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BI-MODAL EFFECT OF PIPERONYL BUTOXIDE ON THE *o*- AND *p*-HYDROXYLATIONS OF BIPHENYL BY MOUSE LIVER MICROSOMES*

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Abstract—Piperonyl butoxide, immediately after intraperitoneal injection into adult male Swiss mice, produces a bi-modal effect of a transient stimulation of *o*-hydroxylation and a concomitant suppression of *p*-hydroxylation of biphenyl by their liver microsomes. Based on the initial kinetic relationship between these two activities and the characterization of their respective responses to varying dosages of piperonyl butoxide, it is shown that this bi-modal effect is due to a shift in equilibrium between the two microsomal enzyme activities, presumably through an isozymic transformation.

PIPERONYL butoxide (PB), a synthetic methylenedioxyphenyl derivative, is widely used as a synergist with various pesticides, particularly pyrethrins.[1] It acts, presumably, by blocking detoxifying microsomal enzymes in insects.[2,3] PB also apparently inhibits drug-hydroxylating systems in mammalian liver.[4–8] Although PB *per se* is relatively nontoxic to insects[9] and mammals,[10,11] combined administration with Freons in neonatal mice produced synergistic acute toxicity and hepatocarcinogenicity;[11] enhancement of acute toxicity was also produced with benzo[*a*]pyrene and griseofulvin.[12] These considerations heightened awareness of potential toxic hazards to man of PB and related naturally occurring methylenedioxyphenyl compounds.[6,12]

We report here a specific stimulatory effect of PB on *o*-hydroxylation of biphenyl of mouse liver microsomes occurring concomitantly and bi-modally† with inhibition of *p*-hydroxylation of the same substrate.

EXPERIMENTAL

Adult (30 g) male Swiss albino mice (ICR/Ha) were maintained on Purina chow and water *ad libitum*. Aqueous suspensions of PB in 1% Tween 20 (v/v) were injected ip. in 0·1-ml volume in groups of three or four mice at 10, 40, 160 and 640 mg/kg. Test and control animals were killed by cervical dislocation at intervals ranging from 15 min to 96 hr. Experiments were replicated on a minimum of three occasions. Crude microsomal fractions were prepared as described[8] from liver homogenates in 0·25 M

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† The term "bi-modal," alluding to the concurrent manifestation of the stimulation and inhibition of two distinct enzyme functions, must not be confused with the term "bi-phasic," which is descriptive of the inductive process relating to a single enzyme function that is initially inhibited and subsequently stimulated after the injection of a microsomal enzyme inhibitor.[13,14]

sucrose–phosphate buffer at pH 7·4. Activities of these fractions with respect to *o*- and *p*-hydroxylations of biphenyl were determined[15] with an isocitric dehydrogenase system[16] at pH 7·4. Both isomers were measured fluorimetrically in an Aminco–Bowman spectrophotofluorimeter at 345 and 415 mμ, with respective excitation at 275 and 295 mμ.

RESULTS

A transient stimulation of *o*-hydroxylation and concomitant suppression of *p*-hydroxylation of biphenyl occurred bi-modally and immediately after administration of PB (Fig. 1). Both activities, expressed as micromoles of biphenyl hydroxylated per gram of liver per hour, were dose-dependent (Fig. 1) and accorded with a log dose–

