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ENHANCED BILIARY EXCRETION OF THYROXINE GLUCURONIDE IN RATS PRETREATED WITH BENZPYRENE*

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Abstract—Pretreatment of male or female rats with a single injection of benzpyrene 48 hr before cannulation of the bile duct produced a 3- to 4-fold increase in biliary excretion of exogenously administered or endogenously produced labeled thyroxine. Almost all of the increase could be accounted for by enhanced excretion of thyroxine glucuronide.

The effect of benzpyrene on thyroxine glucuronide excretion could not be demonstrated 4·5–9·5 hr after administration of the drug. This is in contrast to results obtained with compounds such as sodium salicylate and butyl-4-hydroxy-3,5-diiodobenzoate, which acutely promote increased biliary excretion of thyroxine by displacing it from its binding sites on plasma proteins. It also contrasts with the effect of drugs such as propylthiouracil, which increase thyroxine excretion in bile by blocking the major alternate pathway of metabolism, deiodination.

Liver slices and homogenates from benzpyrene-treated rats displayed a several-fold increase in capacity to form thyroxine or *o*-aminophenol glucuronides. Although optimal conditions for assaying thyroxine glucuronyl transferase in liver homogenates were not achieved, the several-fold increase in glucuronyl transferase activity observed in liver homogenates from benzpyrene-treated rats seems best explained by assuming a corresponding increase in the level of the enzyme. The augmented biliary excretion of thyroxine in benzpyrene-treated rats is also best explained on the basis of an increase in liver glucuronyl transferase.

Phenobarbital pretreatment produced a small increase in labeled thyroxine excretion in bile, which was not limited to the glucuronide alone. There was no effect on thyroxine conjugation *in vitro*, although a slight increase in *o*-aminophenol conjugation could be demonstrated. The slight effect which occurred *in vivo* with thyroxine appeared to be correlated with an increase in bile flow rather than with an increase in glucuronyl transferase.

A NUMBER of drugs and compounds of physiological importance are metabolized by conjugation with glucuronic acid in a reaction involving the liver microsomal enzyme (or series of enzymes), glucuronyl transferase, and a soluble cofactor, uridine diphosphoglucuronic acid (UDPGA§).[1, 2]

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§ The following abbreviations are used: uridine-5′-diphosphoglucuronic acid (UDPGA); uridine-5′-diphosphoglucose (UDPG); thyroxine (T_4); *o*-aminophenol (OAP); butyl-4-hydroxy-3,5-diiodobenzoate (BHDB); 2-mercapto-1-methyl-imidazole (MMI).

It has been demonstrated during the past several years that the glucuronide-forming system of the liver can be stimulated by treatment of animals with a variety of drugs. For example, the formation *in vitro* of glucuronides of *o*-aminophenol[3] and bilirubin[4] by liver microsomes in the presence of added UDPGA is enhanced by pretreatment of the animals with 3,4-benzpyrene. This stimulatory effect probably involves an increase in glucuronyl transferase.

Another possible mechanism by which drug treatment may increase glucuronide formation *in vivo* is through stimulation of uridine diphosphoglucose (UDPG) dehydrogenase, which is involved in the conversion of UDPG to UDPGA.[5] An increase in the dehydrogenase was reported for rats treated with chloretone or barbital, but not for rats treated with benzpyrene or methylcholanthrene.[6]

Since formation and biliary excretion of thyroxine glucuronide are major pathways for thyroxine metabolism in the rat,[7] we became interested in the possibility that glucuronide conjugation of thyroxine might also be stimulated by pretreatment with drugs. Two compounds, phenobarbital and 3,4-benzpyrene, were tested for this effect in the present investigation.

METHODS

Drugs and chemicals

^{131}I-thyroxine (^{131}I-T_4) was purchased from Abbott Radiopharmaceuticals. The radiopurity, routinely checked by paper chromatography as described below, was 80–88 per cent. The presence of 5–10% inorganic ^{131}I and 5–10% of other ^{131}I-moieties would not be expected to invalidate the essential conclusions of this study. *o*-Aminophenol (OAP) was purchased from Eastman Organic Chemicals; *o*-aminophenol glucuronide, *n*-butyl-3,5-diiodo-4-hydroxybenzoate (BHDB), and 2-mercapto-1-methyl-imidazole (MMI) from Aldrich Chemical Company; 3,4-benzpyrene (hereafter benzpyrene) from Mann Research Laboratories; NADP from Pabst Laboratories; UDPGA from Sigma Chemical Co. and Calbiochem; and ^{131}I$^-$ from Oak Ridge National Laboratory. SKF 525-A (β-diethylaminoethyl diphenylpropylacetate) was a gift from Smith, Kline & French Laboratories.

Animals

Male and female Sprague–Dawley (Holtzman) rats, weighing from 150 to 300 g, were maintained on a modified McCollum diet.[8] They were injected i.p. with 10 mg benzpyrene in 0·5 ml corn oil 48 hr prior to surgery or tissue assay. Controls received corn oil concurrently. The phenobarbital-treated groups received sodium phenobarbital (50 mg/kg) i.p. in saline daily for 5 days prior to surgery or tissue assay. Controls were injected with saline.

Bile collection and chromatography

Animals were anesthetized with ether, and the bile duct was cannulated with fine polyethylene tubing (Clay-Adams PE 10 or 20). After recovery from the anesthetic, each rat received a single i.v. injection of ^{131}I-thyroxine (^{131}I-T_4), approximately 0·1 μg/100 g body wt., via the lateral tail vein. To permit collection of bile, the animals were placed in restraining cages, with access to water but not to food. Bile was collected in 12-ml graduated centrifuge tubes over the intervals 0–2 hr and 2–4 hr after the ^{131}I-T_4 injection.

