Role of Bipyridinium Salts in Generation of Photocurrent by *Rhodospirillum rubrum* Cells

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An anodic photocurrent was generated when an SnO_2 electrode, which was immersed in a culture medium of a photosynthetic bacterium, *Rhodospirillum rubrum*, or in a buffer suspending the cell body, was illuminated under potentiostatic conditions. The anodic photocurrent was enhanced about four times with addition of methyl viologen (MV) to the cell suspension, but was suppressed with addition of 1,1′-ethylene-2, 2′-bipyridinium dibromide (EBP). On the other hand, it was observed spectrophotometrically that EBP was reduced by *Rs. rubrum* cells about ten times faster than MV. The disagreement in both data of photocurrent and of reduction rate suggests this to be due to the difference of penetrability of the salts through the cell wall and membrane. This suggestion was confirmed from cyclic voltammetric results of the reagents dissolved in the cell suspension.

Serious environmental problems, including air pollution with NO_x and SO_x and release of vast amounts of CO_2 into atmosphere, limit the utility of fossil fuels. The development of solar energy technology has therefore been desired, promising a substantial future contribution to growing energy demands. The primary process of photosynthesis is the most efficient in converting solar energy to chemical energy and, consequently, could possibly be used as the basis for modified photosynthetic processes applicable in solar energy conversion.[1] On the basis of this viewpoint, many studies[2] have demonstrated the possibility of using photoactive biological components in photoelectrochemical cells, in connection with studies on the mechanism of photosynthetic primary processes as well as solar energy conversion projects. We have been studying photoelectrochemical behavior using a photosynthetic apparatus, chromatophores, of a photosynthetic bacterium, *Rhodospirillum rubrum*, and a cathodic photoresponse was observed at a Pt electrode under potentiostatic conditions,[3] but an anodic one was obtained at an SnO_2 electrode in the presence of ascorbate.[4] However, the photoelectron-transfer activity between chromatophores and the electrodes could not be maintained for a long time because of the inactivation of the electron transport system bound to chromatophores. To overcome this problem, living cell bodies of *Rs. rubrum* were used directly as reported earlier.[5] This paper deals with a photoelectrochemical act of *Rs. rubrum* cells at an SnO_2 electrode immersed in a culture medium or in a buffer, and with an effect of bipyridinium salts added to the cell suspension on the photocurrent generation.

Experimental

The carotenoid-less blue-green mutant (G-9) of *Rs. rubrum* was used throughout this study. Flat glass bottles (about 1.4 liters) were filled with the culture medium and inoculum. The inoculated media were incubated at 30 °C in the dark for one day and then in the light for 3—4 d. The light was provided by 60 W tungsten lamps, the light intensity on the surface of the bottles being about 3000 lx. Hydrogenase activity was assayed by gas chromatography as described previously.[6] The cells were harvested by centrifugation at 4°C and washed with 0.1 M (M=mol dm^{-3}) sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES) buffer (pH 8.0). In addition, to separate to subcellular fractions,[7,8] the harvested cells were washed twice with 0.1 M HEPES buffer (pH 8.0). The washed cells were treated for 2 h at 30 °C with the same buffer containing 0.5 mg ml^{-1} lysozyme, 5 mM EDTA, and 10% sucrose. The cell suspension was then centrifuged at $3000\times g$ for 20 min. The resultant supernatant was used as "periplasm". The resultant precipitate was washed twice with and suspended in the same buffer, followed by sonication and by centrifugation at $100000\times g$ for 2 h. The resultant supernatant was used as "cytoplasm", and the precipitate as "chromatophores". Proteins in periplasm and cytoplasm fractions was measured by the Biuret method using bovine serum albumin as a standard. The density of cell and chromatophore suspensions is expressed in terms of absorbance of bound bacteriochlorophyll at 873 nm (A_{873}; corresponding to 7.3 mg ml^{-1} ${A_{873}}^{-1}$ of wet weight for cells and 1.9 mg ml^{-1} ${A_{873}}^{-1}$ of wet weight for chromatophores, respectively). A photocurrent was measured under potentiostatic conditions at an SnO_2 (2×3 cm^2 of the surface area) for the cells or chromatophores suspended in the culture medium or in the buffer at 25 °C.[4]

