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INHIBITION OF MICROSOMAL DRUG-METABOLIZING ENZYMES FROM RAT LIVER BY VARIOUS 4-HYDROXYCOUMARIN DERIVATIVES

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Abstract—The effects of some 4-hydroxycoumarin derivatives = 4-HCD (4-hydroxycoumarin, dicoumarol, tromexan, warfarin, marcoumar and sintrom) on some liver microsomal enzymes were studied. All 4-HCD with the exception of 4-hydroxycoumarin itself inhibited the hydroxylation of aniline, the *N*-demethylation of aminopyrine and the *O*-demethylation of *p*-nitroanisole. The same pattern of inhibition was obtained with cytochrome P-450 reduction, while cytochrome *c* reduction was not inhibited by 4-HCD. On the other hand reduction of 2,6-dichlorophenolindophenol was inhibited by all 4-HCD. Lineweaver–Burk plots of aniline hydroxylation without inhibitors were linear, while in the presence of the inhibitors dicoumarol or warfarin they were non-linear. The binding of 4-HCD to liver microsomes were studied spectrophotometrically. Dicoumarol, warfarin and marcoumar elicited modified type II spectral changes, while 4-hydroxycoumarin and tromexan did not produce any spectrophotometrical change at all. The binding of dicoumarol to liver microsomes was examined quantitatively and the spectroscopic dissociation constant was found to be $6{\cdot}5 \times 10^{-5}$ M. The mechanism of the inhibitory effect of 4-HCD on liver microsomal drug oxidation is discussed.

THE EXISTENCE of an electron transport system in liver microsomes involving the sequential interaction of at least NADPH, a flavoprotein and the hemoprotein cytochrome P-450 is generally accepted.[1,2] The participation of the flavoprotein NADPH-cytochrome *c* reductase in this electron transport system is suggested although by no means definitely settled.[1,3] Cytochrome P-450 is the terminal oxidase of this electron transport pathway and is responsible for both oxygen activation[4–6] and the binding of substrate.[7,8] Many foreign compounds are oxidatively attacked by this system.[9] A similar electron transport system responsible for the 11β-hydroxylation of steroids has been detected in mitochondria of adrenocortical tissue.[6,10–13]

Many compounds are known to inhibit the microsomal electron transport system *in vitro*. Among these are some (e.g. deoxycholate, urea, phospholipase, sulfhydryl reagents, organic solvents) which promote the decomposition of cytochrome P-450 to cytochrome P-420 with an associated loss of hydroxylase activity.[9,14] Other types of inhibitors are, however, known (e.g. SKF 525A, other lipid soluble amines and hydrazine derivatives, methylenedioxyphenyl insecticides).[9,15–19]

It is interesting that both the microsomal and the mitochondrial electron transport systems mentioned above may be inhibited by the same compounds. Thus it has been reported that metyrapone, a well known inhibitor of the adrenal mitochondrial system,[20,21] also inhibits the microsomal system *in vitro*.[22] As it was recently reported that dicoumarol is a potent inhibitor of the mitochondrial system[23] we considered

that it may exert a similar effect on the microsomal system. The present communication substantiates an inhibitory action of dicoumarol and related compounds on the *in vitro* activity of some liver microsomal drug oxidizing enzymes. Some evidence is presented that the effect is localized to the terminal oxidase, cytochrome P-450, of the electron transport system.

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").

Preparation of microsomes

The animals were sacrificed by decapitation, the livers removed as quickly as possible, weighed, and then homogenized with 2 vol. of ice-cold 1·15% KCl in 0·02 M tris buffer pH 7·4 in a motor-driven Teflon glass homogenizer. All succeeding tissue manipulations were performed at 0–4°. The homogenates were centrifuged at 10,800 *g* for 15 min in a Sorvall RC 2 centrifuge. The supernatants were removed and again centrifuged at 104,000 *g* for 60 min in a Spinco L-50 ultracentrifuge. The microsomal pellets were suspended in KCl–tris buffer pH 7·4 by gentle manual homogenization with a Teflon glass homogenizer to give the equivalent of 0·33 g liver per ml of buffer. All enzyme assays were performed immediately following the preparation of microsomes.

