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INFLUENCE OF DRUGS AND CHEMICALS UPON HEPATIC ENZYMES AND PROTEINS—I.

STRUCTURE–ACTIVITY RELATIONSHIP BETWEEN VARIOUS BARBITURATES AND MICROSOMAL ENZYME INDUCTION IN RAT LIVER*

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Abstract—The effect of phenobarbital, three *N*-phenylbarbiturates and three *N*-cyclohexylbarbiturates on microsomal protein content, cytochrome P-450, cytochrome b_5, aniline hydroxylase, aminopyrine demethylase, *p*-nitrophenol-glucuronyltransferase and the ultrastructure of hepatic cells in rat liver were studied to elucidate the influence of barbiturate structure on enzyme-inducing activity. Smooth endoplasmic reticulum increased after administration of phenobarbital, phetharbital or bucolome. Phenobarbital and phetharbital, especially phenobarbital, induced cytochrome P-450 and glucuronyltransferase. On the other hand, the other barbiturates showed little enzyme-inducing activity. From these results, the type and spatial position of barbiturate substituents are considered important for hepatic microsomal enzyme induction. Aniline hydroxylase and aminopyrine demethylase activities changed coincidentally with cytochrome P-450 content in almost all rat livers. However, quantitative agreement of the changes in cytochrome P-450 content and drug-metabolizing activity could not be obtained, suggesting the possibility of substrate inhibition or of intrinsic inhibitors in the microsomal fraction. Bucolome, one of the *N*-cyclohexylbarbiturates, has been reported to be effective in the reduction of serum unconjugated bilirubin level in Gilbert's syndrome. However, in spite of the increased smooth endoplasmic reticulum, there was a reduction in the microsomal protein content, cytochrome P-450 and glucuronyltransferase after bucolome administration. This would seem to indicate that the serum bilirubin-reducing mechanism of bucolome is different from that of phenobarbital and phetharbital.

More than 200 chemical compounds have been reported to show microsomal drug-metabolizing enzyme- and glucuronyltransferase-inducing activity in animal liver [1]. This enzyme induction should be taken into account, when decreases in plasma drug level are observed after long-term administration or when the interaction of drugs occurs [2,3]. On the other hand, some enzyme-inducing chemical compounds have been used therapeutically in unconjugated hyperbilirubinemia [4–9] and in Cushing's syndrome [10–13]. All of these enzyme-inducing chemical compounds are lipid-soluble, but no relationship has been reported between their chemical structures and enzyme-inducing activities. Since some derivatives of barbiturate such as phenobarbital (PB) and phetharbital (PT) have been recognized as enzyme inducers [1,14], the present study investigated the relationship between structures of various barbiturates and hepatic microsomal enzyme-inducing activities in order to elucidate to some degree the mechanism of microsomal enzyme induction.

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MATERIALS AND METHODS

Chemical compounds

The following derivatives of barbiturate were studied: PB, 5-diethyl-1-phenyl-2,4,6-trioxoperhydropyrimidine (NPB I = *N*-phenylbarbital; PT), 5-*n*-butyl-1-phenyl-2,4,6-trioxoperhydropyrimidine (NPB II), 5-ethyl-1-phenyl-2,4,6-trioxoperhydropyrimidine (NPB III), 5-diethyl-1-cyclohexyl-2,4,6-trioxoperhydroprimidine (NCB I = *N*-cyclohexylbarbital), 5-*n*-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine (NCB II = bucolome; BC) and 5-ethyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine (NCB III). All barbiturates other than NCB I were sodium salts. For convenience, the above-designated abbreviations will be used in this report. Chemical structures of these barbiturates are presented in Fig. 1. PB was a product of the Dai-ichi Pharmaceutical Co.; NPB I (PT) was a gift from Burroughs Wellcome & Co. and the other barbiturates were kindly supplied by Dr. H. Izumi of the Gifu College of Pharmacy.

Experimental method

Male Wistar albino rats (about 100 g) were maintained on commercial laboratory chow and water *ad lib.* for 3–5 days prior to use.