As the light source, a 60 W tungsten lamps was used with a Toshiba Y-46 filter. Reduction of bipyridinium salts, such as methyl viologen (MV), benzyl viologen (BV), 1,1′-ethylene-2,2′-bipyridinium dibromide (EBP), its 4,4′- and 5,5′-dimethyl derivatives (4MEBP and 5MEBP), 1,1′-trimethylene-2,2′-bipyridinium dibromide (TBP), its 5,5′-dimethyl derivatives (5MTBP), and 1,10-ethylene-1,10-phenanthrolinium dibromide (EPH), by *Rs. rubrum* cells was done in a Thunberg-type cuvette under argon, and the increase in the absorbance at absorption maxima of the reduced form of the analogs was measured at 25 °C. These rates thus obtained were compared with the photocurrent. These 2,2′-bipyridinium salts were synthesized and purified by the methods described in the literature.[9] Cyclic voltammetry was done at 25 °C in a conventional three-electrodes cell after incubating the reagents with *Rs. rubrum* cell suspension under argon for 30 min at 30 °C. The working electrode was an amalgamated copper disk (ϕ=2 mm), and all measurements were made in deaerated HEPES buffer (pH 8.0).

Results and Discussion

Only a small anodic photocurrent was observed on illumination for the cell-cultivating medium under anodic polarization (Fig. 1), presumably due to insulation of the electron-transfer site to the electrode by cell envelopes. In general, the presence of an electron mediator is required to facilitate the transfer of electrons from the cell body to the electrode, and the requirements for suitable mediators are:[10] 1) The mediator should not be toxic to microorganisms. 2) The reagent should be able to penetrate, at least, the cell wall of the microorganism to react with the source of electrons. 3) It should be electrochemically active at the electrode. 4) The formal redox potential should be near to that of the redox couple providing the reducing action within microorganism, which is likely in many cases to be NAD^+/NADH. 5) The reagent should be chemically stable in the solution for practical application over long periods of time. 6) The mediator must be reasonably soluble in buffer solutions, which are usually around pH 7. So, we have used here some bipyridinium salts as the mediators, some of which satisfy these requirements except that many of analogs are toxic to the bacterium at higher concentration because the reagents were developed as herbicides. Figure 1 also shows typical profiles of electrolytic currents on illumination in the presence of MV or EBP. The increase of the electrolytic current immediately after illuminating was taken as the value of photocurrent. The anodic photocurrent was enhanced about four times with addition of MV to the culture medium, but was suppressed with addition of EBP. The photocurrent depended on the electrode potential, showing maximum at +200—+300 mV vs. NHE in the presence of each mediator. Figure 2 shows the relationship between cell growth and photocurrent for a sample (ca. 5

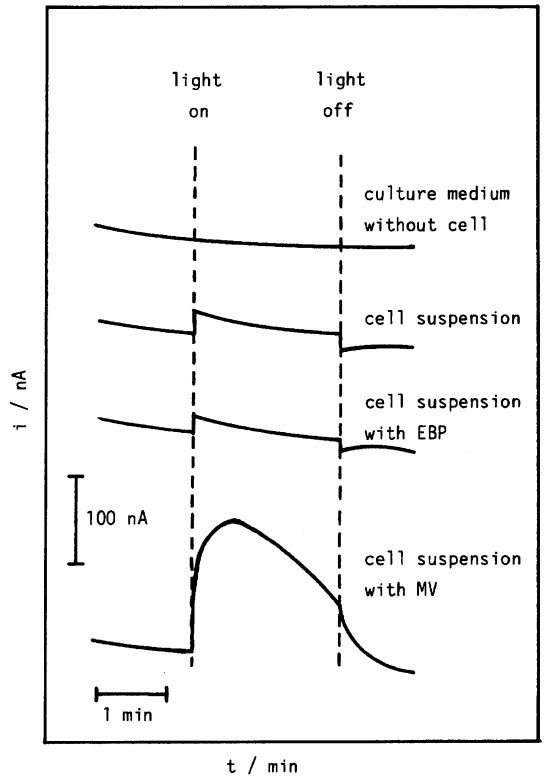

Fig. 1. Typical time profile of electrolytic current. *Rs. rubrum* cells were incubated at 30 °C in a culture medium for one day in the dark and then for one day in the light. The density of the cells was adjusted to be A_{873}=2. The concentrations of MV and EBP were 1 mM. The current-time profiles were measured at +650 mV of the applied potential. Illuminance was 6000 lx.

