Significance of Surface Potential in Interaction of 8-Anilino-1-naphthalenesulfonate with Mitochondria: Fluorescence Intensity and ζ Potential†

Toshihiro Aiuchi, Naoki Kamo,* Kenzo Kurihara, and Yonosuke Kobatake

ABSTRACT: The 8-anilino-1-naphthalenesulfonate (Ans) fluorescence in nonenergized and energized mitochondria was measured at various concentrations of Ans and KCl. Under the same experimental conditions, the ζ potential was determined from measurements of the electrophoretic mobility of mitochondria. The fluorescence intensity under various conditions was represented quantitatively in terms of the Langmuir adsorption isotherm where the electrostatic interaction acting between Ans and mitochondria was properly taken into account. The values of qN (q, proportionality constant related to quantum yield of Ans; N, the maximum number of the adsorption site) and ΔG (nonelectrical part of the free-energy change due to the binding of Ans to mitochondria) were constant irrespective of difference in energy state and in ionic strength in media. It was concluded that changes in the Ans fluorescence in mitochondria are mainly attributed to changes in the surface potential of mitochondria.

The fluorescence dye 8-anilino-1-naphthalenesulfonate (Ans)[1] exhibits virtually no fluorescence in water but fluoresces strongly in nonpolar solvents or when adsorbed to certain proteins, lipids, or membranes (Weber and Lawrence, 1954; Brand and Gohlke, 1972, Radda and Vanderkooi, 1972). A number of important studies have demonstrated the extreme usefulness of Ans as a fluorescence probe of the functional state of mitochondria (Chance, 1970; Datta and Penefsky, 1970; Harris, 1971; Azzi and Santato, 1972; Layton et al., 1974; Schäfer and Rowhl-Quisthoudt, 1975; Ferguson et al., 1976; references cited below). According to these studies, addition of respiratory chain substrates or ATP to mitochondria brings about a decrease in Ans fluorescence and that of uncouplers or inhibitors reverses this effect. These changes of fluorescence intensity observed with mitochondria have been interpreted in terms of changes in quantum yield of Ans (Nordenbrand and Ernster, 1971; Brocklehurst et al., 1970), the extent of Ans binding (Azzi et al., 1971), or electrophoretic transport of Ans across the membrane (Jasaitis et al., 1971). As far as the authors are aware, no agreement has been obtained for interpretation of mechanism of Ans fluorescence changes in mitochondrial suspension.

In a previous paper (Kamo et al., 1977), we analyzed quantitatively the interaction between Ans and liposomes by measuring fluorescence changes and ζ potentials of liposome under the presence of salts of various species and/or concentrations and concluded that changes in fluorescence intensity of Ans in liposomal suspension are attributed to changes in the surface potential of liposomes. It is recently shown that the negative charges appear when mitochondria are energized by substrates or ATP and disappear when mitochondria are treated with respiratory inhibitors or uncouplers (Kamo et al., 1976). Then, similar analysis employed with the liposomal suspension is applied to clarify the interaction between Ans and mitochondria in the present study. The fluorescence and ζ potential are measured with mitochondria of energized or nonenergized state at various concentrations of Ans or KCl. It is shown that changes in Ans fluorescence intensity during energization as well as those caused by changes in ionic strength in medium are attributed to changes in the surface potential of mitochondria.

Experimental Section

Mitochondria were isolated from rat liver. The liver was homogenized with a solution of 0.25 M sucrose and 0.2 mM EDTA, and nuclei and cell debris were removed by centrifugation for 10 min at 600g. Mitochondria were sedimented at 5500g (Hogeboom, 1955). The respiratory control ratio of mitochondria used in the present study was 4–7 when succinate was used as a substrate. Sedimented mitochondria were stocked in a solution containing 0.3 M mannitol, 0.2 mM EDTA, and 10 mM Tris-HCl (pH 7.4) at 0 °C. For measurements of the ζ potential and fluorescence of mitochondria, the stocked mitochondria were suspended in a solution containing 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 0.2 mM EDTA, and 0.25 M sucrose. In order to alter the ionic strength, a part of or whole sucrose was substituted for KCl keeping the tonicity constant.

