Kinetics of Proton-Hydroxyl Transport across Lecithin Vesicle Membranes as Measured with a Lipoid pH-Indicator

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When unilamellar vesicles are prepared in the presence of 3-Palmitoyl-7-oxy-coumarin (abbreviation 3P-UBF) this lipoid pH-Indicator is anchored by its fatty acid chain to the membrane and can be used to measure pH-changes at the outer and inner membrane surface (ranging from pH 5 to pH 9.5). By rapidly-changing the pH of the outer aqueous phase a pH-gradient is set up across the vesicle membrane. The rate of the subsequent H^+ or OH^- influx into the vesicles can be measured as a change of the 3P-UBF absorbance at 424 nm. This was done with a stopped-flow-spectrophotometer at temperatures between 10 °C and 50 °C.

Suspensions of vesicles prepared from egg-lecithin or L-dipalmitoyl-lecithin were investigated in buffered salt solutions. The influence of Na^+, K^+, Cl^-, SO_4^{2-} and valinomycin on the rate of absorbance changes was studied at different temperatures.

It was found that the rate of the pH-equilibration between the aqueous phase outside and inside the vesicles depends on the direction of the pH-gradient. This new result together with a high H^+/OH^- permeability of vesicle membranes found in recent studies from other laboratories and confirmed by this investigation is interpreted to indicate a higher permeability of the vesicle membrane to OH^--ions compared to H^+-ions. (Calculated values are: $P_{OH} = 1.4 \times 10^{-4}$ cm/s at pH 9 and $P_H = 8.3 \times 10^{-7}$ cm/s at pH 5 and 20 °C.) All data described in the literature in detail agree with this suggestion but a pH-dependence of P_{OH} and P_H cannot be excluded.

Introduction

The transport of ions across biological membranes plays a central role in proper cellular function. Specific ion gradients are known to be necessary for important processes *e. g.* nerve impulse transmission or energy storage. Proton and hydroxyl transport across cellular membranes is generally accepted to drive ATP-synthesis according to the chemiosmotic theory [1]. In addition a number of intracellular processes may be strongly influenced by the intracellular pH- *e. g.* enzyme function and regulation, cell division and growth. Therefore it seems essential to understand mechanisms by which proton gradients are built up, maintained and dissipated. Because the lipid bilayer represents the major barrier to ionic permeation in most biological membranes, lipid model membranes my be used to study passive proton-hydroxyl transport. Large [2, 3] and small [4, 5] vesicles and planar bilayer membranes [6] have been used recently as model systems. pH-changes occurring on one side of the membrane after changing the pH on the other side of the membrane were measured with a glass electrode [3, 6] or with water soluble pH-indicators [2, 4, 5, 7]. The finding of these studies that vesicles show an unexpectedly high H^+/OH^- permeability compared to planar lipid membranes is very interesting in view of the biological importance of pH-gradients as mentioned above. A mechanism explaining all aspects of passive H^+/OH^- transport across lipid bilayers is not known until now. Therefore it seems useful to study the problem using different methods. One possibility which was chosen in this investigation is the use of probes bound to the membrane surface. In an earlier study [8] it has been found with the lipoid pH-indicator 4-pentadecyl-7-oxy-coumarin (4P-umbelliferon, abbreviation C_{15}-UBF) that pH-equilibration across vesicle membranes occurs within < 30 s. Meanwhile a new lipoid pH-indicator 3P-UBF (3-palmitoyl-7-oxy-coumarin or -umbelliferon) has been synthesized [9] which has some advantages compared to C_{15}-UBF and was used in this study.

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Materials and Methods

Chemicals

Egg-phosphatidylcholine (egg-PC) was prepared by K. Janko (University Konstanz). Dipalmitoyl-phosphatidylcholine (DP-PC) was purchased from Sigma Chem. Comp. Inorganic salts of analytical grade and buffer solutions were products from Merck, Darmstadt. 3P-UBF was synthesized by H. Alpes (University Konstanz). Valinomycin (abbr. VAL) was obtained from Calbiochem.

Vesicle preparation and stationary spectral measurements

20 mg lecithins and 0.1 mg 3P-UBF were mixed in ethanolic solution and dried under a stream of N_2. 2 ml of a salt solution containing either 0.1 M NaCl, KCl, Na_2SO_4, K_2SO_4 and 10 mM citrate buffer (pH 5) or 10 mM carbonate-bicarbonate buffer (pH 9.5) were added to the lipid. The sonication procedure has been described elsewhere [10, 11]. After about 60 min sonication the vesicles were diluted to 20 ml with the same buffer solution used for sonication. The resulting vesicle suspension will be called pH 5-vesicles and pH 9-vesicles. Absorption spectra were measured as described [11]. The vesicle suspensions were stable at least for one day. The absorption maxima of 3P-UBF decreased by about 10% during this period. Typical spectra are shown in Fig. 1 of the results section.

