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Simultaneous determination of membrane potential and pH gradient by photodiode array spectroscopy

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Membrane potential ($\Delta\psi$) and pH difference (ΔpH) were simultaneously determined in liposomes using a photodiode array spectrophotometer. By the use of a cyanine dye (DiS-C_3(5)) and 9-aminoacridine for $\Delta\psi$ and ΔpH probes, respectively, both changes of $\Delta\psi$ and ΔpH could be successfully determined by photodiode array spectrometry. Each dye did not disturb the fluorescence spectrum of the other probe when its concentration was lower than 5 μM. The K^+-diffusion potential-driven, FCCP(protonophore)-mediated H^+-influx process in the K^+-loaded liposomes was analyzed by this method. Results indicate that the kinetic behavior of H^+ influx changes at a FCCP concentration of approx. 30 nM. The rate of ΔpH formation increased quantitatively with increasing concentrations of FCCP up to 30 nM, but was markedly enhanced at higher concentrations, although the maximal ΔpH attained was about 3 pH units in any case when a K^+-diffusion potential of -180 mV was applied.

Introduction

It is well known that in the cells of bacteria or eucaryotes, secondary ion transport systems such as Na^+/H^+ antiporter participate in important cellular physiological functions [1–4] such as cytoplasmic pH regulation, maintenance of $\Delta\psi$, regulation of cell proliferation or nutrients uptake. The mechanism of activation and the functional regulation of the secondary ion transport systems are considered to be strictly related to $\Delta\psi$ and/or ΔpH created by the primary ion pumps across the membrane. Thus, a precise kinetic analysis of the initial phase of $\Delta\psi$ and ΔpH changes following the primary energy charge is a prerequisite for the understanding of the precise mechanism of coupling between the primary and secondary ion transport systems and also for the mechanistic and functional analysis of the secondary ion transport systems, such as the gating mechanism.

Several methods for determining the $\Delta\psi$ and ΔpH are known such as the one using an ion specific electrode [5]. However, the response time of a conventional electrode is not short enough to study the initial phase of $\Delta\psi$ and ΔpH changes. Moreover, it is usually difficult to determine the two quantities simultaneously because of electrical interference of the electrodes used for the $\Delta\psi$ and ΔpH assay.

Abbreviations: PDAD, photodiode array detector; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; ΔpH, transmembrane pH difference; $\Delta\psi$, membrane electrical potential; diS-C_3(5), 3,3′-dipropyl-2,2′-thiadicarbocyanine iodide; $\Delta\tilde{\mu}_{H^+}$, proton motive force; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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Distribution of radio-labelled lipophilic cations [6] or amines [7] is also frequently used to evaluate $\Delta\psi$ and ΔpH, respectively, but the method is also not suitable for kinetic studies.

The fluorescent probes are widely used for determining the kinetic change of $\Delta\psi$ [8] and ΔpH [9] in both the biological and artificial membrane systems, because the method is quite simple and has a quick response. A conventional fluorescence detector system is, however, not feasible for the simultaneous determination of $\Delta\psi$ and ΔpH because of its monochromatic excitation-detection mechanism and its response time.

The photodiode array spectroscopy we are describing here made it possible to determine both components of $\Delta\tilde{\mu}_{H^+}$ by selecting the proper fluorescent probes for $\Delta\psi$ and ΔpH without any interference between them.

Materials and Methods

Liposome preparation. Large unilamellar liposomes were prepared by the reverse-phase evaporation method reported by Szoka and Papahadjopoulos [10] with some modifications. Phosphatidylcholine (66 μmol) and cholesterol (7.3 μmol) were dissolved in 6 ml of diethyl ether/chloroform mixture (5 : 1, v/v), then the organic solvent was removed under reduced pressure by a rotary evaporator at room temperature to obtain a thin film of lipids. After the lipids were redissolved in 6 ml of diethyl ether, 2 ml of 0.2 mM Hepes buffer containing 150 mM KCl (pH 7.4) was added as the loading buffer. The resulting two phase system was, then, sonicated in a bath-type sonicator for 3 min at 0°C. The homogeneous lipid emulsion obtained was treated under reduced pressure (100 mmHg) to remove the bulk of the ether until a stable gel formed. The residual ether was further evaporated off at about 700 mmHg for 10 min. The liposomes dispersion thus obtained was centrifuged for 20 min at $12\,000 \times g$ to remove aggregated materials. K^+-gradient was generated by passing the liposome suspension through a Sephadex G-50 column (1×25 cm) with 150 mM NaCl and 20 mM Hepes buffer (pH 7.4).