Determination of ζ Potential. The ζ potential, ζ, of mitochondria was calculated from the electrophoretic mobility with the aid of Helmholtz–Smoluchowski equation (Verwey and Overbeek, 1948)

$$\zeta = 4\pi\eta u/D \qquad (1)$$

Here, u is the mobility of mitochondria, and η and D are the viscosity and dielectric constant of the dispersion medium, respectively. The viscosity was measured by an Ostwald viscometer. The electrophoretic mobilities were measured by a microelectrophoretic apparatus (Cytopherometer, Karl Zeiss, West Germany). Measurements were made by the direct observation of the velocity of mitochondria at the "stationary layer" in a flat cell regulated at 25 °C. The quantity of mitochondria required for the measurements of electrophoretic velocity is less than that for the fluorescence. Then, an aliquot

† From the Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan. *Received July 22, 1976.*

[1] Abbreviations used: Ans, 8-anilino-1-naphthalenesulfonate; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; DDA^+, dibenzyldimethylammonium ion.

TABLE I: ζ Potential of Nonenergized and Energized Mitochondria in Various Media.[a]

Medium	ζ Potential (mV)
Buffer only	-21 ± 2
Buffer + 25 mM KCl	-15 ± 2
Buffer + 125 mM KCl	-8 ± 1
Buffer + 1 mM ATP	-26 ± 2
Buffer + 1 mM ATP + 125 mM KCl	-15 ± 2
Buffer + 1 mM succinate	-27 ± 2

[a] The values in the table are the averaged values of the data obtained with 25 mitochondria. The buffer contains 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 0.2 mM EDTA, 0.25 M sucrose, and 1 μM rotenone. The concentration of Ans is 100 μM.

of the mitochondria suspension prepared for the measurements of the fluorescence (described below) was diluted with ten times the volume of the same medium as that of the suspension. The ratio of mitochondria to Ans in the diluted suspension is not identical with that in the suspension for the fluorescence measurements. However, this discrepancy in the ratio produced no experimental error since the presence of Ans scarcely affected the electrophoretic velocity of mitochondria as described in the subsequent section. The observed velocity of mitochondria was proportional to the electric field applied externally and was independent of the size of mitochondria. The strength of the electric field applied was about 5 V/cm. The velocity reached a steady value within 5 min after addition of rotenone, succinate, or ATP. The electrophoretic mobility of the fixed condition was unchanged at least in 3 h after the isolation. Under a given condition, 25 mitochondria were timed in each direction of the field to eliminate the polarization of the electrodes and the averaged value was taken as velocity of mitochondria.

Fluorescence Measurements. The sodium salt of Ans was purified by the method of Weber and Young (1964). Fluorescence was measured with a Hitachi Perkin-Elmer MPF-2A at 25 °C (an excitation wavelength of 375 nm and an emission wavelength of 470 nm). Procedures for fluorescence measurements are as follows. (1) Rotenone (final concentration, 1 μM) was added to mitochondrial suspension (0.3 mg of protein per mL) in the buffer solution; (2) 3 mL of the suspension was pipetted into cuvettes for fluorescence measurements. A given volume of concentrated Ans solution was added to one cuvette and an equal volume of the buffer solution containing rotenone was added to another. (3) ATP or succinate was added to the cuvettes for energization, and the fluorescence in the steady state was measured. Mitochondria have an intrinsic fluorescence around 470 nm and exhibit light scattering, which was affected by addition of rotenone, ATP, or succinate. Hence, it is necessary that the difference between fluorescence of mitochondrial suspension with and without Ans was taken as the fluorescence change of Ans (Azzi, 1974). In order to compare the intensity of emission obtained under various conditions, the fluorescence intensity of a quinine sulfate solution (0.3 mg/L in 0.1 N H_2SO_4) was used as reference. In the subsequent discussion, the fluorescence intensity, f, is defined as

$$f = (f_a - f_m)/f_s \quad (2)$$

where f_a, f_m, and f_s stand for mitochondria-Ans suspension, the mitochondrial fluorescence, and quinine solution fluorescence, respectively.