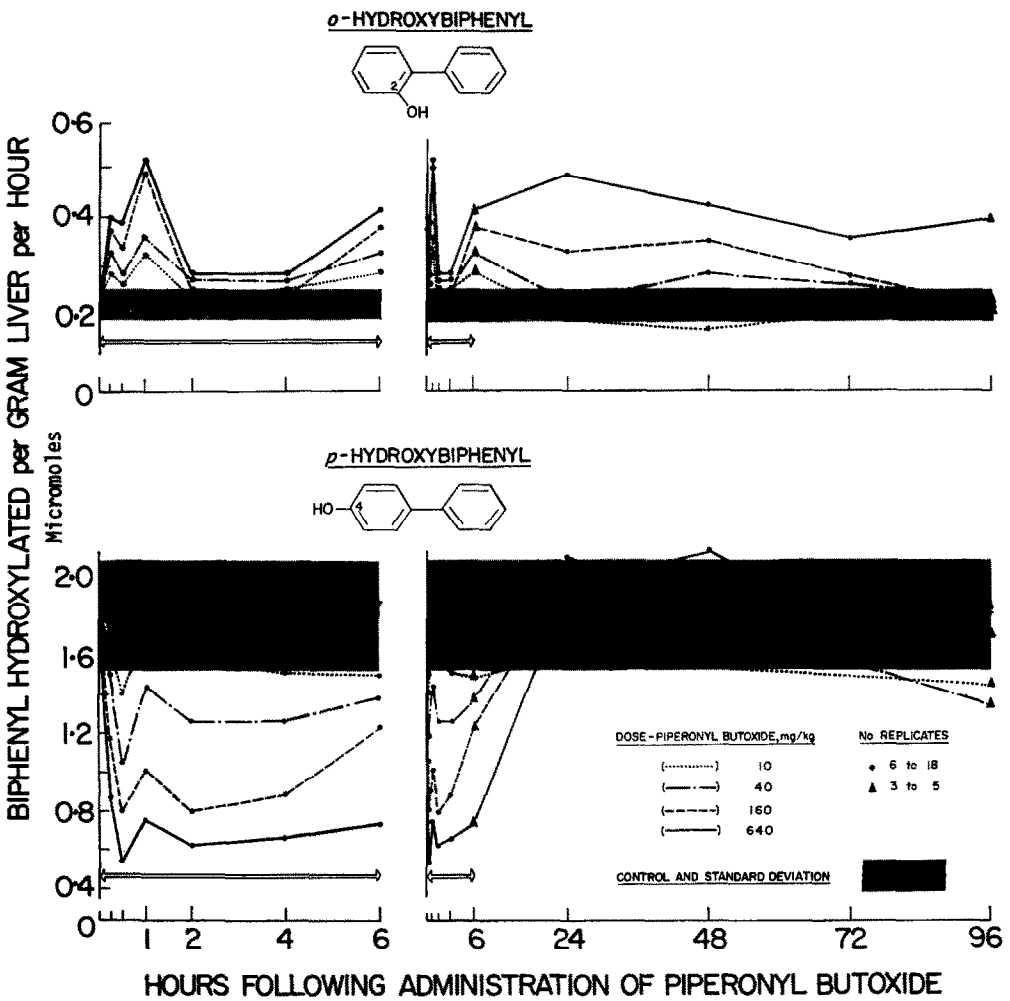

FIG. 1. Time–dose response relationships of *o*- and *p*-biphenyl hydroxylase activities in mouse liver microsomes after a single injection of piperonyl butoxide. The initial kinetics are shown on an expanded time scale at the left, as indicated by the open arrow.

