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OXIDATIVE PHOSPHORYLATION IN *AZOTOBACTER VINELANDII*. ENERGY-LINKED pH CHANGES AND FLUORESCENCE CHANGES OF ATEBRIN AND 1-ANILINONAPHTHALENE-8-SULPHONATE

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SUMMARY

1. Phosphorylating particles from *Azotobacter vinelandii* show a rapid, respiration-induced reversible increase in pH of the suspending medium; this is not found with non-phosphorylating particles.

2. The observed pH response requires the presence of low concentrations of Mg^{2+} or of higher concentrations of Na^+ or K^+.

3. Between 40 and 10 °C the rates of proton influx and efflux have similar temperature coefficients; below 10 °C the effect of temperature is greater on proton efflux.

4. The kinetics of the energy-linked enhancement of fluorescence 1-anilinonaphthalene-8-sulphonate are slower than that of the quenching of the fluorescence of atebrin.

INTRODUCTION

Energy-linked pH changes observed in suspensions of mitochondria[1], submitochondrial particles[2] and chloroplasts[3] are usually interpreted as due to a translocation of protons (or of OH^- moving in the opposite direction) across semipermeable membranes in these organelles[1–4]. It has been shown that under appropriate conditions proton translocation is accompanied by a translocation of cations[5–8] or of anions[9]. The formation of a pH gradient and/or membrane potential are crucial elements of the chemiosmotic theory of oxidative phosphorylation[10,11].

Recently, energy-linked pH changes have been demonstrated in suspensions of cells from *Escherichia coli*[12], *Rhodosperillum rubrum*[13,14] and *Micrococcus denitrificans*[15], in chromatophores from *R. rubrum*[16] and in membrane vesicles from *E. coli*[17].

Changes in the pH of suspensions of cells or vesicles can be measured directly with a glass electrode while the internal pH may be calculated from changes in

Abbreviation: ANS, 1-anilinonaphthalene-8-sulphonate.

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absorbance of certain dyes such as bromothymol blue[18], bromocresol purple[19] or 5,5-dimethyl-2,4-oxazolidione[20] which are distributed across the membrane according to an existing pH gradient.

Other probes, *e.g.* 1-anilinonaphthalene-8-sulphonate (ANS) or auramine-O, interact with biological membranes in such a way that their fluorescence becomes enhanced[21–23] and further enhancement follows energization. Recently, it has been reported that the fluorescence of certain inhibitors of electron transport[24,25] or uncouplers of phosphorylation[26,27] becomes quenched upon interaction with energized submitochondrial particles or chloroplasts. In a previous paper it was reported that the uncoupler atebrin can be used as a fluorescent probe for the energized state in phosphorylating particles from *Azotobacter vinelandii*[27,28]. It has been proposed that atebrin interacts at a site in the membrane of these particles and of chloroplasts that is involved in energy conservation[26–31], although different explanations are given by other authors[32,33].

In the study reported here, energy-linked pH changes in suspensions of phosphorylating particles from *A. vinelandii* have been characterized and the results are discussed in relation to the behaviour of atebrin during interaction with the particles under energized conditions.

MATERIALS AND METHODS

Phosphorylating and non-phosphorylating particles from *A. vinelandii*, as well as preparations of the soluble factor, were prepared as described by Pandit-Hovenkamp[34]. Incubations were carried out in a thermostated cell equipped with a micro glass electrode (Electrofact), connected with a E.I.L. sensitive pH meter (Type 33 B-2). The standard reaction medium consisted of 25 mM sodium or potassium malate, 2.5 mM Tris–HCl or phosphate buffer, $MgCl_2$ (usually 5 mM) and 20 μg catalase. Other conditions are given in the legends. Particles (1–2 mg protein) were preincubated with the medium (adjusted to the pH indicated in the legends) for 5 min. Experiments were started by addition of 3–5 μl of aq. H_2O_2, corresponding to a final concentration of 1–2 mM O_2.

