J. Membrane Biol. 64, 175–179 (1982)

The Journal of
Membrane Biology

# Anilinonaphthalene Sulfonate Fluorescence and Amino Acid Transport in Yeast

Jan Slavík, Jaroslav Horák, Ludmila Říhová, and Arnošt Kotyk
Department of Cell Physiology, Institute of Microbiology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

**Summary.** Fluorescence of 1-anilinonaphthalene-8-sulfonate in yeast membranes appears to be caused predominantly by binding to lipids ($ANS_{protein}:ANS_{lipid} \approx 1:20$) as indicated by the fluorescence lifetime, degree of polarization, and excitation spectra. It was insensitive to short-circuiting the membrane potential. Fluorescence intensity increased as cells (especially after pretreatment with energy donors such as glucose) were exposed to some amino acids, in particular, aspartic and glutamic acids. The character of fluorescence shifted to that of protein-bound ANS, suggesting an exposure of new protein sites accessible to the probe. This shift could be prevented by inhibitors of energy transduction as well as of transport. The $K_{1/2}$ of the shift was at 2.5 mM aspartic acid.

**Key words** fluorescence probe · membrane transport · biological membrane · amino acids · aspartic acid · yeast cells · anilinonaphthalene sulfonate

## Introduction

Amino acid transport in baker's yeast, an active, practically unidirectional process (e.g., Kotyk & Říhová, 1972) has been shown to be accompanied by shifts of protons, and in opposite direction, of potassium ions, at widely ranging stoichiometries (e.g., Eddy, 1978). As the use of pH difference for driving secondary transports practically always has depolarization of the membrane as its corollary, it was thought that a fluorescence probe that has been reported as responsive to membrane energization might shed new light on the role of the membrane potential in amino acid transport by baker's yeast. In fact, some striking fluorescence changes were obtained, but their interpretation is by no means unequivocal.

## Materials and Methods

### *Microorganism and its Cultivation*

*Saccharomyces cerevisiae* K isolated from distillery yeast was used throughout (Kotyk, Ponec & Říhová, 1971). It was grown in a mineral medium with glucose and yeast extract at 30 °C for 20 hr. The $op_1$ mutant, lacking the ADP-ATP transport protein of mitochondria, was a kind gift from Dr. J. Šubík of the Research Institute of Distilleries and Canning in Bratislava. It was grown in the same medium as above.

### *Reagents*

1-Anilinonaphthalene-8-sulfonate (ANS) was a potassium salt from Sigma (USA), 3-chlorophenylhydrazonomalononitrile (CCCP) and 4-(trifluoromethoxy)phenylhydrazonomalononitrile (FCCP) were from Calbiochem (Switzerland), valinomycin and the potassium salt of 2-toluidinonaphthalene-6-sulfonate (TNS) were from Serva (FRG), N-phenyl-α-naphthylamine (NPN) was from ICN Pharmaceuticals (USA), 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Fluka (Switzerland); all other chemicals, including the amino acids, were from Lachema (Czechoslovakia).

Liposomes were prepared by squirting an ethanolic solution of highly purified egg-yolk lecithin (after Singleton, Gray, Brown & White, 1965; 0.02 mg/ml) into physiological saline (Batzri & Korn, 1973). Human serum albumin was from Radioisotope Production and Distribution Centre (USA), bovine serum albumin from Koch-Light (UK).

