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# BINDING OF 8-ANILINO-1-NAPHTHALENE SULFONIC ACID TO VIABLE PULMONARY MACROPHAGES

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## SUMMARY

1. The fluorescent probe, 8-anilino-1-naphthalene sulfonic acid, has been used to study binding properties of viable pulmonary macrophages.

2. When a suspension of macrophages was added to a solution of the probe, enhanced fluorescence, accompanied by a hypsochromic shift of the emission maximum was observed. Double reciprocal plots of fluorescence intensity *vs* macrophage plasma membrane protein concentration resulted in straight lines. Binding values have been calculated from the double reciprocal plot data using the Klotz equation, and studied as a function of probe concentration.

3. Studies indicate it is unlikely that within 10 min and at low concentration, the fluorescent probe enters into the cytoplasm to any appreciable extent. Therefore, the probe is bound primarily to the plasma membrane at these low concentrations.

4. High concentrations of the probe had an adverse effect on macrophage viability which is interpreted as being due to leakage of the probe into the cells.

5. Binding parameters at low concentrations of the probe differ from those found using high concentrations. These differences are attributed to interaction of the probe with intracellular proteins when using high concentrations.

## INTRODUCTION

The fluorescent probe 8-anilino-1-naphthalene sulfonic acid (ANS) is a polycyclic aromatic compound that is virtually non-fluorescent in aqueous solution and which becomes highly fluorescent upon combining with many proteins. In 1954, Weber and Laurence[1] first proposed the use of ANS for the study of protein structure and conformation. Numerous studies have since been carried out using ANS and other fluorescent probes in studying conformation of proteins and enzymes[2–9].

Also in 1954, Newton[10] reported on the use of a similar compound, *N*-tolyl-α-naphthylamine-8-sulfonic acid in studying the competition between the antibiotic

Abbreviation: ANS, 8-anilino-1-naphthalene sulfonic acid.

polymyxin and certain cations for sites on cells of *Pseudomonas aeruginosa*, thus extending the use of fluorescent probes to membranes. In recent years, ANS has been used to study numerous structures, including erythrocyte membranes[11–17], mitochondrial membranes[18–24], submitochondrial particles[25,26], microsomes[27–29], sarcoplasmic reticulum[30,31], chromatophores[32], electroplax cell fragments[33], and crab, lobster, and squid nerves[34].

Not all of these studies dealt with binding parameters and none dealt with the derivation of binding parameters of systems involving intact living cells. Membrane binding studies were limited to isolated membranes, membrane fragments, or subcellular components with the inherent disadvantage that such systems may not be representative of the living cell. This disadvantage can be overcome by studying systems involving living cells. Since macrophage membrane sites play an important role in phagocytosis and antibody response, we decided to explore the feasibility of using ANS as a tool for studying plasma membrane binding properties of viable macrophages. The results of such studies are presented in this report.

## MATERIALS AND METHODS

### *Reagents*

The magnesium salt of ANS was obtained from Eastman Kodak Company and was used without further purification. Since light has a deleterious effect upon dilute solutions of ANS, they were prepared just prior to use from a 0.5 mM stock solution of ANS. The stock solution was stored in a tightly stoppered amber bottle at 4 °C and used for periods not longer than thirty days. All solutions of ANS were prepared in Krebs phosphate buffer without $Ca^{2+}$ or $HCO_3^-$ (ref. 35) and allowed to warm to room temperature before being used.

Quinine sulfate, as the hemi-sulfate, (Sigma Chemical Company), was used to calibrate the fluorescence spectrophotometer. A stock solution of quinine sulfate containing 1 mg/ml was prepared in 0.05 M $H_2SO_4$. Working solutions of 1, 2, and 5 $\mu$g/ml in 0.05 M $H_2SO_4$ were prepared at 1–2-week intervals from the stock solution. All quinine solutions were stored at 4 °C in amber-colored flasks and allowed to warm to room temperature before being used.

### *Collection of macrophages*

Rabbit pulmonary macrophages were collected as described previously[36]. The final number of cells was adjusted to a concentration of $5 \cdot 10^8$ cells per ml of Krebs phosphate buffer using a hemacytometer for counting. Packed cell volumes were determined using an International Model MB Hematocrit Centrifuge. Cell suspensions were kept at 0–4 °C until used. They were used within 4–6 h after collection.

### *Protein determinations*

The total protein content of intact cells was determined by the biuret method[37] and the protein content of the cell supernatant was determined by the method of Lowry *et al.*[38]. A solution of bovine albumin was used as the standard in both assays. The amount of protein in the plasma membrane was estimated to be 10% of the total cell protein[39].