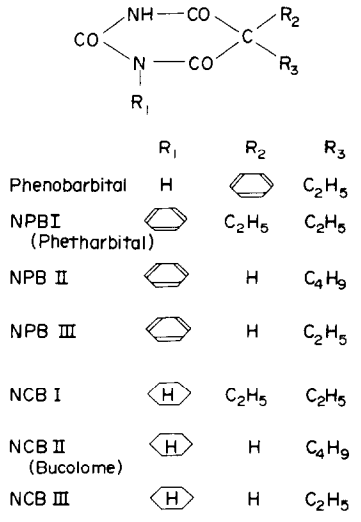

Fig. 1. Structures of barbiturates studied. Chemical abbreviations are described in Materials and Methods.

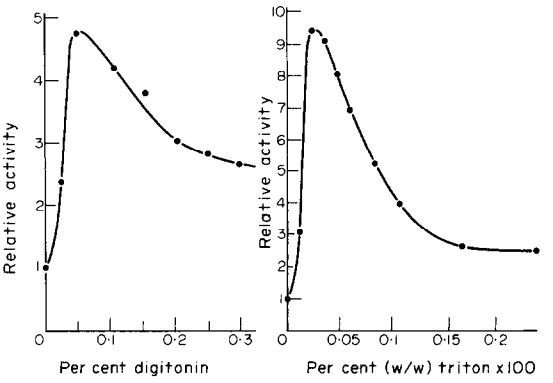

Fig. 2. Activation of *p*-nitrophenol-UDP-glucuronyltransferase by digitonin (left) and Triton X-100 (right). Digitonin or Triton X-100 was added to the incubation mixture prior to UDP- glucuronic acid and preincubation was performed before determining the enzyme activity.

Subcutaneous injection study. The rats were divided into seven groups of equivalent weights for the studies. The control group received daily subcutaneous injections of isotonic saline solution (5 ml/kg/day) for 5 days. The other groups received daily subcutaneous injections of PB, NPB I, NPB II, NPB III, NCB II and NCB III, respectively, for 5 days. The doses of PB and NPB I were 50 mg/kg/day, and those of NPB II, NPB III, NCB II and NCB III were 200 mg/kg/day. The rats were injected once daily at 9 a.m.

Oral administration study. The rats were divided into two groups of equivalent weights. One group received daily oral administration of NCB I (100 mg/kg/day) as an emulsion in water, while the control group received water (5 ml/kg/day) through a stomach tube. The emulsion of NCB I or water was administered once daily at 9 a.m. for 7 days.

Tissue preparation. The animals were sacrificed 24 hr after the last administration of chemicals by stunning and subsequent cervical dislocation. The liver was perfused rapidly *in situ* with ice-cold isotonic saline, excised and homogenized with 3 vol. of 0·25 M sucrose–0·01 M potassium phosphate buffer (pH 7·4) with a Potter–Elvejhem glass homogenizer. The homogenate was centrifuged at 9000 *g* for 20 min in a refrigerated centrifuge. The 9000 *g* supernatant fraction was further centrifuged at 105,000 *g* for 120 min to produce the pellet of microsomes. The pellet was washed once in 1·15% KCl–0·01 M Tris buffer (pH 7·4), centrifuged at 105,000 *g* for 45 min, and resuspended in 1·15% KCl–0·01 M Tris buffer (pH 7·4). Each ml of the resuspension contained microsomes from 0·25 g liver.

Enzyme assay methods

Para-nitrophenol-UDP-glucuronyltransferase. An incubation mixture of about 1 mg microsomal protein, 1 μmole triethanolamine, 0·75 μmole *p*-nitrophenol, 10 μmoles $MgCl_2$ and 0·6 mg digitonin was prepared up to a volume of 1 ml (pH 7·4). This mixture was kept at 0° for 30 min to activate the enzyme. The activation of the enzyme was studied with digitonin and Triton X-100 (Fig. 2). Digitonin was used because of the broader range of concentrations near the maximal activation level. After 30 min, the mixture was preheated at 37° for 3 min and 2 μmoles UDP-glucuronic acid dissolved in 0·2 ml water was added to start the reaction. After shaking for 20 min in a 37° water bath, 1 ml trichloroacetic acid (6·7%) was added. The mixture was centrifuged and a 1-ml aliquot was obtained. Four ml NaOH (0·2 N) was added to the aliquot and the remaining *p*-nitrophenol in the samples was determined spectrophotometrically at 400 nm with a Shimazu UV200 spectrophotometer.

