*Biochimica et Biophysica Acta*, 691 (1982) 233–239
Elsevier Biomedical Press



BBA 71278

# MEASUREMENT OF THE QUANTUM YIELD OF 8-ANILINO-1-NAPHTHALENE SULPHONATE BOUND ON PLANT MICROSOMES

## CRITICAL APPLICATION OF THE METHOD OF WEBER AND YOUNG

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(Received March 15th, 1982)

*Key words: Anilinonaphthalene sulfonate spectroscopy; Quantum yield; (Plant membrane)*

**The method of Weber, G. and Young, L. ((1964) J. Biol. Chem. 239, 1415–1423) allows one to evaluate by graphical means the maximum fluorescence intensity of a fixed amount of anilinoaphthalene sulphonate (ANS) in presence of increasing concentrations of proteic sites for this probe. This value is used to estimate the quantum yield of the fixed ANS. The method is based on the linear extrapolation of the double reciprocal plot of fluorescence intensity vs. protein concentration. We show by experimental means and critical analysis that such an extrapolation is not valid unless two specific conditions are fulfilled: the fractional saturation of the sites at the beginning of the titration must be low and the fractional fixation of the probe at the end of the titration must be high. Due to the low affinity of membranes for ANS, the latter condition is not easily satisfied, which may lead to erroneous interpretations. We propose a procedure which overcomes this drawback. When applied to microsomes of roots from horse bean, it reveals that the quantum yield of bound ANS is insensitive to pH in the pH range 2–8, and to the presence of $Ca^{2+}$.**

## Introduction

The first step in the interaction of ions and biological membranes involves variations of the surface charge density and the surface potential at the water-membrane interface. It possibly results in structural modifications as demonstrated for artifical bilayers [1] and natural membranes [2].

The fluorescent molecule 8-anilino-1-naphthalene sulfonate (ANS) is currently used for probing the dielectric and the electrostatical characteristics of the surface [3–20] and conformational changes [21–25] of the membrane. The conformational changes are monitored by measuring the variations of the quantum yield of the dye bound on the membranes [26]. On another hand, the use of ANS as a purely electrostatic probe implies that the quantum yield be constant in all experimental conditions or at least that its variations would be known. In some rare cases, direct measurements of the quantum yield have been obtained by the nanosecond fluorescence technique [10–13] or by the spectroscopic method [14]. In all other cases [13–24], the quantum yield has not been estimated but the constancy of the fluorescence efficiency (to which it is releated) has been checked by the empirical method of Weber and Young [27]. A given amount of ANS is titrated by proteins or membranes and the maximum fluorescence intensity at infinite concentration is estimated by graphical extrapolation. This method was originally developed by studying the interaction of ANS with bovine serum albumin which has high affinity receptors for the probe. The receptors on biological membranes have a lower affinity for ANS. It has been argued that this is responsible

for an underestimation of the quantum yield by the empirical method [10]. On the other hand, it has been shown that this method may lead to an overestimation of the quantum yield [24] possibly due to the electrostatical potential created by the adsorbed ANS itself [6].

The quantum yield of ANS bound on animal cell membranes appears to be insensible to the ionic conditions (e.g. Refs. 12 and 13). The data on plant membranes are scarce. In contrast to animal membranes which generally bath in well controlled mediums, plant membranes must face to large ionic fluctuation in the external medium as well as in vacuolar compartments [28–33] and perhaps in the cisternae of the endoplasmic reticulum [34,35]. In order to study the response of such membranes to the ionic conditions, we used microsomes from roots of horse bean. Horse bean is a calcicole leguminosae adapted to neutral or basic soils, and excluded from acidic ones. A dramatic increase of the passive permeability of its roots occurs when the pH is lowered to 4.0 in absence of $Ca^{2+}$ [41,42]. We have studied the effect of the pH on the quantum yield of ANS bound on the microsomes, by using an original procedure which allows to overcome the above described drawbacks of the classical method.

