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Effect of the alkyl chain length of monocarboxylic acid on the permeation through bilayer lipid membranes

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Abstract

Electrically silent hydrogen ion fluxes across a planar bilayer lipid membrane (BLM) induced by an addition of monocarboxylic acid at one side of BLM were studied by measuring pH changes in the unstirred layers near the BLM surface. The pH changes were assayed by recording protonophore-dependent potentials as well as by direct measurements of pH shifts in the unstirred layers close to the membrane by the pH microelectrode. It was shown that the mechanism of the acid transport changed qualitatively upon the increase of the hydrophobic chain length of the acid. In the case of short-chain acids at $pH < pK_a$, the total transport was limited by diffusion of the anionic form of the acid across the unstirred layers, while at the alkaline pH ($pH >> pK_a$) the transport was limited by diffusion of the neutral form across the membrane. In the alkaline pH range the pH shifts induced by short-chain acids were sensitive to the presence of cholesterol in the BLM as well as to the stirring conditions in the cell. However, in the case of long chain acids (more than 8 carbonic atoms) the transport was limited by diffusion of the anionic form of the acid in the whole range of pH studied. In the latter case, pH changes in the unstirred layers did not depend on the presence of cholesterol in the membrane, and moreover pH shifts were not dependent on the thickness of the unstirred layer. It was proposed that the peculiarities of the long-chain acid-induced proton transport were associated with the formation of micelles of the acid in bathing solutions.

Keywords: Permeability; Fatty acid; Bilayer lipid membrane; Unstirred layer; Monocarboxylic acid

1. Introduction

Monocarboxylic acids represent an important minor component of cells. Long-chain acids (called fatty acids) are important substrates for cell energetics; in addition they play a regulatory role in the organism [1,2]. The mechanism of monocarboxylic acid permeability across membranes was studied in a series of papers [3–10] both on natural and artificial membranes. Gutknecht and co-workers studied the process of permeation of short chain acids (from formic (C1) to caproic (C6)) across planar bilayer lipid membranes (BLM) [4,5] and showed that depending on the experimental conditions there were three limiting cases: (1) diffusion of the neutral form of the acid across the unstirred layers (USL) at low pH; (2) diffusion of the anionic form across the USL at intermediate pH; (3) diffusion of the neutral form across the membrane at alkaline pH. It might be concluded that the process of the acid binding to BLM was not a kinetically significant step. In the other series of papers the mechanism of fatty acid permeation across membranes of liposomes was studied [3,9–11]. It was shown by different methods that the neutral form of fatty acids can easily penetrate through membranes, and an important step of permeation is the acid binding to the membranes. It may be proposed therefore that the mechanism of acid permeation across the membrane depends on the length of the hydrocarbon chain of the acid.

The aim of the present study was to investigate the effect of the hydrocarbon chain length on the mechanism of permeation of monocarboxylic acids (from acetic acid (C2) to lauratic acid (C12)) across planar bilayer lipid membranes. Acid fluxes were determined by recording protonophore-dependent potentials [6,7] as well as by direct measurements of pH shifts in the unstirred layers close

Abbreviations: TTFB, a protonophore tetrachlorotrifluoromethylbenzeimidasole; CCCP, a protonophore carbonylcyanide *m*-chlorophenylhydrazone; MES, 2-[*N*-morpholino]ethanesulfonic acid; BLM, bilayer lipid membrane.

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to the membrane by the pH microelectrode [12]. It may be concluded that the permeability of short chain monocarboxylic acids (less than C6) across the bilayer lipid membrane can be satisfactorily described in terms of the conventional model of the transport [17]. Acids with a longer hydrocarbon chain length have specific transport properties which may be attributed to their ability to form micelles.

