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IN VITRO HYDROXYLATION OF DIPHENYLHYDANTOIN AND ITS INHIBITION BY OTHER COMMONLY USED ANTICONVULSANT DRUGS

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Abstract—Using a crude 9,000 *g* rat liver microsomal preparation, *in vitro* studies were carried out on (a) the metabolism (hydroxylation) of diphenylhydantoin (DPH), (b) the effect of other commonly used anticonvulsants on this hydroxylation. DPH hydroxylation exhibited saturation kinetics at a DPH substrate concentration of approximately 10^{-4} M. Mean K_m and V_m values for the reaction were 9.3×10^{-5} M and 23.3 μg/ml respectively. The linear Hill plot with an interaction coefficient of 1.0 suggests that there is no cooperativity between different DPH molecules during DPH receptor binding process. The anticonvulsants ethosuximide, sodium phenobarbitone, sodium valproate and sulthiame all exhibited inhibition of DPH hydroxylation to varying degrees. K_i inhibition constants for the four anticonvulsants were respectively 1.1×10^{-2}, 9×10^{-4}, 1.8×10^{-2} and 8.8×10^{-4} M. Inhibition of DPH hydroxylation by sodium phenobarbitone and sulthiame was strong and competitive in nature. Ethosuximide showed a weak competitive type of inhibition and sodium valproate a weak uncompetitive type of inhibition.

The administration of more than one anticonvulsant drug to patients with epilepsy as an aid to the management of their fits is now a common clinical practice. Diphenylhydantoin (DPH) is considered by many clinicians to be the drug of choice in most forms of epilepsy with the exception of the petit mal group and is, therefore, frequently included in a combined drug therapy.

Due to the chronic nature of treatment of patients with epilepsy, the possibility that one anticonvulsant drug may interfere with the metabolic degradation of another must always be considered. Phenobarbitone, for example, strongly induces the formation of liver hydroxylating enzymes [1], which results in an increased rate of detoxication of many other drugs, including DPH, with a subsequent decrease in plasma half life.

Apart from induction, it is known that some compounds, including phenobarbitone, interfere directly with the metabolism, which is primarily hydroxylation [2], of DPH [3, 4]. By contrast, observations based on the use of *in vivo* measurements such as DPH plasma half life, plasma concentration and DPH urinary metabolites suggest that sulthiame is a strong inhibitor of DPH hydroxylation [5–7].

In this report we present a direct assessment of the *in vitro* hydroxylation of DPH, using a crude 9,000 *g* rat liver microsomal enzyme fraction. Data is presented on the type of competition for the microsomal DPH receptor site, between DPH and various other commonly used anticonvulsant drugs. The affinity of these anticonvulsant drugs for the DPH receptor, which is a measure of ability to inhibit DPH hydroxylation, was compared quantitatively by measurement of K_i inhibition constants.

MATERIALS AND METHODS

A. *Preparation of* 9,000 *g microsomal fraction.* Male Wistar albino rats weighing 200–300 g and kept on a normal laboratory diet (diet 4B, H. B. Styles Ltd., London) were sacrificed by decapitation. Their livers were removed immediately, weighed and placed on ice. The livers were homogenized in a Potter type homogenizer at 4°. For each 1.0 g of tissue, 1.5 ml of 0.25 M sodium phosphate buffer (pH 7.4) was used. The whole homogenate was centrifuged at 9,000 *g* for 15 min in a MSE Superspeed 50 centrifuge. After centrifugation, the waxy upper layer was removed and the middle zone was then poured into a collecting flask, leaving the sediment in the centrifuge tube to be discarded. The 9,000 *g* microsmal fraction was always used on the day of its preparation, being stored at 4° prior to use.

B. *Incubation mixture.* The incubation mixtures (final volume 1.0 ml), as modified from Kutt and Verebelly [3], comprised: 0.4 ml, 9,000 *g* microsomal fraction, 1.0 ml DPH (sodium salt) in 0.01 M NaOH; 0.1 ml sodium valproate or phenobarbitone (sodium salt) or ethosuximide made up in distilled water or sulthiame in 0.01 M NaOH; 0.1 ml of 0.25 M phosphate buffer pH 7.4 and 0.1 ml each of NADP, NAD and ATP made up in 0.25 M phosphate buffer pH 7.4 to give final concentration of 8.2×10^{-3} M, 9.3×10^{-3} M and 3.9×10^{-2} M respectively. Anticonvulsant drug concentration used varied from 5×10^{-5} M to 10^{-3} M. Samples were incubated for 15 min in a shaking water bath at 37° with an oxygen flow rate of 1 litre/min.

