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Ligand-Induced Self-Association of Human Chorionic Gonadotropin. Positive Cooperativity in the Binding of 8-Anilino-1-naphthalenesulfonate†

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ABSTRACT: Human chorionic gonadotropin (hCG) self-associates to form higher molecular weight species in the presence of the fluorescence probe 8-anilino-1-naphthalenesulfonate (ANS). Sedimentation equilibrium and fluorescence titration data have been analyzed in terms of a monomer–dimer–tetramer model in which the various oligomers have different affinities and/or capacities for the ligand. The results indicate that the ligand affinities are in the order tetramer > dimer > monomer whereas the numbers of ligand binding sites per mole of hCG are in the reverse order. Consequently, addition of ANS first shifts the equilibrium from monomer to tetramer and gives rise to positive cooperativity in the titration curves. At sufficiently high ANS concentration (~0.5 mM), the equilibrium shifts back to the dimer because of its greater binding capacity. This is manifested by a second phase in the titration curve and a decrease in the polarization of ANS fluorescence. The results are discussed in terms of the general problem of ligand controlled protein association and are contrasted to results reported in the previous paper for the homologous protein, human luteinizing hormone.

Human chorionic gonadotropin (hCG)[1] and human luteinizing hormone (hLH) are glycoproteins comprised of two nonidentical subunits, α and β, held together by noncovalent bonds (Canfield et al., 1971; Morgan and Canfield, 1971; Ward et al., 1973). There is extensive homology between the primary structures of the two hormones and they have similar biological properties. They have been shown to compete with each other for binding to receptor sites on target tissue membranes (Catt, et al., 1972; Lee and Ryan, 1973; Rao, 1974).

In the previous paper (Ingham et al., 1975) we reported the results of a detailed study of the interaction of the fluorescence probe, 8-anilino-1-naphthalenesulfonate (ANS), with hLH. It was shown that hLH self-associates to form dimers $(\alpha\beta)_2$ in the presence of ANS, due to the presence of a single high affinity site ($K > 10^6\ M^{-1}$) on the dimer while binding of ANS to the monomer was too weak to be observed. This led to negative cooperativity in the ANS binding and to a dependence of that binding on hormone concentration. In this paper we report the results of a similar study with hCG which, in spite of its extensive homology with hLH, exhibits marked differences in its interaction with ANS.

Materials and Methods

Highly purified hCG (CR117) was obtained from Dr. R. Canfield of Columbia University through the Center for Population Research, National Institute of Child Health and Human Development, NIH. hCG concentrations were determined optically using ϵ 1.2×10^4 l. per mol per cm at 276 nm. This molar extinction coefficient was calculated from the amino acid composition (Bellisario et al., 1973; Carlsen et al., 1973; Morgan et al., 1973) as described previously (Ingham et al., 1975). Concentrations determined in this manner were within 10% of those determined either by refractive index measurements or by the weight of lyophilized hormone assuming a molecular weight of 37,900 (Bellisario et al., 1973; Carlsen et al., 1973). The fluorescence spectra of hCG solutions had maxima near 305 nm with no indication of any tryptophan emission indicating that preparations were free of contaminating proteins which contain tryptophan.

The magnesium salt of ANS was obtained from Eastman

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[1] Abbreviations used are: hCG, human chorionic gonadotropin; hLH, human luteinizing hormone; ANS, 8-anilino-1-naphthanesulfonate.

and used without further purification. Concentrations of ANS were determined using ϵ_{350} 6240 l. per mol per cm (Ferguson and Cahnmann, 1975). All of the experiments described herein utilized either 0.01 or 0.001 *M* potassium phosphate buffer, as indicated, at pH 7.0.

Gel filtration, fluorescence, and fluorescence polarization measurements were made as previously described (Ingham et al., 1975). Care was taken to keep the optical density at the excitation wavelength as low as possible to avoid errors due to absorption artifacts ("internal filter" effects). In order to measure ANS fluorescence polarization over a broad range of ANS concentration, two different excitation wavelengths were used. For the high ANS concentration range (3.0×10^{-5}–2.0×10^{-3} *M*), an excitation wavelength of 430 nm was used, whereas for ANS $<3.0 \times 10^{-5}$ *M*, 360 nm was used. By contrast to the results reported with hLH (Ingham et al., 1975), the fluorescence of ANS in the presence of hCG exhibited no time effects.