2. Materials and methods

BLM was formed on a hole in a Teflon partition 1.2 mm in diameter, by a conventional method [13]. A membrane-forming solution contained 20 mg phosphatidylcholine from soy beans (asolectin, Sigma) and 10 mg cholesterol (Merck) in 1 ml of n-decane unless otherwise stated. The pH gradients on the BLM were measured by the method of the measurements of open-circuit potential in the presence of a protonophore (10 μM TTFB at pH < 8 and 10 μM CCCP at pH > 8) [6]. The experiments were carried out at room temperature (21–23°C). Protonophores were added at both sides of the BLM.

The measurements of pH shifts near the BLM were made by means of an antimony pH microelectrode according to [12]. Typically the electrode tip was about 10 μm. The process of BLM formation, microelectrode movements and touching the BLM were observed through the transparent window in the front side of the cell. Smooth approach of the microelectrode to the membrane was carried out by means of a micromanipulator.

Acetic acid (C2), butyric acid (C4), caproic acid (C6), caprylic acid (C8), capric acid (C10), undecanoic acid (C11) and lauric acid (C12) were from Sigma. Tris, MES, β-alanine, KCl, CCCP were from Serva; TTFB was a gift of Prof. E.A. Liberman; other chemicals were extra pure grade from Reakhim (Russia).

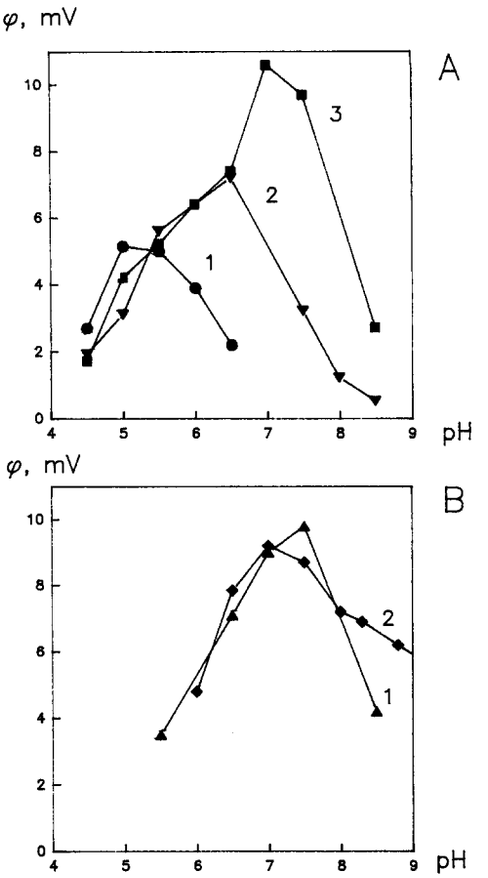

Fig. 1. pH dependence of the formation of the potential on BLM induced by the addition of monocarboxylic acid at one side of the membrane (100 μM, *cis* side) in the presence of a protonophore (10 μM TTFB at pH < 8 and 10 μM CCCP at pH > 8). The potential had plus sign on the *cis* side corresponding to the acidification of the *cis* side and/or the alkalinization of the *trans* side. The solutions was 1 mM citrate, 1 mM Tris, 1 mM β-alanine, 0.5 mM KCl, 20 mM choline chloride, pH as indicated on horizontal axis. A, curve 1, acetic acid (C2); 2, butyric acid (C4); 3, caproic acid (C6); B, curve 1, caprylic acid (C8); 2, capric acid (C10).

3. Results

Monocarboxylic acids were added at one side of the BLM. The formation of the concentration gradient of the acid resulted in pH shifts near the surfaces of the membrane in its USLs. Fig. 1 shows the dependence of the BLM potential in the presence of a protonophore (which corresponds to the pH gradient on the membrane) on pH in the bathing solutions for acetic acid (C2, Fig. 1A,1), butyric acid (C4, A,2), caproic acid (C6, A,3), caprylic acid (C8, B,1) and capric acid (C10, B,2). It is seen that all dependencies have a maximum. One can divide the series of dependencies into two groups. In the first group (C2-C6) an increase in the acid chain length resulted in a shift of the pH dependence to the alkaline side and a concomitant increase in the maximum value of the potential. In the second group (C8 and longer) the change of the acid chain length did not affect the pH dependence. Experiments with undecanoic acid (C11) and lauric acid (C12) revealed that in the pH range from 7 to 9 the values of the acid-induced BLM potentials coincided with the potentials induced by capric acid (C10) (data not shown). It is worth noting that the two acids are insoluble in water and we added an anionic detergent, sodium dodecylsulfate (SDS), to make them soluble. It was not possible to dissolve these two acids at pH < 7 even in the presence of SDS under our experimental conditions. Control experiments showed that in the presence of a protonophore SDS did not generate potentials on BLM at concentrations less than 100 μM. Besides, an addition of SDS to the system with the capric acid gradient (C10) did not change the value of the potential on BLM.