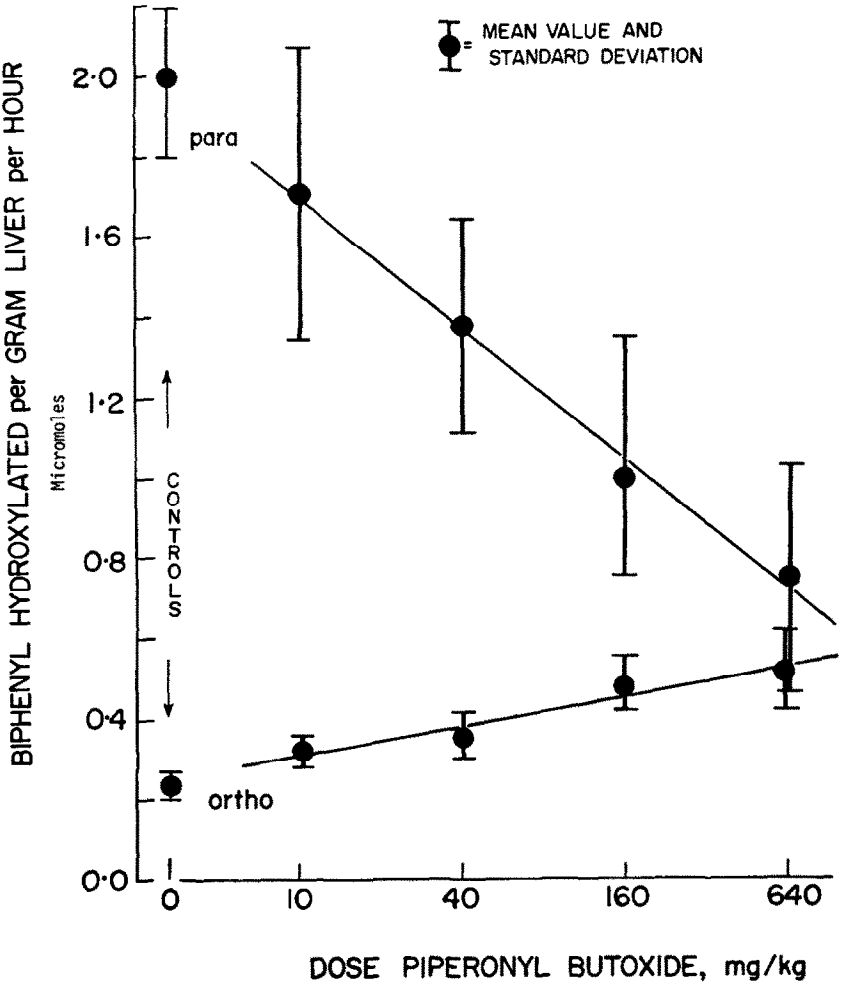

FIG. 2. Dose-response relationships of *o*- and *p*-biphenyl hydroxylase activities 1 hr after injection of piperonyl butoxide.

response function (Fig. 2), derived from the following regression lines calculated from the data obtained at 1 hr:

$$o\text{-hydroxylation: } (a_2) = 0{\cdot}20 + 0{\cdot}12 \log_{10}(\text{PB}) \quad (1)$$

$$p\text{-hydroxylation: } (a_4) = 2{\cdot}24 - 0{\cdot}54 \log_{10}(\text{PB}) \quad (2)$$

A prolonged stimulation of *o*-hydroxylation subsequently developed at 4 hr which, for the highest PB dose, peaked at 24 hr and thereafter gradually decayed to normal ranges (Fig. 1). There was a parallel and dose-dependent rise in *p*-hydroxylation, which returned to normal at 24 hr.

The early bi-modal effect of PB (Fig. 1) can be resolved into two distinct kinetic phases (Fig. 3); (1), a transient stimulation of *o*-hydroxylation, peaking at 15 min, which is accompanied by a transient suppression of *p*-hydroxylation, maximal at 30 min; (2), a slower stimulation of *o*-hydroxylation and inhibition of *p*-hydroxylation, which terminated and plateaued, respectively, at 2 hr.

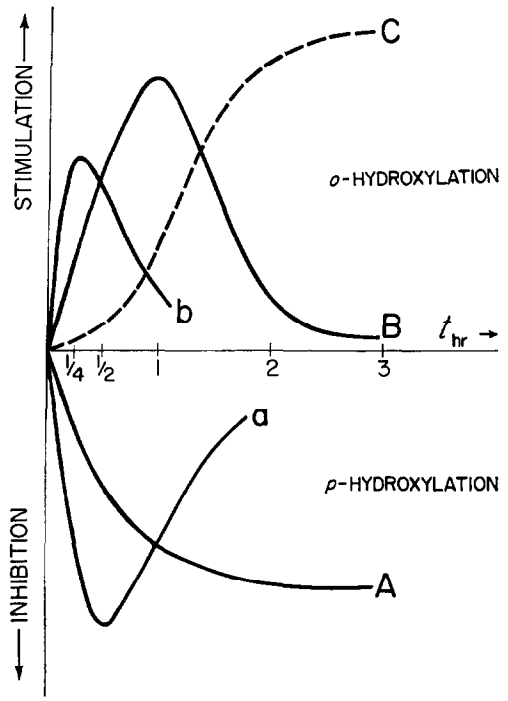

FIG. 3. Schematic resolution of the kinetics of the initial bi-modal effect of piperonyl butoxide. Two concurrent effects are depicted: (1), an early transient response of both hydroxylase activities, as indicated by curves a and b; and (2), a set of corresponding slower responses, curves A and B. The kinetics of the latter are characteristic of a consecutive reaction process. $A \rightleftarrows B \rightarrow C$, where A and B correspond to *p*- and *o*-biphenyl hydroxylase, respectively, and C is an uncharacterized reaction product.

