Temperature-Induced Alterations in 8-Anilino-1-naphthalenesulfonate Fluorescences with Membranes from *Mycobacterium phlei*†

H. N. Aithal,‡ Vijay K. Kalra, and Arnold F. Brodie*

ABSTRACT: Electron transport particles (ETP) from *Mycobacterium phlei* exhibit an increase in 8-anilino-1-naphthalenesulfonate (ANS) fluorescence upon energization. The energized fluorescence was found to be sensitive to respiratory inhibitors and anaerobiosis. Energization of the membranes results in an increase in ANS binding to the membrane, an increase in the relative quantum yield, and a decrease in the apparent K_D of the dye–ETP complex. Depleted ETP, devoid of particulate-bound coupling factor, exhibited the same level of energized fluorescence as observed with untreated ETP. An ATP-dependent energized fluorescence was observed only after trypsin treatment of ETP. The energized fluorescence elicited by ATP in ETP was found to require Mg^{2+} ions. Short-term heat treatment of ETP, which results in an increased level of phosphorylation, elicited an increased level (2–3-fold) of energized fluorescence. The increase in the energy-dependent ANS response in heat-treated membrane vesicles, as compared to untreated ETP, was shown to be result of an increase in the affinity of membrane binding sites for ANS and an increase in the relative quantum yield (3.6–5-fold) without changes in the number of irreversible or slowly reversible binding sites. These results indicate that heat treatment induced a change in the structure of the membranes which increased the efficiency of coupled phosphorylation. However, a quantitative relationship between the increased level of phosphorylation and the energized fluorescence was not established.

A number of fluorescent dyes have been used as probes of biological membranes (Edelman and McClure, 1968; Chance, 1971; Brand and Gohlke, 1972; Waggoner and Stryer, 1970; Vanderkooi and Martonosi, 1971; Kasai *et al.*, 1969; Radda, 1971; Secrist *et al.*, 1972). The dye, 8-anilino-1-naphthalenesulfonic acid (ANS),[1] has been utilized for probing the structure of the hydrophobic regions of macromolecules (Weber and Lawrence, 1954; Stryer, 1965, 1968; McClure and Edelman, 1966; Tasaki *et al.*, 1971). Characteristic changes in ANS fluorescence occur in mitochondria and submitochondrial particles upon the addition of ATP or oxidizable substrates (Azzi *et al.*, 1969, 1971; Azzi and Vainio, 1971; Datta and Penefsky, 1970; Avi-Dor *et al.*, 1970; Nordenbrand and Ernster, 1971; Brocklehurst *et al.*, 1970). The changes in ANS fluorescence have been attributed either to alterations of the membrane potential or to changes in the conformation of the membrane (Chance, 1970; Brocklehurst *et al.*, 1970; Nordenbrand and Ernster, 1971; Azzi *et al.*, 1971).

Bacterial membrane preparations which carry out oxidative phosphorylation have also been observed to exhibit similar fluorescent properties with ANS, as those described for mitochondrial systems (Chance, 1970; Kalra *et al.*, 1972). The present report describes in detail changes in fluorescence that occur upon energization of the membrane preparations from *Mycobacterium phlei* which have been subjected to heat treatment. Heat treatment has been observed to affect membrane structure and influence the level of oxidative phosphorylation (Bogin *et al.*, 1970a; Aithal *et al.*, 1971). An increase in the level of energy-dependent ANS fluorescence and an increase in the level of oxidative phosphorylation was observed with heat-treated membrane preparations. However, a direct correlation between the level of oxidative phosphorylation and the energized fluorescence of the membrane preparations subjected to different temperatures was not observed.

Experimental Procedures

Materials. M. phlei ATCC 354 was grown and harvested according to the method described previously (Brodie and Gray, 1956). The electron transport particles (ETP) capable of coupled phosphorylation were prepared by sonic disruption of washed cells (Brodie, 1959).

Preparation of Depleted ETP. The bound coupling factor (BCF_4) was removed from the ETP by washing the ETP two times with water in the absence of Mg^{2+} ions according to the method of Higashi *et al.* (1969). The resulting depleted ETP (DETP) were capable of oxidation but unable to couple phosphorylation to substrate oxidation (Higashi *et al.*, 1969).

Reconstituted ETP. Depleted ETP (8.0 mg of protein) were incubated with BCF_4 (4.0 mg of protein) in the presence of 4 mM $MgCl_2$ at 30° for 5 min. Centrifugation at 144,000*g* for 30 min yielded reconstituted electron transport particles. The reconstituted ETP were suspended in water before use.

Heat Treatment of ETP. ETP preparations, suspended in water (15 mg of protein/ml), were treated as previously reported (Bogin *et al.*, 1970a) for 10 min at either 40, 50, 60, or 70° and then were allowed to cool slowly and kept on ice until used. Unless otherwise stated the heat-treated ETP

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‡ Present address: Department of Pathological Chemistry, University of Western Ontario, London, Ontario, Canada.

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; ETP, electron transport particles; DETP, ETP depleted of bound coupling factor; H-ETP, heat-treated ETP at 50° for 10 min; BCF_4, particulate-bound coupling factor; F_3CCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Cl_5PhOH, pentachlorophenol; Ph_4B, tetraphenylboron; NE_f, nonenergized fluorescence; E_f, energized fluorescence.