Kinetic measurements by stopped-flow spectrophotometry

Measurements were done on a Durrum instrument modified by H. Merkle in the group of P. Kroneck (University Konstanz) and on an apparatus constructed by the group of S. Ghisla (University Konstanz). The setups had a dead time of about 2 and 3.5 ms and were used in the absorption mode at 424 nm (bandwidth about 6 mm). Signals were stored with a Nicolet-Signal-Averager or a storage oszilloscope and plotted with a Hewlett-Packard or Servogor X-Y-recorder. By two 2 ml sample syringes vesicle suspensions were mixed with buffer solutions in a volume ratio 1:1. Before stopped-flow measurements were done the pH of the buffer solutions were adjusted such that the final 1:1 mixtures had either a pH between 9.5 and 10.0 or between 5.0 and 5.5. Stopped-flow experiments are designated by the pH of the initial vesicle preparation and the pH of the final mixture (*e.g.*: $5 \rightarrow 9$ corresponds to an initial pH ~ 5 and final pH ~ 9.5).

Results

Fig. 1 shows the absorption spectra of 3P-UBF incorporated into egg-PC vesicles and its dependence on the pH of the aqueous phase. The structure of the chromophore is also shown. When the hydroxyl-group is deprotonated the absorbance at 424 nm increases. The schematic drawing shows the incorporation of the chromophore into the vesicle membrane. Probe molecules on the outer surface will indicate the outside pH, probe molecules on the inside the inside pH. The "outside population" will react faster after a sudden change of the outside pH. All experiments support this notion. Fig. 2. shows some results from typical experiments.

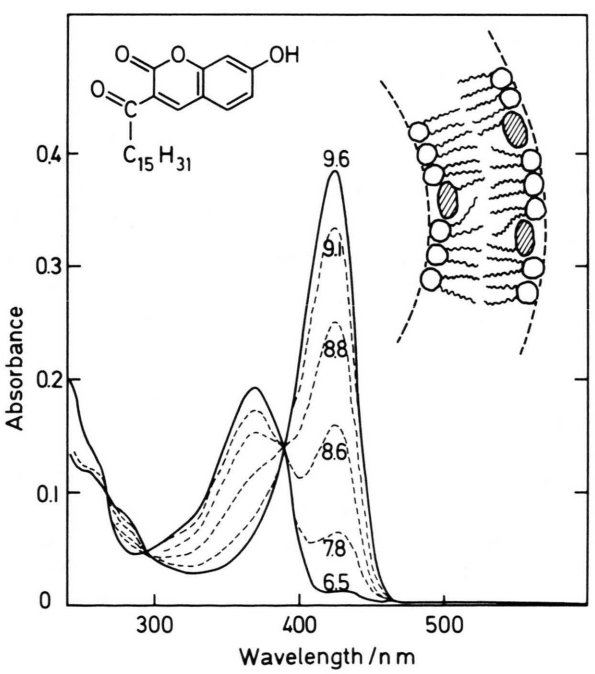

Fig. 1. Absorption spectra of 3-palmitoyl-7-oxy-coumarin (3P-UBF) in egg-PC-microvesicles. The pH of the aqueous phase is indicated. Upper left: structure of 3P-UBF. Upper right: schematic section of a vesicle showing the incorporation of the probe.

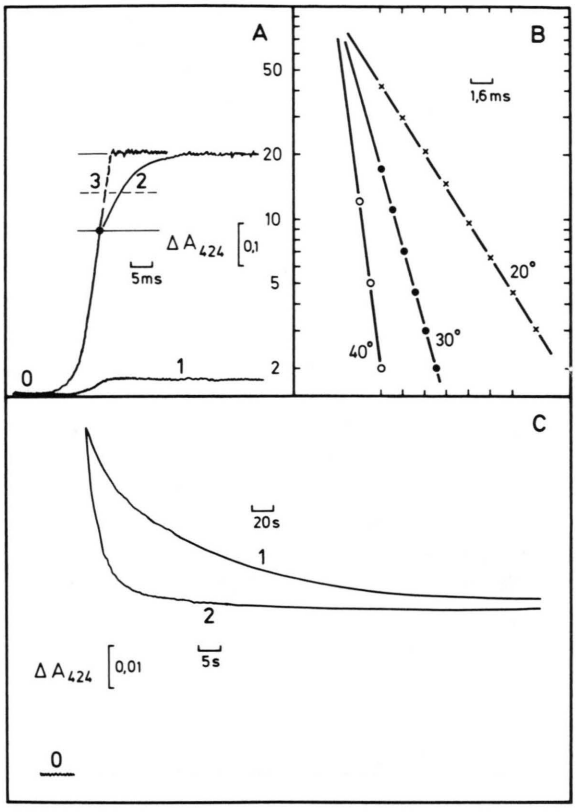

Fig. 2. A, B: Absorbance change at 424 nm during pH 5 → 9 experiments. For details see section "Results". All egg-PC-vesicles. A: Line 0 is the absorbance of pH 5 buffer. Curve 1: pH 5-vesicles were mixed with pH 5-buffer. Curve 2: pH 5-vesicles were mixed with pH 9-buffer. Curve 3: pH 9-vesicles were mixed with pH 9-buffer. B: Semilogarithmic plot of curve 2 taken at different temperatures as indicated. C: Absorbance change at 424 nm during pH 9 → 5 experiments. Line 0: buffer without vesicles. Curve 1: pH 9-K_2SO_4-vesicles were mixed with pH 5-K^+-buffer. Curve 2: the same experiment after addition of valinomycin (VAL:PC = 1:60).

