Odani, S., and Ikenaka, T. (1973b), *J. Biochem. (Tokyo) 74*, 857.
Odani, S., Koide, T., and Ikenaka, T. (1972), *J. Biochem. (Tokyo) 71*, 839.
Ruhlman, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973), *J. Mol. Biol. 77*, 417.
Schroeder, D. D., and Shaw, E. (1968), *J. Biol. Chem. 243*, 2943.
Seidl, D. S., and Liener, I. E. (1971), *Biochim. Biophys. Acta 251*, 83.
Seidl, D. S., and Liener, I. E. (1972a), *Biochim. Biophys. Acta 258*, 303.
Seidl, D. S., and Liener, I. E. (1972b), *J. Biol. Chem. 247*, 3533.
Stevens, F. C., and Feeney, R. E. (1963), *Biochemistry 2*, 1346.
Tanford, C. (1961), Physical Chemistry of Macromolecules, New York, N. Y., Wiley, p 465.
Tschesche, H. (1974), *Angew. Chem., Int. Ed. Engl. 13*, 10–28.

Negative Cooperativity in the Binding of Thyroxine to Human Serum Prealbumin†

Robert N. Ferguson, Harold Edelhoch,* Harry A. Saroff, and Jacob Robbins

with an addendum

Preparation of Tritium-Labeled 8-Anilino-1-naphthalenesulfonic Acid

Robert N. Ferguson and Hans J. Cahnmann*

ABSTRACT: The binding of thyroxine (T_4) and 8-anilino-1-naphthalenesulfonic acid (ANS) to human serum prealbumin was measured by equilibrium dialysis at pH 7.4 in 0.05 M phosphate–0.10 M NaCl at 25°. The data were analyzed for the binding constants based on equations for (1) two independent sites and (2) two identical sites with negative interaction. Evaluation by the independent site model gave the following association constants: for T_4 binding, $K_{T1} = 1.0 \times 10^8\ M^{-1}$, $K_{T2} = 9.5 \times 10^5\ M^{-1}$; for ANS binding, $K_{A1} = 9.5 \times 10^5\ M^{-1}$, $K_{A2} = 2.1 \times 10^5\ M^{-1}$. The interactive model gave constants $k_T = 5.5 \times 10^7\ M^{-1}$ and $k_A = 5.5 \times 10^5\ M^{-1}$. Interaction factors, α, defined such that $-RT \ln \alpha$ is the energy of interaction, were: $\alpha_T = 0.041$ and $\alpha_A = 0.62$ for T_4 and ANS, respectively. The "best fit" values for the number of sites were 2.0 and 1.6 for T_4 and ANS, respectively. The binding of T_4 to human prealbumin was competitive with ANS, and the binding constants evaluated from competition experiments were in agreement with those found for each ligand when studied separately. On the basis of analysis of X-ray data of human prealbumin (Blake *et al.*) there appear to be two identical T_4 sites. It is therefore evident that the binding of T_4 represents a case of negative cooperativity which is presumably due to interaction between ligands.

Interest in thyroxine binding to human serum prealbumin has been stimulated by recent studies on the structure of this protein. PA[1] is a very stable tetramer of identical subunits (Branch *et al.*, 1971b; Morgan *et al.*, 1971; Gonzales and Offord, 1971) with a twofold axis of symmetry and a channel through the center of the molecule formed by the subunits (Blake *et al.*, 1971, 1974). Although there appear to be four binding sites for retinol-binding protein (through which PA participates in vitamin A transport) (Van Jaarsveld *et al.*, 1973), recent reports have described only a single site for thyroxine (Pages *et al.*, 1973; Raz and Goodman, 1969). Nilsson and Peterson (1971) found three low affinity sites for T_4 in addition to one strong site, but the method used did not permit an accurate evaluation of the binding parameters.

Since PA exhibits twofold symmetry, it is somewhat unexpected to find only a single site for T_4 binding. In our previous work (Pages *et al.*, 1973) we found that various T_4 analogs were apparently bound to either one or two high affinity PA sites. Working with higher concentrations of T_4 we have now found a second, much weaker binding site for T_4. In preliminary studies (Branch *et al.*, 1971a) we observed that the fluorescence probe, 8-anilino-1-naphthalenesulfonic acid, was bound to two equivalent sites and that ANS fluorescence was quenched by T_4 in a manner suggesting competition for the same site or sites. Due to the complexity of the T_4–ANS competition a more direct method of measuring this interaction was necessary; therefore, the equilibrium dialysis technique was applied to a study of [^{3}H]ANS binding and also to the T_4–ANS interaction. The results reported herein are compatible with the presence of two sites on PA for both T_4 and ANS. The constants for the interaction of T_4 with PA based on two models are evalu-

† From the Clinical Endocrinology Branch and the Laboratory of Biophysical Chemistry, National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received June 17, 1974*.

[1] Abbreviations used are: PA, serum prealbumin; T_4, L-thyroxine; T_3, 3,5,3′-triiodo-L-thyronine; ANS, 8-anilino-1-naphthalenesulfonic acid; Mg-ANS and NH_4-ANS, magnesium and ammonium salt of ANS, respectively; DNS-glycine, 1-dimethylaminonaphthalene-5-sulfonylglycine.

ated: (1) binding to two independent sites or (2) binding to two identical sites with negative cooperativity.

Methods and Materials

Prealbumin. The human prealbumin was obtained from Behring Diagnostics and was purified by preparative polyacrylamide gel electrophoresis as described previously (Branch *et al.*, 1971b) yielding protein migrating as a single band with a higher mobility than albumin. Prealbumin concentrations were determined spectrophotometrically at 280 nm ($E_{1\,cm}(1\%) = 14.1$) (Raz and Goodman, 1969). All the dialysis data were obtained on a single preparation of prealbumin. Differences in the number of ligands bound, therefore, cannot be attributed to differences in the quality of prealbumin.

L-*Thyroxine.* The sodium salt of T_4 was obtained from Calbiochem. Purity was checked by thin-layer chromatography (tlc) on silica gel (Ogawara and Cahnmann, 1972) and by gas-liquid partition chromatography (Funakoshi and Cahnmann, 1969). The concentration of T_4 was determined from its absorption in 0.01 M NaOH using a molar extinction coefficient at 325 nm (in 0.01 M NaOH) of 6180 (Edelhoch, 1962). [^{125}I]T_4 (in 50% propylene glycol) was purchased from Abbott Laboratories. Chemical and radiochemical purity was checked by tlc and autoradiography. Iodide was usually the only contaminant and was removed immediately before use by ion exchange chromatography on AG50W-X12 (Bio-Rad).