Fluorescence was measured as previously described[26,27]. In these experiments 0.1–0.2 mg particles were suspended in a medium containing 25 mM phosphate buffer (pH 7.4), 1 mM EDTA, 5 mM $MgCl_2$, 20 μM catalase and 25 mM sodium malate.

Enzymes, ADP and ATP were purchased from Boehringer (Mannheim, Germany), L-malate from Koch and Light, D-lactate from Sigma and other chemicals were obtained from BDH (analytical grade).

Valinomycin and recrystallysed anilinonaphthalene-8-sulphonate were kindly supplied by Dr C. W. Pettinga and Dr G. K. Radda, respectively. Nigericin was a gift from Eli. Lilly Cy. Indianapolis, U.S.A.

RESULTS

The kinetics of the respiration-driven pH pulse in a suspension of phosphorylating *Azotobacter* particles, with malate as substrate, is shown in Fig. 1 (upper trace). The pH of the suspension increases rapidly until a steady state is reached and decays exponentially upon anaerobiosis. Non-phosphorylating particles[34] show little response

(Fig. 1, lower trace). Proton translocation varied between 15 and 50 nmoles per mg protein and was found to be remarkably constant for a given preparation. It is possible to repeat a pH response 15 times in the same incubation before a decrease in extent occurs. The direction of the proton movement indicates that the polarity of the membrane in *Azotobacter* is the same as in chloroplasts, submitochondrial particles[2] and chromatophores[16] and opposite to that in intact mitochondria[1,10,11]. The rate of proton translocation is comparable with observations in other systems[17,35].

A reversible translocation of protons is also associated with the oxidation of D-lactate (Table I). The initial rate of proton influx, the total amount of proton translocation and, consequently, the rate of proton efflux were all lower than with L-malate as substrate. This is to be expected since the rate of oxidation of lactate

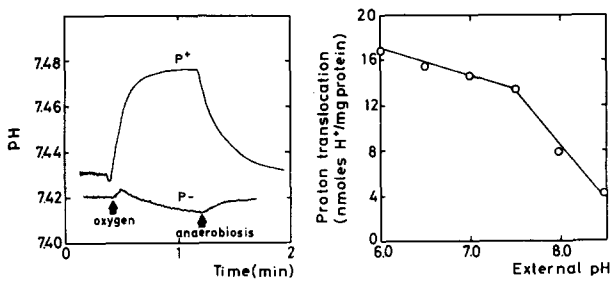


Fig. 1. Respiration-induced pH response in suspensions of phosphorylating and non-phosphorylating particles from *A. vinelandii*. Medium: 25 mM potassium malate, 3 mM $MgCl_2$, 2.5 mM Tris–acetate buffer, 20 μg catalase and 1.9 mg (protein) phosphorylating particles (upper trace) or 2.2 mg non-phosphorylating particles (lower trace) (prepared as described in ref. 34). Addition of O_2 where shown. Proton translocation was calculated by calibration with a known amount of oxalic acid at the end of each experiment.

Fig. 2. Effect of initial pH on proton translocation with malate as substrate. Conditions as in Fig. 1 except that the $MgCl_2$ concentration was 5 mM. Phosphorylating particles, prepared in 125 mM sucrose, 50 mM KCl and 2.5 mM phosphate buffer (pH 7.0), had been stored in liquid N_2 before use.

TABLE I

PROTON TRANSLOCATION WITH L-MALATE AND D-LACTATE AS SUBSTRATES

Reaction medium: 2 mM $MgCl_2$, 0.5 mM EDTA, 20 mM sodium L-malate or 15 mM sodium D-lactate, 50 μg catalase, 2 mg phosphorylating particles. Final volume, 1.0 ml; initial pH, 7.4.

	Malate	*Lactate*
Oxidation rate* (μatoms O/min per mg protein)	4	0.7
Net proton translocation (nmoles H^+/mg protein)	35	12
Initial rate of proton influx (nmoles H^+/min per mg protein)	750	283
Initial rate of proton efflux (nmoles H^+/min per mg protein)	550	168

* Estimated from the time required to reach anaerobiosis.

was only 20% of that of malate and the P/O ratios for the two substrates are the same[36].

