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Betamethasone-Mediated Activation of Biphenyl 2-Hydroxylation in Rat Liver Microsomes

Studies on Possible Mechanisms

MARK STEVEN MILLER,[1] MOU-TUAN HUANG,[2] ALAN M. JEFFREY, AND ALLAN H. CONNEY[2]

Department of Pharmacology, Columbia College of Physicians and Surgeons, New York, New York 10032, and Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, New Jersey 07110

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SUMMARY

The addition of the steroid betamethasone to intact or detergent-solubilized rat liver microsomes caused a concentration-dependent increase in the rate of biphenyl 2-hydroxylation. Betamethasone (100 μM) increased the apparent V_{max} for 2-hydroxybiphenyl formation 2- to 4-fold but had no effect on the apparent K_m when either the biphenyl or NADPH concentration was varied. Betamethasone had little or no effect on the apparent V_{max} or apparent K_m of the 3- and 4-hydroxylations of biphenyl. The steroid did not enhance biphenyl 2-hydroxylation through a peroxidative mechanism. Betamethasone had little or no effect on the rate of the NADPH-dependent reduction of cytochrome *c* or total microsomal cytochrome P-450. The addition of purified NADPH-cytochrome P-450 reductase to cholate-solubilized liver microsomes increased the rate of hydroxylation of biphenyl in positions 2 and 4. Betamethasone (100 μM) decreased the apparent K_m for purified cytochrome P-450 reductase by 48% and increased the apparent V_{max} of 2-hydroxybiphenyl formation by 2-fold when the concentration of cytochrome P-450 reductase was varied. The steroid did not alter the K_m or V_{max} values for the 4-hydroxylation of biphenyl. The data suggest that betamethasone enhances the interaction between the reductase and the form(s) of cytochrome P-450 responsible for the 2-hydroxylation of biphenyl.

INTRODUCTION

During the last two decades, several compounds have been identified which enhance the catalytic activity of monooxygenases found in liver microsomes (1, 2). Recently, Bridges and his associates have shown that the *in vitro* addition of certain glucocorticoids to hepatic microsomal preparations can activate the mixed-function oxidase systems of rats (3–8), hamsters (6), and humans (8) responsible for the hydroxylation of biphenyl. Although hydroxylation of biphenyl in position 2 was enhanced, these steroids had little or no effect on the hydroxylation of biphenyl in position 3 or 4 (3, 5, 8).

Of the many steroids tested, betamethasone was the most potent activator of monooxygenase activity in rat liver microsomes (3, 5, 6), whereas progesterone was the most potent activator of hamster liver microsomes (6). No relationship was found between the stimulatory effect of several glucocorticoids on biphenyl 2-hydroxylation and their glucocorticoid activity (3). In studies with rat liver microsomes, Benford *et al.* (6) found that when the concentration of biphenyl was varied in the presence of betamethasone, a sigmoidal curve was obtained. Both the apparent K_m and apparent V_{max}[3] increased as compared with the linear control curve when the data were calculated from a Lineweaver-Burk plot. The authors suggested that these results could be explained by the presence of two different enzymes, one of which is selectively affected by betamethasone.

Recent studies on the mechanism of activation of benzo[*a*]pyrene hydroxylation by 7,8-benzoflavone suggested that this flavonoid stimulated benzo[*a*]pyrene hydroxylation by enhancing the flow of electrons between NADPH-cytochrome P-450 reductase (EC 1.6.2.4) and cytochrome P-450 (9). Since both 7,8-benzoflavone and betamethasone activate biphenyl 2-hydroxylation in rat hepatic microsomes (7), we initiated studies to determine the mechanism by which betamethasone causes its stimulatory effects. In the present report, we provide evidence suggesting that betamethasone enhances the interaction

[1] Predoctoral Fellow at Columbia University. The data in this paper represent part of the requirements for the fulfillment of the doctoral thesis for the degree of Doctor of Philosophy in Pharmacology at the Graduate School of Arts and Sciences.

[2] Hoffmann-La Roche Inc.

[3] The terms K_m and V_{max} as used in this paper refer to the apparent K_m and apparent V_{max}.

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between cytochrome P-450 reductase and the form(s) of cytochrome P-450 that is responsible for the hydroxylation of biphenyl in position 2.

METHODS

Chemicals. Biphenyl and 2-hydroxybiphenyl were purchased from Aldrich Chemical Company (Milwaukee, Wisc.), and 3-hydroxybiphenyl and 4-hydroxybiphenyl were obtained from ICN Pharmaceuticals, Inc. (Plainview, N. Y.); DEAE-Sepharose A-25 and Sephadex G-25 were purchased from Pharmacia Fine Chemicals (Piscataway, N. J.); agarose-hexane-adenosine 2′,5′-diphosphate Type 2 was obtained from P-L Biochemicals, Inc. (Milwaukee, Wisc.); calcium phosphate gel was obtained from Bio-Rad Laboratories (Richmond, Calif.); Emulgen 913 was purchased from Kao-Atlas Company, Ltd. (Tokyo, Japan); Triton N-101 was obtained from Rohm and Haas Company (Philadelphia, Pa.); HPLC[4] grade isooctane and acetonitrile were purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.); and cumene hydroperoxide was purchased from MC/B Manufacturing Chemists (Norwood, Ohio).

Animals. Three-week-old male Long Evans rats were obtained from Blue Spruce Farms, Inc. (Altamont, N. Y.). The rats were housed for 1 week in wire-mesh cages. They were maintained on a 12-hr light/dark cycle and allowed free access to food (Purina rodent laboratory chow) and water.

