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Proton concentration jumps and generation of transmembrane pH-gradients by photolysis of 4-formyl-6-methoxy-3-nitrophenoxyacetic acid

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Proton concentration gradients across membranes are important for many biological energy transducing processes. The kinetics of proton dependent processes can be studied by pH-jump methods in which protons are photochemically released. In the following we describe the synthesis and the properties of photolabile 4-formyl-6-methoxy-3-nitrophenoxyacetic acid, a 'caged proton'. The synthesis is based on vanillin, which is alkylated with chloroacetic acid to give a carboxylic acid (pK = 2.72). In a second step a nitro group *ortho* to the formyl group is introduced. Photochemical proton release occurs by a reaction mechanism analogous to the well known photochemical formation of 2-nitrosobenzoic acid from 2-nitrobenzaldehyde. The pK values of the photoproduct are 0.75 and 2.76, respectively, thus allowing the use of the compound in a wide pH-range. The quantum yield is 0.18, lower than in the case of the 2-nitrobenzaldehyde / 2-nitrosobenzoic acid system (Φ = 0.5). The release of the proton in a flash photolysis experiment occurs within less than 1 μs. The spectrum of photolabile compound has absorption maxima at 263 nm and 345 nm, respectively. Its permeability across a lipid bilayer membrane is very low (permeability coefficient $P_d \approx 10^{-9}$ cm · s^{-1} at pH 8) so that transmembrane proton concentration gradients can be generated.

Introduction

The method of proton-concentration jumps may be used for the study of proton dependent biological processes. Conventional mixing techniques are often limited because of the difficulty of introducing the reactants to their sites of reaction in a time shorter than the characteristic time of the reaction under investigation. During the last years studies on ATP consuming reactions were carried out successfully using the technique of (laser-) flash photolysis for removing a protecting 2-nitrobenzyl group at the γ-phosphate position of ATP [1]. The reaction takes a few milliseconds at physiological conditions, and concentration jumps up to the millimolar concentration range were reached [2]. Following the work on caged ATP, 2-hydroxylphenyl-1-(2-nitro)phenylethyl phosphate, a caged proton, was synthesized [3] and successfully used for investigations on the bacterial flagellar motor [4].

Alternatively, proton pulses can be produced with intensive laser pulses applied to aromatic alcohols [5]. But only transient pulses (duration: 100–200 μs) can be obtained by this method and a stable acidification of the solution is not possible.

Another well established method for generating rapid concentration changes is the stopped-flow-technique. In this case the typical time limit is in the range of one or a few milliseconds. But many

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experimental problems can arise due to the high flow rates and the mechanical shear forces working on sensitive biological probes (cells, lipid vesicles, etc.).

Starting from the known photochemistry of 2-nitrobenzaldehyde (Fig. 1, Scheme 1) [6–8], we synthesized 4-formyl-6-methoxy-3-nitrophenoxyacetic acid (caged-H^+). This compound which has an ionized carboxyl group ($pK = 2.72$) has a low membrane permeability. Furthermore, a second absorption peak, compared to 2-nitrobenzaldehyde, shifted to longer wavelengths ($\lambda_{max} = 345$ nm) is observed.

For the determination of the quantum yield and time course of proton release we measured the absorption change of the pH-indicator phenol red after photolysis of caged-H^+. The ability of caged-H^+ to generate proton gradients across lipid membranes was tested using large unilamellar vesicles made by the detergent dialysis method [9,10]. Proton permeation across the vesicular membrane was monitored by a pH indicator encapsulated in the interior of the vesicle [11,12]. The permeability of caged-H^+ across lipid bilayers can be estimated by observing photoinduced protonation of the internal indicator after various incubation times.