Determinations of $\Delta\psi$ and ΔpH in liposome by photodiode array spectrometry. A photodiode array detector (PDAD)(MCPD-100, Union Giken Co. Ltd.) connected with a computer system (PC 9801Vm, NEC) was used. A sample cuvette was illuminated with a 500 W Xe actinic light source through a silica fiber light guide (30×0.5 cm ∅) without using any filter. The fluorescence signals were measured at 90° to the axis of excitation light and sent to the PDAD also through a silica fiber light guide. Typically, 2 ml of K^+-loaded liposomes (6 μg lipid P) suspended in 20 mM Hepes buffer (pH 7.4) containing 150 mM NaCl was taken in the cuvette and was stirred with a magnetic stirrer. After a background spectrum was recorded, each 2-μl aliquot of 9-aminoacridine (1 mM aqueous solution) and diS-C_3(5) (1 mM ethanol solution) were added successively. The fluorescence levels were stabilized within 2 min. 2-μl aliquot of valinomycin (0.3 mM in ethanol) was injected to the liposome suspension with a microsyringe to generate K^+-diffusion potential, then an aliquot of FCCP (ethanol solution) was added at 1 or 2 min after valinomycin addition to induce $\Delta\psi$-driven, FCCP-mediated ΔpH formation in the liposomes. Through the experimental procedures described above, the whole fluorescence spectra covering the wavelength range between 400 and 800 nm were determined with a sampling time of 1 s and stored in a disk memory. Net fluorescence intensity was adjusted by changing the actinic light intensity or the sampling time. All the data stored in the computer were processed later on using the program developped for MCPD-350PC HPLC analysis system (Union Giken Co. Ltd.).

For the kinetic studies on the $\Delta\psi$-driven, FCCP-mediated H^+-influx process, FCCP (5 nM) was added to the liposome suspension before each experiment. After addition of fluorescence probes, various amounts of valinomycin (3–300 nM final concentrations) were injected to the liposome suspension with a microsyringe to initiate the reaction.

FCCP and 9-aminoacridine purchased from Tokyo Kasei Co., Ltd. diS-C_3(5) was from Japanese Research Institute for photosensitizing Dyes. Gramicidin and phosphatidylcholine (99% pure) were from Sigma Co. Ltd. Cholesterol and all other reagents used were purchased from Wako Co. Ltd.

Results and Discussion

Fig. 1 shows the whole fluorescence spectrum in the wavelength from 400 to 800 nm of the liposome suspension after addition of both 9-aminoacridine and cyanine dye (diS-$C_3(5)$). Two fluorescence peaks appeared at around 455 and 690 nm, which correspond to the fluorescence spectra of 9-aminoacridine and diS-$C_3(5)$, respectively. The concentration of either of two probes was kept constant (1 μM), then the concentration of the other probe was varied form 0.1 μM to 5 μM. However, the fluorescence spectrum of 9-aminoacridine or cyanine dye was not affected at all by the other probe. Accordingly, there was no interaction between these two fluorescence probes in the concentration range used above. Since the maximum response to $\Delta\psi$ appeared at 1 μM of diS-$C_3(5)$, this concentration was routinely used for the following experiments.

The fluorescence spectra from both of 9-aminoacridine and diS-$C_3(5)$ after addition of K^+-ionophore valinomycin, and protonophore FCCP, were recorded with a sampling time of 1 s at room temperature and stored in the computer.