### *Fluorescence Measurements*

Washed cells diluted in distilled water to a density of approximately 20 per nl were stained with ANS (final concentration 50 μM) and placed in an aerated thermostatic 2-ml cuvette with a diameter of 13 mm. The measurement itself was done in a right-angle arrangement. A high pressure 200-W mercury lamp HBO 200 (wavelength 365 nm) or 500-W xenon lamp XBO 500 (excitation spectra), both from Narva (GDR), in connection with a grating monochromator (MONOSPEC 600, Hilger & Watts, UK) were used as the excitation light source with a bandwidth of 2 nm. The spectral purity of the excitation light was further improved by inserting interference filters (Optice Technology, USA). The emission was analyzed by means of a triple quartz prism spectrograph (Steinheil GH, BRD) connected to an EMI 6256 S photomultiplier and photon-counting apparatus (Photometric readout system PC 1, Spex, USA). Dichroic polarizers (Polacoat, USA) were employed for polarization measurements. The emission spectra were fully corrected for the spectral responses of both monochromator and the photomultiplier; likewise, all necessary corrections of polarization measurements were made (Parker, 1968). The quantum yield is expressed relative to that of ANS in ethanol, taking an average value from literature references 0.38 (Slavík, 1982). To assess quantitatively the intensity of excitation energy transfer from protein to ANS, we employed the fluorescence intensity increase in the

0022-2631/82/0064-0175 $01.00

**Table 1.** Fluorescence of free and bound ANS

| Property | Units | Free | Bound to | | |
|---|---|---|---|---|---|
| | | | Lecithin liposomes | Albumin | *Escherichia coli* |
| Spectral maximum | nm | 516 | 490 | 480 | 490 |
| | eV | 2.40 | 2.53 | 2.58 | 2.53 |
| Spectral band width | eV | 0.38 | 0.45 | 0.44 | 0.45 |
| Quantum yield | – | 0.005 | 0.4 | 0.9 | – |
| Lifetime (mean) | nsec | 0.6 | 5.6[a] | 16 | 5.4 |
| Degree of polarization | – | 0.01 | 0.15 | 0.30 | 0.25 |
| Polarization anisotropy | – | 0.01 | 0.11 | 0.22 | 0.18 |
| Intensity of excitation energy transfer[b] | Relative units | 1.4 | 1.8 | 3.5 | 1.9 |
| Rotational relaxation time[b] | nsec | 0.1 | 9 | 100 | 21 |

[a] Exponential decay
[b] *cf.* Material and methods.

emission maximum on shifting excitation wavelength from 365 to 275 nm (Wallach et al., 1970). The rotational relaxation time $\rho$ was calculated formally from Perrin's equation $(1/p - {}^1/_3) = (1/p_o - {}^1/_3)(1 - 3\,\tau/\rho)$ using the known lifetime $\tau$ and the degree of polarization $p$; $p_o$ was taken as 0.43 (Slavík, 1982).

## Results

### *Fluorescence of Yeast Cells and ANS*

The various fluorescence parameters of ANS were first tested with ordinary aerated yeast and compared with ANS fluorescence of lecithin liposomes, an aqueous solution of albumin and previously investigated *Escherichia coli* 15 TAU (Slavík & Vondrejs, 1981) (Table 1).

Fluorescence of yeast cells alone (when excited at 365 nm without ANS) was very weak without a pronounced maximum (*cf.* Fig. 3), the lifetime being 4.9 nsec. After addition of ANS the intensity rose steeply within a few seconds and remained constant for up to several hours. The constancy was also preserved in the fluorescence spectrum. The rise of fluorescence intensity thus appears to be a one-step process. The fluorescence decay was approximately exponential; a detailed deconvolution analysis revealed a major component with $\tau \approx 5$ nsec and a minor (less than 5% of total fluorescence intensity) component with $\tau \approx 13$ nsec (Slavík & Razjivin, 1978). The concentration dependence of the mean fluorescence lifetime observed in a phase fluorimeter indicates the same course of saturation of binding sites with ANS molecules in yeast cells and in lecithin liposomes (Fig. 1).

The fluorescence intensity was practically independent of temperature between 25 and 35 °C (a change of less than 3%). Addition of 10 μM FCCP or 0.25 μM valinomycin had no observable effect on fluorescence intensity.