Materials and Methods

Materials

Reagents for the synthesis and salts were purchased from Merck (Darmstadt, F.R.G.). Phenol red was obtained from Aldrich Chemicals (Steinheim, F.R.G.). 8-Hydroxypyrene-1,3,6-trisulfonic acid, trisodium salt (Pyranine) was from Molecular Probes (Junction City, OR, U.S.A.). Carbonylcyanide 4-trifluoro methoxyphenylhydrazone (FCCP) and valinomycin were obtained from Serva (Heidelberg, F.R.G.). *n*-Octyl-β-D-glucopyranoside (octyl glycoside) was purchased from Calbiochem. (Frankfurt, F.R.G.). Dioleoyl-α-L-phosphatidylcholine (DOPC) was obtained by Avanti Polar Lipids (Birmingham, AL, U.S.A.). Bio-Beads SM 2 adsorbent were obtained from Bio-Rad Lab. (München, F.R.G.) and were activated or regenerated by several washings with organic solvents and water [13]. Bovine serum albumin, essentially fatty acid- and globulin-free, was obtained from Sigma (München, F.R.G.). Dialysis tubings (Spectrapor 2, 10 mm flat width, Type 'high speed') came from Spectrum Medical Industries Inc. (Los Angeles, CA, U.S.A.).

Static spectra were recorded on a Lambda 5 UV/VIS spectrophotometer (Perkin Elmer). ^{1}H-NMR and ^{13}C-NMR spectra were obtained in C^2HCl_3 at 250 MHz and 500 MHz with tetramethylsilane as internal reference on a WM-250 spectrometer (Bruker).

Synthesis (cf. Fig. 1, Scheme 2)

Synthesis of 4-formyl-2-methoxyphenoxyacetic acid (II). To 17.0 g KOH (0.3 mol) in 60 ml H_2O were added 20.0 g vanillin (0.13 mol) and 12.5 g chloroacetic acid (0.13 mol) during stirring. The mixture was heated under reflux for 4 h, cooled to room temperature, acidified with conc. HCl, and the product collected by filtration. The solid was washed with water, and recrystallized from water and once from ethanol. Yield: 15.0 g, 55%; m.p. 178 °C; $C_{10}H_{10}O_5$ (210.2), mass spectrum: 210 $(M)^+$.

Synthesis of 4-formyl-6-methoxy-3-nitrophenoxyacetic acid, 'caged proton' (III). 5.0 g II was added at 0 °C to a mixture of 30 ml nitric acid (1.4) and 10 ml fuming nitric acid in protions by stirring. The mixture was stirred at 0 °C for 30 min and at room temperature for 15 min. The reaction mixture was then poured into 350 ml of ice water under stirring. The solid was collected by filtration, washed with cold water and dried to yield 5.5 g. This raw product was once recrystallized in ethanol. Yield: 5.0 g, 82%, yellow crystals; m.p. 180 °C; $C_{10}H_9NO_7$ (255.2). Analysis:

Calcd.:	C 47.1%;	H 3.5%;	N 5.5%
Found:	C 47.1%;	H 3.6%;	N 5.3%.

Thin-layer chromatography gave one spot in different solvents, on Silica Gel (Merck, Darmstadt, F.R.G.) precoated plates.

Absorption maxima: 263 nm ($\epsilon = 15\,490$), 345 nm ($\epsilon = 5\,130$) at pH 5.

^{1}H-NMR: (d_6-DMSO): CH_3 $\delta = 4.02$, CH_2 5.02, aryl 7.41, 7.69, HC = O 10.23, COOH 13.3.

^{13}C-NMR: (d_6-DMSO): CH_3 $\delta = 57.9$, CH_2 66.9, C-6 110.2, C-3 111.9, C-4 126.7, C-5 144.5, C-2 151.7, C-1 154.1, COOH 170.5, HC = O 189.7 (gated decoupling).

The pK values of compounds (III) and (IV) were determined spectroscopically [14]: (III) has a pK of 2.72 ± 0.17. The pK values of the photoproduct (IV) were determined to be 0.75 ± 0.05 and 2.76 ± 0.25, respectively. The caged-H^+ (III) is soluble in aqueous solutions up to 300 mM (at pH 8).