To confirm the idea that the potentials in the presence of a protonophore correspond to pH gradients in the USL, we measured pH shifts near the BLM directly by the pH microelectrode. Fig. 2 shows the pH dependence of the

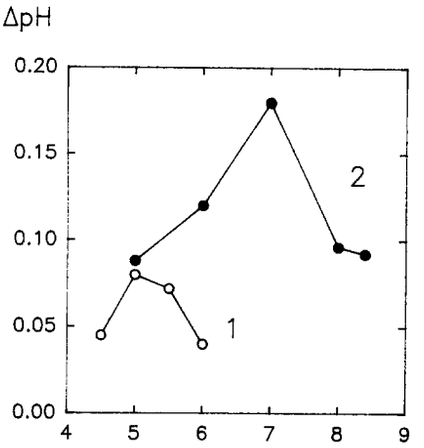

Fig. 2. pH dependence of the pH gradient on BLM induced by the addition of 100 μM acetic acid (C2, curve 1) or capric acid (C10, curve 2) at one side of the BLM. Measurements were performed with the help of a pH microelectrode. The points represented the sums of pH shifts at the cis and the trans sides of the BLM. The solution was as in the caption to Fig. 1.

sum of pH shifts at both sides of the BLM induced by acetic (C2, curve 1) and caproic acids (C6, curve 2). Fig. 2 confirms the main result of the experiments with the measurements of the BLM potential, namely the shift of the maximum to the alkaline side and a concomitant increase in its magnitude upon the increase in the acid chain length from acetic (C2) to caproic acid (C6).

In other series of experiments, the fluxes of different acids were compared with that of acetic acid at different pH values. Fig. 3A shows a typical example of such experiments. After the potential on the BLM had attained a steady-state value in the presence of the caproic acid (C6) gradient and a protonophore, we formed a reverse gradient of acetic acid and increased this gradient until the BLM potential reached zero. Under these conditions the ratio of the concentrations of the caproic and acetic acids is a measure of the caproic acid flux. Fig. 3B shows the pH dependence of the ratio for butyric acid (C4, curve 1), caproic acid (C6, curve 2) and caprylic acid (C8, curve 3). In the case of butyric acid, the S-type curve was obtained, while in the cases of caproic (C6) and caprylic (C8) acids the ratio increased gradually with a pH increase.

Pohl et al. [14] introduced a relatively simple test which enabled one to distinguish between two possibilities of the transport limitations under the given experimental conditions: unstirred layers or the membrane itself. The test was based on the effect of the rate of the solution stirring on the value of the BLM potential induced by a weak acid in the presence of a protonophore. More intensive solution stirring should reduce the BLM potential when the transport is limited by the membrane, whereas this should have no effect on the potential when the transport is limited by the USL. This can be explained as follows. An increase in the rate of solution stirring facilitates diffusion of buffer molecules across the USL concomitant with the diffusion of the charged form of the acid, which maintains constant the value of the pH gradient when diffusion of the charged form is the rate-limiting step. In another extreme case when diffusion across the membrane is the rate-limiting step, an increase in the stirring rate enhances diffusion of buffer molecules, while the flux of protons across the membrane is constant and decreases the magnitude of the pH gradient (see Section 4).