Proton translocation with malate as substrate decreases slowly as the external pH raised from 6.0 to 7.4–7.5, above which there is a sharp decrease (Fig. 2).

Fig. 3A shows the effect of temperature on the rates of proton influx and efflux and on the rate of malate oxidation. Influx and efflux have a similar temperature coefficient between 40 and 10 °C but below 10 °C proton efflux is more dependent on temperature than the influx, suggesting that at lower temperatures either the rate-determining step of efflux or the mechanism of efflux itself may be different. Fig. 3B shows that the net translocation shows a plateau between 20 and 30 °C. A plateau might be expected if we assume that the extent of translocation is determined by a dynamic equilibrium between proton influx and proton efflux (*cf.* ref. 37). However, this assumption does not seem to be true at temperatures below 10 °C or above 30 °C.

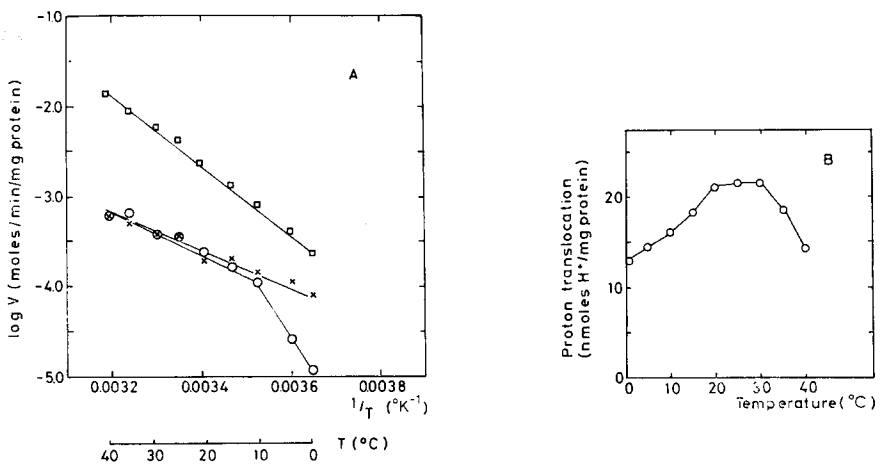
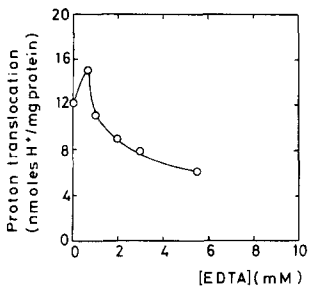


Fig. 3. (A) Arrhenius plot of the rates of malate oxidation (□—□), proton influx (× — ×) and proton efflux (○—○) of phosphorylating particles. All velocities are expressed in nmoles/min per mg protein. Experimental conditions as described in Materials and Methods and Fig. 2. (B) Net proton translocation as function of temperature.

Below 10 °C one might expect a higher net translocation than actually observed, in view of the strongly reduced rate of proton efflux, whereas above 30 °C the temperature coefficients of influx and efflux are the same as between 10 and 30 °C.

It is to be expected that proton translocation is balanced by a compensating translocation of either cations or of anions. In mitochondria, Ca^{2+} and K^+ can exchange with protons (*cf.* ref. 38). Low concentrations of EDTA stimulate and higher concentrations inhibit proton translocation in *Azotobacter* particles (Fig. 4). The translocation could be restored completely by addition of a slight excess of $MgCl_2$ (10 mM) at the end of the titration, but not by adding $CaCl_2$ (not shown here). The initial stimulation (Fig. 4) may be explained by the removal of inhibitory cations (perhaps Ca^{2+}) and the inhibition at higher concentrations by removal of Mg^{2+}, but there seems to be no absolute requirement for Mg^{2+}. Addition of valinomycin in the presence of high concentrations of K^+ restored proton translocation in the presence of EDTA (Table II) suggesting that, as in particles from beef heart mitochondria[39,40], a proton–monovalent cation exchange can occur in *Azotobacter* particles.