Time-dependent change of the whole fluorescence spectra in the wavelength between 400 and 800 nm was read out as an average of every 4 s

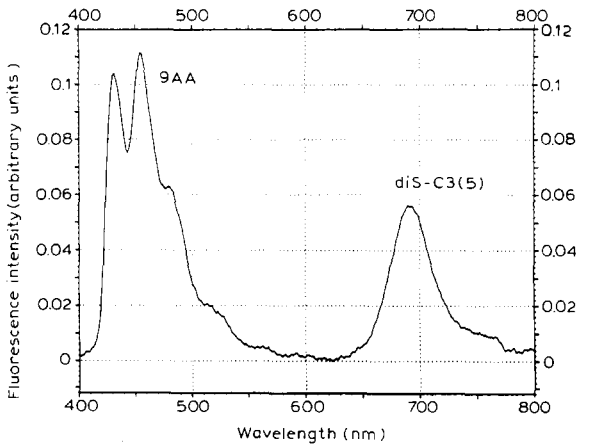

Fig. 1. Fluorescence spectra of 9-aminoacridine (9AA) and diS-$C_3(5)$ in liposome suspension. The medium contains phosphatidylcholine/cholesterol liposomes (6 μg lipid P) loaded with 150 mM KCl, 20 mM Hepes (pH 7.4) and 150 mM NaCl. 9-Aminoacridine was added as an aqueous solution and diS-$C_3(5)$ as an ethanol solution, final concentrations 1 μM. Sampling time 1 s.

and is shown in Fig. 2a. The contour map of the fluorescence intensities of both probes is also given in Fig. 2b. From this, it is easily recognizable that the fluorescence from diS-$C_3(5)$ sharply decreased after addition of valinomycin according to the formation of K^+-diffusion potential. Subsequent addition of FCCP initiated the quenching of 9-aminoacridine fluorescence responding to the formation of inside-acidic ΔpH due to the $\Delta\psi$-driven, FCCP-mediated H^+ influx into the liposomes. Without FCCP addition, such change of 9-aminoacridine fluorescence could not be detected at all, even though it was previously reported that a significant H^+ influx occurred when valinomycin-K^+ diffusion potential (more than -60 mV) was applied in the liposomes prepared with the lipids extracted from *Bacillus alcalophilus* or phosphatidylcholine [11]. Therefore, the liposomes we prepared here are less permeable to H^+ and hence are more profitable for the study of ion transport. Further addition of small amount of gramicidin restored both fluorescence intensities completely to the original levels. At the same time, it is also notable that the significant volume change of the liposomes did not occur during the ion flux process since any drift of the base line due to the light-scattering change could not be observed.

From Fig. 2b, we are able to determine the wavelengths which give the maximal spectral change of each probe and obtain the kinetic changes of the fluorescence intensity of them as shown in Fig. 2c. Cyanine dye itself is rather unstable under the experimental condition and the fluorescence intensity decreased about 9.4% during 10 min, probably due to the decomposition of diS-$C_3(5)$.

For the simulation of the $\Delta\psi$-coupled secondary H^+-transport process, $\Delta\psi$-driven, FCCP-mediated H^+-influx process was analyzed by the present method. Since it is reported that FCCP quenches cyanine dye fluorescence [9], the effects of FCCP on the fluorescence spectra of 9-aminoacridine and diS-$C_3(5)$ were examined in the presence of liposomes (Fig. 3). The fluorescence of diS-$C_3(5)$ was not significantly affected by the addition of FCCP at concentrations lower than 10 nM, but at higher concentrations the fluorescence was considerably reduced without any change in its spectral shape. The quenching levels were lin-