Sedimentation equilibrium measurements were made as previously described (Ingham et al., 1975) except as follows. For run A (no ANS) and run B (1.0×10^{-5} *M* free ANS), low-speed (8000 rpm) sedimentation equilibrium was used. Synthetic boundary experiments were performed to determine the total hormone concentration in the cell. A higher speed (20,000 rpm) was used for run C (8.0×10^{-4} *M* free ANS) and the boundary conditions were established by the meniscus depletion method of Yphantis (1964). A partial specific volume of $\bar{v} = 0.704$ was calculated for hCG from the known composition (Carlsen et al., 1973; Bellesario et al., 1973) utilizing the data of Cohn and Edsall (1943) for amino acids and of Gibbons (1966) for carbohydrate.

Sedimentation equilibrium and fluorescence titration data were fit to a monomer–dimer–tetramer model (see Theoretical Section). The approach used to assess the uniqueness of the resulting parameter values was previously described (Ingham et al., 1975).

Theoretical Section

A. Model. The data have been analyzed according to a monomer (P_1)–dimer (P_2)–tetramer (P_4) model:

$$2P_1 \rightleftarrows P_2 \qquad K_d = [P_2]/[P_1]^2 \tag{1}$$

$$4P_1 \rightleftarrows P_4 \qquad K_t = [P_4]/[P_1]^4$$

or

$$(2P_2 \rightleftarrows P_4 \qquad K_x = K_t/K_d^2)$$

in which P_1, P_2, and P_4 are assumed to have respectively N_1, N_2, and N_4 equivalent and independent sites for binding the ligand, L, with corresponding intrinsic association constants K_1, K_2, and K_4. In the absence of ligand, the total molar concentration of protein, P_0, in units of monomer is given by

$$P_0 = [P_1] + 2[P_2] + 4[P_4] \tag{2}$$

or, utilizing eq 1

$$P_0 = [P_1] + 2K_d[P_1]^2 + 4K_t[P_1]^4 \tag{3}$$

In the presence of ligand, eq 3 becomes

$$P_0 = (1 + K_1L)^{N_1}[P_1] + 2K_d(1 + K_2L)^{N_2}[P_1]^2 + 4K_t(1 + K_4L)^{N_4}[P_1]^4 \tag{4}$$

where L is the molar concentration of free ligand. The concentration of bound ligand, L_b, is given by

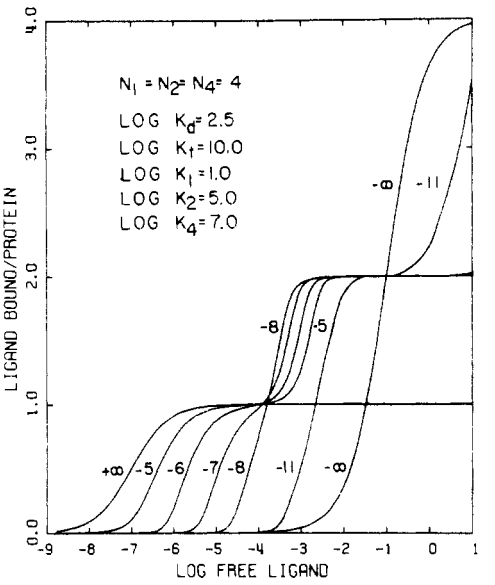

FIGURE 1: Theoretical binding curves for a monomer–dimer–tetramer successive "half-of-the-sites" model as described in the Theoretical Section of the text using the parameter values indicated in the figure. The numbers next to the curves are the logarithms of the total molar protein concentration, P_0, in units of monomer.

$$L_b = N_1K_1L(1 + K_1L)^{N_1-1}[P_1] + N_2K_dK_2L(1 + K_2L)^{N_2-1}[P_1]^2 + N_4K_tK_4L(1 + K_4L)^{N_4-1}[P_1] \tag{5}$$

Given values for the K's and N's, eq 4 can be solved numerically for $[P_1]$ for any value of L. The quantity $\bar{v} = L_b/P_0$ can then be calculated using eq 5.

