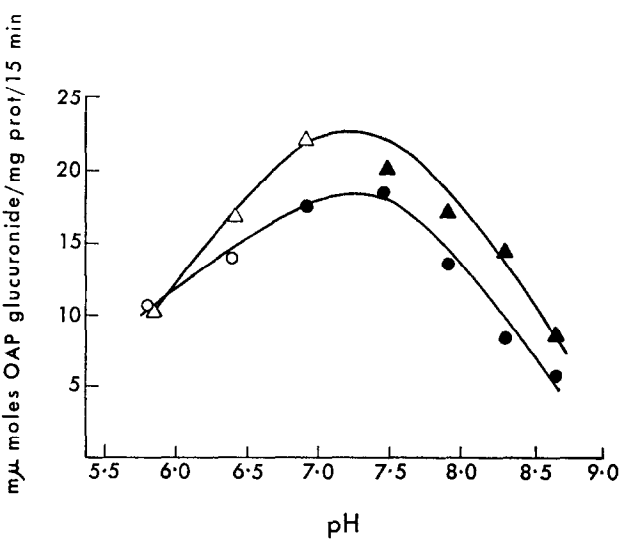

FIG. 2. Effect of pH on guineapig UDP-glucuronyltransferase. The circles (○—●) represent the pH curve of microsomes from control animals. The triangles (△—▲) represent the pH curve of microsomes from MC treated animals. The assay medium contained 1·0 μmole OAP, 0·5 μmole UDPGA, 15 μmoles $MgCl_2$ and either 10 μmoles Na-phosphate buffer (open circles and triangles) or 50 μmoles tris–HCl buffer (closed circles and triangles) in a final volume of 1·0 ml.

K_m of the enzyme from MC-treated guinea pigs was significantly higher ($P < 0.05$) than control, indicating a decrease in relative affinity for the substrate. The Michaelis constants of both species treated with MC were very similar (7·9 and 8·8 $\times$ 10^{-5} M respectively).

Recently, Rickert and Fouts[16] reported that the pH optimum of aniline hydroxylation was increased after administration of benzpyrene to rats. We found that the pH optimum for the guineapig liver microsomal glucuronyltransferase to be about 7·3 for both control and treated animals (Fig. 2). Surprisingly, the pH optimum of glucuronyltransferase in both control and treated rats was found to be about 9·2 (Fig. 3). This was very different from the reported pH optimum which ranges between 7·3 and 7·6.[17] In order to rule out non-enzymatic formation of OAP-glucuronide, OAP and UDPGA were incubated without microsomes at pH 9·2. No detectable conjugation occurred in this system. OAP was also incubated in the presence of boiled microsomes with and without UDPGA at 9·2. In both of these systems formation of OAP-glucuronide failed to occur. A similar pH curve was obtained when sodium barbital buffer was employed, indicating that the pH optimum of 9·2 was not a glycine buffer effect.

DISCUSSION

The significant increase in glucuronyltransferase activity of weanling rats and young guinea pigs following MC administration is in accord with the results of Inscoe and Axelrod.[1] However, our inability to demonstrate enhanced OAP conjugation following treatment with agents other than MC is not in agreement with the results of other workers. Phenobarbital has been reported to increase OAP conjugation in liver slices

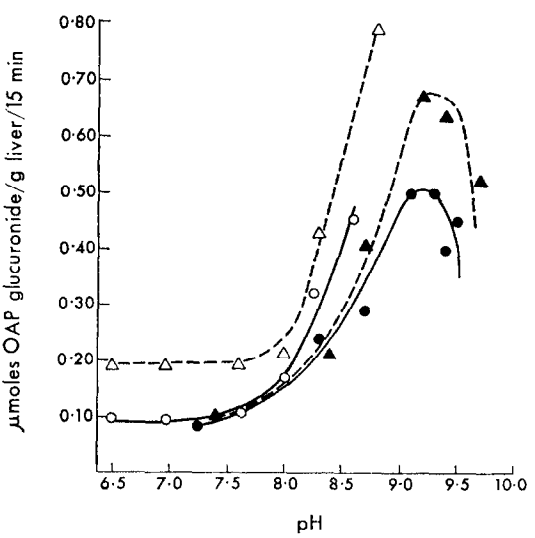

FIG. 3. Effect of pH on rat UDP-glucuronyltransferase. The circles (○—○, ●—●) represent the pH curves of microsomes from control animals. The triangles (△- -△, ▲- -▲) represent the pH curves of microsomes from MC-treated animals. The assay medium contained 1·0 μmole OAP, 2·0 μmoles UDPGA, 15 μmoles $MgCl_2$ and either 40 μmoles glycine buffer (closed circles and triangles) or 50 μmoles tris–HCl buffer (open circles and triangles) in a final volume of 1·0 ml.