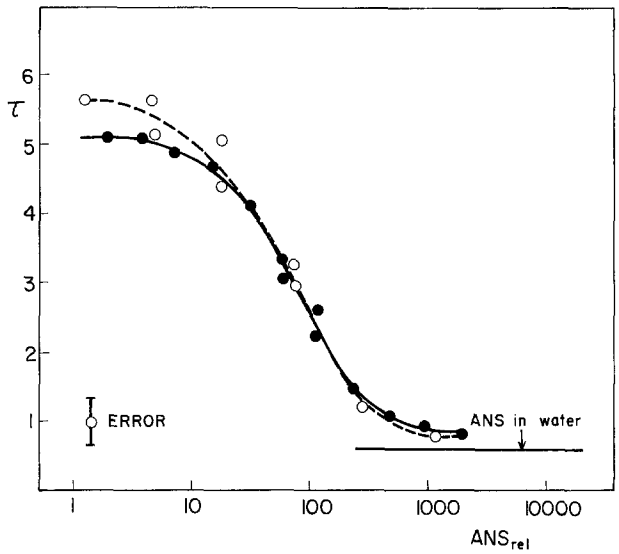


**Fig. 1.** Dependence of the mean fluorescence lifetime $\tau$ in nsec (phase-shift method) of ANS bound to yeast cells (solid line, filled circles) and to lecithin liposomes (broken line, open circles) on the relative ANS concentration (per cell or lipid) in arbitrary units ($ANS_{rel}$)

### *Fluorescence Changes Induced by Amino Acid Transport*

Addition of amino acids to a yeast preincubated in water had a minor effect on fluorescence intensity, the only exception being observed with aspartic and glutamic acids. This might be connected to a pH effect on ANS membrane fluorescence (*cf.* Discussion). However, when the cells had been preincubated for 1 hr with 1% glucose or a similar sugar-type source of energy there were some striking changes in fluorescence intensity observed (Table 2). The increase observed after addition of L-aspartic or L-glutamic acid was not instantaneous but was completed in only 20 min (Fig. 2) and it involved a shift in the

**Table 2.** Relative changes in fluorescence intensity of ANS bound to yeast cells[a]

| | | | |
|---|---|---|---|
| Glutamic acid | 3.8 | Cysteine | 0.9 |
| Aspartic acid | 3.8 | Isoleucine | 0.9 |
| Histidine | 1.5 | Leucine | 0.9 |
| Valine | 1.4 | Tyrosine | 0.9 |
| Lysine | 1.2 | Phenylalanine | 0.9 |
| Methionine | 1.0 | Glycine | 0.9 |
| Glutamine | 1.0 | Serine | 0.9 |
| Asparagine | 1.0 | Threonine | 0.9 |
| Arginine | 1.0 | Tryptophan | 0.8 |
| 2-Aminoisobutyric acid | 0.9 | Alanine | 0.8 |

[a] Measured 5 min after addition of 4 mM amino acid (L-form where applicable).

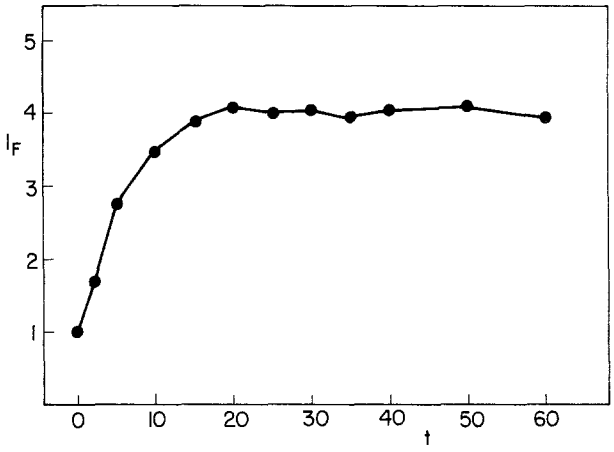


**Fig. 2.** Dependence of fluorescence intensity $I_F$ (measured at spectral maximum) of ANS bound to yeast cells after addition of 4 mM aspartic acid, in relative units, on time $t$ in min

emission maximum (Fig. 3) and some other fluorescence parameters (Table 3). The deconvolution analysis of fluorescence decay curve revealed a marked increase of the component with $\tau \approx 14$ nsec.