Preparation of microsomes. Four-week-old rats were killed by decapitation, and the livers were quickly removed and washed with a solution of cold 1.15% KCl. Liver microsomes were prepared from a 33% (w/v) homogenate in 50 mM Tris-HCl buffer (pH 7.4) and 1.15% KCl as described previously (10). The microsomal pellets were resuspended and washed with 1.15% KCl containing 10 mM EDTA. The microsomes were repelleted by centrifugation, suspended in 0.25 M sucrose, and stored at −90°. The protein content of the microsomes ranged from 39.0 to 72.2 mg/ml, and hydroxylase activity of the microsomes was stable for at least 1 year.

Preparation of cholate-solubilized microsomes. A 1-ml aliquot of the microsomal suspension was added to 7.5 ml of a 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 40% glycerol. A 20% sodium cholate solution (cholate was recrystallized from 50% ethanol/water) was added to achieve a final detergent concentration of 0.8%. The suspension was stirred for 20 min at 4°, and insoluble material was collected by centrifugation for 1 hr at 105,000 × *g*. The supernatant fraction contained about 84% of the original cytochrome P-450. In experiments in which solubilized microsomes were used, the microsomes were added to obtain a final concentration of 0.5 nmole of cytochrome P-450 per incubation mixture.

NADPH-dependent biphenyl hydroxylation. The hydroxylation of biphenyl was measured by a modification of methods described elsewhere (4). Reaction mixtures contained the following components (unless otherwise stated): 50 mM potassium phosphate buffer (pH 7.6), 5 mM $MgSO_4$, 3.5 mM DL-isocitric acid, 0.8 unit of isocitric dehydrogenase, 0.5 mM NADPH, and 2 mg of microsomal protein. Betamethasone was added in 10 μl of DMF, and the final steroid concentration was 100 μM. Control incubation mixtures contained 10 μl of DMF alone. The reaction was started by addition of 10 μl of DMF containing biphenyl to provide a final substrate concentration of 2.0 mM in the incubation mixture and a final volume of 2 ml. In experiments where the NADPH concentration was varied, the reaction was started by addition of NADPH. The reaction mixtures were shaken gently for 10 min at 37°, and reactions were terminated by addition of 0.5 ml of 4 M HCl. The samples were then extracted for 20 min in a bottle shaker or reciprocating shaker with 7 ml of isooctane containing 1.5% (v/v) of either *n*-amyl or isoamyl alcohol. Following centrifugation for 10 min at 1,500 × *g*, 5-ml aliquots of the upper organic layers were evaporated under a stream of nitrogen in the presence of 10 μl of triethylamine. The samples were stored for no more than 4 days at −20° until subsequent analysis by HPLC. Zero-time samples had either biphenyl or NADPH added after the microsomal enzymes were inactivated with acid. Recoveries of authentic monohydroxylated standards were greater than 85%.

The samples were analyzed by the HPLC method of Burke and Prough (11). The evaporated extracts were dissolved in 200 μl of acetonitrile, and 50-μl samples were injected into a Waters Associates ALC/GLC 204 high-pressure liquid chromatograph equipped with a Waters Associates NH_2-μBondapack analytical column (4 mm × 30 cm). A guard column containing Bondapack AX/Corasil was used in some experiments. The injected samples were eluted isocratically with a solvent mixture consisting of isooctane-acetonitrile-*n*-amyl or isoamyl alcohol (100:4:4) at a flow rate of 2 ml/min. The hydroxylated derivatives in the effluent stream were monitored with a flow-through spectrophotometer measuring the UV absorbance of these compounds at 254 nm. The tracings were recorded on a Soltec Model 220 recorder (Soltec Corporation, Sun Valley, Calif.), and peak heights were measured and compared with authentic standards injected into the HPLC. Corrections were made for blanks and percentage recoveries as determined in each experiment.

Cumene hydroperoxide-dependent biphenyl hydroxylation. The cumene hydroperoxide-dependent hydroxylation of biphenyl was assayed by a modification of a method described earlier (12). The reaction mixtures contained 50 mM potassium phosphate buffer (pH 7.6), 5 mM $MgSO_4$, and 4 mg of microsomal protein in a final volume of 2 ml. Various amounts of betamethasone were added simultaneously with biphenyl (final concentration, 2.0 mM) in 10 μl of DMF; control incubation mixtures had the biphenyl added in 10 μl of DMF. The reaction was initiated by addition of 10 μl of cumene hydroperoxide diluted in DMF to a final concentration of 50 or 500 μM in the incubation mixture. The reaction mixtures were shaken gently for 3 min at 37°, and reactions were terminated by addition of 0.5 ml of 4 M HCl. Zero-time samples had the cumene hydroperoxide added after the enzymes were inactivated with acid. Formation of 2-, 3-, and 4-hydroxybiphenyl was determined as described above for the NADPH-dependent hydroxylation of biphenyl.

NADPH-dependent cytochrome c reduction. The reduction of cytochrome *c* by liver microsomes was determined by a modification of the assay described by Phillips and Langdon (13). The reaction mixtures contained the following components in a final volume of 1 ml: 0.33 M potassium phosphate (pH 7.7), 50 μM cytochrome *c*, 0.072 mg of microsomal protein, 0.1 mM NADPH, and various amounts of betamethasone added in 10 μl of DMF. Cytochrome *c* reductase activity was measured spectrophotometrically by monitoring the increase in absorbance at 550 nm in a Gilford spectrophotometer at 22°. One unit of NADPH-cytochrome *c* reductase activity is defined as the amount of enzyme catalyzing the reduction of 1 nmole of cytochrome *c* per minute at 22°.