## Materials and Methods

The roots were excised from 8-day-old seedlings of horse bean (*Vicia faba* L., var. *minor*) grown in the dark at 25°C on a dilute saline solution [36]. The microsomal fraction was obtained by classical differential centrifugations after homogeneization of the whole roots with a Pascall tripple-roll-mill. Fluorescence measurements were performed on a Jobin-Yvon JY3D spectrofluorimeter. The excitation and emission wavelengths were 390 nm and 480 nm, respectively. The scattered incident light was stopped by a cut-off filter. A peristaltic pump maintained a constant cycling of the sample between the microcell (optical length: 3 mm) and a batch, both thermostatically controlled at 25°C. The output signal was recorded graphically. The measurement of FI was made by automatic integration for 15–60-s periods. When the probe absorbance was higher than 0.05, the fluorescence intensity was corrected with the method of Beyer et al. [37]. The standardization of the fluorescence intensity was made with a Perkin-Elmer standard device. The quantum yield was determined by comparing the fluorescence efficiency of the ANS adsorbed on membranes to that of ANS in methanol [38]. Dialysis experiments were performed on a Dianorm equilibrium teflon cell with cuprophan membranes. The free ANS concentration in dialysis experiments was determined by fluorescence measurement following adsorption on an excess concentration of bovine serum albumin. Protein concentration was determined by the method of Lowry et al. [39].

## Results

Fig. 1 shows the results of an application of the classical Weber and Young method [27] for measuring the quantum yield of ANS fixed on microsomes. The experimental conditions were typical of those currently used [15–17]: the membrane concentration was lower than $0.1\,g \cdot l^{-1}$ of proteins and the ANS concentration was 25 or 50 $\mu$M. The double reciprocal plot was linear. The ordinate intercept is considered as being equal to the reciprocal of the maximum fluorescence intensity (corresponding to an infinite sites concentration).

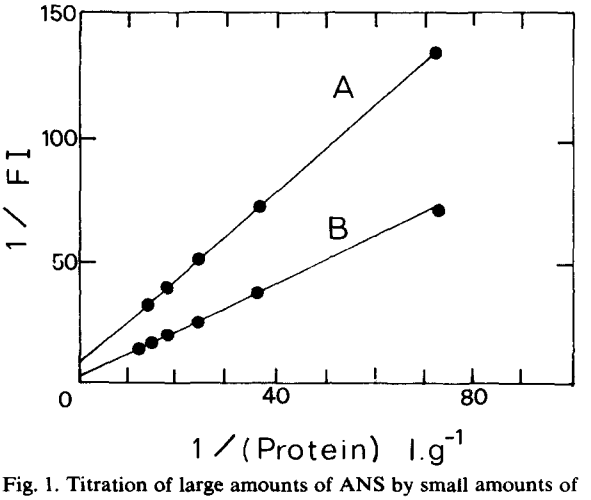


Fig. 1. Titration of large amounts of ANS by small amounts of microsomal membranes: double reciprocal transformation of fluorescence intensity (FI) vs. protein concentration. The media contained 3 mM $CaCl_2$ and their pH was 7.4. (A) 25 $\mu$M ANS; (B) 50 $\mu$M ANS.

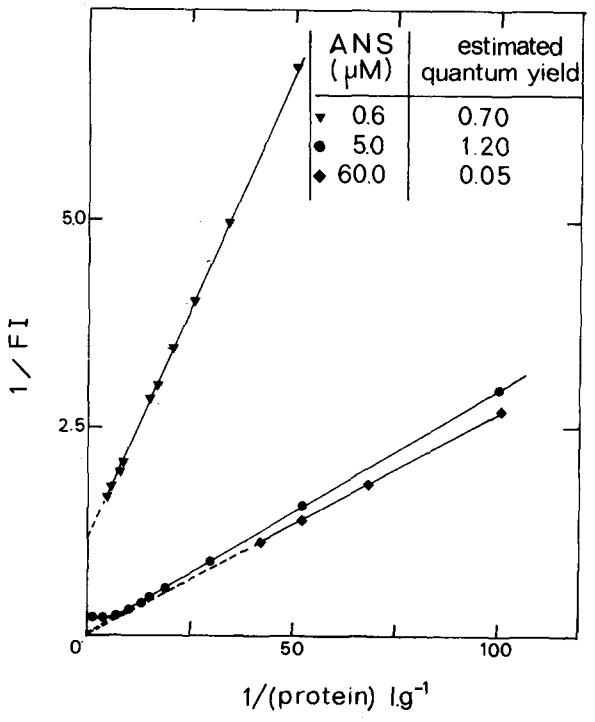


Fig. 2. Titration of fixed amounts of ANS (insert) by bovine serum albumin: double reciprocal transformation of the fluorescence intensity (FI) vs. bovine serum albumin concentration. The extrapolations of the linear segments are considered as estimations of the reciprocal of the maximum fluorescence intensity (i.e. FI which would be observed at infinite sites concentration). The quantum yields calculated from these values are given in the insert.