Total radioactivity of the bile was measured in a Nuclear Chicago scintillation well counter. A 50-μl sample of bile was delivered on filter paper strips (Whatman 3 MM) for ascending chromatography in collidine–3N NH_4OH (100 : 33), as previously described for plasma.[9] The chromatograms were placed in contact with X-ray film and, with the resulting radioautograms as a guide, the radioactive sections of the chromatogram corresponding to origin, T_4–glucuronide, T_4 and I^- were excised and counted. In a few preliminary experiments 5 μl T_4 carrier (25 μg) and 30 μl of 0·01 N KI carrier (38 μg) were added to the sample of bile before chromatography. The T_4 marker was visualized by spraying with diazotized sulfanilic acid. The I^- marker was visualized by spraying with palladium chloride. The glucuronide was identified from its position on the radioautogram.[7]

In vitro *conjugation of* ^{131}I-T_4

Liver slices. Liver slices, 400 mg, from control or benzpyrene-treated rats were incubated at 37° with shaking in 3 ml Krebs–Ringer bicarbonate buffer. ^{131}I-T_4 was added at a level of 0·01 μg/ml, or 0·11 μg/ml. After 2 hr of incubation, the slices were homogenized in their own incubation medium. Acid butanol extraction of the homogenate, concentration of the butanol extract, and paper chromatography in collidine–NH_4OH were performed as previously described for plasma.[9]

Liver homogenate. Rats were decapitated and the livers quickly removed and weighed. A portion of the chilled liver was homogenized with 4 vol. of cold sucrose (0·25 M) with a teflon-glass homogenizer. In most instances, the incubation vessels contained 100 μmole Tris buffer (pH 7·4), 30 μmole $MgCl_2$, 0·3 μmole UDPGA, 0·33 μg ^{131}I-T_4 and liver homogenate equivalent to 400 mg wet wt. of liver from normal or drug treated rats in a final volume of 3 ml. Samples were incubated in air with shaking for 30 min at 37°, and the reaction was stopped by placing the incubation vessels in the freezer at −20°. After a few hours or when convenient, 30 μl of 1 % MMI was added to the thawed incubation mixture and it was centrifuged at low speed for 3 min. A 200-μl sample of the supernatant was applied to a filter paper strip over a 7·0 cm line, in 2 successive deliveries of 100 μl, and ascending chromatography was performed in collidine–3N NH_4OH, as described above. Since the ^{131}I-T_4 was added in 50 μl propylene glycol, the effect of this solvent on the liver glucuronide-forming system was tested separately on glucuronide conjugation of *o*-aminophenol (see below). No appreciable effect was observed.

In vitro *conjugation of* o-*aminophenol*

A previously described method for estimation of *o*-aminophenol conjugation by mouse liver homogenates[10] was adapted for rat liver homogenates. Rat liver homogenates were prepared as described above. In most experiments, the incubation vessels contained 100 μmole Tris (pH 7·4), 30 μmole $MgCl_2$, 0·15 μmole UDPGA, 0·42 μmole OAP, and liver homogenate equivalent to 200 mg wet wt. of liver in a final volume of 3 ml. Incubation was carried out in air at 37° for 30 min with shaking. The method of Dutton and Storey[11] was used for measuring OAP glucuronide. Standards containing OAP glucuronide were prepared with each run. In later experiments the incubation time was shortened to 20 min for a better estimate of initial rates, and incubation was carried out under N_2 to minimize contributions of UDP-glucose dehydrogenase.

Determination of optimal conditions for assay of glucuronyl transferase

In most experiments the above described conditions were used for measurement of OAP and T_4 conjugation. However, in order to relate observed differences between control and drug-treated animals to possible effects on the level of glucuronyl transferase, conditions were sought which would make this enzyme rate limiting in glucuronide formation. Comparisons were made, therefore, between liver homogenates from control and drug-treated animals with varying substrate (OAP or T_4) or cofactor (UDPGA) concentrations. To conserve the expensive cofactor, UDPGA, the incubation volume was cut in half (to 1·5 ml), and the amount of liver homogenate was reduced to the equivalent of 50 mg wet wt. of liver.

Metabolism of hexobarbital in vitro

Liver homogenates were prepared by homogenizing tissue with 4 vol. of cold KCl (1·15%), and the homogenate was centrifuged in the cold at 9000 *g* for 15 min. Activity of the 9000 *g* supernatant toward hexobarbital was determined by the procedure of Kato and Gillette,[12] except that a 1-hr incubation period and 2 μmole of substrate were used rather than a 30-min incubation period and 4 μmole of substrate. In one experiment, both procedures were followed with tissue from both control and phenobarbital-treated animals, and the ratio of hexobarbital metabolized by phenobarbital-treated vs. control animals was similar (5:6) for the two procedures. Generally, two livers from identically treated animals were pooled for an assay. Both treated and control animals were assayed on the same day. Incubations were made in triplicate. Hexobarbital disappearance was measured by the method of Cooper and Brodie.[13] Standard curves were made with each hexobarbital assay.

RESULTS

Effect of pretreatment of rats with benzpyrene or phenobarbital on excretion of ^{131}I in bile after administration of ^{131}I-T_4

The percentage of the injected dose of ^{131}I excreted in the bile in 4 hr was very similar for males and females. Pretreatment with benzpyrene for 48 hr increased biliary ^{131}I excretion from 6·4 $\pm$ 1·6 per cent to 23·4 $\pm$ 8·6 per cent (3·7-fold) for females, and from 6·5 $\pm$ 0·3 to 15·1 $\pm$ 2·4 per cent (2·3-fold) for males (Table 1). This increase was primarily the result of enhanced glucuronide excretion. The percentage of the injected dose of ^{131}I excreted as glucuronide was increased 5·5-fold in females and 3·2-fold in males. The excretion of ^{131}I-T_4 and $^{131}I^-$ was essentially unchanged. This was reflected in the relative increase of glucuronide as a percentage of the ^{131}I in the bile, from 44 per cent to 72 per cent in the 0- to 2-hr samples and from 55 per cent to 77 per cent in the 2- to 4-hr samples in females. A parallel increase occurred in males.