of adult rats.[18] We employed weanling rats and determined OAP conjugation in a system fortified with UDPGA. Arias *et al.*[19] have reported that chlorcyclizine enhanced OAP conjugation in both newborn and pregnant Wistar rats. They treated the adult animals for 10 days and found a 50 per cent increase in glucuronyltransferase activity. We treated male weanling rats with chlorcyclizine for only 3 days and found no difference between control and treated animals. Arias' group treated the newborn rats for 3 days and found a 2·5-fold increase in glucuronyltransferase activity. However, newborn rats are known to possess low enzyme activities and exhibit a much greater response to the stimulatory effect of foreign compounds.[1,15,20] Although nikethamide has been reported to increase the urinary excretion of D-glucuronic acid in rats,[21] we did not find that it had an effect on OAP conjugation by rat liver microsomes.

The latent period of several hours before increased glucuronyltransferase activity could be detected suggested that MC did not exert its effect directly on the enzyme protein, which was confirmed by addition of MC *in vitro*. Similar results were reported by Juchau *et al.*[22] who found that rat microsomal benzpyrene hydroxylase and zoxazolamine hydroxylase were not enhanced significantly until at least 6·5 hr had elapsed after 3,4-benzpyrene administration. The lack of immediate effect of MC, coupled with the fact that prior administration of protein synthesis inhibitors blocked the stimulation of enzyme activity strongly indicates that the increase in enzyme activity is due to synthesis of new enzyme protein.

To our knowledge a pH optimum as high as 9·2 has not been reported for glucuronyltransferase regardless of the species or substrate employed. The highest reported optimal pH is 8·0–8·2 for rabbit liver microsomal glucuronyltransferase using estrone as the substrate.[23] Although MC produced major changes in the kinetic constants of the enzyme, it had no effect on the pH optimum in either species.

The determination of kinetic constants in a heterogenous system, such as isolated microsomes, does not permit a detailed analysis of the reaction studied. However, the data we obtained appeared to follow Michaelis–Menton kinetics and we, therefore, felt that a comparison of the kinetic constants of treated and control animals would be profitable. However, we should point out that until glucuronyltransferase is solubilized and purified, these values can only be regarded as the "apparent" kinetic constants.

Our results indicate several marked differences between the rat and guinea pig in regard to the properties of the enzyme. The guineapig enzyme has a significantly lower pH optimum, appears to have a greater affinity for OAP and has a faster rate of conjugation than the rat enzyme. Our kinetic data confirm the observations of Temple, Clement and Done.[14] Although they employed different strains of guinea pigs and rats than in our experiments, they also found that the guineapig enzyme exhibited a smaller K_m and greater V_{max} than the rat enzyme.

In both species, MC administration increased the rate (V_{max}) of OAP-glucuronidation. This result suggests an increase in enzyme protein or an increase in the turnover number (catalytic activity) of the enzyme. However, the results with protein synthesis inhibitors would tend to support the former hypothesis.

In the rat, MC administration doubled specific activity from about 6 to 13 nmoles/mg prot./15 min, whereas the V_{max} was only increased 1·5-fold. There was even less agreement for the increases in specific activity and V_{max} in the guinea pig; the former

was increased 1·5 times and the latter three times. This may be partly explained by the increase in K_m which suggests that the treated guineapig enzyme may not have been saturated with OAP at a concentration of 0·1 mM. Furthermore, treatment with MC may have resulted in changes in the requirements for UDPGA which were not determined.

The significant difference in the K_m of the enzyme from control and treated animals suggests that the polycyclic hydrocarbon caused the appearance of a different enzyme with a separate and distinct ability to bind OAP. These data also indicate that, at least in the case of glucuronyltransferase, MC can effect the kinetic constants of a microsomal enzyme independent of any changes in the microsomal electron transport chain.

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