Figure 1 shows the results of such a calculation for a particular choice of parameters which qualitatively illustrates the hCG–ANS system (see Results). This is a successive "half-of-the-sites" model (Levitzki et al., 1971; Bernhard and MacQuarrie, 1973) in which $K_1 < K_2 < K_4$ and $N_1 = N_2 = N_4 = 4$. Thus, the number of sites per mole of protein monomer is successively reduced while the affinity increases going from monomer to dimer to tetramer. At infinite protein concentration, only the tetramer is present and a statistical[2] saturation curve is obtained with 50% saturation at $L = K_4^{-1}$. As the protein is diluted, the curve shifts to the right, becomes steeper (a manifestation of positive cooperativity), and at sufficiently high L, a second phase of binding is observed. The latter is due to a shift of the equilibrium from tetramer to dimer which has a greater number of sites per mole of protein. Further dilution of protein results in a similar effect as dimer dissociates into monomers (for the same reason) and at infinite dilution, one observes statistical binding to the monomer with 50% saturation at $L = K_1^{-1}$.

B. Analysis of Sedimentation Data. At sedimentation equilibrium, the radial distribution of each species, P_i, in the centrifuge cell is given by

$$[P_i]_r = [P_i]_b \exp(iMA[r^2 - b^2]) \tag{6}$$

where r is the radial distance, b is the value of r at the cell bottom, and M is the molecular weight of the monomer. The quantity $A = (1 - \bar{v}\rho)\omega^2/2RT$ is a constant for a given angular velocity, ω; $\bar{v}$ is the partial specific volume and ρ is

[2] By statistical we mean a normal unperturbed saturation curve. See previous paper (Ingham et al., 1975) for details.

Table I: Summary of Sedimentation Equilibrium Analysis.

Expt	Concn (M)		Parameters[b]		Distribution (%)[c]		
	hCG[a]	ANS	Log K_d	Log K_t	Monomer	Dimer	Tetramer
A	1.44×10^{-4}	0.0	≤ 4	10.3 ± 0.5	80.4	0	19.5
			log K_d'	log K_t'			
B	3.6×10^{-5}	1.0×10^{-5}	$3.5^{+1}_{-\infty}$	12.7 ± 1	60.1	10.8	29.1
C	6.0×10^{-6}	8.0×10^{-4}	7.0^{+1}_{-2}	19^{+1}_{-2}	2.6	84.1	13.3

[a] hCG concentration, before sedimenting. [b] Values corresponding to theoretical curves in Figure 3. See eq 7 and 8 for definitions of K_d' and K_t'. [c] Calculated values obtained by integration of theoretical curves.

the density of the solvent. From the measured concentration of protein at the cell bottom, $[P_0]_b$, and initial estimates of the parameters K_d and K_t, one can solve for $[P_1]_b$, $[P_2]_b$, and $[P_4]_b$ using eq 1-3. Then, using eq 6, the concentration of each species can be calculated for any value of r and their sum compared to the observed value. The values of K_d and K_t are then allowed to vary until the best fit is obtained. Note that the apparent values of K_d and K_t obtained in the presence of ANS (i.e., K_d' and K_t') have an entirely different meaning than those obtained in its absence. Comparison of eq 3 and 4 shows that

$$K_d' = K_d(1 + K_2L)^{N_2} \quad (7)$$

$$K_t' = K_t(1 + K_4L)^{N_4} \quad (8)$$

where the primes indicate the values in the presence of ANS.

Results

A. Gel Filtration. Evidence for ANS-induced self-association of hCG was obtained from gel filtration on Sephadex G-100 at 4°C. The results were similar to those shown in the previous paper for hLH (Ingham et al., 1975). In the absence of ANS, the hormone eluted as a single band centered at $V_e/V_0 = 1.26$. When the column was equilibrated with 1.0×10^{-4} M ANS, elution occurred earlier at $V_e/V_0 = 1.08$. Thus, the effective molecular radius of the hormone increased in the presence of ANS indicating that aggregation had occurred.

B. Sedimentation Equilibrium Measurements. Preliminary experiments indicated that the sedimentation coefficient ($s_{20,w}$) of hCG increased in the presence of ANS, qualitatively confirming the gel filtration results. In order to analyze the ligand-induced self-association in detail, the influence of ANS on the sedimentation equilibrium behavior of hCG was investigated. Three experiments were performed at the hCG and ANS concentrations indicated in Figure 2 and Table I. The results were analyzed in terms of the monomer-dimer-tetramer model as described above. Analysis of the data using a trimer instead of a tetramer gave less satisfactory results. One could obviously include both trimer and tetramer at the expense of introducing a third parameter but little additional information would be gained.