Optical measurements

For the photolysis of the samples an excimer laser was used (Lambda Physik, Model EMG 100, XeCl-line: $\lambda = 308$ nm, $E_{max} = 100$ mJ/flash). Time-resolved measurements were carried out with a home-made kinetic spectrophotometer and fluorophotometer consisting of commercially available optical components. For the detectors silicon photodiodes (OSD 15-5 and OSD 60-5, Model Centronic, Laser Components, Gröbenzell, F.R.G.) were used in connection with an operation amplifier (AD 50 J, Analog Devices, München, F.R.G.) working as a current-to-voltage converter. The time and amplitude resolution of the absorption detector was 1 μs and $\Delta A/A = 10^{-5}$, respectively. For the fluorescent detector the time and amplitude resoluton were 10 μs and $\Delta F/F_o = 3 \cdot 10^{-3}$, respectively. The voltage at the detector's output was digitized, stored and analyzed with a storage oscilloscope (Nicolet Mod. 4094, Nicolet Oscilloscope Division, Madison, VI, U.S.A.).

Optical blocking against the wavelength of the photolysing laser flash was performed with appropriate filters (UV-blocking filter and interference filter ($\lambda_{max} = 570$ nm), Deutsche Balzers GmbH, Geisenheim, F.R.G.) or OG 515 and BG 18 (Schott, Mainz, F.R.G.)). In the case of the kinetic fluorescence measurements, the total blocking of the intensive UV-laser flash is not possible, because the fluorescence indicator is strongly excited by the flash. Therefore this artefact cannot be blocked optically and the detector comes into saturation. In the timescale of milliseconds, however, this effect is negligible due to the time resolution (10 μs) of the detector.

For the kinetic measurements we modified commercially available quartz cuvettes by polishing the lateral surfaces. Thus, cuvettes with two optical pathlengths of 2 mm and 10 mm, respectively, were obtained. This geometry was found to be usefull for optimizing the excitation and detection conditions: The optical pathway of the flash was split in such way that excitation beams entered from both sides of the cuvette (along the 2 mm dimension) to achieve a more homogeneous excitation. The fluorescence was excited also along the 2 mm dimension and, perpendicular to this, the emitted fluorescence was detected along the 10 mm dimension. In the case of the kinetic absorption measurements, the measuring beam and the detector were aligned to the 10 mm dimension of the cuvette.

Preparation of liposomes

The lipid-detergent mixture was prepared from stock solutions of lipid and detergent in chloroform. The organic solvent was evaporated under vacuum and the remaining lipid-detergent film was kept under vacuum for 2–3 h at 35–40 °C. The film was dissolved in buffer I (75 mM potassium sulfate/0.1 mM EDTA/1 mM pyranine/5 mM imidazole (pH 7.3)). The final lipid concentration was 10 mg/ml and the lipid-detergent ratio was 1 : 10. 1 ml of the micellar solution of lipid and detergent was transferred into the dialysis tubing and dialyzed against 25 ml of buffer I and 2–3 g of Bio-Beads. The beads were equilibrated in buffer I prior to use. Dialysis tubing, buffer and heads were enclosed in a 30 ml test tube and fastened on a device rotating with a speed of 20–30 rpm. An air bubble in the dialysis tube and in the outer dialysis buffer caused mixing of the solutions during dialysis. After 24 h buffer I was exchanged for buffer II (i.e., buffer I with 5 mg/ml bovine serum albumin instead of imidazole as buffering component and adjusted to pH 8). Buffer II was changed 3–4 times at 8–12 h intervals. Finally the pyranine outside the liposomes was removed by gel filtration (Sephadex G-25) or by several changes of the dialysis buffer containing no pyranine. In this way liposomes were prepared under defined pH-conditions having not other buffering agents inside except the indicator pyranine.

Determination of the quantum yield

The following method was used for an estimate of the quantum yield: First, the quanta absorbed by a solution containing caged-H^+ and phenol red were determined by an energy-meter (Gentech

Inc., Ste-Foy, Canada, Mod. ED 200). The absorption caused by the presence of phenol red was measured separately. Then the number of the released protons was determined spectroscopically from the same sample. By comparing these two numbers the quantum yield was obtained. The known quantum yield of 2-nitrobenzaldehyde ($\Phi = 0.5$ [8]) could be reproduced by this assay.