This allows one to calculate the fluorescence efficiency and then the quantum yield. Results of Fig. 1 gave quantum yield values lower than 0.04, which were dependent on ANS concentration.

Direct measurements of the amounts of ANS adsorbed on the membranes were performed by equilibrium dialysis with various ANS concentrations in the range 5–200 $\mu$M. The Scatchard plot of the results indicated that the mean number of sites on membranes was 42 $\mu mol \cdot g^{-1}$ of proteins. The curves fluorescence intensity vs. (ANS concentration) were obtained in parallel runs. The maximum fluorescence intensity at infinite ANS concentration was estimated from the Scatchard plots of the results. The comparison of this value with the number of sites determined by dialysis indicated that the quantum yield is 0.43. This value is within an order of magnitude higher than our first estimation (above) and within the range of the values generally given for biological membranes [10–13]. Fig. 2 shows titrations of fixed amounts of ANS with bovine serum albumin. The double reciprocal plots were linear only when the ANS concentration was low compared to the intrinsic dissociation constant (approx. 4 $\mu$M). When the concentration of the probe was near this value, the graph was curved in the high protein concentration region. For higher ANS concentration, only a linear region of the graph may be easily explored. This limitation is due to the high values of the fluorescence intensity, which would attain the saturation limit of the detector at high protein concentration. The extrapolation of the linear segments gave different estimations of the quantum yield (Fig. 2, insert). The exact value was estimated to be 0.75 from direct saturation with bovine serum albumin. Weber and Young [27] report exactly the same value. In the titration experiments of Fig. 2, the correct value was obtained only for the lowest ANS concentrations (approx. 1 $\mu$M). Increasing this concentration up to 60 $\mu$M gave first an overestimation (with an irrealistic

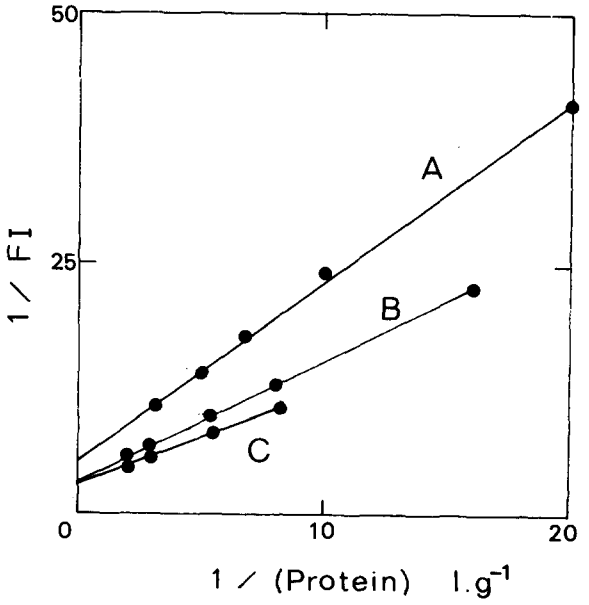


Fig. 3. Titration of small amounts of ANS by large amounts of microsomal membranes: double reciprocal transformation of fluorescence intensity (FI) vs. protein concentration. (A) 2.5 $\mu$M ANS, 1.5 M KCl, pH 7.4; (B) 4 $\mu$M ANS, 1.5 M KCl, pH 3.0; (C) 4 $\mu$M ANS, 3 mM $CaCl_2$, pH 3.0.

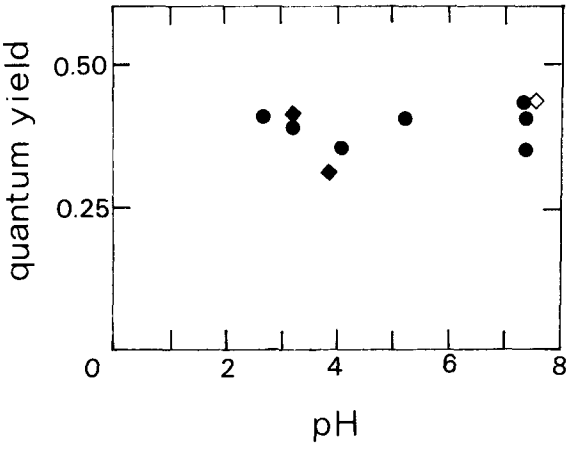


Fig. 4. Quantum yield of ANS fixed on microsomal membranes in function of the pH of the medium. The closed symbols refer to evaluations of the quantum yield by the method of Weber and Young under the conditions described in the text as situation three. The open symbol refers to direct comparison of the measured fluorescence of the fixed ANS molecules to their number determined by equilibrium dialysis. The mediums contained 1.5 M KCl (circles) or 3 mM $CaCl_2$ (squares). The pH values were adjusted with HCl.

high value) and then an underestimation of the quantum yield. In the 25–50 $\mu$M concentration range, the underestimation factor was the same as the one observed with microsomes (1/10).