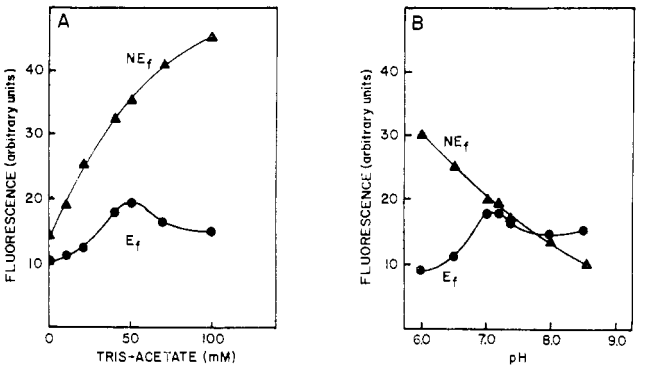

FIGURE 1: The effect of Tris-acetate concentration (A) and pH (B) on ANS fluorescence under nonenergized and energized conditions with ETP from *M. phlei*. The reaction mixture contained, in a final volume of 3 ml, ETP (1.0 mg of protein), 83.3 μM anilinonaphthalenesulfonate (ANS), and Tris-acetate buffer. Either the concentration of Tris-acetate buffer (pH 7.2) (A) or the pH of 50 mM Tris-acetate (B) was varied as indicated. ANS fluorescence was measured at 480 nm with an excitation wavelength of 368 nm. Energized fluorescence was measured after addition of 17 mM Tris-succinate (pH 7.2).

(H-ETP) refers to ETP heated to 50° for 10 min and then allowed to cool slowly to room temperature and then placed in ice until used.

Measurement of Oxidation and Phosphorylation. Oxygen uptake was measured by conventional manometric technique using a Gilson differential respirometer at 30°. Following termination of the reaction with 10% CCl_3COOH, the inorganic orthophosphate was determined by the method of Fiske and Subbarow (1925).

Measurement of Fluorescence. Changes in ANS fluorescence were measured with a Baird-Atomic Fluorispec SF-100 fluorimeter (Kalra *et al.*, 1972) at 25°. With succinate as an electron donor, the excitation wavelength was 368 nm and emission was measured at 480 nm. The increase in fluorescence upon the addition of ETP (1.0 mg of protein) to a medium containing 0.25 μmol of ANS and 50 μmol of Tris-acetate (pH 7.2) in a total volume of 3 ml is referred to as nonenergized fluorescence, NE_f. A further increase in the fluorescence observed upon the addition of succinate or other oxidizable substrates is referred to as energized fluorescence, E_f (Nordenbrand and Ernster, 1971). Arbitrary units used to measure NE_f and E_f were the same for all experiments.

Dye-Binding Determination. ANS binding was measured as follows. The reaction mixture of 3 ml containing 50 mM Tris-acetate (pH 7.2), 3.3 mM $MgCl_2$, ETP, or H-ETP (1.68 mg of protein), and ANS varying from 19.29 to 333.2 μM, were incubated at 25° for 5 min in the presence of 17 mM succinate or 17 mM succinate + 2 mM KCN. After 5 min, the samples were rapidly cooled to 0° and centrifuged at 144,000*g* for 30 min. The concentration of protein used in the experiment was low enough to keep the membrane system in the oxidized state during centrifugation. The amount of dye in the supernatant (unbound) and in the pellet was determined in the presence of saturating amounts of bovine serum albumin as reported by Azzi *et al.* (1971). The number of binding sites in ETP and heat-treated ETP, under nonenergized and energized conditions, was obtained by using Scatchard plots as described by Azzi and Santato (1971).

The apparent dissociation constants (K_D) of the ETP–ANS complex and heat-treated ETP complex, under nonenergized and energized conditions, were determined according to the method of Cheung and Morales (1969) by a double-reciprocal plot of fluorescence *vs.* ANS concentrations. The relative quantum efficiencies were determined by plotting a double-reciprocal graph of fluorescence *vs.* protein concentrations similar to that reported by Brocklehurst *et al.* (1970).

Prolonged Sonication of ETP. The ETP from *M. phlei* are generally prepared by sonication of washed whole cells for 4 min at 4° (Brodie, 1959). Aliquots of ETP were further sonicated for 40 min and assayed for changes in fluorescence.

Escherichia coli Preparations. ETP from *E. coli* W cells were prepared by sonication of the washed cells according to the method of Kashket and Brodie (1963).

Protein Determination. Protein was estimated by the biuret method using deoxycholate for solubilization of the membrane preparations (Gornall *et al.*, 1949).

ANS was obtained from Eastman Kodak Co. and the Mg^{2+} salt was prepared as described by Weber and Young (1964).

Results

Effect of pH and Ionic Strength on ANS Fluorescence of ETP. The addition of membrane preparations (ETP) to a reaction mixture of ANS and Tris buffer (pH 7.2) caused an increase in fluorescence (nonenergized fluorescence, NE_f) and a blue shift from 530 to 480 nm. The addition of succinate further increased fluorescence (energized fluorescence) and resulted in a blue shift of 4 nm (476 nm). This energized fluorescence (E_f) decreased to the nonenergized level when the system became anaerobic (Kalra *et al.*, 1972). The effects of pH and buffer concentrations on NE_f and E_f are shown in Figure 1. The NE_f increased with an increase in the concentration of Tris-acetate buffer. However, the E_f was enhanced by increasing the concentration of Tris-acetate buffer (maximum E_f between 40–50 mM), but then decreased at higher buffer concentrations (Figure 1A). The NE_f decreased as the pH was increased from 6.0 to 9.0. However, the E_f exhibited a peak at pH 7.2 (Figure 1B). Thus, the system was assayed at pH 7.2 with 50 mM Tris-acetate buffer.

Effect of Inhibitors of Oxidation and Uncoupling Agents. In mitochondrial systems, it has been demonstrated that both inhibitors of oxidation and uncoupling agents cause a decrease in the energized fluorescence (Chance, 1970; Nordenbrand and Ernster, 1971; Azzi *et al.*, 1969). The effect of various inhibitors and uncoupling agents was tested on the E_f with the ETP from *M. phlei*. The results are presented in Table I. Respiratory inhibitors, cyanide and azide, inhibited the E_f. Irradiation at 360 nm, which has been shown to inhibit succinate and NAD^+-linked oxidations (Kurup and Brodie, 1966; Brodie *et al.*, 1957), also inhibited the E_f. A variety of uncouplers (Table I) inhibited the E_f with the exception of *N,N'*-dicyclohexylcarbodiimide. *N,N'*-dicyclohexylcarbodiimide was used at a concentration which has been shown to uncouple phosphorylation from oxidation in ETP from *M. phlei* (Kalra and Brodie, 1971). It would appear that the energized fluorescence is disrupted by uncoupling agents and respiratory inhibitors. The inhibitors did not significantly affect the NE_f.

