Thyroxine-Binding Globulin: Characterization of the Binding Site with a Fluorescent Dye as a Probe

Abstract. *The fluorescent dye 1,8-anilinonaphthalenesulfonate competed with thyroxine for binding to thyroxine-binding globulin. Fluorescence analysis indicated that the dye bound to the globulin in a molar ratio of 1 : 1 and with an association constant (at 23°C) of* $4.19 \times 10^6 M^{-1}$, *and that thyroxine bound to the globulin in a molar ratio of 1 : 1 and with an association constant (at 23°C) of* $2.35 \times 10^{10} M^{-1}$. *Displacement of globulin-bound dye by thyroxine was shown by fluorescence quenching, and displacement of globulin-bound thyroxine by dye was demonstrated by ultrafiltration.*

Since the observation nearly two decades ago that thyroxine (T_4) avidly binds to a protein migrating electrophoretically in the region between α_1 and α_2 globulins, little progress has been made toward defining the nature of the interaction. The few reports on the purification of thyroxine-binding globulin (TBG) have provided quantitative data neither on the T_4 binding site nor on the process by which T_4 is bound (*1, 2*). Affinity chromatography with T_4-substituted Sepharose has been developed in this laboratory for the purification of TBG (*2*). This method has yielded a product which is pure as judged by disc gel electrophoresis, binds T_4, and has the same mobility as TBG in whole human serum.

The *N*-arylaminonaphthalenesulfonate dyes adsorb to functionally important sites on several proteins (*3, 4*). Because these fluorescent dyes undergo spectral changes when their environment is altered, they have been widely used as probes of the adsorbing regions of proteins. In particular, 1,8-anilinonaphthalenesulfonic acid (ANS) undergoes a marked increase in fluorescence quantum yield and a hypsochromic shift in emission maximum when it is transferred from an aqueous to a nonpolar solvent (*5*). Similar changes that occur when the dye is adsorbed to proteins have led to its use as a probe of hydrophobic sites on proteins. We report that ANS competes with T_4 for binding to TBG.

The TBG was prepared as reported (*2*). The molecular weight, as determined by ultracentrifugation and by polyacrylamide gel electrophoresis, was 63,000 (*6*). Titrations of ANS and T_4 were followed spectrofluorimetrically with an Aminco-Bowman spectrofluorometer equipped with cuvette temperature regulation and an *X-Y* recorder. Solutions of ANS and T_4 were added to the test solution in small (10 μl) portions. Relative fluorescence values were uniformly corrected for dilution and for attenuation of the exciting and emitted energy (*7*). Complete emission spectra were recorded at each observation. Except in determinations of quantum yield, all spectral readings were uncorrected for instrumental response. All assays were carried out at 23°C unless otherwise noted.

Figure 1 shows the uncorrected fluorescence emission and excitation spectra of free and of bound ANS. At the dye concentrations used, emission energy was not dependent on dye concentration. In the presence of TBG, the fluorescence of ANS was nearly 150 times that of ANS alone in aqueous solution at the same concentration. The quantum yield of ANS in aqueous solution has been reported as 0.004 (*4*). The quantum yield of the dye in the presence of TBG was calculated as 0.62 after the spectral recordings were corrected for photomultiplier response and the area under the fluorescence emission curve of ANS alone was compared with that of ANS in the presence of TBG.

The emission maximum of ANS, 525 nm (uncorrected), underwent a marked hypsochromic shift to 475 nm (uncorrected) when TBG was present (Fig. 1). The excitation spectrum for ANS in the presence of TBG showed an additional peak with a maximum at about 280 nm, the absorption maximum for the protein.

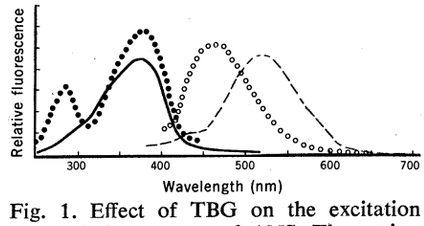

Fig. 1. Effect of TBG on the excitation and emission spectra of ANS. The excitation spectrum of ANS ($10^{-4}M$) (——) was recorded by emission at 520 nm, and that of ANS ($5.0 \times 10^{-7}M$) in the presence of TBG ($7.35 \times 10^{-7}M$) (●●●) was recorded by emission at 470 nm. Emission spectra of the same ANS (- - -) and ANS plus TBG (○○○) solutions were recorded in response to excitation at 375 nm. Protein solutions contained 0.05*M* tris(hydroxymethyl)aminomethane (tris) and 0.10*M* NaCl and were at *p*H 7.3. Spectra are uncorrected.