Phenobarbital pretreatment increased the percentage of the injected dose of ^{131}I excreted in bile in 4 hr to a small extent (1·5-fold for both males and females). The percentage of the injected dose of ^{131}I excreted as glucuronide was also increased 1·5-fold. However, bile flow was increased by the same factor. Therefore, the percentage of ^{131}I present as glucuronide in the bile from females was increased only slightly in the 2-hr sample and was not significantly different from control in the 4-hr sample. Neither sample was significantly higher than the control for phenobarbital-treated males.

TABLE 1. EFFECT OF PRETREATMENT OF RATS WITH BENZPYRENE OR PHENOBARBITAL ON EXCRETION OF ^{131}I IN BILE AFTER ADMINISTRATION OF ^{131}I-T_4*

Sex	Treatment	No. of animals	Bile flow in 4 hr (ml)	Per cent injected ^{131}I excreted in 4 hr as				Glucuronide as % total ^{131}I in bile	
				Total	Glucuronide	Thyroxine	Iodide	0–2 hr sample	2–4 hr sample
F	Corn oil	8	2·3 ± 0·3	6·4 ± 1·6	3·2 ± 1·1	1·06 ± 0·29	0·70 ± 0·15	44·5 ± 5·0	55·1 ± 4·8
F	Benzpyrene	10	2·6 ± 0·4†	23·4 ± 8·6‡	17·6 ± 7·2‡	1·44 ± 0·59†	0·93 ± 0·26†	72·1 ± 3·8‡	76·7 ± 6·8‡
M	Corn oil	7	2·5 ± 0·3	6·5 ± 0·3	3·3 ± 0·2	1·04 ± 0·36	0·53 ± 0·11	48·9 ± 5·4	55·2 ± 6·5
M	Benzpyrene	11	2·6 ± 0·6†	15·1 ± 2·4‡	10·7 ± 4·3‡	1·15 ± 0·35†	0·71 ± 0·22†	65·4 ± 7·5‡	74·0 ± 8·0‡
F	Control	12	2·6 ± 0·5	8·2 ± 1·3	4·2 ± 0·2	1·55 ± 0·58	0·92 ± 0·16	45·8 ± 5·0	60·0 ± 3·2
F	Phenobarbital	14	3·4 ± 0·8§	12·4 ± 2·2‡	7·0 ± 1·1‡	1·75 ± 0·45†	1·12 ± 0·39†	53·0 ± 5·7‖	62·2 ± 5·4†
M	Control	5	2·3 ± 0·4	7·6 ± 1·3	4·4 ± 0·6	1·19 ± 0·51	0·64 ± 0·16	53·2 ± 5·8	66·6 ± 4·1
M	Phenobarbital	4	3·4 ± 0·3‡	11·0 ± 3·0§	6·7 ± 1·2‖	1·30 ± 0·30†	0·87 ± 0·12§	53·8 ± 4·2†	67·6 ± 3·0†

* Rats were injected i.p. with 10 mg benzpyrene in 0·5 ml corn oil 48 hr prior to bile duct cannulation, or with sodium phenobarbital (5 mg/100 g body wt.) in saline for 5 days with the last injection 24 hr prior to bile duct cannulation. Controls were injected concurrently with vehicle. A single i.v. injection of ^{131}I–T_4 (0·1 μg/100 g body wt.) was given after recovery from anaesthetic and bile collection was begun immediately. Each value is expressed as the mean ± S.D.

† Not significantly different from controls, $P > 0.05$.

‡ Significantly different from controls of same sex, $P \leqslant 0.001$.

§ Significantly different from controls of same sex, $P \leqslant 0.05$.

‖ Significantly different from controls of same sex, $P \leqslant 0.01$.

Effect of benzpyrene on excretion of endogenously formed ^{131}I-T_4

To provide further evidence that benzpyrene pretreatment affects the metabolism of circulating T_4, even when the hormone is present at a physiological level, experiments were performed with rats that had been treated with $^{131}I^-$ instead of ^{131}I-T_4. They were maintained on a special low iodine diet for 10–14 days before the start of the experiment to increase the rate of conversion of injected $^{131}I^-$ to circulating ^{131}I-T_4. Further details are given in Table 2, together with the results.

Thyroid ^{131}I uptakes were similar for the benzpyrene-treated and control rats and ranged from 34–61 per cent. Despite considerable variation within each group, the per cent of the injected dose of ^{131}I excreted in the bile was significantly higher in the benzpyrene-treated animals. The difference between the groups was more obvious when the ^{131}I distribution patterns in bile were examined. In the benzpyrene-treated rats, glucuronide comprised 75·8 per cent of the total ^{131}I in bile, whereas the corresponding figure in control animals was only 57·0 per cent. Likewise, the percentage of ^{131}I present as T_4 was 7·1 in the former group and 15·3 in the latter.

It is clear, therefore, that glucuronide conjugation and biliary excretion of thyroxine are stimulated by pretreatment with benzpyrene even at normal physiological levels of thyroid hormone.

Effect of benzpyrene pretreatment on thyroxine glucuronide formation by liver slices

Table 3 shows that pretreatment of female rats with benzpyrene caused a 4-fold increase in the per cent conversion of ^{131}I-T_4 to ^{131}I-glucuronide by liver slices. The per cent conversion was the same for the two ^{131}I-T_4 concentrations used, indicating that the system was not saturated with substrate.