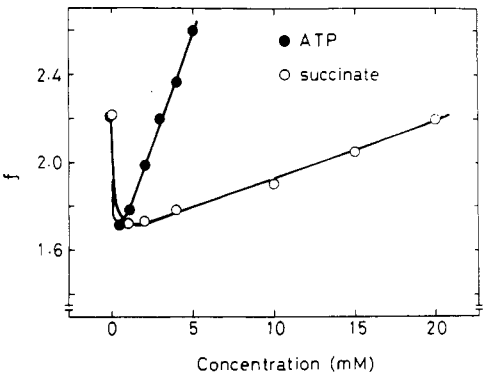

FIGURE 1: Fluorescence intensity (f) of Ans as a function of concentration of ATP or succinate added to mitochondrial suspension. The definition of f is given in eq 2. The concentration of Ans is fixed at 50 μM. (○) Succinate; (●) ATP.

Results and Discussion

Table I represents the ζ potential of mitochondria under various conditions. The ζ potential of nonenergized mitochondria was -21 ± 2 mV in the buffer containing rotenone and addition of KCl to the buffer decreased the magnitude of the potential. On the other hand, addition of ATP or succinate increased the magnitude of the potential. The surface charge density calculated from the electrophoretic mobility is -1.2 and -0.7 $\mu C/cm^2$ for energized and nonenergized states, respectively (Kamo et al., 1976). The data shown in Table I were obtained in the presence of 100 μM Ans, but the data in the presence of Ans were practically identical with those in the absence of Ans. This indicated that adsorption of Ans to mitochondria did not affect the ζ potential owing to high inherent charge density of mitochondria and/or to little binding of Ans to mitochondria. The amount of Ans bound to mitochondria was at most 10% of Ans in bulk solution for all cases examined.

In Figure 1, the fluorescence changes at the fixed concentration of Ans are plotted against concentration of ATP or succinate. As seen in the figure, the fluorescence decreases during energization by addition of 1 mM ATP but this decrease of fluorescence was reversed by further increase of ATP. Above 3 mM ATP, the fluorescence intensity exceeded the original level without energization. Similar phenomenon was also observed with succinate. In this case, about 20 mM succinate was needed to reverse the fluorescence intensity to the original level. These results suggest that the fluorescence changes may not reflect only the energy state of mitochondria since it is unlikely that higher concentration of ATP or succinate brings about deenergization in mitochondria. As will be described later, the effect of ATP or succinate to increase Ans fluorescence is attributed to the increase of ionic strength in the medium which facilitates the binding of Ans to mitochondria.

In Figure 2a, fluorescence changes in nonenergized mitochondria in the presence of rotenone are plotted against Ans concentration in media of different ionic strength. Curves 1, 2, and 3 represent fluorescence changes in the buffer only, the buffer plus 25 mM KCl, and the buffer plus 125 mM KCl, respectively. For each curve, fluorescence approaches respective maximum levels with increase of Ans concentration. Fluorescence intensity becomes large with increase of ionic strength at equal concentration of Ans. Curve 4 represents fluorescence changes in the presence of 1 mM succinate. Fluorescence intensity decreases during energization by succinate (compare curve 4 with 1). Figure 2b represents fluo-

