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CORRECTION OF THE APPARENT MICHAELIS CONSTANT, BIASED BY AN UNSTIRRED LAYER, IF A PASSIVE TRANSPORT COMPONENT IS PRESENT

D. WINNE

Abteilung für Molekularpharmakologie, Pharmakologisches Institut der Universität Tübingen, Wilhelmstrasse 56, D-7400 Tübingen (G.F.R.)

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Summary

An unstirred layer shifts the permeation curve due to a carrier-mediated transport system (non-passive component) to the right, so that a higher K_m value is determined (apparent Michaelis constant biased by an unstirred layer). If a substance is transported simultaneously by a non-passive and a passive mechanism, and if the non-passive component can be inhibited, the permeation curve due to the non-passive component can be obtained by subtraction of the curve due to the passive component alone from the curve obtained with intact passive and non-passive component. But in the presence of an unstirred layer the difference curve lies always below the curve which would be obtained in the presence of the non-passive component alone. This error increases with increasing unstirred layer thickness and increasing magnitude of the passive transport component. By means of an appropriate equation the apparent Michaelis constant biased by an unstirred layer and determined from the difference curve can be corrected, if the unstirred layer thickness and area are known.

Introduction

It has been shown, theoretically [1—8] and experimentally [3,9—12], that an unstirred layer introduces a bias into the determination of the apparent Michaelis constant of a carrier-mediated transport process. The permeation curves (permeation rate versus bulk phase concentration) are shifted to the right, so that the concentration, corresponding to the half-maximal value, is higher than the apparent Michaelis constant. The use of the double reciprocal plot causes an additional error [5]. The biased Michaelis constant can be corrected by an appropriate equation [1,5], if the unstirred layer thickness and area are known. Sometimes a substance is transported simultaneously by a non-passive and a passive mechanism, so that the analysis of experimental data is more complicated [13,14]. If the non-passive component can be inhibited, it is possible to determine the permeation curve of the passive component separately. Subsequently, the curve for the non-passive component can be obtained by subtraction. In the absence of an unstirred layer the transport rates due to the passive and non-passive component are additive. But, as shown below, this is not true in the presence of an unstirred layer. The difference between the permeation rate, measured with intact passive and non-passive component, and the rate, measured after inhibition of the non-passive component, is smaller than the permeation rate which would be obtained by the non-passive component alone. Therefore, the equation for correcting the unstirred layer biased Michaelis constant published earlier [1,5] cannot be applied to data obtained by subtraction as described above. An appropriate correction equation is derived in the present paper.

Theory

At zero water net flux the permeation rate of a substance through the unstirred layer of a plane membrane can be described by the following equation:

$$\dot{n}_{\mathrm{UL}} = A_{\mathrm{UL}} \frac{D}{\delta} (C_{\mathrm{b}} - C_{\mathrm{S}}) \tag{1}$$

$\dot{n}_{\mathrm{UL}}$, permeation rate through the unstirred layer (mol/min); A_{UL}, area of the unstirred layer (cm^2); D, diffusion constant of the substance in the unstirred layer (cm^2/s); δ, effective thickness of the unstirred layer (cm); C_{b}, concentration of the substance in the well-mixed bulk phase (M); C_{S}, concentration at the surface of the plane membrane (M). The boundary between bulk phase and unstirred layer is not sharp. Therefore, δ is used as an operational quantity [15] and represents an "effective" thickness. The permeation through the membrane by the carrier-mediated transport system (= non-passive component) is described here by the simplest equation:

$$\dot{n}_{\mathrm{np}} = A_{\mathrm{M}} \frac{VC_{\mathrm{S}}}{K_{\mathrm{m}} + C_{\mathrm{S}}} \tag{2}$$

