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Comparison of passive drug transport through Caco-2 cells and artificial membranes

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Abstract

Impregnated artificial membranes have been used to study the mechanism of passive diffusion by trans- and para-epithelial transport routes of a structurally diverse set of compounds through Caco-2 cells. The selected compounds of which Caco-2 permeability data have been reported in the literature differed in their physicochemical properties, such as 1-octanol/water distribution coefficient, charge and molecular weight. Experimentally observed Caco-2 permeability-lipophilicity relationships are rationalized as a function of permeation pathway and the above-mentioned physicochemical properties. Impregnated artificial membranes appear to mimic the passive diffusion through in vitro monolayer cell cultures. © 1997 Elsevier Science B.V.

Keywords: Caco-2 cell culture model; Diffusion cell; Artificial membranes; Passive diffusion; Permeability

1. Introduction

Cell culture models are of current interest for the prediction of oral drug absorption (Gan et al., 1994; Conradi et al., 1996). In particular, the human intestinal epithelial cell line Caco-2 has been recommended for such biological in vitro studies, since these cells express various biological membrane properties, including enzymatic and transporter systems (Arturrson, 1991; Hillgren et al., 1995). It is assumed that the great majority of drugs cross biological barriers by a passive diffusion mechanism. Some compounds, including di- and tripeptides and related peptidomimetics, may use active transport systems. Permeation may also be hindered by efflux systems involving P-glycoproteins (Hunter et al., 1993; Schinkel et al., 1995). There are two possible pathways for permeation, the transcellular and paracellular route. The paracellular route refers to the tight-junctional pathway between cells, which can be con-

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sidered as pores filled with water (Tanaka et al., 1995). Negative charges at the membrane components near the paracellular route are postulated to contribute to an ion-selective permeation (Rubas et al., 1994; Lennernäs, 1995; Conradi et al., 1996).

As recently reviewed, passive permeation through a biological membrane depends to a large extent on three interdependent physicochemical properties, namely lipophilicity (partition and distribution coefficient), polarity (charge, hydrogen bonding) and molecular size (Camenisch et al., 1996). It has also been demonstrated that lipophilicity ($\log P$, $\log D$) involves molecular size and a factor related mainly, but not uniquely, to the hydrogen bonding capacity of the solute (Testa and Seiler, 1981; El Tayar et al., 1992). The diffusion coefficient $\bar{D}$ of a solute within a biological membrane is a function of solute size (Lieb and Stein, 1971; Walter and Gutknecht, 1986; Xiang and Anderson, 1994), while in water this dependence is relatively minor.

The paracellular diffusion through biological membranes and monolayers such as Caco-2 cells is, because of the restricted dimensions of the tight junctions, also dependent on molecular size (Leahy et al., 1989). Generally, the dimension of the tight junctions has been estimated to be in the range 3–10 Å (Lennernäs, 1995) and may vary with disease state (Noach, 1994). For intestinal mucosa, a contribution to permeability via pore diffusion of small hydrophilic compounds up to a maximal molecular weight of about 200 was proposed (Lennernäs, 1995). The molecular weight (MW) is often used as a simple molecular size descriptor (Leahy et al., 1989; Camenisch et al., 1996). However, molar volume or surface area may be good alternatives. In summary, three different size effects influence membrane transport of a drug, namely diffusion, sieving and distribution effects.

The most general model for the relationship between permeability through a membrane and the lipophilicity of a series of compounds is a sigmoidal function (Ho et al., 1977, 1983; De Haan and Jansen, 1985; Leahy et al., 1989). The plateau at high lipophilicity values is believed to be due to hindered diffusion through stagnant aqueous diffusion layers in front of the membrane (Stehle and Higuchi, 1972). In contrast, the frequently observed plateau at low lipophilicity values is believed to be due to the uptake of small hydrophilic compounds via the aqueous pore pathway (Ho et al., 1977, 1983). This additional route through the pores is considered to be relatively inefficient compared with the transcellular pathway, because the intercellular space has been estimated to be very small compared with the entire surface area (ca. 0.01% for Caco-2 cells) (Arturrson, 1991; Lennernäs, 1995). Generally, outliers from such a general sigmoidal relationship have been explained in terms of molecular size, charge or solubility (Yalkowsky et al., 1974; Leahy et al., 1989; Camenisch et al., 1996). However, the recent discovery of active transport mechanisms and P-glycoprotein efflux systems further complicate the rationalization of experimental permeability data (Fenstermacher et al., 1981; Karlsson et al., 1993).