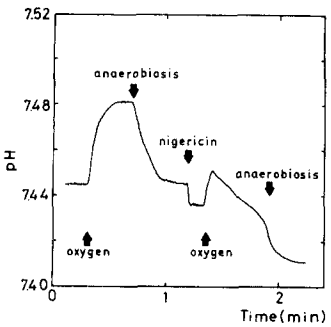


Fig. 4. Effect of EDTA on proton translocation by *Azotobacter* particles. Conditions as in Fig. 1, except that $MgCl_2$ was omitted. 2.3 mg protein was added.

Fig. 5. Effect of nigericin on the pH of suspensions of *Azotobacter* particles. Addition of 1 μg nigericin and 2 mM H_2O_2 where shown. Further conditions as in Fig. 1.

TABLE II

EFFECT OF EDTA AND VALINOMYCIN ON PROTON TRANSLOCATION

Reaction medium: 100 mM KCl, 1 mM EDTA, 25 mM potassium L-malate, 20 μg catalase and 0.6 mg particle protein. Phosphorylating particles were prepared in 50 mM KCl, 2.5 mM phosphate buffer (pH 7.0) and 125 mM sucrose, and were stored in liquid N_2 before use. Temperature during incubation, 25 °C. Initial pH, 7.4.

Valinomycin ($\mu g/ml$)	*EDTA* (*mM*)	*Proton uptake* (*nmoles/mg protein*)
—	1	22
—	11	13
1.25	11	30

As is to be expected, phosphorylation, as measured by the rate of pH increase after addition of 50 μM ADP (*cf.* Nishimura *et al.*[41]), was slightly decreased by valinomycin in the presence of K^+. Nigericin, known to facilitate a proton – K^+ exchange[40], causes a slow outward translocation of protons that is superimposed on the reversible proton uptake associated with respiration (Fig. 5). The net inward translocation and efflux (after anaerobiosis) are consequently decreased by the addition of nigericin. The slow, nigericin-induced outward translocation may be due to an exchange with external K^+. As shown in Table III, nigericin alone has little effect on phosphorylation (*cf.* ref. 42), whereas together with valinomycin the phosphorylation was inhibited by 84%. This is probably due to a cyclic transport of protons, resulting in a loading of the particles with K^+ at the expense of respiratory energy.

The effect of nigericin *plus* nitrate (Table III) can be explained in a similar way. Nitrate itself stimulates proton translocation since it is a permeant anion and nigericin causes a subsequent exchange of K^+, the net result being an energy-consuming inward translocation of KNO_3.

TABLE III

COMPARISON OF THE BEHAVIOUR OF PROTON TRANSPORT AND PHOSPHORYLATION UNDER VARIOUS CONDITIONS

Experimental conditions as described in Materials and Methods and Table I. Proton translocation is expressed as percentage of the value observed before additions were made. P/O ratios were calculated from the rates of ATP synthesis and oxidation in a series of parallel experiments under strictly similar conditions.

Additions	*Proton uptake (% of control)*	*P/O ratio (% of control)*
None	100	100
20 mM nitrate	326	86
20 mM nitrate + 1 μg nigericin	78	35
1.25 μg valinomycin	178	82
1.25 μg valinomycin + 1 μg nigericin	45	16
1 μg nigericin	25	75

TABLE IV

EFFECT OF ANIONS ON PROTON UPTAKE

Reaction medium: 5 mM $MgCl_2$, 1 mM EDTA, 25 mM sodium L-malate, 20 μg catalase and 2.45 mg protein, in a final volume of 2.65 ml. Phosphorylating particles were prepared in 50 mM KCl and 2.5 mM phosphate buffer (pH 7.0). Temp., 1 °C; initial pH 7.4. The anion (23 mM) was added as sodium salt.