Effect of ATP and Mg^{2+}. ATP has been shown to elicit energized fluorescence in submitochondrial particles (Chance, 1970; Datta and Penefsky, 1970; Nordenbrand and Ernster, 1971). The addition of ATP produced a decrease in nonenergized fluorescence in *M. phlei* ETP (Figure 2A) and a further decrease was observed upon the addition of Mg^{2+} ions (Figure 2B). The effect of ATP and Mg^{2+} on E_f was also examined with succinate as substrate (Figure 2C,D). The succinate-induced E_f decreased upon the addition of ATP and this effect

TABLE I: Effects of Inhibitors and Uncoupling Agents on the E_f and NE_f of ANS Fluorescence with ETP from *M. phlei*.[a]

Inhibitors	Concn (M)	NE_f[b]	Changes in Fluorescence (Arbitrary Units) E_f Before Addn of Inhibitor	E_f After Addn of Inhibitor
Cyanide	1×10^{-2}	23	11	2
F_3CCP	5×10^{-5}	26	10	2
Dicyclohexylcarbodiimide	5×10^{-5}	22	10	10
Gramicidin	2 μg	24	12	3
Dnp	1×10^{-4}	25	10	1
Cl_5PhOH	1×10^{-4}	23	11	2
Ph_4B	5×10^{-5}	21	11	3
Azide	1×10^{-2}	26	9	4
Irradiation (360 nm)		17	11[c]	0

[a] The reaction mixture contained, in a final volume of 3 ml, 50 mM Tris–acetate (pH 7.2) and 83.3 μM anilinonaphthalenesulfonate (ANS). The addition of particles (ETP, 0.74 mg of protein) resulted in fluorescence (NE_f). Further addition of succinate (17 mM) caused a further increase in fluorescence (E_f). Inhibitors, at concentrations indicated, were added after the steady-state level of energized fluorescence was attained (usually 1–2 min after the addition of succinate). Irradiation of ETP was carried out at 4° for 20 min at 360 nm. [b] The NE_f was determined before the addition of the inhibitors. [c] Determined before irradiation.

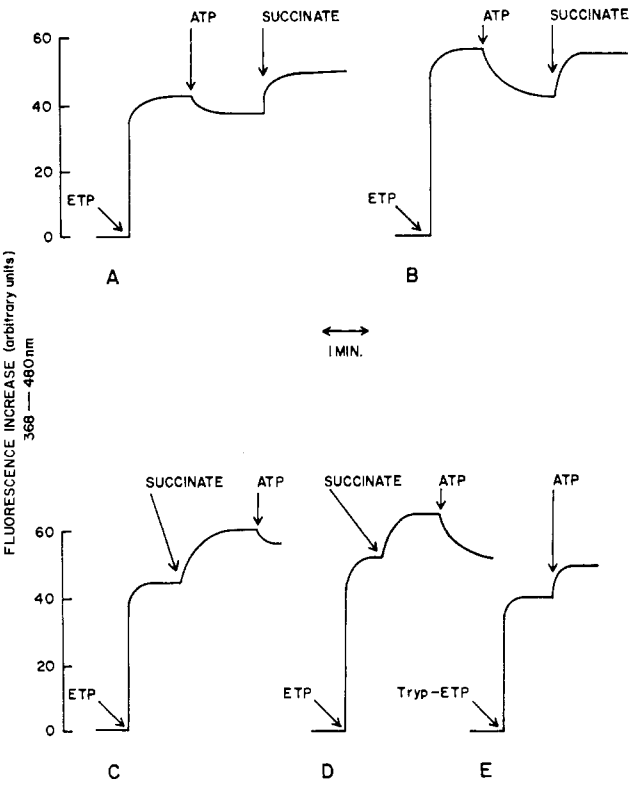

FIGURE 2: The effect of ATP and Mg^{2+} on anilinonaphthalenesulfonate (ANS) response of the ETP from *M. phlei*. The reaction mixture contained, in a final volume of 3 ml, ETP (1.0 mg of protein), 83.3 μM anilinonaphthalenesulfonate (ANS), 50 mM Tris–acetate buffer (pH 7.2), and where indicated 1.7 mM ATP and 17 mM succinate (final concentrations) was added (A–D). In parts B and D, the reaction mixture in addition contained 1 mM $MgCl_2$. In part E, the ETP (2.0 mg of protein) were trypsinized in 50 mM Tris–acetate buffer (pH 7.2) and 1 mM $MgCl_2$ with trypsin (150 μg of protein) at 30° for 10 min and then the reaction was terminated with the addition of trypsin inhibitor (300 μg of protein). Trypsinized ETP (1.2 mg of protein) were added to the reaction mixture containing 83.3 μM ANS, 50 mM Tris–aceate buffer (pH 7.2), and 1 mM $MgCl_2$, in a final volume of 3 ml. ATP was added at a final concentration of 1.7 mM as shown.

was potentiated by the presence of Mg^{2+} ions (Figure 2C,D). ATP was only observed to enhance the ANS fluorescence in ETP when the particles were trypsinized (Figure 2E). This ATP-enhanced fluorescence was Mg^{2+} dependent. These results are of interest since efforts to demonstrate ATP-driven energy-linked reactions have been futile with the *M. phlei* system (Murthy and Brodie, 1964).