For the simulation of permeation processes different non-biological in vitro systems have been proposed. One approach uses a diffusion cell consisting of impregnated artificial membranes streched between two chambers filled with aqueous medium (De Meere and Tomlinson, 1984; Tsai et al., 1992). The thickness of the stagnant aqueous diffusion layers on each side of the artificial membranes is influenced by mechanical mixing of the chambers (Levich, 1962; Hayton, 1980). An alternative with well-defined hydrodynamics is a rotating diffusion cell (Albery et al., 1976). To mimic biological membranes different organic fluids have been chosen such as the amphiphilic 1-octanol or isopropylmyristate. Tetrafluorethylene or dimethylpolysiloxane are often used as artificial membranes, since they have been shown to maintain the integrity in the presence of buffers and the above-mentioned fluids cannot be rinsed by aqueous phases (Amidon et al., 1982; De Meere and Tomlinson, 1984).

In the present paper the transport of a heterogeneous set of compounds (differing in lipophilicity, molecular size and degree of ionisation) through a biological membrane (Caco-2 cells) and different impregnated artificial membranes (hydrophilic mixed cellulose ester) is compared, in

order to rationalize observed sigmoidal permeability-lipophilicity relationships and potential outliers. Two organic solvents were tested as possible simulation systems for passive Caco-2 cell permeation.

2. Materials and methods

2.1. Methods

Using Caco-2 cells, permeability is measured as the sum of the distribution, diffusion and sieving effect (Fig. 1a). For a better understanding of the underlying processes it is necessary to study separately the two processes, diffusion through the pores and diffusion through the membrane, which is associated with the distribution step. Moreover, it appears reasonable to neglect initially the sieving effect. These conditions can be simulated with the help of a diffusion cell using artificial membranes with large pores (unable to hold back through sieving any compound used in this study). Transcellular diffusion can be simulated by impregnation of the membrane pores with an appropriate lipophilic solvent (Fig. 1b) while paracellular diffusion can be simulated by using the artificial membrane without primary treatment (Fig. 1c).

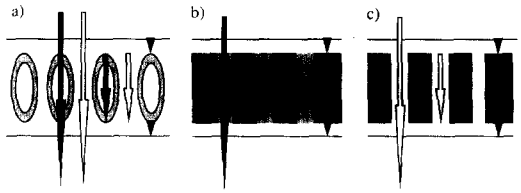

Fig. 1. Transport barriers for passive diffusion through: (a) Caco-2 cell monolayers; (b) an artificial membrane with organic impregnation; and (c) an artificial membrane without any impregnation. Permeabilities determined through non-impregnated membranes and through the tight junctions of the Caco-2 cells are denoted as $\log P_{erm}^{app,hyd}$ (large open arrow), which includes the permeability through the stagnant water layers on both sides of the membrane $\log P_{erm}^{aq}$ (▼) and the permeabilty through the water-filled pores (= pore diffusion) $\log P_{erm}^{hyd}$ (small filled arrow). In Caco-2 cells $\log P_{erm}^{hyd}$ can be influenced by the molecular size (= sieving effect). Permeabilities determined with impregnated membranes and through the lipoidal part of the Caco-2 cell monolayers are symbolized by $\log P_{erm}^{app,org}$ (large filled arrow), including all steps of distribution, the permeability through the stagnant water layers and the permeability through the lipophilic organic solvent (= membrane diffusion) $\log P_{erm}^{org}$ (small filled arrow).

2.2. Data set and chemicals

Caco-2 cell permeability data and experimental $\log D_{oct}$ values were taken from the literature (Artursson and Karlsson, 1991). The 16 compounds cover a range of molecular weight from 100 up to 400 g/mol and a relevant range of lipophilicity. At pH 7.4, some of the selected compounds are fully ionized, while others are neutral. An overview of the experimental and physicochemical data is presented in Table 1. The following compounds are considered: corticosterone (Co), testosterone (Te), propranolol (Pr), alprenolol (Al), warfarin (Wa), metoprolol (Me), felodipine (Fe), hydrocortisone (Hy), dexamethasone (De), salicylate (Sa), acetylsalicylate (Ac), practolol (Pa), terbutaline (Tb), atenolol (At), sulphasalazine (Su) and olsalazine (Ol).

