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# QUANTITATIVE STRUCTURE–ACTIVITY RELATIONSHIP OF COUMARIN DERIVATIVES

## INVOLVEMENT OF PARTITION BETWEEN AQUEOUS AND MEMBRANE PHASE

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SUMMARY

In order to understand the substrate behaviour of several 7-alkoxycoumarins and 7-alkoxy-4-alkylcoumarins towards the liver microsomal monooxygenase system, their lipophilic properties have been examined. As a model for the lipophilicity the reversed-phase liquid chromatographic retention parameter $\log k_w$ has been used. In a system with methanol–water as the mobile phase and RP-18 (octadecylsilica) as the stationary phase, we found a quadratic relationship between the volume fraction of the organic solvent and the logarithm of the capacity factor ($\log k'$). The extrapolation to a pure aqueous phase reveals a linear relationship of the theoretical capacity factor $\log k_w$ with the chain length. This holds for 1–12 carbon atoms in the alkoxy chain and for zero to three carbon atoms in the alkyl chain. Moreover, the incremental effect of the methylene residues on the lipophilicity of the compounds ($\Delta \log k_w / \Delta CH_2$) is found to be $0.60 \pm 0.01$.

If the coumarin derivatives are used as substates for the liver microsomal monooxygenase system, no systematic dependence of the enzymic data (Michaelis–Menten constant $K_m$) on the lipophilic data ($\log k_w$) can be demonstrated.

The metabolism of these compounds by the microsomal monooxygenase system seems not to be limited by the partition between the membrane and the aqueous phase. Whether other factors, *e.g.* the lateral diffusion of the substrates *versus* the membrane-bound enzyme system or enzyme active-site characteristics, govern the metabolism remains to be investigated.

## INTRODUCTION

In the case of membrane-bound enzymic reactions, enzyme mechanistic data, such as the Michaelis–Menten constant $K_m$ and the spectral dissociation constant $K_s$, are related to the partition coefficient of a substrate between the membrane and the aqueous phase, as has been pointed out by Hansch *et al.*[1]. This should hold for the liver microsomal monooxygenase system, which is deeply integrated into the membrane of the endoplasmic reticulum.

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To characterize the lipophilicity of a compound, the partition coefficient $P$ is often used. This is usually obtained from an $n$-octanol–water partitioning system according to eqn. 1:

$$P = \frac{C_{\text{oct.}}}{C_{\text{water}}} \quad i.e. \quad \log P = \log C_{\text{oct.}} - \log C_{\text{water}} \tag{1}$$

where $C_{\text{oct.}}$ and $C_{\text{water}}$ are the concentrations of the compound in the octanol phase and the water phase, respectively. Instead of $n$-octanol, other hydrocarbons such as cyclohexane, $n$-hexane or oils may be used[2]. Because of the limited applicability ($\log P \leqslant 4$) and experimental difficulties of determining $P$, especially when a compound is not soluble in one of the solvent phases, other methods have been developed.

Boyce and Milborrow[3] used thin-layer chromatography (TLC) (paraffin-coated silica gel plates). They referred to the terms $R_{\text{m}}$ and $R_F$, which had been introduced by Bate-Smith and Westall[4] and by Martin[5], respectively. These terms are related to the partition coefficient $P$;

$$P = k\left(\frac{1}{R_F} - 1\right) \tag{2}$$

$$R_{\text{m}} = \log\left(\frac{1}{R_F} - 1\right) \tag{3}$$

where $k$ is the empirical constant for the system and $R_F$ is the relative migration rate, which is given by the distance moved by solute divided by the distance moved by solvent front.

The $R_{\text{m}}$ values depend on the composition of the mobile phase, and this must be taken into consideration when different data are compared. Furthermore, the proportion of acetone to water (0.7, v/v) chosen by Boyce and Milborrow[3] seems to be somewhat arbitrary. In contrast to this, Braumann and Grimme[6] have introduced the model parameter $\log k_{\text{w}}$:

$$\log k_{\text{w}} = \log k' - S\varphi_{\text{B}} \tag{4}$$

where $k'$, a capacity factor, is given by

$$k' = (t_R - t_0)/t_0 \tag{5}$$

where $t_R$ is the chromatographic retention time of the substance and $t_0$ is the "dead time" of the chromatographic system. $\varphi_{\text{B}}$ represents the volume fraction of the mobile phase and $S$ is a parameter that depends on the nature of the organic part of the mobile phase and on the substrate used[7].