The solid curves in Figure 2 are theoretical curves corresponding to the best fit with the chosen model. The values of the parameters obtained from this analysis are summarized in Table I along with the calculated distribution of monomer, dimer, and tetramer. Note that the fraction of hormone in the form of monomer decreases with increasing ANS, even though the total hCG is diluted. The addition of ANS shifts the equilibrium toward the higher molecular weight forms, first to the tetramer and then, at higher ANS concentration, back to dimer.

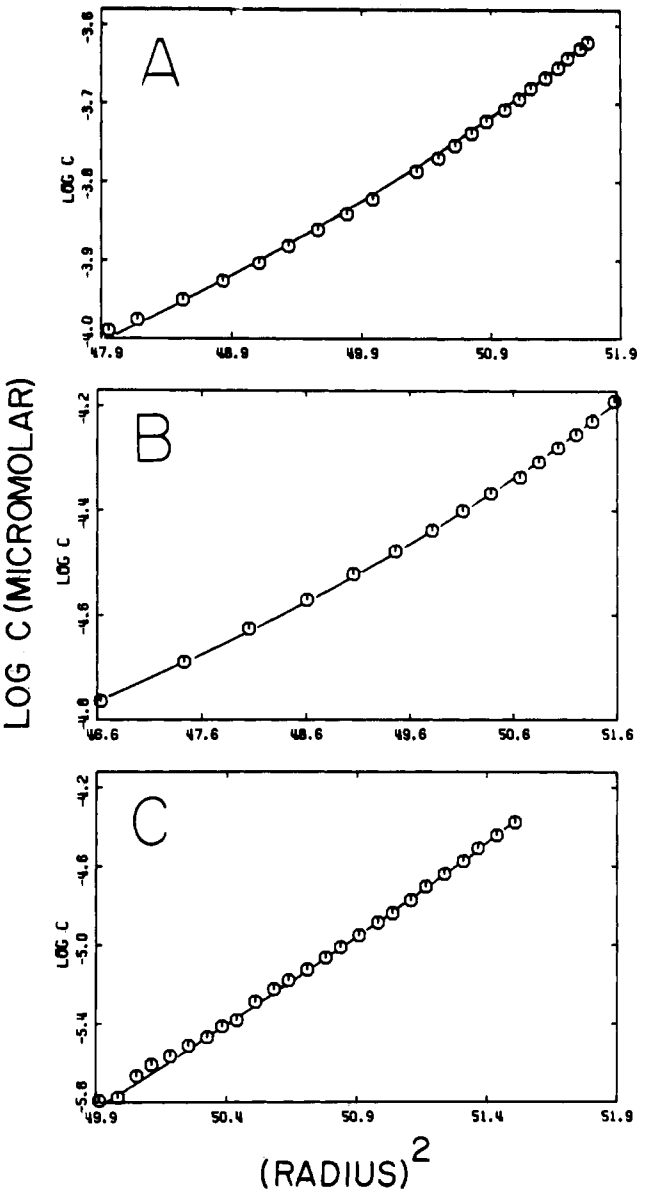

FIGURE 2: Sedimentation equilibrium measurements of different solutions of hCG and ANS at 25°C in 10^{-3} M phosphate buffer (pH 7.0). The lines are theoretical curves corresponding to the best fits of the experimental points to a monomer-dimer-tetramer model. (A) hCG = 1.44×10^{-4} M, no ANS; (B) hCG = 3.6×10^{-5} M, ANS = 1.0×10^{-5} M (free); (C) hCG = 6.0×10^{-6} M, ANS = 8.0×10^{-4} M (free).

Figure 3 illustrates the results of the parameter depen-

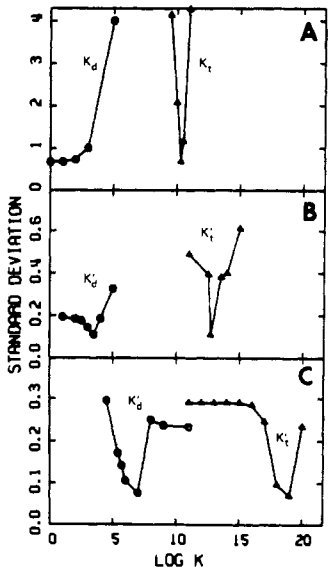

FIGURE 3: Effect of arbitrary changes in K_d and K_t on the quality of the fit of the sedimentation data in Figure 3 to the monomer–dimer–tetramer model as measured by the standard deviation. K_d' and K_t' are apparent constants defined by eq 7 and 8.

