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Biochimica et Biophysica Acta 1416 (1999) 333–338

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

A ^{1}H-NMR study of the permeation of glycolic acid through phospholipid membranes

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Received 11 June 1998; received in revised form 16 November 1998; accepted 18 November 1998

Abstract

The transmembrane permeability coefficient of the α-hydroxyacid, glycolic acid, has been measured for egg phosphatidylcholine large unilamellar vesicles. The determination of the vesicle concentration independent first-order rate constant for membrane transport and the permeability coefficient were made using an NMR technique employing shift agents. Both the temperature dependence and the dependence on cholesterol content were investigated. The activation energy and the Arrhenius pre-exponential factor were found to be dependent on the cholesterol content. A marked increase in both parameters was observed up to 20 mol% cholesterol, with a further, small increase up to 50%. The pH dependence of permeability was also investigated. An increase in permeability is observed with a decrease in pH, providing a possible explanation for the effectiveness of glycolic acid in skin treatment.

Keywords: Transmembrane permeability; Glycolic acid; ^{1}H-NMR; Acne; Aging of the skin; Cholesterol

1. Introduction

Glycolic acid (hydroxyacetic acid) is the smallest in a series of naturally derived α-hydroxyacids (AHAs) which includes lactic, malic, tartaric, and citric acid. Their structures are illustrated in Fig. 1. The motivation for studying the permeability of glycolic acid was two-fold. Firstly, having shown the usefulness of NMR to examine permeants, such as DMA [1], with permeability coefficients greater than 10^{-9} m s^{-1}, we wished to expand this range to cover faster permeating molecules. Alger and Prestegard [2], showed the permeability of acetic acid in phospholipid membranes to be about 10^{-6} m s^{-1}, from which it can be estimated that glycolic acid should permeate at a similar, or perhaps slightly slower rate. This presumption is based on the fact that glycolic acid (log P = −1.11) is more hydrophilic than acetic acid (log P = −0.31) as measured by octanol/water partition coefficients (*P*) [3].

Secondly, glycolic acid is used within the cosmetic industry and is reported to improve acne as well as premature aging of the skin. [4,5]. Information on its permeability may be of practical use to dermatologists as they attempt to decipher the mechanisms of its action. Glycolic acid is hydrophilic and may therefore diffuse freely throughout the intercellular phase in plasma and skin, without the need for proteins to act as carriers. This has been proposed as an explanation for the skins mild secondary reactions to

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0005-2736/99/$ – see front matter
PII: S0005-2736(98)00236-3

glycolic acid compared to the harsh effects of more hydrophobic skin products [6]. Also, glycolic acid has been shown to have a pH-dependent ability to stimulate cell renewal [7]. Its maximal stimulation was observed at about pH 3, while very little stimulation was observed above pH 6. As well, glycolic acid has been shown to exhibit its effects quickly, possibly due to its ability to penetrate the skin rapidly.

By studying the passive permeability of glycolic acid, explanations for some of its observed effects may be provided.

2. Materials and methods

All materials used throughout this work were purchased in the purest form available from Sigma-Aldrich Canada (Oakville, ON, Canada) unless otherwise stated.

2.1. EPC storage and use

Egg phosphatidylcholine (EPC) is received as 1 g of powder in vials (Northern Lipids, Vancouver, BC, Canada). Once the vial is opened, the EPC is dissolved in 10 ml chloroform and stored in a freezer (−18°C). When needed, the EPC solution is measured by volume, dried first by rotary evaporation, then under vacuum.

2.2. LUV preparation

LUVs were prepared by the extrusion procedure of Cullis and colleagues [8,9]. A 1.5-ml solution of glycolic acid (8 mg ml^{-1}) in D_2O was adjusted with NaOH or HCl to the desired pH as monitored by a pH meter. This solution was used to hydrate 0.1 g of a mixture of dried EPC and cholesterol. The mixture was vortexed to produce a multilamellar suspension and was allowed to equilibrate, at room temperature, overnight. This suspension was frozen in liquid nitrogen (30 s) and thawed in warm water (40°C) five times to increase the bilayer unilamellarity [8]. This freeze–thawed suspension was transferred into an extruder (Lipex Biomembranes, Vancouver, BC, Canada) and passed, ten times, through two polycarbonate filters (Nucleopore Canada, Toronto, ON, Canada) under 600 psi nitrogen gas to produce LUVs.