1-Octanol has been proposed as a simulation system for biological membranes (Fujita et al., 1964; Smith et al., 1975) and was used for the impregnation of the artificial membranes. As an alternative organic fluid, the more polar water-saturated isopropylmyristate (IPM), usually used to simulate skin membranes, was tested (Tanaka et al., 1978; Tsai et al., 1992).

For the measurements in the diffusion cell, all compounds except for olsalazine (Pharmacia AB, Sweden), testosterone (Roche Bioscience, USA), felodipine (Astra Hässle AB, Sweden) and practolol (Zeneca Pharmaceuticals, UK) were ordered from Sigma Chemie (Switzerland). 1-Octanol, IPM and ethanol came from Merck (Germany). The permeation studies were carried out in 0.15 M Sörensen-phosphate buffer (pH 7.4).

2.3. Diffusion cell and materials

The diffusion cell used (F. Hoffmann-La Roche Ltd., Switzerland) was made of Teflon (Fig. 2). The sealing consisted of chemically and mechanically resistant Kalrez. Both compartments were stirred with a synchronized stirrer and each had a

Table 1
Physicochemical properties and absorption characteristics of the drugs

Symbol[a]	M_W (g/mol)	Log D_{oct}^{b}	Charge at pH 7.4	Log $P_{erm}^{app,Caco-2}$ (cm/s)[c]	Log $P_{erm}^{app,hyd}$ (cm/s)[d]	Log $P_{erm}^{app,org,oct}$ (cm/s)[d]	Log $P_{erm}^{app,org,IPM}$ (cm/s)[d]
Sa	138	−2.14	−	−4.924	−4.010	−5.840	−6.735
Ac	180	−2.57	−	−5.620	−4.033	−6.421	−6.882
Tb	225	−1.40	+	−6.420	−4.069	−5.007	−6.539
Al	249	1.00	+	−4.393	−4.180	−4.297	−4.334
Pr	259	1.54	+	−4.378	−4.125	−4.261	−4.253
Pa	266	−1.40	+	−6.046	−4.042	−4.907	−6.430
At	266	−2.14	+	−6.700	−4.039	−5.866	−6.793
Me	267	0.07	+	−4.569	−4.158	−4.442	−5.484
Te	288	3.31	0	−4.286	−4.204	−4.178	−4.187
Ol	302	−4.50	− −	−6.959	−4.040	−6.989	−6.997
Wa	308	0.12	−	−4.417	−4.172	−4.368	−4.697
Co	346	1.89	0	−4.263	−4.207	−4.185	−4.193
Hy	362	1.53	0	−4.668	−4.159	−4.645	−4.633
Fe	384	3.48	0	−4.644	−4.160	−4.638	−4.727
De	392	1.74	0	−4.903	−4.179	−4.832	−4.997
Su	398	−0.13	−	−6.886	−4.159	−6.699	−6.956

[a] Compound names see Section 2.2.
[b] Experimental 1-octanol/water distribution coefficient at pH 7.4 according to (Artursson and Karlsson, 1991).
[c] Permeability coefficient through Caco-2 cells according to (Artursson and Karlsson, 1991).
[d] Experimental permeability coefficients through artificial membrane. The standard deviations were always less than 5%.

volume of 70 ml. The surface area between the two compartments of the diffusion cell was 2.85 cm^2.

The artificial membranes (Millipore, Switzerland, Cat.No. GVHP 00010) were composed of hydrophilic mixed cellulose ester (nitrate + acetate) with a pore size of 0.22 μm, a filter thickness of 150 μm and a porosity of 75%. They have a partial negative character.

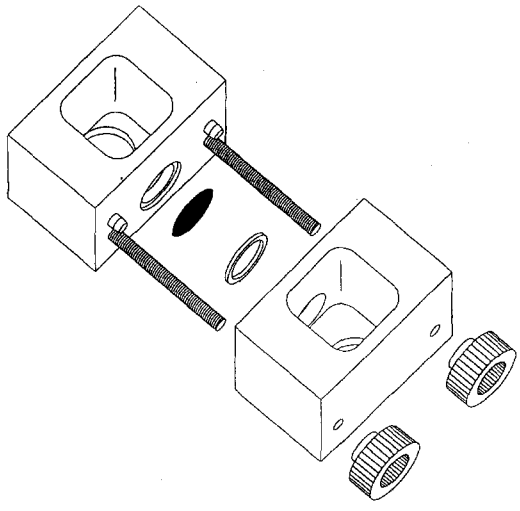

Fig. 2. Diffusion cell used to study transport through artificial membranes.