Mixed function oxidase activity

This activity was determined for the hydroxylation of aniline, the *N*-demethylation of aminopyrine and the *O*-demethylation of *p*-nitroanisole. All incubations were performed in duplicate in 25 ml conical flasks, a Dubnoff type metabolic shaking apparatus ("Heto") being used for the incubations. All samples were incubated in air at 37° for 10 min (aniline hydroxylation and *p*-nitroanisole *O*-demethylation) or 5 min (aminopyrine *N*-demethylation). Each incubation flask contained, if not otherwise stated, substrate (aniline 3 μmoles, aminopyrine 10 μmoles, *p*-nitroanisole 5 μmoles), inhibitor (1 μmole), $MgCl_2$ (15 μmoles), glucose-6-phosphate (20 μmoles), NADP (1·5 μmoles), glucose-6-phosphate dehydrogenase (2 units), and tris–HCl (100 μmoles, pH 7·4 at 37°). Substrate (1 ml) was added followed first by 0·1 ml inhibitor dissolved in 0·1 M NaOH and then 0·1 ml 0·1 M HCl. The other compounds were added (0·5 ml) and the volume brought to 2 ml with water. Flasks without inhibitors were treated in the same way as those with inhibitors (including the addition of 0·1 ml 0·1 M NaOH and 0·1 ml 0·1 M HCl) and were used as controls. In the experiments with aminopyrine *N*-demethylation flasks without substrate were also included to allow correction for production of endogenous chromogenic material. The reactions were started by the addition of 1 ml of enzyme suspension (equivalent to 0·33 g liver) to give a final volume of 3 ml.

The reactions were stopped after the appropriate time by placing the flasks in an ice bath (aniline hydroxylation), by the addition of 2 ml of 0·6 M perchloric acid to 1 ml of the incubation mixture (aminopyrine *N*-demethylation) or by adding an equal

volume of trichloroacetic acid 10% to the incubation mixture (*p*-nitroanisole *O*-demethylation).

The formation of *p*-aminophenol from aniline was determined as described by Holtzman and Gillette.[24] The formation of formaldehyde from aminopyrine was determined according to the method of Nash[25] as modified by Cochin and Axelrod.[26] The determination of *p*-nitrophenol produced from *p*-nitroanisole was performed by the method of Fouts as described by Gram *et al.*[27]

The NADPH mediated reductions of cytochrome *c*, 2,6-dichlorophenolindophenol and cytochrome P-450 were all performed at 25° ± 0·1. The activities were determined in duplicate with a Shimadzu MPS-50 L recording spectrophotometer relative to controls which were treated exactly in the same way as the samples but without inhibitors.

NADPH mediated reduction of cytochrome c

This was measured as the change in absorbance at 550 nm by a modification of the method of Williams and Kamin.[28] To a spectrophotometric cuvette with a 1-cm optical path the following were added: 1 ml of 0·15 mM cytochrome *c* in 0·02 M tris buffer pH 7·4, 1 ml 3 mM KCN in tris, 1 ml microsomal suspension (0·3–0·5 mg protein per ml of tris), 0·1 ml 10 mM inhibitor in 0·1 M NaOH, 0·1 ml 0·1 M HCl and 0·50 mg NADPH in 0·05 ml tris. The reference cuvette was treated in an analogous way except that NADPH was omitted. The cuvettes were allowed to equilibrate for 5 min in the spectrophotometer and the reaction was started by the addition of NADPH. As the reaction was linear with time for about 4 min the activity was determined from the initial (30 sec) slope of the curve. The reaction was linear with respect to microsomal protein concentration and saturated with respect to NADPH and cytochrome *c*.

NADPH mediated reduction of 2,6-*dichlorophenolindophenol*

This was determined from the decrease in absorbance at 600 nm by a modification of the method of Williams and Kamin.[28] To each cuvette was added: 1 ml 0·30 mM, 2,6-dichlorophenolindophenol in tris buffer 0·02 M pH 7·4, 2 ml microsomal suspension (0·1–0·3 mg protein per ml of tris), 0·1 ml 10 mM inhibitor in 0·1 M NaOH and 0·1 ml 0·1 M HCl. The reaction was started after 5 min of equilibration in the spectrophotometer by the addition of NADPH (0·5 mg in 0·05 ml tris) while the reference cuvette received 0·05 ml tris without NADPH. As the reaction was linear with time for about 4 min the activity was determined from the initial (30 sec) slope of the curve. The reaction was linear with respect to microsomal protein concentration and saturated with respect to NADPH and 2,6-dichlorophenolindophenol.