NADPH-dependent cytochrome P-450 reduction. The reduction of cytochrome P-450 was measured by a modification (9) of the method of Gigon *et al.* (14). A 2.5-ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.6), 5 mM $MgSO_4$, 3.5 mM isocitric acid, 1.0 unit of isocitric dehydrogenase, 20% glycerol, 2.5 mg of microsomal protein, 2.0 mM biphenyl, and 100 μM betamethasone was placed in an anaerobic cell (American Instrument Company). In some experiments, biphenyl and/or betamethasone were replaced by DMF solvent alone, which was present at a concentration of 1% in all experiments. CO was bubbled through the mixture for 5 min, and 50 μl of a 25 mM solution of NADPH saturated with CO were then added. The reduction of cytochrome P-450 was monitored at room temperature for 3 min in an Aminco DW-2A spectrophotometer set in the dual wavelength mode (Δ 450–490 nm). A full-scale absorbance setting of 0.05 was used with the response control in the fast setting and a recorder scan rate of 20 sec/in.

Preparation of purified NADPH-cytochrome P-450 reductase. NADPH-cytochrome P-450 reductase (cytochrome *c* reductase) was solubilized from liver microsomes of phenobarbital-treated rats and chromatographed on a DEAE-Sepharose A-25 column as described previously (15) except that Triton N-101 was substituted for Renex 690. Following calcium phosphate gel fractionation, the reductase was further purified by affinity chromatography on a 2′,5′-ADP Sepharose 4B

[4] The abbreviations used are: HPLC, high-pressure liquid chromatography; DMF, *N,N*-dimethylformamide.

column as described elsewhere (16). Buffer consisting of 0.1 M potassium phosphate (pH 7.7), 0.1 mM dithiothreitol, 0.1 mM EDTA, 0.4% sodium cholate, and 20% glycerol was used to wash the column. Reductase was eluted from the column with 25 ml of the same buffer containing 10 mM NADP. The fractions containing reductase were concentrated over an Amicon XM-50 membrane, and the reductase was passed through a Sephadex G-25 column (90 × 1.5 cm) previously equilibrated with 50 mM potassium phosphate buffer (pH 7.7). The final preparation had a specific activity of 47,300 units of NADPH-cytochrome *c* reductase activity per milligram of protein.

Determination of cytochrome P-450 and protein content. The concentration of cytochrome P-450 was determined by the method of Omura and Sato (17). Protein content was assayed by the procedure of Lowry *et al.* (18).

Statistical analysis. The K_m and V_{max} values for each experiment were determined by the computer program developed by Cleland (19), with weighting of local variance as described by Ottaway (20). A paired Student's *t*-test was used to determine statistical significance.

RESULTS

Effect of betamethasone on the 2-hydroxylation of biphenyl by untreated and cholate-solubilized rat liver microsomes. Addition of betamethasone to untreated or cholate-solubilized microsomal preparations caused a concentration-dependent increase in the rate of biphenyl hydroxylation at position 2 (Fig. 1). The stimulatory effect of betamethasone on biphenyl 2-hydroxylation by untreated liver microsomes occurred at concentrations as low as 1 μM. At the highest concentration tested (1 mM), betamethasone caused a 4-fold activation in the rate of 2-hydroxybiphenyl formation in untreated microsomes and a 2.5-fold activation in cholate-solubilized microsomes. Betamethasone had little or no effect on the amount of 3- or 4-hydroxybiphenyl produced over the concentration range tested, except at the highest concentration, where there was some inhibition (about 20%) of biphenyl hydroxylation at positions 3 and 4. Since steroids can serve as substrates for the cytochrome P-450 enzyme system, this inhibition may have been caused by competition between the betamethasone and biphenyl for binding sites on the microsomal enzymes. In subsequent experiments, a 100 μM concentration of betamethasone was used since this was the highest amount of the steroid capable of enhancing biphenyl 2-hydroxylation without inhibiting the formation of the 3- and 4-hydroxybiphenyls under optimal conditions of substrate concentration.

Effect of biphenyl concentration on the betamethasone stimulation of biphenyl 2-hydroxylation. The K_m for biphenyl and the V_{max} of biphenyl hydroxylation were determined in the presence and absence of 100 μM betamethasone. The biphenyl concentration was varied from 50 to 500 μM. K_m and V_{max} values were obtained from double-reciprocal plots of the rate of formation of the 2-, 3-, and 4-hydroxybiphenyl metabolites versus the concentration of biphenyl. Figure 2 shows the Lineweaver-Burk plot for the formation of 2-hydroxybiphenyl, and it can be seen that betamethasone caused a 4-fold stimulation of the V_{max} of biphenyl 2-hydroxylation. The V_{max} was increased from 437 to 1902 pmoles of product formed per minute per nanomole of cytochrome P-450 ($p < 0.05$). Betamethasone, however, did not significantly affect the K_m for biphenyl with respect to the 2-hydroxylation reaction ($p > 0.05$).

The results of our studies also indicated that betamethasone had no effect on the V_{max} of 3-hydroxybiphenyl and 4-hydroxybiphenyl formation, nor did the steroid alter the K_m for biphenyl with respect to the 4-hydrox-

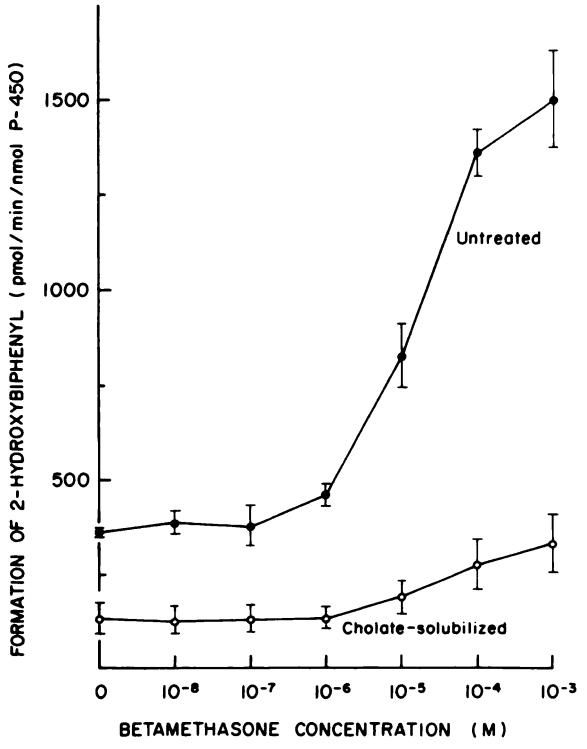

FIG. 1. *Effect of betamethasone concentration on the 2-hydroxylation of biphenyl by untreated and cholate-solubilized rat liver microsomes.*