C. *Assay procedure.* All ingredients of the incubation mixture, except for the microsomes were added to the incubation tubes. The reaction was started by the addition of 0.4 ml of the microsomal fraction. At the end of the 15 min incubation period, microsomal enzyme activity was terminated by the addition of 0.5 ml of 1 N HCL and this at the same time served as the first step in the extraction of DPH. 0.2 ml of the internal standard 5-(*p*-tolyl)5-phenylhydantoin

was added and the total incubation mixture transferred to 20 ml extraction tubes with two 5 ml chloroform washes. DPH was estimated by a combination of the extraction method of Toseland *et al.* [8] and the GLC method of MacGee [9] as described by Goldberg *et al.* [10]. DPH was assayed by gas–liquid chromatography using a Perkin–Elmer F11 gas chromatograph. Protein determinations were carried out according to Lowry *et al.* [11].

RESULTS

Kinetic studies. The rate of disappearance of DPH in the *in vitro* incubation mixture was used as an indication of enzyme activity. We found that in agreement with Kutt and Verebely [4] the rate of DPH metabolism accorded with first order kinetics with respect to time. The reaction velocity though is only linear for the first 20 min. We chose a 15 min incubation period. The product of DPH metabolism was found to be 5-(*p*-hydroxyphenyl)5-phenylhydantoin (pHPPH), suggesting a hydroxylation reaction. The dihydrodiol derivative, 5-(3,4-dihydroxy-1, 5-cyclohexadien-1-yl)-5-phenylhydantoin, which is normally present with pHPPH as an excretory product of DPH metabolism in rat urine was not detected.

The rate of DPH hydroxylation was found to be dependent upon the concentration in the reaction mixture (Fig. 1). Transition between first and second order kinetics occurs at a DPH concentration of approximately 10^{-4} M and it is at this DPH concentration that the DPH hydroxylating enzyme is beginning to exhibit saturation kinetics (Fig. 1).

The values of maximal velocity (V_m) for the incubation mixture per 15 min incubation period for 10 mg of 9,000 *g* microsomal protein ranged from 20 to 30 μg/ml DPH with a mean of 23.3. Figure 2 shows the range of Michaelis constant (K_m 6.8×10^{-5} to 1.18×10^{-4} M) obtained from different Hofstee plots.

Interaction of DPH with enzyme binding site. Figure 3 is a Hill plot derived from the Hill equation [13]. It presents an analysis of the same data used to plot the Hofstee plot (Fig. 2) and it can be seen that log $v/V - v$ varies as a linear function of log of DPH concentration.

Inhibition studies. When the anticonvulsants ethosuximide, sodium phenobarbitone, sodium valproate and sulthiame were included in the DPH-microsome incubation mixture DPH hydroxylation was inhibited to varying degrees (Fig. 4). On a molar to molar basis, sodium phenobarbitone and sulthiame showed strong inhibition whilst ethosuximide and sodium valproate were weak inhibitors with sodium valproate being the weakest.

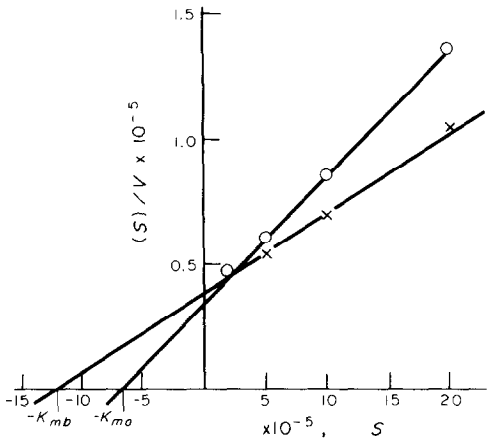

Fig. 2. Hofstee plot showing the range of the Michaelis constant (K_m) obtained from 12 different experiments. K_{ma} and K_{mb} shown by intercepts on *x*-axis represent the range in K_m values obtained and are respectively 6.3×10^{-5} M and 12×10^{-4} M with a mean of 9.3×10^{-5} M. [S] represents the molar concentration of DPH. *V* represents the rate of DPH metabolism during a 15 min incubation period in μgDPH/ml of incubation mixture per 10 mg of liver 9,000 *g* microsomal protein.