Equilibrium Dialysis. The method used was a modification of the procedure described by Raz and Goodman (1969). Dialysis bags were prepared from No. 20 cellulose dialysis tubing (Union Carbide) which had been boiled for 30 min in 0.2 M Na_2CO_3–1 mM EDTA and then washed extensively with distilled water. The bags were stored in 50% v/v ethanol–water. The prepared bags were filled with 1.5 ml of the protein solution, and placed in cellulose nitrate tubes (Beckman No. 302236) containing 5 ml of the buffer and ligand. The tubes were stoppered, placed upright in a horizontal shaker, and agitated for 20–24 hr at room temperature (25 ± 1°).

Preliminary experiments were performed both with and without protein and showed equilibrium of T_4 and ANS under these conditions. Adsorption of ANS to either the tubing or cellulose nitrate tubes was negligible as shown by recovery of 95–102% of added radioactivity. Recovery of T_4 was also excellent at lower concentrations but fell to 85–95% above 3 μM T_4 because of low solubility at pH 7.4. Since most data were obtained at lower T_4 concentrations the error from this source was relatively small and was not used in corrections for recovery. In the T_4 experiments a correction was made for radioactivity present as $^{125}I^-$ at the end of the dialysis. The method of Sterling and Brenner (1966) was used and gave 1–4% contamination by $^{125}I^-$. The assumption was made that there was no appreciable binding of I^- to prealbumin. With both ANS and T_4 it was shown that the same equilibrium distribution was obtained whether the ligand was originally inside or outside of the bag.

After dialysis, 1-ml aliquots of both inside and outside solutions were removed for counting. [^{125}I]T_4 samples were counted in a well-type scintillation counter; 1 ml of [^{3}H]ANS samples was added to 10 ml of Aquasol (New England Nuclear) and counted in a liquid scintillation counter. Response was linear over the ANS concentrations used in these experiments and no correction was required for difference in counting samples in the presence of prealbumin (~1 μM). Calculations were carried out as described by Raz and Goodman (1969). In the competitive binding experiments, labeled T_4 and unlabeled ANS were used.

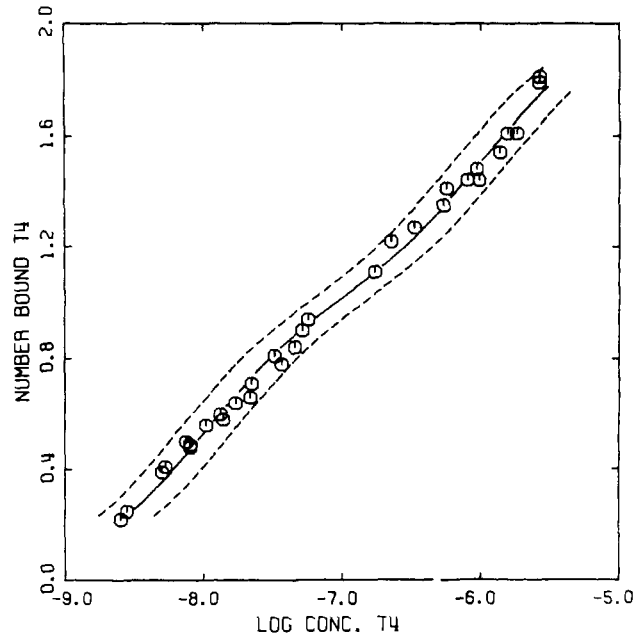

FIGURE 1: Binding of [^{125}I]thyroxine to human serum prealbumin. Concentration of PA, 0.50–0.94 μM; (—) Calculated from eq 1 with constants of Table I; (- - -) calculated to show effects of ±0.2 on values of log K_{T1} and log K_{T2} of Table I. The abscissa (log concentration T_4) is log c_T and the ordinate (number bound T_4) is $\bar{\nu}_T$.

Buffer. The buffer used for all of the experiments was 0.05 M potassium phosphate–0.1 M NaCl–1 mM Na_2EDTA, adjusted to pH 7.4.

Results

Binding of T_4 to Prealbumin. Two molecules of T_4 bind to PA at physiological pH in the concentration range of free T_4 varying from 2.5×10^{-9} to 2.5×10^{-6} M (Figure 1). The upper concentration limit is close to the solubility of T_4 in aqueous solutions at neutral pH. The binding constants of T_4 were determined by the method of least squares[2] for a model containing two independent sites according to

$$\bar{\nu}_T = \frac{(n_T/2)K_{T1}c_T}{1 + K_{T1}c_T} + \frac{(n_T/2)K_{T2}c_T}{1 + K_{T2}c_T} \qquad (1)$$

where K_{T1} and K_{T2} are the apparent association constants for the binding of T_4 to each site, c_T is the molar concentration of T_4 free in solution, n_T is the apparent number of binding sites, and $\bar{\nu}_T$ is the molar ratio of bound T_4 to total PA. The data are presented as $\bar{\nu}_T$ *vs.* log c_T in Figure 1. The values of the apparent constants determined by the method of least squares are listed in Table I. In order to display the difference in binding constants in the more conventional form, a Scatchard plot is shown in Figure 2.

Figure 1 also shows two dashed curves which are estimated error boundaries for the data and which reflect an assigned uncertainty of ±0.2 log unit in K_{T1} and K_{T2}. n_T is set at the value shown in Table I, *i.e.*, 2.04. It should be noted that the value of n_T becomes an important parameter in later analysis, as will be seen in the discussion of the error in n_T in the combined fit model.

[2] All references to "best fit" refer to the method of data analysis by least squares.

Table I: Constants for the Binding of T_4 and ANS to Prealbumin.