Returning to microsomes with these informations, we measured the fluorescence intensity in titration experiments with ANS concentrations 10-fold lower and membrane concentration 10-fold higher than previously (Fig. 3). This procedure was applied with different ionic conditions. It gave estimations of the quantum yield which are in good accordance with the results of the dialysis experiments (Fig. 4). Furthermore, the quantum yield was insensitive to the pH in the range 2–8, and to the nature of the cation ($K^+$ or $Ca^{2+}$).

*Methodological analysis*

The fundamental observation is that in some cases the linear extrapolation used to estimate the maximum fluorescence intensity is not valid. Harris [24] made the same observation on submitochondrial particles. The fixation of ANS on bovine serum albumin and on membranes may be described by an action mass law [10,27]. The fluorescence intensity of the adsorbed probe is then

$$FI = \phi \left( \frac{[ANS]_{tot}(\text{concentration of free sites})}{K_d + (\text{concentration of free sites})} \right) \quad (1)$$

where $K_d$ is the dissociation constant, $[ANS]_{tot}$ is the concentration of total ANS (free plus bound) and $\phi$ is the fluorescence efficiency.

In the method of Weber and Young, the concentration of free sites is unknown and replaced by the total protein concentration, which is proportional to the total concentration of sites. The linear double reciprocal plot $(FI)^{-1}$ vs. (protein concentration)$^{-1}$ is then considered as a proof of the empirical rectangular hyperbolic relation:

$$FI = \phi \frac{[ANS]_{tot}(\text{concentration of proteins})}{K_e + (\text{concentration of proteins})} \quad (2)$$

where $K_e$ is an empirical parameter. By comparing relations 1 and 2, it may be shown that:

$$K_e = \frac{K_d + [ANS]_{free}}{n} \quad (3)$$

where $n$ is the number of sites per g of proteins. It may be easily seen from relations 2 and 3 that the double reciprocal plot will be linear only when $K_e$ remains constant during the titration of the fixed amount of ANS by the proteins. This is obtained in two cases, when $[ANS]_{free}$ is negligible as compared to $K_d$, and when it remains constant. The latter condition is satisfied when the protein concentration is very low, so that the sites are saturated by a small fraction of the total ANS.

The relations 2 and 3 may be used to simulate the fluorescence intensity data obtained during ANS titration by proteins. When the results are plotted as are the experimental ones in the method of Weber and Young (Fig. 5), it appears that the critical factors are the fractional saturation of the sites at the beginning of the titration and the fractional fixation of the probe at the end of the titration. Three different situations may be recognized. In the first one, the fractional saturation of the sites is already high after the first addition of proteins. That implies that the concentration of free ANS is larger than $K_d$, so that $K_e$ is large. When the titration progresses, the concentration of the free ANS is lowered, so that $K_e$ decreases and the graph of the double reciprocal plot bends (Fig. 5A). In this case, no extrapolation is possible. The

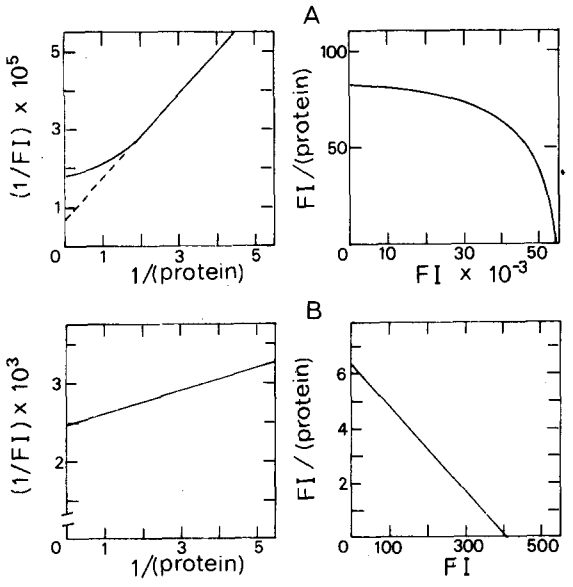


Fig. 5. Simulatation of the results of ANS titrations by proteins performed as in the Weber and Young method. The simulation was made with the help of relations 2 and 3 in the text. The concentration of free ANS was computed by solving the action mass law which describes the first-ordered fixation of ANS on a variable amount of proteins with a dissociation constant $K_d = 4\ \mu M$. (A) Situations one and two (see text); (B) situation three. The results (fluorescence intensity vs. protein concentration) are presented as double reciprocal plot (left), and Scatchard plot (right).