$\dot{n}_{\mathrm{np}}$, permeation rate through the membrane by the non-passive component (mol/min); A_{M}, membrane surface (cm^2); V, apparent maximal transport rate of the non-passive component per unit membrane area (mol $\cdot$ min^{-1} $\cdot$ cm^{-2}); K_{m}, apparent Michaelis constant (M). The concentration of the substance on the opposite side of the membrane is assumed to be low, so that the flux in the reversed direction can be neglected. The permeation through the membrane by the passive component is described by the following equation:

$$\dot{n}_{\mathrm{p}} = A_{\mathrm{M}} P C_{\mathrm{S}} \tag{3}$$

$\dot{n}_{\mathrm{p}}$, permeation rate through the membrane by the passive component (mol/min); P, permeability coefficient of the passive transport component (ml $\cdot$ min^{-1} $\cdot$ cm^{-2}). In the case of a plane membrane the unstirred layer area is equal to the membrane area ($A_{\mathrm{UL}} = A_{\mathrm{M}}$). In the intestine we have a villous membrane, so that the membrane area is larger than the unstirred layer area ($A_{\mathrm{M}} > A_{\mathrm{UL}}$). The following derivations are valid only for plane membranes. The application of the derived correction equation to villous membranes has to be regarded as approximation.

In the steady state the permeation rate through the unstirred layer is equal to the permeation rate through the membrane. The latter rate is the sum of the passive and non-passive transport rate:

$$\dot{n}_{\mathrm{UL}} = \dot{n}_{\mathrm{p}} + \dot{n}_{\mathrm{np}} \tag{4}$$

The unknown concentration of the substance at the membrane surface C_{S} is lower than the bulk phase concentration. After introducing Eqns. 1, 2, and 3 into Eqn. 4 we get for C_{S} the following equation of second order:

$$C_{\mathrm{S}}^2 + C_{\mathrm{S}}q\left[\frac{K_{\mathrm{m}}}{q} + \frac{V\delta}{\rho D} - C_{\mathrm{b}}\right] - qK_{\mathrm{m}}C_{\mathrm{b}} = 0 \tag{5}$$

with

$$\rho = A_{\mathrm{UL}}/A_{\mathrm{M}} \tag{6}$$

and

$$q = \frac{\rho D/\delta}{P + \rho D/\delta} = \frac{1}{1 + \dfrac{P\delta}{\rho D}} \tag{7}$$

In practice the permeation rate is standardized to wet tissue weight, dry weight, serosal length, serosal area, or other easily measurable quantities. Here, we choose the wet tissue weight as standardizing quantity and define a practical permeation rate:

$$\phi = \dot{n}/W \tag{8}$$

ϕ, permeation rate standardized to wet tissue weight ($\mathrm{mol \cdot min^{-1} \cdot g^{-1}}$); W, wet tissue weight (g). Introducing the solution of Eqn. 5 into Eqn. 1 and using Eqn. 8 we obtain the permeation rate of a substance through a membrane with a non-passive and passive transport component in the presence of an unstirred layer:

$$\phi_{\mathrm{T}} = \frac{A_{\mathrm{UL}}}{W}\frac{D}{\delta}\left\{C_{\mathrm{b}} + \tfrac{1}{2}q\left[\frac{K_{\mathrm{m}}}{q} + \frac{V\delta}{\rho D} - C_{\mathrm{b}}\right] - \left[\tfrac{1}{4}q^2\left(\frac{K_{\mathrm{m}}}{q} + \frac{V\delta}{\rho D} - C_{\mathrm{b}}\right)^2 + qK_{\mathrm{m}}C_{\mathrm{b}}\right]^{1/2}\right\} \tag{9}$$

In Eqn. 9 only the sign of the square root is given which results in positive concentrations. The permeation rate ϕ_{np} due to the non-passive component alone ($P = 0$) can be obtained from Eqn. 9 by setting $q = 1$. The permeation rate ϕ_{p} due to the passive component alone ($V = 0$) is given by:

$$\phi_{\mathrm{p}} = \frac{A_{\mathrm{M}}}{W}qPC_{\mathrm{b}} = \frac{A_{\mathrm{UL}}}{W}q\frac{P}{\rho}C_{\mathrm{b}} \tag{10}$$

For two special cases simplier equations can be derived:

First case. $C_{\mathrm{S}} << K_{\mathrm{m}}$, that is the region where the permeation by the non-passive component depends nearly linearly on the concentration at the membrane surface. With $K_m + C_S \approx K_m$ we obtain from Eqns. 1—4:

$$\phi_T = \frac{A_M}{W} \frac{C_b}{\dfrac{\delta}{\rho D} + \dfrac{1}{V/K_m + P}} \tag{11}$$

and in the presence of the non-passive component alone:

$$\phi_{np} = \frac{A_M}{W} \frac{C_b}{\dfrac{\delta}{\rho D} + \dfrac{K_m}{V}} \tag{12}$$

The permeation rate due to the passive component alone is given by Eqn. 10.