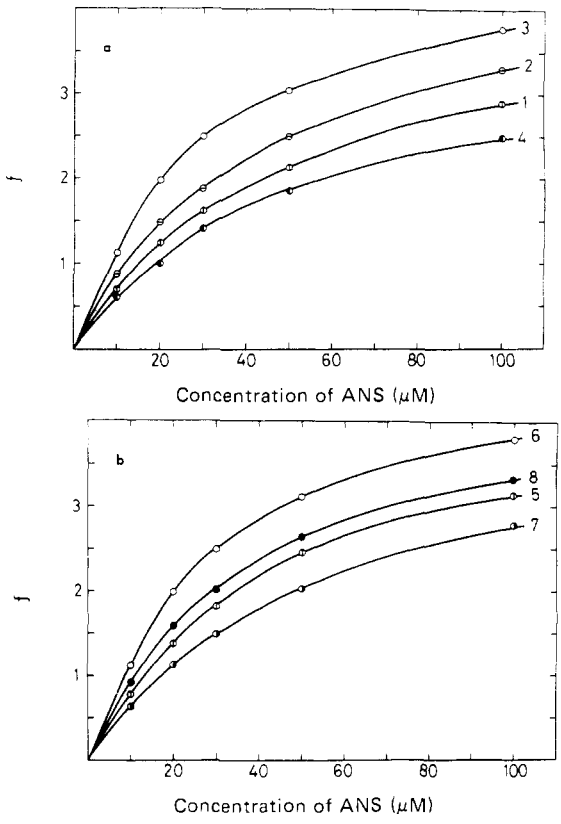

FIGURE 2: Relation between fluorescence intensity and Ans concentration for nonenergized and energized mitochondria in various media. In a: 1 (the buffer only); 2 (the buffer + 25 mM KCl); 3 (the buffer + 125 mM KCl); 4 (the buffer + 1 mM succinate). In b: 5 (the buffer only); 6 (the buffer + 125 mM KCl); 7 (the buffer + 1 mM ATP); 8 (the buffer + 125 mM KCl + 1 mM ATP). The buffer composition is 10 mM potassium phosphate (pH 7.4), 1 mM $MgCl_2$, 0.2 mM EDTA, 0.25 M sucrose, and 1 μM rotenone.

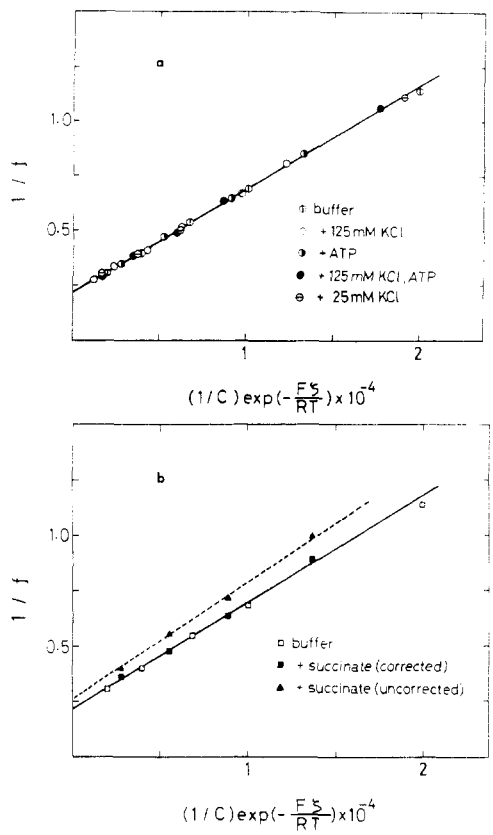

FIGURE 3: Plots of $1/f$ against $(1/C)\exp(-F\zeta/RT)$ according to eq 4. The data were taken from Figure 2. (a) Energized (by addition of ATP) and nonenergized mitochondria in media of different ionic strength. The notations are written in the figure. (b) Mitochondria in the presence or absence of 1 mM succinate. Closed squares represent the values corrected for the quenching effect by 1 mM succinate. The quenching effect in the presence of 1 mM succinate was evaluated from measurements of the Ans fluorescence in mitochondria inhibited by antimycin A at various concentrations of Ans.

rescence changes against Ans concentration in the presence and absence of 1 mM ATP. Comparing each pair of the curves (curves 5 for nonenergized and 7 for energized mitochondria in the buffer only; curves 6 for nonenergized and 8 for energized mitochondria in the buffer plus 125 mM KCl), it is evident that the fluorescence decreases during energization by ATP in both media. Note that the fluorescence intensity for curve 8 (energized state) is larger than that for curve 5 (nonenergized state) at equal molar concentration of Ans. The example that the fluorescence intensity did not reflect the energy state was also seen in Figure 1.

The above results indicate that the fluorescence intensity of Ans in both nonenergized and energized mitochondria increases with increase of salt concentration and approaches the respective plateau levels with increase of Ans concentration. These results are similar to those obtained with liposomes in a previous paper (Kamo et al., 1977). Hence, theoretical analysis similar to that proposed in the previous paper was applied to the data obtained in the present study as shown below.