T_4 (31 points)		Ans (40 points)		
		Independent Sites		a
Log K_{T1}	8.02 M^{-1}	Log K_{A1}	5.98 M^{-1}	5.85 M^{-1}
Log K_{T2}	5.98 M^{-1}	Log K_{A2}	5.32 M^{-1}	5.26 M^{-1}
n_T	2.04	n_A	1.64	2.00
$\Sigma\Delta^2$ [b]	0.043	$\Sigma\Delta^2$	0.049	0.048
		Interactive Model		
Log k_T	7.74 M^{-1}	Log k_A	5.74 M^{-1}	5.65 M^{-1}
α_T	0.041	α_A	0.619	0.681
n_T	1.99	n_A	1.64	2.00
$\Sigma\Delta^2$	0.042	$\Sigma\Delta^2$	0.048	0.073

[a] Value of binding constants when n is fixed at 2.00 with a correction factor for concentration of ANS equal to 1.23. [b] The symbol $\Sigma\Delta^2$ is the sum of the squares of the differences between the experimental and calculated values.

Table II: Relative Concentrations of Various Species for Interactive Model for the Binding of T_4 to Prealbumin.

Species	Relative Concn
Zero T_4 bound	1
One T_4 bound	$2k_Tc_T$
Two T_4 bound	$k_T^2c_T^2\alpha$

The difference between the constants for T_4 is a factor of about 100. Since PA is a tetramer of identical subunits it is reasonable to assume that the two binding sites could be equivalent and that the difference in binding results from an interaction between the two molecules of T_4 when bound to PA. If an interaction factor α is defined such that $-RT$ ln α is the energy of interaction then the various species may be defined as listed in Table II where the symbol k_T is the constant for the binding of T_4 to one of the two equivalent sites. These species then give the eq 2[3] for the binding of T_4 to PA.[4]

$$\bar{\nu}_T = \frac{n_T k_T c_T (1 + k_T c_T \alpha_T)}{1 + 2k_T c_T + k_T^2 c_T^2 \alpha_T} \quad (2)$$

Binding of ANS to Prealbumin. The data for the binding of ANS to prealbumin are illustrated in Figures 2 and 3. These data are also interpreted to give a maximum of two molecules of ANS bound per tetramer of PA in the region of free ANS concentration varying from 2.0×10^{-7} to 5×10^{-5} M. When the data were treated in the same way as those for T_4, the two apparent constants listed in Table I, *i.e.*, 9.55×10^5 and 2.09×10^5 M^{-1}, were obtained. The value of the fitting parameter n_A evaluated by the curve fitting procedure was 1.64. The best fit and estimated error boundary curves, constructed for the ANS data in the same manner as for the T_4 data, are shown in Figure 3. Analysis

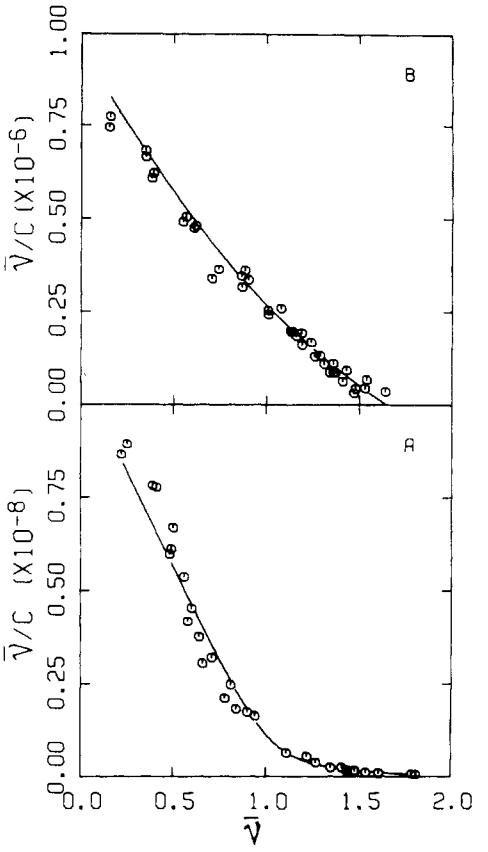

FIGURE 2: Scatchard plot of (A) the data in Figure 1 and (B) the data in Figure 3. The lines are the calculated curves from the constants in Table I.

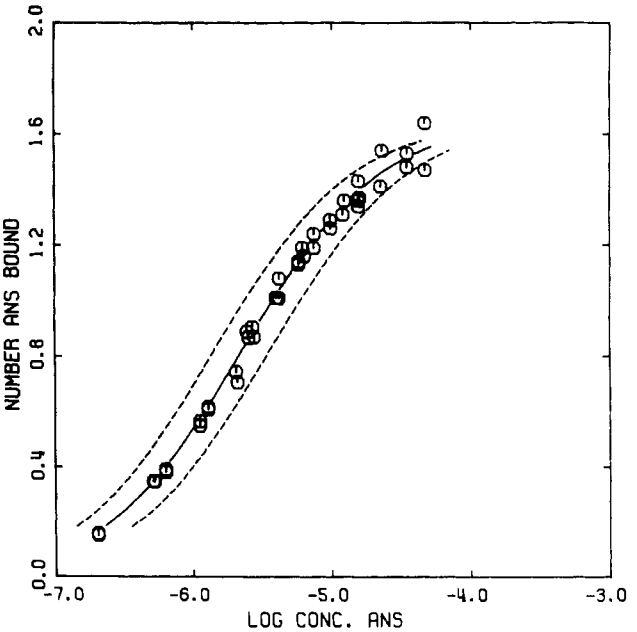

FIGURE 3: Binding of [^{3}H]ANS to human serum prealbumin. Concentration of PA, 1.7-7.3 μM. (—) Calculated from eq 1 with constants shown in Table I. (- - -) calculated to show effects of ±0.2 on values of log K_{A1} and log K_{A2} of Table I.

[3] There are three basically different ways of applying an interactive model to achieve a good fit to the data (Saroff and Yap, 1972). These are: (1) an unperturbed constant for the binding of the first ligand (K_1) where the equation is as given in the text; (2) an unperturbed constant for the binding of the last ligand where the equation is

$$\bar{\nu}_T = \frac{n_T k_T c_T \alpha_T (1 + k_T c_T)}{1 + 2k_T c_T \alpha_T + k_T^2 c_T^2 \alpha}$$

and (3) an unperturbed apparent ligand affinity where the equation is

$$\bar{\nu}_T = \frac{n_T k_T c_T (\alpha_T + k_T c_T)}{1 + 2k_T c_T \alpha_T + k_T^2 c_T^2}$$

The first choice is considered most reasonable because of the likelihood of ligand-ligand interactions as discussed in the text.