While the choice of octadecylsilica as the stationary phase and the extrapolation to the pure aqueous phase may reflect the partition behaviour in a biological system, the choice of an organic part for the mobile phase remains somewhat arbitrary and may be made to suit experimental convenience. Measurements made by Braumann *et*

*al.*[8] show that the correspondence of log $k_w$ values, obtained from methanol–water systems, with the log *P* values is better than those obtained from acetonitrile–water or tetrahydrofuran–water systems.

We decided to apply this method to a high-performance liquid chromatographic (HPLC) system because many elutions may be performed on one column, whereas TLC is limited by the dimensions of the plates and interplate variations are a source of considerable error[3].

We are investigating coumarin derivatives as substrates of the liver microsomal monooxygenase system[9]. They represent a set of compounds that is well suited to discriminate the different molecular properties that govern the metabolism of the substrates by the microsomal monooxygenase system.

The properties to be considered are the partition of the substrate between the aqueous phase and the membrane, the mobility of the substrate (and the enzymes) in the membrane, the binding characteristics of the substrate to the active site of the enzyme and different reaction properties determined by the enzyme.

Here only the initial point will be considered and treated under its more common aspects.

## MATERIALS AND METHODS

Table I lists the substances that we investigated. Most of the substances were synthesized in our laboratory by the method described by Will and Beck[10] or, in the case of the 7-hydroxy derivatives, according to Pechmann and Duisberg[11]. Pure coumarin was purchased from Merck (Darmstadt, F.R.G.). The column (125 mm × 4.6 mm I.D.) (Merck) was slurry-packed with LiChrosorb RP-18, 10 μm (Merck).

TABLE I

LIST OF 7-HYDROXY-(4-ALKYL)COUMARINS AND 7-ALKOXY-(4-ALKYL)COUMARINS INVESTIGATED

| *Abbreviation* | *Name* | *Source* |
|---|---|---|
| 7HOC | 7-Hydroxycoumarin | E. Merck, Darmstadt, F.R.G. |
| 7HO4MC | 7-Hydroxy-4-methylcoumarin | Aldrich, Steinheim, F.R.G. |
| 7HO4EC | 4-Ethyl-7-hydroxycoumarin | Our laboratory |
| 7HO4PC | 7-Hydroxy-4-propylcoumarin | Our laboratory |
| 7MOC | 7-Methoxycoumarin | Aldrich, Steinheim, F.R.G. |
| 7EOC | 7-Ethoxycoumarin | Sigma, Deisenhofen, F.R.G. |
| 7POC | 7-Propoxycoumarin | Our laboratory |
| 7BOC | 7-Butoxycoumarin | Our laboratory |
| 7HexOC | 7-Hexyloxycoumarin | Our laboratory |
| 7OcOC | 7-Octyloxycoumarin | Our laboratory |
| 7DecOC | 7-Decyloxycoumarin | Our laboratory |
| 7-DodeOC | 7-Dodecyloxycoumarin | Our laboratory |
| 7MO4MC | 7-Methoxy-4-methylcoumarin | Roth, Karlsruhe, F.R.G. |
| 7EO4MC | 7-Ethoxy-4-methylcoumarin | Our laboratory |
| 7HexO4MC | 7-Hexyloxy-4-methylcoumarin | Our laboratory |
| 7EO4EC | 7-Ethoxy-4-ethylcoumarin | Our laboratory |
| 7EO4PC | 7-Ethoxy-4-propylcoumarin | Our laboratory |

Carbon tetrachloride was used as the suspending medium. To introduce the suspension into the column we used a Haskel pump at 360 bar.