Drug-metabolizing enzymes. The oxidative *N*-demethylation of aminopyrine was measured as described by Cochin and Axelrod [15]. The method of Imai *et al.* [16] was used to determine the *p*-hydroxylation of aniline. The content of cytochrome P-450 was determined from the CO difference spectra of dithionite-reduced samples with an extinction coefficient of 91 cm^{-1} mM^{-1} as described by Omura and Sato [17]. The content of cytochrome b_5 was determined from the difference spectra produced by NADH reduction with an extinction coefficient of 185 cm^{-1} mM^{-1} [18].

Protein content of microsomal suspension. The protein content was determined by the method of Lowry *et al.* [19] as modified by Miller [20]. Data were analyzed statistically with Student's *t*-test; the level of significance was $P < 0.05$.

Electron microscopic observation

For the electron microscopic study, nonperfused livers were fixed in 5% glutaraldehyde for 1 hr and in 1% osmic acid for 1·5 hr, dehydrated in graded ethanol and embedded in Epon-812. Thin sections were cut on an ultramicrotome, placed on 200 mesh copper grids, stained with lead nitrate and uranyl acetate, and examined with a Hitachi HU-11D electron microscope.

RESULTS

Liver weight (per cent of body weight) (*Table* 1). In the PB and NPB I groups, liver weight increased significantly. On the other hand, liver weight decreased

Table 1. Effect of various barbiturates on liver weight, microsomal protein content, cytochromes and enzyme activities in rat

Treatment	N	Liver wt (% of body wt)	Microsomal protein (mg/g liver)	Cytochrome P-450 (nmoles/mg protein)	Cytochrome b_5 (nmoles/mg protein)	Aniline hydroxylase (nmoles/mg protein/20 min)	Aminopyrine demethylase (nmoles/mg protein/20 min)
Saline (s.c.)†	17‡	5·10 ± 0·26	17·8 ± 2·1	0·480 ± 0·058	0·437 ± 0·029	30·9 ± 3·4	109·2 ± 43·8
PB	5	6·33 ± 0·63§	20·3 ± 0·9‖	1·148 ± 0·124§	0·478 ± 0·031¶	40·3 ± 2·8§	204·0 ± 42·7§
NPB I	5	5·74 ± 0·33§	20·1 ± 2·8	0·679 ± 0·124‖	0·452 ± 0·060	34·3 ± 3·1	113·4 ± 38·1
NPB II	5	5·41 ± 0·63	16·3 ± 2·0	0·441 ± 0·032	0·442 ± 0·040	28·7 ± 1·7	59·1 ± 3·4‖
NPB III	5***	5·18 ± 0·43	16·6 ± 1·7	0·499 ± 0·039	0·431 ± 0·019	34·5 ± 3·9	76·4 ± 6·3¶
NCB II	5	4·50 ± 0·37‖	16·2 ± 3·3	0·404 ± 0·045¶	0·377 ± 0·050¶	30·8 ± 1·7	73·8 ± 26·0¶
NCB III	4	4·36 ± 0·11§	17·5 ± 3·8	0·527 ± 0·030	0·457 ± 0·026	45·0 ± 0·9§	116·4 ± 2·3
Water (p.o.)†	5	5·02 ± 0·12	18·0 ± 2·3	0·431 ± 0·108	0·450 ± 0·066	31·2 ± 4·1	104·3 ± 18·6
NCB I (p.o.)	5	5·54 ± 0·29††	14·2 ± 1·0‡‡	0·471 ± 0·049	0·426 ± 0·029	28·7 ± 4·7	78·7 ± 28·3

* Values are means ± S.D.

† No significant difference was observed between the data of saline (s.c.) and water (p.o.) groups.

‡ N = 16 in the estimation of aniline hydroxylase and aminopyrine demethylase activites.