*Fluorescence measurements*

Fluorescence measurements were made at room temperature using a Baird Atomic Model SF-1 fluorescence spectrophotometer. At the beginning of each experiment and immediately prior to each reading, a solution of quinine sulfate was read at the maximum excitation and emission wavelengths determined for quinine (354 and 456 nm, respectively). Any deviation from the fluorescence intensity for which the instrument had been calibrated, was corrected for with the fine gain or by repeaking the lamp. In this manner, fluctuations in readings due to positional variations of the arc in the xenon lamp were avoided. Solutions of ANS, excited at 396 nm, had an emission maximum at 536 nm. After addition of macrophage suspension, the emission maximum shifted to 504 nm. Therefore, these settings were used for reading the ANS–macrophage system.

*ANS titrations*

Usually, titrations are carried out by adding increments of a membrane suspension to a tube containing the ANS solution and recording the fluorescence intensity after each addition. This method is feasible when the increase in fluorescence is instantaneous and does not change with time. In our case, however, the fluorescence intensity did change with time. The rate of change was greatest up to about 8 min after addition of macrophage suspension (Fig. 1). We therefore elected to wait 10 min, when the rate of increase was negligible, before reading the fluorescence intensity. Under these conditions, the usual titration would have necessitated a 10 min interval before reading the fluorescence intensity after each addition of cell suspension. Consequently, the cells from the initial additions would have been in the titration mixture for a considerable period of time before completion of the titra-

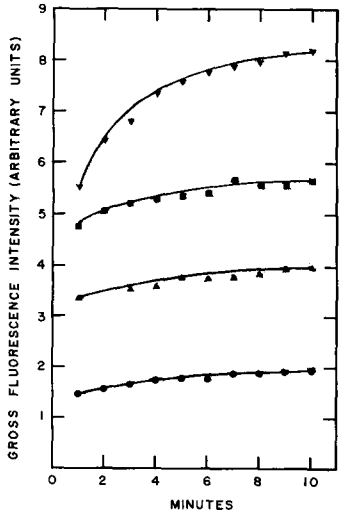



Fig. 1. Increase in fluorescence as a function of time at various ANS concentrations. 0.2 ml of macrophage suspension ($1 \cdot 10^8$ cells) was added to 2.8 ml of ANS solution and fluorescence intensities were followed with time. Mixtures were stirred prior to each reading. The excitation wavelength was 396 nm and emission intensity was measured at 504 nm. The concentrations of ANS used were: ●, 10 $\mu$M; ▲, 25 $\mu$M; ■, 50 $\mu$M; ▼, 100 $\mu$M.

tion. To avoid this, we carried out the titrations by adding increasing amounts of cell suspension (25–150 $\mu$l) to fixed volumes (6.0 ml) of ANS contained in a series of tubes. After addition of the macrophage suspension to the ANS solution, the tube was thoroughly stirred. 3.0 ml of the mixture were then transferred to a cuvet and allowed to remain at room temperature. The mixture was again thoroughly stirred for 15 s prior to being read in the spectrofluorometer 10 min after initial mixing. Gross fluorescence intensities were measured and recorded for the contents of each tube.

The supernatant from the macrophage suspension contained enough protein to contribute to the overall fluorescence. Therefore, it was necessary to determine the fluorescence from this source and to subtract it from the total fluorescence readings. Since the volume of the supernatant fluid in the suspension was known from the packed cell volume, a portion of the macrophage suspension was centrifuged at $1000 \times g$ at 4 °C for 5 min and the supernatant recovered. Another titration was carried out in the same manner using those amounts of supernatant corresponding to the amount of cell suspension used in the initial titration. These fluorescence intensity values were subtracted from the gross fluorescence readings.

The small contribution to fluorescence from the cell suspension alone in buffer was also deducted. Also, a small correction factor due to increases in total volume upon adding the cell suspension to ANS solutions was applied.

