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THE COMPLEXITY OF THE INHIBITORY ACTION OF DICOUMAROL ON THE MIXED FUNCTION OXIDASE SYSTEM OF RAT LIVER MICROSOMES

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Abstract—The inhibitory effect of dicoumarol on the mixed function oxidase system of liver microsomes from phenobarbital treated rats were studied. Lineweaver–Burk plots of *p*-nitroanisole *O*-demethylation were non-linear in the presence of dicoumarol but linear in its absence. At high concentrations of *p*-nitroanisole the inhibition was partially overcome indicating a competition between the substrate and dicoumarol for the enzyme. Furthermore, it was found that dicoumarol promotes a time and concentration dependent conversion of cytochrome P-450 to P-420 and that it inhibits cytochrome P-450 reduction. While these fenomena seem directly related to the inhibitory action of dicoumarol on the overall *p*-nitroanisole demethylase reaction, an inhibitory effect of dicoumarol on NADH : ferricyanide reductase, on the contrary, was shown not to be related to the inhibition of the demethylation process.

IT HAS been shown that dicoumarol is metabolized by the mixed function oxidase system of liver microsomes[1] and that it inhibits the action of this system on various substrates.[2] The inhibition is not of the competitive type but of a complex nature as is evident from the non-linearity of Lineweaver–Burk plots.[2] In order to elucidate the mode of the inhibitory action of dicoumarol various possibilities for the site of its action were considered; particularly, in relation to the model for the electron transport system of liver microsomes as proposed by Hildebrandt and Estabrook.[3] In this model the reduction of cytochrome P-450 is brought about in two steps, one electron being donated via a NADPH-dependent flavoprotein and the other via a NADH-dependent flavoprotein and cytochrome b_5. Partly on the basis of this model we considered the following possibilities for the inhibitory action of dicoumarol. (1) It promotes a conversion of cytochrome P-450 to P-420. (2) It inhibits the NADPH-dependent reduction of cytochrome P-450. (3) It inhibits the NADH-cytochrome b_5-mediated reduction of oxygenated ferro-cytochrome P-450. (4) It competes with other substrates for the active site of cytochrome P-450. This analysis disclosed more than one site for the inhibitory action of dicoumarol and further analysis of the relative contributions of the various sites to the total inhibitory effect on the mixed function oxidase was therefore indicated.

MATERIALS AND METHODS

Animals. White male rats of an inbred strain L from our animal stock were used throughout the experiments. The animals had free access to water and a laboratory food source (Altromin).

Unless otherwise stated phenobarbital treated rats were used. The animals received 80 mg/kg of the drug i.p., daily for 4 days, the last dose being administered 20 hr before sacrifice.

Preparation of microsomes. These were prepared from the livers of two animals. The animals were killed by decapitation and the livers immediately perfused *in situ* with ice-cold 0·15 M NaCl (10 ml). They were then removed, weighed and homogenized with 3 vol. of ice-cold 0·25 M sucrose in 0·05 M Tris buffer, pH 7·4, in a motor driven Teflon glass homogenizer. From the homogenate, microsomes were prepared, as described by Franklin and Estabrook.[4] The pellet resulting from the last 103,000 *g* centrifugation was resuspended in 0·25 M sucrose–0·05 M Tris buffer, pH 7·4, by gentle manual homogenization in a Teflon glass homogenizer to give the equivalent of 0·5 g liver/ml of buffer. This preparation had a low content of cytochrome P-420 and was used immediately.

