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THE KINETICS OF INHIBITION OF DRUG METABOLISM *IN VITRO* BY SOME NATURALLY OCCURRING COMPOUNDS

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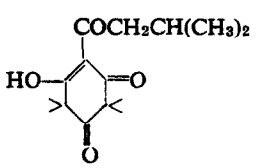
Abstract—Kinetic studies with two naturally occurring ketones, leptospermone and grandiflorone, which inhibit oxidative metabolism of drugs in liver homogenates together with parallel studies with SKF-525A and piperonyl butoxide have been carried out. The inhibition appears to be competitive. However, except for leptospermone, the Lineweaver–Burk plots show a downward curvature. This is attributed to metabolism of the inhibitor. Rapid inactivation of the inhibitor was shown to occur when inhibitor and enzyme were incubated together for varying times before addition of substrate. This effect did not take place with leptospermone which may not be metabolised by liver homogenates.

IN A PREVIOUS paper[1] we have shown that a number of naturally occurring compounds are inhibitors of oxidative drug metabolising enzyme systems *in vivo* and *in vitro*. In an attempt to determine the mechanism of inhibition we have carried out kinetic studies with two ketones, leptospermone (I) and grandiflorone (II). In addition we have carried out parallel studies with two known inhibitors, piperonyl butoxide (III) and SKF-525A (IV). The reactions examined were hydroxylation of aniline and demethylation of aminopyrine.



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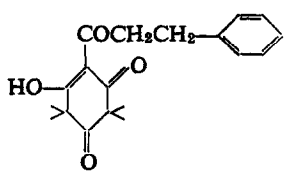

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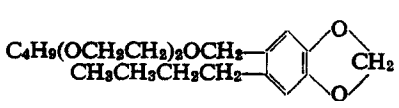

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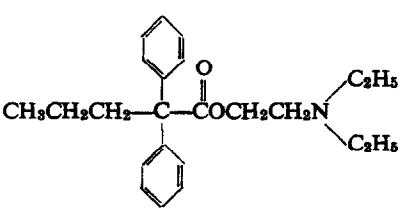

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MATERIALS AND METHODS

Samples of grandiflorone and leptospermone were obtained from the Museum of Applied Arts and Sciences, Sydney. SKF-525A was donated by Smith, Kline and French Laboratories, Australia, and piperonyl butoxide by William Cooper and Nephews Pty. Ltd., Australia.

The *in vitro* inhibition of aminopyrine demethylation and aniline hydroxylation was studied, using a rat liver 9000 *g* supernatant preparation as enzyme source. The incubation and assay procedures have been described[1] previously. Where incubation volumes were greater than 3 ml the amounts of cofactors and enzyme were adjusted so that concentrations remained the same.

Kinetic studies

The effect of variation in substrate concentration on rate of metabolism in the presence and absence of inhibitor was examined, and the results plotted according to Lineweaver and Burk.[2]

Preincubation studies

The inhibitor was preincubated at 37° with the liver supernatant and cofactors for varying periods of time prior to 30 min incubation with the substrate. Control flasks were preincubated for the same period of time. In most experiments preincubation was aerobic; when carried out under anaerobic conditions evacuated Thunberg tubes were used. At the completion of the anaerobic preincubation period, 3 ml aliquots were transferred to flasks containing the substrate, and aerobic incubation (30 min) was carried out in the usual manner. In addition to preincubation studies, the effect of duration of incubation on inhibitory activity of piperonyl butoxide was evaluated.

RESULTS

The Lineweaver–Burk plot for the inhibition of aniline hydroxylation by piperonyl butoxide and grandiflorone is shown in Figs. 1 and 2. In each case the plot, in the presence of inhibitor, was curved at the lower substrate concentrations (Figs. 1 and 2). Over a high substrate concentration range it appears that inhibition is competitive. Similar results were obtained for inhibition of aminopyrine demethylation by piperonyl butoxide, grandiflorone and SKF-525A. Over the substrate concentration range used, leptospermone appeared to be a purely competitive inhibitor of aminopyrine demethylation. The double reciprocal plots for inhibition of aminopyrine demethylation by leptospermone and grandiflorone are shown in Fig. 3, inhibition by SKF-525A is illustrated in Fig. 4.

The extent of inhibition by piperonyl butoxide was found to vary with time. As the incubation proceeded, percent inhibition fell (Table 1). This indicates that the inhibitor is being inactivated in some way during the course of the incubation.