2.4. Permeability measurements

Before performing permeability studies through the diffusion cell, the polymeric membranes were impregnated with buffer, IPM or 1-octanol for 1 h. These prepared membranes were then rinsed with distilled water and subsequently streched in the diffusion cell. The amount of compound used for the permeability coefficients determination was limited by the solubility in the buffer and the sensitivity of detection by the photometer. Both the donor and acceptor compartment could accommodate a volume of 40 ml buffer. Preliminary studies demonstrated that presaturation with organic solvent is not required. The permeation through the membrane was determined at room temperature and a stirring speed of 150 revs./min over a maximum period of 12 h (Kamagami et al.,

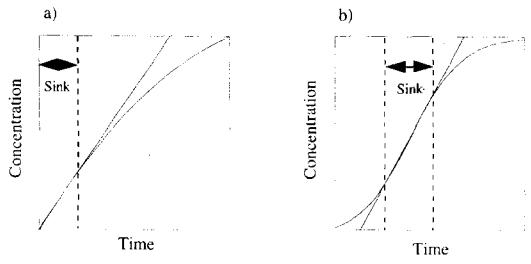

Fig. 3. Determination of the linear range of diffusion defining sink conditions: (a) for non-impregnated artificial membranes; (b) for impregnated artificial membranes, where the increase in drug concentration in the acceptor compartment is observed after a certain lag-time (Flynn, 1990).

1991). Aliquots of 0.5 ml were taken from the acceptor compartment at appropriate intervals and their concentrations were determined photometrically (Uvikon 860, Kontron Instruments). The volumes removed from the acceptor compartment were replaced with buffer. The permeability was determined in each case as the average of three measurements (S.D. < 5%). The experimental data are presented in Table 1.

2.5. Calculations

Transport through a membrane can be described by the apparent permeability coefficient P_{erm}^{app} (cm/s). From linear kinetics, P_{erm}^{app} can be determined by (Hilgers et al., 1990; Camenisch et al., 1996):

$$P_{erm}^{app} = \frac{V_A}{A \cdot (C_D - C_A)} \cdot \frac{dC_A}{dt} \quad (1)$$

where $(dC_A)/dt$ is the increase of drug concentration in the acceptor chamber over the time-period considered ($mg \cdot s^{-1} \cdot ml^{-1}$), A the membrane surface (cm^2), V_A the solvent volume in the acceptor chamber (ml), C_A the initial solute concentration in the acceptor chamber (mg/ml) and C_D the initial concentration of solute in the donor chamber (mg/ml). The slope is determined over the linear diffusion range. This is the range where sink conditions more or less apply so that back-diffusion can be neglected (Fig. 3a and b). A lag-time is apparent for lipophilic compounds, partly due to an adsorption to the membrane. Assuming sink conditions in the acceptor chamber, comparable with the removal of drug by blood flow, C_A is zero. Whereas diffusion through the non-impregnated membranes as well as diffusion through the impregnated membranes is possible, the compounds can cross the artificial membrane only through the pores (Fig. 1b and c). The effective surface A, corrected for a membrane porosity of 75%, is 2.14 cm^2.

For a biological membrane, having an aqueous (*hyd*) and lipoidal (*org*) pathway in parallel, P_{erm}^{app} is made up as follows (Flynn et al., 1974; De Haan and Jansen, 1985):

$$P_{erm}^{app} = P_{erm}^{app,org} \cdot f^{org} + P_{erm}^{app,hyd} \cdot f^{hyd} \quad (2)$$

where f^{org} and f^{hyd} are the fractional areas of the parallel hydrophilic and lipophilic diffusional routes, respectively.