The activity of the mixed function oxidase system of rat liver. This was estimated from its *p*-nitroanisole *O*-demethylase activity. This activity was determined from incubation experiments of 10 min duration unless otherwise stated and in the presence of a NADPH-generating system.[2] The amount of *p*-nitrophenol liberated during the reaction was estimated by the method of Fouts as described by Gram *et al.*[5] Dicoumarol was added to the incubation mixture as previously described.[2]

Cytochromes P-450 and P-420. These were determined by the method of Omura and Sato.[6] The difference spectra (CO-reduced minus reduced) were recorded by a Shimadzu MPS-50L spectrophotometer. For calculation of the amount of cytochrome P-450 an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in absorption between 450 and 490 nm was used. Likewise the cytochrome P-420 content was calculated using an extinction coefficient for the difference in absorption between 420 and 490 nm of 111 mM^{-1} cm^{-1}. To correct for the contribution of cytochrome P-450 to the absorption at 420 nm an extinction coefficient of -41 mM^{-1} cm^{-1} for the absorption of cytochrome P-450 at 420 nm was used.[7]

NADPH-cytochrome P-450 reductase activity. This was measured by the method of Gigon *et al.*[8] as previously described.[2]

NADH mediated reduction of ferricyanide. This was estimated by a modification of the method of Strittmatter and Velick[9] and used as a measure of NADH–cytochrome b_5 reductase activity. To a cuvette with a light path of 1 cm was added 2·5 ml of microsomes (0·2–0·3 mg protein/ml) in 0·05 M Tris pH 7·4 containing 1 mM EDTA, followed first by 0·1 ml of dicoumarol dissolved in 0·1 M NaOH and then 0·1 ml of 0·1 M HCl. The reference cuvette received the same components. To the sample cuvette was then added 0·3 μmole NADH dissolved in 50 μl Tris buffer, and to the reference cuvette was added 60 μl of Tris. The reaction was initiated by the addition of 10 μl of $K_3Fe(CN)_6$ (0·6 μmole) in Tris buffer using a special plunger system permitting mixing to be performed within less than 2 sec. The decrease in absorption at 340 nm was recorded for the following 20 sec. The activity was estimated from the initial slope of the curve, using the following extinction coefficients at 340 nm: NADH 6·30 mM^{-1} cm^{-1}, $K_3Fe(C)_6$ 0·55 mM^{-1} cm^{-1} and $K_4Fe(CN)_6$ 0·25 mM^{-1} cm^{-1}.

All spectrophotometric assays were performed in thermostated cuvettes at 25°.

Protein. This was determined by the method of Lowry *et al.*[10] with bovine serum albumin as a standard.

Chemicals. NADH, NADPH, NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from C.F. Boehringer & Soehne, Mannheim, Germany. Dicoumarol [3,3′-methylene-*bis*(4-hydroxycoumarin)], Tromexan [= aethylibis-coumacetate, 3,3′-carboxy-methane-*bis*(4-hydroxycoumarin) ethyl ester], Warfarin [3-(α-acetonylbenzyl)-4-hydroxycoumarin], Marcoumar [= Phenprocoumon, 3-(α-ethylbenzyl)-4-hydroxycoumarin], Sintrom [= Acenocoumarol, 3-(α-acetonyl-4-nitrobenzyl)-4-hydroxyocumarin] were obtained from commercial sources. 4-Hydroxy-coumarin was obtained from Fluka. Other chemicals used were analytical reagents.

RESULTS

Inhibition of mixed function oxidase activity by dicoumarol. This is apparent from Fig. 1 showing a Lineweaver–Burk diagram of *p*-nitroanisole *O*-demethylase activity in the presence and absence of a fixed concentration of dicoumarol. Without dicoumarol a straight line was repeatedly obtained while in the presence of dicoumarol the curve tended to be non-linear with an upward convex curvature. The inhibitory effect of dicoumarol at low concentrations of *p*-nitroanisole is evident while it is partly abolished at the highest concentrations of substrate.