pH 5 → 9 experiments (Fig. 2 A, B)

When vesicles are prepared in buffer solutions at pH 5 and mixed with high pH buffer to increase the outside pH suddenly to about 9.5 the absorbance at 424 nm increases biphasic to its final value. 65% of the increase occurs within the dead time of the stopped flow spectrophotometer. 35% of the increase occurs exponentially in the ms-range in egg-PC vesicles. This result is interpreted to show 3P-UBF at the vesicle outside and inside respectively, sensing the outside and inside pH. The rate of the absorbance change was the same in buffers containing Na^+ or K^+ as predominant cations and Cl^- or SO_4^{2-} as predominant anions. It was not influenced by the addition of VAL (50 μM). The temperature dependence of the absorbance increase is summarized in Table I. Above 40 °C the process became too fast to be resolved. In DP-PC vesicles the rate was only measured in buffers containing 0.1 M K_2SO_4. At 20 °C it was about 1000 times slower than in egg-PC vesicles (see Table I). The absolute absorbance apparently increased at temperatures above 30 °C. Experiments with VAL could not be done because the DP-PC vesicles precipitated.

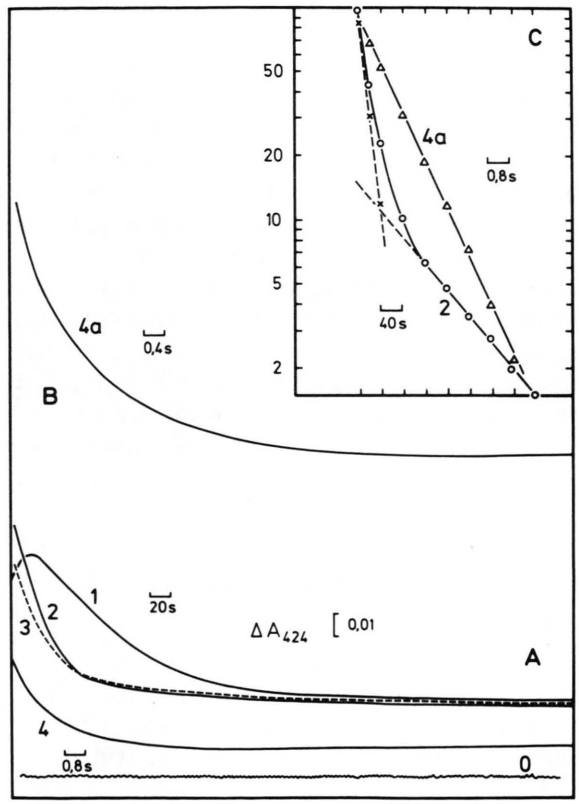

Fig. 3. Absorbance change at 424 nm during pH 9 → 5 experiments. Influence of Na^+, K^+ and valinomycin. All egg-PC-vesicles. A: Line 0 from buffer without vesicles. Curve 1: pH 9-Na_2SO_4-vesicles were mixed with pH 5-Na^+-buffer. Curve 2: as 1 but in the presence of valinomycin (1:25 PC). Curve 3: as 2 but Na^+-vesicles mixed with K^+-buffer. Curve 4: pH 9-Na_2SO_4-vesicles were incubated with pH 9-K_2SO_4 buffer (1:1) and than mixed with pH 5-K_2SO_4-buffer (note faster time scale). B: Curve 4a: as A curve 4 but 3 experiments summed in the signal-averager and faster time scale. C: semilogarithmic plot of A curve 2 and B curve 4a. Note that 4a can be seperated in a fast ($t_{1/2}$ = 15 s) and a slow ($t_{1/2}$ = 96 s) component.

Table I. Rate of the absorbance increase when pH 5-vesicles are rapidly brought to pH 9.5[a]. Dependence of $t_{1/2}$[b] from type of lipid, salt and temperature.

Egg-PC-vesicles			Temp.	DP-PC-vesicles	
NaCl or KCl	K_2SO_4	K_2SO_4 + VAL	[°C]	K_2SO_4	K_2SO_4 + VAL
–	9.5 ± 2.0 ms	–	10	–	
3.5 ± 0.5 ms	3.0 ± 0.5	3.2 ± 0.8 ms	20	4.0 ± 1.0 s	vesicles
–	–	1.7 ± 0.5	25	1.5 ± 0.5	aggregate
–	1.3 ± 0.5	1.1 ± 0.5	30	0.4 ± 0.1	
–	–	0.8 ± 0.5	35	–	
–	0.6 ± 0.3	0.4 ± 0.3	40	3.0 ± 1.0 ms	
	not resolved	–	50	≪ 1 ms	