Effects of Heat Treatment. It was previously demonstrated (Kalra *et al.*, 1972) that heat treatment of *M. phlei* ETP at 50° for 10 min resulted in a 3-fold increase in the E_f as compared to untreated ETP whereas the NE_f increased 1.5-fold. There was also an increased level of phosphorylation in the heat-treated ETP (Bogin *et al.*, 1970a; Aithal *et al.*, 1971). However, a quantitative correlation could not be obtained between the increased E_f and the increased level of phosphorylation due to heat treatment. In the present studies, the effect of different temperatures on the increase in E_f was studied in relation to oxidative phosphorylation in ETP. The ETP were heated at different temperatures and allowed to cool slowly as described in Experimental Procedures. Fluorescence studies were carried out on these preparations at constant temperature of 25°. The pattern of changes in E_f and coupled phosphorylation to oxidation of substrates with ETP subjected to different temperatures was similar (Figure 3). It should be noted that heat treatment resulted in a slight decrease in the rate of oxidation (2–5%), whereas the level of phosphorylation was increased 1.4–2-fold.

E_f Induced by Different Energy Sources in ETP and H-ETP. The effect of heat treatment on membrane vesicles from *M. phlei* on the E_f with different energy sources was examined. It can be seen from Figure 4 that either exogenous NADH or ascorbate–phenazine methosulfate as electron donor was found to elicit the E_f with ETP. Heat-treated ETP (H-ETP) exhibited a greater level of E_f than that observed with untreated ETP in the presence of these energy sources. Thus, when NADH was used as a substrate, the increase in fluorescence of ANS (405 → 580 nm) was about 3-fold in H-ETP as compared to untreated ETP (Figure 4A). Heat treatment of ETP at 50° for 10 min also resulted in a 5-fold increase in E_f when ascorbate–phenazine methosulfate was used as an energy source (Figure 4B). The E_f induced by NADH and ascorbate–phenazine methosulfate with ETP was completely inhibited by F_3CCP (5×10^{-5} M), whereas with H-ETP, F_3CCP (5×10^{-5} M) inhibited the E_f by 70%.

Studies with Coupling Factor Depleted Membranes. Earlier observations have shown that *M. phlei* ETP could be depleted of particulate-bound coupling factors (BCF_4) by washing with sucrose or water in the absence of either Mg^{2+} ions or KCl (Higashi *et al.*, 1969). The depleted ETP (DETP) are capable of substrate oxidation, but do not exhibit coupled phosphorylation. With succinate as electron donor, the DETP had a similar NE_f and E_f as that of ETP (Table II). Reconstituted ETP (DETP + BCF_4) exhibited oxidative

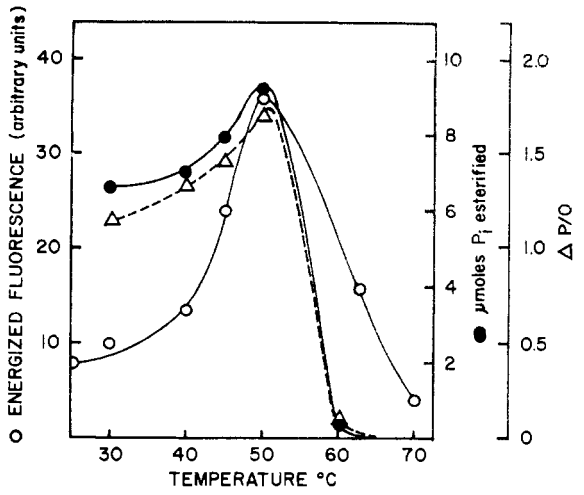

FIGURE 3: Relationship between the changes in E_f and phosphorylation with *M. phlei* ETP subjected to different temperatures. The reaction mixture consisted of ETP or ETP heat treated at different temperatures (2.0 mg of protein), 100 μmol of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-KOH buffer (pH 7.4), 50 μmol of glucose, 15 μmol of orthophosphate, 30 μmol of $MgCl_2$, 3.0 mg of hexokinase, 2.5 μmol of AMP, 25 μmol of KF, and 50 μmol of succinate in a final volume of 2.0 ml. The oxygen uptake was measured for 15 min using a Gilson differential respirometer and the reaction was stopped by the addition of 1 ml of 10% CCl_3COOH. The samples were centrifuged and aliquots of the resulting supernatant were used for the phosphate determination. Heat treatment of ETP was carried out as described in Experimental Procedures. Fluorescence assays of ETP or heat-treated ETP were carried out at 25°. The reaction mixture for determination of E_f was similar to that described under Figure 1B: (○) fluorescence; (●) P_i esterified; (△) P/O ratio.

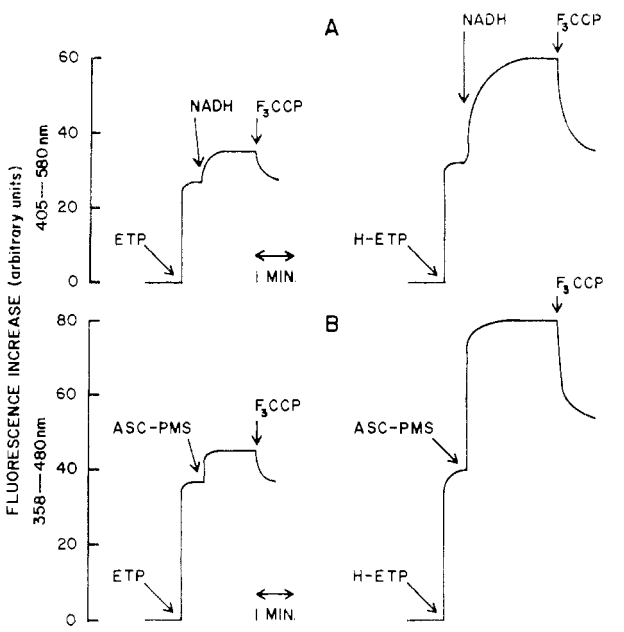

FIGURE 4: Anilinonaphthalenesulfonate (ANS) response of ETP and heat-treated ETP with either (A) NADH or (B) ascorbate + phenazine methosulfate (PMS) as substrate. The reaction mixture contained, in a final volume of 3 ml, ETP or heat-treated ETP at 50° for 10 min (H-ETP) (1.0 mg of protein), 83.3 μM ANS, 50 mM Tris–acetate (pH 7.2), and where indicated 13.5 mM NADH or a mixture of 10 mM ascorbate and 10 μM PMS were added. (A) ANS fluorescence was measured at 580 nm with an excitation wavelength of 405 nm. (B) ANS fluorescence was measured at 480 nm with an excitation wavelength of 358 nm. The concentration of F_3CCP was 5×10^{-6} M where indicated.