It is assumed that the fluorescence intensity of Ans in mitochondrial suspension, f, is proportional to the amount of Ans bound to mitochondria (note that the quantum yield of Ans in water is negligibly small) and the Langmuir adsorption isotherm may be applicable to the binding of Ans to mitochondria. Since Ans bears a negative charge, the electrostatic interaction acting between Ans and mitochondria must be taken into account. Thus, we obtain the following equation

$$f = qN\frac{KC\exp(F\psi/RT)}{1 + KC\exp(F\psi/RT)} \tag{3}$$

where q is a proportionality constant related to quantum yield of Ans, N, the maximum number of the Ans binding site in mitochondria, and C, the molar concentration of unbound Ans. F, R, and T have their usual thermodynamical significances. In eq 3, K is given by $K = (1/55.6)\exp(-\Delta G/RT)$, where ΔG is the change in the nonelectrical part of the free-energy change due to the binding. The meaning of ψ in eq 3 is the difference in the electrostatic potential between the binding site and the bulk solution, which is, however, not accessible experimentally. In the present paper, the ζ potential, ζ, is used as an experimental approximation to ψ. Putting ζ in place of ψ and rewriting eq 3, we obtain eq 4

$$\frac{1}{f} = \frac{1}{qN}\left[1 + \frac{1}{KC}\exp(-F\zeta/RT)\right] \tag{4}$$

Equation 4 states that the plot of $1/f$ against $(1/C)\exp(-F\zeta/RT)$ gives a straight line and values of qN and K can be determined from the ordinate intercept and the slope of the straight line.

In Figure 3, $1/f$ is plotted against $(1/C)\exp(-F\zeta/RT)$ according to eq 4 where the data shown in Figure 2 and Table I are used. The value of C, which is Ans concentration in bulk solution, was determined by measuring the optical density of the supernatant obtained by centrifugation of mitochondrial

suspension. As seen in Figure 3a, all data obtained with nonenergized and energized mitochondria induced by addition of 1 mM ATP fell on a single straight line, although the ionic strength of the medium was significantly varied when the data of fluorescence were plotted in accordance with eq 4.

Closed triangles in Figure 3b show the plot of the data obtained with energized mitochondria by 1 mM succinate. The data fell on a straight line, which is, however, different from that for nonenergized mitochondria (open square). Addition of succinate also brought about a decrease of Ans fluorescence even when the respiratory chain was arrested by antimycin A. Oxygen consumption was completely stopped under this condition. This suggests that the presence of succinate quenches Ans fluorescence. In fact, a quenching effect of succinate was also observed when succinate was added to bovine serum albumin–Ans system. The data shown by the closed triangle in Figure 3b were corrected by adding the value of the quenched fluorescence in the presence of 1 mM succinate, which was evaluated from the measurements of the Ans fluorescence in mitochondria inhibited by antimycin. The data corrected for the quenching effect fell on the same straight line as that for nonenergized mitochondria.

The results in Figure 3 indicate that eq 4 holds in all cases where Ans concentration, ionic strength, or energized state is changed. Furthermore, the ordinate intercept and the slope of the straight line were almost identical for all cases; that is, qN and ΔG were constant irrespective of difference in energy state or ionic strength. The above results indicate that changes of the Ans fluorescence under various conditions are mainly attributed to changes in the electrostatic potential of mitochondria. The results presented here are also consistent with the fact that the maximum wavelength of the Ans fluorescence in mitochondria was practically independent of energy state. Ferguson et al. (1976) also reported that the maximum wavelength and the lifetime of excited Ans were the same irrespective of the energized or nonenergized submitochondria. Harris (1971) described that the ordinate intercept of the plot of $1/f$ vs. the reciprocal of the concentration of the submitochondria is the same both for energized and nonenergized states when Ans is sufficiently concentrated. He concluded that the results drawn by the experiments employing low Ans concentration were not reliable. Actually, the change in the intrinsic fluorescence (f_m in eq 2) is the origin of error for Ans extrinsic fluorescence especially when the concentration of Ans is low. From the straight line in Figure 3, the value of ΔG was calculated to be -7.4 kcal/mol. This value of ΔG was a little smaller than that for liposomes (-9 to ca. -10 kcal/mol, Kamo et al., 1977).