§ Significantly different from saline (s.c.) group ($P < 0·001$).

‖ Significantly different from saline (s.c.) group ($P < 0·01$).

¶ Significantly different from saline (s.c.) group ($P < 0·05$).

*** N = 4 in the estimation of aminopyrine demethylase activity.

‡‡ Significantly different from water (p.o.) group ($P < 0·001$).

†† Significantly different from water (p.o.) group ($P < 0·01$).

Table 2. Effect of various barbiturates on *p*-nitrophenol (pNP) glucuronyltransferase activity in rat liver microsomes*

Treatment	N	pNP conjugated (nmoles/min/mg protein)	pNP conjugated (nmoles/min/g liver)	pNP conjugated (μmoles/min/100 g body wt)
Saline (s.c.)†	17	27·5 ± 3·8	486 ± 77	2·42 ± 0·48
PB	5	28·1 ± 1·0	571 ± 16‡	3·62 ± 0·39‡
NPB I	5	33·1 ± 1·6‡	662 ± 67‡	3·81 ± 0·54‡
NPB II	5	26·2 ± 3·3	424 ± 61	2·29 ± 0·41
NPB III	5	26·5 ± 2·6	441 ± 78	2·33 ± 0·52
NCB II	5	20·3 ± 3·8§	321 ± 53‡	1·43 ± 0·15‡
NCB III	4	30·5 ± 1·0	521 ± 138	2·27 ± 0·61
Water (p.o.)†	5	27·1 ± 4·0	476 ± 18	2·39 ± 0·12
NCB I (p.o.)	5	28·2 ± 2·2	396 ± 46‖	2·21 ± 0·34

* Values are means ± S.D.
† No significant difference was observed between the data of saline (s.c.) and water (p.o.) groups.
‡ Significantly different from saline (s.c.) group ($P < 0·001$).
§ Significantly different from saline (s.c.) group ($P < 0·01$).
‖ Significantly different from water (p.o.) group ($P < 0·01$).

significantly in the NCB II and NCB III groups. In the NPB II and NPB III groups, the changes in liver weight were not significant. With the oral treatment, liver weight increased slightly in the NCB I group.

Microsomal protein content (*Table* 1). Average protein content of the microsomal fractions in the saline (s.c.) and water (p.o.) groups was 17·8 and 18·0 mg/g of liver respectively; this difference was not significant. In the PB and NPB I groups protein content increased (significantly in the PB group), whereas in the NCB I group the protein content decreased significantly. In the other groups (NPB II, NPB III, NCB II and NCB III) the protein content decreased slightly.

Microsomal cytochrome P-450 content (*Table* 1). The averages of the saline (s.c.) and water (p.o.) control groups were 0·480 and 0·431 nmole/mg of microsomal protein respectively; this difference was not significant. In the PB and NPB I groups, cytochrome P-450 increased 2·4 and 1·4-fold respectively. There was a significant decrease in the NCB II group. Cytochrome P-450 increased slightly in the NPB III and NCB III groups, but tended to decrease in the NPB II group.

Microsomal cytochrome b_5 content (*Table* 1). The averages of the cytochrome b_5 content of the saline (s.c.) and water (p.o.) control groups were 0·437 and 0·450 nmole/mg of microsomal protein respectively; this difference was not significant. Cytochrome b_5 increased significantly in the PB group and decreased