The fluorescence intensity stimulation was concentration-dependent with a half-saturation constant of about 2.5 mM (close to the $K_T$ of transport of aspartic acid by stationary yeast cells). It was apparently an actively generated process: 1 mM sodium azide, 0.1 mM uranyl nitrate and 10 μM CCCP had a pronounced (with uranyl nitrate 100%) blocking effect on the increase.

Somewhat unexpectedly, 1 mM N-ethylmaleimide and 2 mM sodium fluoride in fact stimulated the intensity increase. The presence of 5 mM $Na_2SO_4$ or 5 mM $Na_2SO_3$ caused about 30% decrease of the aspartic acid fluorescence enhancement.

Cells of the $op_1$ mutant responded with about 30% intensity increase as compared to the wild-type strain; the blocking effect of inhibitors was the same in both strains.

The temperature dependence of the aspartic acid

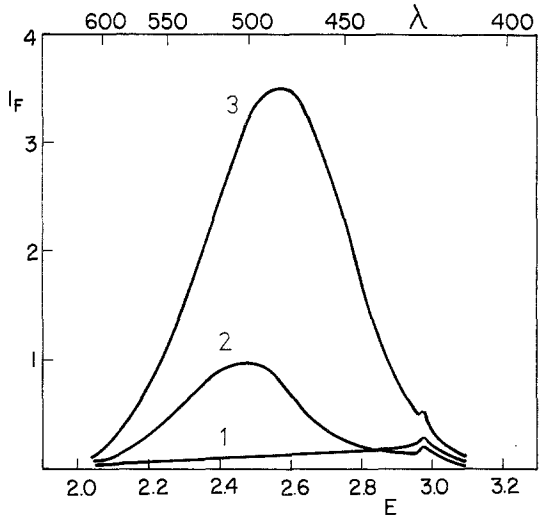


**Fig. 3.** The fluorescence spectrum of yeast cells alone (excitation wavelength 365 nm) (curve *1*), after addition of 50 μM ANS (curve *2*), 20 min after further addition of 4 mM aspartic acid (curve *3*), expressed as quanta per unit energy interval against energy ($E$, in eV), in relative units ($I_F$). For clarity, the corresponding wavelengths are shown on the upper abscissa

**Table 3.** Fluorescence of ANS bound to yeast cells

| Property | Units | Control | After addition of | |
|---|---|---|---|---|
| | | | 4 mM aspartic acid[a] | 1 mM hydrochloric acid[b] |
| Spectral maximum | nm | 500 | 481 | 490 |
| Spectral maximum | eV | 2.48 | 2.58 | 2.53 |
| Spectral bandwidth | eV | 0.43 | 0.47 | 0.45 |
| Intensity | Relative units | 1 | 4 | 3.5 |
| Lifetime (mean) | nsec | 5.2[c] | 8[d] | 5.2[c] |
| Degree of polarization | – | 0.15 | 0.23 | 0.10 |
| Polarization anisotropy | – | 0.11 | 0.17 | 0.07 |
| Intensity of excitation energy transfer[e] | Relative units | 2.0 | 3.8 | 1.7 |
| Rotational relaxation time[e] | nsec | 9 | 25 | 5 |

[a] pH attained was 3.3
[b] pH attained was 3.2
[c] exponential decay
[d] average value from two exponentially decaying components
[e] *cf.* Materials and Methods.

effect was little pronounced between 5 and 22 °C, the size of the effect representing some 30–50% of that at 30 °C.

Since it was striking that the acidic amino acids showed the highest effect and since ANS binding to

cell membranes was known to be pH-dependent (Freedman & Radda, 1969; Vanderkooi & Martonosi, 1969; Feinstein, Spero & Felsenfeld, 1970; Gomperts, Lantelme & Stock, 1970; Hasselbach & Heimberg, 1970; Flanagan & Hesketh, 1973), several other acids were tested in this respect. Succinic, fumaric, aconitic, 2-oxoglutamic, citric, malonic as well as hydrochloric acid itself caused in fact similar and even greater increases of fluorescence intensity than the amino acids. However, the "lipid" character of the binding (particularly fluorescence lifetime) remained unaltered (Table 3).