*Calculations*

Calculation of binding parameters were carried out based on the findings of Klotz[40], discussed by Weber and Young[2], and used by several other investigators in their membrane studies[12,13,16]. The adsorption of ANS on a protein molecule may be characterized by the quantities $n$ and $\bar{K}_{app}$ as defined by the equation:

$$\frac{P_0}{xD_0} = \frac{1}{n}\{1 + \bar{K}_{app}[(1-x)D_0']^{-1}\} \quad (1)$$

where $n$ is the number of moles of ANS adsorbed per mg of membrane protein, $\bar{K}_{app}$ is the statistical average apparent dissociation constant for the binding of ANS to the $n$th sites, $P_0$ is the total mg of membrane protein, $D_0$ is the ANS concentration in moles, $D_0'$ is the ANS concentration expressed in molarity, and $x$ is the fraction of ANS bound to membrane protein. $x$ is obtained from a double reciprocal plot of the membrane protein concentration (mg/ml) *vs* net fluorescence intensity by extrapolating the resulting straight line to infinite protein concentration. From the fluorescence intensity at this extrapolated point ($F_0$) and the fluorescence intensity ($F$) at each point of the titration, one can calculate $x$ as the ratio $F/F_0$.

## RESULTS AND DISCUSSION

Fig. 2 represents three typical plots of the change in fluorescence as a function of membrane protein concentration at constant ANS values. Increased fluorescence is obtained with increasing amounts of macrophage suspension. This increased fluorescence along with the observed shift of emission maximum to a lower wavelength has been interpreted as the interaction of the probe with hydrophobic regions in the membranes[11,12,41,42]. Other workers have evaluated this phenomenon in terms

of ANS binding to lipids in the cell membrane[13,26,28,30]. On the basis of these studies, we have assumed that ANS, in 10 min and at low concentrations, binds primarily to hydrophobic sites encountered on or within the predominantly lipoidal plasma membrane and after binding to these regions, is unable to penetrate into the cytoplasm to any great degree. According to this hypothesis, we are dealing with plasma membrane binding sites and not with the binding of ANS to intracellular proteins.

We tested this assumption by determining the fluorescence intensity of macrophages homogenized and dissolved in 7.3 M urea and comparing the results with those obtained from the same amount of intact cells under the same conditions. The dissolved macrophages exhibited a net fluorescence intensity 4 times greater in 50 $\mu$M ANS than intact cells exposed to the same concentration of ANS for 10 min. Similar results were observed when intact cells were compared with cells which had been frozen and thawed 6 times. The fluorescence intensity of ANS in the presence of 7.3 M urea was negligible. The increased fluorescence observed for both dissolved and ruptured macrophages over intact cells indicates that ANS is binding to released intracellular material. If the ANS were penetrating into the interior of the macrophages during the 10-min incubation, one should expect no appreciable differences in fluorescence intensity between intact cells and those whose contents had been exposed to ANS.

Udenfriend *et al.*[43] in their fluorescence studies of amino acid transport using sarcoma 37 ascites mouse tumor cells, also reported evidence that ANS does not enter cells. After exposing these cells to ANS, and examining them by fluorescence microscopy, they observed that the cells appeared enclosed in a fluorescent envelope showing that the ANS was located primarily on or in the cell membrane. Further evidence is provided by Tasaki *et al.*[34]. They found that ANS, when introduced into a giant squid axon by the internal perfusion technique, is localized within the interior indicating that ANS does not penetrate the membrane. They infer from this, that ANS applied outside the axon does not enter the cell and consequently their observed fluorescence change does not involve intracellular material.

TABLE I

EFFECT OF ANS ON MACROPHAGE VIABILITY

25 $\mu$l of macrophage suspension ($1.25 \cdot 10^7$ cells) were added to 2.0 ml of ANS solution and stirred. After 10 min at room temperature, 0.5 ml of 0.5% trypan blue solution was added to the suspension and mixed. The criterion used was the ability of viable cells to exclude dye.

| *ANS concentration* ($\mu M$) | *Cell viability* (%) |
|---|---|
| 0 | 98 |
| 15 | 98 |
| 30 | 94 |
| 60 | 88 |
| 90 | 60 |
| 120 | 46 |
| 180 | 19 |
| 300 | 2 |

The question now arises as to what happens when higher concentrations of ANS are used. Does leakage of ANS into the cells occur? If so, toxic effects on metabolic processes should take place. An examination of the effect of ANS concentration on cell viability, as judged by exclusion of trypan blue (Table I), reveals that high concentrations of ANS have a toxic effect on pulmonary macrophages. Cell viability dropped below 50% at ANS concentrations greater than 90 $\mu$M and virtually all cells were dead at 300 $\mu$M ANS concentration. The results of our viability studies are consistent with the premise that at high concentrations, ANS enters the macrophage thereby causing cell death. At low concentrations (below 60 $\mu$M), ANS interactions are limited mainly to the membrane and therefore a greater percentage of cells survive within the 10 min of exposure to ANS.