The chromatographic system consisted of an Altex pump (Model 100-A) and a UV detector (Laboratory Data Control Model 1203 UV III) with the detection wavelength fixed at 254 nm.

The mobile phase was methanol p.a.–water (twice distilled over a quartz column) in different volume fractions. Aliquots of 5–10 $\mu$l of the $10^{-5}$–$10^{-2}$ $M$ sample solutions (solvent methanol) were injected using a 10-$\mu$l precision syringe with the aid of a Rheodyne Model 7105 sample valve (Rheodyne, Berkeley, CA, U.S.A.). The retention times were measured with a stopwatch. There was no significant relation between the retention time and the substrate concentration or the sample volume.

When substances with long retention times (0.5–2 h) were eluted, derivatives with shorter retention times could be investigated simultaneously, and no comigration or other influences were observed. This allowed more measurements in the same time, even when some substances were "overtaking" each other.

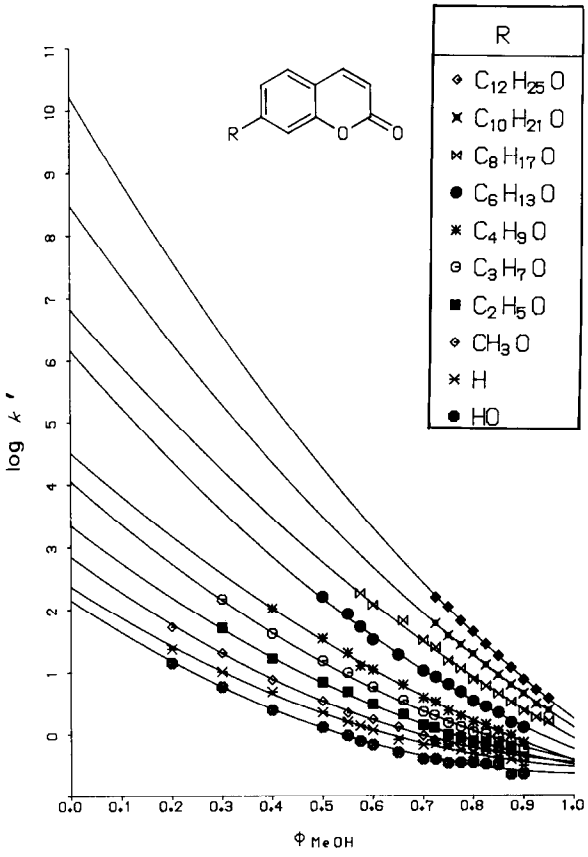



Fig. 1. Chromatographically determined partition of coumarin derivatives in a methanol–water system as the mobile phase. The capacity factor $k'$ as defined by eqn. 5 is plotted against the mobile phase volume fraction of methanol $\varphi_{MeOH}$ (for symbols see inset).

The "dead time" ($t_0$) of the system was determined with potassium nitrate dissolved in methanol. For each substance at least three measurements were carried out for each composition of the mobile phase. The "dead times" were determined at least three times for all mobile phase compositions by three measurements each.

To evaluate the log $k_w$ values we developed a PASCAL-program on a microcomputer (Apple IIe). A precondition for the regression was a subroutine with an accuracy of 35 numerals.

The details of the enzymic investigations will be described elsewhere[9].

## RESULTS AND DISCUSSION

The graphic representation of the relationship between the log $k'$ values and the volume fraction ($\varphi_B$) of the mobile phase, as shown in Figs. 1 and 2a and b, indicates a quadratic behaviour, which can be described by the following function:

$$\log k' = \log k_w + S\varphi_{MeOH} + S'\varphi^2_{MeOH} \quad (6)$$

This is in good agreement with measurements of Schoenmakers *et al.*[12] for some substituted benzenes. Table II summarizes the data obtained by us.