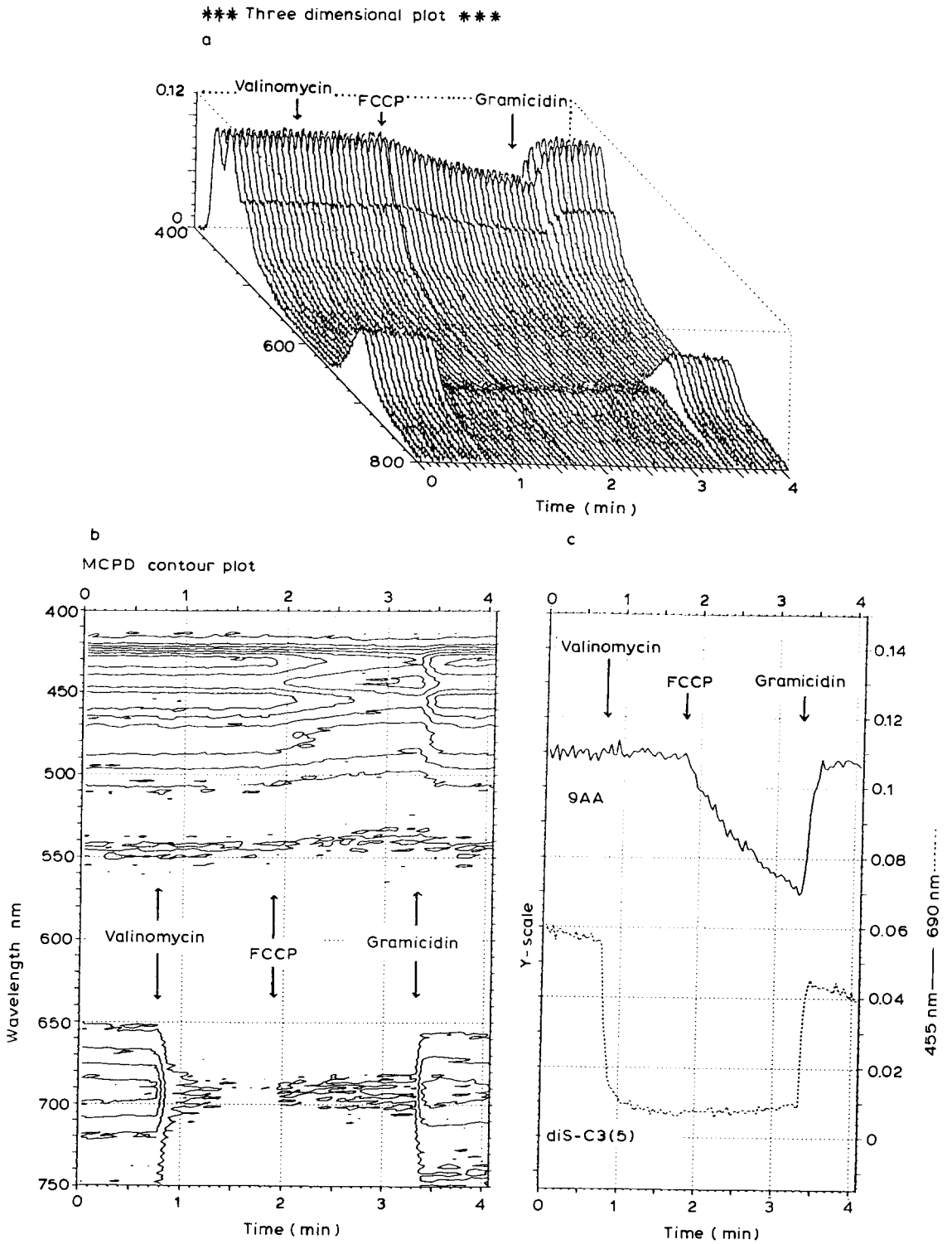

Fig. 2. (a) Three-dimensional display of the kinetic changes of the fluorescences of ΔpH and $\Delta\psi$ probes in K^+-loaded liposomes after additions of valinomycin and FCCP. Reaction mixture is the same as in Fig. 1. Valinomycin, FCCP and gramicidin were added as ethanol solutions with a microsyringe to give a final concentration of 0.3 μM, 1 nM and 3 nM, respectively. Sampling time 1 s, each spectrum is the average of four measurements. (b) Contour map of the fluorescence intensity changes. (c) Kinetic changes of the fluorescence intensities of $\Delta\psi$ and ΔpH probes. Fluorescence intensities of 9-aminoacridine (9AA) and diS-C_3(5) were monitored at 455 and 690 nm, respectively.