In addition, the results in Fig. 1 show that the increase in fluorescence during the first 10 min is quite different in the 10–50 $\mu$M ANS range than with 100 $\mu$M ANS. At the lower ANS concentrations, the rate of increase is slow and the overall change is small. However, at 100 $\mu$M ANS, the rate of increase is more rapid and the overall change is greater, indicating that readily accessible membrane sites are occupied, after which, excess ANS enters the cell giving rise to a rapid increase in fluorescence before levelling off.

Fig. 3 shows that the double reciprocal plots of the data obtained in Fig. 2 result in straight lines, indicating that the fluorescence enhancement of each molecule of ANS is the same[44]. The points were fitted by the method of least squares[45] and the fluorescence value at infinite protein concentration was determined from the equation of the ensuing straight line.

The variations in the intercepts of these plots at infinite protein concentration represents a difference in quantum yield of bound ANS[46]. Intercept values increase with increasing concentrations of ANS but not in proportion to the ratio of ANS concentrations as found by DiAugustine *et al.*[27] with rat hepatic microsomes in the 5–20 $\mu$M ANS range.

Fig. 4 shows that the straight line fits of $P_0/xD_0$ *vs* $[(1-x)D_0']^{-1}$ plotted by the method of least squares using the data of Fig. 3 are in agreement with Eqn 1. By determining the value of $P_0/xD_0$ when $[(1-x)D_0']^{-1}$ is equal to zero, one can solve Eqn 1 for $n$. With this value of $n$, the equation can now be solved for $\bar{K}_{app}$ since $P_0/xD_0$ can be determined for any non-zero value of $[(1-x)D_0']^{-1}$ from the straight line equation. In this manner, the values of $n$ and $\bar{K}_{app}$ were calculated for a number of experiments at various constant values of ANS, the results of which are summarized in Table II. Radda[44] in his review on the use of fluorescent probes discusses the effects responsible for fluorescence enhancement, as well as the validity and limitations of using fluorescent probes to measure $n$ and $\bar{K}_{app}$.

From the average of eight experiments, it was found that each mg of plasma membrane protein represented $1.03 \cdot 10^8$ cells ($\pm 10\%$). Using this value, the value of $n$, and Avogadro's constant, it was possible to calculate the number of ANS molecules bound to each macrophage (Table II).

Since we have shown it unlikely that ANS enters the cell at 50 $\mu$M ANS concentration, we can consider the binding values obtained for 15 $\mu$M ANS (Table II) being representative of binding mainly to plasma membrane. The value of $\bar{K}_{app}$ at this ANS concentration is of the same order of magnitude as $\bar{K}_{app}$ values of 30–54 $\mu$M found for isolated erythrocyte membranes by several workers[11–14]. Their

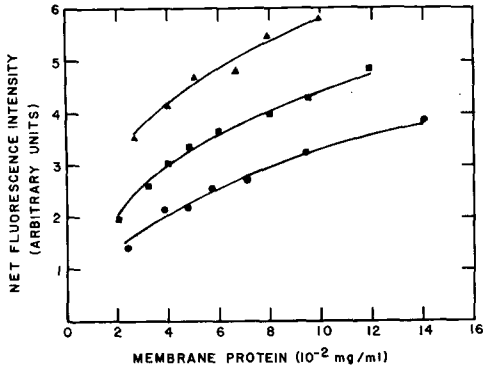
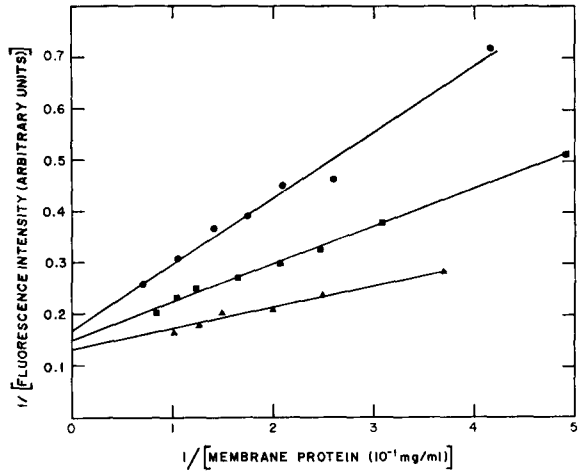



Fig. 2. Typical plots of fluorescence intensity *vs* plasma membrane protein concentration. The excitation wavelength was 396 nm and emission intensity was measured at 504 nm. Plasma membrane protein concentration was estimated to be 10% of total cell protein[39]. The concentrations of ANS used were: ●, 15 $\mu$M; ■ 60 $\mu$M; ▲, 90 $\mu$M.