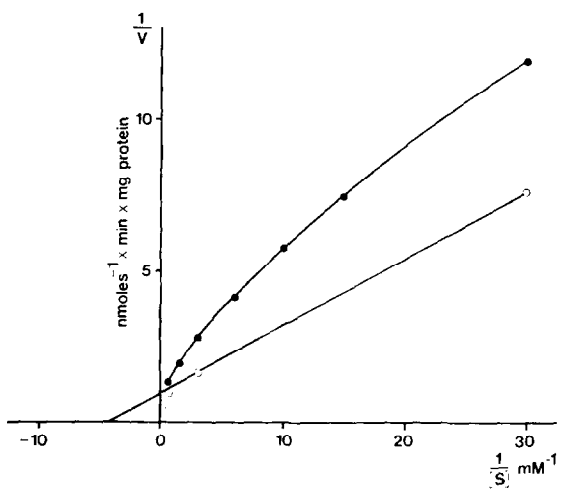

FIG. 1. Lineweaver–Burk diagram illustrating effect of dicoumarol on *p*-nitroanisole demethylase. Without dicoumarol (○) and with added dicoumarol (final concentration 0·33 mM) (●).

Evidence for conversion of cytochrome P-450 to P-420 by dicoumarol. This was obtained from experiments of the type shown in Fig. 2. A time-dependent decrease in the absorption at 450 nm was observed in the CO-difference spectrum, while the absorption at 420 nm increased. In Fig. 3 the quantitative relationship between the amounts of the cytochromes P-450 and P-420 and the time elapsed from adding dicoumarol is shown. Dicoumarol evidently promotes a time dependent conversion of cytochrome P-450 to P-420 tending to decline with increasing time. Thus after about 30 min further conversion of cytochrome P-450 to P-420 was only negligible. The total amount of the cytochromes P-450 and P-420 remained essentially unchanged during the reaction.

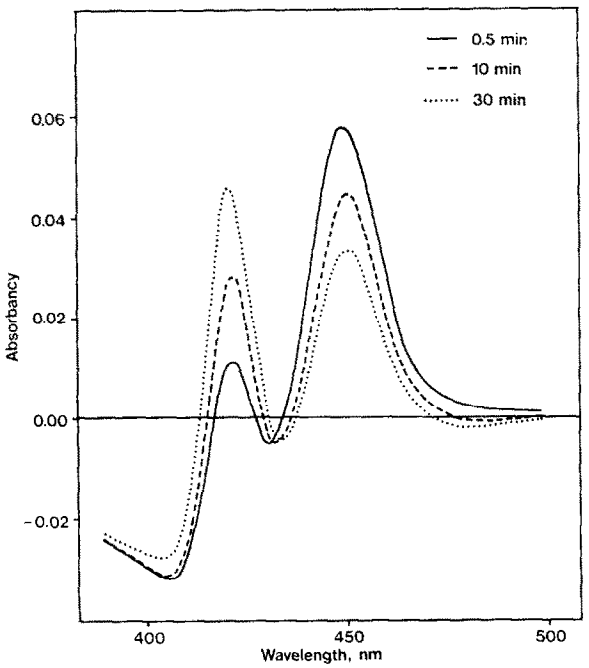

FIG. 2. The time dependent effect of dicoumarol on cytochrome P-450. The sample cuvette contained 3 ml of liver microsomes (0·5 mg protein/ml) in 0·05 M Tris–HCl, pH 7·4 to which a few crystals of sodium dithionite were added. The suspension was bubbled with CO for 30 sec and then received 100 μl of 0·1 M HCl. The reaction was initiated (zero-time) by the addition of 100 μl of dicoumarol (1·2 μmole) in 0·1 M NaOH. The reference cuvette was treated in the same way as the sample cuvette except that CO bubbling was omitted. The difference spectra were recorded at the times indicated.