Fig. 4 shows the recordings of the BLM potentials induced by acetic acid (C2, curves A and B) and capric acid (C10, curves C,D) at low (curves A,C) and high (curves B,D) pH values. In agreement with the results of [15], the increase in the rate of the solution stirring did not affect the acetic acid-induced potential at low pH and reduced significantly the value of the potential at neutral

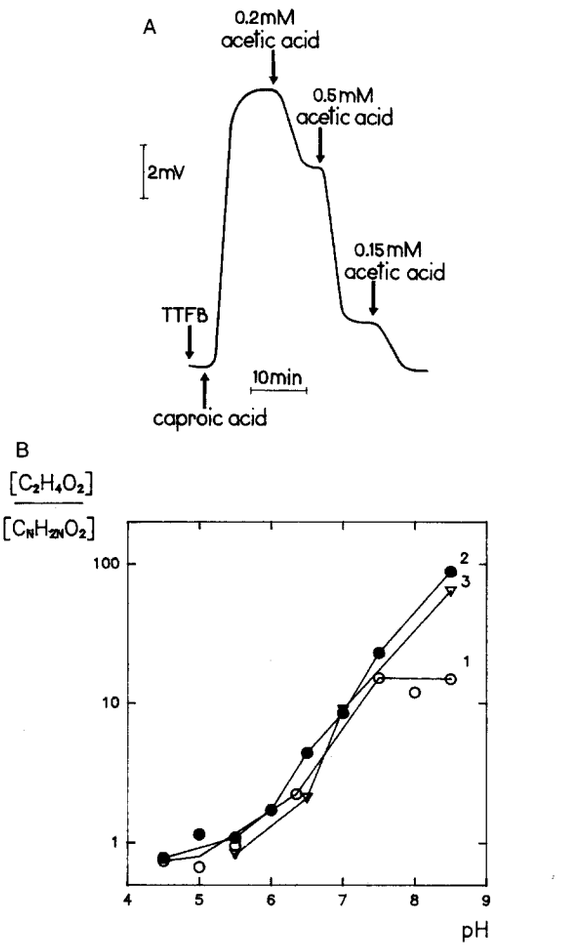

Fig. 3. (A) An example of the measurement of an acid flux as compared to the flux of acetic acid. The potential was generated by the addition of 100 μM caproic acid (C6) at the '*cis*' side (10 μM TTFB was in the solution) and was zeroed gradually by additions of acetic acid at the '*trans*' side. The numbers of concentrations referred to the concentrations of the additions so the final acetate concentration was 0.65 mM. The solution was as in the caption to Fig. 1 (pH 7.0). (B) The effect of the solution pH on the ratio of the concentration of acetic acid which zeroed BLM potential (see A) and the acid. The potential was induced by butyric acid (C4, curve 1), caproic acid (C6, curve 2) or caprylic acid (C8, curve 3).