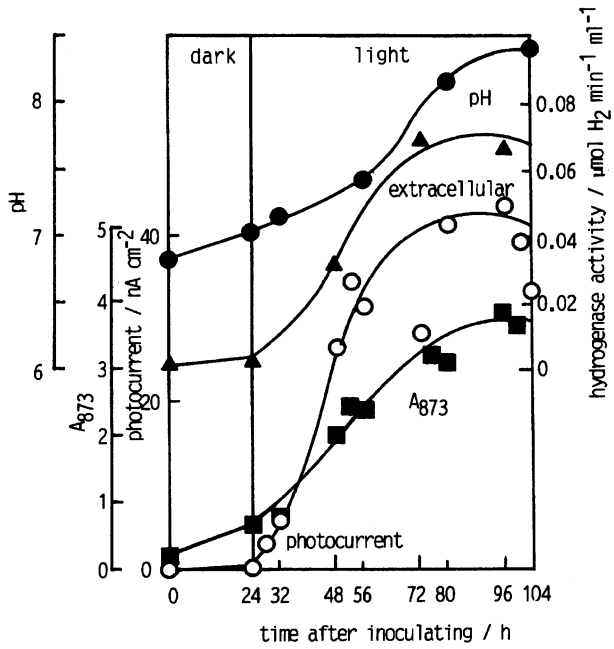

Fig. 2. Relationship between cell growth and photocurrent by the cells in culture medium. Photocurrent was measured for an aliquot (ca. 5 ml) of culture medium containing 1 μM MV at +850 mV of the applied potential and at 6000 lx of illuminance.

ml) of culture medium containing 1 μM MV, together with medium pH and extracellular hydrogenase activity. Agreement of the magnitude of the photocurrent with that of hydrogenase activity indicates that the extracellular hydrogenase[11] or related proteins[12] takes some roles in the generation of photocurrent. Since Saeki et al.[8] found earlier that the light-grown cells of *Rs. rubrum* could catalyze the reduction of MV by NADH, it is expected that this enzyme may directly participate in the generation of photocurrent. However, the magnitude of the photocurrents was also dependent on light intensity, cell density, concentration of mediators, and solution pH (data not shown). Therefore, more detailed discussion is impossible under such varying experimental conditions (Fig. 2). So, all of the succeeding measurements were done in a HEPES buffer (pH 8.0). The similar results to those in the culture medium were obtained as summarized in Table 1. The photocurrent was enhanced about four times with addition of MV, but not in the presence of 5MEBP. In addition, the photocurrent was suppressed by about half with addition of EBP, as described above. The magnitude of the photocurrent was also a function of the wavelength of the incident light. The resulting photocurrent action spectrum (Fig. 3) has three maxima at 600, 800, and 875 nm in agreement with the maxima (588, 804, and 873 nm) of the absorption spectrum of the cell suspension. This suggests that the photocurrent is generated by the cell body itself through the photoresponse within the cell, and is independent of the photoexcitation of the bipyridinium salts. Moreover, a photocurrent was measured for chromatophore suspension containing the reagents. The results are also shown in Table 1. The photocurrent in the presence of EBP was enhanced about three times in contrast to the above results. That is, EBP could be reduced by an enzyme in the cell, and could facilitate the electron transfer between chromatophores and the electrode in the absence of cell wall and membrane. Therefore, this disagreement in the data is suggested to be due to the difference of penetrability of the reagents

Table 1. Photocurrents by *Rs. rubrum* Cells and by Chromatophores

Additives	E_0'	Photocurrent	
		by Cells	by Chromatophores
	mV	nA cm^{-2} A_{873}^{-1}	nA cm^{-2} A_{873}^{-1}
None		2.5	7.8
MV	−446	10	15
EBP	−367	1.3	23
5MEBP	−490	2.3	8.5

The experimental conditions were as follows; $A_{873}=50$ in 0.1 M HEPES buffer (pH 8.0) containing 1 mM of reagents. The density of cell or chromatophore suspension was estimated from an absorbance for the diluted suspension to 250 times. Photocurrent was measured at +650 mV of the applied potential and at 6000 lx of illuminance.