TABLE II: ANS Response in Depleted ETP.[a]

	Untreated		Heat Treated	
	NE_f[b]	E_f	NE_f	E_f
ETP	27	+9	38	+33
DETP	29	+10	36	+35
Reconstituted ETP	28	+9	36	+33

[a] The concentration of ETP, DETP, and reconstituted ETP used in the experiment was the same (0.8 mg of protein). ETP, DETP, and reconstituted ETP were heated for 10 min at 50° and then were allowed to cool slowly and kept on ice until used. Other experimental conditions are described under Experimental Procedure. [b] All NE_f and E_f values are in arbitrary units.

phosphorylation similar to ETP with succinate and NAD^+-linked substrate. Furthermore, the reconstituted ETP elicited the same level of NE_f and E_f as that exhibited by ETP or DETP (Table II). Heat-treated DETP at 50° for 10 min exhibited similar increases in E_f as that observed with H-ETP. Heat treatment of reconstituted ETP at 50° for 10 min showed the same level of E_f as that observed with heat-treated ETP or DETP at 50° for 10 min. Thus, it appears that particulate-bound coupling factor (BCF_4) is not required in eliciting energized fluorescence in the *M. phlei* ETP. Similar observations have been made for active transport of proline in ETP (Brodie *et al.*, 1972); active transport of proline occurred in electron transport particles depleted of coupling factor(s).

Binding Parameters. The succinate-induced enhancement of ANS fluorescence may be due to an increase in the binding of dye molecules arising from either the creation of new binding sites or an increased affinity of the dye to the membrane. It is also possible that there is a change in the quantum yield upon binding of the dye, which may be responsible for the enhanced fluorescence. A double-reciprocal plot of fluorescence *vs.* protein concentrations at a constant ANS concentration is shown in Figure 5. The curve was extrapolated to infinite concentration to estimate the amount of dye fluorescence at the limit where all the dye is bound as described by Brocklehurst *et al.* (1970). Under nonenergized conditions, the quantum efficiency of ANS bound to ETP or H-ETP was the same (Figure 5). The value for heat-treated ETP (H-ETP) under energized conditions is about 3.6- to 5-fold greater than the corresponding value in the energized untreated ETP. A difference was observed in the intercepts which would suggest a change in the quantum yield. Thus, the enhanced fluorescence in the energized state of H-ETP was the result of an increase in the relative quantum yield of the bound ANS.

In order to determine whether the affinity for ANS changed with the different ETP preparations and thus caused the increase in energized fluorescence with H-ETP, fluorescence was measured as a function of ANS concentration at a constant concentration of protein. A linear double-reciprocal plot was obtained (Figure 6) and the apparent dissociation constant (K_D) under energized conditions of the dye–ETP complex was extrapolated. It was shown earlier (Kalra *et al.*, 1972) that the K_D of the dye–ETP complex decreases with energization. The present results indicate that under energized conditions, the K_D of untreated ETP was 2.4-fold greater than that of heat-treated ETP.

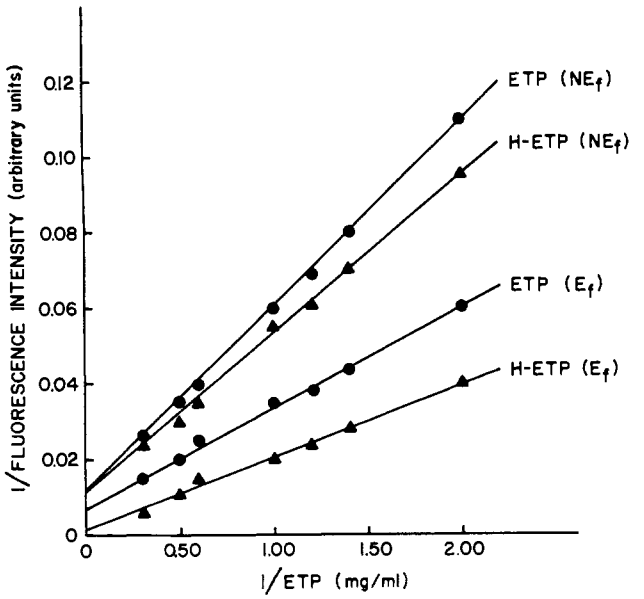

FIGURE 5: Anilinonaphthalenesulfonate (ANS) response at different concentrations of ANS with ETP or heat-treated ETP under energized conditions. The reaction mixture contained, in a final volume of 3 ml, ETP or heat-treated ETP (1.0 mg of protein), 50 mM Tris–acetate (pH 7.2), and different concentrations of ANS. Energization was induced by the addition of 17 mM Tris–succinate (pH 7.2). The reciprocal of the fluorescence intensity was then plotted *vs.* the reciprocal of the ANS concentration. The resulting data were treated by the method of least squares and plotted as shown.

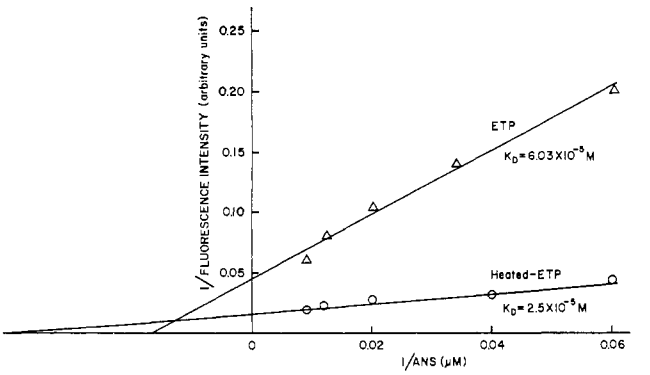

FIGURE 6: Dependence of nonenergized and energized ANS fluorescence on the concentration of ETP or H-ETP. The reaction mixture was the same as described in Figure 1B, except the concentration of ETP or heat-treated ETP was varied as shown in the figure.

