compound into lipoidal phases. This process is likely to occur in the intestine as demonstrated both *in vitro* and *in vivo*. Since absorption of the compound occurs from regions of the GI tract which have pH values >6, it is suggested that ion pair formation with naturally abundant cations such as sodium plays a significant role in the absorption of proxicromil.

An alternative explanation for the GI absorption is that there is a difference between bulk and surface pH. The data of Lei *et al.* (15) suggest the real surface pH might be lower than that expected from bulk pH. To overcome surface pH effects in the intestinal perfusion experiments, we have equilibrated the gut for 10 min by perfusion of buffer at the appropriate pH at 1 mL/min. Under these conditions, surface pH and bulk pH should have tended toward an equilibrium (16), the true pH of which would be very close to that of the measured eluant buffer (see Table I). The linear rate of absorption at each pH indicated in Fig. 7 also demonstrates that an equilibrium has been achieved, since a decreasing rate of absorption would be expected at the lower pH values as the surface pH gradually increased. The significance of a surface pH of 6.5 in explaining absorption of acidic compounds is also of much greater relevance for compounds such as *n*-butyric acid (pK_a 4.9) (16) than proxicromil (pK_a 1.93).

The ion pair behavior described here is in marked contrast to the normally held concept (17). Proxicromil possesses intrinsic lipophilicity and requires only charge neutralization to partition into lipoidal phases. Endogenous ions such as sodium and potassium readily form such neutral lipophilic ion pair complexes. This can be contrasted to the facilitation of partitioning of a nonlipophilic drug by the incorporation of a lipophilic counterion, the concept of which has led Jonkman and Hunt (17) to consider ion pair absorption as more fiction than fact.

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Relationship Between Molecular Structure and Cytochrome P_{450}–Metabolic Intermediate Complex Formation, Studied with Orphenadrine Analogues

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Received April 4, 1983, from the *Department of Pharmacology and Pharmacotherapy, Faculty of Pharmacy, State University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands.* Accepted for publication July 29, 1983.

Abstract □ Complexation of ferrous cytochrome P_{450} by metabolic intermediates formed during NADPH-catalyzed metabolism of compounds structurally related to orphenadrine was studied. This so-called metabolic intermediate complexation was determined in rat liver microsomes, obtained from phenobarbital-pretreated rats, at 455 nm using 33 μM of the orphenadrine derivatives. Using secondary amine derivatives with various *N*-alkyl substituents, a parabolic relationship between the logarithm of percentage of cytochrome P_{450} complexation and hydrophobic fragmental constant was observed. The derivative with a bulky tertiary butyl group, however, was devoid of metabolic intermediate-complexing activity. This indicates that steric factors besides lipid solubility may govern the complexing activity; also substitution at the phenyl group affects metabolic intermediate complex formation.

Keyphrases □ Cytochrome P_{450}—complexation with metabolic intermediates, orphenadrine analogues □ Orphenadrine—analogues, metabolic intermediate Complexation with cytochrome P_{450} □ Complexation—metabolic intermediate, orphenadrine analogues with cytochrome P_{450}

Several types of nitrogenous compounds have been shown to undergo cytochrome P_{450}-catalyzed metabolic conversions leading to metabolic intermediates that complex with cytochrome P_{450} (1–3). This complexed cytochrome P_{450} is inactive. Metabolic intermediate complex formation may therefore inhibit metabolic reactions (4). Among the nitrogenous compounds which elicit this complex formation, there are several classes of therapeutically important drugs.

Amphetamine derivatives form a metabolic intermediate complex with hepatic microsomal cytochrome P_{450} *in vitro*, but not *in vivo* (4, 5). With regard to amphetamine analogues, relationships between molecular structure and *in vitro* complexation have been described (4, 6). Recently, metabolic intermediate complex formation produced by macrolide antibiotics have gained much interest (7, 8). With a series of macrolide antibiotics, structural features which are important for the formation of metabolic intermediate complexes have been determined (9). However, with regard to compounds related to proadifen (like propoxyphene, desipramine, and orphenadrine) which produce such complexation both *in vitro* and *in vivo* (10, 11), little is known about the relationship between molecular structure and the ability to evoke complexes