[4] Though eq 1 and 2 are based on completely different models they cannot be used to analyze the data in order to distinguish between the two models since they are mathematically equivalent, *i.e.*, $K_2 = k_T[1 - (1 - \alpha)^{1/2}]$, $K_1 = k_T[1 + (1 - \alpha)^{1/2}]$ when $n = 2$.

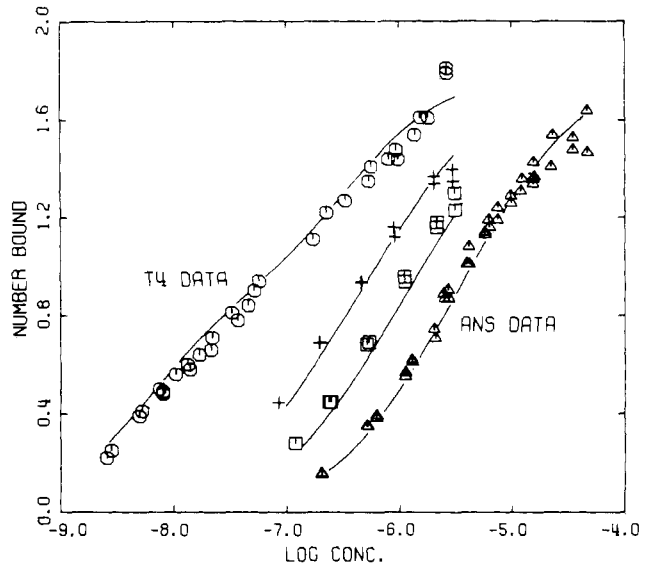

FIGURE 4: Data for the competitive binding of [^{125}I]T_4 and unlabeled ANS to prealbumin. Concentration of PA, 0.99–1.6 μM. (○) Binding of T_4 to PA; (+) binding of T_4 to PA when total concentration of ANS = 2.88×10^{-5} M; (□) binding of T_4 to PA when total concentration of ANS = 7.74×10^{-5} M. Abscissa for these three sets of data is the log concentration of free T_4. (△) binding of ANS to PA, log concentration refers to free ANS. All curves calculated from eq 3 with constants of Table IV, part IA.

of the data by the interactive model gave $k_A = 5.50 \times 10^5$ and $\alpha_A = 0.619$. Whereas the data for the binding of T_4 to PA show a marked difference in the constants for the two ligands ($K_{T1}/K_{T2} = 110$), those of ANS show only a small difference in the two constants (the ratio of $K_{A1}/K_{A2} = 4.6$). Equation 1 is expressed so that identical sites would give the same value for K_1 and K_2.

Competitive Binding of T_4 and ANS. The data presented so far have shown that both T_4 and ANS bind to two sites on PA. This binding was clearly shown to be competitive by dialysis experiments with both ligands present. These experiments showed the expected displacement of the T_4 binding curve due to the concentration-dependent competition of ANS for the T_4 sites on PA. The same conclusion was drawn from data derived from fluorescence and absorption experiments (to be published elsewhere[5]). Furthermore, [^{3}H]ANS was shown to be largely displaced from PA by T_4 where the initial concentrations of ANS gave $\bar{\nu}_A > 1$, indicating competition at both sites. Consequently, we have developed models based on the competitive nature of the ligand binding to PA which quantitatively describe the system. The equation for the competitive binding of T_4 may be formulated by considering the relative concentrations of the various species listed in Table III. When the simultaneous binding of T_4 and ANS to their respective strong or weak sites is not allowed (Table III), the binding of T_4 to PA is given in

$$\bar{\nu}_T = \frac{(n/2)(K_{T1}c_T + K_{T2}c_T + K_{T1}c_TK_{A2}c_A + K_{T2}c_TK_{A1}c_A) + nK_{T2}c_T^2}{1 + K_{T1}c_T + K_{T2}c_T + K_{T1}K_{T2}c_T^2 + K_{A1}c_A + K_{A2}c_A + K_{A1}K_{A2}c_A^2 + K_{T1}c_TK_{A2}c_A + K_{T2}c_TK_{A1}c_A} \quad (3)$$

[5] Unpublished data by the authors.

Table III: Relative Concentrations of Species for the Simultaneous Binding of T_4 and ANS to Prealbumin.

Species	Relative Concn
Zero binding	1
1 T_4 on site 1	$K_{T1}c_T$
1 T_4 on site 2	$K_{T2}c_T$
2 T_4	$K_{T1}K_{T2}c_T^2$
1 ANS on site 1	$K_{A1}c_A$
1 ANS on site 2	$K_{A2}c_A$
2 ANS	$K_{A1}K_{A2}c_A^2$
1 T_4 on site 1, 1 ANS on site 2	$K_{T1}c_TK_{A2}c_A$
1 T_4 on site 2, 1 ANS on site 1	$K_{T2}c_TK_{A1}c_A$
1 T_4 on site 1, 1 ANS on site 1	Not allowed
1 T_4 on site 2, 1 ANS on site 2	Not allowed

Table IV: Competitive Binding of T_4 and ANS.[a]

	Log K_{T1} (M^{-1})	Log K_{T2} (M^{-1})	Log K_{A1} (M^{-1})	Log K_{A2} (M^{-1})	n	$\Sigma\Delta^2$ (93 points)
I. Independent Sites						
A. Uncorrected for ANS concentration	8.23	6.44	5.92	5.06	1.79	0.287
B. Corrected for ANS concentration	8.10	6.00	5.69	5.18	2.1[b]	0.453

	Log k_T (M^{-1})	α_T	Log k_A (M^{-1})	α_A	α_{AT}	n	$\Sigma\Delta^2$ (93 points)
II. Interactive Model							
A. Uncorrected for ANS concentration	7.87	0.054	5.71	0.27	0.26	1.88	0.284
B. Corrected for ANS concentration	7.79	0.032	5.60	0.55	0.56	2.1[b]	0.539

[a] Data collected at two concentrations of ANS: 7.74×10^{-5} and 2.88×10^{-5} M. [b] Maximum allowed value.