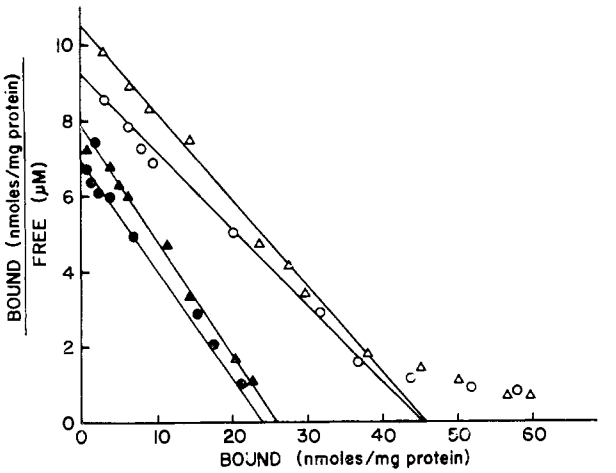

FIGURE 7: Scatchard plots of anilinonaphthalenesulfonate binding to ETP and H-ETP under nonenergized and energized conditions. The reaction mixtures of 3 ml containing 50 mM Tris–acetate (pH 7.2), 3.3 mM $MgCl_2$, ETP or H-ETP (1.68 mg of protein), and ANS varying from 19.29 to 333.2 μM were incubated at 25° for 5 min in the presence of 17 mM succinate (energized conditions) or 17 mM succinate + 2 mM KCN (nonenergized conditions). After cooling and centrifugation ANS was determined in supernatants and pellets (see Experimental Procedures). Nonenergized conditions: (●) ETP and (▲) H-ETP; energized conditions: (○) ETP and (△) H-ETP.

The binding of ANS to untreated ETP and to heat-treated ETP under nonenergized and energized conditions was measured as described under Experimental Procedures. Scatchard plots (Figure 7) based on the measurement of the amount of ANS bound to particles at different concentrations of ANS in both the energized (succinate) and nonenergized (succinate + KCN) states revealed a difference in the binding characteristics between ETP and H-ETP. In the nonenergized state, the binding capacity was 23 nmol of ANS/mg of ETP protein and 26 nmol of ANS/mg of H-ETP protein. Energization with succinate as a substrate caused a 2-fold increase in the binding capacity of ANS for both ETP and H-ETP (44 nmol of ANS/mg of ETP protein and 46 nmol of ANS/mg of H-ETP). Therefore, it appears that under energized conditions the binding sites of ANS for ETP and H-ETP remain the same as measured by this method. However, this method measures only the binding sites which are irreversible or slowly reversible and not the rapidly dissociating sites.

The contribution of rapidly dissociating binding sites of ANS for ETP or H-ETP on energized fluorescence enhancement was examined. ETP exhibited an energized fluorescence of 9 units (arbitrary) with succinate. However, if ETP were incubated with ANS and succinate for 5 min and then centrifuged to remove the unbound ANS, the resulting pellet (ANS bound to ETP) showed an energized fluorescence of only 4 units (arbitrary). Under similar conditions, H-ETP exhibited an energized fluorescence of 18 units (arbitrary) as compared to ANS bound to H-ETP which showed fluorescence enhancement of 5 units (arbitrary). The fluorescence enhancement in ANS-bound ETP or H-ETP possibly is due to irreversible or slowly reversible binding sites of ANS on the ETP or H-ETP. These results indicate that rapidly dissociating binding sites of ANS for ETP or H-ETP may also be contributing to the fluorescence enhancement upon energization.

Thus, the increase in energized fluorescence of H-ETP as compared to ETP seems to result from an increase in the relative quantum yield, an increased affinity for ANS and an increase in the possibly rapidly reversible binding sites. There is no apparent change in the number of irreversible or slowly reversible binding sites.

Effect of Succinate Concentration. It was of interest to study whether the increased energized fluorescence in heat-treated ETP resulted from an increased affinity of these membranes for succinate, which served as the energy source. A double-reciprocal plot (Figure 8) revealed that the apparent K_m value for succinate is decreased by 4.6-fold in heat-treated ETP. This indicated that the affinity for the energy source was increased in heat-treated ETP. The y intercept (fluorescence at an infinite substrate concentration) was also different for heat-treated ETP. These results suggest that heat treatment caused an increase in the affinity of the membrane binding sites for succinate.

Effect of Prolonged Sonication on the Heat-Induced Increase in E_f. Prolonged sonication of ETP from *M. phlei* has been

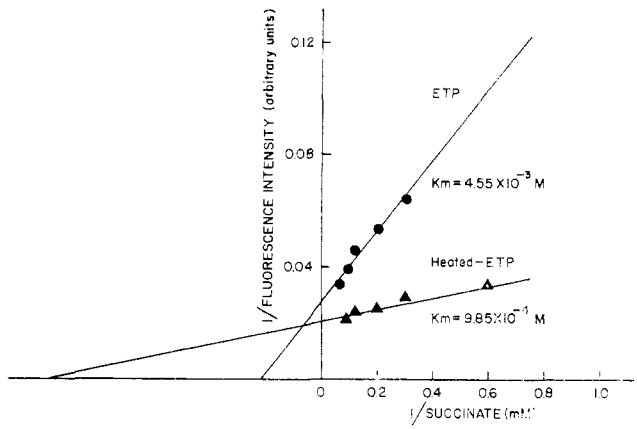

FIGURE 8: Double-reciprocal plot of energized fluorescence of ANS (induced by succinate) *vs.* succinate concentration. The reaction mixture contained, in a final volume of 3 ml, ETP or heat-treated ETP (1.0 mg of protein), 50 mM Tris–acetate (pH 7.2), and 83.3 μM ANS. The energized fluorescence of ANS was induced by the addition of different concentrations of succinate. The reciprocal of the fluorescence intensity was then plotted *vs.* the reciprocal of the succinate concentration. The resulting data were treated by the method of least squares and plotted as shown.

shown to result in structural damage as evidenced by a decrease in oxidative and phosphorylative activities (Brodie and Gray, 1957). Prolonged sonication of ETP resulted in a decreased level of energized fluorescence. However, the increase in energized fluorescence observed with heat-treated ETP (H-ETP) was not observed after prolonged sonication (Table III).