2.3. NMR sample preparation

In a typical experiment, 0.4 ml of the LUVs were added to an NMR tube. To this was added 3 μl of 50 mM $PrCl_3$ in D_2O as a shift reagent.

2.4. NMR data collection

^{1}H-NMR spectra were accumulated with a Bruker AM400 NMR spectrometer using quadrature detection. The temperature was controlled with a BVT-100 temperature controller. Spectra were acquired with two dummy scans followed by eight scans of 8192 data points, 2-s relaxation delay, a 90° detection pulse, and a 3800-Hz sweep width.

2.5. NMR data analysis

NMR data was analyzed using the Bruker WIN-NMR program (version 940401). Typically, no line broadening was applied to the FIDs. After Fourier transformation, and phase correction, a 5th order baseline correction was applied to the spectrum. Peak widths at half height (accurate to ±1 Hz) of the internal glycolic acid resonance were analyzed using either the AM400s Aspect 3000 console (EP mode), or the WIN-NMR deconvolution routine.

3. Results

The glycolic acid region of a typical ^{1}H-NMR spectrum is depicted in Fig. 2a. A single resonance is observed with a natural linewidth of about 2.5 Hz.

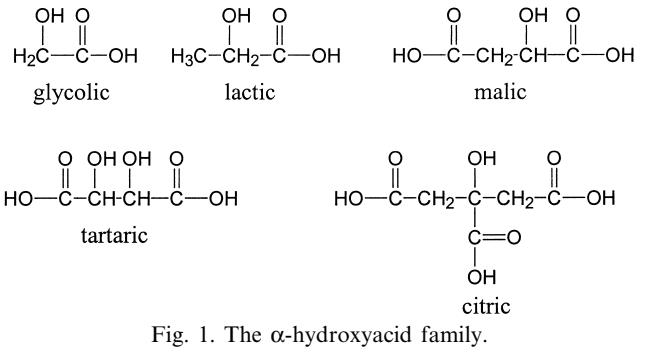

Fig. 1. The α-hydroxyacid family.

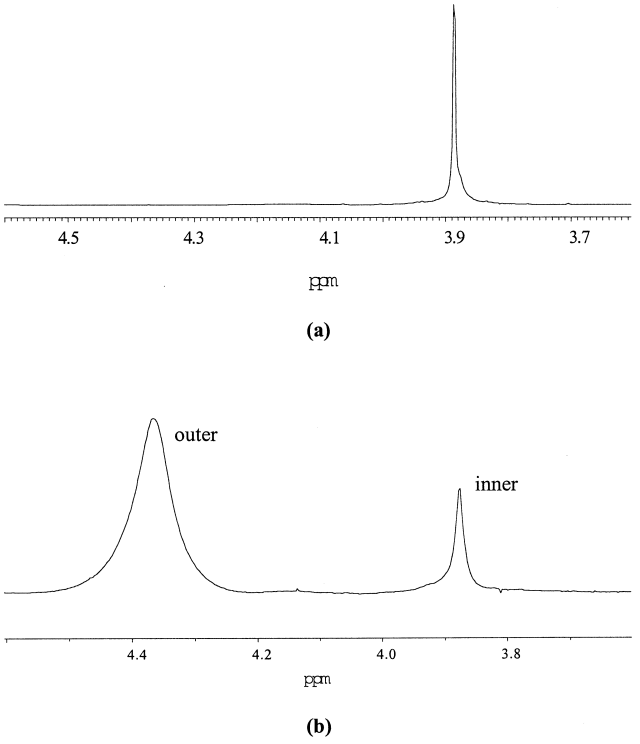

Fig. 2. Glycolic acid resonance before (a), and after (b), the addition of a shift agent to the vesicle system. pH 3.94, T= 303 K, 100 nm vesicle diameter.