Second case. $C_S >> K_m$, that is the region of saturation of the non-passive component. With $K_m + C_S \approx C_S$ we obtain from Eqns. 1—4:

$$\phi_T = \frac{A_M}{W} q(PC_b + V) \tag{13}$$

and in the presence of the non-passive component alone:

$$\phi_{np} = \frac{A_M}{W} V \tag{14}$$

The permeation rate due to the passive component alone is given by Eqn. 10.

If the permeation rate ϕ_p, measured after inhibition of the non-passive component (Eqn. 10), is subtracted from the rate, measured with intact passive and non-passive component (Eqn. 9), we obtain the difference:

$$\Delta\phi = \phi_T - \phi_p = \frac{A_{UL}}{W} \frac{D}{\delta} \left\{ \tfrac{1}{2} q C_b + \tfrac{1}{2} K_m + \tfrac{1}{2} q \frac{V\delta}{\rho D} - \left[\tfrac{1}{4} q^2 \left(\frac{K_m}{q} + \frac{V\delta}{\rho D} - C_b \right)^2 + q K_m C_b \right]^{1/2} \right\} \tag{15}$$

With increasing bulk phase concentration the difference approaches asymptotically $\Delta\phi_m$ (see Eqns. 13 and 10):

$$\Delta\phi_m = \frac{A_M}{W} q(C_b P + V) - \frac{A_M}{W} P q C_b = \frac{A_M}{W} q V = \frac{A_{UL}}{W} \frac{q}{\rho} V \tag{16}$$

By setting in Eqn. 15 $\Delta\phi = 0.5\ \phi_m$ the relationship between K_m, the apparent Michaelis constant of the non-passive component, and C_b^+, the bulk phase concentration for achieving the half-maximal value of the difference curve (= apparent Michaelis constant biased by an unstirred layer), is obtained:

$$K_m = q\left(C_b^+ - \frac{1}{2} \frac{V\delta}{\rho D} \right) \tag{17}$$

For practical use Eqn. 17 can be modified:

$$K_m = \left[1 - \frac{bW\delta}{A_{UL} D} \right] C_b^+ - \frac{1}{2} \frac{\Delta\phi_m W\delta}{A_{UL} D} \tag{18}$$

with

$$b = \frac{d\phi_p}{dC_b} = \frac{A_M}{W} Pq \tag{19}$$

To determine the unbiased apparent Michaelis constant of a carrier-mediated transport system in the presence of a passive transport component and an unstirred layer the following quantities are needed: $\Delta\phi_m$, the maximal value of the difference curve; C_b^+, the bulk phase concentration for achieving the half-maximal value of the difference curve (= apparent Michaelis constant biased by an unstirred layer); b, the regression coefficient (steepness) of the permeation curve obtained after inhibition of the non-passive component; A_{UL}/W, the unstirred layer area standardized to wet tissue weight or other relevant quantities (the permeation rate has to be standardized to the same quantity); D, the diffusion constant; δ, the effective unstirred layer thickness. Since in the presence of an unstirred layer the double reciprocal plot of the permeation data does not yield a straight line, an additional error is introduced by use of this transformation [5], so that $\Delta\phi_m$ has to be determined from the non-transformed difference curve. C_b^+ is obtained by interpolation using only points near the half-maximal value of the difference curve. For this procedure the double reciprocal plot can be applied, since in this small region the transformed curve can be approximated by a straight line.