NADPH mediated reduction of cytochrome P-450

This was measured as the change in absorbance at 450 nm by a modification of the method of Gigon *et al.*[29] Carbon monoxide, which had been deoxygenated by passage through an alkaline dithionite solution, was bubbled for 5 min through 2 ml of microsomal suspension (3–4 mg protein per ml of 0·05 M tris buffer pH 7·4) in a 1 × 1 cm spectrophotometric cuvette. This was then closed with a rubber stopper equipped with a plunger system and two inlet tubes, one for the continuous aeration of the gas phase and the other for the aeration (30 sec) of the sample with carbon monoxide

which had its exit through the plunger-shaft. With the chart running a zero line was recorded and the reaction was started by the addition of NADPH (0·5 mg in 0·01 ml tris). Simultaneously 0·01 ml tris without NADPH was added to the reference cuvette which was equipped with a similar plunger system. The activities were determined from the initial slope of the curves. The reaction was linear with respect to microsomal protein and was saturated with respect to NADPH.

Binding of 4-hydroxycoumarins to liver microsomes

Microsomes were diluted with 0·1 M tris–HCl buffer pH 7·4 to give a protein content of usually 2 mg/ml of buffer. Three-ml portions were placed in each of two cuvettes with a light path of 1 cm and a zero line was recorded with the Shimadzu MPS-50 L recording spectrophotometer in the absorbance range $\pm$0·100. To the reference cuvette 0·1 ml 0·1 M NaOH was added followed by 0·1 ml 0·1 M HCl. To the measuring cuvette was added 0·1 ml of the 4-hydroxycoumarin compound to be studied dissolved in 0·1 M NaOH and immediately thereafter 0·1 ml 0·1 M HCl. This way of adding the 4-hydroxycoumarins was found necessary because of the low solubility of many of these compounds in ordinary solvents. The spectrum was recorded in the range 370–600 nm. The quantitative determination of dicoumarol binding was estimated from the change in absorbance at 420 nm in relation to the varying concentrations of dicoumarol.

Determination of protein was performed according to the method of Lowry *et al.*[30] with bovine plasma albumin as a standard.

Chemicals

Dicoumarol (3,3′-methylenebis(4-hydroxycoumarin)), Tromexan (=aethyli biscoumacetate, 3,3′-carboxy-methanebis(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-hydroxycoumarin) were obtained from commercial sources. 4-Hydroxycoumarin was obtained from Fluka. NADP, NADPH, horse heart cytochrome *c*, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were purchased from C. F. Boehringer & Soehne, Mannheim, Germany. Other chemicals used were analytical reagents.

RESULTS

The effect of various derivatives of 4-hydroxycoumarin on the activity of NADPH: cytochrome c reductase is shown in Table 1. Of the compounds studied only dicoumarol exhibited a weak inhibitory action on this enzyme system, while the others did not show any effect. From the same table it is, however, evident that the NADPH-mediated reduction of the dye, 2,6-dichlorophenolindophenol, by liver microsomes is inhibited to an extent of 21–37 per cent by the 4-hydroxycoumarins studied (concentration 0·31 mM). It is especially noticeable that 4-hydroxycoumarin itself inhibits this activity to the same extent as the other hydroxycoumarins in contrast to what was observed with the enzyme activities to be described.

The inhibitory action of the 4-hydroxycoumarins on the activity of NADPH-cytochrome P-450 reduction is apparent from Table 1. With the inhibitor concentration studied

TABLE 1. EFFECT OF 4-HYDROXYCOUMARINS ON ACTIVITIES OF NADPH:CYTOCHROME *c* REDUCTASE, NADPH:2,6-DICHLOROPHENOLINDOPHENOL REDUCTASE AND NADPH:CYTOCHROME P-450 REDUCTASE

Inhibitor†	Per cent inhibition*		
	NADPH:cytochrome *c* reductase activity	NADPH:2,6-dichloro-phenolindophenol reductase activity	NADPH:cytochrome P-450 reductase activity
Dicoumarol	14·6 ± 1·4	37·2 ± 8·0	59·5 ± 4·4
Tromexan	2·8 ± 3·9	21·5 ± 5·1	39·3 ± 3·4
Warfarin	0·3 ± 3·7	29·3 ± 3·7	31·0 ± 4·1
Marcoumar	−3·3 ± 3·7	26·4 ± 3·5	40·5 ± 4·0
Sintrom	1·4 ± 4·0	29·0 ± 4·3	46·4 ± 5·8
4-Hydroxycoumarin	6·7 ± 0·3	27·4 ± 3·0	0·8 ± 5·7

* Results are given as means ± S.D.
The values are based on three separate determinations each performed in duplicate.
† Final concentration of inhibitor 0·31 mM in all experiments, except in the NADPH:cytochrome P-450 reductase experiments where the final inhibitor concentration was 0·45 mM.
For details of determinations see methods.