O-Aminophenol vs. thyroxine conjugation by the same liver homogenate after benzyyrene or phenobarbital pretreatment

Benzpyrene pretreatment significantly increased OAP conjugation and thyroxine conjugation by liver homogenates from female rats as shown in Table 4. Phenobarbital pretreatment, on the other hand, did not increase OAP conjugation significantly, and thyroxine glucuronide conjugation was unchanged.

Hexobarbital metabolism by the 9000 g supernatant after benzpyrene or phenobarbital pretreatment

To determine whether phenobarbital treatment affected other drug-metabolizing enzymes in these rats, the metabolism of hexobarbital in the 9000 *g* supernatant of phenobarbital-treated, benzpyrene-treated and control rats was studied. As seen in Table 4, hexobarbital metabolism was stimulated 4-fold in phenobarbital-treated rats. However, no significant stimulation occurred in benzpyrene-treated rats. From these data, we conclude that the dose of phenobarbital used in these experiments was sufficient to induce drug-metabolizing enzymes.

Effect of benzpyrene and phenobarbital on liver weight

The liver weight/body weight ratio for control, benzpyrene-treated and phenobarbital-treated female rats was 0·034 $\pm$ 0·005 (19 animals), 0·041 $\pm$ 0·007 (13 animals) and 0·039 $\pm$ 0·003 (10 animals) respectively. This is approximately a 21 per cent increase for benzpyrene-treated animals and a 15 per cent increase for phenobarbital-treated animals. The two pretreated groups were significantly different from controls ($P < 0·01$).

TABLE 2. BILIARY EXCRETION OF ENDOGENOUSLY FORMED ^{131}I-T_4 AFTER ^{131}I-ADMINISTRATION TO BENZPYRENE-TREATED AND CONTROL FEMALE HOLTZMAN RATS*

Treatment	No. of animals	Per cent injected ^{131}I excreted in 4 hr	Per cent injected ^{131}I excreted as glucuronide	Per cent ^{131}I in bile as		^{131}I uptake by thyroid (%)
				Glucuronide	Thyroxine	
Benzpyrene	6	$1{\cdot}6 \pm 1{\cdot}0$ ($P < 0{\cdot}05$)	$1{\cdot}26 \pm 0{\cdot}76$ ($P < 0{\cdot}05$)	$75{\cdot}8 \pm 5{\cdot}0$ ($P < 0{\cdot}001$)	$7{\cdot}1 \pm 2{\cdot}1$ ($P < 0{\cdot}01$)	48 (range 34–56)
Control	5	$0{\cdot}6 \pm 0{\cdot}2$	$0{\cdot}39 \pm 0{\cdot}15$	$57{\cdot}0 \pm 4{\cdot}2$	$15{\cdot}3 \pm 5{\cdot}3$	51 (range 38–61)

* Rats were maintained on a special low iodine diet (General Biochemicals Co.) for 10–14 days before beginning the experiment. Each rat received 10 mg benzpyrene in 0·5 ml corn oil or 0·5 ml corn oil alone i.p. 48 hr before surgery. An i.v. injection of 100 μc $^{131}I^-$ was given 24 hr before bile duct cannulation. One 4-hr bile sample was collected immediately after recovery from anesthetic. Each value is expressed as the mean $\pm$ S.D.

Effect of varying substrate and cofactor concentrations on o-aminophenol conjugation

To determine conditions for making the level of glucuronyl transferase rate limiting in the formation of OAP glucuronide, the effects of varying substrate and cofactor concentrations were measured as described under Methods. Fig. 1 shows the effect of increasing the concentration of OAP at a fixed high level of UDPGA. At all levels of

TABLE 3. EFFECT OF PRETREATMENT OF FEMALE RATS WITH BENZPYRENE ON THYROXINE GLUCURONIDE FORMATION BY RAT LIVER SLICES *in vitro**

Experiment No.	Thyroxine concentration (μg/ml)	Glucuronide as % total ^{131}I		Ratio benzpyrene/ control
		Control	Benzpyrene-treated	
1	0·01	1·6	6·0	3·7
1	0·01	1·6	6·7	4·1
2	0·01	1·5	5·2	3·5
2	0·11	1·8	7·3	4·1
2	0·11	1·7	7·2	4·2
2†	0·11	1·8	7·5	4·2

* Conditions: Each experiment included one control and one benzpyrene-treated animal. Individual values represent results of a single incubation vessel. Values for duplicate flasks are given in experiment 1 and for triplicates in experiment 2. Each incubation vessel contained 400 mg liver slices in 3 ml Krebs–Ringer bicarbonate buffer and ^{131}I-T_4 in the concentrations listed above. Incubation was for 2 hr under 95% O_2–5% CO_2 at 37° with shaking.

† UDPGA, 0·1 μmole/ml, added.

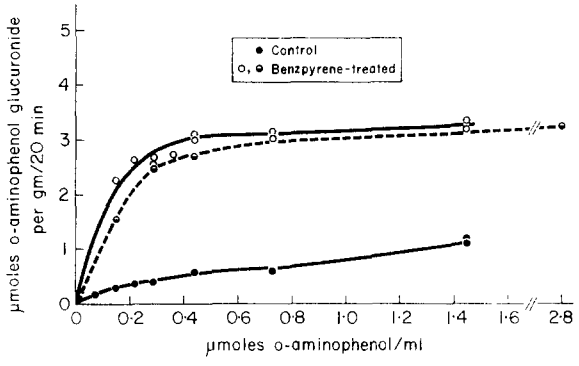

FIG. 1. *o*-Aminophenol glucuronide formation by liver homogenates from benzpyrene-treated and control female rats as a function of substrate concentration. Incubation vessels contained 50 mg tissue and 0·6 μmole UDPGA in a final volume of 1·5 ml. The dotted line, connecting half-closed circles, represents vessels containing 25 mg tissue from a benzpyrene-treated animal and 1·35 μmoles UDPGA in a final volume of 1·5 ml. Incubation was for 20 min under N_2 with shaking at 37°.