On the assumption that all the anticonvulsant drugs under study underwent metabolic degradation similar to DPH, competing for the same metabolic site on the enzyme, experiments were carried out to determine the inhibitor constant (K_i) in the presence of DPH using the graphical method described by Dixon [12]. The results (Figs 5–8) show that whilst ethosuximide, sodium phenobarbitone and sulthiame exhibit a competitive type of inhibition, sodium valproate inhibition is uncompetitive in nature.

DISCUSSION

The Hill interaction coefficient, *n*, was found to be 1.0 (Fig. 3) and this indicates that there is no positive

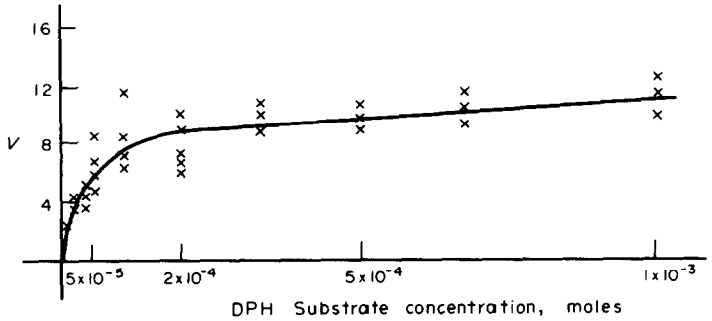

Fig. 1. DPH microsomal hydroxylation showing saturation kinetics with respect to substrate concentration. Saturation of microsomal enzyme occurs at a DPH concentration of approximately 10^{-4} M. Each point represents the mean of four experiments. *V* represents the concentration of DPH, in μg/ml of incubation mixture per 10 mg of liver 9,000 *g* microsomal protein, metabolised during a 15 min incubation period.

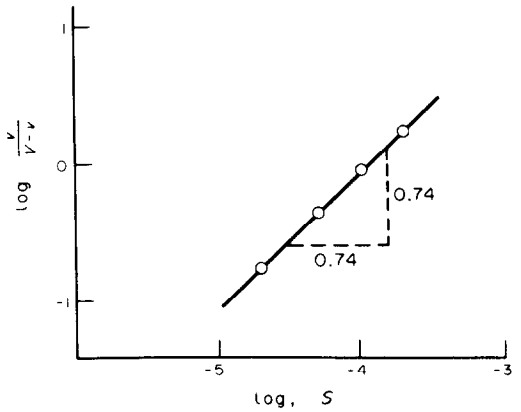

Fig. 3. Hill plot. The Hill interaction coefficient, N, is given by the gradient of the plot and was found to be 1.0. For the significance of this see text. V represents the maximum velocity of the reaction. v represents the velocity of reaction at particular substrate [S] concentration. Units as in Fig. 2.

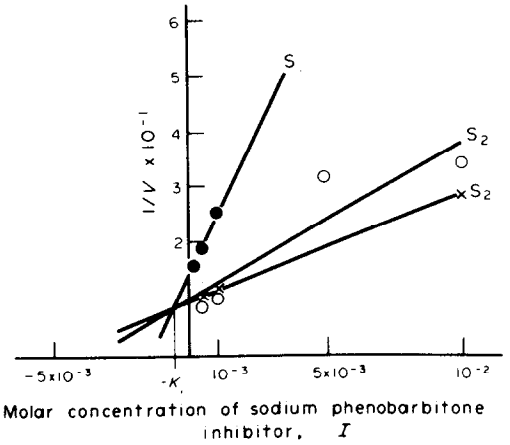

Fig. 5. Inhibition of DPH microsomal hydroxylation by sodium phenobarbitone. A competitive type of inhibition is observed with a mean K_i of 9×10^{-4} M. For clarity the range of K_i obtained from three separate experimental determinations is not shown. Also not all experimental data used for determining K_i is shown. S and S_2 represent respectively DPH substrate concentrations of 3.3×10^{-5} M and 10^{-4} M. V as in Fig. 2.

or negative cooperativity of binding. Since the Hill coefficient cannot exceed the number of binding sites on the receptor molecule [14] it follows that the receptor of DPH has only one binding site.