The inhibitory activity of grandiflorone and piperonyl butoxide was also reduced if these inhibitors were preincubated with liver preparations under aerobic conditions prior to incubation with substrate (Table 2). That the latter is not entirely due to a protective effect of the inhibitors on the drug metabolising enzymes is suggested by the previous results (Table 1). Furthermore, since preincubation of the liver preparation with grandiflorone and piperonyl butoxide under anaerobic conditions leads to no

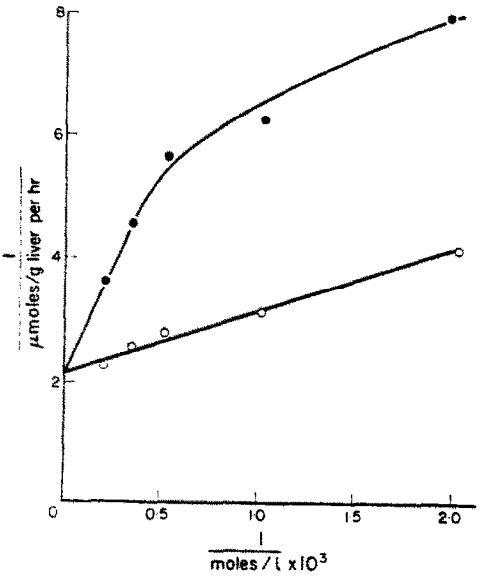

FIG. 1. Lineweaver–Burk plot for the inhibition of aniline hydroxylation by piperonyl butoxide (5×10^{-4} M). The formation of *para*-aminophenol was measured.
○ Control ● + Inhibitor

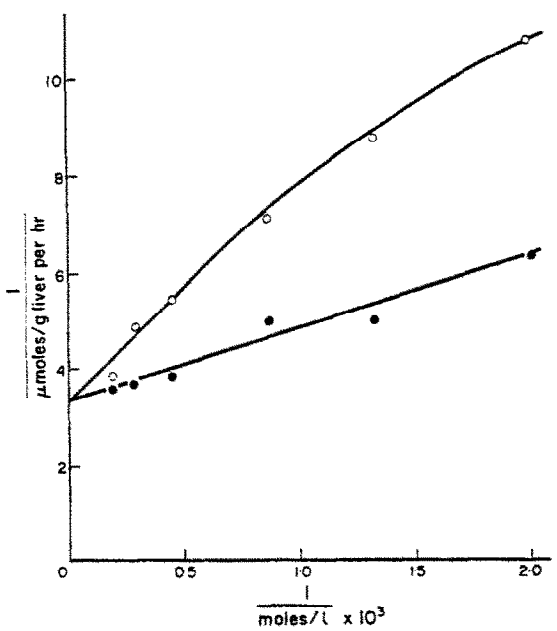

FIG. 2. Lineweaver–Burk plot for the inhibition of aniline hydroxylation by grandiflorone (4×10^{-4} M). Substrate range 5×10^{-3} M to 5×10^{-4} M. The formation of *para*-aminophenol was measured.
● Control, ○ + Inhibitor.

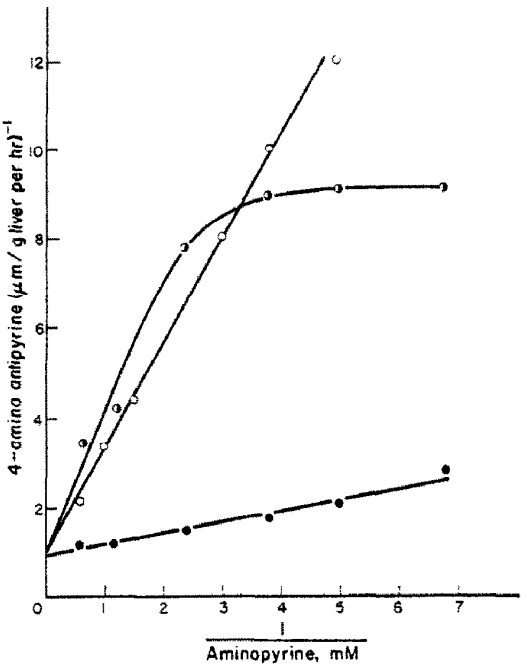

FIG. 3. Double reciprocal plot for inhibition of aminopyrine demethylation by leptospermone 4×10^{-4} M (○) and grandiflorone 10^{-4} M (◑). Control (●).