This equation was applied to the following data simultaneously to give the best fit: (a) T_4 binding data, $c_A = 0$; (b) ANS binding data, $c_T = 0$; (c) competitive binding data when $c_A = 7.74 \times 10^{-5}$ M and $c_A = 2.88 \times 10^{-5}$ M. Since $c_A \gg$ PA, the concentration of free ANS was considered to be constant. The results of this fit to the combined data are given in Figure 4 and Table IV, part IA, where it can be seen that the values for the four association constants are similar to the values in Table I. The best value for n in the combined data analysis is 1.79. When eq 3 was evaluated with the terms $K_{T1}c_TK_{A2}c_A$ and $K_{T2}c_TK_{A1}c_A$ replaced by those not allowed in Table III, the model did not fit the data.

Table IV, part IIA, includes the constants for the equation for two identical and interacting sites for both T_4 and

ANS binding. With this interpretation of the data an added factor, α_{AT}, for the interaction between the two ligands, T_4 and ANS, is required. The interactive equation for the binding of T_4 and ANS is

$$\bar{\nu}_T = \frac{(n/2)(2k_T c_T + 2k_T c_T k_A c_A \alpha_{AT}) + n k_T^2 c_T^2 \alpha_T}{1 + 2k_T c_T + k_T^2 c_T^2 \alpha_T + 2k_A c_A + k_A^2 c_A^2 \alpha_A + 2k_T c_T k_A c_A \alpha_{AT}} \quad (4)$$

The value of $\alpha_{AT} = 0.26$ is reasonable for this model. However, the drop in α_A from 0.62 (virtually no interaction) for the binding of ANS to PA in the absence of T_4 (Table I), to 0.27 in the presence of T_4 (Table IV, part IIA) requires some explanation. This drop appears to be related to the distortion of the binding curve introduced by variation in the apparent number bound, n, discussed below.

Error Analysis. There is no apparent reason for the difference in the parameter n for the two ligands, 2.04 and 1.64 for T_4 and ANS, respectively (Table I). Since the value of T_4 was close to 2, one assumption was that an error in ANS concentration was the reason for the low ANS value. However, when the ANS concentration was adjusted to give $n = 2.0$ by introducing another parameter reflecting a constant error in ANS concentration, no improvement in the fit of the data to either eq 1 or 2 was found since $\Sigma\Delta^2$ remained the same or became larger (Table I).

In the case of the fit to the competitive data with both models (Table IV, parts IA and IIA) the values found for n were 1.79 and 1.88. These values constitute a significant shift from those obtained for the independent data (Table I) where $n_T \simeq 2.00$ and $n_A = 1.64$. This makes it difficult to compare the values for the association constants and interaction terms, α, derived from the data uncorrected for ANS concentration in Tables I and IV. When ANS concentration was adjusted as described in the preceding paragraph and n was fixed at 2.0 ± 0.1, a second set of parameters was obtained (Table IV, parts IB and IIB). The fits to the data are not as good as those with the concentration of ANS remaining uncorrected since $\Sigma\Delta^2$ is now larger. However the value of α_A is now closer to that derived from the uncorrected data for ANS alone (0.55 in Table IV, part IIB compared to 0.62 in Table I).

Discussion

In a recent study of the binding characteristics of PA we have reported (Pages *et al.*, 1973) that two molecules of 4-hydroxy-3,5-diiodobenzaldehyde are bound whereas only one molecule appeared to be bound for each of the following compounds: T_4, T_3, 3,5-diiodo-3′,5′-dinitro-L-thyronine, 3-(4-hydroxy-3,5-diiodophenyl)propionic acid, and 4-hydroxy-3,5-diiodocinnamic acid. All the iodinated analogs had binding constants similar to that of T_4, including the aldehyde analog where two molecules are bound with similar affinity, *i.e.*, 5.5×10^7 M^{-1}. Fluorescence studies with ANS and equilibrium dialysis with [^{3}H]DNS-glycine showed two sites for each molecule with similar binding constants. The results obtained with ANS by fluorescence[5] have been confirmed by equilibrium dialysis. In the present study, [^{3}H]ANS was used and gave an affinity constant somewhat greater than that derived from fluorescence measurements. The binding constant[5] of [^{3}H]DNS-glycine was 3.0×10^5 M^{-1} and n was equal to 2.0.

The binding of ANS was observed to be competitive with T_4 since the fluorescence of the bound ANS could be eliminated by T_4 (Branch *et al.*, 1971a). Attempts to analyze the fluorescence data based on competition between two binding sites for ANS and one for T_4 were unsuccessful. It has now been found, however, using greater concentrations of T_4 than previously, that T_4 binds to a second site, although with a much reduced affinity. The competition between T_4 and ANS has now been evaluated directly by equilibrium dialysis.

Recent information concerning the structure of PA helps to clarify the ligand binding studies. It is almost certain that the four subunits (M_r 13,500) have the same amino acid sequence (Morgan *et al.*, 1971; Gonzales and Offord, 1971). The three-dimensional structure of PA has also been largely resolved by the work of Blake and colleagues (1971, 1974). Circular dichroic spectra (Branch *et al.*, 1971b) reveal a large content of β structure which appears from the X-ray studies to form a considerable part of the surface of the subunits. According to Blake *et al.* the tetramer is formed by rotating the dimer about the horizontal twofold axis. The interface between the two dimers then forms a channel which contains the binding sites. It is of interest that the second site of T_4 binding was independently found by binding studies and predicted by X-ray studies by Blake *et al.* (personal communication). Thus, with a depiction of the binding sites by the X-ray work, an explanation of the variable number of binding sites for different ligands can be attempted.

The second site for T_4 with its lower association constant has, therefore, a free energy of binding 2.8 kcal higher than that of the first site. The shape of the binding curve may be defined by K_{T2}/K_{T1} which is about 0.01 (negative cooperativity). The interaction energy by the model presented above is $-RT \ln \alpha$ or 1.9 kcal. It is reasonable to assume that a second site with a much lower affinity also exists for T_3 and the other analogs and we have verified this for the case of T_3 by equilibrium dialysis.[5] It is of interest in this connection to note that 4-hydroxy-3,5-diiodobenzaldehyde, which binds to two high affinity sites, differs from 3-(4-hydroxy-3,5-diiodophenyl)propionic acid and 4-hydroxy-3,5-diiodocinnamic acid, which bind to only one high affinity site, in the size of the side chain on the diiodophenol ring. If the side chain is situated in the interior of the channel and the phenolic hydroxyl on the outside, as seems likely since the apparent pK_i values for T_4 and T_3 are not very different from those found in their free states (Nilsson and Peterson, 1971), then there may not be enough space for both side chains in the cases of the propionic and cinnamic acid analogs. Alternatively, the negative charge of the carboxyl group could inhibit the binding of the second ligand by electrostatic effects without requiring actual coverage by one ligand of part of the second site. In the case that the steric overlap or electrostatic interaction is marked, the binding of the second ligand may be so weak that it is not observable in the concentration range of ligand allowable by its solubility.