Biphenyl hydroxylation was determined with untreated microsomes (●) or cholate-solubilized microsomes (○) in the presence of various concentrations of betamethasone. The amount of cytochrome P-450 in each reaction mixture was 0.5 nmole. Each value represents the mean ± standard deviation from three experiments.

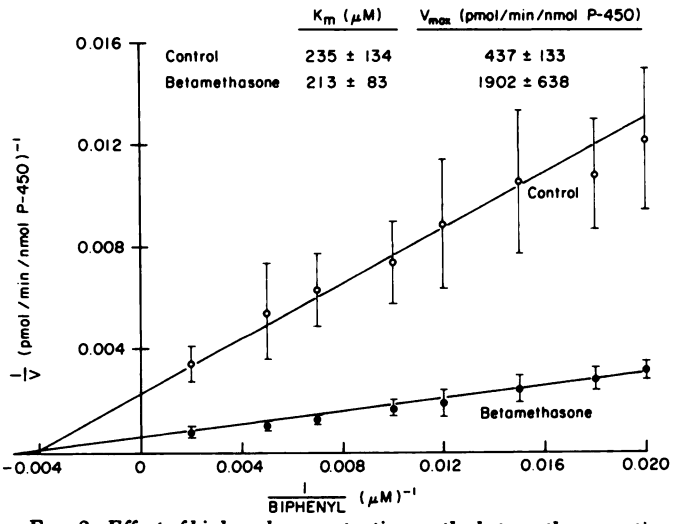

FIG. 2. *Effect of biphenyl concentration on the betamethasone stimulation of biphenyl 2-hydroxylation in rat liver microsomes.*

The rate of biphenyl hydroxylation was determined in the presence (●) and absence (○) of 100 μM betamethasone. The biphenyl concentration was varied from 50 to 500 μM. Each value represents the mean ± standard deviation from four experiments.

ylation reaction (data not shown). Betamethasone, however, may competitively inhibit the formation of 3-hydroxybiphenyl, since the K_m for biphenyl with respect to the 3-hydroxylation reaction was significantly increased by 37% from 54 to 74 μM (data not shown).

Effect of NADPH concentration on the betamethasone stimulation of biphenyl 2-hydroxylation. The K_m for NADPH and the V_{max} of biphenyl hydroxylation were determined in the presence and absence of 100 μM betamethasone. The NADPH concentration was varied from 1 to 10 μM. Betamethasone increased the V_{max} of biphenyl 2-hydroxylation 4-fold from 432 to 1767 pmoles of product formed per minute per nanomole of cytochrome P-450 ($p < 0.05$) but did not have any significant effect on the K_m for NADPH with respect to the 2-hydroxylation reaction (Fig. 3). The V_{max} values for 3-hydroxybiphenyl and 4-hydroxybiphenyl formation were not altered by the addition of betamethasone. The K_m for NADPH with respect to the 3-hydroxylation of biphenyl was increased by 29% from 5.35 to 6.88 μM ($p < 0.05$), whereas the K_m for NADPH with respect to the 4-hydroxylation of biphenyl was increased by 20% from 5.01 to 6.03 μM ($p < 0.05$).

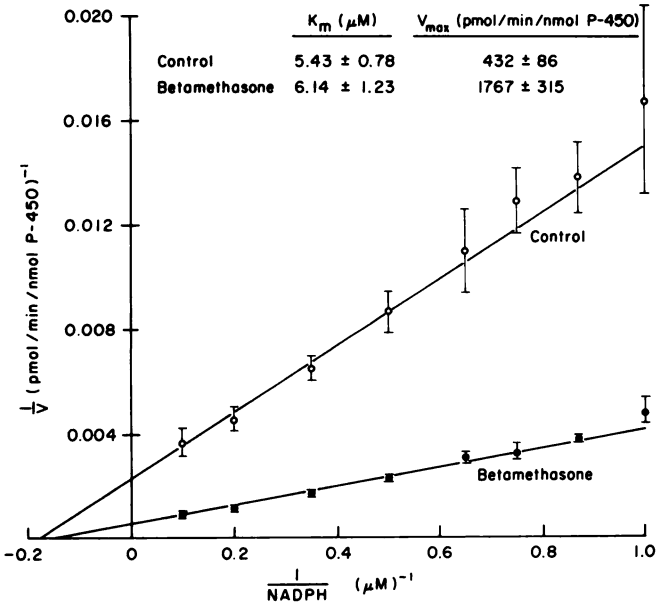

FIG. 3. *Effect of NADPH concentration on the betamethasone stimulation of biphenyl 2-hydroxylation in rat liver microsomes.*

The rate of biphenyl hydroxylation was determined in the presence (●) and absence (○) of 100 μM betamethasone. The NADPH concentration was varied from 1 to 10 μM. The concentration of NADPH added to the reaction mixtures was measured spectrophotometrically at 340 nm prior to each experiment. Each value represents the mean ± standard deviation from three experiments.