DISCUSSION

The transient character of the initial stimulation of the *o*-hydroxylation appears to contraindicate either enzyme synthesis *de novo* or a specific enzyme activation, since either process would have led to a sustained elevation of activity. However, the stimulatory effect appears directly associated with the concurrent suppression of the *p*-hydroxylation and from the characteristic kinetic profiles of the two activities, it can be inferred that they are related by a consecutive reaction process: $A \rightleftarrows B \rightarrow C$ (Fig. 3). This implies conversion of the "*p*-hydroxylase" (A) moiety into a metastable "*o*-hydroxylase" (B), which is then transformed into an uncharacterized end-product (C). The apparent shift of the equilibrium, from one mode of hydroxylation to another, induced by PB thus suggests a ligand-induced conformational enzyme alteration similar to that effected by *p*-chloromercuribenzoate on the comparable equilibrium of the conformational isozymes of L-amino acid oxidase ($\alpha \rightleftarrows \beta \rightarrow \gamma$).[17] Since from a steric viewpoint *p*-hydroxylation is "end-on" and *o*-hydroxylation is "edge-on," these orientations must reflect the relative spatial disposition of the substrate molecule with respect to two different configurations of the active hydroxylating center in the microsomal enzyme complex. These configurational modifications can arise from differences in the state of aggregation or conformation of the discrete enzyme moieties containing the active center.[18,19] That there are at least two stereospecific arrangements

of the active hydroxylating center is further indicated by the fact that methylcholanthrene, which is a potent microsomal enzyme inducer, at 24 hr effects a stimulation of the *o*-hydroxylation of biphenyl[20] and the 7(α)-hydroxylation of testosterone,[21] both of which are "edge-on" hydroxylations; methylcholanthrene also concurrently effects a suppression of the *p*-hydroxylation of biphenyl[20] and the 16(α)-hydroxylation of testosterone,[21] which are both "end-on" hydroxylations. The hypothesis thus seems warranted that both of these two stereospecific activities bear an isozymic relationship to each other and that an allosteric change is associated with their respective activation and inhibition by PB, and probably by methylcholanthrene and like agents as well. Since the enzyme moieties constitute an integral part of the membrane structure of the endoplasmic reticulum from which they probably cannot be dissociated without profound alteration of their structures, it may well be that the incorporation of the enzyme protein into the membrane structure has the functional significance of facilitating conformational or aggregational interconversions that otherwise would be extremely slow or energetically disallowed in the free state of the enzyme.[18] This speculation provides a plausible explanation for the extraordinary multiplicity of the hydroxylating functions associated with the endoplasmic reticulum[22] and also the peculiar lability of these activities in microsomal fractions under various storage conditions.[23] The mobility of such molecular interconversions could be effected either directly by the interaction of ligand molecules with the metastable enzyme entities or indirectly in response to a structural alteration of the enveloping matrix induced by some agent or agency. PB very likely is representative of a class of compounds that can exert both actions, since it serves as a substrate for the microsomal hydroxylating system[24] and is a synthetic structure that was purposely modified to be an effective emulsifier for insecticidal oily mixtures and highly hydrophobic fluorocarbon propellants.[25] This dualistic action possibly could account for the two kinetic phases manifested in the early bi-modal effect induced by PB (Figs. 1 and 3).

A direct correlation between the two activities can be derived from the above dose–response equations (1) and (2) by eliminating the common variable, $\log_{10}(PB)$, whence the following relationship is obtained:

$$9(a_2) + 2(a_4) = 6{\cdot}28. \tag{3}$$

This relationship can be re-expressed in the form of a partition function as:

$$(a_2)/0{\cdot}70 + (a_4)/3{\cdot}14 = 1 \tag{4}$$

where the denominators correspond to the limiting activities $(a_m)_2$ and $(a_m)_4$ of the *o*- and *p*-hydroxylations respectively. This equation thus provides a statement of the interconversion of the two specific enzyme activities in terms of the relative fraction each comprises of an invariant total "biphenyl hydroxylase" activity.