TABLE III: Effect of Extensive Sonication and Heat Treatment on ANS Fluorescence in *M. phlei* Membranes.[a]

Sonication Time of ETP (min)	Untreated ETP		Heat-Treated ETP	
	NE_f[b]	E_f	NE_f	E_f
4	26	+9	30	+31
40	10	+3	7	+0

[a] 4-min sonicated ETP refer to ETP prepared by sonication of whole cells for 4 min (Brodie, 1959). 40-min sonicated ETP refer to ETP subjected to an additional 36-min sonication. The experimental conditions were the same as those described in Table I, except the ETP concentration used in this experiment was 0.8 mg of protein. [b] All NE_f and E_f values are in arbitrary units.

It was observed that heat treatment of extensively sonicated ETP (40 min) did not show increased phosphorylation compared to the ETP prepared by normal sonication procedures (4 min) (H. N. Aithal, V. K. Kalra, and A. F. Brodie, unpublished results).

Energized Fluorescence of Heat-Treated ETP from E. coli. ETP prepared from *E. coli* showed a similar E_f in the presence of succinate as was observed in *M. phlei* ETP (Table IV). However, in contrast to *M. phlei* ETP, the ETP from *E. coli* failed to show an increase in E_f following heat treatment at 50° for 10 min. Heat treatment of *E. coli* ETP produced a decrease in energized fluorescence. It was also observed that heat treatment of ETP from *E. coli* did not cause an increase

TABLE IV: Effect of Heat Treatment on ANS Fluorescences with *E. coli* W ETP.[a]

Treatment	Changes in Fluorescence	
	NE_f	E_f
None	17	7
Heat treated	11	3

[a] The reaction mixture was similar to that in Table I. ETP (1.5 mg of protein) prepared from *E. coli* cells were used. Succinate (20 mM) was used as an electron donor. [b] NE_f and E_f values are in arbitrary units.

in phosphorylation but rather caused a decrease in oxidative phosphorylation.

Discussion

Membrane vesicles from *M. phlei* exhibit ANS fluorescence which increases upon energization with substrates such as succinate, NADH, and ascorbate + phenazine methosulfate. This energized fluorescence is sensitive to respiratory inhibitors and anaerobiosis. The enhancement of ANS fluorescence upon energization can be due to an increase in the number of binding sites, an increase in the quantum efficiency of bound ANS to the membrane, or an increase in the affinity of the membrane binding sites for ANS. It was observed in membrane vesicles from *M. phlei* that energization resulted in an increase in the number of membrane binding sites for ANS (from 23 nmol/mg of protein to 44 nmol/mg of protein), an increase in the relative quantum yield of ANS (1.5-fold) and a decrease in the apparent K_D of the membrane binding sites for ANS (from 16.7×10^{-5} to 6.03×10^{-5} M). The changes in binding parameters of the dye–membrane complex (relative quantum yield and K_D) observed upon energization of the membrane can be attributed to either a conformational change of the membrane or a change in the electrical potential associated with the membrane (Brocklehurst *et al.*, 1970; Azzi *et al.*, 1971).

Heat treatment of ETP has been shown to result in an increased P/O ratio due to an increased level of phosphorylation coupled to oxidation (Bogin *et al.*, 1970a; Aithal *et al.*, 1971). This may be due to a structural change of the membrane and thus was studied with ANS as a membrane probe. The energized fluorescence of ANS with heat-treated ETP was increased 2–3-fold compared to untreated ETP. Furthermore, a comparison of the binding sites, the relative quantum yield and the apparent K_D values showed significant differences in these parameters. Although the quantum yield of ANS with either ETP or heat-treated ETP was the same prior to substrate addition, the quantum yield increased 3.6–5-fold upon energization in heat-treated ETP when compared to untreated ETP. Furthermore, under energized conditions, the apparent K_D for heat-treated ETP decreased to 2.5×10^{-5} M compared to untreated ETP (6.03×10^{-5} M). The number of irreversible or slowly reversible binding sites under energized condition of heat-treated ETP and untreated ETP remained the same. These changes in the relative quantum yield and the affinity of ANS in heat-treated ETP as compared to untreated ETP under energized conditions can be attributed to structural changes of these membranes upon heat treatment. Thus, in *M. phlei* ETP, heat treatment appears to induce a change in the membrane structure which is reflected in increased phosphorylation and an increase in the

E_f of ANS. Whether this structural change is a result of changes in the electrical potential associated with the membrane upon energization is not clear at the present time (Azzi *et al.*, 1971; Avi-Dor *et al.*, 1970).

One difference between *M. phlei* ETP and mitochondrial systems is the inability of ATP to elicit energized fluorescence in the bacterial membrane vesicles. This may be related to the inability to observe the ATP-driven energy-linked reactions, *i.e.*, the energy-dependent reversal of electron transport and the ATP-dependent reduction of $NADP^+$ by NADH (Murthy and Brodie, 1964). However, trypsin treatment of membrane vesicles, which also increases latent ATPase activity (Bogin *et al.*, 1970b), resulted in an ATP-induced ANS response. This ATP-dependent enhanced fluorescence was Mg^{2+} dependent as is latent ATPase activity. However, attempts to demonstrate ATP-driven energy-linked reactions in trypsinized ETP have been futile. The ETP from *M. phlei*, depleted of particulate-bound coupling factor (lacking latent ATPase activity), exhibit energized fluorescence to the same level as untreated ETP, indicating that particulate-bound coupling factor (BCF_4) is not required for energized fluorescence. This is in accord with the lack of effect of dicyclohexylcarbodiimide on the succinate-induced enhancement of fluorescence. These results suggest that a high-energy phosphorylated intermediate as proposed by the chemical hypothesis (Lardy and Ferguson, 1969) may not be involved in eliciting the enhanced fluorescence observed upon energization; however, the involvement of a nonphosphorylated high-energy intermediate cannot be ruled out.