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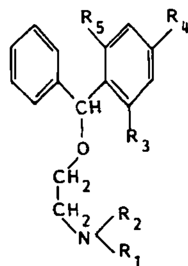
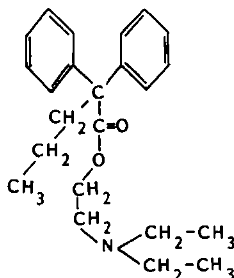
Table I—Complexation with Cytochrome P_{450}



Compound	Substituents[a]					Cytochrome P_{450} Complexed[b], %
	R_1	R_2	R_3	R_4	R_5	
I	H	H	H	H	H	0.8
II	H	H	Me	H	H	—[c]
III	Me	H	H	H	H	1.8
IV (Diphenhydramine)	Me	Me	H	H	H	10.0
V (Tofenacine)	Me	H	Me	H	H	14.0
VI (Orphenadrine)	Me	Me	Me	H	H	8.8
VII (Pytamine)	Me	Me	Et	H	Et	—
VIII	Et	H	H	H	H	9.4
IX	*n*-Pr	H	H	H	H	16.7
X	*i*-Pr	H	H	H	H	10.4
XI	*i*-Pr	H	Me	H	H	6.4
XII	*i*-Pr	H	H	Me	H	—
XIII	*n*-Bu	H	H	H	H	21.0
XIV	*t*-Bu	H	H	H	H	—
XV (N—OH Tofenacine)	Me	OH	Me	H	H	50.0[d]
XVI (Proadifen)[e]						8.5

[a] Key: Me, methyl; Et, ethyl; Bu, butyl; *i*, iso-; *n*, normal; *t*, tertiary. [b] Determined using 2.5 μM cytochrome P_{450} and 33 μM of the compounds. [c] Not detectable. [d] Based on previous data. [e] Refers to the formula:



(1, 4). However, because of metabolic intermediate complex formation, peculiar kinetics (12) and adverse drug interactions may arise (4). On the other hand selective inhibition of cytochrome P_{450} subforms provides an interesting tool to manipulate these subforms. In this manner, formation of toxic metabolites might be reduced.

In the present study, therefore, we investigated the relationship between molecular structure and metabolic intermediate complex formation of several orphenadrine derivatives. In this way both structural features important in the formation of complexes as well as insight into the mechanism of complexation with this class of compounds were established.

EXPERIMENTAL SECTION

Chemicals—Orphenadrine and its derivatives were gifts[1]; NADPH was purchased[2]. All other chemicals and solvents used were of analytical-grade purity.

Preparation of Microsomes and Pretreatment of Animals—Liver microsomes were prepared from Wistar rats weighing 250–280 g, as described previously (13). The rats used were pretreated with phenobarbital, dissolved in saline, inasmuch as metabolic intermediate complex formation is specifically enhanced after induction with phenobarbital (4). The phenobarbital-pretreatment consisted of three daily injections of 80 mg/kg ip. On the fourth day the rats were killed by decapitation.

Spectral Measurements—All spectrophotometric observations were performed with the microsomal fraction (2 mg of protein/mL) suspended in 50 mM phosphate buffer (pH 7.4) containing 0.1 mM EDTA. The spectral determinations were performed with UV-visual spectrophotometer[3] at 37°C.

Metabolic intermediate complex formation was measured with the microsomal suspension containing substrate as indicated and 4.2 mM $MgCl_2$. The reaction, started with 400 μM NADPH, was followed by dual-wavelength spectrometry (455 nm *versus* 490 nm) unless stated otherwise in the text. To quantitate the percentage of cytochrome P_{450} complexed, a molar extinction coefficient (ϵ) of 65 mM^{-1} cm^{-1} was used (14, 15). The ϵ is merely a proportionality constant and not of decisive importance in this study. However, use of ϵ provides quantitation of the sequestered amount of cytochrome P_{450}.

Protein concentration (16) and cytochrome P_{450} (17) were determined according to standard procedures.

RESULTS

Scanning spectrometry (430–500 nm) with the spectrophotometer in the split-beam mode (11) was used to establish whether the compounds studied at 33 μM (Table I) exhibited a Soret absorption maximum at 455 nm due to metabolic intermediate complex formation. Inasmuch as this was the case[4], employment of dual wavelength (455–490 nm) spectrometry is valid to study this complex formation *in vitro*.