The binding constants for T_4 and ANS have therefore been determined by two models, referred to as the "independent sites" and "interactive" models. The two models cannot be distinguished by analysis of the data since they are formally equivalent.[4] However, a distinction between the two models may be made by considering additional data. Thus, two molecules of 4-hydroxy-3,5-diiodobenzaldehyde bind to PA with similar and high affinity and two molecules of both ANS and DNS-glycine bind with ap-

proximately equal but with much lower affinities. These data and those from the X-ray results make it reasonable to consider that the difference in the binding energies of T_4 result from an interaction either between two ligands (steric or electrostatic) or between the two sites (conformational).

It is difficult to distinguish between ligand-ligand and site-site interactions in the absence of X-ray data which preclude the former. Subunit interactions are usually invoked in the case of hemoglobin because the heme groups are isolated from each other by the globin molecules and cannot interact directly by contact (Perutz, 1970). Site-site interactions through a ligand-induced conformational change have been invoked to explain "half-of-the-site" reactivity of numerous multisubunit enzymes (Lazdunski, 1972). Site interactions have also been suggested as a mechanism to explain the binding of insulin to its weaker membrane-receptor binding sites (De Meyts *et al.*, 1973).

It is intrinsically very difficult if not impossible to rule out a conformational effect, since it could be very small and involve only a very limited number of residues. There are two considerations which could be cited against a conformational control of binding. The first is that two molecules of 4-hydroxy-3,5-diiodobenzaldehyde, which is a T_4 analog, are bound almost with the same affinity as the first T_4. The second is less direct and relates to the stability of PA. It has been shown (Branch *et al.*, 1972) that at neutral pH, PA is one of the most stable proteins known since it resists denaturation (and dissociation) in 8 M urea–0.10 M sodium dodecyl sulfate, and concentrations of guanidinium hydrochloride close to 6 M. Thus, it seems unlikely that site-site interactions explain the complexity of binding of T_4 and its analogs to PA.

Addendum: *Preparation of Tritium-Labeled 8-Anilino-1-naphthalenesulfonic Acid*

In contrast to unlabeled ANS which has been used in previous dialysis experiments (Stryer, 1965; Brand *et al.*, 1967) radioactive ANS gives precise and accurate data over a wide concentration range. However, it is not commercially available and the only literature procedure for its preparation (Parker and Osterland, 1970) yields an unpure product of low specific activity.

The present work describes a simple method for the preparation of chemically and radiochemically pure [^{3}H]ANS of high specific activity. Other than normal laboratory equipment all that is required is a simple glass apparatus connected to a diffusion pump which can produce a vacuum of 10^{-4} Torr.

Materials and Methods

Mg-ANS[1] (Eastman Kodak, Rochester, N.Y.) was 81.5% pure based on its absorption at 351 nm (see Results); NH_4-ANS (Pierce Chemical Co., Rockford, Ill.) was 93.0% pure by the same criterion. ^3H_2O (10 mCi/μl) was obtained from New England Nuclear, Boston, Mass; Platinum Black (fuel cell grade) was from Engelhard Industries, Newark, N.J.; basic aluminum oxide (alumina catalyst AL-0109-P) was from Harshaw Chemical Co., Cleveland, Ohio; the cation exchange resin AG50W-X4, 30-35, μ, 1.2 mequiv/ml resin bed, from Bio-Rad Laboratories, Richmond, Calif.

Thin-Layer Chromatography. Solvent 1, toluene–methanol–acetic acid (8:1:1), freshly prepared for each run; solvent 2, upper phase of ethyl acetate–methanol–0.1 M NH_4OH (5:2:3). Silica gel plates (Q1 and Q1-F) were obtained from Quantum Industries, Fairfield, N.J. Chromatograms were sprayed with a mixture of 470 ml of toluene, 40 ml of 95% ethanol, and 40 ml of Liquifluor (New England Nuclear). The plates were dried and exposed to Kodak X-ray film SB-54 at −20 to −30°.

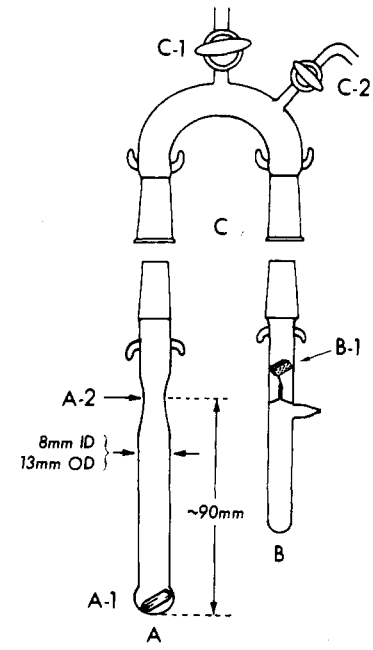

FIGURE 5: Glass apparatus for preparation of exchange reaction. (A) Reaction vessel containing a magnetic stirring bar A-1; (B) sealed ^3H_2O container; (B-1) magnetic bar; (C) adapter to connect A with B and both to a vacuum line (at C-1); C-2 is an air inlet.

Labeling Procedure. Since ANS is somewhat light-sensitive,[6] the entire procedure was carried out in the dark or in subdued light. The crude Mg-ANS (104 mg) and Pt Black (150 mg) were placed in the bulb of the reaction vessel A (Figure 5). A constriction was then made at A-2 and 250 μl of 20 mM ethanolic NaOH, prepared by mixing 1 volume of 1 M NaOH and 49 volumes of absolute ethanol, was added. Both A and B, the latter containing 200 mCi of ^3H_2O in a volume of 20 μl, were connected to C. The lower part of A was immersed in liquid nitrogen and the ^3H_2O was distilled into it at a vacuum of $<10^{-4}$ Torr using standard procedures. Oxygen remaining in the reaction mixture was removed by three successive freeze–thaw cycles. Finally, A was sealed at A-2 with a glassblower's torch at a vacuum of $<10^{-4}$ Torr.