The extrapolated log $k_w$ values show a linear relationship with the 7-alkoxy chain

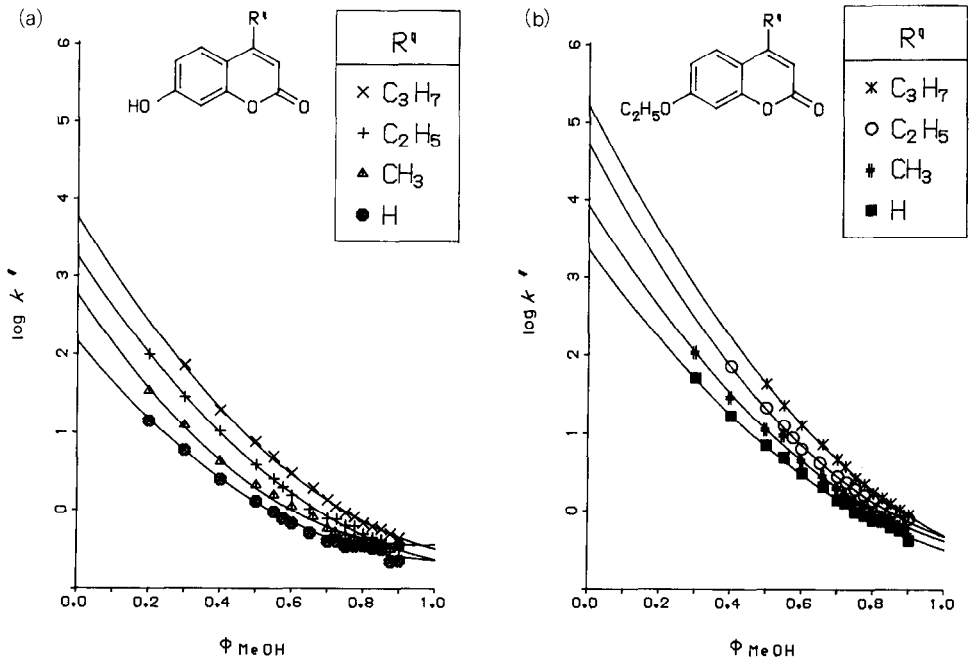



Fig. 2. Chromatographically determined partition of (a) 7-hydroxy-4-alkylcoumarins and (b) 7-ethoxy-4-alkylcoumarins, in a methanol–water system as the mobile phase. The capacity factor $k'$ as defined by eqn. 5 is plotted against the mobile volume fraction of methanol $\varphi_{MeOH}$ (for symbols see insets).

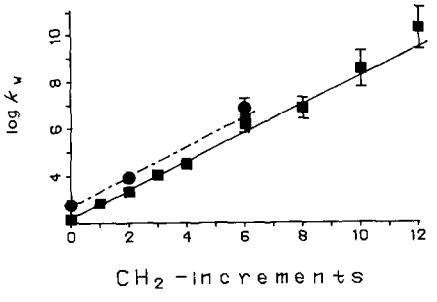
TABLE II

LIPOPHILIC–HYDROPHILIC PARTITION OF COUMARIN AND ITS DERIVATIVES

log $k_w$, $S$, and $S'$ data (± standard error) fitting eqn. 6 with the correlation coefficient $r^2$; $n$ = number of various compositions of the mobile phase.