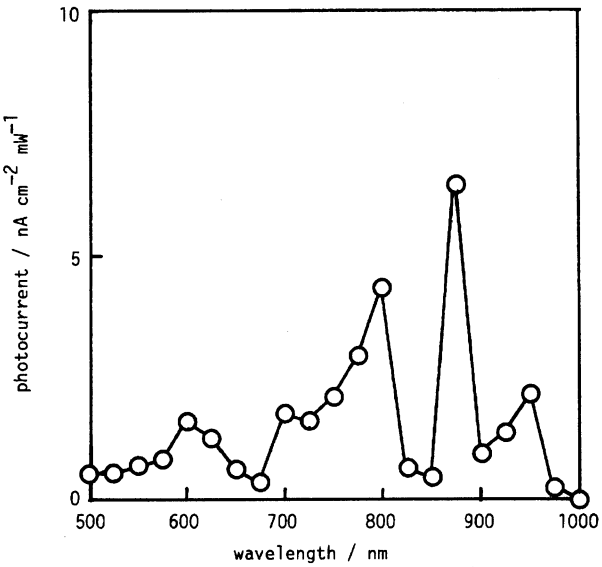

Fig. 3. Photocurrent action spectrum for *Rs. rubrum* cell suspension. The experimental conditions were as follows; $A_{873}=50$ in 0.1 M HEPES buffer (pH 8.0) in the presence of 1 mM MV, and +650 mV of the applied potential.

through the cell wall and membrane. Furthermore, the photocurrent by chromatophores was not enhanced by addition of 5MEBP, similar to that by the cells. The redox potential of 5MEBP is 120 and 40 mV lower than those of EBP and MV, respectively[13] (Table 1). It may be indicated that 5MEBP was hardly reducible by a reducing component within the cell if the reagent could penetrate the cell wall and membrane. In other words, the reducing power of a reductant in the cell may be limited to near the redox potential of 5MEBP.

To confirm the reduction mechanism by the cell, the reduction of bipyridinium salts by the cell was spectrophotometrically measured in some detail. Most of these reagents could be reduced by the cell as shown in Table 2. The reduction of these salts was started in a Thunberg-type cuvette by adding the salts to the cell suspension, which had been evacuated and flashed with argon for removal of dissolved oxygen, and the rate of reduction was followed for about 15 min, while approaching a steady state of reduction. Then the reduction was again started by adding NADH into the cell suspension. The reduction rates of the salts in the presence of NADH are also listed in Table 2. Similar results were obtained in the presence of NADH, although the values were somewhat larger. In addition, the reduction rates depended on redox potentials of the salts,[13] indicating that the salts could be reduced by the same enzyme in the cell, the reduction of which was achieved by NADH and, maybe, NADH:MV reductase as pointed out earlier.[8] These reduction rate were also dependent on the density of the cells (approaching maxima at $A_{873}=3$—5), on the concentrations of the salts (as typically shown in Fig. 4) and NADH (up to

Table 2. Reduction of Bipyridinium Salts by *Rs. rubrum* Cells

	E_0'	Reduction rate $\mu M\ min^{-1}$	
	mV	without NADH	with NADH
EPH	−240	5.07	6.00
BV	−360	3.84	5.88
EBP	−367	2.29	4.68
MV	−446	0.29	0.93
4MEBP	−491	0.13	0.48
5MEBP	−490	0.10	0.40
TBP	−555	ca. 0	ca. 0
5MTBP	−667	ca. 0	ca. 0

The experimental conditions were as follows; $A_{873}=3$ in 0.1 M HEPES buffer (pH 8.0) containing 1 mM bipyridinium salt with or without 2 mM NADH.