Results and Discussion

Synthesis

The photolysis of 2-nitrobenzaldehyde leads to the formation of 2-nitrospbenzoic acid (Fig. 1, Scheme 1) [6–8]. Several investigators have examined this reaction in detail, in attempts to understand the mechanism of this transformation [8,15]. Thus, it is known that this reaction proceeds intramolecularly and that it can take place in both the solid state and in solution. Besides 2-nitroaldehydes, even substrates containing a suitable C-H functionality in the *ortho* position of the aromatic nitro compound have been found to undergo this type of rearrangement [16]. As a starting material for the synthesis of the photolabile compound we used vanillin (4-hydroxy-3-methoxybenzaldehyde (I), Fig. 1, Scheme 2) wich has both a phenolic and an aldehyde group. As a phenol, vanillin forms esters and ethers, and the nucleus is easily substituted by a nitro group. Chloroacetic acid reacts smoothly with phenols in aqueous alkali hydroxide giving good yields of the alkali salts of aryloxyacetic acids. The acids themselves are crystalline solids which are easily purified by recrystallization from water or ethanol [17,18]. When the hydroxide group of vanillin is blocked by esterification or etherification, substitution takes place preferentially in the *ortho* position to the formyl group [19,20].

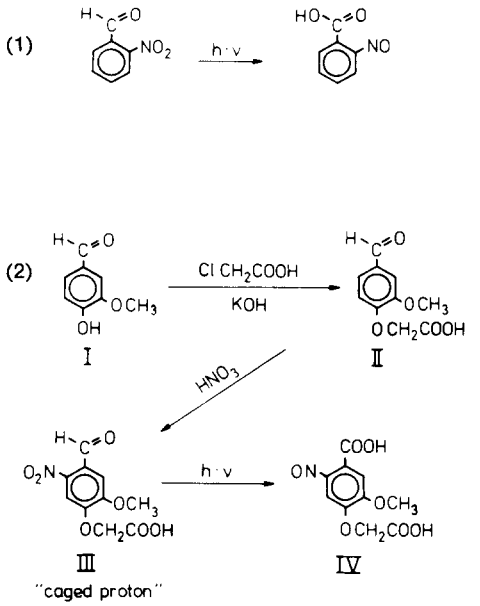

Fig. 1. Scheme 1: The principle photochemical reaction used: Photolysis of 2-nitrobenzaldehyde leads to 2-nitrosobenzoic acid with a concomitant fast release of a proton.
Scheme 2: Synthesis of 'caged proton' (III). Vanillin (I) is substituted with an acetic acid group by chloroacetic acid and then a nitro group *ortho* to the formyl group is introduced by reaction with nitric acid. Photolysis of 'caged proton' (III) leads to the nitroso compound (IV).

Static measurements of optical parameters

Fig. 2A shows the static spectra of 4-formyl-6-methoxy-3-nitrophenoxyacetic acid (caged-H^+, $c = 300\ \mu M$) in a water solution containing 50 mM K^+-phosphate (pH 7.3) before ('O') and after photolysis with up to 100 successive laser flashes ('1', '3', '10', '30' and '100', respectively). In the inset the difference spectra are shown. An advantage using the 308 nm line of an excimer laser should be noted: During photolysis a homogeneous excitation of the sample can be obtained. The spectrum little changes at this wavelength (Fig. 2A, isosbestic points at 258 nm and 314 nm, respectively). The molar absorption coefficient at 308 nm is approx. 5000. This should be taken into account for a suitable choice of the concentration and optical pathlength of the cuvette. The absorbance of the solution should not exceed the value of 0.3 in order to avoid inhomogeneous excitation and local concentration gradients in the cuvette.