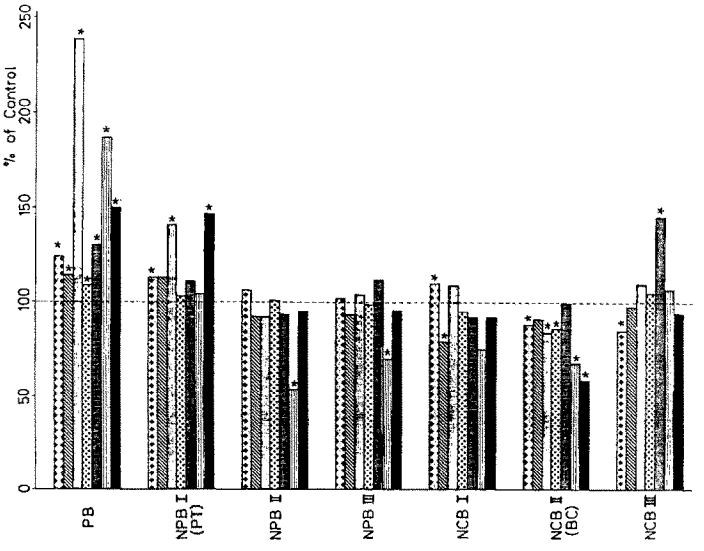

Fig. 3. Effect of various barbiturates on liver weight, microsomal protein content, cytochromes and enzyme activities in rat. Bars marked with asterisks differed significantly ($P < 0·05$) from control values (expressed as 100%). ▨ Liver weight; □ hepatic microsomal protein content; ▨ cytochrome P-450; ▨ cytochrome b_5; ▩ aniline hydroxylase; ▩ aminopyrine demethylase; ■ *P*-nitrophenol-glucuronyltransferase. Cytochromes, aniline hydroxylase activity and aminopyrine demethylase activity are expressed per mg of microsomal protein, whereas glucuronyltransferase activity is expressed per 100 g of body weight.

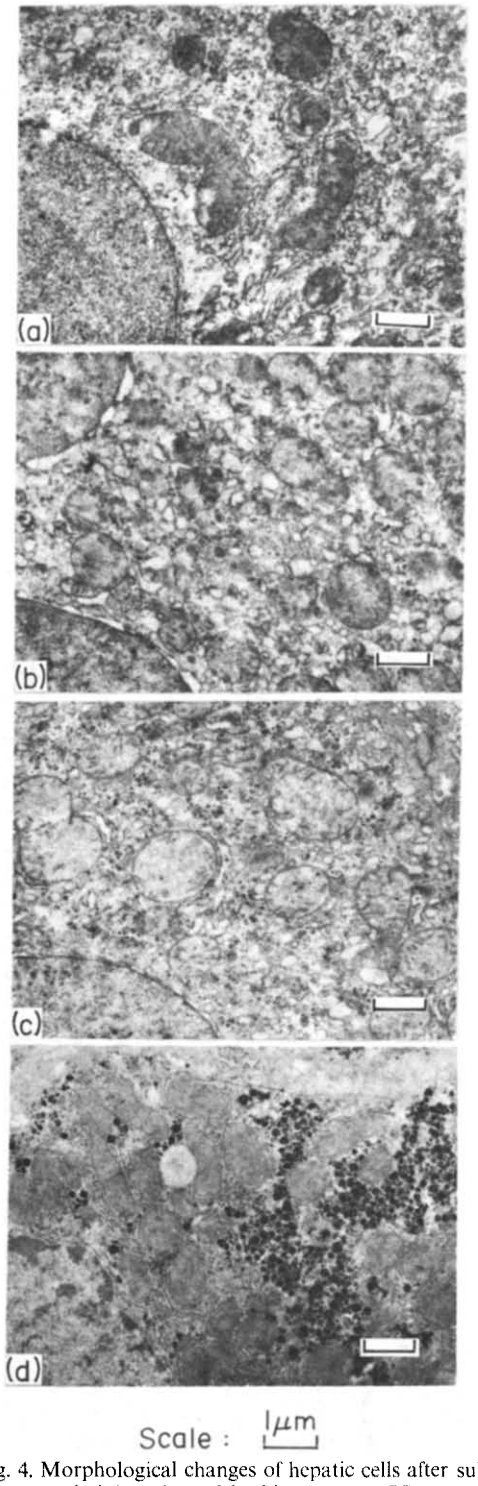

Fig. 4. Morphological changes of hepatic cells after subcutaneous administration of barbiturates. A, PB group; B, NCB II group; C, NPB I group; D, NPB III group.

significantly in the NCB II group. In the other groups, the cytochrome b_5 content remained constant.