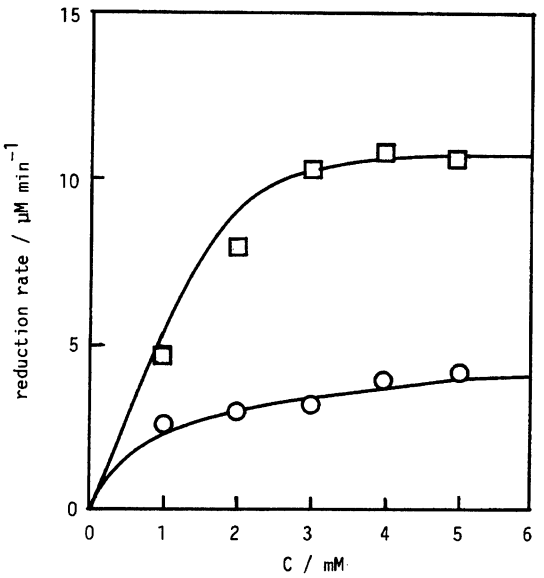

Fig. 4. Dependence of reduction rate by intact cells on concentration of EBP. ○ and □ represent the values in the absence and the presence of 2 mM NADH. respectively. Reduction rate was estimated from the absorbance change at 378 nm (absence) and 441 nm (presence). The other experimental conditions were as follows; $A_{873}=3$ in 0.1 M HEPES buffer (pH 8.0).

about 2 mM), and on the pH of the cell suspension (maximum at pH 8.0—8.2). Then, EBP could be again reduced by the cells about 5—8 times faster than MV, similar to the measurement of photocurrent by chromatophores, and in contrast to that by cells. Moreover, 5MEBP and 4MEBP were barely reduced, and TBP and 5MTBP, which had the lowest redox potentials among the reagents tested, could not be reduced, again indicating that the reducing power of NADH:MV reductase may be limited to near −490 mV. Furthermore, the reduction rates of MV and EBP by disrupted cells with sonication were measured in the presence of 1 mM 5MEBP and 2 mM NADH. The reciprocal of the reduction rates was plotted against that of concentration of the salts. The reduction rates were depressed with addition of 5MEBP. The Lineweaver–Burk's plots are shown in Fig. 5. In the presence of 5MEBP, the slopes for both MV and EBP reduction were different from those without addition of 5MEBP, while both intercepts were equal. These results indicate that 5MEBP competitively inhibited the reduction of MV and EBP. That is, the reduction of a series of the salts seems again to be catalyzed by the same enzyme.

Before explain the disagreement in the EBP reducing activity by the cell measured photoelectrochemically and spectrophotometrically, the cells were divided into subcellular fractions to search for the distribution of the enzyme, and the fractions were subjected to the measurments of reducing MV and EBP in the presence of NADH. The results are summarized in Table 3. The periplasm fraction showed almost no activity for MV or EBP reduction. On the other hand, the cytoplasm and chromatophore fractions showed high activity, as expected from the results.[8] It is indicated that the enzyme catalyzed the reduction of the salts by NADH was located within the cell membrane. Therefore, the results suggest that, to have the electron transfer between the cell and the electrode, these reagents must once penetrate the cell wall and membrane (or at least only the cell wall) to react with some sources of electrons in the cell (or on the membrane surface of the periplasmic side), and then repenetrate it to transfer the electron to the electrode. Earlier, Bagyinka et al.[14]

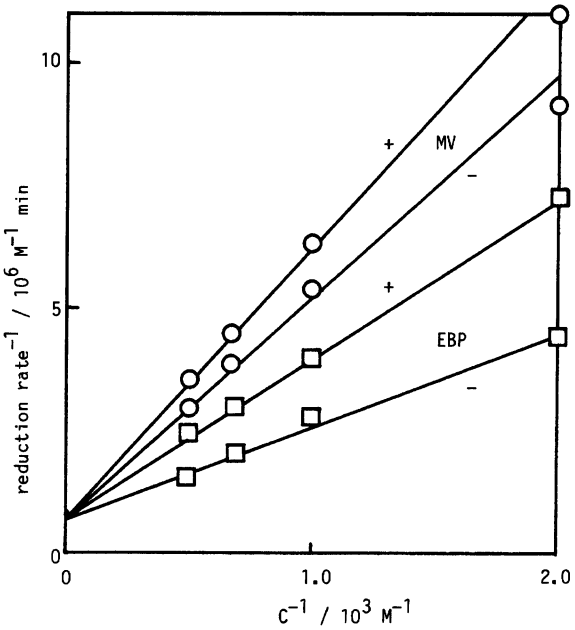

Fig. 5. Lineweaver–Burk's plots for reduction of MV or EBP by disrupted cells. ○ and □ represent the values for reduction of MV and EBP, and the symbols + and − represent those in the presence and the absence of 1 mM 5MEBP, respectively. The experimental conditions were as follows; $A_{873}=1$ in 0.1 M HEPES buffer (pH 8.0) containing 2 mM NADH.