Substrate concentration dependency has been observed both for rate and extent of metabolic intermediate complex formation (11, 18). We therefore compared the extent of cytochrome P_{450} complexation with each derivative ($\Delta A_{455-490\ nm}$) at three substrate concentrations. Maximal complexation was achieved at 33 μM for each compound (Fig. 1). Moreover, using a concen-

[1] Gist-Brocades N.V., Delft, The Netherlands.
[2] Boehringer/Mannheim GmbH, Mannheim, F.R.G.

[3] Model DW2; Aminco.
[4] Unpublished results.

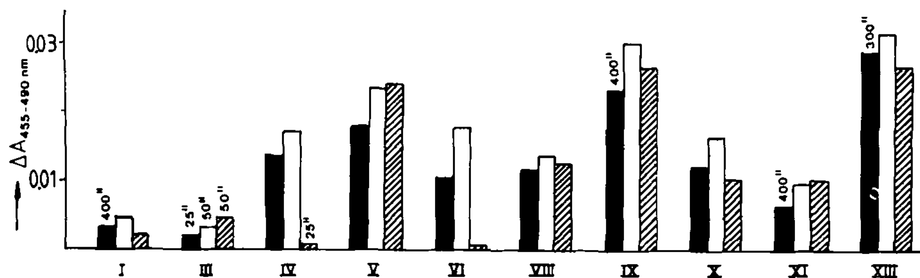


Figure 1—*Concentration dependency of extent of metabolic intermediate complexation ($\Delta A_{455-490\ nm}$) produced by various compounds (see Table I). For each compound, three concentrations were tested. Incubation has been performed for 650 s, unless stated otherwise in the figure. Key: (■) 10 μM; (□) 33 μM; (▨) 300 μM.*

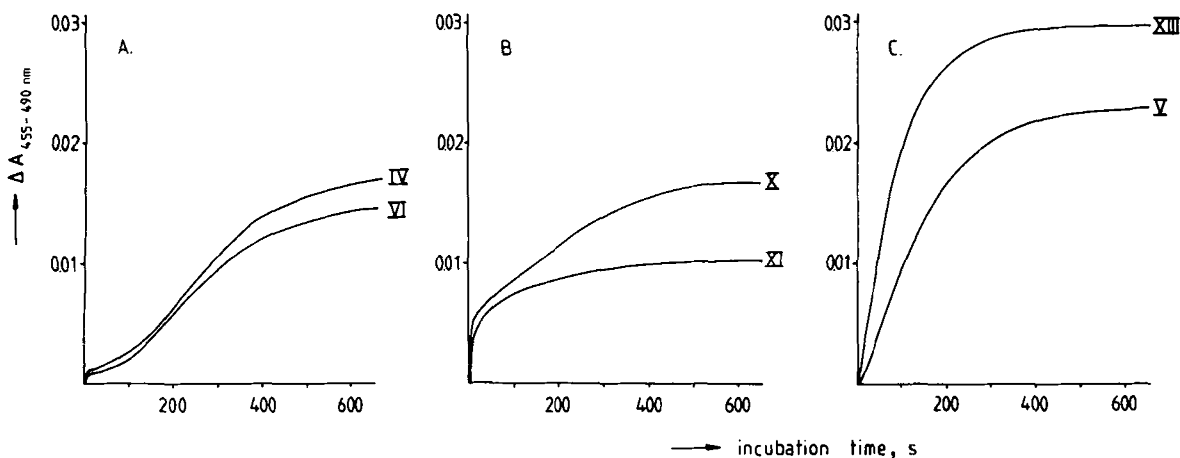


Figure 2—*Time course of metabolic intermediate complex formation, indicated by $\Delta A_{455-490\ nm}$ (see Table I). Experimental conditions as described in the* Experimental Section.

tration of 33 μM, maximal absorption at 455 nm was accomplished within an incubation time of 650 s and remained stable thereafter. The only exception, III, shows maximal absorption in 50 s, which decreased thereafter. Maximal complexation was used for further evaluation.