The addition of a shift agent, in this case $PrCl_3$, causes the glycolic acid on the outside of the vesicles to shift downfield, as is seen in Fig. 2b. Both resonances are exchange broadened, but still have separate frequencies, exhibiting that diffusion is still in the slow exchange regime. The increase in linewidth of the inner resonance as a result of exchange is given by

$$\Delta\nu = \frac{k_{app}}{\pi} \quad (1)$$

where $\Delta\nu$ is the line broadening (in Hz) arising from exchange processes and k_{app} is the pH-dependent, vesicle concentration independent rate constant of transport across the membrane (in s^{-1}). In order to calculate $\Delta\nu$, the linewidth in the absence of exchange, ν_0, is subtracted from the observed linewidth, ν. As the temperature decreased, the linewidth decreased, but no further decrease in ν was observed below 283 K ($\nu_0 \approx 5$ Hz). Hence, ν_o was taken to be the linewidth at this temperature. Fig. 3 illustrates the effect of the temperature on the broadening of

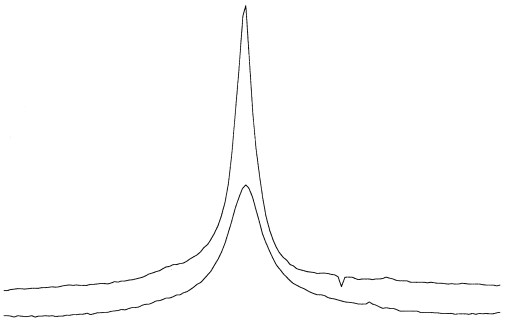

Fig. 3. The effect of temperature on the inner, exchange broadened, glycolic acid resonance. The top trace was taken at 283 K and has a linewidth at half height, ν_o, of 5.2 Hz, the wider, bottom trace was taken at 303 K and has a linewidth, ν, of 12.0 Hz. pH 3.94, 100 nm vesicle diameter.

the inner peak as the temperature is increased from 283 to 303 K. From the difference in linewidth k_{app} is calculated to be 22 ± 4 s^{-1}.

The pH dependence of k_{app} is analogous to that observed previously in our studies of dimethylarsinic acid (DMA) [1]. The pH dependent rate constant is related to the rate constant for permeation of the uncharged acid (k_a), the negatively charged base (k_b), and the fraction of undissociated acid, α, as in Eq. 2 [1].

$$k_{app} = (k_a - k_b)\alpha + k_b \quad (2)$$

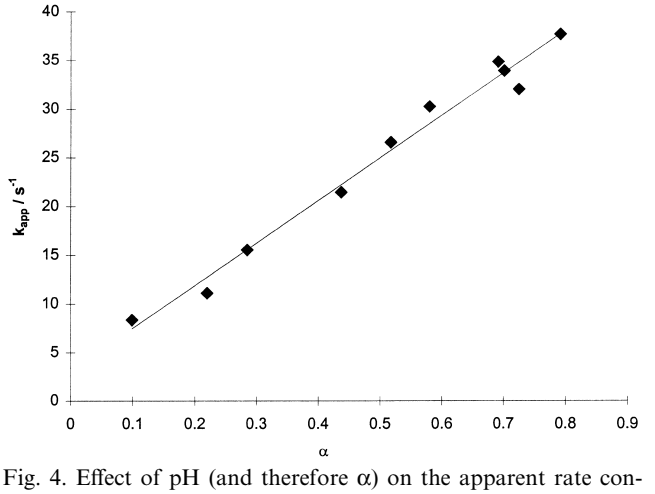

Fig. 4. Effect of pH (and therefore α) on the apparent rate constant of permeation, k_{app}. The observed data points are represented by ♦, and the line represents linear regression analysis. A slope of 43.7 s^{-1} (SD = 2.1 s^{-1}) and intercept of 3.1 s^{-1} (SD = 1.2 s^{-1}) were obtained. ($R^2 = 0.982$) T = 303 K.