substrate, the rate of OAP conjugation by the liver homogenate from the benzpyrene-treated rat exceeded that from the control rat several-fold. Fig. 2 shows the effect of increasing the concentration of UDPGA at a fixed high level of OAP. At all levels of UDPGA, the liver homogenate from the benzpyrene-treated rat was again several times more active than the liver homogenate from a control or phenobarbital-treated rat. These results indicate that the observed increases in OAP glucuronide formation

TABLE 4. EFFECT OF BENZPYRENE OR PHENOBARBITAL PRETREATMENT ON GLUCURONIDE CONJUGATION AND HEXOBARBITAL METABOLISM*

Treatment	*o*-Aminophenol glucuronide formed (μmole/g liver/30 min)	^{131}I-T_4 (% converted to glucuronide/30 min)	Hexobarbital metabolized (mμmole/g liver/hr)
Corn oil	0·19 ± 0·05 (5)	1·0 ± 0·4 (4)	513 ± 225 (4)
Benzpyrene	0·70 ± 0·07 (6) $P < 0·001$	2·7 ± 0·9 (5) $P < 0·05$	577 ± 141 (4) N.S.
Saline	0·20 ± 0·05 (4)	1·3 ± 0·1 (6)	714 ± 310 (3)
Phenobarbital	0·30 ± 0·10 (7) N.S.	1·1 ± 0·1 (9) $P < 0·01$	3028 ± 290 (3) $P < 0·001$

* Benzpyrene 10 mg, was given 48 hr prior to assay or 50 mg/kg phenobarbital was given for 5 days with assay on day 6. Each value represents the mean ± S.D. with the number of animals included in the assay in parentheses. Assays of glucuronide conjugation were performed with whole homogenate and assays of hexobarbital metabolism with the 9000 *g* supernatant. Conditions for assay of *o*-aminophenol conjugation, thyroxine conjugation and hexobarbital metabolism are described under Methods.

in benzpyrene-treated rats were most likely due to an increase in the level of glucuronyltransferase.

By using optimum substrate and cofactor concentrations suggested by Figs. 1 and 2, the effect of phenobarbital pretreatment on OAP conjugation was rechecked. As shown in Table 5, there was a 70 per cent increase under these conditions, which was statistically significant ($P < 0.01$).

TABLE 5. GLUCURONIDE CONJUGATION OF *o*-AMINOPHENOL BY LIVER HOMOGENATES OF PHENOBARBITAL-TREATED FEMALE RATS*

Treatment	OAPG (μmole/g/ 20 min)	Liver wt. (g)	Body wt. (g)
Saline (6)	0·64 ± 0·11	7·08 ± 0·85	212 (195–233)
Phenobarbital (6)	1·10 ± 0·33 ($P < 0.01$)	8·98 ± 0·84 ($P < 0.01$)	213 (197–232) N.S.

* Phenobarbital (50 mg/kg) was administered i.p. for 5 days and assays were performed on day 6. The vessels contained 0·44 μmole/ml OAP, 0·27 μmole/ml UDPGA and 50 mg whole homogenate in 1·5 ml of total incubation volume. Incubation was for 20 min at 37° with shaking under N_2. Each assay was performed in duplicate. The number of animals per group is listed in parentheses after the group. Each value is expressed as the mean ±S.D.

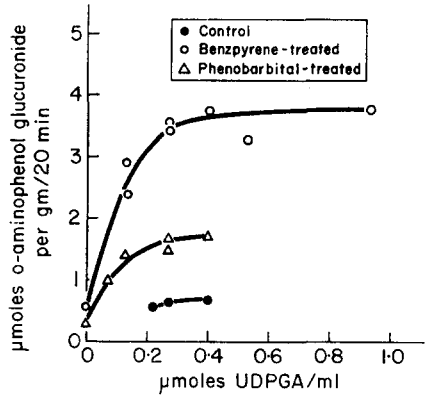

FIG. 2. Determination of optimum UDPGA concentration for *o*-aminophenol conjugation by benzpyrene-treated, phenobarbital-treated and control female rats. Incubation flasks contained 0·44 μmole *o*-aminophenol/ml and 50 mg liver homogenate in a final volume of 1·5 ml. Incubation was under N_2 with shaking for 20 min at 37°.

Effect of varying substrate and cofactor concentrations on thyroxine conjugation

The effect of varying UDPGA concentration on thyroxine glucuronide formation is shown in Fig. 3 for liver homogenates from benzpyrene- and phenobarbital-treated rats. When the concentration of UDPGA was increased 10-fold, the rate of thyroxine glucuronide formation was approximately doubled. However, the ratio of benzpyrene to normal activity remained fairly constant at approximately 3 to 4. Thyroxine glucuronide formation/30 min/400 mg liver by liver homogenates from benzpyrene-treated female rats was increased to 9 per cent after addition of 1·0 μmole/ml UDPGA. This value was higher than the conversion observed in liver slices after 2 hr of incubation (Table 2).