Within the series of compounds tested (Table I), the time course of development of absorption at 455 nm differed. Figure 2A shows that complexation produced by the tertiary amine structures (IV and VI) have a prominent initial lag phase. Compounds X and XI, containing an isopropyl substituent, display a biphasic increase in 455 nm absorption (Fig. 2B). With the secondary amines, in which an *n*-alkyl substituent is present on the nitrogen atom, (*e.g.*, V and XIII), neither a lag phase nor biphasic kinetics occur (Fig. 2C).

Using a concentration of 33 μM, the percentage of cytochrome P_{450} complexed was established for each compound (Table I). Evaluation of data of Table I reveals a possible parabolic dependence of metabolic intermediate complexation on lipophilicity. The latter was expressed by the fragmental constant (*f*) of the —NHR moiety of I, III, VIII, IX, X, and XIII (19) (Fig. 3). As shown in Table I, XIV, with a bulky tertiary butyl group, does not produce metabolic intermediate complexation. Substitutions on the aromatic part of the molecules give large variations in complex formation. Comparison of II with I, V with III, and XI with X shows that the effect of substitution at R_3 depends on the substituent at R_1. Moreover, XII, with a methyl substituent at R_4 and an isopropyl group at R_1, did not form a metabolic intermediate complex (Fig. 4).

DISCUSSION

The optimum concentration for metabolic intermediate complexation has often been found to be 33 μM, irrespective of whether hepatic microsomes were obtained from untreated or phenobarbital-pretreated rats (1). The same optimum concentration was established not only with different microsomal preparations, but also with different compounds (1, 11). Accordingly, the present data also suggest that 33 μM can be used for comparative purposes (Fig. 1). The concentration dependency, explained by substrate inhibition (11), hinders use of K_m values. Biphasic kinetics observed with X and XI (Fig. 2B) suggest that different forms of cytochrome P_{450} are involved in metabolic intermediate complex formation. The lag time observed with tertiary amines (Fig. 2A) is probably explained by initial *N*-demethylation, which has to occur before the complexing species can be formed (11). Because of the different time courses of metabolic intermediate complexation, we did not use the initial rate but rather the extent of such complexation.

A trend toward a parabolic relationship between percentage of cytochrome P_{450} complexed and lipophilicity was observed. The possible bilinear relationship describes transport of the substrate and, in addition, may also reflect a hydrophobic interaction (20). Dependency on the hydrophobic fragmental constant may reflect the fact that the active sites of cytochrome P_{450} are located in a hydrophobic pocket, submerged in the membrane (21). Transport between hydrophilic and hydrophobic phases as a rate-limiting step has also been suggested for *N*-oxidation of amphetamine derivatives (6). Compound X, which contains an isopropyl group, deviates (Fig. 3). This may indicate that steric factors besides lipid solubility may govern metabolic intermediate-complexing activity as well. This notion is substantiated by the fact that XIV, which has a bulky tertiary butyl group, is devoid of such complexing activity. Similar dependencies arise from literature reports, in which *N*-dealkylation of secondary amines have been described (22, 23). This suggests that the distinct correlation, which has been found between the rate of *N*-demethylation and metabolic intermediate complex formation (11, 24) can be extended to *N*-dealkylation and metabolic intermediate complexation. The differences in complex formation with secondary amines may therefore reflect *N*-dealkylation. This would correlate well with the suggestion that the actual

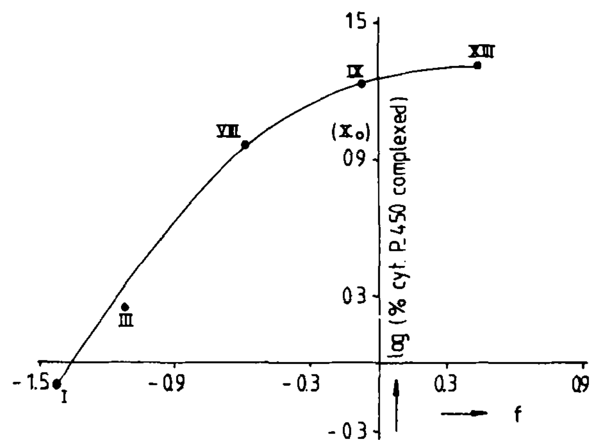


Figure 3—*Plot of the logarithm of percentage of cytochrome* P_{450} *complexed (obtained from Table I) against the sum of the hydrophobic fragmental constant of the substituted amine function (see text) for several compounds which vary in their amine function.*