If only a carrier-mediated transport system is present, Eqn. 18 simplifies to:

$$K_m = C_b^+ - \frac{1}{2} \frac{\phi_m W \delta}{A_{UL} D} \tag{20}$$

where ϕ_m is the asymptotic maximal permeation rate. Eqn. 20 corresponds to Eqn. 9 of Winne [5]. The correct maximal transport rate of the non-passive component can be obtained by:

$$\phi_{npm} = \frac{\Delta\phi_m}{q} = \frac{\Delta\phi_m}{1 - \dfrac{bW\delta}{A_{UL}D}} \tag{21}$$

Results

Fig. 1 shows graphically the influence of an unstirred layer on the apparent kinetics of a carrier-mediated transport system (non-passive component) in the presence of a passive transport component. Curve 1 represents the linearly increasing permeation rate due to the passive component alone (non-passive component inhibited) in the absence of an unstirred layer. Curve 2 describes the permeation rate due to the non-passive component alone (passive component and unstirred layer absent). If a passive and a non-passive transport component are present (unstirred layer absent), the non-linear curve 3 results. At high concentrations this curve approaches asymptotically an ascending straight line. As proposed by Curran [13], the fraction of the permeation rate due to the passive component can be obtained from the steepness of the asymptotic straight line (equal to b in Eqn. 19). Curves 4, 5, and 6 are the analogous permeation rate curves in the presence of an unstirred layer. Since the unstirred

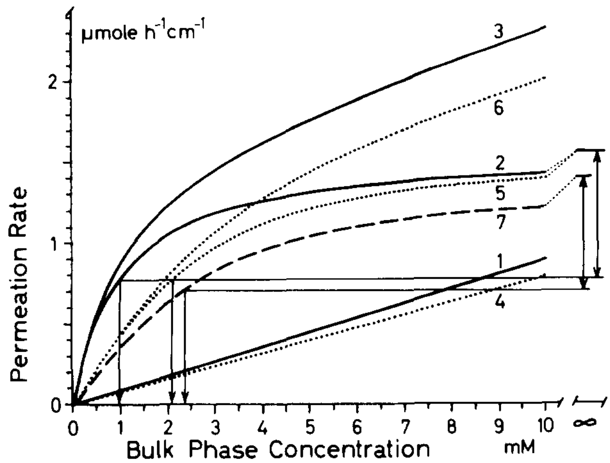

Fig. 1. Influence of an unstirred layer on the apparent kinetics of a carrier-mediated transport system in the presence of a passive transport component. Based on data of Rider et al. [21] as presented by Curran [13] (glucose absorption in rat ileum in vivo) and Winne (unpublished) (unstirred layer thickness and area in rat small intestine in vivo). The permeation rate (ordinate) is standardized to gut length (W = gut length). $V = 1.12\ \mu\text{mol} \cdot \text{h}^{-1} \cdot \text{cm}^{-2}$; $K_m = 1$ mM; $A_{UL}/W = 1.41\ \text{cm}^2/\text{cm}$; $P = 0.064$ cm/h; $D/\delta = 0.49$ cm/h ($D = 0.68 \cdot 10^{-5}\ \text{cm}^2/\text{s}$, $\delta = 500\ \mu\text{m}$). The curves represent the absorption rates under following conditions: curves 1 and 4, passive component alone; curves 2 and 5, carrier-mediated transport component (non-passive component) alone; curves 3 and 6, non-passive and passive component together; curves 1, 2, and 3, unstirred layer absent; curves 4, 5, and 6, unstirred layer present; curve 7, curve 4 subtracted from curve 6 = difference curve.

layer represents an additional resistance, these curves are lying below the corresponding curves obtained in the absence of an unstirred layer. The point of the half-maximal value is shifted to the right (compare curves 2 and 5). Only curve 5 approaches the same asymptotic value as curve 2 does. At high bulk phase concentrations the concentration at the membrane surface, though lower than the bulk phase concentration, is high enough to saturate the non-passive transport component and the permeation rate reaches its maximal value, provided only the non-passive component is present. Curve 7, the difference curve (curve 6 minus curve 4), runs always below curve 5 indicating that in the presence of an unstirred layer the permeation rate, obtained with intact passive and non-passive transport component, is smaller than the sum of the rates which would be measured in the presence of the transport components alone. The reason for this is that in the presence of an unstirred layer the transport rate through the passive pathway is diminished, if a non-passive transport component is present. In this case the concentration at the membrane surface is lower (Table I), since it is the result of the substance flux through the unstirred layer and through the passive and non-passive pathway in the membrane. The presence of a non-passive transport component increases the total flux through the membrane and reduces the concentration at the membrane surface and thereby the flux through the passive pathway. The point of the half-maximal value of the difference curve is located at a higher bulk phase concentration (compare curves 5 and 7) demonstrating the additional error inherent in the difference curve.