[a] Vesicle suspensions were prepared in aqueous solutions containing 0.1 M salts, which are given above and 10 mM citrate buffer pH 5. The suspensions contained 1 mg PC/ml and 5 µg 3P-UBF/ml. When VAL was present it was added in a ratio VAL:PC = 1:25 during the sonication. Vesicle suspensions were rapidly mixed in the stopped-flow-apparatus with buffers of the same salt concentration but with 10 mM carbonate-bicarbonate buffer. The pH was adjusted such that the final 1:1 mixture had a pH of about 9.5 (see Methods).
[b] $t_{1/2}$ was determined from semilogarithmic plots of the final absorbance increase (see Fig. 2B). The limits of error given above are derived from different vesicle preparations (at least 3). Consecutive "shots" with the same vesicles are much better reproducible (< 5%).

pH 9 → 5 experiments (Fig. 2C, Fig. 3, Fig. 4)

When vesicles are prepared in buffer solutions at pH 9.5 and mixed with low pH buffer to decrease the outside pH suddenly to about 5 the absorbance at 424 nm decreases biphasic to its final value. 85% of the decrease occurs within the dead time of the instrument. 15% of the decrease occurs in the range of seconds up to 3 min in egg-PC vesicles. Again this is interpreted to show the pH-change of the outside respectively inside aqueous phase. The rate of the absorbance change depended on the ionic composition of the buffer. It was slower with Na^+ as predominant cation compared to K^+ and slower with SO_4^{2-} as predominant anion compared to Cl^-. Under certain conditions the absorbance decrease is triphasic. After the initial very fast decrease a small increase occurs which is followed by the final slow decrease to the end value (Fig. 3A curve 1, Fig. 4A curves 1 and 2, Fig. 4B curves 3 and 4). The same observation has been made by Clement and Gould [4a] who described it as "noninstrumental overshoot". In our experiments the phase of slow increase disappeared at higher temperatures or in the

Table II. Rate of the absorbance decrease when pH 9-vesicles are rapidly brought to pH 5[a]. Dependence of $t_{1/2}$ (in s)[b] from type of salt and temperature. Only egg-PC-vesicles are shown in this table.

Temp. [°C]	NaCl	Na_2SO_4	Na_2SO_4 + VAL	Na_2SO_4 + VAL + K_2SO_4[c]	Na_2SO_4 + VAL + K_2SO_4[d]	K_2SO_4	K_2SO_4 + VAL
20	6 ± 4	88 ± 5	30 ± 5	26 ± 5	1.04 ± 0.10	54.4 ± 5.0	0.50 ± 0.05
25	–	51 ± 2	see Fig. 3A		–	–	–
30	–	35 ± 2	curve 2	curve 3	0.50 ± 0.10	24.0 ± 2.0	0.23 ± 0.03
35	–	21 ± 2			–	–	–
40	–	13 ± 2			0.18 ± 0.05	11.2 ± 2.0	0.11 ± 0.01
45	–	9 ± 2			–	–	–
50	–	6 ± 2			0.09 ± 0.05 see Fig. 3A, 3B curves 4, 4a	–	–

[a] Vesicles were prepared as described in Table I and in the section "Materials and Methods". Vesicle suspensions and mixing buffers were 0.1 M in the salts given above, except in the cases described under [c] and [d].
[b] $t_{1/2}$ is the time necessary for half of the absorbance change between the maximal absorbance on the curve and the final value. This was done because most curves are not simple exponential.
[c] Vesicles prepared in Na_2SO_4-buffer pH 9.5 were mixed with K_2SO_4-buffer pH 5 in the stopped-flow-apparatus.
[d] Vesicles prepared in Na_2SO_4-buffer pH 9.5 were mixed 1:1 with K_2SO_4-buffer pH 9.5 and incubated for 10 ± 5 min. After this incubation they were mixed in the stopped-flow-apparatus with K_2SO_4-buffer pH 5.