Effect of cytochrome P-450 reductase concentration on the betamethasone stimulation of biphenyl 2-hydroxylation by solubilized liver microsomes. The addition of purified cytochrome P-450 reductase to cholate-solubilized liver microsomes stimulated the rate of biphenyl hydroxylation in positions 2 and 4. The K_m for reductase and the V_{max} of biphenyl hydroxylation were determined in the presence and absence of 100 μM betamethasone. The total cytochrome P-450 reductase concentration (endogenous and exogenous) was varied from 200 to 2000 units. Betamethasone caused a 2-fold increase in the V_{max} of 2-hydroxybiphenyl formation from 697 to 1321 pmoles of product formed per minute per nanomole of cytochrome P-450 ($p < 0.05$) and concomitantly decreased the K_m for reductase with respect to the 2-hydroxylation reaction by 48% from 957 to 497 units ($p < 0.05$). From the double-reciprocal plot of 2-hydroxybiphenyl formation shown in Fig. 4, it is apparent that the percentage of activation caused by betamethasone decreased as the reductase concentration was increased. At lower concentrations of reductase, betamethasone caused a 2.5- to 3-fold stimulation of biphenyl 2-hydroxylation, whereas at higher concentrations of reductase the activation was only 1.5- to 2-fold. Betamethasone had no

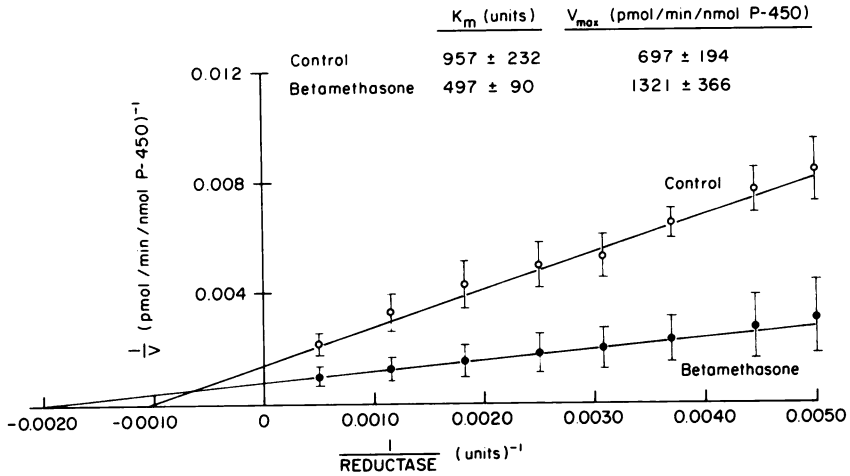

FIG. 4. *Effect of cytochrome P-450 reductase concentration on the betamethasone stimulation of biphenyl 2-hydroxylation by cholate-solubilized rat liver microsomes.*

Various concentrations of purified cytochrome P-450 reductase in 50 μl of 50 mM potassium phosphate buffer (pH 7.7) were added to cholate-solubilized microsomes containing 0.5 nmole of cytochrome P-450. The final volume was 150 μl, and the mixture was kept at 4° for 60 min. Cofactors were then added and the reaction was started by the addition of biphenyl. The rate of biphenyl hydroxylation was determined in the presence (●) and absence (○) of 100 μM betamethasone. The reductase concentration was varied from 200 to 2000 units. Values for total reductase concentration represent the sum of endogenous and exogenously added cytochrome P-450 reductase. Each value represents the mean ± standard deviation of five experiments.

significant effects on the kinetic parameters of biphenyl 4-hydroxylation when the amount of reductase was varied. The rate of 3-hydroxybiphenyl formation in cholate-solubilized microsomes was too low for the accurate determination of turnover numbers.

Lack of effect of betamethasone on the rate of reduction of cytochrome c and total cytochrome P-450. Since the above data suggested that betamethasone stimulates biphenyl 2-hydroxylation by enhancing the interaction between cytochrome P-450 reductase and the cytochrome P-450 enzyme(s) responsible for the 2-hydroxylation of biphenyl, studies were initiated to determine whether betamethasone could stimulate the rate of reduction of either total cytochrome P-450 in microsomes or cytochrome *c*, which can also accept electrons from cytochrome P-450 reductase. Although biphenyl (2 mM) stimulated the reduction of cytochrome P-450, betamethasone (100 μM) had little or no effect on the over-all rate of reduction of total microsomal cytochrome P-450 in the presence or absence of biphenyl (Fig. 5). In addition, concentrations of betamethasone ranging from 10 to 200 μM did not alter the rate of reduction of cytochrome *c* by rat liver microsomes (data not shown).

Lack of effect of chemicals that influence hydrogen peroxide formation and breakdown on the activation of biphenyl 2-hydroxylation by betamethasone. Since it is possible that betamethasone enhances the microsomal metabolism of biphenyl through a peroxidative mechanism, the effect of altering the concentration of hydrogen peroxide on the NADPH-dependent hydroxylation of biphenyl was examined in the presence and absence of 100 μM betamethasone (Table 1). Compounds which increase hydrogen peroxide formation (i.e., glucose and glucose oxidase) or prevent its breakdown by catalase (i.e., potassium cyanide or sodium azide) did not enhance the formation of 2-hydroxybiphenyl in the absence or presence of betamethasone. These agents either had no effect or inhibited the basal rates of biphenyl 2-hydroxylation and did not alter the percentage activation obtained in reaction mixtures containing betamethasone versus matched-reaction mixtures lacking this steroid. In addition, catalase at concentrations ranging from 10 to 100 units had no effect on the 2-hydroxylation of biphenyl in the presence or absence of betamethasone.

Effect of betamethasone concentration on the cumene hydroperoxide-dependent hydroxylation of biphenyl.