3. Results

The plot of the measured Caco-2 cell permeability data $\log P_{erm}^{app,Caco-2}$ (symbol ○) versus experimental $\log D_{oct}$ values and an empirical sigmoidal curve is shown in Fig. 4. Six compounds diverge from this curve, the two smallest compounds in this data set (Ac and Sa) lie above

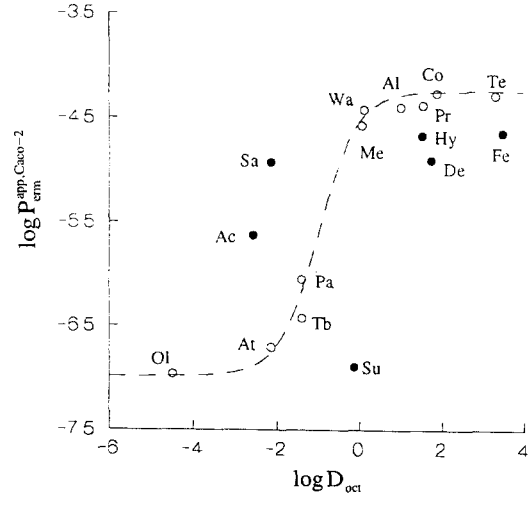

Fig. 4. Experimental permeability-lipophilicity relationship for Caco-2 cell permeation fitted to an empirical sigmoid function.

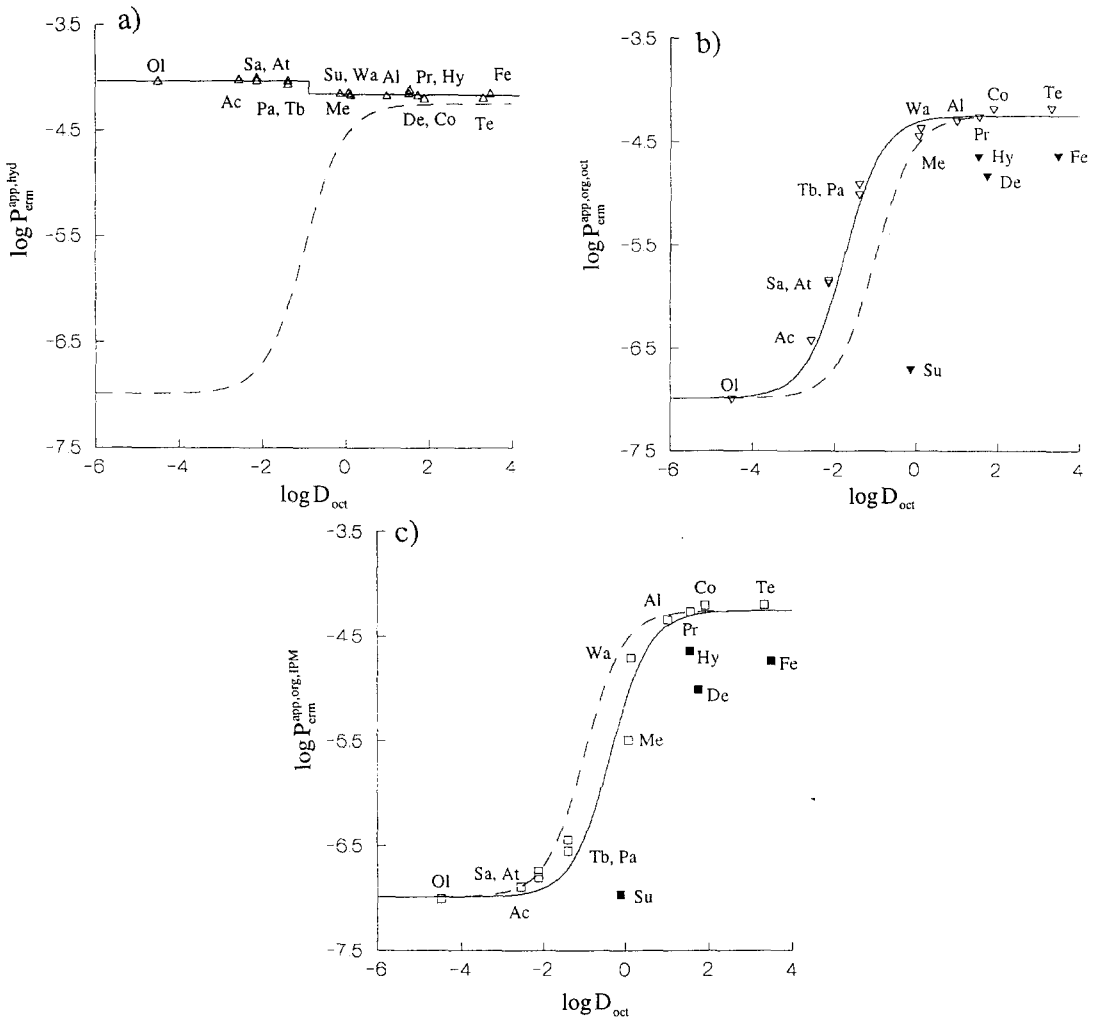

Fig. 5. Superpositions of the sigmoid dependence for Caco-2 cell permeability (- - -) and log D_{oct} with, (a) permeabilities through non-impregnated artificial membranes (△,——); (b) permeabilities through 1-octanol impregnated artificial membranes (▽,——); (c) permeabilities through IPM impregnated membranes (□,——).

the sigmoid curve and the four largest ones (Su, Hy, De and Fe) lie below it.