Fig. 2B shows a similar experiment, but here the released protons were detected by the pH-indicator phenol red. Three successive flashes were applied to a 200 μl-sample containing 300 μM caged-H^+ and 40 μM phenol red initially adjusted to pH 9. In addition, the solution contained 75 mM potassium sulphate and 0.1 mM EDTA (i.e., buffer I, without buffering components). In the region below 400 nm the spectral changes result

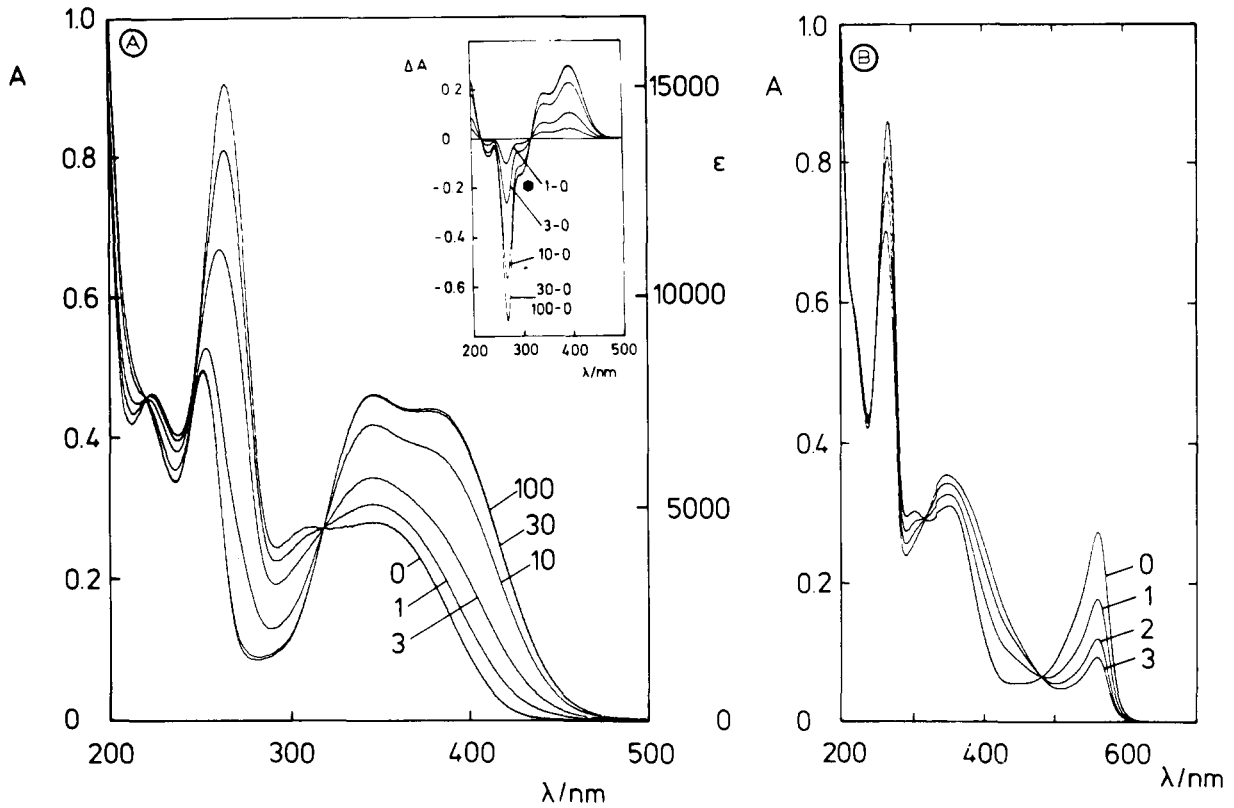

Fig. 2. Spectra of caged proton before and after photolysis with UV-laser flashes. (A) Spectra before ('0') and after 1, 3, 10, 30 and 100 ('1', '3', '10', '30', and '100', respectively) successive flashes (30 mJ/flash; $\lambda = 308$ nm; solution: [caged-H^+] = 300 μM in 50 mM K^+-phosphate buffer (pH 7.3; $d = 2$ mm). Inset: Difference spectra. (B) Spectroscopic determination of the amount of released protons after photolysis with three successive UV-laser flashes. Calibration was carried out using the dye phenol red as pH-indicator ($pK = 7.6$). Experimental conditions: [caged-H^+] = 300 μM, [phenol red] = 40 μM, [K_2SO_4] = 75 mM, [EDTA] = 0.1 mM, adjusted to pH 9 with KOH before the first flash was applied. $d = 2$ mm; 20 mJ/flash; $\lambda = 308$ nm. The number of released protons were evaluated by comparing the flash-induced absorption changes with the changes obtained by titration with acid.