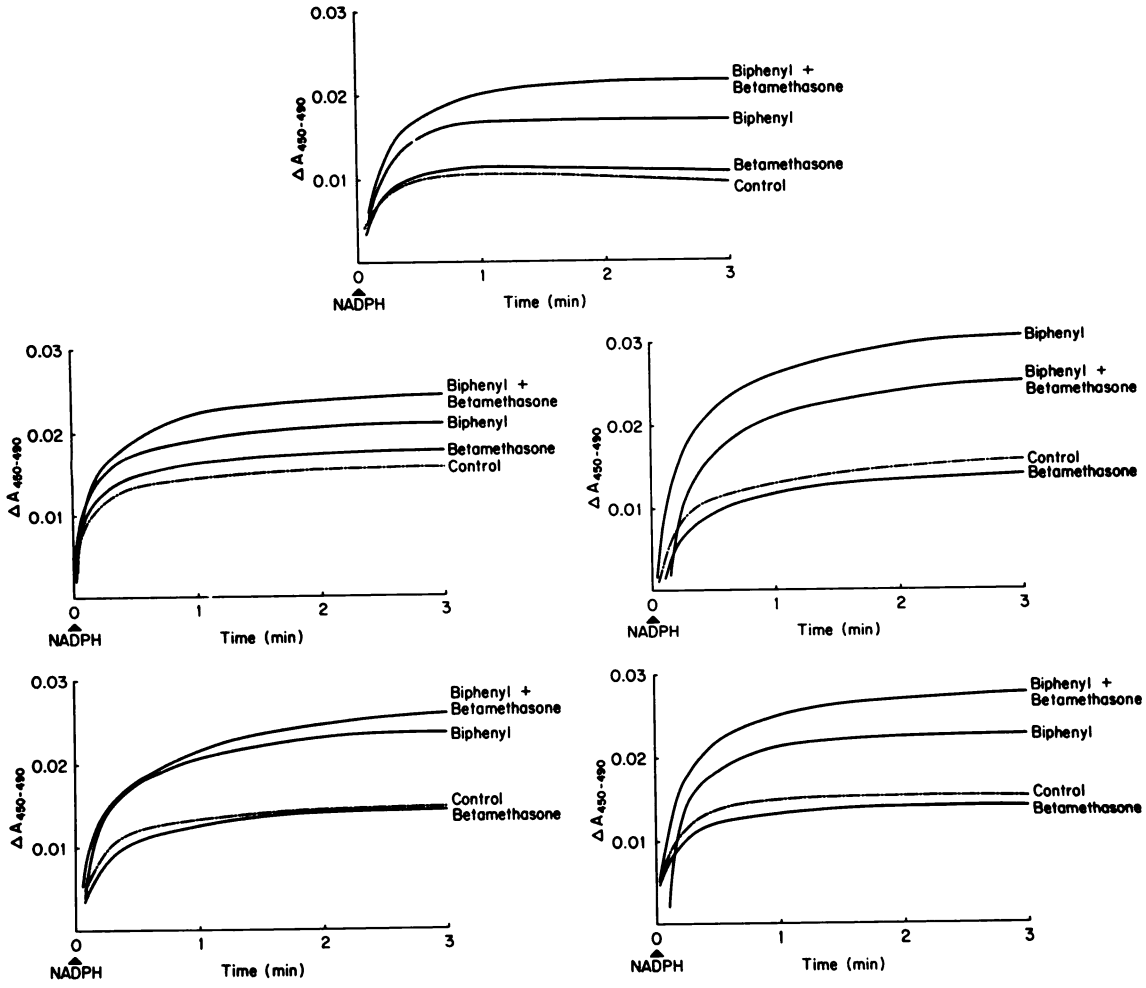

FIG. 5. *Effect of biphenyl and betamethasone on the reduction of cytochrome P-450 in rat liver microsomes.*
The rate of reduction of cytochrome P-450 in hepatic microsomes was determined under anaerobic conditions in the presence and absence of biphenyl (2 mM) and/or betamethasone (100 μM). Reduction of cytochrome P-450 in the presence of carbon monoxide was monitored at room temperature for 3 min as described under Methods. Each panel represents a separate experiment.

TABLE 1

Effect of chemicals that influence hydrogen peroxide formation and breakdown on the activation of biphenyl 2-hydroxylation by betamethasone

Rat liver microsomes were incubated with a complete NADPH-generating system in the absence or presence of glucose (2 mM) and glucose oxidase (12 units), potassium cyanide (10 mM), sodium azide (1 mM), or catalase (10–100 units). Turnover numbers are expressed as the mean ± standard deviation of three experiments.

Addition to complete incubation system	Betamethasone added	2-Hydroxybiphenyl formed	% Control	% Activation
	μM	*pmoles/min/nmole P-450*		
None (control)	0	297 ± 46	100	—
Glucose, glucose oxidase	0	148 ± 10	50	—
Cyanide	0	147 ± 42	49	—
Azide	0	297 ± 46	100	—
Glucose, glucose oxidase, cyanide	0	92 ± 28	31	—
Glucose, glucose oxidase, azide	0	80 ± 12	27	—
Catalase (10 units)	0	287 ± 42	97	—
Catalase (30 units)	0	293 ± 38	99	—
Catalase (100 units)	0	294 ± 52	99	—
None	100	1311 ± 27	441	341
Glucose, glucose oxidase	100	440 ± 8	148	197
Cyanide	100	779 ± 141	262	430
Azide	100	1047 ± 89	353	253
Glucose, glucose oxidase, cyanide	100	440 ± 55	148	378
Glucose, glucose oxidase, azide	100	198 ± 18	67	148
Catalase (10 units)	100	1361 ± 19	458	374
Catalase (30 units)	100	1362 ± 62	459	365
Catalase (100 units)	100	1299 ± 25	437	342

TABLE 2

Effect of betamethasone concentration on the cumene hydroperoxide-dependent hydroxylation of biphenyl by rat liver microsomes

The cumene hydroperoxide-dependent hydroxylation of biphenyl was determined in the presence of varying concentrations of betamethasone as outlined under Methods. Turnover numbers are expressed as the mean ± standard deviation of three experiments.