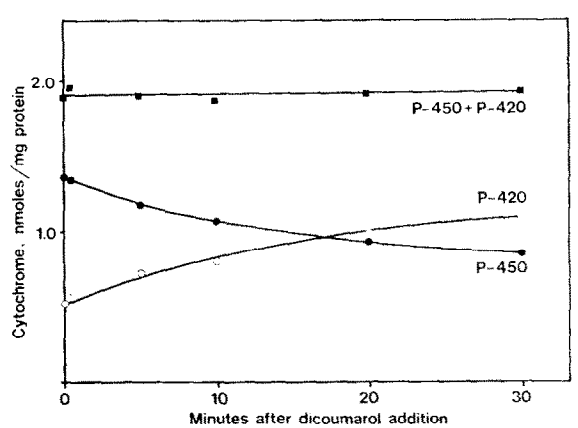

FIG. 3. The time-dependent effect of dicoumarol on the content of cytochromes P-450 and P-420 of liver microsomes. The total amount of cytochromes P-450 and P-420 is also shown. Cytochromes P-450 and P-420 were determined from experiments of the type shown in Fig. 2 and the amounts were calculated as described in Materials and Methods. The concentration of dicoumarol was fixed at 0·38 mM.

From Fig. 4 it is evident that conversion of cytochrome P-450 to P-420 is dependent on the concentration of dicoumarol provided that the determinations are made after a fixed time of incubation. Even at the highest concentrations of dicoumarol which could be obtained only about 75 per cent of cytochrome P-450 were converted to P-420 in 30 min.

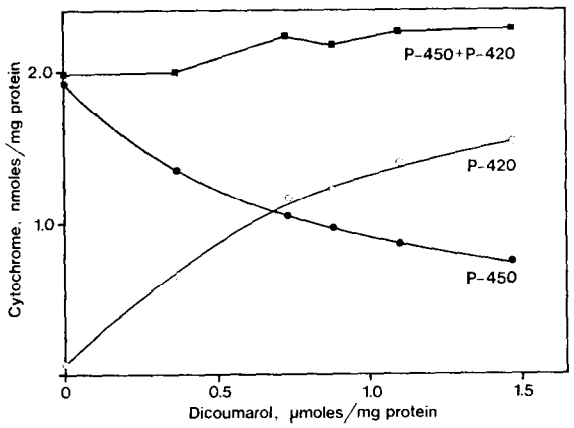

FIG. 4. The conversion of cytochrome P-450 to P-420 as a function of dicoumarol concentration. The total amount of cytochromes P-450 and P-420 is also shown. The determinations were performed as described in Fig. 3 except that the time was fixed at 30 min, and the dicoumarol concentration was varied.

Relationship between the inhibition of p-*nitroanisole demethylase activity and inactivation of cytochrome P-450 by dicoumarol.* As only cytochrome P-450 and not the degraded form P-420 is active in mixed function oxidase processes it would be expected that the decrease in cytochrome P-450 content during the influence of dicoumarol would parallel the decrease in *p*-nitroanisole *O*-demethylase activity. However, from Fig. 5 it is evident that the demethylase activity was depressed much more, especially in the initial phase of the reaction than could be accounted for by the loss of cytochrome P-450.

Inhibition of NADPH-cytochrome P-450 reductase activity by dicoumarol. This is evident from Fig. 6. It was found that *p*-nitroanisole partially protected the cytochrome P-450 reductase from inactivation by dicoumarol. Thus 50 per cent inhibition of the reductase was obtained at a dicoumarol concentration of 0·08 μmole/mg protein while in the presence of *p*-nitroanisole this degree of inhibition was observed at 0·5 μmole dicoumarol/mg protein. It is also evident from Fig. 6 that in the presence of *p*-nitroanisole the reductase activity was depressed to nearly the same extent by dicoumarol as the *p*-nitroanisole *O*-demethylase activity. The two curves, however, do not overlap exactly.

The inhibition of NADH-ferricyanide reductase activity by dicoumarol. This is also shown in Fig. 6. The activity is depressed to a somewhat lesser extent than the *p*-nitroanisole *O*-demethylase activity. Thus the 50 per cent inhibition values for dicoumarol were 0·63 μmole/mg protein for the demethylase activity and 0·77 μmole/mg protein for the reductase activity.