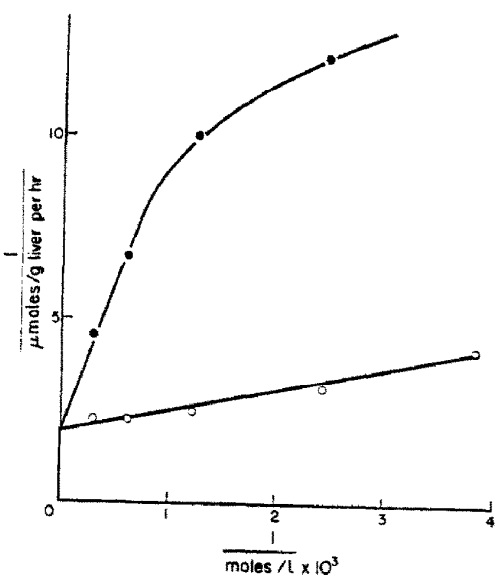

FIG. 4. Lineweaver–Burk plot for the inhibition of aminopyrine demethylation by SKF-525A (5×10^{-6} M). The formation of 4-aminoantipyrine was measured. ○ Control, ● + Inhibitor.

TABLE 1. THE EFFECT OF INCUBATION TIME ON DEGREE OF INHIBITION OF AMINOPYRINE DEMETHYLATION BY PIPERONYL BUTOXIDE

Incubation time (min)	μmoles/g liver		Percent inhibition
	Control	Inhibitor present	
0–20	0·280	0·095	66
20–40	0·280	0·186	34

Inhibitor concentration (6×10^{-5} M).
Substrate concentration ($6{\cdot}7 \times 10^{-4}$ M).
Incubations were carried out at 37°.

TABLE 2. THE EFFECT OF PREINCUBATION ON THE INHIBITORY ACTIVITY OF PIPERONYL BUTOXIDE, LEPTOSPERMONE AND GRANDIFLORONE

Substrate	Inhibitor	Preincubation conditions	Preincubation time (min)	μmoles × 10g liver/hr		Percent inhibition
				Control	inhibitor present	
Aniline	Grandiflorone 4×10^{-4} M	aerobic	0	3·7	2·8	25
			40	2·1	2·1	0
Aminopyrine	Piperonyl butoxide 6×10^{-5} M	aerobic	0	8·3	4·2	50
			40	3·0	2·8	7
,,	,,	anaerobic	0	8·0	4·3	47
			30	0·5	0·3	44
,,	Leptospermone 4×10^{-4} M	aerobic	0	5·5	2·7	51
			40	1·8	0·7	60

Rat liver preparations at 37° were preincubated in the presence or absence of inhibitor, prior to aerobic incubation at 37° in the presence of substrate.

reduction in inhibitory potency, the decreased inhibitory potency after aerobic preincubation is probably due to the oxidative metabolism of the inhibitors. In contrast with grandiflorone and piperonyl butoxide, the purely competitive inhibitor, leptospermone, did not lose inhibitory potency during preincubation. The enzyme activity fell rapidly during the second incubation period of the preincubation experiments. This is consistent with the known *in vitro* instability of the oxidative drug metabolising enzymes of the rat.[3]

DISCUSSION

Kinetic studies of inhibition of an enzyme-catalysed reaction are an important means of determining how an inhibitor may act. A large number of inhibitors of drug metabolism have been studied by various authors. The kinetic data reported by no means suggests that these inhibitors have a common mode of action. The kinetic studies of inhibition of oxidative drug metabolism discussed in this paper suggests that analysis of results may be complicated, and may lead to erroneous conclusion concerning the mode of action of the inhibitors.