from photolyis of caged-H^+. The released protons can be followed by the decrease of the 558 nm peak of the indicator. By comparing the decrease of the absorption peak with changes resulting from the addition of known quantities of acid, the number of released protons can be determined. The quantitative evaluation shows that 50 μM protons are released per flash (i.e., 10 nmol protons) under the conditions used. pH changes up to one unit can be achieved under these conditions. Even larger changes can be obtained when buffering components are left out.

Considering the biological application of caged-H^+, its solubility in aqueous solutions allows the use also at higher concentrations. But a possible inhomogeneous excitation must always be taken into account and should be compensated by diminishing the optical pathlength of the excitation.

The same method for the determination of released protons was used for the evaluation of the quantum yield of the photochemical reaction. The number of emitted quanta per laser flash was determined with a laser energy-meter. The energy meter was mounted behind the cuvette and the absorbed quanta were calculated by subtracting the measured value from the average value obtained in the absence of caged-H^+. The number of protons released in this experiment was determined spectroscopically as described above. The quantum yield is then obtained as the ratio of the number of released protons divided by the number of absorbed quanta. The observed value of the quantum yield was: $\Phi = 0.182 \pm 0.015$ ($N = 9$).

The magnitude of the proton concentration changes can be estimated in the following way: A flash with an energy of 10 mJ contains $2.7 \cdot 10^{16}$ or 45 nmol photons (308 nm). Assuming an absorption of 50% and a quantum yield of approx. 20%, 10% of the photons will be 'converted' to protons (= 4.5 nmol). In a volume of 100 μl a concentration change of $\Delta c = 45$ μM is thus obtained. With a laser flash of 100 mJ and excitation from both the sides (leading to absorption of 90% of the photons in a solution volume of 90 μl) a H^+-concentration jump of $\Delta c = 1$ mM is estimated.

Kinetic measurements

Fig. 3 (lower trace) shows the time course of the protonation of the indicator phenol red after photolysis of caged-H^+ by a single laser flash. The upper trace shows the flash artefact in a control experiment in which all components except caged-H^+ were present. When only the two components,

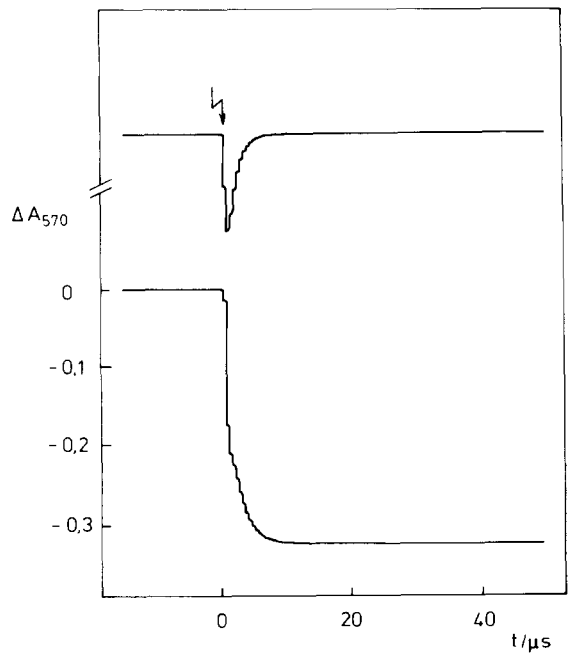

Fig. 3. Time course of pH changes. (Upper trace) Control experiment in the absence of 'caged proton'. (Lower trace) Time course of proton release in the solution; phenol red was used for monitoring the pH changes. Experimental conditions: [phenol red] = 40 μM, [caged-H^+] = 300 μM, the pH was adjusted to 9 before the flash was applied; $d = 10$ mm, 20 mJ/flash, $\lambda = 308$ nm.