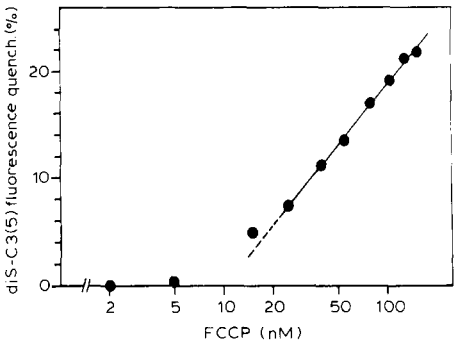

Fig. 3. Interaction of FCCP with diS-$C_3(5)$. Liposome suspensions containing 1 μM each of 9-aminoacridine and diS-$C_3(5)$ were titrated with different aliquots of FCCP without addition of valinomycin. Fluorescence quenching was recorded at 1 min after FCCP addition.

early correlated to the logarithmic concentration of FCCP, suggesting that FCCP interacts directly with diS-$C_3(5)$. On the other hand, 9-aminoacridine fluorescence was not affected by FCCP at all. Therefore, FCCP concentrations lower than 10 nM were used for the kinetic studies.

In order to know the kinetic relationship between $\Delta\psi$ formation and the $\Delta\psi$-driven H^+ influx into liposomes, $\Delta\psi$ was generated by adding various amounts of valinomycin to the K^+-loaded liposomes in the presence of 5 nM FCCP. Then, the fluorescence changes of both 9-aminoacridine and diS-$C_3(5)$ were simultaneously recorded. Typical example of the kinetic changes of the fluorescences from 9-aminoacridine and diS-$C_3(5)$ is given in Fig. 4. The results show that the ΔpH formed synchronously as $\Delta\psi$ formed and no significant lag time between them could be observed.

As the valinomycin concentrations were increased, the rate constants, $k(\text{min}^{-1})$ for the fluorescence changes of both 9-aminoacridine and

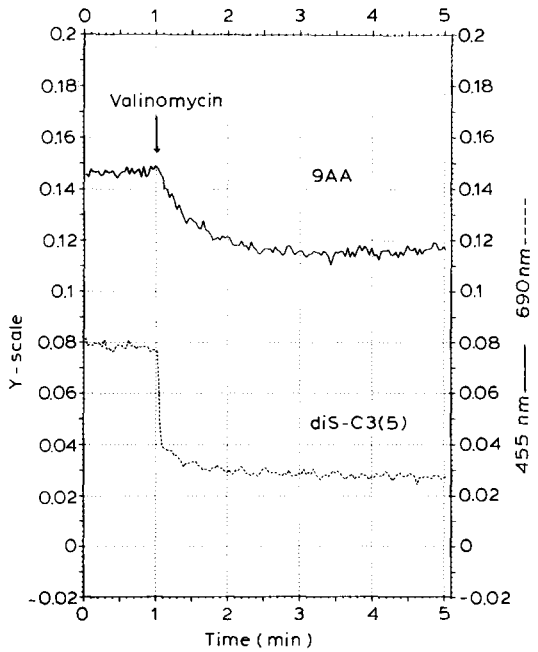

Fig. 4. Typical kinetics of $\Delta\psi$ and ΔpH formation after valinomycin addition in K^+-loaded liposomes. Reaction medium is the same as in Fig. 1 except that to the liposomes 5 nM FCCP was previously added. Valinomycin (15 nM) was added to initiate the reaction with a microsyringe.