(0·45 mM) the inhibition ranged from 60 (dicoumarol) to 31 per cent (warfarin). However, no inhibition was obtained with 4-hydroxycoumarin itself.

In preliminary experiments it was shown that dicoumarol did not exert any inhibitory effect on the NADPH-generating system used in the assays of mixed function oxidases.

The effect of the 4-hydroxycoumarins on aniline hydroxylase of liver microsomes is evident (Table 2) except for 4-hydroxycoumarin itself. The inhibition obtained with various 4-hydroxycoumarin anticoagulants at a concentration of 0·33 mM ranged from 39·5 to 48·5 per cent. The effect of dicoumarol and warfarin on aniline hydroxy-

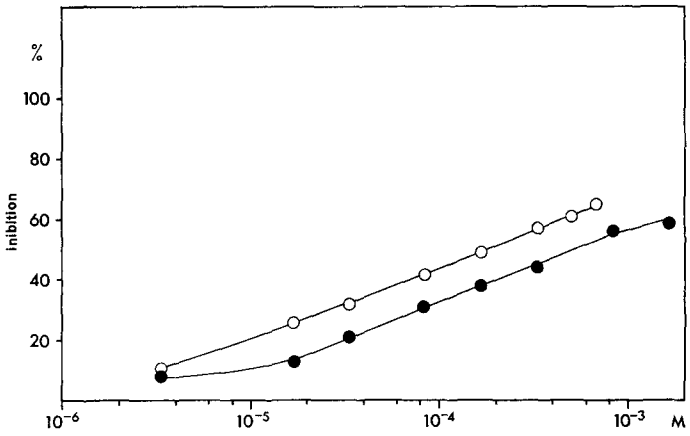

FIG. 1. Inhibition of aniline hydroxylase activity as a function of the concentrations of dicoumarol (○) and warfarin (●). Determinations were performed as described in materials and methods except that the concentrations of dicoumarol and warfarin were varied.

TABLE 2. INHIBITION OF ANILINE HYDROXYLASE, *p*-NITROANISOLE *O*-DEMETHYLASE AND AMINOPYRINE *N*-DEMETHYLASE BY VARIOUS 4-HYDROXYCOUMARINS

Inhibitor†	Per cent inhibition*		
	Aniline hydroxylase	*p*-Nitroanisole *O*-demethylase	Aminopyrine *N*-demethylase
Dicoumarol	44·8 ± 4·2 (3)	32·1 ± 5·8 (3)	68·8 ± 3·8 (4)
Tromexan	41·5 ± 3·6 (3)	23·7 ± 4·9 (3)	61·0 ± 7·2 (4)
Warfarin	39·5 ± 3·3 (3)	11·7 ± 2·0 (3)	25·6 ± 3·5 (5)
Marcoumar	47·8 ± 3·4 (3)	32·4 ± 1·6 (3)	42·3 ± 2·6 (2)
Sintrom	48·5 ± 3·5 (3)	29·2 ± 1·4 (3)	40·5 ± 5·0 (4)
4-Hydroxycoumarin	3·3 ± 2·3 (3)	−3·0 ± 6·7 (3)	7·7 ± 3·7 (4)

* Results are given as means ± S.D.
Number in parentheses indicate number of separate experiments each performed in duplicate.
† Final concentration of inhibitor 0·33 mM in all experiments.
For details of determinations see methods.

lase was further analysed (Fig. 1). The inhibitory action was recognizable at a concentration as low as 3×10^{-6} M and was 50 per cent at a concentration of about 2×10^{-4} M for dicoumarol and 6×10^{-4} M for warfarin.

The inhibition of aniline hydroxylase by dicoumarol was also studied at varying concentrations of aniline. The results are given in a Lineweaver–Burk diagram (Fig. 2).