caged-H^+ and the indicator are present, the time constant of the absorption change is in the range of 1 μs. This is the limit of the time resolution of our kinetic spectrophotometer. In the time range below 1 μs the protonation of the indicator is the time limiting reaction. The photochemical release of protons from 2-nitrobenzaldehyde in water was estimated to be very fast ($\tau = 0.6$ ns [8]). It should be noted that in the presence of other buffering components besides the indicator the observable protonation rate of the indicator is further decreased. This results from the transient protonation of the buffer followed by proton transfer from a protonated buffer molecule to an indicator. A time constant of about 150 μs in the presence of 1 mM buffer have been observed. As expected, the magnitude of the absorption change of the indicator was diminished under these conditions.

To create proton gradients across lipid membranes we used large unilamellar liposomes prepared by detergent dialysis [9,10]. In the interior of the vesicles the dye 8-hydroxypyrene-1,3,6-trisulfonic acid (pyranine) was enclosed, a fluorescent and water-soluble pH indicator ($pK =$ 7.2–7.6 [11]). The fluorescence of pyranine is pH dependent: Lowering the pH lowers the fluorescence intensity. The results are summarized in Fig. 4: Aliquots of liposomes were incubated at pH 8 with various concentrations of the protonophore carbonylcyanide 4-trifluoromethoxyphenylhydrazone (FCCP) to introduce an artificial proton permeability into the membranes. In addition, valinomycin was added in order to increase the electrical conductance of the membrane (the internal and external medium contained 150 mM K^+). Under these conditions protons equilibrate across the vesicle membrane, so that the vesicle interior is stabilized to pH 8. Then caged-H^+ was added to the vesicle suspension and the fluorescence changes caused by protonation of intravesicular pyranine after flash-induced proton release were measured. The upper trace was recorded from an experiment in which only a small concentration of FCCP ($c = 0.6$ nM) was present. After the flash a fast decrease of fluorescence can be observed, followed by a much slower decline. The rate of the slow decline increases with increasing concentrations of FCCP (lower traces, Fig. 4). The small fluorescence decrease immediately after the flash prob-

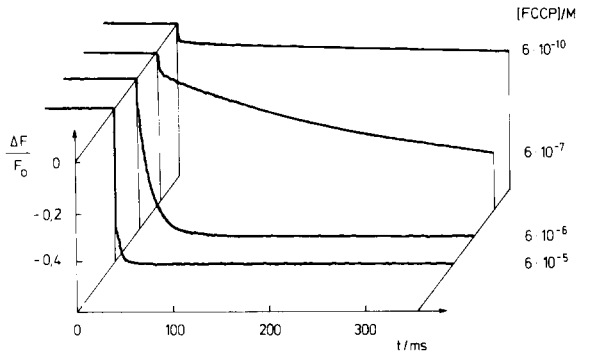

Fig. 4. Generation of proton gradients across lipid membranes by photolysis of 'caged proton'. The pH-indicator pyranine was enclosed in lipid vesicles. With the uncoupler FCCP an artificial proton permeability is introduced. After photochemical release of H^+, protons permeate across the membrane and protonate the indicator in the inner aqueous space. With increasing FCCP concentration faster permeation is observed. Experimental conditions: The solution contained 75 mM K_2SO_4 and 0.1 mM EDTA; concentration of entrapped pyranine 1 mM (refered to enclosed volume); lipid concentration 1 mg/ml; the pH of the medium was 8 and 7 before and after the flash, respectively; [valinomycin] = 1 μM, [FCCP] = $6 \cdot 10^{-10}$ M; $6 \cdot 10^{-7}$ M; $6 \cdot 10^{-6}$ M and $6 \cdot 10^{-5}$ M, respectively; [caged-H^+] = 300 μM, 25 mJ/flash (each coming from both the sides of the cuvette, i.e., total: 50 mJ/flash).

ably comes from indicator molecules adsorbed at the outer surface of the vesicles (see below). At higher FCCP concentrations, the vesicle interior is accessible to externally produced protons which permeate with rates dependent on the FCCP concentration. It is pertinent to mention that the indicator pyranine itself acts as a proton emitter after flash excitation [5,21]. This effect is negligible however, in the time range of our experiments ($t \geq 1$ ms).