The inhibition of DPH hydroxylation by the four anticonvulsants studied (Fig. 4) can be categorized as follows:

1. Strong and competitive as observed with phenobarbitone (Fig. 5) and sulthiame (Fig. 6).
2. Weak and competitive, as observed with ethosuximide (Fig. 7).
3. Weak and uncompetitive as observed with sodium valproate (Fig. 8).

The K_i inhibition constants obtained suggest that phenobarbitone and sulthiame have a high affinity for the DPH receptor site whilst ethosuximide and sodium valproate have low affinities.

The fact that sodium valproate exhibited a different form of inhibition, viz: uncompetitive, as compared with all the other drugs is interesting, in view of its dissimilar molecular configuration. This difference is all the more significant when considered against the remarkable similarity of all the other anticonvulsants studied, including sulthiame when viewed stereochemically [15]).

The results presented here should be considered in the light of clinical reports of drug interactions in patients on DPH. In all cases reported [5, 6] sulthiame has been shown to raise plasma DPH levels and to decrease urinary metabolites, implying strong inhibition of DPH hydroxylation by either sulthiame itself or one of its metabolites. In this study we have observed directly that sulthiame itself is, indeed, a strong inhibitor. Whether the metabolites of sulthiame produce any additional inhibition has yet to be determined.

Phenobarbitone is known to be a strong inducer of liver microsomal enzymes [1]. It would be expected, therefore, that plasma DPH levels of patients on chronic treatment with DPH would be

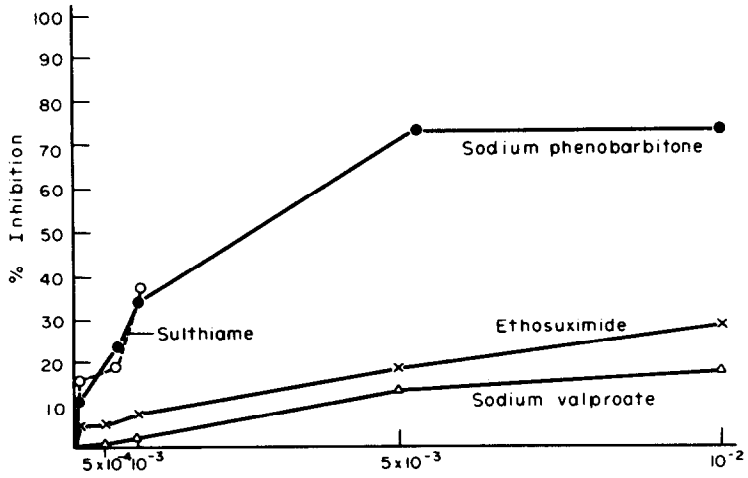

Fig. 4. Comparative inhibition of DPH hydroxylation, expressed as a percentage, in the presence of varying molar concentrations of other anticonvulsant drugs.

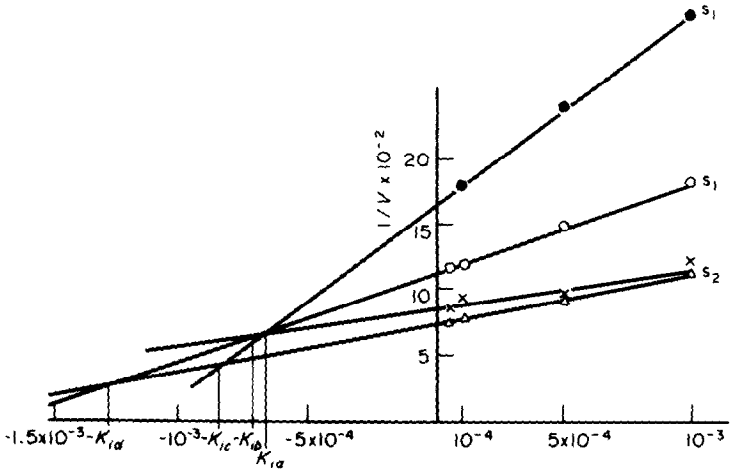

Fig. 6. Inhibition of DPH microsomal hydroxylation by sulthiame. A competitive type of inhibition is observed with a mean K_i of 8.8×10^{-4} M. K_i (a)–(d) represent the range of K_i's obtained in four experiments. S_1 and S_2 represent respectively DPH substrate concentrations of 5×10^{-5} M and 10^{-4} M. V as in Fig. 2.