From a nomogram of the above partition function (Fig. 4), it can be estimated that, in the controls, 1/3 of the total hydroxylase activity effects *o*-hydroxylation and 2/3 effects *p*-hydroxylation, and at the highest dosage of PB (640 mg/kg), the corresponding approximate fractions are 3/4 and 1/4 respectively. The shift of the equilibrium between the two specific activities induced *in vivo* by PB would effectively constitute a *metabolic shunt*, which possibly could be involved in its synergistic action.

As previously indicated, while the initial enhancement of the *o*-hydroxylation activity cannot be due to enzyme synthesis *de novo*,[13] the delayed stimulation peaking at

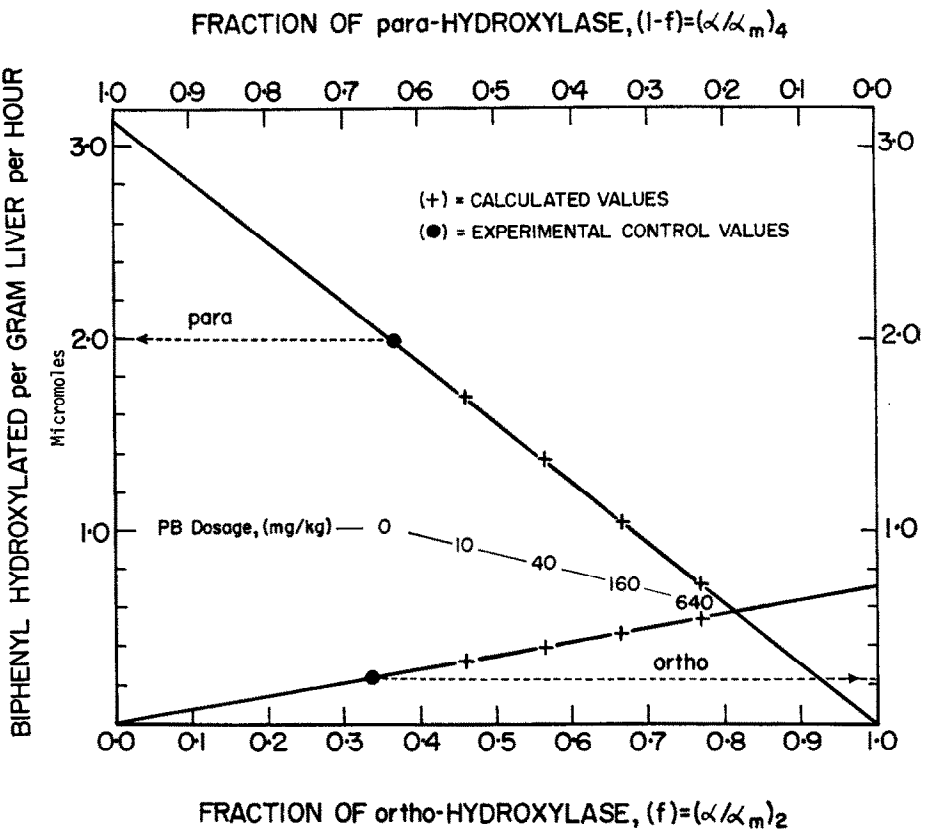

FIG. 4. Nomogram of the partition of the *o*- and *p*-biphenyl hydroxylase activities with respect to dosage of piperonyl butoxide.

24 hr, however, presumably arises from enzyme induction;[20] this accords with the delayed induction by PB[6] and SKF 525-A (β-diethylaminoethyldiphenylpropyl acetate HCl).[14,26]

The concept of isozyme transformations may have wider implications in microsomal enzyme function and to other membrane-bound enzyme systems. Certain other presumptive enzyme inhibitors of the methylenedioxyphenyl class have recently been demonstrated to have a similar bi-modal effect on the *o*- and *p*-hydroxylations of biphenyl.*

* H. Jaffe, K. Fujii, H. Guerin, M. Sengupta and S. S. Epstein, unpublished observations.

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