The results presented in this communication indicate that heat treatment of membrane vesicles from *M. phlei* induced structural changes of the membrane. It is worthwhile to mention that Kasai *et al.* (1969) have reported structural rearrangements of membrane components occurring between 30 and 50° in the electric organ of *Electrophorus electricus*. The structural changes brought about by heat treatment in *M. phlei* membranes probably lead to the secondary effect of increased phosphorylation which results in increased P/O ratios. Changes in binding parameters, particularly the quantum efficiency, which have been implicated in the change in conformation further lend support to the concept of changes in membrane structure. It was reported that freezing *M. phlei* membranes and then slowly thawing them decreased the level of phosphorylation accompanying substrate oxidation (Aithal *et al.*, 1971). This decrease in the level of phosphorylation was reversed by heat treatment of the membrane (Aithal *et al.*, 1971). Freezing and thawing are known to result in changes in membrane structure leading to altered properties of membrane-bound enzymes (Aithal and Ramasarma, 1969; Chelsan *et al.*, 1965; Camerino and King, 1965; Fishbein and Stowell, 1968). The reversal of the freezing and thawing effect by heat treatment might represent some degree of structural reorganization of the membrane.

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Metabolism of Isolated Chick Small Intestinal Cells. Effects of Ammonia and Various Salts†

Ronald L. Prior,‡ Douglas C. Topping,§ and Willard J. Visek*

ABSTRACT: The metabolic responses to ammonia were studied in cells isolated from the small intestine of the chick. Control cells produced $^{14}CO_2$ and lactate and incorporated ^{14}C from [6-^{14}C]glucose into lipid and protein linearly with time. However, their incorporation of ^{14}C from glucose into RNA and DNA declined with time. Ammonia caused a generalized stimulation of glycolysis and of the tricarboxylic acid cycle. This was demonstrated by an increased rate of lactate and $^{14}CO_2$ production from [U-^{14}C]glucose and an increased rate of $^{14}CO_2$ production from [2-^{14}C]pyruvate, [1,5-^{14}C]citrate, [1,4-^{14}C]succinate, and α-[1-^{14}C]ketoglutarate. Cells from chicks beyond 10–15 days of age responded to ammonia by producing more lactate than cells from younger chicks. Ammonia concentrations as low as 1 mM stimulated glucose and citrate metabolism. Ammonia concentrations in the small intestine were 1.2–1.9 mM and reached 6–10 mM in the ceca. It is concluded that quantities of ammonia normally present in the gastrointestinal tract can stimulate carbohydrate metabolism by intestinal cells and alter their life span.

The effects of ammonia (*i.e.* NH_3 plus NH_4^+) upon the metabolism of brain and liver have been extensively studied (Saheki *et al.*, 1971; Worcel and Erecinska, 1962; McKhann and Tower, 1961). Normally peripheral tissues are protected from this highly toxic metabolite by detoxication mechanisms which maintain tissue fluid ammonia below 0.1 mM. Cells continuously exposed to unusually high concentrations of ammonia are those of the gastrointestinal mucosa. Endogenous and bacterial enzymes acting upon nitrogenous substrates release large amounts of ammonia in the bowel (Warren and Newton, 1959; Wilson *et al.*, 1968a). *In vivo* dialysates of human colon contents have been shown to range from 2.7 to 44 mM in ammonia (Wrong *et al.*, 1965). Such concentrations are sufficient to destroy other cells of the body (Dang and Visek, 1968). Normally from 15 to 30% of the urea synthesized by the liver in simple stomached mammals is recycled from the blood into the gastrointestinal tract where it is hydrolyzed to ammonia and CO_2 (Walser and Bodenlos, 1959). This process releases about 3.5 g of ammonia N per day in man. During uremia, ammonia in colon dialysates of human patients has been as high as 75 mM (Wilson *et al.*, 1968b; Wrong *et al.*, 1970).

The present studies were conducted with isolated cells from the small intestine of chicks. The purpose was to examine their response to varying concentrations of ammonia and to gain information about their metabolic characteristics which allow them to function in an environment containing ammonia concentrations which would destroy or seriously impair the function of other cells. The results are compared to those of similar studies conducted concurrently in our laboratory with isolated brain cells (Gibson, 1973). In most of the experiments ammonia was generated from urea by urease since this is a usual and probably major source of ammonia in the gastrointestinal lumen.

Experimental Procedure

Materials. Male, white, 7- to 49-day-old, Leghorn chickens obtained from Dekalb-Marshall Hatcheries, Ithaca, N. Y., provided all of the cells. In exploratory studies a Krebs–Ringer phosphate buffer was used which contained: NaCl, 120 mM; KCl, 4.8 mM; $CaCl_2 \cdot H_2O$, 2.6 mM; K_2HPO_4, 12.5 mM; KH_2PO_4, 3.1 mM; and $MgSO_4 \cdot 7H_2O$, 1.2 mM. Subsequently, a modified Krebs–Ringer phosphate buffer essentially as described by Ram *et al.* (1963) with Ca added proved more satisfactory. The latter contained: NaCl, 12.5 mM; Na_2HPO_4, 85 mM; KCl, 5 mM; $CaCl_2 \cdot 2H_2O$, 2.5 mM; and $MgSO_4 \cdot 7H_2O$, 1.3 mM. Its pH was adjusted to 7.4 with HCl. All buffers and solutions contained 1 mg/ml of bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, Mo.). Buffers used for cell isolation contained 1 mg/ml of hyaluronidase. All cell preparations and experiments were carried out

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‡ Present address: U. S. Meat Animal Research Center, Clay Center, Neb. 68933.
§ National Science Foundation Predoctoral Fellow.