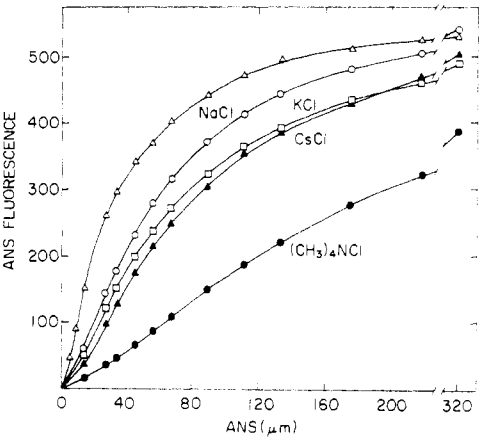

FIGURE 4: Effects of monovalent cations on the fluorescence titration of hCG with ANS at 25°C in 0.001 *M* phosphate buffer (pH 7.0). (△) Data obtained in the absence of added salt. The salt concentration in each remaining case was 0.3 *M*. The excitation and emission wavelengths were 415 and 480 nm, respectively. The Hill coefficients are 1.08 (△), 1.21 (○), 1.35 (□), 1.39 (▲), and 1.57 (●).

dency study which was performed as described in the previous paper (Ingham et al., 1975). In the absence of ANS (Figure 3A), the value of K_t is precisely determined while K_d has only an upper limit. The physical interpretation of this result is that the data are "best fit" by a mixture of monomer and tetramer with little or no dimer allowed. The addition of 10^{-5} *M* ANS (Figure 3B) increases the apparent value of K_t (see eq 8) reflecting the greater fraction of hormone present as tetramer. In this case also, the data are fairly well described in terms of a monomer–tetramer system with very little dimer required. Although the introduction of a small amount of dimer (11%) improves the quality of the fit as reflected by the minimum in the K_d' curve, this minimum may be too shallow to be considered significant. At much higher ANS (Figure 3C), the values of both parameters are further increased and there is a definite minimum in the K_d' curve. At this high ANS concentration (8×10^{-4} *M*), substantial amounts of dimer are required to obtain a good fit. Figure 3C illustrates that if one chooses a value of $K_d' \geq 10^8$ as an initial estimate in the curve-fitting program, the best fit will not be obtained because lowering the value of K_d' causes a slight rise in the error such that the program does not find the true minimum. This further illustrates the necessity of the parameter dependency study.

C. ANS Fluorescence Titrations. Typical fluorescence titrations of hCG with ANS are illustrated in Figure 4. In the absence of added salt (triangles) the curve is almost hyperbolic but becomes sigmoidal upon addition of various salts to an extent which correlates with the size of the cation ($Me_4N^+ > Cs^+ > K^+ > Na^+$). This implies that the salt effects do not arise simply from changes in ionic strength but perhaps reflect more specific interactions with the hormone. Scatchard (1949) type plots of the data in Figure 4 exhibit the downward concavity characteristic of positive cooperativity (Hammes and Wu, 1974; Frieden and Colman, 1967), and Hill coefficients (Brown and Hill, 1923) range from 1.08 to 1.57 (see legend). Since the Mg^{2+} salt of ANS was used throughout these experiments, its effect was also examined. Concentrations of $MgCl_2$ up to 1.0 m*M* (the highest concentration of ANS employed) had no effect on ANS fluorescence.

In order to estimate the stoichiometry of the hCG–ANS interaction, a fluorescence titration was performed at high hCG concentration (2.0×10^{-4} *M*). The results are illustrated in Figure 5. Extrapolation of the ascending linear portion of the curve to the saturation level gives a value of 1.1 mol of ANS bound/mol of hCG. The results of the sedimentation studies suggest that at high hCG concentration, ANS is bound primarily to the tetramer. We thus conclude that the tetramer has four ANS binding sites.