From Eqns. 7 and 17 it follows that in the absence of an unstirred layer (δ =

TABLE I

CONCENTRATION AT THE MEMBRANE SURFACE AND FLUXES THROUGH THE PASSIVE AND NON-PASSIVE PATHWAY OF THE MEMBRANE IN THE PRESENCE OF AN UNSTIRRED LAYER FOR THE SPECIAL CASES: $C_S \ll K_m$ AND $C_S \gg K_m$

Case	Presence of		Surface concentration C_S	Flux through passive pathway	Flux through non-passive pathway
	Non-passive component	Passive component			
$C_S \ll K_m$	+	+	$\dfrac{C_b}{1+\dfrac{P\delta}{\rho D}+\dfrac{V\delta}{K_m\rho D}}$	$\dfrac{A_M}{W}\,\dfrac{C_bV/K_m}{1+\dfrac{V\delta}{K_m\rho D}}\cdot\dfrac{1}{1+\dfrac{P}{\rho D/\delta+V/K_m}}$	$\dfrac{A_M}{W}PqC_b\cdot\dfrac{1}{1+\dfrac{V/K_m}{P+\rho D/\delta}}$
	+		$\dfrac{C_b}{1+\dfrac{V\delta}{K_m\rho D}}$	$\dfrac{A_M}{W}\,\dfrac{C_bV/K_m}{1+\dfrac{V\delta}{K_m\rho D}}$	0
		+	$\dfrac{C_b}{1+\dfrac{P\delta}{\rho D}}$	0	$\dfrac{A_M}{W}PqC_b$
$C_S \gg K_m$	+	+	$qC_b - q\,\dfrac{V\delta}{\rho D}$	$\dfrac{A_M}{W}V$	$\dfrac{A_M}{W}PqC_b-\dfrac{A_M}{W}Pq\,\dfrac{V\delta}{\rho D}$
	+		$C_b-\dfrac{V\delta}{\rho D}$	$\dfrac{A_M}{W}V$	0
		+	qC_b	0	$\dfrac{A_M}{W}PqC_b$

0) the bulk phase concentration C_b^+ for achieving the half-maximal value of the difference curve is identical with the apparent Michaelis constant K_m of the carrier-mediated transport system. The deviation from the correct value increases with increasing unstirred layer thickness δ, increasing maximal transport rate V of the non-passive component, increasing magnitude of the passive component (increasing permeability coefficient P), and decreasing diffusion constant D (Table II).

The application of the correction equation shall be explained shortly by the example shown in Fig. 1. The maximal value of the difference curve $\Delta\phi_m$ amounts to 1.4 μmol $\cdot$ h^{-1} $\cdot$ cm^{-1} (standardized to serosal length = W). 0.08 cm^2/h is obtained for the steepness b of the permeation curve after inhibition of the non-passive component (curve 4). C_b^+ amounts to 2.28 mM. With D/δ = 0.49 cm/h and A_{UL}/W = 1.41 cm^2/cm it follows $K_m = (1 - 0.1158) \cdot 2.28 - 1.013 = 1$ mM; $\phi_{npm} = 1.4/(1 - 0.1158) = 1.583$ μmol $\cdot$ h^{-1} $\cdot$ cm^{-1}.

An alternative method to determine the apparent Michaelis constant in the presence of a non-passive transport component and an unstirred layer is to apply non-linear regression methods to Eqns. 9 and 10. K_m, V/ρ, and P/ρ are the unknown parameters, while D/δ and A_{UL}/W are constants. The latter quantities have to be determined separately.