## Discussion

Judging from fluorescence microscopy of ANS-exposed yeast cells, the probe is not transported into the cells but remains predominantly bound on the cell surface (ring-like appearance of cells). The binding of ANS is, in principle, caused by two types of affinity, one for membrane proteins, the other for membrane lipids (e.g., Zierler & Rogus, 1978). The lipid binding shows a relatively low selectivity, all lecithins and sphingomyelins being active in this respect (Feinstein et al., 1970), choline being required for the binding (Eling & Di Augustine, 1971; Haynes & Staerk, 1974). Hence as a model of ANS binding to membrane lipids one may conveniently use lecithin liposomes. The protein binding is reportedly caused by a specific, highly polar, binding site at the protein surface (Radda & Vanderkooi, 1972; Weber, Tulinsky, Johnson & El-Bayoumi, 1979). The affinity of ANS for proteins is greater than that for lipids (Träuble & Overath, 1973; Zierler & Rogus, 1978). The blue spectral shift and the high quantum yield in the case of protein-bound ANS are not due to hydrophobicity of the binding site but rather to the low mobility of the polar groups near the probe with which it interacts more strongly in the excited state. These groups ["mobile dipoles" (Brand & Gohlke, 1972)] prevent rapid relaxation while the excited state of ANS persists. Therefore, being water-soluble, albumin appears to be as good a model as any with regard to ANS binding to proteins (Slavík, 1982).

The character of ANS fluorescence in yeast membranes before addition of aspartic or glutamic acid (particularly its decay, excitation spectrum and degree of polarization) indicates that we are dealing here with binding to lipids (*cf.* Table 1 and Fig. 1). Minor differences may well be explained as influence of ergosterol in yeast membranes on ANS binding. The fraction bound to membrane proteins may be assessed to represent about 3% of total membrane-bound ANS (respecting lower quantum yield of lipid-bound ANS with respect to that of protein-bound ANS). Proceeding from analogies with *Escherichia coli* (*cf.* Table 1), from the assumption that a protein molecule is surrounded by roughly 600 lipid molecules in the membrane (Träuble & Overath, 1973) and from the estimate of 1–3 binding sites for ANS per average protein molecule and about 5–20 sites per 100 average lipid molecules (Sackmann & Träuble, 1972; Radda & Smith, 1973), we arrive at a ratio of $ANS_{protein}/ANS_{lipid}$ equal to 1:10 – 1:100. Our results with yeast lie within this range, at about 1:20.

When discussing the effect of adding aspartic acid on ANS fluorescence, the role of lowering the pH must be carefully examined. This is known to increase the number of membrane lipid-bound ANS molecules, the effect of pH decrease on ANS fluorescence of yeast cells being similar to that of liposomes. The fluorescence of ANS bound to albumin is pH-independent until the denaturation limit is reached. The interaction of ANS with biological membranes, including the pH effects, is thoroughly discussed in a separate review (Slavík, 1982).

There is no doubt that fluorescence is enhanced by lowering the pH through addition of aspartic acid. However, as compared with the addition of hydrochloric acid (thought to be essentially involved only in decreasing the pH) other fluorescence parameters change in the opposite way after addition of aspartic acid (*cf.* Table 3). The mean fluorescence lifetime (an intensification of the slowly decaying component with $\tau \approx 13$–15 nsec), an increase of fluorescence polarization, an increase of excitation energy transfer from proteins to ANS, and a powerful rise of the rotational relaxation time suggest that in the case of aspartic acid addition we are dealing with an augmentation of the ANS-binding sites on proteins. This type of fluorescence gradually predominates. The greatest increase of fluorescence intensity is observed at lower ANS concentration (about 10–20 μM) which again is characteristic for protein binding sites: greater binding affinity, lower number of binding sites in comparison to lipid binding sites (Träuble & Overath, 1973; Zierler & Rogus, 1978). One may assess the number of newly exposed protein binding sites as $10^8$ per cell, which corresponds to 1 site per about 10–20 $nm^2$.