A key feature of the proposed model is its prediction of a shift in the position of the binding curve with protein concentration, as illustrated by the theoretical curves in Figure 1. Thus, titrations were performed at two concentrations of hCG differing by a factor of 10. An excitation wavelength of 440 nm was chosen in order to extend the titration to very high levels of ANS. At this wavelength, a 1.0×10^{-3} *M* solution of ANS has an absorbance less than 0.1 and no absorption corrections are necessary. The results (Figure 6) indicate a pronounced effect of protein concentration on the position and shape of the titration curves. The curve, corresponding to the more concentrated hCG solution, shows a definite inflection near 10^{-4} *M* ANS which is less apparent in the more dilute curve. The two curves resemble the curves corresponding to 10^{-7} and 10^{-8} *M* protein in Figure 1 and suggest that the inflection point corresponds to saturation of the tetramer. The additional rise in fluorescence at high ANS concentration would then correspond to shifting the equilibrium back toward the dimer due to its greater number of sites per mole of hCG. Such a conclusion is compatible with the sedimentation results (Table I) which indicate 84% dimer at hCG = 6×10^{-6} *M* and ANS = 8×10^{-4} *M*.

Further support for this interpretation was obtained from ANS fluorescence polarization measurements as a function of ANS concentration. Figure 7 illustrates a gradual decrease in polarization between 10^{-6} and 10^{-4} *M* ANS followed by a more dramatic decrease in the range corresponding to the second phase of the binding curve (Figure 6). The initial gradual decrease occurs in the range of conversion from monomer to tetramer and suggests that binding of ANS to monomers is substantially weaker than to tetramers. Otherwise, one would expect the polarization to

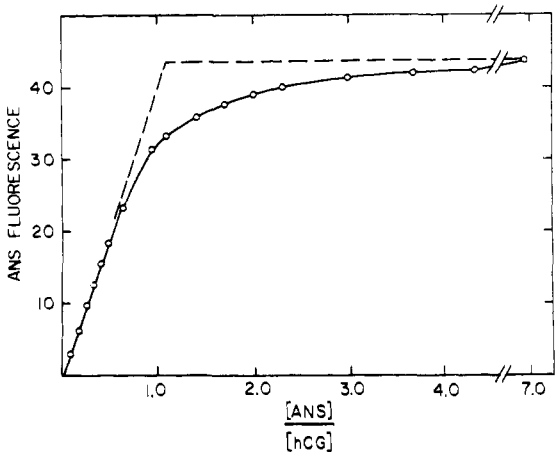

FIGURE 5: Stoichiometric fluorescence titration of hCG with ANS at 25°C in 0.01 *M* phosphate buffer (pH 7.0). The hCG concentration was 2.0×10^{-4} *M*. Excitation and emission wavelengths were 430 and 480 nm, respectively.

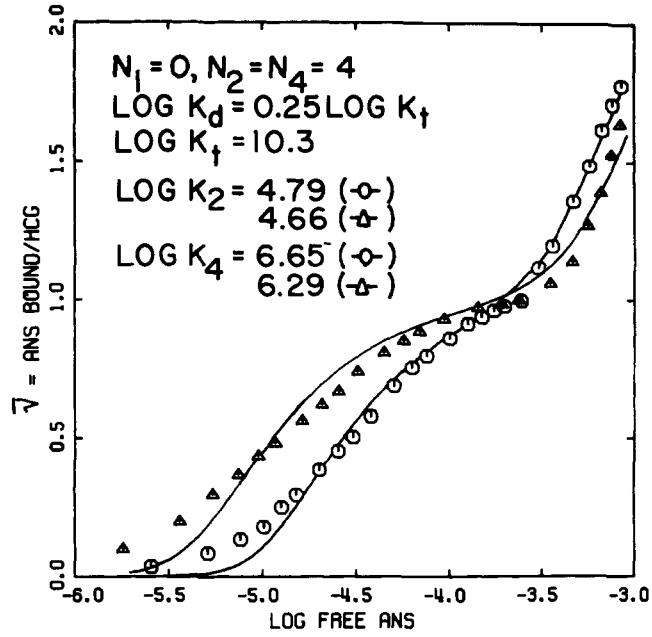

FIGURE 6: Effect of a tenfold variation in hCG concentration on the fluorescence titration with ANS at 25°C in 0.001 *M* phosphate buffer (pH 7.0). The hCG concentration was 7.7×10^{-6} *M* (Δ) and 7.7×10^{-7} *M* (O). Excitation and emission wavelengths were 440 and 480 nm, respectively. The solid lines are theoretical curves obtained by fitting each set of experimental points to the monomer-dimer-tetramer model as described in the text. The corresponding parameter values are indicated in the figure.