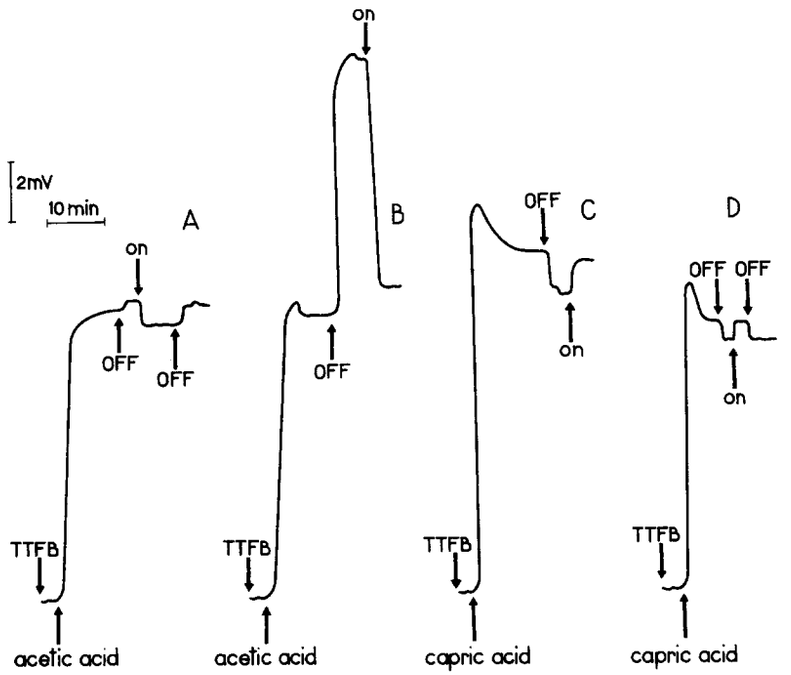

Fig. 4. Effect of the rate of the solution stirring on the value of the BLM potential induced by a weak acid (the addition on one side of the BLM) in the presence of a protonophore. A, 200 μM acetic acid (C2) (pH 4.5); B, 5 mM acetic acid (pH 6.5); C, 100 μM capric acid (C10) (pH 6.5); D, 100 μM capric acid (C10) (pH 8.0). The solution was as in the caption to Fig. 1.

pH. However, in the case of capric acid (C10) the variation of the rate of the solution stirring did not change the BLM potential both at acidic and alkaline pH. According to the above consideration this result suggests that in the case of a long chain acid the permeation is limited by diffusion through the USLs in the whole pH range.

Further evidence in favor of this conclusion was obtained by studying the effect of cholesterol on the BLM potential in the presence of a protonophore. Cholesterol is a widely used agent which is able to decrease substantially the membrane permeability for nonelectrolytes [15]. Fig. 5 shows that while the presence of cholesterol in the membrane-forming solution decreases the acetic acid-induced BLM potential at pH higher than pH_{max}, cholesterol does not change the potential induced by capric acid (C10) regardless of the pH.

4. Theoretical background

The present work dealt with pH gradients on the BLM which arose in the unstirred layers upon an addition of weak acids at one side of the membrane (the *cis* side). The unstirred layer is a region of a poorly mixed solution, in which solution transport occurs only by diffusion [16]. For the case of the planar BLM, the thickness of the USL varies from 100 μm upon vigorous stirring to about 500 μm (mild stirring) [12]. The formation of pH gradients was accounted for by the permeation of the neutral form of the acid through the BLM while the charged form was impermeable. The reaction $T^- + H^+ \rightarrow TH$ which took place on the *cis* side increased the pH values, while the reaction $TH \rightarrow T^- + H^+$ (the *trans* side) decreased the pH values [17]. A quantitative description of the relationship between the acid flux and local pH shifts was rather complicated and led to equations which assumed only the numerical solution [7]. However, as proposed in our previ-

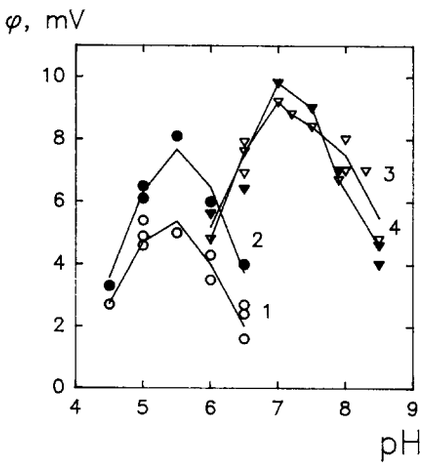

Fig. 5. Effect of cholesterol on the BLM potential induced by acetic acid (C2, curves 1,2; circles) or capric acid (C10, curves 3,4; triangles) in the presence of a protonophore. The membrane-forming solution contained 20 mg asolectin in 1 ml of n-decane (curves 2,4; filled symbols) or 20 mg asolectin and 10 mg cholesterol in 1 ml n-decane (curves 1,3; hollow symbols). The acids concentration on one side of the BLM was 100 μM. The solution was as in the caption to Fig. 1.

ous paper [6], analytical solutions could be derived using several simplifications. The main one was the assumption about small values of pH shifts in the USLs.