Anion	*Proton translocation (percentage of control)*	*Initial rate (percentage of control)*		*Velocity constant of proton efflux* (s^{-1})
		Proton influx	*Proton efflux*	
None	100	100	100	0.015
NO_3^-	250	283	350	0.021
CNS^-	216	445	309	0.035
I^-	268	176	320	0.015
Acetate	103	76	118	0.016
SO_4^{2-}	119	107	140	0.013
Cl^-	175	128	169	0.013

The effect of other permeant anions is shown in Table IV. All anions were added as their potassium salts and the experiments were performed at 1 °C to facilitate a comparison of the rates of proton influx and efflux. As is the case in submitochondrial particles[35], nitrate, thiocyanate and iodide stimulate proton influx, whereas sulphate and acetate have only little effect. Chloride has a slight effect. The influence of the anions on the velocity constants of proton eflux is less than in the experiments

of Papa *et al.*[35], possibly due to the lower temperature at which our experiments were carried out. The slight enhancement of proton translocation by KCl may be related to the previously reported inhibition of phosphorylation by chloride[36], similar to the uncoupling effect of this anion in submitochondrial particles reported by Christiansen *et al.*[43] and Papa *et al.*[39].

As reported previously[27], the fluorescence of the uncoupler atebrin can be quenched almost completely by phosphorylating particles energized by malate oxidation. The quenching in chloroplasts has been explained by Kraayenhof in terms of an interaction with the enzymes directly involved in energy-conservation[26,29–31], whereas Schuldiner and Avron[32]. Gromet-Elhanan[33] believe that it is due to formation of protonated atebrin. However, the fact that the fluorescence intensity of atebrin changes by less than 50% when the pH is changed from about 10 to 2 (*cf.* ref. 44) indicates that the complete quenching of fluorescence observed when atebrin interacts with energized *Azotobacter* particles[27,28] or with chloroplasts[26,29] cannot be due to protonation of the probe within the membrane. In this respect it should be noted also that other acridines with pK values well above 9 show a complete quenching[31]. The excitation and emission spectra shown in Fig. 6 also indicate that the observed quenching cannot be explained by a shift in the absorption or emission peaks.

Table V shows that atebrin is bound to some extent to phosphorylating particles under non-energized conditions, but a large increase in binding sites occurs in the energized state.

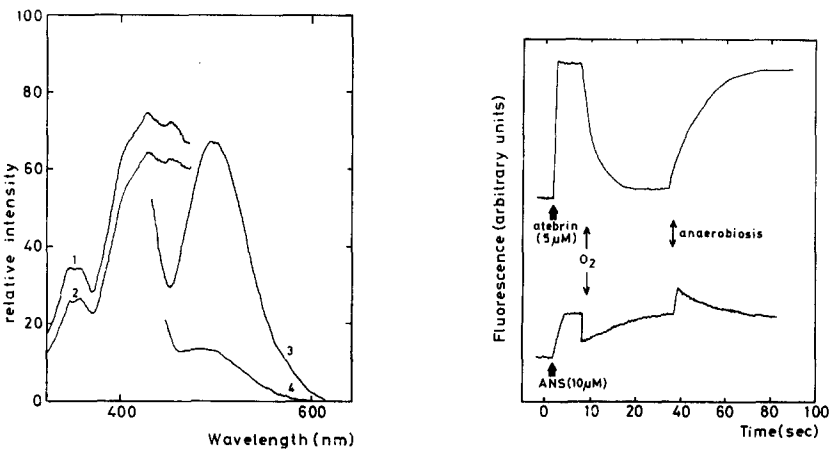


Fig. 6. Excitation (Curves 1 and 2) and emission (Curves 3 and 4) spectra of atebrin in energized and non-energized phosphorylating particles. Spectra were measured on a Aminco-Bowman spectrofluorimeter. Experimental conditions as described in Materials and Methods. Curves 1 and 3 were recorded with 10 μM atebrin added to the suspension in the absence of O_2; Curves 2 and 4 are recordings of the same suspension after addition of O_2. For convenience in plotting, the excitation spectrum shown in Curve 2 is drawn on a different scale from that of Curve 1. The emission spectra (Curves 3 and 4) are drawn on the same scale.