Table 3. Reduction Rates of MV and EBP by Subcellular Fraction

Fraction	NADH added	Quantity	Reduction rate $\mu M\,min^{-1}$	
	mM	mg wet weight	MV	EBP
Intact cell	0	88[a)]	0.29	2.29
Intact cell	2	88[a)]	0.93	4.68
Periplasm	2	17[b)]	ca. 0	ca. 0
Disrupted cell	2	88[a)]	11.4	86.2
Chromatophore	2	23[a)]	4.35	37.2
Cytoplasm	2	46[b)]	3.67	41.8

The experimental conditions were as follows; the fractions were suspended in 4 ml of 0.1 M HEPES buffer (pH 8.0) containing 1 mM MV or EBP. a) The quantity was estimated from 4 ml of the suspension with $A_{873}=3$, and b) was determined by the Biuret method, as described in text.

had reported a method for locating of the hydrogenase in the photosynthetic membrane using MV or BV, and discussed the membrane penetrability of oxidized and reduced form of these reagents. So, we have tried to examine the penetrability of these bipyridinium salts in a similar manner, but the attempts have been unsuccessful because of the difficulties of maintaining the anaerobic condition in a centrifuge tube. Second, we have tried polarographically to measure the remaining reagents in the buffer solution (outside of the cell body), but have also failed in the attempts because the cell body had been weakly adsorbed on a mercury drop to form a granular mercury pool and finally the polarographic test solution (ca. 1 ml) had been buried in the granular pool.

Cyclic voltammetric measurements were, therefore, done using an amalgamated copper disk electrode to explore the disagreement in the data of the photocurrent by the cells and of reduction rate. The quantity of the reduced reagents by the cells and the effective concentration of the reagents in buffer solution (i.e. the quantity of the reagents taken into the cell from the solution) can be estimated from the difference of the voltammetric peak current for anodic and cathodic waves at different densities of cell bodies. Figure 6 shows typical cyclic voltammograms of MV in the presence and the absence of the cells. These bipyridinium salts have been reported to have quasi-reversible redox waves with one-electron transfer in a buffer solution,[13)] and the redox behavior was kept quasi-reversible even in the presence of the cells. If the salts are easily reducible by the cells and both oxidized and reduced form of the salts can penetrate rapidly through the cell wall and membrane, the anodic peak current increases with increasing density of the cells, while the cathodic peak disappears rapidly because the oxidized form of the salts generated on the electrode is immediately re-reduced by the cells (catalytic current). In addition, if only the oxidized form of the salts is penetrable, both the anodic and cathodic peak currents, then decrease gradually with increasing cell density because the reduced form of the