The reaction mixture was magnetically stirred in an oil bath at 90° for 6 days. Initially it was semisolid, but soon turned into a green liquid (except for suspended Pt Black). After 6 days the original green color had assumed a slight yellowish tint. Stirring was continued without heating for another 6 days.

After cooling, the ampoule was opened and the reaction mixture, after dilution with ~10 ml of cold methanol, was centrifuged (3 min at 6000 rpm). The clear supernatant was evaporated and easily exchangeable tritium in the residue removed by two dissolution–evaporation steps using 0.8% acetic acid in methanol followed by one step using methanol as solvent. All solutions were permitted to stand for 0.5–1 hr prior to evaporation.

[6] When a 1.6×10^{-4} M aqueous solution of ANS was kept for 6 weeks at room temperature in normal laboratory light, its absorbance at 351 nm decreased by 9.9%. A similar solution kept in the dark lost only 0.3% of its original absorbance.

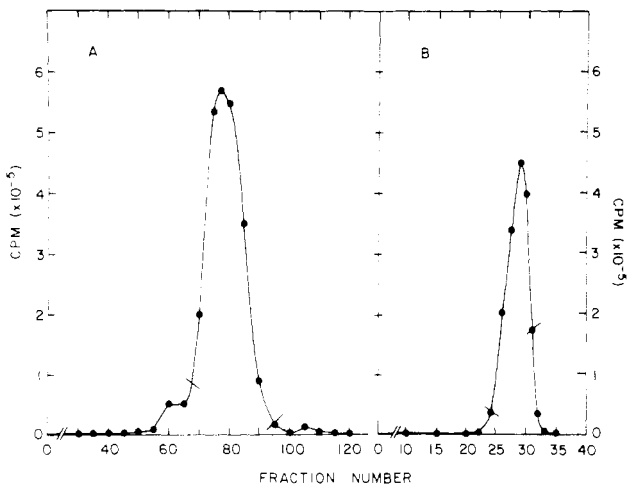

FIGURE 6: Elution profiles of [^{3}H]ANS. Adsorbent and eluent, see Materials and Methods. (A) Column 1: flow rate, 23 ml/hr; 2.3-ml fractions. (B) Column 2: flow rate, 12 ml/hr; 3.5-ml fractions. The slash lines indicate the limit of the pooled fractions.

Purification of [^{3}*H*]*ANS.* The crude [^{3}H]ANS was purified by two successive column chromatographic separations on activated alumina which had been thoroughly washed with the eluent (solvent 2 for thin-layer chromatography; contamination with its lower phase should be avoided). The eluate fractions separated into two phases on standing. Before aliquots for tlc or for counting purposes were removed, 0.5–1 ml of methanol was added to obtain a single phase. Aliquots for counting of radioactivity were mixed with 10 ml of Aquasol (New England Nuclear).

The crude [^{3}H]ANS dissolved in 2 ml of eluent was applied to column 1 (2.2 × 160 cm, 525 g of alumina). The eluate fractions containing the bulk of the ANS were combined (Figure 6A) and evaporated. The residue was dissolved in 2 ml of methanol and 1.5 ml of this was removed and stored for future use. The remaining 0.5-ml aliquot was evaporated and applied in 0.5 ml of eluent to column 2 (1.6 × 78 cm, 135 g of alumina). The individual ANS fractions were evaluated by tlc and autoradiography. The pure fractions were combined (Figure 6B), evaporated, and dissolved in 5 ml of 95% ethanol. After a further purity check by spectrophotometry and determination of the specific activity the solution was stored in small sealed ampoules, in the dark, some at −30° and others at −196° (liquid nitrogen).

Unlabeled ANS. Pure unlabeled ANS was prepared as a reference standard. Crude NH_4-ANS (0.5 g) was dissolved in 10 ml of hot 95% ethanol. The solution was pumped through a column (0.9 × 15 cm) of freshly recycled AG50W-X4 (H^+ form). The jacketed column was poured and eluted with 95% ethanol at 70°. The ANS-containing eluate fraction (25 ml) was evaporated, the residue was crystallized from methanol, and the yellow needles were dried at room temperature in a vacuum desiccator.

Spectra. Absorption spectra were recorded with a Cary 14 spectrophotometer which was calibrated for absorbance with neutral density filters prior to use.

Results

Table V shows that the recovery of ANS in the final product was 77% and that 6.8% of the radioactivity used had been incorporated into [^{3}H]ANS. From the data given in Table V a specific activity of 64 Ci/mol is calculated for the pure [^{3}H]ANS.

Table V: Chemical and Radiochemical Yields of ANS.

Preparation Step	Activity (mCi)	Per Cent of Initial Activity	Amount (mg)	Per Cent of Initial Amount[a]
Crude Mg-ANS + ^3H_2O (before exchange)	200	100	81.8	100
Crude [^{3}H]ANS (after exchange)	21.9	11		
Pooled [^{3}H]ANS fractions (first column)	19.2	9.6		
Pure [^{3}H]ANS (after second column)[b]	13.5	6.8	63.2	77

[a] Amounts calculated as pure ANS. [b] A 25% aliquot of the pooled [^{3}H]ANS fractions from the first column was put on the second column (see text). Figures shown are adjusted to 100%.

The calculation of chemical and radioactive yields is based on the molar extinction coefficient of ANS in water at 351 nm. A value of 6240 was found using a pure reference standard. The purity of the standard was ascertained by elemental analysis and by tlc in both an acid and alkaline solvent. Values of C, H, and N were within 0.2% of theory. Tlc revealed no trace of impurity, even with large applications. The crystals of ANS melted sharply at 226–227° on the Kofler hot stage.

The ultraviolet absorption spectrum of ANS in water shows peaks at 351, 266, and 216 nm. The ratio of the extinction at these wavelengths is 1:3.1:7.9. The spectra of our [^{3}H]ANS and of the unlabeled reference compound were superimposable between 210 and 400 nm. Since any absorbing impurity would alter the extinction ratios, this is a good indication that the [^{3}H]ANS was also pure. Its purity was further checked by tlc and by autoradiography. No labeled or unlabeled impurity could be detected.