Table 1
Vesicle diameter effect on the permeation of glycolic acid through phospholipid vesicles, pH 3.46

k_{app} at 303 K/±4 s^{-1}	Filter size (nm)	Actual diameter [9] (nm)	$P_{app}/\pm 1\times10^{-7}$ m s^{-1}
34	100	103	5.4
20	200	151	4.7

$$\alpha = \frac{[H^+]}{[H^+] + K_a} \tag{3}$$

For a monoprotic acid, such as glycolic acid ($K_a = 3.83$), α is given by Eq. 3. The plot of k_{app} vs. α, depicted in Fig. 4, exhibits a linear relationship. Applying Eq. 2, a pH-independent rate constant of $k_a = 41 \pm 4$ s^{-1} was obtained for the undissociated form of glycolic acid. As judged by the magnitude of the intercept, the anionic base permeates with a rate constant of 3 ± 2 s^{-1}, approximately zero when compared to the undissociated species.

$$P_{app} = \frac{k_{app} V_i}{A_{mem}} \tag{4}$$

As previously discussed [1], the rate constant, k_{app}, is related to the trapped volume/inner vesicular surface area ratio, V_i/A_{mem}, as in Eq. 4. Since the vesicles formed using the extruder are of uniform size [9], this ratio is approximately constant. Assuming that all the vesicles are spherical, we obtain the equation:

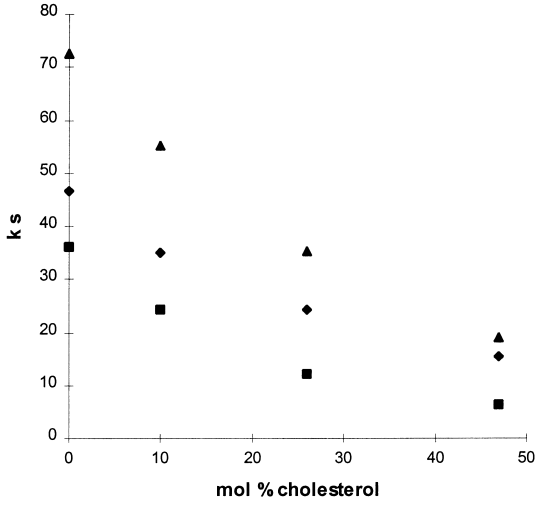

Fig. 5. The effect of cholesterol on the apparent rate constant for permeation (k_{app}) of glycolic acid through EPC LUVs at 299 K (■), 303 K (◆), and 308 K (▲). pH 3.46, 100 nm vesicle diameter. pH 3.46, 100 nm vesicle diameter.

$$\frac{V_i}{A_{mem}} = \frac{\frac{4}{3}\pi r_i^3}{4\pi r_i^2} = \frac{r_i}{3} = \frac{d_i}{6} = \frac{d_o - 2\Delta r}{6} \tag{5}$$

where r_i, and d_i are the inner radius and diameter of the vesicles, respectively. The outer diameter of the vesicles is represented by d_o, and Δr is the membrane thickness. The outer radius of the vesicles is calculated from light scattering methods [9]. For unilamellar, EPC membranes Δr is approximately 40 Å. As an example, for an outer diameter of 100 nm, the ratio of V_i to A_{mem} is 15.3 nm.

The permeability coefficient may now be defined as:

$$P_{app} = \frac{k_{app}(d_o - 2\Delta r)}{6} \tag{6}$$

The results for two different vesicle sizes are tabulated in Table 1. Permeability did not change with vesicle size within error which was on the order of 1×10^{-7} m s^{-1}.

The effect of membrane composition on the permeability of glycolic acid was investigated by the addition of varying amounts of cholesterol to the lipids used to form the LUVs. At all cholesterol levels, the linewidth, ν_o, at low temperature (283 K), varied only slightly (6 ± 1 Hz). The subsequent cholesterol effect on the rate constant of permeation is depicted in Fig. 5.