TABLE II

BIAS OF THE APPARENT MICHAELIS CONSTANT DUE TO AN UNSTIRRED LAYER IN THE ABSENCE AND PRESENCE OF A PASSIVE TRANSPORT COMPONENT

C_b^+/K_m, ratio of the biased Michaelis constant to the correct one; PK_m/V = ratio of passive to non-passive transport at low concentrations (region of proportionality). The parameter $V/K_mD\rho$ multiplied by δ represents the "permeability" ratio of the non-passive transport component at low concentrations and the unstirred layer. Data calculated by Eqn. 17.

$V/K_mD\rho$ (μm^{-1})	δ (μm)	$P K_m/V$				
		0	0.1	0.5	1	2
$1 \cdot 10^{-3}$	50	1.03	C_b^+/K_m = 1.03	1.05	1.08	1.13
	100	1.05	1.06	1.10	1.15	1.25
	200	1.10	1.12	1.20	1.30	1.50
	500	1.25	1.30	1.50	1.75	2.25
	1000	1.50	1.60	2.00	2.50	3.50
$5 \cdot 10^{-3}$	50	1.13	1.15	1.25	1.38	1.63
	100	1.25	1.30	1.50	1.75	2.25
	200	1.50	1.60	2.00	2.50	3.50
	500	2.25	2.50	3.50	4.75	7.25
	1000	3.50	4.00	6.00	8.50	13.5
$1 \cdot 10^{-2}$	50	1.25	1.30	1.50	1.75	2.25
	100	1.50	1.60	2.00	2.50	3.50
	200	2.00	2.20	3.00	4.00	6.00
	500	3.50	4.00	6.00	8.50	13.5
	1000	6.00	7.00	11.0	16.0	26.0
$5 \cdot 10^{-2}$	50	2.25	2.50	3.50	4.75	7.25
	100	3.50	4.00	6.00	8.50	13.5
	200	6.00	7.00	11.0	16.0	26.0
	500	13.5	16.0	26.0	38.5	63.5
	1000	26.0	31.0	51.0	76.0	126

Discussion

The equations derived here show that in the presence of an passive transport component the unstirred layer bias of the apparent Michaelis constant is greater, if the difference curve is used for determining this constant. By an appropriate equation the biased Michaelis constant can be corrected. The error introduced by the unstirred layer depends on its thickness and, therefore, on the special experimental conditions. Sometimes the effect of the unstirred layer is negligible [16,17]. In intestinal absorption experiments the apparent Michaelis constant diminishes by a factor of 1.6—3.4 [11] or 14.8 [12], if the unstirred layer is reduced by increasing the stirring or shaking rate. The relative high Michaelis constants of intestinal transport systems determined in vivo [14, 18—23] are probably caused at least partly by a considerable unstirred layer. An increase of the perfusion rate reduces the K_m values [24,25] presumedly by reduction of the unstirred layer thickness. Depending on the stirring rate the thickness of the unstirred layer in the intestine in vitro amounts to 110—330 μm [11,26,27]; in the perfused rat jejunum in vivo it amounts at least to 500 μm (Winne, D., unpublished).

To apply the correction equation the unstirred layer thickness and area are needed. If this information is lacking, the magnitude of these quantities has to be guessed by theoretical considerations. Subsequently, the influence of the unstirred layer and an eventual bias can be evaluated or ruled out. In biological membranes the unstirred layer thickness can be calculated from the time course of the potential difference after changing the osmolarity of the bulk phase [11,15,28,29] or from the permeability coefficient of highly permeable substances [11,27,30]. The unstirred layer area in the intestine can be obtained by histological measurements after fixation [11] or by measuring the intestinal circumference in situ and correcting for the wall thickness (Winne, D., unpublished). The method of Dugas et al. [12] circumvents the determination of the unstirred layer dimensions. These authors increased stepwise the shaking rate and determined the unbiased K_m and V by extrapolation to infinite shaking rate (corresponding to zero unstirred layer thickness).

The correction equations for the biased Michaelis constant in this paper and elsewhere [1,5] have been derived for plane membranes. Their application to villous membranes has to be regarded as an approximation. In this case the concentration gradient in the intervillous space is neglected. The correction by the equation for plane membranes is too small. The significance of this error decreases as the unstirred layer thickness increases. If the site of absorption is located predominantly in the tips of the villi (see ref. 27), the error caused by the application of the incorrect equation is reduced further.

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