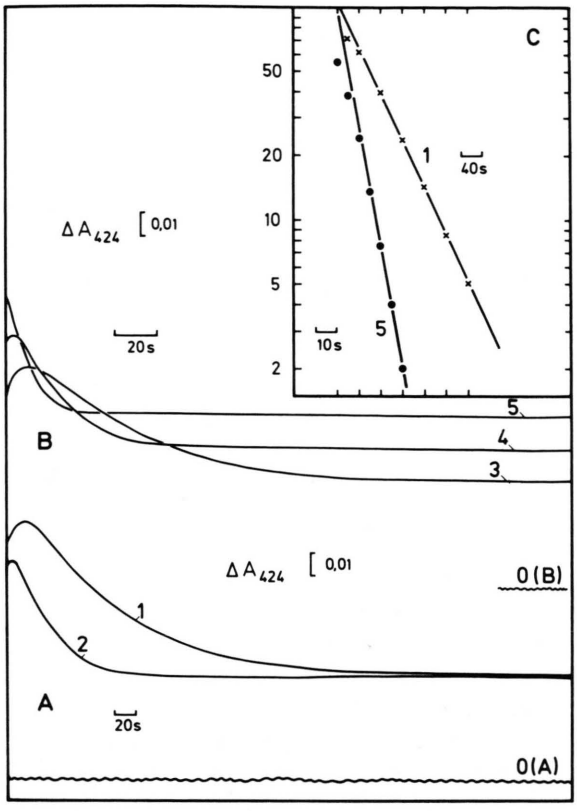

Fig. 4. Absorbance change at 424 nm during pH 9 → 5 experiments. Influence of the temperature. All egg-PC-vesicles. Lines 0: from buffer without vesicles. A: Curve 1: pH 9-Na_2SO_4-vesicles were mixed with pH 5-Na^+-buffer at 20 °C. Curve 2: at 30 °C. B: as A but at a different time scale. Curve 3: at 25 °C. Curve 4: at 35 °C. Curve 5: at 45 °C. C: semilogarithmic plot of curves 1 and 5.

presence of VAL (Fig. 3A curves 2, 3 and 4, Fig. 4B curve 5). In both cases the rate of the absorbance decrease became faster. The absolute absorbance apparently increased with increasing temperature while the total slow absorbance change remained constant. VAL had only a small effect when the predominant cation was Na^+ but the rate was increased up to 100 times in K^+ buffers (Fig. 2C curve 2, Fig. 3A curve 4, 3B curve 4a). When vesicles were prepared in pH 9.5-Na^+ buffer in the presence of VAL and mixed with pH 5-K^+-buffer (Fig. 3A curve 3) only a small rate change was observed. When the pH 9.5-Na^+-vesicles with VAL were mixed with pH 9.5-K^+-buffer, incubated 5 min and afterwards mixed with pH 5-K^+-buffer, the fast rate as observed in the all K^+-system was found (Fig. 3A curve 4, 3B curve 4a).

These observations clearly support the postulation of Gould, Clement and Biegel [4, 5] that the slow phase of the internal pH-decrease comes from a counterion-limited electroneutral proton-counterion exchange. The influence of Na^+, K^+, VAL and temperature on the rate of absorbance decrease is summarized in Table II. Experiments with DP-PC-vesicles were only done with buffered K_2SO_4 solutions. The results with identical vesicle suspensions prepared at different times did not agree as well as in the case of egg-PC-vesicles. Qualitatively the absorbance decrease seemed triphasic between 20 °C and 35 °C. An initial fast absorbance decreases in the ms-range was followed by a small increase in the s-range and a very slow final decrease which could not be followed completely. At the phase transition (40 °C) and above the initial fast decrease was absent. It may be speculated that the initial process is the protonation of 3P-UBF at the outside of the vesicles because the chromophore in the gel state might be not as easily accessible to protons as in the liquid crystalline state.

Interpretation and Discussion

In the following calculations it will be assumed that all vesicle suspensions used in our investigation have the following properties:

1. Each vesicle consists of ~ 3000 PC-molecules, the outer monolayer contains ~ 2000, the inner monolayer ~ 1000 PC-molecules.

2. The outer radius of each vesicle is 12.5 nm, the inner radius 8.5 nm and the bilayer is 4 nm thick. This gives an inner volume $v_i = 2.6 \times 10^{-18}$ cm³ per vesicle. These values are slightly simplified from the original data of Huang [12] for egg-PC-vesicles. DP-PC-vesicles kept at 20 °C for several hours will be larger [10, 13].

3. The aqueous phase inside the vesicles has the same composition as the outside aqueous phase when the sonication procedure is finished. A straightforward calculation shows that a vesicle will on the average contain 1.5 particles of a substrate which is dissolved in the aqueous phase at a concentration of 1 mM. Since in the pH-range studied concentrations of protons and hydroxylions are much lower, this poses a very interesting problem from the theoretical point of view. It is assumed here that the presence of buffering substances (added salts and

3P-UBF) determine a meaningful pH inside the vesicles.

4. The probe 3P-UBF is completely incorporated into the vesicle membranes. This is supported by the finding that the probe cannot be seperated from vesicles by gel filtration. One probe molecule is incorporated per 100 lecithins and each vesicle contains 30 probe molecules. This does not change the vesicle structure or the H^+/OH^- permeability of the vesicle membrane. The probe exists only in two forms as given by the equation $ROH + OH^- \rightleftharpoons RO^- + H_2O$. The spectra of the protonated form ROH and the deprotonated form RO^- are shown in Fig. 1. It is assumed that Lambert-Beers law is applicable for these species. This assumption is probably too simple for DP-PC vesicles below the phase transition when probe molecules apparently aggregate. The difficulty can be circumvented by decreasing the probe concentration. This will be easily possible when the fluorescence of 3P-UBF will be used instead of its absorbance. When these experiments were done only the absorbance could be measured with the stopped-flow-apparatus.