Para-nitrophenol-UDP-glucuronyltransferase (*Table* 2). When expressed per mg of microsomal protein, a significant increase of enzyme activity was observed only in the NPB I group. In the NCB II group the enzyme activity decreased significantly, while in the other groups, including the PB group, no change in enzyme activity was observed. However, when the enzyme activity was expressed per g of liver or per 100 g of body weight, a significant increase in enzyme activity was observed in the PB group as well as in the NPB I group, whereas there was a significant decrease in activity in the NCB II group. The enzyme activity per g of liver decreased significantly in the NCB I group, while for the other groups it was not significantly different from that of the control saline group.

Aniline hydroxylase (*Table* 1). Aniline hydroxylase activity was increased significantly in the PB and NCB III groups. In the groups of NPB I, NPB II, NPB III, NCB I and NCB II, neither an increase nor a decrease in enzyme activity was observed.

Aminopyrine N-demethylase (*Table* 1). The enzyme activity increased significantly only in the PB group. In the NPB I, NCB I and NCB III groups the activity did not show any significant change, and in the NPB II, NPB III and NCB II groups the activity decreased. All the results described above are summarized in Fig. 3.

Electron microscopic study (*Fig.* 4). All groups except the NPB II group were studied. Smooth endoplasmic reticulum (SER) increased in the PB, NPB I and NCB II groups, but no increase of SER was observed in the remaining groups.

DISCUSSION

Increase of liver weight and microsomal protein content, and induction of cytochrome P-450 and glucuronyltransferase were observed after the PB and NPB I treatments, but the enzyme-inducing activity of NPB I was less than that of PB. These results have also been confirmed after oral treatment with the chemicals [21]. No change of these parameters was observed in the NPB II and NPB III groups. Considering the results obtained in experiments using NPB I–III, the type of substituents at the fifth position of the barbiturate, and the structural relation of these groups to the *N*-phenyl residue appear to be very important for microsomal enzyme induction. When a cyclohexyl substituent took the place of an *N*-phenyl group of NPB I, yielding NCB I, the microsomal protein- and enzyme-inducing activities of NPB I were lost. Therefore, the *N*-phenyl substituent also appears to be important, as are the groups at the fifth position. Liver weight, microsomal cytochrome P-450 content and glucuronyltransferase activity were reduced significantly after NCB II treatment, and it was presumed that NCB II is toxic to the liver. NCB II, which is widely used in Japan as an anti-inflammatory drug and which has no hypnotic effect, has been reported to be effective in the treatment of unconjugated hyperbilirubinemia in Gilbert's syndrome [22]. Also, because of the structural similarity of NCB II to PB, there has been speculation on the enzyme-inducing action of NCB II. However, as shown here, NCB II has no enzyme-inducing activity, and it has been reported by the present authors [23] that it binds strongly with albumin and displaces bilirubin from albumin in serum. Therefore, the mechanism of the serum bilirubin-reducing

effect of NCB II appears to be different from that of PB and NPB I. On the other hand, NCB II increases SER and salicylamide glucuronide formation [24]. It is also possible that other enzymes which were not studied, such as bilirubin-UDP-glucuronyltransferase, might be induced by NCB II. Aniline hydroxylase activity and aminopyrine demethylase activity were altered along with the change in cytochrome P-450 content, except for aminopyrine demethylase activity in the NPB III group, but a quantitative parallel relationship was not obtained. The slight discrepancy in enzyme activities with cytochrome P-450 content might be due to the presumed inhibition of the administered compounds or to inhibitors in the microsomal fraction. The enzyme-inducing activities of various barbiturates have been reported [25–28], but there have been few reports concerning structure–activity relationships. In a study by Levin *et al.* [25], it was reported that barbiturates containing allyl groups destroy microsomal cytochrome P-450. Therefore, it is concluded that any minor change in barbiturate substituents influences significantly the ability to induce hepatic microsomal protein and enzymes. However, no clearcut relationship of structure to activity has been observed thus far in the present research. Pelkonen and Kärki [26] reported a positive relationship between enzyme-inducing ability and lipid solubility or a relatively long metabolic half-life. Lipid solubility of *N*-phenyl and *N*-cyclohexylbarbiturates should be studied further for a more precise interpretation of the present results.

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