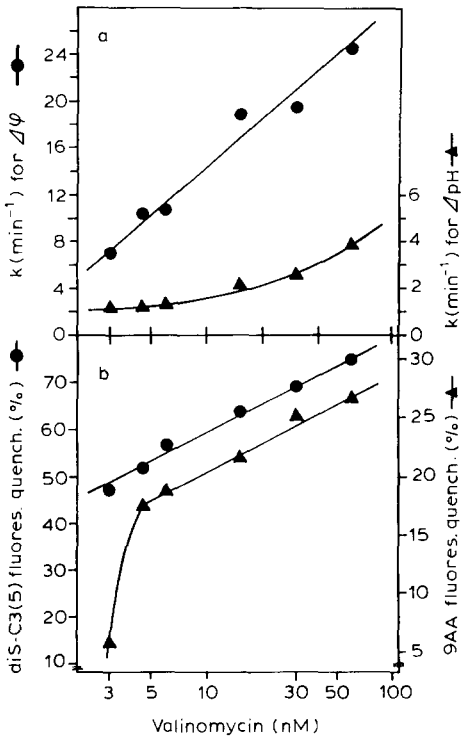

Fig. 5. Valinomycin-dependent formation of $\Delta\psi$ and ΔpH in K^+-loaded liposomes. (a) Rate constants for the fluorescence changes of 9-aminoacridine and diS-$C_3(5)$ after addition of elevated concentrations of valinomycin. Reaction conditions are the same as in Fig. 3. ▲, 9-aminoacridine; ●, diS-$C_3(5)$. (b) Maximum quenching percent of 9-aminoacridine (9-AA) and diS-$C_3(5)$ fluorescence after addition of valinomycin. Reaction conditions are the same as above. ▲, 9-aminoacridine; ●, diS-$C_3(5)$.

diS-$C_3(5)$ increased. In contrast to the rate of $\Delta\psi$ formation, that of ΔpH formation was not linearly correlated to the valinomycin concentration. The rate was more accelerated at the higher concentrations (Fig. 5a). On the other hand, the maximum fluorescence quenching of both probes was linearly correlated to the valinomycin at concentrations higher than 3 nM. Since the ratio of the maximum quenching of both probes is constant over the valinomycin concentration range 4.5–60 nM, it is recognized that the maximum change in ΔpH is governed directly by the $\Delta\psi$ formed by valinomycin-K^+, independently of the rate of H^+ influx. At the concentration less than 3 nM valinomycin, 9-aminoacridine fluorescence was not quenched at all, although cyanine dye fluorescence was quenched (data not shown). From this observation and also the non-linear correlation of H^+-influx rate to the $\Delta\psi$ formation shown in Fig. 5a, it is suggested that a certain threshold potential is required for driving the FCCP-mediated H^+ transport. Further studies are in progress to clarify this problem.

When the $\Delta\psi$ levels were varied by changing the K^+ concentration in the external medium, the percent quenching of the cyanine dye fluorescence obtained by adding 0.3 μM valinomycin was linearly correlated to the $\Delta\psi$ values (calculated from the Nernst equation) from 0 to approx. -100 mV and then became saturated at higher membrane potential values (data not shown) in agreement with the results obtained previously by conventional fluorometry [12]. Although the extent of maximum quenching of the probes was variable, depending on the amounts of either valinomycin or FCCP, the initial rates of $\Delta\psi$ and ΔpH formation were calibrated as about 13.5 mV and 0.1 pH unit per s, respectively, under the experimental conditions (a K^+-diffusion potential of -180 mV, 0.3 μM valinomycin and 1 nM FCCP).

The relationship between the initial rate of $\Delta\psi$-driven, FCCP-mediated H^+ influx into liposomes and the concentration of FCCP under a constant $\Delta\psi$ value (calcd. -180 mV) was studied by the PDA spectroscopy. The results shown in Fig. 6 revealed that the initial rate of H^+ influx was linearly correlated to the logarithmic concentration of FCCP up to approx. 30 nM, but at concentrations of FCCP higher than 30 nM, the

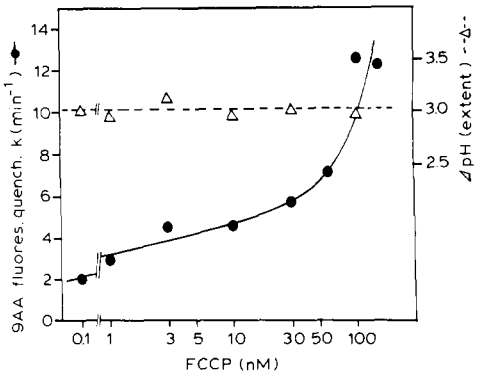

Fig. 6. Relationship of FCCP concentration and the initial rate of $\Delta\psi$-driven, FCCP-mediated H^+ influx into liposomes. Reaction conditions are the same as in Fig. 4 except that the concentration of FCCP was varied. FCCP was added 2 min before valinomycin addition in every case. 9AA, 9-aminoacridine.