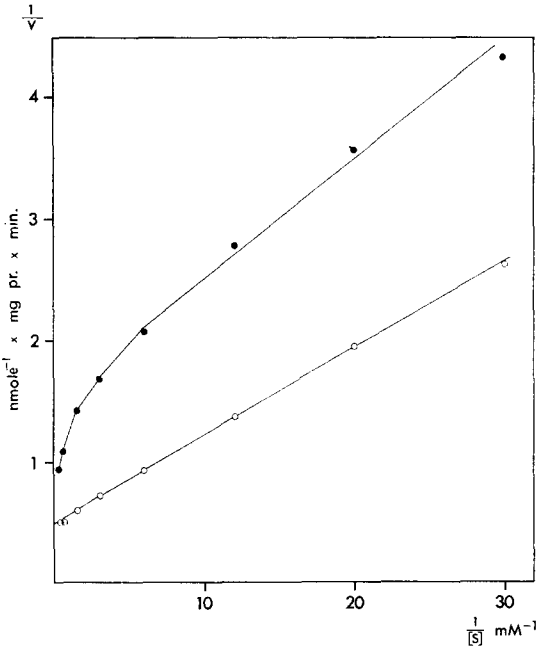

FIG. 2. Lineweaver–Burk diagram illustrating effect of dicoumarol on aniline hydroxylase. Without dicoumarol (○) and with added dicoumarol (final concentration 0·33 mM) (●). The incubations were carried out as described in materials and methods except that the aniline concentrations (S) were varied.

Without dicoumarol the plots of reciprocal velocity as a function of reciprocal aniline concentration resulted in a straight line. With the addition of dicoumarol the plots consistently turned out to be nonlinear at high concentrations of aniline while a straight line was obtained at low concentrations. The same results were observed when warfarin was used as the inhibitor.

The effect of the 4-*hydroxycoumarins on* p-*nitroanisole* O-*demethylation* (Table 2) was more varied, the inhibition ranging from 12 per cent for warfarin to 32 per cent for dicoumarol and again with no inhibitory action of 4-hydroxycoumarin itself.

The inhibition of aminopyrine N-*demethylation by the* 4-*hydroxycoumarins* is also apparent from Table 2. The inhibition varied from 26 per cent for warfarin to 69 per cent for dicoumarol. A very slight inhibitory action on the *N*-demethylase activity was noted with 4-hydroxycoumarin.

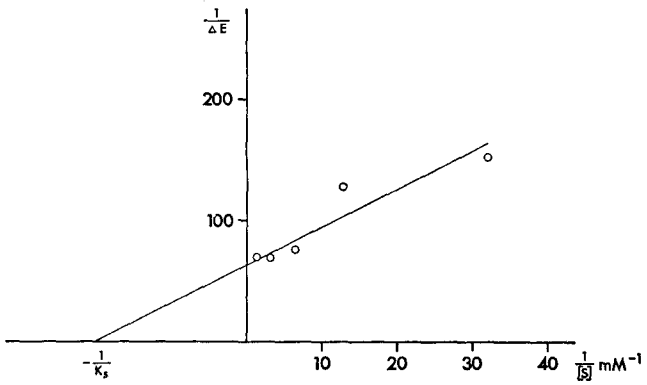

FIG. 3. Graph used for determination of K_s for dicoumarol. The change in absorbance at 420 nm as a function of the concentration of dicoumarol was determined as described in the section on materials and methods. Concentrations of protein 2 mg/ml in all experiments. $K_s = 6{\cdot}5 \times 10^{-5} \pm 2{\cdot}4 \times 10^{-5}$ M (n = 6).

The binding of the 4-*hydroxycoumarins to microsomal cytochrome P*-450 was evident from the difference spectra obtained after the addition of the compounds to suspensions of liver microsomes. Dicoumarol, warfarin and marcoumar elicited modified type II spectral changes according to the classification given by Schenkman *et al.*[8] with a maximum at 419–420 nm and a minimum near 390 nm. Tromexan, curiously, and 4-hydroxycoumarin did not produce any significant changes. The changes produced by sintrom could not be recognized because of its own absorption at the wavelengths studied.

In order to obtain quantitative information about the binding of dicoumarol to liver microsomes the spectral change (ΔE) at 420 nm was related to the concentrations of dicoumarol. The spectroscopic dissociation constant, K_s, was obtained from a graph of reciprocal values of ΔE as a function of reciprocal dicoumarol concentrations (Fig. 3). The value of K_s was determined from six such experiments, K_s being $6{\cdot}5 \times 10^{-5}$ M.