Excitation at 295 nm of TBG alone gave the expected protein fluorescence peak at about 340 nm. When ANS was added, this protein fluorescence peak was quenched, with the concomitant appearance of the characteristic ANS fluorescence maximum at 475 nm (uncorrected). This apparently reflects energy transfer from aromatic residues in the protein to the bound ligand. Thus, ANS was shown to bind to TBG by the criteria of quantum yield enhancement, shift of maximum emission, and energy transfer.

Figure 2A shows the change in relative fluorescence at 475 nm of a solution of TBG when ANS was added in small portions. A plateau is seen at a relative fluorescence value of 178. The failure of additional ANS to cause further enhancement of fluorescence apparently indicates saturation of the ANS binding sites of TBG. If the initial slope of the curve is extrapolated to this plateau, the intercept yields a saturation value N of 0.90 ± 0.03 mole of ANS per mole of TBG. Thus 1 mole of TBG binds 1 mole of ANS.

Extrapolation of the initial slope of the curve produced a line representing moles of bound ANS per mole of TBG. This line was used, in the region of significant dissociation of ligand and protein, to determine the amount of free ANS and the amount of bound ANS at any observed point. These values were then graphed according to the method of Scatchard (*8*) to yield an association constant (K_{assoc}) for the interaction of TBG and ANS at 23°C of $4.19 \pm 0.16 \times 10^6 M^{-1}$. The solid line in Fig. 2A is the theoretical binding curve calculated with this K_{assoc} value.

The experiment was repeated with the same conditions except that temperature was held at 37°C, and a K_{assoc} of $2.09 \pm 0.16 \times 10^6 M^{-1}$ was found. The enthalpy of the reaction, determined with a Van't Hoff plot, was −0.4 kcal/mole. The change in entropy was then calculated to be +28.8 eu at 23°C and +27.5 eu at 37°C. Thus, the reaction appears to be driven almost entirely by entropic energy, and the thermodynamic values suggest an almost purely hydrophobic association. This thermodynamic analysis supports the widespread assumption that ANS is a probe of hydrophobic sites.

The ANS was then added to a TBG

solution until the fluorescence reached a plateau, indicating a saturation of the dye-binding sites, and T_4 was added in small increments. The T_4 titration was monitored fluorimetrically by excitation at 400 nm, a wavelength at which TBG and T_4 show no appreciable absorption. The resulting relative fluorescence at 475 nm versus moles of added T_4 per mole of TBG is graphed in Fig. 2B. The T_4 appears to quench ANS fluorescence stoichiometrically. If the initial slope of the quenching curve is extrapolated to a relative fluorescence of 0, it is seen that 1 mole of bound T_4 per mole of TBG would completely quench ANS fluorescence. This supports the conclusion that ANS competes with T_4 and that there is one major binding site for T_4 on each TBG molecule. The low amount of residual fluorescence was assumed to result from an equilibrium between T_4 with a high binding affinity and ANS with a lower affinity. This assumption was tested by two experiments.

First, when excitation at 295 nm was measured in a solution of TBG in which the ANS binding sites were saturated, no additional quenching of intrinsic protein fluorescence was seen when T_4 was added to the TBG-ANS complex. If T_4 were binding to a site on TBG different from that of ANS, additional quenching of protein fluorescence would be expected. Therefore, T_4 must displace ANS as it binds. Optical rotatory dispersion spectra showed no detectable change when either T_4 or ANS, or both, were added to a solution of TBG. This observation is consistent with single-site competition rather than with an alternative model in which a major protein conformational change accompanies binding at widely separated sites.

Second, evidence for competition of the two ligands was obtained when an ultrafiltration cell (*6*) was used to determine directly the amount of T_4 bound to TBG in the presence of added ANS. In a typical experiment, a solution of protein in tris buffer (0.05*M* tris, 0.10*M* NaCl; *p*H 7.4) was allowed to equilibrate with added radioactive [^{131}I]T_4 (*9*). The solution was then diluted with the same buffer containing a known concentration of ANS, and portions were subjected to ultrafiltration. The T_4 activity remaining with the protein on the membrane was determined.