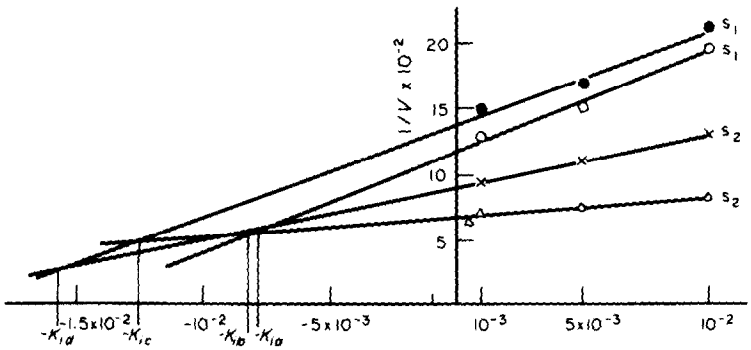

Fig. 7. Inhibition of DPH microsomal hydroxylation by ethosuximide. A competitive type of inhibition is observed with a mean K_i of 1.1×10^{-2} M. K_i (a)–(d) represent the range of K_i's obtained in four experiments. S_1 and S_2 represent respectively DPH substrate concentrations of 5×10^{-5} M and 10^{-4} M. V as in Fig. 2.

lowered by the addition of phenobarbitone to their regime on a long-term basis. However, occasional instances have been reported [16–19] in which plasma DPH levels have either not changed or been raised by addition of the second drug. Our results could explain these observations if in the chronic situation, the inhibition effect prevails over induction.

The strong inhibition of DPH hydroxylation by ethosuximide and its consequential elevation in plasma was suspected in one patient by Frantzen *et al.* [20]. Preliminary results [21] obtained from analysis of plasma DPH levels and DPH urinary metabolites of Wistar rats before and after ethosuximide was added to a regime of DPH showed no significant change. The evaluation of the true clinical interaction of ethosuximide has to await further clinical trials but our data suggests that its inhibition of DPH hydroxylation is probably not important.

The inhibition of DPH hydroxylation by sodium valproate as measured by plasma DPH levels and DPH urinary metabolites has been shown not to be significant in the rat [7]. Furthermore, sodium valproate has no cytochrome P450 inducing proper-

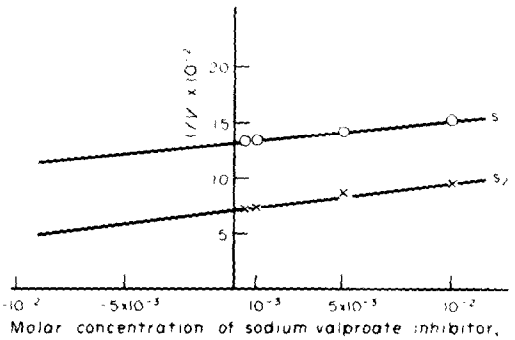

Fig. 8. Inhibition of DPH microsomal hydroxylation by sodium valproate. Uncompetitive type of inhibition is observed with a mean K_i (from three experiments) of 1.8×10^{-2} M. S_1 and S_2 represent respectively DPH substrate concentrations of 3.3×10^{-5} M and 10^{-4} M. V as in Fig. 2.

ties [21, 22]. To date no clinical evidence exists to suggest that this may be different in man. Thus in the light of our present findings it can be concluded that inhibition of DPH hydroxylation by sodium valproate, in man, will probably not be of great clinical importance.

SUMMARY

The *in vitro* hydroxylation of DPH and its inhibition by other commonly used anticonvulsants has been studied, using a 9,000 *g* rat liver preparation. One high affinity, saturable, DPH binding site was found on the enzyme.

All the anticonvulsants studied exhibited various types and strengths of inhibition and this has been discussed in relation to possible important clinical interactions.

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