Concentration of cumene hydroperoxide added	Concentration of betamethasone added	2-Hydroxybiphenyl formed		4-Hydroxybiphenyl formed	
		Rate	% Control	Rate	% Control
μM	*μM*	*pmoles/min/nmole P-450*		*pmoles/min/nmole P-450*	
50	0	199 ± 32	100	145 ± 27	100
50	0.01	200 ± 18	101	148 ± 25	102
50	0.1	175 ± 20	88	132 ± 28	91
50	1	172 ± 9	86	133 ± 15	92
50	10	178 ± 15	89	151 ± 20	104
50	100	176 ± 21	88	172 ± 26	119
50	500	162 ± 30	81	168 ± 39	116
500	0	833 ± 40	100	548 ± 61	100
500	0.01	809 ± 45	97	533 ± 59	97
500	0.1	843 ± 40	101	538 ± 61	98
500	1	817 ± 78	98	530 ± 59	97
500	10	815 ± 74	98	565 ± 67	103
500	100	859 ± 103	103	654 ± 76	119
500	500	934 ± 74	112	733 ± 85	134

The effect of betamethasone on the cumene hydroperoxide-dependent hydroxylation of biphenyl was studied in order to determine whether the steroid acted at some step after the reduction of cytochrome P-450 by the reductase. In this system, the hydroperoxide supplies oxygen directly to the cytochrome P-450 enzymes, eliminating the need for NADPH, cytochrome P-450 reductase, and molecular oxygen. At both suboptimal (50 μM) and optimal (500 μM) concentrations of cumene hydroperoxide, betamethasone had no effect on biphenyl 2-hydroxylation over the entire concentration range tested (Table 2). At higher concentrations of betamethasone (100–500 μM) there is some indication of an enhancement of biphenyl 4-hydroxylation by 15–30%, but further experimentation is required before any definitive conclusions can be drawn. The formation of 3-hydroxybiphenyl could not be determined in the presence of cumene hydroperoxide since a contaminating uv-absorbing compound eluted with 3-hydroxybiphenyl from the HPLC column.

DISCUSSION

The results presented here confirm earlier reports (3–8) indicating that the *in vitro* addition of betamethasone to rat liver microsomes activates the 2-hydroxylation of biphenyl without stimulating the hydroxylation of this substrate in position 3 or 4. A kinetic study was initiated to determine the site(s) in the drug-oxidation pathway that may be influenced by betamethasone. Possible sites of action of betamethasone are shown in Fig. 6.

The lack of effect of betamethasone on the K_m for biphenyl during the measurement of 2-hydroxybiphenyl formation (Fig. 2) indicates that the steroid did not increase the affinity of cytochrome P-450 for the substrate (Fig. 6, Site *1*). Betamethasone also had no effect on the K_m for NADPH during the measurement of biphenyl 2-hydroxylation (Fig. 3), suggesting that this steroid did not affect the interaction between NADPH and cytochrome P-450 reductase (Fig. 6, Site *2*). This observation, taken together with data on the lack of effect of betamethasone on the NADPH-dependent reduction of cytochrome *c* by rat liver microsomes, suggests that betamethasone does not have a direct stimulatory effect on cytochrome P-450 reductase.

The activation of biphenyl 2-hydroxylation by betamethasone occurred in both untreated and cholate-solubilized microsomal preparations, indicating that intact membranes are not required for activation to occur (Fig. 1). The addition of sodium cholate to hepatic microsomes resulted in a 63% decrease in the basal levels of biphenyl 2-hydroxylation in the absence of any exogenously added cytochrome P-450 reductase. The addition of increasing amounts of purified cytochrome P-450 reductase increased the hydroxylation of biphenyl in positions 2 and 4. Although betamethasone decreased the K_m for reductase by 48% and increased the V_{max} by 2-fold when the 2-hydroxylation of biphenyl was measured (Fig. 4), the steroid had considerable specificity since no effect on the K_m for reductase or the V_{max} was observed when the 4-hydroxylation of biphenyl was measured. These results suggest that betamethasone enhances the interaction between the reductase and the specific form(s) of cytochrome P-450 that is responsible for biphenyl 2-hydroxylation (Fig. 6, Site *3*). It is possible that betamethasone enhances the flow of electrons from cytochrome P-450 reductase to the form(s) of cytochrome P-450 that hydroxylates biphenyl in position 2. It was of interest that betamethasone had little or no effect on the rate of reduction of total cytochrome P-450 by rat liver microsomes (Fig. 5). Since spectral determinations of cytochrome P-450 reduction measure the combined reduction of all the forms of the hemoprotein, a possible stimulatory effect of betamethasone on the reduction of only one or two minor cytochrome P-450 isoenzymes could not be demonstrated by spectral measurements in a microsomal preparation containing a mixture of many isozymes. Several laboratories have demonstrated complex formation between cytochrome P-450 reductase and cytochrome P-450 (22–25). Miwa and Lu (23) have shown that lipid stimulates hydroxylation activity by enhancing the association between cytochrome P-450 and cytochrome P-450 reductase; interestingly, the K_m for lipid was not decreased during their studies on the oxidation of benzphetamine. It is not known whether betamethasone and lipid have similar mechanisms of action in stimulating microsomal hydroxylations.