The data shown in Figure 1 can be explained as follows. The ζ potential, ζ, is a function of surface charge density, σ, and ionic strength, I, according to the following approximate equation (Verwey and Overbeek, 1948)

$$\zeta = \frac{2RT}{F}\sigma\sqrt{\frac{500\pi}{IDRT}}$$

As noted before, the value of σ becomes negatively larger when ATP or succinate is added. This implies that the conformational change in mitochondrial outer membrane is induced by ATP or succinate. The change in σ led to increase in magnitude of ζ and hence the amount of Ans bound to mitochondria is decreased, which brought about a decrease of fluorescence. Further increase of ATP or succinate concentration increases ionic strength in the medium, which decreases magnitude of ζ. The decrease of ζ decreases the electrostatic repulsion force between Ans and mitochondria and hence the amount of Ans bound to mitochondria increases again at higher concentration of ATP or succinate. Since the ionic strength of ATP solution is much larger than that of succinate solution at equal molar concentration, the curve for ATP in Figure 1 starts to increase at lower concentration than that for succinate. The quenching effect of succinate on Ans fluorescence partially contributes to the gradual rising of the curve.

Grinius et al. (1970) and Harold and Papineau (1972) showed that lipid-soluble ions such as dibenzyldimethylammonium (DDA^+) permeate membranes and distribute in accordance with the membrane potential. Recently, we devised the selective electrode for DDA^+ by which DDA^+ concentration in the medium can be measured easily and demonstrated that DDA^+ uptake occurs when intact mitochondria are energized (Muratsugu et al., 1977). Contrary to the data shown in Figure 1, the degree of DDA^+ uptake by addition of 0.5 mM ATP or 2 mM succinate reached a plateau level and did not decrease with further addition of ATP or succinate. Thus, it is evident that the data obtained with DDA^+ uptake due to the membrane potential are different from the Ans fluorescence.

It is shown that valinomycin induced the Ans fluorescence change in mitochondria and submitochondria (Azzi et al., 1971; Jasaitis et al., 1971; Ferguson et al., 1976). Jasaitis et al. (1971) reported that valinomycin induced the fluorescence change of Ans in the presence of NaCN and that the magnitude of the fluorescence change due to valinomycin plus K^+ is enough to explain that due to energization. We were not able to observe any fluorescence change of Ans by addition of valinomycin under the conditions where respiration was arrested by rotenone or CN^-. In the present condition, the concentration of K^+ in the medium is relatively high, which seems to be the reason for no change in fluorescence. However, we observed only a small change even in K^+-free Tris-HCl buffer. Moreover, Ferguson et al. (1976) reported that 2-(*N*-methylanilino)naphthalene-6-sulfonate, which responds to energization by ATP or respiratory chain substrates, exhibited no fluorescence change by addition of valinomycin to mitochondria. Thus, the mechanism of the Ans fluorescence changes in mitochondria induced by valinomycin does not seem simple. Further study is needed to clarify the mechanism of the Ans fluorescence changes induced by valinomycin.

The submitochondria, which are considered to have a morphological polarity opposite to that of intact mitochondria, exhibit the opposite response of the Ans fluorescence to mitochondria under various conditions. If the above mechanism on the Ans fluorescence changes in mitochondria is applicable to submitochondria, the surface charge density of submitochondria would change to positive direction on energization contrary to mitochondria. The membrane potential of the submitochondria, which was determined by the lipid-soluble ions, deflects to opposite direction of that in intact mitochondria, i.e., interior positive with respect to the medium (Grinius et al., 1970). It is inferred that the energization brings about the "charge separation" in mitochondrial membrane, which is linked with the membrane potential. Thus, the direction of the change in membrane potentials seems to correspond to that of surface charges of the membrane. The change in surface charges leads to the change in surface potential, which is the significant factor for Ans fluorescence.

The results in the present study, together with those in the previous studies (Kamo et al., 1977) on the interaction of Ans with liposomes, indicated that the fluorescence of Ans could be used as an indicator of the surface potential. Many studies have demonstrated that the fluorescence of Ans changes cor-

relatively with generation of the function of various biological membranes (Tasaki et al., 1969; Partric et al., 1971; Vanderkooi and Martonosi, 1971; Cohen, 1973; Phillips and Cramer, 1973; Grinius and Brazenaite, 1976). For interpretation of changes in the fluorescence intensity of Ans in those biological membranes, changes in the surface potential of the membrane should be considered as one of the significant factors affecting the fluorescence intensity. Significance of the surface potential in the function of biological membranes was discussed elsewhere (Kamo et al., 1974a,b; Hato et al., 1976; Aiuchi et al., 1976).

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