The pH 5 → 9 experiments described in the text, Table I and Fig. 2A, B are interpreted as follows. From 30 3P-UBF molecules incorporated into each pH 5-vesicle about 20 are on the outside and about 10 on the inside. At pH 5 the number of free H^+ and OH^- ions inside of one vesicle is very small (see point 3 above). When the pH is increased outside OH^- ions will diffuse into the vesicles and deprotonate 3P-UBF. As the process seen is mainly due to OH^- flux its time course can be used to calculate the OH^- permeability of the vesicle membrane. Ignoring electrical forces the change of the inside concentration of OH^- from the initial value c_i to the final value c_0 which is equal to the outside concentration is described by the following equation:

$$c_i = c_0(1 - e^{-t/t_{1/2}}) . \tag{1}$$

For spherical vesicles $t_{1/2}$ is given by:

$$t_{1/2} = \frac{r}{3\,P_{OH}} . \tag{2}$$

From the known vesicle radius r and the measured $t_{1/2}$ we can calculate the hydroxyl-permeability P_{OH}. The value at 20 °C is:

$$P_{OH} = \frac{12.5 \times 10^{-9}\ \text{m}}{3 \cdot 3 \times 10^{-3}\ \text{s}} = 1.4 \times 10^{-6}\ \text{m/s} .$$

This is equal to the combined proton-hydroxyl permeability $P_{net} = P_{OH} + P_H$ as measured by Nichols *et al.* [2, 3]. These authors determined $P_{net} = 1.4 \times 10^{-4}$ cm/s.

This possibility of an extremely high hydroxyl-permeability of vesicle membranes is a completely new aspect. Although the use of (1) and (2) may be an oversimplification the obtained result is worth considering it as likely. As we will see later it may explain several findings which were difficult to understand until now. The only point which does not fit into the picture is that Clement and Gould [4a] mentioned: "a very similar pattern of fluorescence changes, of opposite direction, was also observed in vesicle suspensions equilibrated at mildly acidic pH and pulsed with NaOH (data not shown)." But since this quotation is all what they reported it is impossible to decide wether any contradictory results exist at all*. It will be very interesting to compare directly the methods of water soluble and membrane bound pH-indicators.

The pH 9 → 5 experiments described in the text, Table II and Figs. 2C, 3 and 4 are interpreted as follows. From 30 3P-UBF molecules incorporated into each pH 9-vesicle about 25 are on the outside and about 5 on the inside. This may be due to the negative charge of the probe at this pH which could favour the incorporation on the larger outside surface of the vesicle. It may also be that this difference compared to pH 5-vesicles results from an incorrect extrapolation of the absorbance changes to time zero. The absolute value of the absorbance change is not a very important point because we are interested in the proton-hydroxyl-transport which is given by the rate of the absorbance change.

The time course of the absorbance decrease is in general agreement with the work of Clement, Gould and Biegel [4, 5]. On the average the $t_{1/2}$ found in the absence of VAL in this investigation are slightly shorter compared to their values. These small differences may be due to the different lipid composition of the vesicle preparations or to the relatively high 3P-UBF content of my vesicles. As mentioned before it will be possible to reduce the 3P-UBF content considerably by using its fluorescence instead of the absorbance. Following the same arguments as above the absorbance decrease is mainly due to the H^+-influx into the vesicles. As suggested

* See Note added in proof.

by Biegel and Gould [5] the H^+-influx seems limited by the rate of charge-compensating counterion-redistribution. VAL reduces $t_{1/2}$ very effectively when K^+ is present inside and outside of the vesicles. Under these conditions the H^+-influx can be compensated by the K^+-efflux. When the K^+ and H^+ concentration on the outside are increased at the same time $t_{1/2}$ is only slightly influenced (see Fig. 3A curves 2 and 3 or Table II) because a K^+-efflux is not possible in this case. When the K^+ concentration is increased first and the vesicles incubated for 10 ± 5 min, K^+ will equilibrate between the inner and outer aqueous phase. An increase of the outside H^+ concentration is now followed by a faster H^+-influx indicated by a 30 times shorter $t_{1/2}$ (Fig. 3A curve 3 and 4). Under these conditions a K^+-efflux is possible. The small decrease of $t_{1/2}$ in all-Na^+-vesicles with VAL compared without VAL may come from a small Na^+-flux mediated by VAL or from a structural change in the vesicles (by the high VAL content, 1 VAL per 25 PC). The decrease of $t_{1/2}$ in NaCl solutions compared to Na_2SO_4 solutions (see Table II) may be explained by the high permeability for HCl [6]. In the presence of K^+ and VAL $t_{1/2} = 0.50$ s at 20 °C and 0.23 s at 30 °C were obtained in excellent agreement with the value 0.3 s at 25 °C given by Biegel and Gould [5]. Using equation (2) we obtain at 20 °C:

$$P_H = 8.3 \times 10^{-9} \text{ m/s.}$$

P_H is about 150 times smaller than P_{OH} and can be neglected when calculating P_{net}.