second situation is analogous to the former but the curvature region is not experimentally explored owing to technical difficulties associated with the use of high concentrations of sites (excessive turbidity and intrinsic fluorescence [5]). In this case the extrapolation will be erroneous in spite of the apparently linear graph.

In the third situation, the first points correspond to a low fractional saturation of the sites. This means that $[ANS]_{free}$ is low as compared to $K_d$, so that $K_e$ may be considered as constant in relation 3. The final fractional fixation of ANS must be high. This is necessary for the explored equilibrium range to be sufficiently large to provide a correct accuracy for the extrapolation (Fig. 5B). This third situation is the only one which allows a correct estimation of the maximum fluorescence intensity and hence the quantum yield.

Biological membranes generally have low affinity for ANS. Furthermore, their turbidity is relatively high so that only low concentrations may be used. Both these facts explain that the conditions for the situation three were not satisfied in a number of cases. The analysis of the published data reveals that most of them have been obtained in experimental conditions corresponding entirely or in part to situation one [18,19,23,24] or two [13,15–19,23,24]. In some cases [21–23], it has been experimentally shown that this procedure had led to erroneous conclusions [7,24].

In situation two, the entire titration is made in limitant sites conditions. Due to this fact, the fluorescence intensity vs. (proteins) graph as well as the double reciprocal plot are quasi linear with near zero-ordinate intercepts. On the other hand, the slope of the graphs depends on the ionic conditions because $K_d$ in the $K_e$ parameter is an apparent dissociation constant which is affected by the surface potential [5–8,10–13]. This commonly leads to a family of straight lines converging toward an extremely low ordinate intercept (i.e. Ref. 15–17). This apparently common intercept is taken as an indication of the constancy of the quantum yield. Our analysis shows that it has no real significance. The Scatchard plot would allow to overcome this confusion because in situation one it gives a quasi horizontal line (Fig. 5A).

Our conclusion is that the correct procedure for using the method of Weber and Young is as follows: (i) choosing the ionic condition in order to minimize the absolute value of the surface potential and hence the value of the apparent $K_d$; (ii) measuring the apparent $K_d$ by titration of the membrane with ANS, in order to determine the maximum ANS concentration that can be used. This concentration is to be an order of magnitude lower than $K_d$; (iii) choosing the membrane concentration as high as possible with respect to their turbidity and intrinsic fluorescence [7]. The fractional fixation of ANS at the end of the titration must be sufficiently high (approx. 30%). This may be done by comparing the highest attained fluorescence intensity value and the maximum fluorescence intensity estimated by extrapolation; (iv) using the Scatchard plot instead of the double reciprocal one, in order to increase the accuracy of the linear extrapolation.

In these conditions, the method of Weber and Young gives a simple means to estimate the aver-

age quantum yield of the probe adsorbed on membranes. Furthermore, the use of a low fractional saturation range of the sites minimizes complications due to the interactions between ANS molecules [5,6,10,40].

## Discussion

The quantum yield of ANS bound on horse bean microsomes is nearly the same as the one measured on animal membranes by nanosecond technique [12,13]. It has been made increasingly clear in the recent years that only one main binding site exists for ANS and that it is phospholipidic [12]. This may explain why little variation appears in the measured values of the quantum yield. The quantum yield is thougth to reflect the polarity and the microviscosity at the binding site [26]. The fact that is does not vary when the pH is lowered below 4.0 suggests that the loss of the control of the passive permeability observed in vivo in this condition [41] is not related to structural perturbations of the membrane. The same conclusion may be reached from the insensitivity of the quantum yield to $Ca^{2+}$ at $pH < 4.0$, since in vivo this cation protects the horse bean against the effect of the acidic pH [42]. We are led to suppose that the effect of the ionic conditions on the horse bean membranes only involves electrostatic surface phenomena.

## Acknowledgement

This work was supported by Institut National de la Recherche Agronomique and Centre National de la Recherche Scientifique (E.R.A. 618).

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