The amount of T_4 retained by TBG fell as ANS concentration was increased. In the presence of $2.5 \times 10^{-3}M$ ANS, only 16.0 ± 0.1 percent of the control value for T_4 remained associated with TBG. By measuring the amount of T_4 displaced by a known concentration of ANS from a known solution of TBG and by assuming simple competition, the K_{assoc} for T_4 at 23°C was calculated as $2.35 \pm 0.43 \times 10^{10}M^{-1}$. This agrees well with the K_{assoc} of $2.24 \pm 2.1 \times 10^{10}M^{-1}$ calculated by directly measuring the affinity of T_4 for TBG by ultrafiltration.

In qualitative studies, ANS also underwent a marked increase in quantum yield in the presence of human serum prealbumin (HSP) and human serum albumin (HSA). In addition, binding of ANS to HSP and HSA was demonstrated by the observation of energy transfer. That is, when the protein solutions were excited at 295 nm, the addition of ANS caused a gradual quenching of the intrinsic protein fluorescence peak; this quenching was accompanied by the appearance of the characteristic ANS fluorescence peak. Finally, ANS demonstrated a hypsochromic shift of fluorescence emission maximum in the presence of each protein.

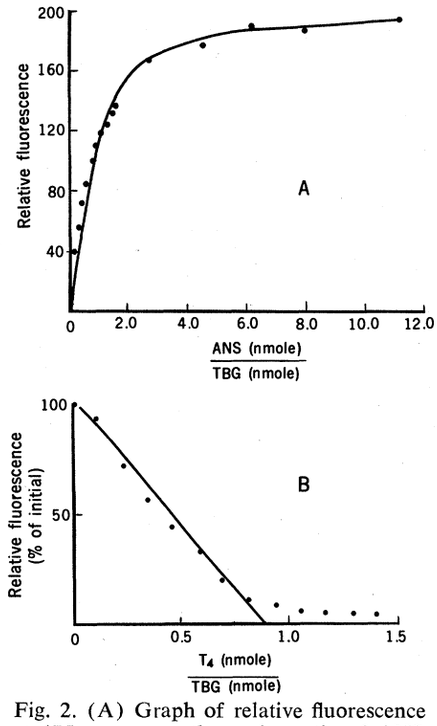

Fig. 2. (A) Graph of relative fluorescence at 475 nm versus the molar ratio of ANS to TBG. Portions of a solution of ANS were added to a solution of TBG ($6.3 \times 10^{-7}M$) in tris buffer (0.05*M* tris, 0.10*M* NaCl; *p*H 7.3). The resulting fluorescence was corrected for dilution and light absorption. Circles are data, and the line was calculated assuming a single binding site with K_{assoc} of $4.2 \times 10^{6}M^{-1}$. Excitation was at 400 nm, and emission was measured at 475 nm. (B) Graph of relative fluorescence at 475 nm versus the molar ratio of T_4 to TBG. Excitation was by light of 400 nm, and emission was measured at 475 nm. Portions of a T_4 solution were added to a solution of TBG ($1.11 \times 10^{-6}M$) and ANS ($1.0 \times 10^{-5}M$) in tris buffer (0.05*M* tris, 0.10*M* NaCl; *p*H 7.3). Circles are data, and the solid line was determined by linear regression analysis and represents the initial slope. In all cases, correlation coefficients are $-.95$ or less. Standard deviations are indicated in the text.

Addition of T_4 to solutions of ANS and either HSA or HSP produced a quenching of ANS fluorescence. With HSP, almost complete ANS quenching occurred when 1 mole of T_4 was added per mole of protein; this result confirmed the existence of one T_4 binding site per molecule, as reported in the literature (*10*). As expected from other studies (*11*), HSA showed a more complicated pattern, which implied the presence of one strong binding site for ANS and T_4 and of several weaker sites. Ultrafiltration data supported these fluorescence results by showing that labeled T_4 was displaced when ANS was added.

The hypsochromic shift of the emission maximum of ANS when the dye was bound to TBG, HSA, and HSP also indicates that the T_4 binding sites are probably relatively hydrophobic. However, restriction of solvent relaxation in a polar medium may also cause fluorescence changes indicative of hydrophobicity (small dipole moment) (*12*).

The binding of a structurally dissimilar dye to the T_4 binding site of TBG, along with earlier reports of the binding of diphenylhydantoin (*13*) *o,p'*-dichlorodiphenyldichloroethane (*14*), salicylate, and dinitrophenol (*7*, *15*) to the same site, indicates that TBG has a striking ability to associate with diverse organic ligands. Conventionally, the physiologic function of TBG has been assumed to be that of T_4 transport, but the binding versatility demonstrated by this protein may indicate a less restricted role.