The pH gradient on the BLM was proportional not to the total acid flux J_t, but rather to the flux of H^+-ions (J_H) across the BLM which was associated with the diffusion of the T^- form of the acid across the USL and the chemical reactions near the membrane surface. The equation for J_H is presented below. pH shifts at the *cis* and *trans* sides of the BLM induce the fluxes of hydrogen ions carried predominantly by buffer molecules across the *cis* and *trans* unstirred layers J_{buf}^{ciS} and J_{buf}^{trans}. According to [18]

$$J_{buf}^{cis} = P_{buf}^{USL cis} \cdot B \cdot \Delta pH^{cis},$$

$$J_{buf}^{trans} = P_{buf}^{USL trans} \cdot B \cdot \Delta pH^{trans} \quad (1)$$

where $P_{buf}^{USL cis}$ and $P_{buf}^{USL trans}$ are USL permeabilities of the buffer molecules, B is the buffer capacity and ΔpH^{cis}, ΔpH^{trans} are pH shifts between the bulk phase and the opposite sides of the BLM.

At a steady state $J_H = J_{buf}^{cis} = J_{buf}^{trans}$ and assuming symmetrical unstirred layers

$$\Delta pH = \Delta pH^{cis} + \Delta pH^{trans} = J_H \cdot \left(2 / P_{buf}^{USL} \cdot B\right) \quad (2)$$

Thus the pH gradient on the BLM is proportional to the J_H flux induced by the acid. The total flux of a weak acid J_t through the unstirred layers and the membrane can be conveniently considered as the sum of two independent fluxes:

$$J_{TH}: TH \overset{cisUSL}{\rightarrow} TH \overset{BLM}{\rightarrow} TH \overset{transUSL}{\rightarrow} TH$$

$$J_H: T^- \overset{cisUSL}{\rightarrow} T^- + H^+ = TH \overset{BLM}{\rightarrow} TH$$

$$= H^+ + T^- \overset{transUSL}{\rightarrow} T^-$$

According to Ref. [4]

$$1/J_t = 1/\left(P_{T^-}^{USL}[T^-] + P_{TH}^{USL}[TH]\right) + 1/P_{TH}^{M}[TH] \quad (3)$$

where P_{TH}^{M} is the membrane permeability of the neutral form of the acid and $P_{T^-}^{USL}$, P_{TH}^{USL} are the USL permeabilities of the two forms of the acid.

In the limiting case of $P_{TH}^{M} > P_{T^-}^{USL}$, the flux J_H can be described by the following simple equation [6]:

$$1/J_H = 1/P_{T^-}^{USL}[T^-] + 1/P_{TH}^{M}[TH] \quad (4)$$

To derive an explicit equation J_H (pH), the concentrations of $[T^-]$ and [TH] should be expressed as a function of the total acid concentration C_0 and pH.

$$J_H = C_0 P_{TH}^{M} P_{T^-}^{USL} \alpha / (1 + \alpha)\left(P_{T^-}^{USL}\alpha + P_{TH}^{M}\right) \quad (5)$$

where $\alpha = 10^{pH-pK} = K/[H^+]$.

Eq. (5) predicts a maximum in the dependence of J_H on pH, whose position is a function of the acid pK and its permeabilities.

$$pH_{max} = pK + \log\left(\sqrt{P_{TH}^{M}/P_{T^-}^{USL}}\right) \quad (6)$$

$$J_H^{max} = P_{TH}^{M} C_0 / \left(1 + \sqrt{P_{TH}^{M}/P_{T^-}^{USL}}\right)^2 \quad (7)$$