Ray[4] and Lewis *et al.*[5] have recently published Lineweaver–Burk plots similar to those presented in this paper. These authors have studied the inhibition of aldrin

epoxidation by several insecticide synergists. The plot for metabolism in the presence of inhibitor only was curved, the double reciprocal plot for control activity being linear. Their explanation is based on the assumption that the metabolism of the substrate is catalysed by two enzymes, each yielding the same product. Theoretical double reciprocal plots for this type of interaction have been discussed by Dixon and Webb.[6] The two enzymes have been assumed to have identical K_m values, but to be inhibited in different ways. There is, however, no direct evidence for the existence of two enzymes able to carry out the aldrin epoxidation. In the absence of such evidence the results of Ray and co-workers[4, 5] can be equally well accounted for by the metabolism of two substrates (aldrin and synergist) by one enzyme. Destruction of the inhibitor during the course of the incubation would also cause curvature of the double reciprocal plot for the inhibited reaction. From the steady-state equation (i), the amount of free enzyme increases as the concentration of substrate is decreased, if the substrate is not present in excess. If the inhibitor is itself metabolised by the same enzyme (ii), the amount of inhibitor metabolised, and hence inactivated, would be expected to increase as the concentration of free enzyme increases.

$$E + S \rightleftharpoons ES \rightarrow P + E \quad \text{(i)}$$

$$E + I \rightleftharpoons EI \rightarrow P' + E \quad \text{(ii)}$$

This would result in a fall in inhibition, provided that there was a significant destruction of available inhibitor in the course of the reaction. In such circumstances the double reciprocal plot in the presence of inhibitor will curve downward at low substrate concentrations.

The inhibitory activity of piperonyl butoxide and grandiflorone were found to fall as the incubation time increased (Tables 1 and 2). It appears that this is due to metabolism of the inhibitor, since there was no such marked decrease in activity when preincubation was carried out under anaerobic conditions. The purely competitive inhibitor, leptospermone, did not lose activity when preincubated for periods up to 40 min prior to incubation with substrate. This indicates that metabolism of leptospermone proceeds too slowly for a significant fall in potency to be observed. In this respect it should be noted that the inhibitors may be bound to microsomal proteins to varying extents, and the degree of binding will determine the availability of the inhibitor.[7] Thus the amount of inhibitor metabolised may be significant only if a large proportion of added inhibitor is unavailable due to non specific protein binding. SKF-525A is very strongly bound to microsomal proteins.[7, 8]

If the activity of the inhibitor falls during incubation, simple Michaelis–Menten kinetics are not applicable. The results presented suggest that determination of whether inhibition is competitive or non-competitive may depend on the substrate range chosen in kinetic studies.

Holmes and Bentz[9] reported that inhibition by SKF-525A of cholesterol synthesis was uncompetitive—the lines of the double reciprocal plot being essentially parallel. However only low substrate concentrations were used, and in fact results similar to those reported by Ray,[4, 5] and those presented here may have been noted, had higher substrate concentrations been used. Various inconsistencies reported in the literature regarding kinetic data may be due to unrecognised curvature of the inhibited Lineweaver–Burk plot. Gillette[10] has reported that aminopyrine demethylation is inhibited non-competitively in the mouse and competitively in the rat. Chloramphenicol is

stated to be a non-competitive inhibitor of acetanilide hydroxylation,[11] but a competitive inhibitor of demethylation.[12] Although Elison *et al.*[13] has reported that nalorphine is a non-competitive inhibitor of morphine *N*-demethylation, Leadbeater and Davies[14] have suggested that inhibition is partly competitive. *N*-demethylation of ethylmorphine is inhibited competitively by a wide range of compounds.[15] Inhibition of hexobarbital metabolism by adrenergic blocking agents is apparently variable, showing both competitive and non-competitive inhibition.[16] The kinetics of the inhibition of amide hydrolysis by SKF-525A are reported to be complex,[17] as are the kinetics of inhibition of oxidative metabolism by diphenyl-propylacetic acid (SKF acid).[15] The results presented in this paper indicate that inhibition is competitive, even though inhibitors and substrates may undergo oxidative metabolism by a variety of pathways. This suggests that the inhibitors are acting as alternative substrates for one non-specific enzyme. Rubin *et al.*[18] in a detailed study of the kinetics of hepatic microsomal drug metabolism have also made this suggestion. As an alternative they propose that the inhibitors could be competing for a common intermediate since they found that substances which were not metabolised did not function as inhibitors. However, this is not necessarily so. In our present work it seems likely that leptospermone, a reasonably efficient inhibitor, is not readily metabolised *in vitro* by liver preparations. More recently it has been found in this laboratory that certain water soluble azo dyes which do not undergo oxidative metabolism also function as inhibitors.[19] More detailed knowledge of the way in which the present group of inhibitors function will require work on their metabolism in liver fractions. However, it seems that no major advances in this field of enzymology will result until methods are available for the isolation and purification of these microsomal oxidative systems.

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