The effect of increasing thyroxine concentration on ^{131}I-T_4 glucuronide formation by liver homogenates from 2 control and 2 phenobarbital-treated female rats with 1·0 μmole/ml UDPGA is shown in Table 6. T_4 concentration was increased up to 20-fold by adding nonradioactive carrier thyroxine to the incubation media. The per cent conversion remained the same in all four cases, indicating that the enzyme was

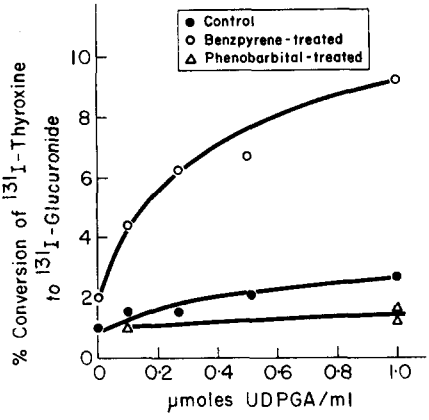

FIG. 3. Effect of UDPGA concentration on ^{131}I-thyroxine conjugation by liver homogenates from benzpyrene-treated, phenobarbital-treated and control female rats. Incubation flasks contained 0·11 μg/ml ^{131}I-thyroxine and 200 mg tissue in a final volume of 1·5 ml. Incubation was for 30 min under air at 37° with shaking. Values for phenobarbital-treated rats were obtained from Tables 4 and 6.

not saturated with substrate at any of the concentrations used. The very limited solubility of thyroxine prevented the use of higher concentrations. At all substrate concentrations, liver homogenate from control rats gave values as high as or higher than those from phenobarbital-treated rats.

TABLE 6. EFFECT OF INCREASING SUBSTRATE CONCENTRATION ON THYROXINE GLUCURONIDE CONJUGATION BY LIVER HOMOGENATES FROM PHENOBARBITAL-TREATED VS. CONTROL FEMALE RATS*

Thyroxine (μg/ml)	Per cent conversion ^{131}I-thyroxine to glucuronide			
	Phenobarbital-1	Phenobarbital-2	Control-1	Control-2
0·11	1·3	1·6	1·5	1·9
0·31	1·5	1·7	1·7	1·9
0·71	1·4	1·9	1·9	1·9
2·11	1·6	2·2	2·4	2·4

* Incubation mixtures contained 200 mg tissue and 1·5 μmole UDPGA in a final volume of 1·5 ml. Incubation was for 30 min at 37° under air with shaking.

Contributions of β-glucuronidase to results in vitro

In addition to glucuronyl transferase, two other enzymes, UDPGA pyrophosphatase and β-glucuronidase, are present in microsomes.[14, 15] Moreover, UDP-glucose dehydrogenase, a supernatant enzyme, could influence results in whole homogenates. The addition of UDPGA in optimum amounts presumably negated the influences of the dehydrogenase and the pyrophosphatase. However, variable levels of

the β-glucuronidase in control, phenobarbital-treated and benzpyrene-treated animals could conceivably affect the results *in vitro*. To study the contribution that β-glucuronidase makes to the results of the system *in vitro*, 0·08 μmole OAP glucuronide was incubated with liver homogenates from control and phenobarbital-treated rats. No hydrolysis of the glucuronide was observed, indicating that β-glucuronidase activity was negligible under these conditions. Inhibition of β-glucuronidase activity therefore, cannot be invoked as an explanation for the stimulation of glucuronide formation observed in benzpyrene-treated rats, nor can the difference between the increase in transferase activity observed *in vitro* after phenobarbital and benzpyrene be attributed to an increase in β-glucuronidase in the phenobarbital-treated animals.

Effect of SKF 525-A on o-aminophenol conjugation in vitro

Cooper *et al.*[16] reported that 2×10^{-4} M SKF 525-A produced an 80 per cent inhibition of morphine glucuronide formation by rat liver slices. *o*-Aminophenol glucuronide formation by a liver homogenate from a benzpyrene-treated female rat was not inhibited by 2×10^{-5}, 2×10^{-4}, or 2×10^{-3} M SKF 525-A in the present study.

Short-term pretreatment with benzpyrene

Other compounds that have been reported to increase biliary excretion of administered labeled thyroxine include butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) and salicylates.[17, 18] In the case of BHDB and salicylates, the primary mechanism probably involves an inhibitory effect on the binding of thyroxine to plasma proteins, thus making relatively more circulating thyroxine available for glucuronide formation.[19, 20] This would explain the observations that these drugs increase biliary thyroxine excretion within a short time after their administration to rats, and that they also increase biliary excretion when added to the perfusing blood of an isolated liver preparation.[21, 22] However, it does not explain the fact that BHDB is active when the isolated liver is obtained from a BHDB-treated animal and the perfusing blood is taken from an untreated animal.[21] It is possible that BHDB alters binding of T_4 to liver proteins as well as to plasma proteins, thereby altering access of substrate to microsomes.

In order to rule out an effect of benzpyrene on binding to plasma or liver proteins, biliary excretion of thyroxine was studied after short-term pretreatment with benzpyrene. Three rats were pretreated with 10 mg benzpyrene in 0·5 ml corn oil i.p., and two were pretreated with 50 mg BHDB orally in 1 ml lipomul. The bile ducts were cannulated 4·5 to 5·5 hr later, and ^{131}I-T_4 was injected as previously described. Two control rats with no pretreatment were cannulated concurrently.

Pretreatment with benzpyrene did not increase the excretion of ^{131}I-containing compounds into the bile 4·5 to 9·5 hr after pretreatment, as shown in Table 7. BHDB pretreatment, however, increased the excretion of the injected dose of ^{131}I approximately 2- to 3-fold (from 9 to 23 and 22 per cent), as observed by previous investigators.[17] Fluorescence due to benzpyrene or its metabolites could be readily observed in the bile of all benzpyrene-treated animals, ruling out the possibility that it had not been absorbed in this time period.