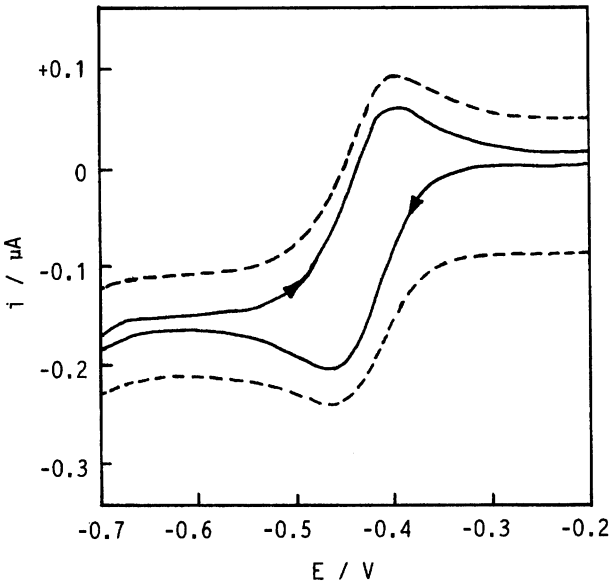

Fig. 6. Typical cyclic voltammograms of MV in the presence (dotted, $A_{873}=5$) and the absence (solid) of *Rs. rubrum* cells. The concentration of MV was 0.5 mM in 0.1 M HEPES buffer (pH 8.0), and potential sweep rate was 5 $mV\,s^{-1}$. The potential was initially swept from −0.2 V to a negative direction, and then reversed. The voltammogram obtained in the presence of *Rs. rubrum* cells is shown arbitrarily with respect to i, and its residual current was fundamentally similar to that obtained in the absence of cells.

salts remain taken into the cell body and becomes electrochemically inactive. Moreover, if the salts are hardly reducible by the cells, both the peak currents are almost independent on the cell density, regardless of the penetrability. These three examples are, of course, ideal, and the actual dependence of the magnitude of anodic and cathodic peak currents on the density of cell bodies will become complicated to a certain extent, due to the relative magnitude between the reduction rate of the reagents by the cell and the penetrating velocity. Figure 7 shows the actual cyclic voltammetric results thus

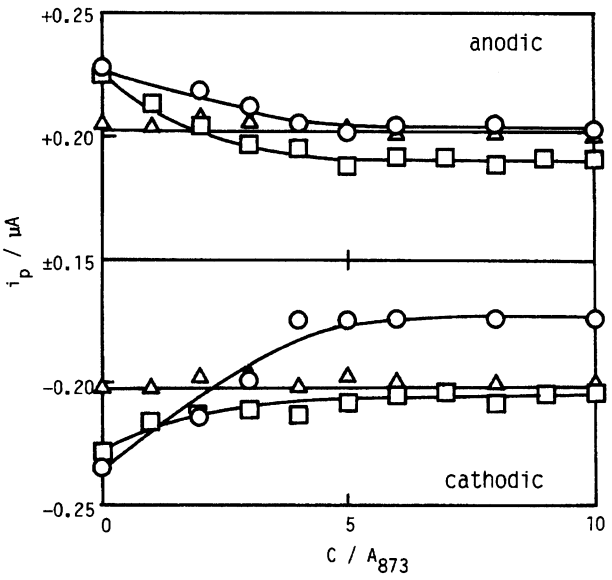

Fig. 7. Dependence of peak current on density of *Rs. rubrum* cells. ○, □, and △ represent the data obtained in the presence of 0.5 mM MV, EBP, and 5MEBP, respectively. Potential sweep rate was 5 $mV s^{-1}$.

obtained. A cathodic peak current for MV decreased more rapidly than that for EBP, maybe due to the catalytic effect as described, while an anodic peak current decreased slightly than that for EBP. In addition, both anodic and cathodic peak currents for 5MEBP were independent on the cell density, as expected. So, it is indicated that MV with both oxidized and reduced form can penetrate, at least, the cell wall and the reduced form can penetrate more hardly than the oxidized form. On the other hand, EBP with oxidized form can penetrate the cell wall but more hardly with the reduced form. The difference of penetrability for both oxidized and reduced form of the salts, and for the different mediators has also been discussed in the literature,[14,15] but the knowledge on the penetrating velocity has not, so far as we know. It becomes necessary to obtain the knowledge on the penetrating velocity of the reagents to understand more accurately the penetrability, and we plan to study potentiometrically the time profile of the equilibrium potentials for the cell suspension containing the reagents.

In conclusion, the anodic photocurrent generated at an SnO_2 electrode by *Rs. rubrum* cells was enhanced by the addition of MV, but not with EBP. However, the reductions of MV and EBP by the cells were observed spectrophotometrically. In addition, the enzyme that catalyzed the reduction of the salts (maybe, NADH:MV reductase) was indicated to be located within the cell membrane or on the membrane surface of the periplasmic side. So, the disagreement in both data of photocurrent and of reduction rate are suggested to be due to the difference of penetrability of the salts through the cell wall and membrane, and this suggestion was confirmed from cyclic voltammetric results of the reagents dissolved in the cell suspension.

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