slope of the dose-response curve of the initial rate of the H^+ influx changed and the rate was markedly accelerated. At the same time, the cyanine dye fluorescence began to be significantly quenched (data not shown), so that a wrong conclusion might be extracted as if FCCP-mediated H^+ influx hyperpolarized the membrane secondarily. On the other hand, the maximum ΔpH obtained was approx. 3 pH units in all cases, which is in good agreement with the value expected from the $\Delta\tilde{\mu}_{H^+}$ applied in this system. Although it is known that a large amount of FCCP quenches cyanine dye fluorescence [9], present experiments further revealed that FCCP changes its kinetic behavior of H^+ transport depending on its concentration. When the number of liposomes in the cuvette was calculated from the average liposome diameter of 200 nm which was determined by an electron microscopy and the trapping volume evaluated by calcein-cobalt method [13], a value of $3.4 \cdot 10^{11}$ liposomes per 2 ml of reaction medium was obtained. Therefore, if we suppose all of the FCCP molecules present in the liposome, it can be calculated that approximately 100 molecules of FCCP distributed in one liposome at 30 nM of FCCP, where the kinetic behavior of FCCP-mediated H^+ influx was markedly changed. At the same time, the number of lipid molecules in one liposome was calculated to be approx. $3.5 \cdot 10^5$. Thus, the number of iono-

phore in one liposome is still small enough relative to that of lipid so that the ionophore molecule may not deteriorate the membrane structure to be permeable for H^+. Although we are not able to give a reasonable answer to the real meaning of 100 ionophore molecules per liposome at present, the data described above indicate that the number of FCCP molecules per vesicle is important for the kinetic behavior of FCCP-mediated H^+ transport. Therefore, we should be cautions with the FCCP concentration when studying the kinetic effect of FCCP either on ΔpH or $\Delta\psi$, especially when the cyanine dye is used as a $\Delta\psi$ probe. Otherwise, complex results caused by an FCCP effect, such as the concentration-dependent effect of FCCP, might be observed.

Obviously, there is an argument that the kinetic responses of the fluorescence probes may not necessarily be a measurement for the kinetics of the changes in $\Delta\psi$ and ΔpH, especially for 9-aminoacridine, being a distribution probe which redistributes across the membrane in response to the ΔpH formed, the kinetic behavior of the fluorescence probe thus only reflecting the time response of the probe itself. However, this may be ruled out, because as Fig. 6 shows, the maximum ΔpH attained was about 3 pH units in all the concentration of FCCP ranging from 0.1 to 100 nM when a calculated -180 mV of artificial membrane potential was applied, but the rates of the ΔpH formation were highly dependent on the FCCP concentrations. Additionally, when valinomycin concentrations were varied, the percent changes of ΔpH and $\Delta\psi$ were parallel over the concentration range from 4 to 60 nM, but the kinetic changes of these fluorescence probes differed (Fig. 5). Thus, we think that the observed kinetics of the probe fluorescence may reflect the kinetics of $\Delta\psi$ and ΔpH changes in the liposomes, even though the probe distribution is the rate-limiting step for determination. Anyway, further studies are in progress to clarify the true meaning of the kinetic behavior of the $\Delta\psi$-dependent, carrier-mediated H^+-transport including the threshold potential for the transport.

As described above, PDA spectroscopy made it possible to determine $\Delta\psi$ and ΔpH simultaneously without any special devices. Thus the method might be feasible for the kinetic study on the initial process of $\Delta\tilde{\mu}_{H^+}$ driven secondary ion transport process in biological membrane by choosing a proper dye probe.

A few trials to determine the sequential events occurring in lymphocyte activation, that is, membrane potential change and H_2O_2 burst, were carried out using two kinds of fluorescent dyes [14], the fluorescence changes being determined by a system equipped with a pair of independent lazer excitation beams and detectors. The present method is also applicable to this kind of experiment.

Since the advantage of using PDAD lies in the fact that the whole spectral change in a wide wavelength range can be recorded in a short time period and also that non-filtered actinic light is usable for excitation of different fluorescent probes at once, the method must be quite useful to trace a few different events proceeding simultaneously in a time period of seconds to minutes and to analyze the interrelationship between them.

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