This result can be compared with results from other groups found with different methods. P_{net} given by Nichols *et al.* [2] and obtained in this study is 1.4×10^{-4} cm/s. The equation given by Nichols and Deamer [3] yields J_{net} when electrical forces are ignored for pH 9 → 5 experiments:

$$J_{net} = P_H([H^+]_o - [H^+]_i) + P_{OH}([OH^-]_i - [OH^-]_o) .$$

Inserting the values for pH 9.5, pH 5, P_H and P_{OH} we calculate:

$$J_{net} = J_H + J_{OH} = 8.3 \times 10^{-15} + 4.4 \times 10^{-12} \text{ mol/cm}^2 \text{ s} .$$

Since the number of ions available inside the vesicles is extremely small the main contribution to the observed J_{net} comes from an ion-influx as discussed before. This is J_H ($= 8.3 \times 10^{-15}$ mol/cm^2 s) in the pH 9 → 5 experiments or J_{OH} ($= 4.4 \times 10^{-12}$ mol/cm^2 s) in the pH 5 → 9 experiment. Comparing these values with Fig. 3 of Nichols and Deamer [3] they seem to fit with the line calculated for $P_H = 0$ above pH 7 and better with the curve calculated for $P_{OH}/P_H = 10$ at pH 6 and 5. This supports the suggested pH dependence of P_H and P_{OH}. In general P_{OH} will be larger as P_H. This may also have implications on the fast and slow kinetic components described by Clement and Gould [4a]. An OH^--efflux gives the same effect as a H^+-influx. The fast component may be caused by an OH^--efflux. It seems clear that its contribution would be more prominent at an internal pH 8.2 compared to 7.2. Since the biphasic fluorescence decrease disappeared in the rapid-mixing experiment [5] an alternative explanation will be given (see later in the discussion).

Another experimental result is in good agreement with my suggestion. Crandell *et al.* [14] derived from their studies of erythrocyte membranes a $P_{OH} = 2 \times 10^{-4}$ cm/s at pH 9 and made a much lower P_H likely. Finally the investigations of Gutknecht and Walter [6] showed that planar lipid bilayer membranes showed OH^- selectivity at high pH whereas no H^+ selectivity was found at low pH. These authors reported a $P_H = 3 \times 10^{-9}$ cm/s which is about 250 times smaller than the value given above for vesicles. This difference between planar solvent containing and highly curved solvent-free bilayer membranes seems plausible. Gutknecht and Walter could not measure P_{OH} but give an upper limit of 6×10^{-7} cm/s. In an earlier paper on Ca-flux across planar bilayer membranes [7] we have observed a pH-increase at one side of the membrane when KOH was added to the other side. We were not directly concerned with hydroxyl-permeability in that paper but when the old data are used now to calculate P_{OH} values between 10^{-5} and 10^{-7} cm/s result. Probably in these experiment the OH^--flux became electroneutral by the simultaneous Ca^{2+}-flux. Therefore P_{OH} is larger than in the electrogenic case [6]. Nevertheless both results indicate that also in planar bilayer membranes $P_{OH} > P_H$ applies at high pH. This would also explain the instability of these membranes at high pH [6]. A high P_{OH} means a relatively high concentration of OH^- within the membrane. An accumulation of negative charges will increase the probability of rupture. If this argument also applies to vesicles is unknown and should

be tested by studying the leakiness of vesicles for water soluble markers (*e. g.* glucose) at pH 9–10.

I want to discuss now the question if the interpretation of the results as outlined above is valid and compare the advantages or disadvantages of membrane-bound versus water soluble pH indicators. In agreement with all data in the literature the H^+/OH^- permeability is found much higher than the values determined for other single cations or anions which are in the range of 10^{-10} to 10^{-14} cm/s. The new conclusion that $P_{OH} \gg P_H$ does not seem in conflict with any published experiment. The sum of P_{OH} and P_H as calculated above agrees exactly with P_{net} as determined in a similar vesicle system by Nichols *et al.* [2, 3]. As these authors suggested one or both of the coefficients P_{OH} and P_H may be pH dependent so that the values given above may only be true at high pH for OH^- ions and at low pH for H^+ ions. This problem remains to be solved by further investigations. Another question which has been mentioned before is the possible influence of 3P-UBF on the membrane permeability. It will be possible to reduce the 3P-UBF concentration by a factor of 30 when the fluorescence is measured instead of the absorbance. This should be enough to detect permeability changes introcuded by the probe if there are any.

Comparing the method described in this paper with the pyranine-method [4, 5] the latter seems to be a more direct approach to measure the intenal aqueous hydrogen ion concentration in phospholipid vesicles. This is because one would expect pyranine to probe the aqueous phase directly whereas 3P-UBF probes a region close to the membrane surface with a pH different from the bulk aqueous phase. On the other hand it seems likely that a substantial part of pyranine is adsorbed to the vesicle membrane which would be a second population of probe molecules compared to only one population of membrane-bound 3P-UBF molecules. Although Clement and Gould [4a] suggest that all pyranine molecules associated with the vesicle fraction are free in the inside solution they present data which could also indicate a substantial portion of pyranine molecules adsorbed to the membrane: when vesicles were prepared without pyranine, pyranine added afterwards to the external buffer and the vesicles passed through a Sephadex column they still showed 4–9% of the fluorescence [4a] which was found in vesicles prepared with pyranine, after removal of nonincorporated probe. Since membrane bound indicators show an alkaline shift in the pK_a [8] (about 2 pH units) this residual fluorescence may indicate that about the same amount is adsorbed as is free in solution*. Also the "bulk" concentration of 100 μM pyranine [5] found in vesicles prepared in a buffer containing 2.5 mM pyranine after removal of the nonincorporated probe by gel filtration seems to high to exclude adsorption of the probe in addition to the probe free in the internal aqueous phase. Summarizing these points it seems that the methods of water soluble and membrane-bound indicators have their advantages and disadvantages. Probably the use of both methods will yield an optimal chance to understand H^+ and OH^--transport through lipid bilayer membranes.