The character of these newly exposed binding sites is further confirmed by using other fluorescence probes. N-Phenylnaphthylamine (NPN) – a derivative of ANS without the charged sulfonate group – and 1,6-diphenyl-1,3,5-hexatriene (DPH), neither of which shows appreciable affinity for proteins, displayed no fluorescence change on adding aspartic or glutamic acid to yeast cells. 2-Toluidinonaphthalene-6-sulfonate (TNS), on the other hand, behaved similarly to ANS as it binds to both lipids and proteins.

There remain several problems to be solved. The rate at which the ANS-binding sites are exposed on adding aspartic or glutamic acid is very slow (*cf.*

Fig. 2) to be simply due to a carrier reorientation. Judging from the maximum rates of transport (e.g., Kotyk et al., 1971) and the estimated density of carrier sites (e.g., Opekarová, Kotyk, Horák & Kholodenko, 1975), one arrives at carrier turnover time of about 0.1 msec. It rather resembles a situation where transinhibition of amino acid transport begins to occur, this having a pronounced effect on carrier behavior in membranes (Horák & Kotyk, 1977). This would then lead to a further question, *viz.*, why the effect should be observed only with aspartic and glutamic acid if other amino acids, particularly lysine, cause much more powerful transinhibition (Horák, Kotyk & Říhová, 1977). A plausible explanation of this problem might be connected with the observation (*cf.* competition by sulfate anions) that ANS binds preferentially to anion-binding sites in a membrane (Fortes & Hoffman, 1974; Levinson & Villereal, 1975; Cabantchik, Knauf & Rothstein, 1978).

Be it as it may, ANS fluorescence provided an unexpected means of detecting the exposure of new binding sites that appears to be characteristic for some active transport processes in yeast.

## References


Batzri, S., Korn, E.D. 1973. Single bilayer liposomes prepared without sonication. *Biochim. Biophys. Acta* **298**:1015–1019

Brand, L., Gohlke, J.R. 1972. Fluorescence probes for structure. *Annu. Rev. Biochem.* **41**:843–868

Cabantchik, Z.I., Knauf, P.A., Rothstein, A. 1978. The anion transport system of the red blood cell. The role of membrane protein evaluated by the use of "probes." *Biochim. Biophys. Acta* **515**:239–302

Eddy, A.A. 1978. Proton-dependent solute transport in microorganisms. *In:* Current Topics in Membranes and Transport. F. Bronner and A. Kleinzeller, editors. Vol. 10, pp. 279–360 Academic Press, New York

Eling, T.E., Di Augustine, R.P. 1971. A role for phospholipids in the binding and metabolism of drugs by hepatic microsomes. *Biochem. J.* **123**:539–549

Feinstein, M.B., Spero, L., Felsenfeld, H. 1970. Interaction of a fluorescent probe with erythrocyte membrane and lipids: Effects of local anesthetics and calcium. *FEBS Lett.* **6**:245–248

Flanagan, M.T., Hesketh, T.R. 1973. Electrostatic interactions in the binding of fluorescent probes to lipid membranes. *Biochim. Biophys. Acta* **298**:535–545

Fortes, P.A.G., Hoffman, J.F. 1974. The interaction of fluorescent probes with anion permeability pathways of human red cells. *J. Membrane Biol.* **16**:79–100

Freedman, R.B., Radda, G.K. 1969. The interaction of 1-anilino-8-naphthalene sulfonate with erythrocyte membranes. *FEBS Lett.* **3**:150–152

Gomperts, B., Lantelme, F., Stock, R. 1970. Ion association reactions with biological membranes, studied with the fluorescent dye 1-anilino-8-naphthalenesulfonate. *J. Membrane Biol.* **3**:241–266

Hasselbach, W., Heimberg, K.W. 1970. The interaction of 1-anilino-8-naphthalenesulfonate with the membranes of the sarcoplasmic reticulum and various lipid compounds. *J. Membrane Biol.* **2**:341–350

Haynes, D.H., Staerk, H. 1974. 1-Anilino-8-naphthalenesulfonate: A fluorescent probe of membrane surface structure, composition and mobility. *J. Membrane Biol.* **17**:313–340