At the acidic pH in the limiting case $pH < pK$ Eq. (5) gives

$$J_H = P_{T^-}^{USL} C_0 \alpha \quad (8)$$

A decrease in J_H with a pH decrease is caused by the reduction of the T^- concentration when diffusion of T^- across the USLs is the rate-limiting step. At the acidic pH, the ΔpH values should be independent of the change in the stirring conditions, since the change in the width of the unstirred layers should simultaneously change the $P_{T^-}^{USL}$ *and* P_{buf}^{USL} values (see Eqs. (2) and (8)). In experiments with titration of an acid flux by acetic acid (Fig. 3) the final ΔpH value on the BLM was zero. Therefore the J fluxes induced by the acid and acetic acid were equal. At acidic pH, the ratio of their concentrations was determined by the ratio of their $P_{T^-}^{USL}$ values

$$C_0^1/C_0^2 = P_{T_2^-}^{USL}/P_{T_1^-}^{USL} \quad (9)$$

In the opposite limiting case $pH >> pK$ Eq. (5) gives

$$J_H = P_{TH}^{M} C_0/\alpha \quad (10)$$

The decrease in J_H with a pH increase at alkaline pH corresponded to reduction of the TH concentration when diffusion of TH across the BLM was the rate-limiting step. At alkaline pH the ΔpH values should depend on the change in the stirring conditions, since the change in the width of the unstirred layers should alter the P_{buf}^{USL} values (see Eq. (2)). In experiments with titration of the flux by acetic acid (Fig. 3), the ratio of the concentration of the acid and acetic acid was determined by their P_{TH}^{M} ratio

$$C_0^1/C_0^2 = P_{TH_2}^{M}/P_{TH_1}^{M} \quad (11)$$

Analysis of Eqs. (5)–(7) showed that an increase in the membrane permeability for TH (P_{TH}^{M}) caused by the increase in the acid chain length led to a shift of the maximum to alkaline pH and a rise of J_H^{max} (Fig. 6A). J_H^{max} should reach saturation at high P_{TH}^{M} which is equal to $P_{TH}^{USL} \cdot C_0$. Variation of P_{TH} did not change the values of J_H in the acidic pH range ($pH < pK_\alpha$) where diffusion of T^- through the USLs was the rate limiting step (Fig. 6A). On the other hand, a variation of pK_α without changes in P_{TH} led simply to a shift of the pH dependence along the pH axis which changed the J values at acidic pH (Fig. 6B).

5. Discussion

The analysis of experimental pH dependencies of the BLM potentials (which were proportional to J_H) showed that in a series of short chain acids (C2-C6) an increase in the chain length resulted in a rise of the magnitude of the maximum and a shift of the maximum to alkaline pH in a similar manner as in Fig. 6A. Nonlinear regression of the data of Fig. 1 according to Eq. (1) gave the following values of the P_{TH}^{M} (cm/s) and pK_a parameters: C2 0.024,