Before arriving at the labeling procedure described above (Materials and Methods) pilot experiments were carried out with ^3H_2O of low specific activity (1 mCi/g). These indicated that the solvent must be sufficiently alkaline to prevent excessive decomposition of ANS on heating. Decomposition of ANS in the course of the exchange reaction can be observed by a change of the color of the reaction mixture from green to brown. An exchange reaction should be terminated or the temperature lowered when the solution assumes a yellowish tint. However, if high specific activity is more important than high chemical yield, heating may be continued longer. In order to achieve a high degree of incorporation of radioactivity in ANS, a catalyst with a large surface area (25–35 m^2/g) was used, and all traces of oxygen, a catalyst poison, were removed by applying a vacuum of at least 10^{-4} Torr.

Discussion

The common methods for introduction of tritium into organic compounds include synthesis, reduction, exposure to tritium gas (Wilzbach method), exposure to tritium gas activated by a microwave discharge, and catalytic exchange with ^3H_2O or other tritiated compounds. Preliminary experiments and theoretical considerations led us to choose the latter method.

Catalytic exchange of deuterium of ^2H_2O with hydrogen of aromatic compounds has been investigated by Garnett

and his coworkers (Brown and Garnett, 1958). The procedure is also applicable to tritium exchange with ^3H_2O (Garnett, 1962). In the exchange procedure described in the present paper, Garnett's method has been improved (use of high vacuum) and simplified (omission of prereduction of the catalyst). In this manner a product of high specific activity is obtained in good yield.

An accurate value for the molar extinction coefficient for ANS is essential not only for the determination of the chemical yield and the specific activity of [^{3}H]ANS, but also for a valid interpretation of equilibrium dialysis data obtained with unlabeled ANS. However, various commercially available ANS salts showed differing values for ϵ_{350}. Also, literature values for ϵ_{350} vary widely: 4950 (Weber and Young, 1964), 5000 (Stryer, 1965), 5600 (Nagradova *et al.*, 1972), and 6000 (Dodd and Radda, 1969). We therefore prepared analytically pure ANS, whose ϵ_{351} = 6240 is higher than any previously reported value. It appears that previous preparations of ANS were not pure. This is supported by the finding that the recrystallization of Mg-ANS under various conditions results in a product which contains excess magnesium (Penzer, 1972). We also found that four recrystallizations of commercial Mg-ANS from water led to Mg-ANS with ϵ_{351} = ~6000 which still gave an incorrect elemental analysis. However, ion exchange conversion of NH_4-ANS to the free acid gave a product with an excellent elemental analysis after one crystallization from methanol.

In order to store [^{3}H]ANS for long periods of time (several years) we dissolved it in 95% ethanol which is known to prevent or minimize self-radiolysis of tritiated compounds (Bayly and Evans, 1966).

Acknowledgments

The authors thank William T. Branch for his preliminary work on T_4-ANS competition for PA binding which led to the experiments reported here and Robert A. Pages for assistance in the equilibrium and labeling experiments. A portion of the data on T_4 binding reported in this manuscript is taken from the work of Pages *et al.* (1973).

References

Bayly, R. J., and Evans, E. A. (1966), *J. Label. Compounds 2,* 1.

Blake, C. C. F., Geisow, M. J., Swan, I. D. A., Rerat, C., and Rerat, B. (1974), *J. Mol. Biol. 88,* 1.

Blake, C. C. F., Swan, I. D. A., Rerat, C., Berthou, J., Laurent, A., and Rerat, B. (1971), *J. Mol. Biol. 61,* 217.

Branch, W. T., Edelhoch, H., and Robbins, J. (1971a), *J. Clin. Invest. 50,* 11a.

Branch, W. T., Robbins, J., and Edelhoch, H. (1971b), *J. Biol. Chem. 246,* 6011.

Branch, W. T., Robbins, J., and Edelhoch, H. (1972), *Arch. Biochem. Biophys. 152,* 144.

Brand, L., Gohlke, J. R., and Sethu Rao, D. (1967), *Biochemistry 6,* 3510.

Brown, W. G., and Garnett, J. L. (1958), *J. Amer. Chem. Soc. 80,* 5272, and subsequent papers.

De Meyts, P., Roth, J., Neville, D. M., Gavin, J. R., and Lesniak, M. (1973), *Biochem. Biophys. Res. Commun. 55,* 154.

Dodd, G. H., and Radda, G. K. (1969), *Biochem. J. 114,* 407.

Edelhoch, H. (1962), *J. Biol. Chem. 237,* 2778.

Funakoshi, K., and Cahnmann, H. J. (1969), *Anal Biochem. 27,* 150.

Garnett, J. L. (1962), *Nucleonics 20, No. 12,* 86.

Gonzales, G., and Offord, R. E. (1971), *Biochem. J. 125,* 309.

Lazdunski, M. (1972), *Curr. Top. Cell. Regul. 6,* 267.

Morgan, F. J., Canfield, R. E., and Goodman, DeW. S. (1971), *Biochim. Biophys. Acta 236,* 798.

Nagradova, N. K., Asryants, R. A., and Ivanov, M. V. (1972), *Biochim. Biophys. Acta 268,* 622.

Nilsson, S. F., and Peterson, P. A. (1971), *J. Biol. Chem. 246,* 6098.

Ogawara, H., and Cahnmann, H. J. (1972), *Biochim. Biophys. Acta 257,* 328.

Pages, R. A., Robbins, J., and Edelhoch, H. (1973), *Biochemistry 12,* 2773.

Parker, C. W., and Osterland, C. K. (1970), *Biochemistry 9,* 1074.

Penzer, G. R. (1972), *Eur. J. Biochem. 25,* 218.

Perutz, M. (1970), *Nature (London) 228,* 726.

Raz, A., and Goodman, DeW. S. (1969), *J. Biol. Chem. 244,* 3230.

Saroff, H. A., and Yap, W. T. (1972), *Biopolymers 11,* 957.

Sterling, K., and Brenner, M. A. (1966), *J. Clin. Invest. 45,* 153.

Stryer, L. (1965), *J. Mol. Biol. 13,* 482.

Van Jaarsveld, P. P., Edelhoch, H., Goodman, DeW. S., and Robbins, J. (1973), *J. Biol. Chem. 248,* 4698.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239,* 1415.