| *Substance* | *n* | *log* $k_w$ | $S$ | $S'$ | $r^2$ |
|---|---|---|---|---|---|
| Coumarin | 17 | 2.37 ± 0.03 | −5.23 ± 0.13 | 2.33 ± 0.13 | 1.000 |
| 7MOC | 16 | 2.84 ± 0.03 | −5.95 ± 0.13 | 2.65 ± 0.12 | 1.000 |
| 7EOC | 15 | 3.34 ± 0.07 | −6.24 ± 0.26 | 2.41 ± 0.23 | 0.999 |
| 7POC | 15 | 4.05 ± 0.07 | −7.12 ± 0.28 | 2.65 ± 0.23 | 0.999 |
| 7BOC | 15 | 4.50 ± 0.08 | −7.03 ± 0.28 | 2.11 ± 0.23 | 1.000 |
| 7HexOC | 14 | 6.16 ± 0.36 | −9.51 ± 1.03 | 3.11 ± 0.72 | 0.997 |
| 7OcOC | 14 | 6.82 ± 0.46 | −9.32 ± 1.23 | 2.43 ± 0.81 | 0.998 |
| 7DecOC | 10 | 8.48 ± 0.77 | −11.58 ± 1.84 | 3.20 ± 1.10 | 0.999 |
| 7DodeOC | 10 | 10.21 ± 0.91 | −13.79 ± 2.17 | 3.81 ± 1.29 | 0.999 |
| 7HOC | 17 | 2.15 ± 0.04 | −5.41 ± 0.20 | 2.62 ± 0.19 | 0.999 |
| 7HO4MC | 16 | 2.76 ± 0.08 | −6.71 ± 0.32 | 3.51 ± 0.32 | 0.995 |
| 7HO4EC | 17 | 3.24 ± 0.04 | −6.79 ± 0.19 | 2.92 ± 0.19 | 0.999 |
| 7HO4PC | 15 | 3.75 ± 0.08 | −7.36 ± 0.29 | 3.12 ± 0.24 | 0.999 |
| 7EO4MC | 15 | 3.91 ± 0.09 | −7.19 ± 0.35 | 2.89 ± 0.31 | 0.998 |
| 7EO4EC | 17 | 4.70 ± 0.14 | −8.54 ± 0.44 | 3.52 ± 0.36 | 0.998 |
| 7EO4PC | 13 | 5.19 ± 0.08 | −8.74 ± 0.25 | 3.23 ± 0.18 | 1.000 |
| 7HexO4MC | 13 | 6.83 ± 0.41 | −10.52 ± 1.18 | 3.55 ± 0.83 | 0.998 |

length for 1–12 carbon atoms (Fig. 3) and with the 4-alkyl chain length for zero to three carbon atoms (Fig. 4).



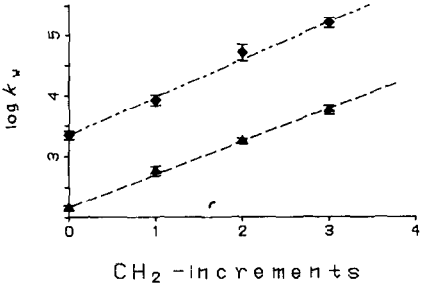




Fig. 3. Differential lipophilicities of the 7-alkoxycoumarins and 7-alkoxy-4-methylcoumarins. The lipophilicity parameter log $k_w$, as defined by eqn. 6, is plotted against the $CH_2$ increments for the 7-alkoxycoumarins (■, measured data; ——, linear regression line, $r^2$ = 0.994) and for the 7-alkoxy-4-methylcoumarins (●, measured data; – – –, linear regression line, $r^2$ = 0.992).

Fig. 4. Differential lipophilicities for the 7-hydroxy- and 7-ethoxy-4-alkylcoumarins. The lipophilicity parameter log $k_w$, as defined by eqn. 6 is plotted against the $CH_2$ increments for the 7-hydroxy-4-alkylcoumarins (▲, measured data; – – –, linear regression line, $r^2$ = 0.996) and for the 7-ethoxy-4-alkylcoumarins (◆, measured data; — · · —, linear regression line, $r^2$ = 0.993).

TABLE III

SUBSTITUENT CONSTANT $\tau_w$ ($\pm$ STANDARD ERROR) AS DEFINED BY EQN. 7 FROM DIFFERENT COUMARIN AND BENZENE DERIVATIVES

$\bar{\tau}_w = \frac{\sum \tau_w(i)}{n}$, where $n$ = number of different basic systems ($i$ = 7HOC, 7EOC, 7HexOC).