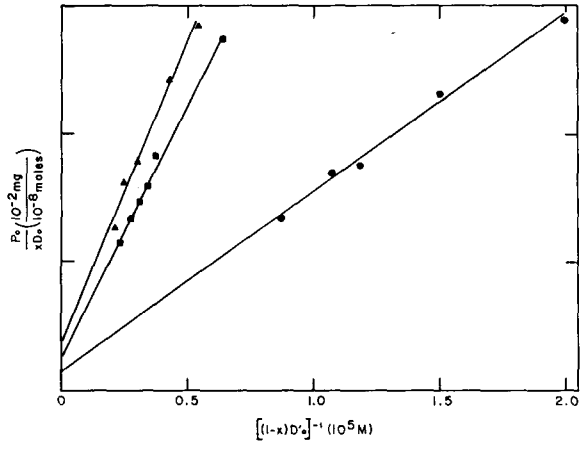




Fig. 3. Typical double-reciprocal plots of fluorescence intensity *vs* plasma membrane protein concentration using the data from Fig. 2. The concentrations of ANS used were: ●, 15 $\mu$M; ■, 60 $\mu$M; ▲, 90 $\mu$M.

Fig. 4. Data of Fig. 3 plotted according to Eqn 1. The concentrations of ANS used were: ●, 15 $\mu$M (ordinate units: 0–15); ■, 60 $\mu$M (ordinate units: 0–3); ▲, 90 $\mu$M (ordinate units: 0–1.5).

TABLE II

SUMMARY OF ANS BINDING PARAMETERS

Values for $n$ and $\bar{K}_{app}$ were calculated from data as in Fig. 4, according to Eqn (1). Means ± S.D. given in parentheses.

| *ANS concentration* ($\mu M$) | $n$ (*μmoles of ANS per mg of plasma membrane protein*) | $\bar{K}_{app}$ ($\mu M$) | *Number of molecules of ANS per macrophage* ($\times 10^{10}$) |
|---|---|---|---|
| 15 | 1.26 | 87.3 | 0.738 |
| | 1.29 | 57.5 | 0.756 |
| | (1.28 ± 0.02) | (72.4 ± 21.1) | (0.747 ± 0.120) |
| 60 | 4.63 | 235 | 2.71 |
| | 3.58 | 138 | 2.10 |
| | 3.85 | 253 | 2.26 |
| | (4.02 ± 0.55) | (209 ± 62) | (2.36 ± 0.32) |
| 90 | 4.81 | 153 | 2.82 |
| | 7.58 | 390 | 4.44 |
| | 5.18 | 120 | 3.03 |
| | (5.86 ± 1.50) | (221 ± 147) | (3.43 ± 0.88) |

values of $n$ varied from 15 to 76 nmoles/mg which averages about 50 times less than our value for pulmonary macrophages. This difference in $n$ can be explained in part by the fact that the plasma membranes involved in this study are metabolically active whereas inert isolated membranes were used in the erythrocyte studies. Functionally important components in the erythrocyte membrane may well have been lost during the isolation process and as a consequence the number of binding sites is reduced. Rubalcava *et al.*[12] roughly calculate that $1.05 \cdot 10^7$ molecules of ANS are bound to each erythrocyte. This is about 700 times less than found in this study. This dissimilarity is most likely due to differences in the number of binding sites and the surface area of macrophages as opposed to erythrocytes.

At higher ANS concentrations, interpretation of results in terms of binding solely to plasma membrane becomes tenuous. At 60 $\mu$M ANS, the value of $n$ rises by a factor of 4 and at 90 $\mu$M, $n$ is still rising, albeit slowly, whereas viability drops from 88% at 60 $\mu$M ANS to 60% at 90 $\mu$M ANS concentration. Between 60 $\mu$M and 90 $\mu$M ANS, $\bar{K}_{app}$ values are essentially the same but different by a factor of three from the value at 15 $\mu$M ANS. This indicates that similar binding is occurring at the higher ANS concentrations but different from that taking place at lower ANS concentrations. These findings are consistent with the concept that at 15 $\mu$M, binding is localized predominantly to plasma membrane sites whereas at the higher ANS concentrations, additional binding with intracellular protein occurs.

Although the question of how much the intracellular proteins are contributing to fluorescence enhancement has not been resolved, the probe technique can be a useful tool in studies with living cells. If one restricts the ANS concentration to less than 50 $\mu$M, it is reasonable to deal with binding values representative of binding to plasma membrane. At high ANS concentrations, it appears that one is dealing with binding to plasma membrane as well as intracellular proteins.

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