Horák, J., Kotyk, A. 1977. Temperature effects in amino acid transport by *Saccharomyces cerevisiae*. *Exp. Mycol.* **1**:63–68

Horák, J., Kotyk, A., Říhová, L. 1977. Specificity of transinhibition of amino acid transport in baker's yeast. *Folia Microbiol.* **22**:360–362

Kotyk, A., Ponec, M., Říhová, L. 1971. Uptake of amino acids by actidione-treated yeast cells. I. Specificity of carriers. *Folia Microbiol.* **16**:432–444

Kotyk, A., Říhová, L. 1972. Transport of α-aminoisobutyric acid in *Saccharomyces cerevisiae*. Feedback control. *Biochim. Biophys. Acta* **288**:380–398

Levinson, C., Villereal, M.L. 1975. Interaction of the fluorescent probe. 1-anilino-8-naphthalene sulfonate, with the sulfate transport system of Ehrlich ascites tumor cells. *J. Cell. Physiol.* **86**:143–154

Opekarová, M., Kotyk, A., Horák, J., Kholodenko, V. 1975. Isolation and properties of an arginine-binding protein from *Saccharomyces cerevisiae*. *Eur. J. Biochem.* **59**:373–376

Parker, C.A. 1968. Photoluminescence of solutions. Elsevier, Amsterdam

Radda, G.K., Smith, D.S. 1973. Human erythrocyte ghosts: Relationship between membrane permeability and binding kinetics of the fluorescent probe 1-anilinonaphthalene-8-sulfonate. *Biochim. Biophys. Acta* **318**:197–204

Radda, G.K., Vanderkooi, J. 1972. Can fluorescent probes tell us anything about membranes? *Biochim. Biophys. Acta* **265**:509–549

Sackmann, E., Träuble, H. 1972. Studies of the crystalline-liquid crystalline phase transition of lipid model membranes. I. Use of spin labels and optical probes as indicators of the phase transition. *J. Am. Chem. Soc.* **94**:4482–4491

Singleton, W.S., Gray, M.S., Brown, M.L., White, J.L. 1965. Chromatographically homogeneous lecithin from egg phospholipids. *J. Am. Oil Chem. Soc.* **42**:53

Slavík, J. 1982. Anilinonaphthalene sulfonate as a probe of membrane composition and function. *Biochim. Biophys. Acta (in press)*

Slavík, J., Razjivin, A.P. 1978. Binding of ANS to the biological membrane. *Stud. Biophys.* **73**:157–158

Slavík, J., Vondrejs, V. 1981. Anilinonaphthalenesulfonate as a fluorescent probe for temperature induced changes in *Escherichia coli* membranes. *Folia Microbiol.* **26**:176–178

Träuble, H., Overath, P. 1973. The structure of *Escherichia coli* membranes studied by fluorescence measurement of lipid phase transitions. *Biochim. Biophys. Acta* **307**:491–512

Vanderkooi, J., Martonosi, A. 1969. Sarcoplasmic reticulum. VIII. Use of 8-anilino-1-naphthalene sulfonate as conformational probe on biological membranes. *Arch. Biochem. Biophys.* **133**:153–163

Wallach, D.F.H., Ferber, E., Selin, D., Weidekamm, E., Fischer, H. 1970. The study of lipid-protein interactions in membranes by fluorescent probes. *Biochim. Biophys. Acta* **203**:67–76

Weber, L.D., Tulinsky, A., Johnson, J.D., El-Bayoumi, M.A. 1979. Expression of functionality of α-chymotrypsin. The structure of a fluorescent probe-α-chymotrypsin complex and the nature of its pH dependence. *Biochemistry* **18**:1297–1303

Zierler, K., Rogus, E. 1978. Fluorescence of 1,8-anilinonaphthalene sulfonic acid bound to proteins and to lipids of sarcolemma. *Biochim. Biophys. Acta* **514**:37–53


Received 19 February 1981; revised 12 June 1981; revised again 22 July 1981