The permeability measurements on the same compounds through the non-impregnated (log $P_{erm}^{app,hyd}$), 1-octanol-impregnated (log $P_{erm}^{app,org,oct}$) and IPM-impregnated (log $P_{erm}^{app,org,IPM}$) polymeric artificial membranes, respectively, are shown in Fig. 5a–c, always compared with the sigmoidal reference permeability curve (Fig. 4) measured for the main group of compounds using the Caco-2 cells. For permeation through the non-impregnated membranes (symbol △) (Fig. 5a), simulating the *paracellular* pathway through the tight junctions without a sieving effect, it is evident that the permeability coefficients of all lipophilic compounds (approximately log $D_{oct} > -1$) are slightly shifted to lower values. For permeation through the 1-octanol-impregnated membranes (symbol ▽) (Fig. 5b), simulating the transcellular pathway, a sigmoidal relationship with four outliers Su, Hy, De and Fe, was obtained. Remarkably, the same outliers appear below the curve as already seen with the Caco-2 permeability. Their measured permeability

values are more or less identical in both systems (see Table 1). No outliers appear above the curve, rather the Caco-2 outliers Ac and Sa now fall on the curve. Superposition of these on the Caco-2 cell data shows that the two sigmoidal curves are not identical. Using a 1-octanol-impregnated membrane the rise in permeability occurs earlier (for less hydrophobic log D_{oct} values) in comparison with the Caco-2 cell permeabilities. Nevertheless, both curves tend towards the same limiting values. For permeation through the IPM-impregnated membranes (symbol □) (Fig. 5c), again for most of the compounds a sigmoidal dependence was apparent, but shifted to the right (higher lipophilicities) compared with the Caco-2 and 1-octanol data. Concerning the outliers, the same remarks can be made as for the 1-octanol-impregnated system (Ac and Sa fall on the curve, while Su, Hy, De and Fe appear as outliers below the curve with almost the same permeability values as in the Caco-2 cells). Again both curves tend towards the same limits.

4. Discussion

The sigmoidal permeability-lipophilicity relationships for the three permeation systems used in this study cannot be superimposed exactly (Fig. 5b and c). The two artificial systems (water/1-octanol/water, water/IPM/water) are qualitative but not quantitative models of passive diffusion through Caco-2 cells. Over the lipophilicity range $-3 < \log D_{oct} < 1$, for the water/1-octanol/water system the permeability is overestimated and for the water/IPM/water system the permeability is underestimated compared with Caco-2 permeation data. Similar shifts in transport-lipophilicity relationships for various organic solvents have also been observed in simple two-phase distribution systems (De Haan et al., 1983; Van de Waterbeemd, 1983).

The diffusion coefficient in water is only weakly dependent of molecular size (Walter and Gutknecht, 1986). The present experimental data for the non-impregnated artificial membrane (Fig. 5a) confirm this unrestricted permeation. In addition, no charge-dependence of permeation is observed, although the artificial membranes have a partial negative character. Lipophilicity seems to have an influence on diffusion through the aqueous pores, as permeability decreases slightly for compounds with $\log D_{oct} > -1$. Adsorption mechanisms at the artificial membrane surface of the more hydrophobic compounds may be one explanation for this observation. Also, stagnant aqueous diffusion layers may be responsible for this observation (Ho et al., 1977; Ho et al., 1983), although the effect seems relatively small.

Fig. 5b and c demonstrate that the observed sigmoidal relationships of permeability and log D_{oct}, for these structurally diverse compounds, is systematic. We assume that this relationship is a function of the diffusion through the lipoidal parts of the membrane, stagnant aqueous layers and the distribution step on both sides of the membrane. Only a low flux ($\log P_{erm}^{app} \cong -7$) is observed for highly hydrophilic compounds over 12 h.