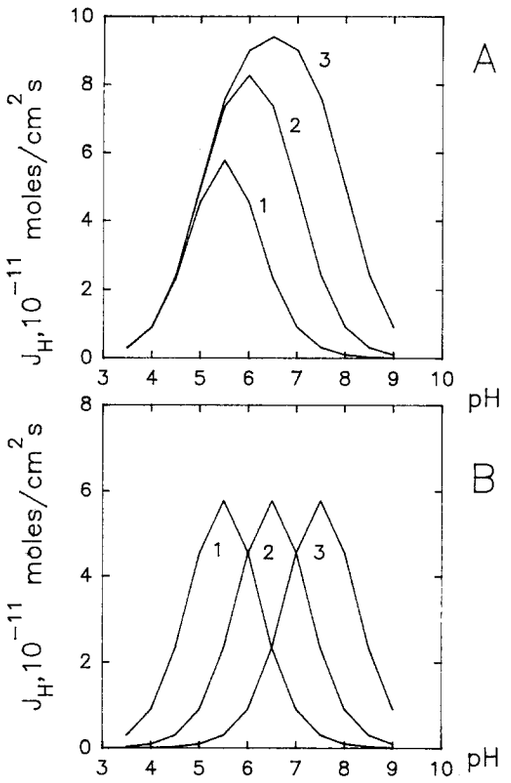

Fig. 6. A, theoretical pH dependences of the J_H fluxes calculated from Eq. (1) at different values of P_{TH}^M. The acid concentration was 100 μM, $P_T^{USL} = 10^{-3}$cm/s, $pK_a = 5.0 \cdot P_{TH}^M = 10^{-2}$cm/s (curve 1), $P_{TH}^M = 10^{-1}$cm/s (curve 2), $P_{TH}^M = 1$ cm/s (curve 3). B, effect of pK_a on the theoretical pH dependence of J_H (Eq. (1)). The acid concentration was 100 μM, $P_T^{USL} = 10^{-3}$cm/s, $P_{TH}^M = 10^{-2}$ cm/s, $pK_a = 5.0$ (curve 1), $pK_a = 6.0$ (curve 2), $pK_a = 7.0$ (curve 3).

4.7; C4 0.122, 5.2; C6 0.970, 5.3; C8 0.173, 6.1; C10 0.221, 6.0. In spite of the fact that the acids under study had similar pK_α values in water solutions (about 4.8 [19]), regression with the fixed value of $pK_\alpha = 4.8$ led to significant deviations from experimental curves. As it has been pointed out in Section 3, the above calculations showed that the series of acids could be divided into two groups. For short chain length acids (C2-C6) an increase in the chain length led to an increase in P_{TH}^M and affected pK_α insignificantly, while medium chain length acids (C8-C10) had high values of the pK_α parameter and low values of P_{TH}^M which did not depend on the chain length. This analysis showed that in the case of fatty acids the J_H value was determined by a process whose pK was more alkaline than the pK_α of the acids in water solution.

In experiments where acid fluxes were compared with that of acetic acid (Fig. 3) qualitatively similar results were obtained: in the case of butyric acid (C4) the pH dependence of the ratio of the butyric and acetic acid concentrations could be described by the above model while for acids with a longer chain length the model was not valid. In fact at low pH the model predicted the ratio being close to unity (Eq. (9)) since the diffusion coefficients of the acids in water should be similar. At high pH the process was limited by diffusion of the acids through the BLM per se, and the ratio would approach the ratio of permeabilities of their neutral forms (Eq. (11)). In the case of butyric acid, the ratio reached the value of 15 (Fig. 3), apparently due to different hydrophobicity of butyric and acetic acids. This value agreed reasonably well with that obtained from radioactive trace experiments where the ratio was 10 [5].

It can be also seen from Fig. 3 that in the case of acids with a longer chain length the ratio rose gradually with a pH increase (Fig. 3, curves 2,3). Since the pH dependencies had no breaking points, it can be assumed that there was no alteration of the limiting steps through the whole pH range studied. The experiments on the effect of the rate of the solution stirring on the BLM fluxes suggested that this limiting step should be the diffusion of the charged form of these acids across the USL. The conclusion that the diffusion of the charged form of these acids across the USL was a rate-limiting step of permeation of long chain acids in the whole pH range was also confirmed by the absence of the cholesterol effect on the BLM potential (Fig. 5). Cholesterol decreased the membrane permeability for the neutral form of acids [15].

The phenomenon observed with long chain acids was apparently due to dimerization [20] and formation of micelles [21]. For example it was shown in [21] that lauric acid (C12) can form micelles in a water solution, and this process is promoted at alkaline pH. This leads to a decrease in the concentration of monomeric forms of the acid. On the other hand, the pK values of an acid on the surface of micelles should be higher compared to that one in a solution. It should be noted that long chain acids (C11, C12) were used in our experiments as mixed micelles with an anionic detergent, SDS.

It may be concluded that the permeability of short chain monocarboxylic acids (less than C6) across the bilayer lipid membrane can be satisfactorily described in terms of an conventional model of the transport [17]. Acids with a longer hydrocarbon chain length have specific transport properties which may be attributed to the ability to form micelles. Therefore besides the two steps which are included in the above mentioned model (proton transfer reactions in the unstirred layers and diffusion of the neutral form across the membrane), an additional stage should be taken into consideration, namely mononer-micelle equilibration in the unstirred layers.

Acknowledgements

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