| *Substituent* | $\tau_w$ *(coumarin)* | $\tau_w$ *(7HOC)* | $\tau_w$ *(7EOC)* | $\tau_w$ *(7HexOC)* | $\bar{\tau}_w$ | $\tau_w$ *(benzene) (ref. 12)* | $\tau_w$ *(benzene) (ref. 8)* |
|---|---|---|---|---|---|---|---|
| 7-HO– | −0.22 ± 0.05 | | | | | −0.92 | −0.82 |
| 7-$CH_3O$– | +0.48 ± 0.04 | | | | | | −0.01 |
| 7-$C_2H_5O$– | +0.98 ± 0.07 | | | | | | |
| 7-$C_3H_7O$– | +1.68 ± 0.08 | | | | | | |
| 7-$C_4H_9O$– | +2.13 ± 0.08 | | | | | | |
| 7-$C_6H_{13}O$– | +3.79 ± 0.36 | | | | | | |
| 7-$C_8H_{17}O$– | +4.45 ± 0.46 | | | | | | |
| 7-$C_{10}H_{21}O$– | +6.12 ± 0.77 | | | | | | |
| 7-$C_{12}H_{25}O$– | +7.85 ± 0.91 | | | | | | |
| 4-$CH_3$– | | +0.61 ± 0.09 | +0.57 ± 0.11 | +0.67 ± 0.55 | +0.60 ± 0.02 | +0.79 | +0.56 |
| 4-$C_2H_5$– | | +1.09 ± 0.06 | +1.35 ± 0.15 | | +1.13 ± 0.09 | +1.23 | +1.02 |
| 4-$C_3H_7$– | | +1.60 ± 0.09 | +1.85 ± 0.11 | | +1.70 ± 0.12 | | +1.66 |

To compare the effect of different functional groups, a substituent constant $\tau_w$ has been defined as

$$\tau_w = \log k_w (X_i) - \log k_w (H_i) \quad (7)$$

where X is a substituent that replaces a hydrogen atom of the parent compound (i).

In Table III the substituent constants are collected. The averaged $\tau_w$ values for the alkyl groups of coumarins are in good agreement with those of benzene, but the values for the hydroxy and alkoxy groups in the coumarin system show significant deviations from the analogous values in the benzene system.

The reason for that may be a principal limitation of the validity range of eqn. 7 for hydroxy and short-chain alkoxy groups, as well as for other substituents that tend to form hydrogen bonds. If such a hydrogen bond-forming substituent is connected to a highly lipophilic molecule, *e.g.* benzene, the hydrophilic contribution may be higher (*i.e.* the $\tau_w$ value may be more negative) than in the case where that particular substituent is connected to a molecule that already forms hydrogen bonds, *e.g.* coumarin.

Furthermore, our set of log $k_w$ values allows one to calculate the differential effect of the methylene group ($CH_2$) of alkoxy and alkyl chains from the slope of the regression lines, as shown in Figs. 3 and 4, in a wide range of involved methylene groups. The numerical results are given in Table IV.

It is obvious that our $\Delta \log k_w/\Delta CH_2$ values are independent (within the error limits) of the chain type and the basis system. This much cannot be said for the values

TABLE IV

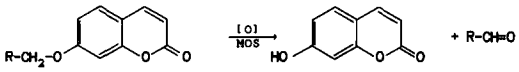
DIFFERENTIAL EFFECT OF $CH_2$ INCREMENTS ON THE LIPOPHILICITY OF DIFFERENT HOMOLOGOUS SERIES OF COUMARINS AND BENZENES

Determined from different methods (the underlined residue indicates the substituent of interest). log $k_w$ has been determined according to eqns. 6 and 4, respectively. (1) log $k_w$ data from Table II ($n$ = 9, see also Fig. 4). (2) log $k_w$ data from Table II ($n$ = 3, see also Fig. 4). (3) log $k_w$ data from Table II ($n$ = 4, see also Fig. 5). (4) log $k_w$ data from Table II ($n$ = 4, see also Fig. 5). (5) log $k_w$ data from ref. 8: phenol ($CH_2$ = 0), 1.34; anisole ($CH_2$ = 1), 2.15. (6) log $k_w$ data from ref. 8: benzene ($CH_2$ = 0), 2.16; toluene, 2.72; ethylbenzene, 3.18; *n*-propylbenzene, 3.82; *n*-butylbenzene, 4.32. (7) log $k_w$ data from ref. 12: benzene ($CH_2$ = 0), 2.31; toluene, 3.09; ethylbenzene, 3.53.