Concerning the possible mechanism of the high H^+/OH^- permeability of vesicles the theory of hydrogen bonded chains transversing a nonaqueous region was put forward by Nagle *et al.* [15] following the basic idea of von Grotthus who explained in 1805 the conductivity of electrolytes by a similar mechanism. This idea of "proton wires" seems very reasonable since it is known that water molecules are more ordered in a hydrophobic environment than elsewhere in solution. The theory has been used by Nichols, Deamer, Clement, Biegel and Gould [2–5] to explain the high H^+/OH^- permeability of vesicles. Transmembrane, hydrogen-bonded H_2O-chains seem to be equally suitable for H_2O, H^+ and OH^- transport It is not surprising that the neutral H_2O molecule has the highest permeability ($P_{H_2O} = 2 \times 10^{-3}$ cm/s). The question is, if the large difference in the OH^- and H^+ permeability suggested in this paper, can be explained in the framework of the "proton-wire-theory". It seems possible that a rearrangement in the hydration-shell of the hydroniumion may be the rate-limiting step for the entrance of H^+ into the hydrogen-bonded H_2O chains or that a negative charge can be more evenly smeared across oriented molecules than a positive charge. Both effects could favour the transport of OH^--ions compared to H^+-ions.

An alternative mechanism for the H_2O, and H^+/OH^- permeability may be the "kinks" discussed by Träuble [16] for neutral, small molecules. If these structural defects can also be intrinsic carriers for hydronium- and hydroxylions has not been investi-

* See Note added in proof.

gated. In combining both views a kink-defect could be envisaged as an incomplete hydrogen-bonded H_2O-chain, which does not reach across the total thickness of the membrane but is shorter and moves across by diffusion. These defects would presumably show a size distribution. Neutral H_2O molecules would be transported in defects of all sizes, OH^- could be transported in small defects which occur more frequently and H^+ only in larger defects which appear rather seldom.

Speculations about the biological significance of $P_{OH} \gg P_H$ seem premature at the present time. It will be necessary at first to proof the suggestion made in this paper by comparative studies with all methods available for the measurement of H^+/OH^- transport across lipid membranes.

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Note added in proof:

I want to thank Drs. Deamer, Gould and Teissié for helpful and critical comments which I received when this article was in press. Dr. Deamer sent me a review chapter on "Proton Permeability in Biological and Model Membranes" to be included in a book on intracellular pH which he is editing. From this review I learned about two papers by Cafiso and Hubble [17] and Nozaki and Tanford [18] who have also studied the proton permeability of vesicles. These authors reported smaller values for P_H. Though arising considerable controversy about the "real" values of the permeabilities the latter paper reports $P_{OH} \gg P_H$ [18] which is supporting my results. Dr. Gould communicated several unpublished and published [19, 20] results which clearly show that pyranine does *not* bind significantly to anionic but to cationic vesicle membranes. My suggestion of a free and adsorbed population of pyranine is therefore unjustified. Dr. Gould has also "consistently noticed that the rate of transmembrane pH equilibration is faster after a NaOH pulse than after an HCl pulse". This supports my suggestion that $P_{OH} > P_H$. Dr. Teissié reminded me that neglecting electrical (surface potential or Nernst potential) and structural effects (influence of the lipid head group, which we have studied before [21, 22]) may be an oversimplification. This should be studied as well as the possibility that 3P-UBF might act as a protonophore.

Finally I want to acknowledge the letter or Dr. Gutknecht, who sent me reprints of his recent research on H^+/OH^- Transport through planar lipid bilayer membranes [23, 24]. He found that P_{H+} at low pH and P_{OH-} at high pH are of similar size, about 10^{-9} cm/s. The lower value of P_{H+} compared to my results has been discussed above. Gutknechts value for P_{OH-} is about five orders of magnitude smaller than my value reported above. One reason for this difference might be the composition of the lipid bilayers (egg-PC : cholesterol 1 : 1 molar ratio in tetradecane) which was the only mixture forming stable membranes above pH 11. It may well be that the presence of cholesterol decreases the concentration of kink-like defects which were discussed above as a possible mechanism of OH^--transport. This should be studied in more detail, to find out if solventfree microvesicles generally show a higher H^+/OH^- permeability than solventfree macrovesicles [18] and planar bilayers containing solvents, as long as the lipid composition is the same in all three model membranes.