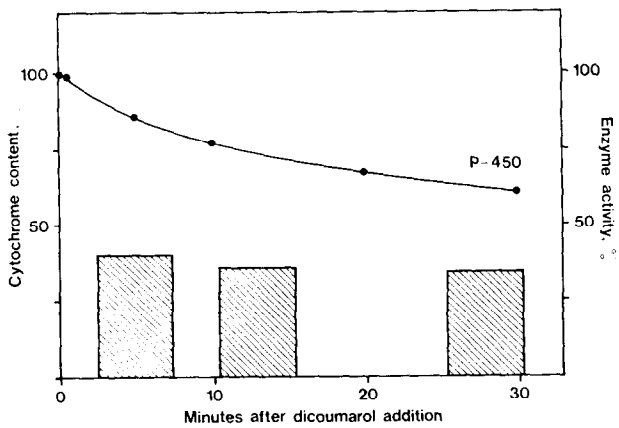

FIG. 5. The time-dependent effect of dicoumarol on cytochrome P-450 (curve) compared with its effect on *p*-nitroanisole *O*-demethylase (columns). The microsomal content of cytochrome P-450 was determined as described in Fig. 2 and the *O*-demethylase activity as described in Materials and Methods except that the incubation time was only 5 min. The concentration of dicoumarol was in both cases 0·81 μmole/mg protein and the temperature 25°. The values are expressed as a percentage of that recorded without dicoumarol.

Table 1 also shows that dicoumarol inhibits the NADH-ferricyanide reductase in both phenobarbital and untreated rats. It is, however, evident from this table that several other 4-hydroxycoumarin derivatives depress the reductase activity to a much lesser extent than does dicoumarol. Indeed, warfarin and 4-hydroxycoumarin did not inhibit the reductase activity at the concentrations studied.

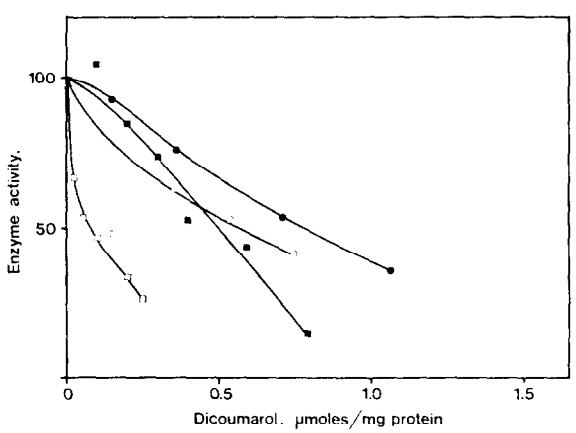

FIG. 6. The relationship between dicoumarol concentration and cytochrome P-450 reductase activity with (■) and without added *p*-nitroanisole (□). Also shown are the NADH:ferricyanide reductase (●) and *p*-nitroanisole *O*-demethylase (○) activities. Determinations as described in Materials and Methods except that the *O*-demethylase activity was determined in the presence of NADPH (3 μmole) instead of the NADPH-generating system. All determinations were performed at 25°.

TABLE 1. EFFECT OF SOME 4-HYDROXYCOUMARIN DERIVATIVES ON THE ACTIVITY OF NADH-FERRICYANIDE REDUCTASE

Inhibitor*	Inhibition (%)	
	Untreated rats	Phenobarbital-treated rats
Dicoumarol	81·4	79·4
Sintrom	12·5	10·1
Tromexan	11·7	5·4
Marcoumar	5·3	9·2
Warfarin	− 0·1	2·4
4-Hydroxycoumarin	3·2	1·1

* Final concentration of inhibitor 0·362 mM in all experiments. The values given for the inhibition are based on three separate determinations.

For details of experiments see Materials and Methods.