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Biogenic Amine Metabolites in Cerebrospinal Fluid of Depressed and Manic Patients

Abstract. *A reduction in 5-hydroxyindoleacetic acid in cerebrospinal fluid was found in depressed and manic patients both while they were symptomatic and also after treatment. The concentration of homovanillic acid was initially reduced and then tended to increase after treatment.*

Considerable attention has been directed to possible abnormalities of biogenic amine metabolism in both depression and mania (*1*). The concentrations of amines and their metabolites in patients' urine have been studied but interpretation is difficult because only a small percentage of urinary amine metabolites originate in the brain. For example, it has been calculated that less than 1 percent of urinary 3-methoxy-4-hydroxymandelic acid in the dog originates in its brain. Urinary 3-methoxy-4-hydroxyphenyl glycol may be an exception to this (*2*).

Consequently, several investigators have examined amine metabolites in cerebrospinal fluid (CSF). A significant decrease in the concentration of CSF 5-hydroxyindoleacetic acid (5HIAA) has been reported in depressed patients (*3*); in addition, the increase in 5HIAA in CSF, usually observed after large doses of probenecid are given, was lower than that in control subjects. This suggests a decrease in the turnover of brain serotonin in depressed patients (*4*). Other studies suggest that 5HIAA concentrations in CSF return to normal after patients recover from depression and that values are normal in hypomanic patients (*3*). Unfortunately, the interpretation of much of this data is made difficult by the uncertain description of the patients studied and the large range of concentrations found.

We studied severely ill patients with affective disorders who were hospitalized on a special research ward. A period of 7 to 21 days lapsed before drug therapy was started. During this period no drugs were given, and a detailed clinical, psychological, and biological evaluation was made. The patients received a diet designed for the study of the metabolism of biogenic amines. Patients were diagnosed as manic-depressive if there was a history of previous hospitalization for both manic and depressive episodes and if two psychiatrists agreed on the current clinical state of the patient. Unipolar, or primary depressive, illness was diagnosed if there was no history of previous manic episodes, and if there was no other significant psychiatric abnormality or organic brain syndrome. A daily clinical evaluation of each patient's behavior was made by a trained research nursing staff (*5*) and recorded on a specially designed form containing symptoms of both mania and depression. These forms were developed for the systematic rating of manic patients (*6*). The ratings can be considered reliable, in view of the fact that there was little variation between the ratings of individuals making the determinations.

Lumbar punctures were performed between 9:00 a.m. and 11:00 a.m. while the patient was in a recumbant position, before he was allowed to rise, and before he had eaten. The first puncture was performed before treatment, and, in most instances, the second was done after clinical recovery and before the patient was discharged from the hospital. Several of the patients had been receiving drugs the second time (indicated in Table 1). The CSF was acidified with HCl and immediately frozen. Homovanillic acid (HVA) was estimated by the method of Gerbode

Table 1. Concentration of 5HIAA and HVA in CSF; before and after treatment.

Patient	5HIAA (ng/ml)		HVA (ng/ml)	
	Before	After	Before	After
		Manic-depressed, depressed		
1	16.9*		84.9*	
2	21.8		6.8	
3	19.8	17.5	7.6	48.5
4	20.9	15.7	23.6	
5	8.2	29.6†	10.6	58.6†
6	< 7.0		25.6	
Mean	15.8‡	20.9‡	26.5	53.6
		Manic-depressed, manic		
7	< 7.0	9.6		
1	< 7.0*	19.4*§		16.7*§
8	12.4||	< 7.0||¶	35.7||	58.1||¶
8	42.1||	20.6||¶	52.7||	98.5||¶
Mean	17.1‡	14.2‡	44.2	57.8
		Unipolar depressives		
9	< 7.0		19.9	
10	< 7.0		57.2	
11	< 7.0		26.7	
12	14.7	21.6#	20.7	68.2#
13	17.8		33.8	
14	< 7.0		8.1	
Mean	10.1‡		27.7	

* These concentrations are found in the same patient (No. 1) at different clinical stages of his illness. † Recovered from depression—moderate anxiety. ‡ These means were calculated with the value of < 7 ng being regarded as 7. The actual values were probably lower and the true means would therefore be lower. § Receiving lithium carbonate. || These concentrations are found in the same patient (No. 8) at different clinical stages of his illness. ¶ Mania remitted; mild depression. # Little change in clinical condition; had received large doses of L-dopamine for 5 weeks.