A fast optical change has previously been observed in experiments in which the internal protonation of small liposomes was studied after a proton pulse [22]. The authors interpreted the fast component as a proton permeation occurring with 200 ms. In a more recent investigation [23] no fast components ($\tau < 1$s) have been found. This difference may result from differences in lipids used for vesicle formation, as well as from different preparation procedures.

A possible explanation for the occurrance of the fast component is that indicator molecules are adsorbed at the outer membrane's surface or are partly incorporated in the polar region of the membrane. This interpretation is supported by the following finding: After release of the entrapped indicator from the vesicles by addition of small amounts of cholate [24] and subsequent removal of the indicator, the fast component remains unchanged. The slower component is diminished and has not the same amplitude as the fast component (not shown). This shows that not removable indicator molecules are adsorbed at the outer and inner membrane surfaces.

With vesicles containing indicator an estimate of the permeability of caged-H^+ across the lipid membrane can be obtained in the following way: By addition of an impermeable buffer such as bovine serum albumin the pH shift in the external medium is nearly totally suppressed. When the experiments described above are repeated under these conditions, only a small and fast fluorescence decrease (0.5–1% of the total fluorescence) can be observed after protons have been released by the flash. This small effect is likely the result from bleaching of the indicator by the intensive ultraviolet flash; it is also observed in the absence of caged-H^+. When now, in the course of time, caged-H^+ molecules permeate into the liposomes, an increase of the fast fluorescence signal should be observable. The concentration of the indicator in the vesicles was 1 mM. The vesicles were incubated with 10 mM caged-H^+ for various times and then diluted for measuring. After 3 h a 2-fold increase of the fast component of the signal was observed. Taking this time as an approximation for the permeation time constant τ and using the known formula for computing the permeability coefficient of spherical vesicles (internal radius $r = 100$ nm [10]), one obtains:

$$P_d = \frac{A}{V \cdot \tau} = \frac{r}{3 \cdot \tau} \approx 10^{-9}\,\mathrm{cm/s}$$

(A is the membrane area and V the internal aqueous volume of the vesicles).

Conclusion

The method for the generation of proton concentration jumps described here is based on the well-known photochemical reaction of 2-nitrobenzaldehyde. By introduction of an acetic acid

residue into the benzene ring the water-solubility of the compound was increased and the membrane permeability decreased. Furthermore, introduction of a methoxy group led to a red shift of the absorption spectrum. The observable time course of the proton release is fast ($\tau \leq 1$ μs), when using a pH indicator for monitoring. This would allow the application of the compound in studies of proton-dependent reactions having time constants up to some tenths of microseconds. Possible applications are studies of biological energy-coupling processes or proton-transfer reactions on surfaces or membranes (micelles, lipid bilayers, etc.). Typical relaxation times for protein reactions involving conformational changes are in the time range of milliseconds (proton uptake from the bulk phase during the photocycle of bacteriorhodopsin [25]). Further possible applications are investigations of H^+-ATPase having synthesis rates up to 100 s^{-1} or showing conformation changes in the time range of milliseconds [26,27]. For ATP-synthesis one often wants the gradients to be acidic inside. Using an appropriate ester instead of the acid in the first step of the synthesis (cf. Fig. 1, Scheme 2) a membrane permeable compound is obtained. Analogous to the known permeable chelators or indicators [28] this compound will be converted into the impermeable acid by cellular esterases. When working with reconstituted systems the caged-H^+ outside the vesicles can be removed by conventional gel filtration.

For the generation of proton concentration gradients it is important to use a compound which is virtually membrane-impermeable. Using intensive UV-laser flashes for photolysis and an optimal geometry of the optical system, fast proton concentration changes up to the millimolar concentration range will be possible.

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