DISCUSSION

In a previous paper[2] it was shown that dicoumarol and warfarin exert a complex inhibitory effect on the aniline hydroxylase activity of microsomes from normal rats. This was evident from the non-linear Lineweaver–Burk diagram of aniline hydroxylation in the presence of the inhibitors. It has now been shown that this is also the case for the *O*-demethylation of *p*-nitroanisole using rat liver microsomes from phenobarbital-treated rats. It is evident from Fig. 1 that the inhibition tends to be partly overcome at high concentrations of *p*-nitroanisole indicating a competition between this compound and the inhibitor, dicoumarol. It has been shown recently that dicoumarol is a substrate of the mixed function oxidase system of liver microsomes[1] and it would therefore not seem unlikely that a competition between *p*-nitroanisole and dicoumarol could occur. However, the non-linearity of the Lineweaver–Burk plots, points to a more complex mechanism for the inhibitory action of dicoumarol than simple competition.

As it has been shown that many compounds may promote a conversion of cytochrome P-450, which is active as a component of the mixed function oxidase system, to the inactive cytochrome P-420 species[10] it seemed reasonable to suggest that dicoumarol might promote such a conversion. Actually it was found that dicoumarol effects a time and concentration dependent conversion of cytochrome P-450 to P-420. This is in accordance with the findings of Ichikawa *et al.*[11] who showed that various substituted phenols caused the conversion of cytochrome P-450 to P-420. They also showed that this conversion is dependent on the hydrophobic character of the compounds. The strong lipophilic property of dicoumarol is in accordance with this observation.

Although dicoumarol evidently provokes an inactivation of cytochrome P-450 the results shown in Fig. 5 revealed that the mixed function oxidase activity was much more depressed than could be accounted for by loss of cytochrome P-450. Therefore, this could only contribute a minor fraction of the total inhibitory effect of dicoumarol on the mixed function oxidase activity.

The finding that the reduction of cytochrome P-450 was apparently inhibited much more than the *O*-demethylase activity (Fig. 6) was surprising as it has been proposed that this step is rate-limiting in the mixed function oxidase process.[3,12] However, the demonstration that *p*-nitroanisole protects the cytochrome P-450 reductase activity against the inhibitory action of dicoumarol to such a degree that the 50 per cent inhibition value is nearly identical for the reductase activity and the *O*-demethylase activity, makes it probable that inhibition of the cytochrome P-450 reduction is a most important factor for the observed effect of dicoumarol on the overall demethylase reaction. The mechanism by which dicoumarol exerts its inhibitory effect on the cytochrome P-450 reduction is not clear. Although a part of the inhibition may be related to the formation of cytochrome P-420 it has previously been shown that the NADPH-cytochrome *c* reductase activity which is implicated in the cytochrome P-450 reduction is not inhibited by dicoumarol.[2] It may then be suggested that the inhibition of cytochrome P-450 reduction is the result of a change in cytochrome P-450 which makes it less accessible for reduction but which has not yet resulted in a spectral change to the totally inactive P-420 species.

In the model of Hildebrandt and Estabrook for the electron transport in microsomes[3] the second electron necessary for reduction of oxygenated ferro-cytochrome P-450 is donated via the flavoprotein NADH-cytochrome b_5 reductase and cytochrome b_5. We assumed that the reductase activity could be measured by the NADH: ferricyanide reductase activity of microsomes[9] and found this activity to be inhibited by dicoumarol. However, other 4-hydroxycoumarin derivatives, which inhibit the mixed function oxidase activity of liver microsomes to about the same degree as dicoumarol[2] did not inhibit the NADH-ferricyanide reductase activity (Table 1). The inhibition by dicoumarol of the NADH-ferricyanide reductase activity does, therefore, not seem to be related to the inhibitory action of dicoumarol on the overall demethylase reaction.

In conclusion, dicoumarol exerts a complex inhibitory action on the mixed function oxidase activity of rat liver microsomes. The inhibition may be partly due to competition between dicoumarol and the substrate at the active site of cytochrome P-450, partly due to conversion of cytochrome P-450 to P-420, and partly due to an inhibition of the reduction of cytochrome P-450 not directly associated with formation of cytochrome P-420.

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