Similarly, the total excretion of ^{131}I-glucuronide remained the same for benzpyrene-treated animals as for controls but increased 2- to 3-fold for the BHDB-treated animals. The distribution of ^{131}I in the bile of the benzpyrene-treated animals appears

TABLE 7. EFFECT OF SHORT-TERM PRETREATMENT WITH BENZPYRENE OR BHDB ON BILIARY EXCRETION OF ^{131}I-THYROXINE BY FEMALE RATS*

Time interval after ^{131}I-T_4	Treatment	per cent injected ^{131}I excreted in bile as				Per cent ^{131}I in bile as			Bile flow (ml)
		Total	Glucuronide	T_4	I^-	Glucuronide	T_4	I^-	
0–2 hr	Control-1	5·3	2·36	0·90	0·72	44·6	17·0	13·6	1·2
	Control-2	5·5	2·63	0·99	0·74	47·9	18·0	13·4	1·4
	BHDB-1	12·3	7·02	1·57	1·08	57·1	12·8	8·8	1·75
	BHDB-2	11·8	6·95	1·17	1·27	58·9	9·4	10·8	2·0
	Benzpyrene-1	3·4	1·79	0·38	0·37	52·5	11·2	11·0	0·9
	Benzpyrene-2	5·1	3·13	0·39	0·49	61·4	7·6	9·6	1·0
	Benzpyrene-3	3·9	2·09	0·48	0·51	53·5	12·4	13·0	1·3
2–4 hr	Control-1	3·5	1·99	0·57	0·34	56·9	16·4	9·6	1·1
	Control-2	4·0	2·40	0·61	0·33	59·9	15·3	8·2	1·4
	BHDB-1	10·9	6·76	1·31	0·65	62·0	12·0	6·0	1·9
	BHDB-2	9·0	5·42	0·99	0·73	60·2	11·8	8·1	2·4
	Benzpyrene-1	3·0	1·98	0·25	0·22	66·0	8·2	7·3	0·95
	Benzpyrene-2	4·1	2·86	0·29	0·31	69·8	7·0	7·5	1·2
	Benzpyrene-3	2·8	1·97	0·18	0·18	70·4	6·6	6·5	1·15

* Rats were injected i.p. with 10 mg benzpyrene in 0·5 ml corn oil or 50 mg butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) was administered orally in 1 ml lipomul 4·5 to 5·5 hr before i.v. injection of ^{131}I-T_4 (0·1 μg/100 g body wt.). Bile collection was begun immediately after injection of ^{131}I-T_4.

to have changed, however. There was a consistent increase in the percentage of the biliary ^{131}I in the glucuronide and a decrease in the thyroxine fraction compared to control values. When viewed as per cent of the injected dose excreted, however, the excretion of thyroxine glucuronide was normal. Excretion of unchanged thyroxine appeared to be low, causing glucuronides to comprise a larger percentage of the total ^{131}I excreted.

It may be concluded, therefore, that the only short-term effect of benzpyrene treatment (4·5 to 9·5 hr after treatment) on biliary excretion of thyroxine by female rats appeared to be a decrease in the excretion of unchanged thyroxine. There was no increase in the excretion of glucuronide conjugates of thyroxine.

DISCUSSION

The data presented here demonstrate that ^{131}I-thyroxine glucuronide formation is markedly stimulated, both *in vivo* and *in vitro*, in the livers of rats 48 hr after a single injection of benzpyrene. Of particular interest is the fact that the stimulation *in vivo* was observed in rats that had received physiological doses of ^{131}I-thyroxine or that had formed endogenous ^{131}I-thyroxine after administration of $^{131}I^-$. This increase in the biliary excretion of the glucuronide of endogenous ^{131}I-T_4 was sufficient to increase significantly the percentage of the injected dose of ^{131}I excreted via the bile. These data indicate not only a change in the pattern of metabolites excreted after benzpyrene, but also an increase in the total clearance of the endogenous T_4 via the bile. Stimulation of the drug-metabolizing system of the liver, therefore, affects the metabolism of thyroxine present at physiological levels in the circulation.

Previous studies have shown that liver microsomes isolated from animals treated with phenobarbital and other drugs display enhanced metabolic acitvity toward adrenal and sex steroids.[23, 24] Phenobarbital and diphenylhydantoin have been reported to increase the excretion of 6 β-hydroxycortisol *in vivo*.[25, 26] However, the result of the increase in steroid hydroxylases may be a change in the pattern of metabolites excreted rather than an increase in the total excretion of steroids, since Werk *et al.*[25] found an increase in the excretion of 6 β-hydroxycortisol after diphenylhydantoin to be concomitant with a decrease in the total output of 17-keto-steroids.

Benzpyrene treatment increased thyroxine glucuronide conjugation by liver slices and whole homogenates from female rats approximately 4-fold. The enhanced activity is best explained by an increase in glucuronyl transferase, as shown by the findings in Fig. 3 and Table 4. However, these results may not accurately depict the magnitude of the increase, since the assays were carried out under conditions where the enzyme was not saturated with substrate. When OAP was used as a substrate, it was possible to saturate the enzyme with both substrate and cofactor. Under these conditions glucuronyl transferase activity was also increased 4- to 5-fold by pretreatment with benzpyrene.

Although Cooper *et al.*[16] and others have reported that SKF 525-A, a nonspecific inhibitor of drug metabolism, inhibited morphine conjugation *in vitro*, no effect of SKF 525-A was found on OAP conjugation *in vitro*. Likewise, thyroxine glucuronide excretion *in vivo* by two control female rats was not inhibited by SKF 525-A (25 mg/kg) given 1 hr before bile duct cannulation.

As mentioned previously, BHDB and salicylate probably increase biliary excretion of administered thyroxine by an inhibitory effect on the binding of thyroxine to plasma

proteins and possibly also to liver proteins.[18, 19, 21] The rapid effect which these compounds have on biliary excretion of T_4 in the isolated perfused liver[21, 22] can hardly involve an increase in glucuronyl transferase. The experiment comparing the acute effects of BHDB and benzpyrene *in vivo* showed no effect of benzpyrene on thyroxine glucuronide excretion at a time when BHDB produced marked increases in the biliary excretion of thyroxine and its metabolites. These data, together with those from the studies *in vitro*, indicate that the enhanced formation of thyroxine glucuronide observed 48 hr after treatment with benzpyrene is related not to an effect on the binding of thyroxine to plasma proteins, but more likely to an increase in liver glucuronyl transferase activity.