DISCUSSION

The present investigation has shown an inhibitory effect of dicoumarol and related compounds on the activities of some mixed function oxidases of rat liver (e.g.

p-hydroxylation of aniline, *N*-demethylation of aminopyrine and *O*-demethylation of *p*-nitroanisole). No effect on these activities was, however, obtained with 4-hydroxycoumarin itself. This might suggest that the inhibitory effect of 4-hydroxycoumarins is dependent on the presence of a side chain at position 3 of 4-hydroxycoumarin confering lipid solubility to the compounds. From other inhibitors of the mixed function oxidase system it is also known that the inhibition is related to the lipid solubilities of the inhibitors (e.g. hydrazine derivatives).[17]

It is interesting to note the lack of inhibitory effect of 4-hydroxycoumarins on the activity of NADPH-cytochrome *c* reductase since this enzyme may be involved in the microsomal electron transport system between NADPH and cytochrome P-450.[3,31] However, the inhibitory effect on the NADPH dependent reduction of 2,6-dichlorophenolindophenol obtained with all the 4-hydroxycoumarin compounds studied may be difficult to explain if it is assumed that this activity resides in the same enzyme as the NADPH-cytochrome *c* reductase activity.[31] The cause of this discrepancy might be attributed to binding of the 4-hydroxycoumarins to cytochrome *c* thereby reducing their effective free concentrations, but this possibility was not further tested. The inhibition of 2,6-dichlorophenolindophenol reduction by 4-hydroxycoumarin itself is noteworthy because this compound has no effect on any of the three oxidase reactions studied (aniline hydroxylation, aminopyrine *N*-demethylation and *p*-nitroanisole *O*-demethylation). The inhibition of 2,6-dichlorophenolindophenol reduction by all the 4-hydroxycoumarins studied, therefore, does not seem to be related to their inhibitory effect on the mixed function oxidase reactions mentioned.

The inhibition of cytochrome P-450 reduction by 4-hydroxycoumarin derivatives with the exception of 4-hydroxycoumarin itself may be fundamentally related to the inhibition of the mixed function oxidase activity of liver microsomes. However, the available experimental information does not permit any definite conclusions to be drawn concerning the mechanism by which these phenomena are related. As already stated dicoumarol and related compounds mostly produced a modified type II spectral change upon interaction with liver microsomes and it has been shown that many compounds producing this type of spectral change inhibit the reduction of cytochrome P-450[32,33] and some of them may also be potent inhibitors of the drug hydroxylating system in microsomes.[9]

The kinetics of the inhibition of aniline hydroxylase by dicoumarol and warfarin were anomalous as the Lineweaver–Burk plots were consistently found to be non-linear especially at high substrate concentrations of aniline. However, without inhibitors the plots for aniline hydroxylase were found to be perfectly linear. A similar situation has been reported by Lewis *et al.*[34] who found departure from linearity in Lineweaver–Burk plots of aldrin epoxidation at high substrate concentrations in the presence of the inhibitors 1,3-benzodioxoles. Interestingly, Wada *et al.*[35] in contrast to our findings observed non-linear reciprocal plots of aniline hydroxylase activity in rat liver microsomes and furthermore found that the curvature was increased in the presence of the inhibitor prednisolone. Such observations may be consistent with the presence of more than one enzyme involved in the aniline hydroxylation being inhibited differently by the inhibitor. However, with the relatively crude preparations used as the source of enzyme in the present investigation the interpretation of the kinetic data is difficult.

As already mentioned dicoumarol has been found to inhibit the 11β-hydroxylation

of deoxycorticosterone in mitochondria of adrenocortical tissue.[23, 36] The inhibition by dicoumarol of this system was 50 per cent at a concentration of dicoumarol of about 7×10^{-5} M while in the present study this degree of inhibition with the aniline hydroxylase was obtained at a concentration of dicoumarol about 2×10^{-4} M. Thus dicoumarol is about three times as potent an inhibitor of the 11β-hydroxylase system in mitochondria of adrenocortical tissue as compared with the aniline hydroxylating system of liver microsomes. Williamson and O'Donnell[36] found the inhibition of the 11β-hydroxylase system to be complex being of the noncompetitive type at low concentrations of dicoumarol and of a mixed type at high concentrations. It is interesting to note that dicoumarol itself is metabolized by the mixed function oxidase system of liver microsomes (Christensen, unpublished). It might therefore be that dicoumarol acts partly as a competitive inhibitor of aniline hydroxylation at the substrate binding site, presumably at cytochrome P-450, and partly as a non competitive inhibitor at an undetermined step in the electron transport chain. However, further studies are needed for a more thorough understanding of the mechanism by which 4-hydroxycoumarins inhibit mixed function oxidase activity of liver microsomes.

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