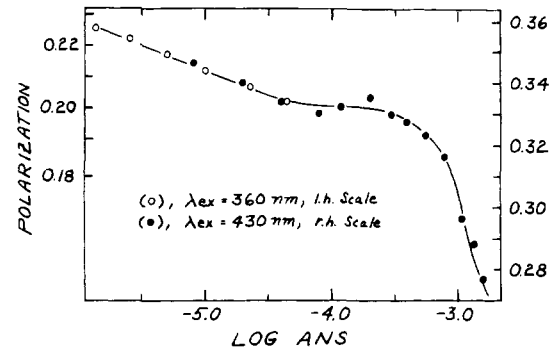

FIGURE 7: Dependence of the polarization of ANS fluorescence on ANS concentration at 25°C in 0.01 *M* phosphate buffer (pH 7.0). The hCG concentration was 3.0×10^{-6} *M* and the different symbols correspond to two different excitation wavelengths, 360 nm (O) and 430 nm (●), as explained in Materials and Methods.

increase in this range, due to the much larger rotational relaxation time of the tetramer (Chen et al., 1969). The gradual decrease could be due to increased energy transfer as the tetramer becomes saturated (Anderson and Weber, 1969). The steeper fall in polarization at higher ANS concentration is compatible with a lower rotational relaxation time for the dimer. In addition, since the average separation between ANS molecules on the dimer should be less than on the tetramer, one might expect a contribution from increased energy transfer. However, this effect should not be too important when excitation occurs at the long wavelength edge of the absorption spectrum (Anderson and Weber, 1969).

Before fitting the fluorescence binding data of Figure 6 to the monomer-dimer-tetramer model, an effort was made to restrict the model as much as possible in order to minimize the number of variable parameters. The assumptions and restrictions will now be presented, along with some of their justifications.

(1) The fluorescence intensity is directly proportional to the concentration of bound ANS. This assumption is commonly used in studying the binding of fluorescent ligands to macromolecules (Brand et al., 1967; Anderson and Weber, 1969; Mooser et al., 1972; Laurence, 1952).

(2) There is no interaction between ANS binding sites. Each site on a given oligomer has the same affinity when corrected for statistical factors. Although we cannot exclude minor site-site interaction or minor heterogeneity, such complications are not necessary to interpret the data.

(3) The only effect of ANS on the self-association of hCG is via mass action. Thus, we fixed log K_1 at its value of 10.3 obtained from the sedimentation results in the absence of ANS (Table I). However, those same results give only an upper limit for log $K_d \leq 4.0$. For the initial curve fitting, we arbitrarily set $K_d = (K_t)^{1/4}$ assuming a square geometry for the tetramer.[3] After the best fits were obtained, K_d was allowed to vary to see if the fits could be improved. The results were found to be insensitive to the value of K_d. This is due to the ability of small changes in K_2 to compensate for large changes in K_d, and arises because, in eq 4, K_2 appears to the 4th power while K_d appears to the 1st power. A similar relation exists between K_t and K_4. However, this was not explored since the sedimentation results gave a precise value for K_t.

(4) The order of affinities of ANS for monomer, dimer, and tetramer are $K_1 < K_2 < K_4$. This relationship is dictated by the sedimentation results which indicate that ANS binds first to the tetramer (at low [ANS]) and later to the dimer (at high [ANS]). In the initial analysis, K_1 was arbitrarily set equal to zero. Once a reasonable fit was obtained, K_1 was varied (assuming $N_1 = 4$) in an effort to improve the fit. No change was observed until $K_1 \geq 10^3$ at which point the quality of the fit was drastically reduced. This result, coupled with the results of the polarization measurements, justifies our neglect of binding of ANS to monomer.

(5) The tetramer was assumed to have four ANS binding

[3] Since, to a first approximation, the free energy ($\Delta G = -RT \ln K$) of self-association can be assumed proportional to the number of monomer-monomer interactions, a square geometry would suggest $K_d = K_t^{1/4}$. Similarly, $K_d = K_t^{1/3}$ for a linear geometry and $K_d = K_t^{1/6}$ for a tetrahedral geometry.