Table 2
Arrhenius parameters obtained from temperature variation of glycolic acid exchange (k_{app}) experiments performed with 100 nm vesicles, pH 3.46

Mass% cholesterol	mol%	ln A	E_a (kJ mol^{-1})
0	0	29.6	60
5	10	31.5	70
15	26	38.2	89
30	47	39.3	93

Standard deviations of 0.1 for ln A and 9 kJ mol^{-1} for E_a were estimated.

The temperature dependence on the rate constant for permeation was investigated at several cholesterol contents. The data from this investigation were analyzed using Arrhenius kinetics with A representing the pre-exponential Arrhenius factor, E_a the activation energy, R the gas constant, and T temperature. The Arrhenius parameters obtained, exhibited a dependence upon membrane composition, as summarized in Table 2.

4. Discussion

The ability to use NMR as a tool for probing membrane permeabilities in LUVs, too fast for the method outlined previously [1] has been demonstrated. By forming vesicles in a solution of glycolic acid, we were able to extract rate constants for permeation under equilibrium conditions. The paramagnetic shift reagent, Pr^{3+}, was employed to resolve the ^1H signals arising from glycolic acid inside and outside the LUVs. The linewidth of the inner signal, being exchange broadened, was measured and compared to the width at low temperature.

A series of experiments were performed to study the effects of pH, temperature, vesicle size, and membrane composition on the rates of permeation of glycolic acid in EPC vesicular membranes. By applying slow NMR exchange kinetics, permeation rate constants (independent of vesicle concentration) were calculated from the data. Using these rate constants, in conjunction with the known vesicle diameter and membrane width parameters, permeability coefficients were obtained. As predicted by the partition coefficients, the permeability coefficient for glycolic acid is about an order of magnitude slower than that of acetic acid.

The correlation between the rate constant for permeation and the fraction of undissociated acid is similar for glycolic acid as has been observed previously for DMA [1] and other systems [10–12]. A rate constant for permeation of 41 ± 4 s^{-1} was obtained for undissociated glycolic acid. This translates into a permeability coefficient of $(6.4 \pm 1) \times 10^{-7}$ m s^{-1}. The anionic form of glycolic acid has a rate constant of permeation of approximately 3 ± 2 s^{-1} and a permeability coefficient of $(5 \pm 3) \times 10^{-8}$ m s^{-1}. At low pH, where a higher percentage of undissociated acid is present, the rate constant is larger than that at a higher pH, where more of the anion is present. This effect has been observed in several studies, and is attributed to the relative inability of the anion to partition into the neutral bilayer when compared with the undissociated species [1,10–12]. This effect also offers an explanation for the pH dependence on glycolic acid's ability to stimulate cell renewal, where effectiveness is enhanced at lower pH [7].

The trends observed here for the transport of glycolic acid across phospholipid membranes with an increased presence of cholesterol are similar as those previously reported by ourselves [1] as well as others [13–30]. The results reported here show that the major effect of cholesterol occurs up to the 20% (mol) level, and that further increase in the cholesterol content has only a limited effect. This behavior is probably due to the balance between the increase in the partition coefficient and decrease in the rate of membrane transport as cholesterol content is increased.

The activation energies observed in this work, for membranes free of cholesterol, are comparable to those observed for the permeability of a variety of charged and uncharged solutes [31]. Being hydrophilic, we would expect that the barrier of diffusion to glycolic acid is the hydrophobic membrane interior. Our observations of an increasing E_a with increasing cholesterol up to 20 mol%, followed by a leveling off is consistent with the growth and leveling of the hydrophobic barrier as described by Subczynski and coworkers [32] and will be the subject of further investigation by ourselves.

Acknowledgements

The Natural Sciences and Engineering Research Council of Canada (NSERC) is thanked for support in the form of research grants to F.G. Herring and a postgraduate scholarship to R.G. Males.

References

[1] R.G. Males, J.C. Nelson, P.S. Phillips, W.R. Cullen, F.G. Herring, Biophys. Chem. 70 (1998) 75–85.
[2] J.R. Alger, J.H. Prestegard, Biophys. J. 28 (1979) 1–14.