Fig. 7. Respiration-induced fluorescence response of atebrin and ANS by *Azotobacter* particles Medium: 25 mM phosphate buffer, 5 mM $MgCl_2$, 1 mM EDTA, 20 μg catalase, 25 mM potassium malate, and either 5 μM atebrin or 10 μM ANS. Final volume. 1.0 ml; temp., 25 °C; pH 7.4. Fluorescence was recorded with an oscillograph connected with an Eppendorf fluorimeter (*cf.* ref. 26). Addition of 1 mM O_2 where shown. Excitation wavelengths were: atebrin, 430 nm; ANS, 360 nm. Emission wavelengths: atebrin, 505 nm; ANS, 470 nm.

The fluorescence of ANS is enhanced on addition to phosphorylating particles and a further enhancement follows energization (Fig. 7), similar to the behaviour of this probe in submitochondrial particles[22,23,29]. The immediate changes after addition of O_2 and at anaerobiosis are probably due to changes in the absorption of the excitation light by redox carriers of the particles (*e.g.* iron–sulphur proteins). The kinetics of the response of fluorescence of ANS are considerably slower as compared with atebrin.

TABLE V

BINDING OF ATEBRIN TO PHOSPHORYLATING PARTICLES UNDER ENERGIZED AND NON-ENERGIZED CONDITIONS

Reaction medium as described in Materials and Methods. Phosphorylating particles were incubated with atebrin in stainless-steel centrifuge tubes (final volume 5 ml) in the presence or absence of 50 mM L-(−)malate. 15 mM H_2O_2 was added and the tubes were immediately centrifuged for 10 min at ($80000 \times g$) in a Spinco ultracentrifuge. Atebrin was measured fluorimetrically in the resultant pellet (*cf.* refs 28 and 48).

Atebrin added (μM)	*Atebrin found in pellet (nmoles/mg protein)*	
	Energized (malate present)	*Non-energized (without malate)*
10	12.5	0.7
25	40	3.5

TABLE VI

EFFECT OF VALINOMYCIN ON THE KINETICS OF QUENCHING OF ATEBRIN FLUORESCENCE

Medium: 2.5 mM Tris–HCl buffer, 5 mM $MgCl_2$, 1 mM EDTA, 25 mM malate, 20 μg catalase and 1.0 mg phosphorylating particles were preincubated for 5 min. Atebrin (5 μM) was added and the experiment was started with addition of 2 mM H_2O_2. Valinomycin (5 μg/ml) was present where mentioned. Temp., 1 °C.

Change of fluorescence	*Pseudo-first-order constant* (s^{-1})	
	Without valinomycin	*With valinomycin*
Decrease (after addition of O_2)	0.07	0.12
Increase (after anaerobiosis)	0.015	0.025

As shown in Table VI, the kinetics of the fluorescence response of atebrin is accelerated by valinomycin, indicating that either a movement of the probe across the membranes may be involved in exchange with K^+, or that the conformational changes responsible for the interaction are faster when membrane permeability for K^+ is enhanced by valinomycin.

Pandit-Hovenkamp[45] found that incubation of non-phosphorylating particles with a "soluble factor" in the presence of salts restored phosphorylation. In the experiment shown in Fig. 8, phosphorylation (with malate as substrate), proton translocation and the atebrin response were measured, after preincubation of non-phosphorylating particles for 45 min with varying amounts of a partly purified preparation of the soluble factor in the presence of 50 mM KCl. Proton translocation and the amount of atebrin bound to the preparation appear to be restored with smaller amounts of the soluble factor than phosphorylation. It was also noticed that the amount of atebrin bound to the particles was approximately double the net proton translocation; the values corresponding to 100% (Fig. 8) being 19.3 nmoles/mg protein and 10.2 nmoles/mg protein, respectively.