Two additional compounds which increase biliary excretion of administered labeled T_4 are propylthiouracil (PTU) and diphenylhydantoin.[27, 28] PTU has been reported to inhibit peripheral deiodination of thyroxine,[29] and the increased biliary excretion of thyroxine observed after its administration may simply be a passive reflection of this inhibitory effect on an alternate metabolic pathway. In the case of diphenylhydantoin, it is not clear what mechanism is involved in stimulating biliary thyroxine excretion. Unlike BHDB and salicylates, diphenylhydantoin does not seem to affect binding of thyroxine to plasma proteins, and its stimulating effect is relatively small.[27]

Since the completion of this work, Newman and Moon[30] reported that when rats receiving 5 μg ^{131}I-T_4/day for 17 days were also given 10 mg methylcholanthrene daily for the first 10 days by intubation, the fecal excretion of ^{131}I (as ^{131}I-T_4) was greater in the methylcholanthrene-treated rats than in the control rats (70 vs. 40 per cent of the daily injected dose/24-hr period) by the fourth day. The excretion of ^{131}I in the urine (as $^{131}I^-$) was decreased from 60 per cent for the control rats to 20 per cent for the methylcholanthrene-treated rats. The protein-bound ^{131}I was also decreased to less than half the control value. Although these authors present no data on glucuronide formation *in vivo* or *in vitro*, our results showing increased thyroxine glucuronide formation *in vitro* and increased biliary excretion of thyroxine glucuronide *in vivo* after benzpyrene provide the most probable explanation for the effects they observed with methylcholanthrene.

In the present study, treatment of rats with phenobarbital (50 mg/kg daily for 5 days) caused a much less striking increase (50 per cent) in biliary thyroxine excretion than did administration of a single 10-mg dose of benzpyrene. Moreover, the increase in ^{131}I excretion in bile of phenobarbital-treated rats was accompanied by a proportionate increase in the bile flow rate, so that the ^{131}I concentration in the bile remained essentially unchanged. The relative amount of biliary glucuronide and free thyroxine resembled that in untreated rats. This is in marked contrast to the results with benzpyrene-treated rats in which the bile flow rate was essentially unchanged, but both the total ^{131}I and the relative amount of ^{131}I present as the glucuronide were markedly increased.

Moreover, no increase was observed in thyroxine glucuronide formation *in vitro* with the liver homogenates from phenobarbital-treated rats, with optimum UDPGA concentrations and a 20-fold range of thyroxine concentrations. On the other hand, the 70 per cent increase in OAP conjugation with optimum cofactor and substrate concentrations was significantly greater than control. Zeidenberg *et al.*[31] recently reported a similar increase in glucuronide conjugation of *p*-nitrophenol after phenobarbital treatment. It is not possible to conclude from the above data whether the fail-

ure to demonstrate an increase in T_4 conjugation *in vitro* represents a real difference between this system and the OAP-conjugating system, or whether it is due to insensitivity of the method or lack of saturation of the enzyme with substrate. Since the concentration of glucuronide in the bile is increased only slightly or not at all and the stimulation *in vitro* is not observed over a wide range of thyroxine concentrations, it is unlikely that the slight increase in biliary thyroxine excretion after phenobarbital results from increased glucuronyl transferase. Roberts and Plaa[32] have observed a similar effect of phenobarbital on bilirubin excretion in rats. Although a 50 per cent increase in bile flow and bilirubin excretion occurred after phenobarbital, there was no change in the concentration of bilirubin in the bile. Liver uptake of bilirubin was also increased in mice. These authors suggested that the increase could possibly occur via increased glucuronyl transferase and that the increase in liver weight might account for the increase in bile flow. Our results with benzpyrene, however, show no increase in bile flow even when glucuronyl transferase is increased 4- to 5-fold, and the increase in liver weight is at least as great as after phenobarbital. Hollman and Touster[6] found increases in both UDP-glucose dehydrogenase and UDPGA pyrophosphatase after 2 days of barbital administration, but demonstrated no effect on glucuronyl transferase. Since increases in the first two enzymes would have opposite effects on the availability of UDPGA *in vivo*, and bile flow may be increased by yet another mechanism, the role of all these factors on excretion of glucuronide *in vivo* becomes difficult to assess.

Inscoe and Axelrod[3] reported that the formation of OAP glucuronide *in vitro* by rat liver microsomes was four times higher for male rats than for females. In addition, the stimulation of OAP conjugation after benzpyrene was much greater in females than in males (400 vs. 33 per cent). No such sex difference in the biliary excretion of thyroxine glucuronide was observed in the present study. It should be noted, however, that the OAP studies involved relatively high concentrations of the drug, whereas biliary thyroxine excretion was studied only with physiological doses. It is possible that a sex difference for thyroxine might have been observed if larger doses of hormone had been injected, but this was not tested. However, in one experiment with liver homogenate from one control and one benzpyrene-treated male rat, thyroxine conjugation by the control male was identical with thyroxine conjugation by females, even though OAP conjugation was much higher than in females. Moreover, the increase in thyroxine conjugation after benzpyrene was similar to that seen in females (4-fold), whereas OAP conjugation was increased much less (60 per cent compared to a 400 per cent increase for females). Here again, however, the thyroxine concentration was relatively low and did not saturate the enzyme.

An attempt to produce goiter by chronic treatment with benzpyrene was unsuccessful. The thyroid weights of 4 rats, injected every third day for 43 days with 10 mg benzpyrene, were no greater than those of control rats. However, these negative results are not conclusive since chronic treatment at this dose level led to ascites, weight loss and diarrhea. Experiments employing lower doses of benzpyrene are indicated, but these were not performed.

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