| *Homologues* | $\frac{\Delta \log k_w}{\Delta CH_2}$ | $r^2$ |
|---|---|---|
| 7-Alkoxycoumarins | (1) 0.598 ± 0.04 | 0.994 |
| 7-Alkoxy-4-methylcoumarin | (2) 0.619 ± 0.06 | 0.992 |
| 7-Hydroxy-4-alkylcoumarins | (3) 0.591 ± 0.05 | 0.999 |
| 7-Ethoxy-4-alkylcoumarins | (4) 0.631 ± 0.21 | 0.993 |
| Average value | 0.60 ± 0.01 | |
| Alkoxybenzenes | (5) 0.81 | |
| Alkylbenzenes | (6) 0.542 ± 0.014 | 0.998 |
| | (7) 0.610 ± 0.098 | 0.975 |

reported by other laboratories and that seems to be a general problem in this field. For all that, the incremental effects of $CH_2$ on the lipophilicity of the coumarins and the benzenes are of the same order even though they are obtained by different methods and from different molecules. Therefore, the log $k_w$ model is well suited to quantify the hydrophobicity of coumarin derivatives. It is our method of choice because of its broad and convenient applicablity. The linear relationship of the log $k_w$ values with respect to the alkoxy and alkyl chain lengths means that values for derivatives that have not been determined can be interpolated or extrapolated.

As mentioned above, our main interest is to compare the lipophilic data of the coumarin derivatives with their substrate properties towards the microsomal monooxygenase system. For this reason we have investigated the enzymic parameters for the type of reaction shown in eqn. 8:




(8)

where MOS is the monooxygenase system from pig liver microsomes.

The relationship between the Michaelis–Menten constant ($K_m$) (the details of the enzymic investigations will be published elsewhere[9]) and the lipophilic parameter (log $k_w$) for a collection of coumarin derivatives is shown in Fig. 5.

It is clear that there is no regular variation in the enzymatic parameter log $K_m$ with the lipophilic parameter log $k_w$, and from a knowledge of the lipophilicity alone no prediction of the substrate properties is possible.

## CONCLUSIONS

Under the assumption that the "log $k_w$ model" reflects the solubility properties of a biological membrane, we can conclude that the partition of coumarin derivatives

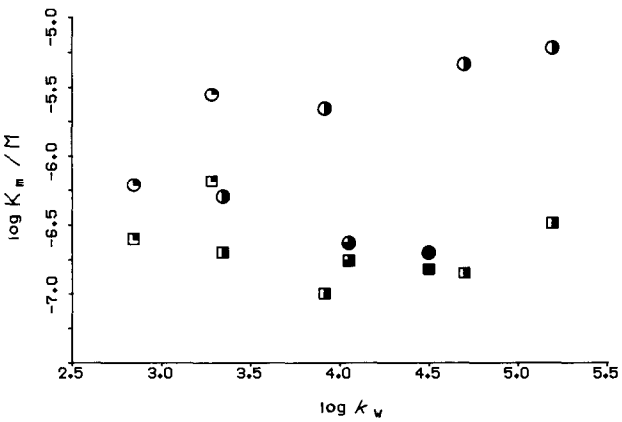



Fig. 5. 7-O-Dealkylation activity of 7-alkoxycoumarins *versus* their lipophilic parameter log $k_w$ (eqn. 6). $K_m$ is the Michaelis-Menten constant, and the number of filled quarters represents the number of carbon atoms of the alkoxy chain; the circles and squares correspond to different microsomal preparations.

between the membrane and the aqueous phase is linearly related to the length of the alkoxy or alkyl chain. But the affinity of these molecules for the metabolizing enzyme cytochrome P-450, expressed by the $K_m$ values, does not vary in a consistent manner.

So, the partition of the coumarin derivatives between the aqueous phase and the biological membrane is not a primary factor governing the kinetics of these compounds. Other parameters, such as the mobility of the substrates in the membrane and enzyme active-site limitations, must be considered.

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