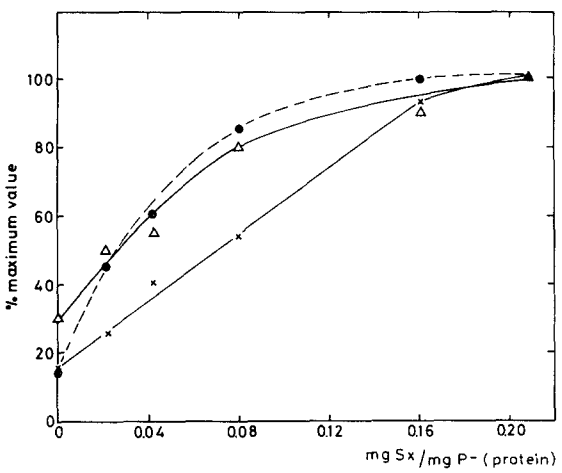


Fig. 8. Reconstitution of phosphorylation, proton translocation and fluorescence response with atebrin by incubation of non-phosphorylating particles with increasing amounts of soluble factor. Conditions: non-phosphorylating particles[34] were incubated for 45 min with various amounts of a partly purified preparation of soluble factor[34,45] (as indicated on the abscissa). Proton translocation and fluorescence of atebrin were determined as described in Materials and Methods. Phosphorylation with malate as substrate was measured as described in ref. 36. The amount of atebrin bound to the particles was calculated as described in ref. 27. ●– – –●, binding of atebrin; △—△, proton translocation; ×—×, P/O ratio with malate.

DISCUSSION

The experiments described in this paper demonstrate that the transition of phosphorylating particles from *A. vinelandii* from a non-energized to an energized state, induced by respiration with malate or lactate as substrates, results in a rapid reversible increase of pH of the suspending medium. It has been shown that the extent of the pH increase is lowered by removal of Mg^{2+} and stimulated by K^+ in the presence of valinomycin, suggesting that a trans-membrane transport of protons (or of OH^-) accompanied by cation transport is involved. This is further confirmed by the effects of nigericin and of permeable anions. The biphasic pH change observed in the presence of nigericin (Fig. 5) suggests that part of the pH change is due to a binding of protons to certain sites on the particles that undergo a change in p*K*

during energization. Such an assumption may also explain the relatively sharp decrease of proton translocation above pH 7.4–7.5. We propose that, in the presence of nigericin, only the proton binding contributes to the pH change observed immediately after energization or anaerobiosis, whereas the exchange of external protons against internal K^+ is reversed by the action of the antibiotic.

It has been suggested[22,46–48] that the negatively charged probe ANS is bound to positive regions in the membranes of submitochondrial particles. The finding that in *A. vinelandii* particles, both ANS and the positively charged probe atebrin are bound under energized conditions may indicate that in these membranes also a separation of positive and negative charges occurs, as proposed by Azzi[46,48] and by Nordenbrand and Ernster[47]. In this respect the apparent stoicheiometry between proton translocation and the amount of atebrin that can be bound to energized particles is interesting, since it suggests that the negatively charged sites generated in the membrane could be responsible for the binding of atebrin as well as for binding of protons.

The slow response of ANS as compared with atebrin (Fig. 7) may be due either to the interaction of this probe with a conformation of the membranes that is formed later than that detected by atebrin, or due to a slow binding (or redistribution) of ANS.

The relatively large amounts of soluble factor required to obtain reconstitution of phosphorylation compared with the amounts that restore proton translocation or the fluorescence response of atebrin may indicate that the "soluble factor" may actually contain several components, one being involved in the primary processes of energy conservation and another responsible for the reactions leading to ATP synthesis. These components may have functions in *A. vinelandii* membranes comparable to those of some of the factors isolated from beef-heart mitochondria[49,50].

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