[3] A. Leo, C. Hansch, D. Elkins, Chem. Rev. 71 (1971) 525–616.
[4] H. Murad, A.T. Shamban, P.S. Premo, Dermatol. Clin. 13 (1995) 285–307.
[5] C.P. Clark III, Clin. Plastic Surg. 23 (1996) 49–56.
[6] R. Hermitte, Cosmet. Toiletries 107 (1992) 63–67.
[7] W.P. Smith, Cosmet. Toiletries 109 (1994) 41–48.
[8] M.J. Hope, M.B. Bally, G. Webb, P.R. Cullis, Biochim. Biophys. Acta 812 (1985) 55–65.
[9] L.D. Mayer, M.J. Hope, P.R. Cullis, Biochim. Biophys. Acta 858 (1986) 151–168.
[10] P.R. Harrigan, K.F. Wong, T.E. Redelmeier, J.J. Wheeler, P.R. Cullis, Biochim. Biophys. Acta 1149 (1993) 329–338.
[11] J.H. Prestegard, J.A. Cramer, D.B. Viscio, Biophys. J. 26 (1979) 575–584.
[12] J. Gutknecht, A. Walter, Biochim. Biophys. Acta 649 (1981) 149–154.
[13] R.A. Cooper, J. Supramol. Struct. 8 (1978) 413–430.
[14] R.A. Cooper, E.C. Arner, J.S. Wiley, S.J. Shattil, J. Clin. Invest. 55 (1975) 115–126.
[15] A. Finkelstein, A. Cass, Nature 216 (1967) 717–718.
[16] R.A. Demel, S.C. Kinsky, C.B. Kinsky, L.L.M. Van Deenen, Biochim. Biophys. Acta 150 (1968) 655–665.
[17] J. de Gier, J.G. Mandersloot, L.L.M. van Deenen, Biochim. Biophys. Acta 150 (1968) 666–675.
[18] J. de Gier, J.G. Mandersloot, J.V. Hupkes, R.N. McElhaney, W.P. van Beek, Biochim. Biophys. Acta 233 (1971) 610–618.
[19] R.N. McElhaney, J. de Gier, E.C.M. Van der Neut-Kok, Biochim. Biophys. Acta 298 (1973) 500–512.
[20] B. Deuticke, C. Ruska, Biochim. Biophys. Acta 433 (1976) 638–653.
[21] N. Haran, M. Shporer, Biochim. Biophys. Acta 426 (1976) 638–646.
[22] V.V. Morariu, G. Benga, Biochim. Biophys. Acta 469 (1977) 301–310.
[23] R.N. McElhaney, J. de Gier, L.L.M. van Deenen, Biochim. Biophys. Acta 219 (1970) 245–247.
[24] R. Fraley, R.M. Straubinger, G. Rule, E.L. Springer, D. Papahadjopoulos, Biochemistry 20 (1981) 6978–6987.
[25] S.H. Koenig, F.A. Quet, R.D. Brown III, M. Lafleur, M. Spiller, E. Unger, C. Tilcock, Magn. Reson. Med. 23 (1992) 275–286.
[26] M.S. Webb, D.V. Lynch, B.R. Green, Plant Physiol. 99 (1992) 912–918.
[27] M.H. Nouri-Sorkhabi, B.E. Chapman, D.R. Sullivan, P.W. Kuchel, Magn. Reson. Med. 32 (1994) 505–510.
[28] A.R. Waldeck, M.H. Nouri-Sorkhabi, D.R. Sullivan, P.W. Kuchel, Biophys. Chem. 55 (1995) 197–208.
[29] T.X. Xiang, B.D. Anderson, J. Membr. Biol. 148 (1995) 157–167.
[30] T.X. Xiang, B.D. Anderson, Biophys. J. 72 (1997) 223–237.
[31] G. Cevc, D. Marsh, Phospholipid Bilayers, John Wiley and Sons, Toronto, 1987.
[32] W.K. Subczynski, A. Wisniewska, J.J. Yin, J.S. Hyde, A. Kusumi, Biochemistry 33 (1994) 7670–7681.