A stimulatory effect of betamethasone on the reduction of a specific isozyme(s) of cytochrome P-450 may result in the increased formation of (a) the reduced oxycytochrome P-450/substrate complex (Fig. 6, Site *4*), (b) the

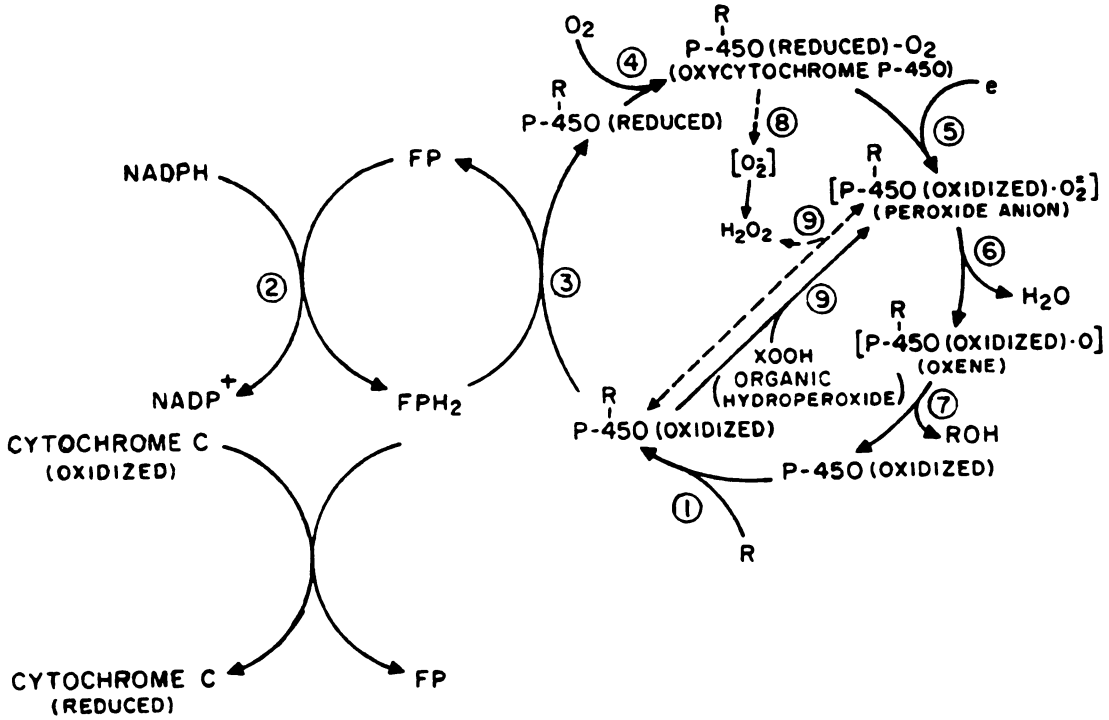

FIG. 6. *Electron transport scheme for the oxidation of foreign chemicals by liver microsomes.* Adopted from the scheme described by Estabrook and Werringloer (21).

oxidized cytochrome P-450/peroxide anion substrate complex (Fig. 6, Site 5), (c) the formation of the oxidized cytochrome P-450 oxene/substrate complex (Fig. 6, Site *6*), and (d) the generation of 2-hydroxybiphenyl (Fig. 6, Site *7*). The possibility that betamethasone could cause its activating effects by stimulating the formation of hydrogen peroxide (Fig. 6, Sites *8* and *9*) was explored, and evidence was presented indicating that betamethasone does not enhance biphenyl 2-hydroxylation by a peroxidative mechanism. Betamethasone had no effect on the formation of 2-hydroxybiphenyl when a cumene hydroperoxide-dependent metabolizing system was employed (Table 2). In addition, chemicals which alter the concentration of hydrogen peroxide in the incubation mixtures did not markedly influence the stimulatory effect of betamethasone on the hydroxylation of biphenyl in position 2. These data suggest that betamethasone does not stimulate a peroxide-mediated formation of oxidized cytochrome P-450 peroxide anion (Fig. 6, Site *9*) or the subsequent steps leading to the formation of 2-hydroxybiphenyl (Fig. 6, Sites *6* and *7*). It is of interest, however, that betamethasone causes a 15–30% increase in the formation of 4-hydroxybiphenyl during the cumene hydroperoxide-dependent hydroxylation of biphenyl.

Several chemicals have been identified that activate the mixed-function oxidase system, and some of these agents appear to act by the same mechanism. Evidence has been presented that several activating agents may stimulate drug metabolism by enhancing the interaction of cytochrome P-450 and reductase. Examples include the stimulation of the *p*-hydroxylation of aniline by ethyl isocyanide and cyanide (26, 27), the activation of acetanilide hydroxylation by metyrapone (28), the enhancement of the *p*-hydroxylation of aniline by acetone (29), the stimulation of aniline hydroxylation by 2,2′-bipyridine (29, 30), and the increased metabolism of benzo[*a*]pyrene by flavone and 7,8-benzoflavone (9). Several lines of research suggest that the reduction of the oxycytochrome P-450/substrate complex by the cytochrome P-450 reductase may be a rate-limiting step for the oxidation of some drugs (reviewed in ref. 31). Thus, the stimulation of cytochrome P-450 reduction by a high concentration of some chemicals may represent an important regulatory mechanism whereby the catalytic activity of specific monooxygenases is enhanced. The observation that endogenous chemicals can activate drug metabolism suggests that the activation phenomenon may be one of the physiological mechanisms by which the body regulates the metabolism of certain endogenous chemicals. Colby *et al.* (32) have recently demonstrated the activation of monooxygenase activity by estradiol in guinea pig adrenal microsomes. Recent studies suggest that the activation of monooxygenases can occur *in vivo*. Flavone has been shown to activate the hydroxylation of zoxazolamine by liver microsomes from neonatal rats, and it was found that the i.p. injection of flavone caused an immediate several-fold stimulation in the total body metabolism of zoxazolamine (33, 34). Further studies are needed to determine whether or not betamethasone can activate the hydroxylation of biphenyl or other chemicals *in vivo*.

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Send reprint requests to: Dr. Allan H. Conney, Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, N. J. 07110.