The outliers below the sigmoidal curve can be rationalized by the diffusion effect through the membrane, since they show restricted membrane diffusion related to their molecular size (Fig. 5a–c). In all systems considered, a diffusion-limiting effect only becomes effective once a certain molecular size is attained. This molecular size is more or less similar for all the systems ($M_W \approx 350$). This corroborates the finding that 1-octanol and IPM are useful model systems for biological membranes. More or less restricted holes in the alkyl chain region of the Caco-2 cell membrane or in the water-saturated organic bulk phases, acting as a kind of sieve, may be a possible explanation for this observation (Lippold and Adel, 1972; Walter and Gutknecht, 1986). Therefore, all compounds lying on the sigmoidal curve show no restriction in their transcellular diffusion. Thus, based on Eq. 2, using this sigmoidal curve for permeability through Caco-2 cells and the permeability data measured through the non-impregnated membrane, the maximum contribution of the paracellular pathway to the Caco-2 permeability can be estimated. This procedure corresponds to a summation of the relationships presented in Fig. 5a, taking into consideration the fractional contribution of the different diffusion pathways. Fig. 6

shows the resulting relationships for different amounts of hydrophilic fractional contributions. For an aqueous pore fraction of 0.01%, which has been estimated for Caco-2 cells (Arturrson, 1991; Lennernäs, 1995), the contribution over the whole lipophilicity range is negligible. With increasing pore fraction (f^{hyd} values), which may occur under certain conditions, the paracellular pathway becomes more important, but only for hydrophilic compounds ($\log D_{oct} < -1$), making a small contribution to transcellular diffusion. The higher than expected permeability values of Ac and Sa, observed in Caco-2 cell studies (see Fig. 4), may thus be partly due to increased pore diffusion. Most probably active transport processes are involved. Indeed, small organic acids are generally suggested to be absorbed by an active transport system (Tamai et al., 1995) and particularly for Sa a carrier-mediated pH-dependent anion exchange mechanism has been described (Takanaga et al., 1994).

The present study demonstrates that passive diffusion through Caco-2 cells, a model for intestinal absorption, can be simulated using impregnated artificial membranes. In predicting absorption through Caco-2 cells a shift in the qualitatively similar permeability-lipophilicity relationships must be taken into consideration and proper calibration is required. With respect to diffusion and partitioning, Caco-2 cells appear to fit somewhere between 1-octanol and isopropylmyristate. The relationship between permeability and lipophilicity is sigmoidal. However, this only becomes apparent when sufficiently hydrophilic ($\log D_{oct} < -2$) compounds are included in the study. Otherwise, the relationship may be approximated by a hyperbolic function (Leahy et al., 1989: Camenisch et al., 1996). The use of such simple simulation models for absorption estimation may be of importance for high-throughput screening of new compounds in the pharmaceutical industry. In particular, in combinatorial chemistry projects, where large numbers of compounds have to be evaluated, this is not feasible on a large scale using in vitro cell cultures. However, the latter are very useful to study mechanistic details, e.g. active transport, efflux systems or paracellular diffusion and metabolic activity during absorption.

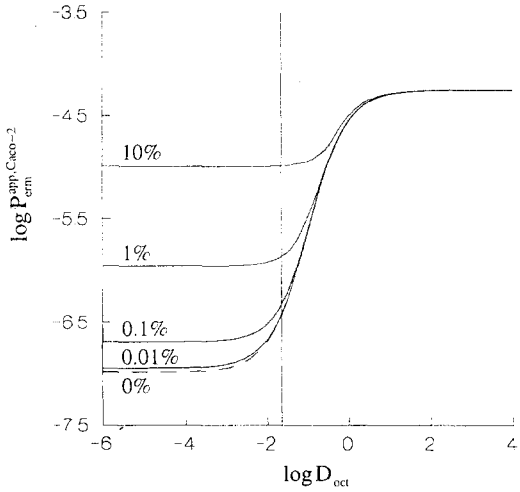

Fig. 6. Theoretical contribution of the paracellular diffusion pathway to non-restricted transcellular Caco-2 cell permeation based on Eq. (2). The curves are given for 0 (- - -), 0.01, 0.1, 1 and 10% (——) hydrophilic (aqueous pores) fractional contributions. The vertical line at $\log D_{oct} = -2$ denotes the approximate lipophilicity value above which hyperbolic, instead of sigmoidal, relationships can be observed.

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