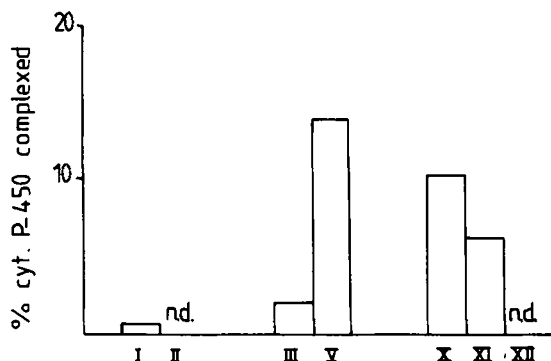


Figure 4—*Effect of substitution in the aromatic part of the substrates on metabolic intermediate-complexing activity, indicated as percentage of cytochrome* P_{450} *complexed (obtained from Table I).*

ligand responsible for such complex formation is an *N* oxidized primary amine, probably a nitroso compound or a nitroxide radical (11). Presumably the main mechanism leading to the ultimate metabolic intermediate ligand is *N*-oxidation of the secondary amines, since *N*-hydroxytofenacine (XV) leads to a large extent to complex formation. *N*-Oxidation of the *N*-hydroxy-*N*-alkylamine may lead to an unstable *N*-hydroxy-*N*-alkylamine *N*-oxide, which after rearrangement is readily *N*-dealkylated. The *N*-dealkylated *N*-hydroxylamine is rather unstable and may give the nitroxide radical. Direct *N*-oxidation of the primary amine does not have an important contribution to metabolic intermediate complex formation inasmuch as I and II do not produce a metabolic intermediate complex to any large degree.

Interpretations of data become more difficult if the effect of substitution at the aromatic portion of the molecule comes into play (Fig. 4). Substitution on one phenyl group greatly affects complexation. Moreover, the substituent on the amine function influences the effect of substitution on the aromatic part in an unpredictable manner. Clearly more compounds are needed to unravel the effect on metabolic intermediate complexation if substitution occurs in different parts of the molecule simultaneously.

In conclusion, evaluation of the relationship between structure and biological activity may be helpful in development of compounds which will show the desired pharmacological effect without metabolic intermediate complexation. One may also design compounds with prominent complexing activity with specific cytochrome P_{450} subforms, which may be used for pharmacological or toxicological purposes.

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Hypolipidemic Activity of 3-*N*-(1′,8′-Naphthalimido)propionic Acid in Rodents

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Received January 31, 1983, from the *Division of Medicinal Chemistry, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514.* Accepted for publication July 29, 1983.

Abstract □ 3-*N*-(1′,8′-Naphthalimido)propionic acid was synthesized and shown to effectively lower both serum cholesterol and triglyceride levels in rats and mice. In hyperlipidemic mice, serum lipid levels were lowered significantly, approaching normal levels of cholesterol and below normal levels of triglyceride. The serum lipid levels were reduced due to accelerated clearance of cholesterol *via* the biliary route as well as a lowering of available acetyl CoA in the cytoplasm for liver *de novo* cholesterol and triglyceride syntheses. The liver regulatory enzyme of fatty acid and triglyceride syntheses were depressed by drug treatment. Significant reduction of liver lipids as well as the lipid content of the lipoprotein fractions were observed. The agent possessed a safe therapeutic index when used as a hypolipidemic agent.

Keyphrases □ 3-*N*-(1′,8′-Naphthalimido)propionic acid—hypolipidemic activity in rodents, cholesterol and triglycerides reduction □ Antihyperlipidemic agents—3-*N*-(1′,8′-naphthalimido)propionic acid, activity in rodents, cholesterol and triglycerides reduction

N-Substituted cyclic imides have been shown to be potent hypolipidemic agents in rodents at the low dose of 20 mg/kg/d. Substitutions of four carbon atoms or an oxygen atom for one of the carbons provided the most active agent of the phthalimide, saccharin, and the 1′,8′-naphthalimide series (1). These agents effectively lowered both serum cholesterol and tri-

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