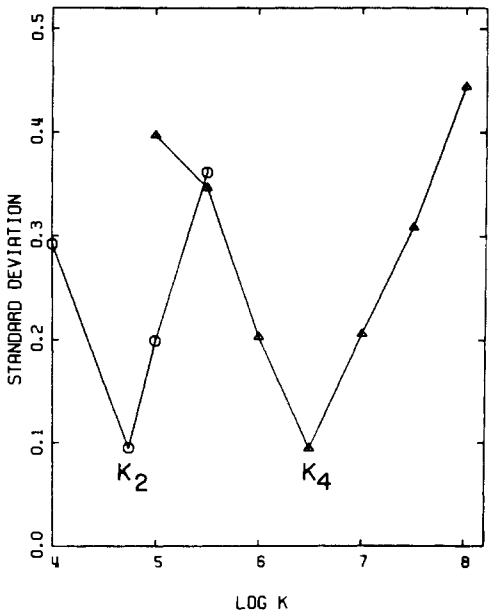

FIGURE 8: Effect of arbitrary changes in K_2 and K_4 on the quality of the fit of fluorescence data on Figure 7 to the monomer–dimer–tetramer model. An abridged set of data consisting of nine experimental points from each titration curve in Figure 7 was used for these calculations. Both sets of data were fit simultaneously.

sites, based on the titration of Figure 5 and the sedimentation results. Thus, the ordinate at the point of intersection of the curves in Figure 6 was fixed at $\bar{\nu} = 1$.

(6) The exact number of ANS sites for the dimer was not determined. The magnitude of the rise in fluorescence beyond the inflection point of Figure 6 suggests at least a doubling of sites on shifting from tetramer to dimer, assuming that ANS has the same fluorescence quantum yield when bound to dimer or tetramer. We therefore fixed $N_2 = 4$. The precise value of N_2 is not critical for the model. It is only necessary that the number of ANS sites *per mole of hCG* be significantly greater for the dimer than for the tetramer.

With the above restrictions, the only remaining parameters are K_2 and K_4. The solid curves in Figure 6 are theoretical curves obtained by fitting each set of data separately. The corresponding values of K_2 and K_4 are indicated in the figure. The agreement between the values of the constants obtained for the two experiments is not unreasonable given the restrictions placed on the model. If one fits both sets of data simultaneously, intermediate values are obtained (log $K_2 = 4.74$, log $K_4 = 6.49$). This was the approach used for the dependency study (Figure 8) which indicates a rather narrow range of values of K_2 and K_4 which are capable of accommodating both sets of data.

It was also possible to estimate values of log $K_2 \geq 4.0$ and log $K_4 = 5.5$ from the sedimentation results (Table I) using eq 7 and 8. Both values are within an order of magnitude of the values obtained by fitting the fluorescence data, lending internal consistency to the results.

Discussion

Ligand-induced association of proteins is a subject of increasing interest, primarily because of the implication of such reactions in biological control mechanisms. The subject has been reviewed recently (Koshland, 1970; Frieden, 1971; Phillips, 1974; Hammes and Wu, 1974; Dunne and Wood, 1975). Although a number of authors have discussed various theoretical aspects of this phenomenon (Nichol et al., 1967; Levitzki and Schlessinger, 1974), there are relatively few examples where the cooperative effects have been shown to derive exclusively from protein association. With hCG we have an experimental example of cooperative ligand binding in which the cooperativity arises predominantly from mass action effects due to oligomeric differences in ligand affinity and capacity. Although the fits are not perfect, the major predictions of the model, i.e., the shapes of the binding curves and their dependence on hormone concentration, are clearly demonstrated in Figure 6. While the model may appear somewhat elaborate, the real system is apparently even more complicated. A more precise evaluation of all the constants involved would require extensive analysis of the extent of self-association, at various concentrations of ANS, as a function of hormone concentration.

The effects of chloride salts (Figure 4) on the binding of ANS to hCG are rather dramatic and the fact that different cations have different effects suggest that this behavior is not due simply to changes in ionic strength. It is possible that the hormone binds the various cations with affinities which are greater for the larger cations. Binding of monovalent cations has been observed with a number of enzymes and the affinities tend to depend on the size of the cation (Suelter, 1972). The various salts tend to accentuate the sigmoidal shape of the titration curves with very little effect on the final fluorescence (Figure 4), suggesting that their major effect is to inhibit the hormone association. This suggestion is supported by recent experiments in which the Sephadex G-100 elution profile of hCG at high concentration (25 mg/ml) was much broader at low ionic strength than in the presence of 0.3 *M* KCl (K. Ingham, unpublished observations). Since ANS fluorescence is obviously sensitive to the oligomeric state of hCG, this system might serve as a useful model for investigating the effects of neutral salts on protein association (Von Hippel and Schleich, 1969).

The physiological significance of the ligand-induced self-association of hCG is not clear. A similar effect has been observed with human luteinizing hormone (Ingham et al., 1975) whose primary structure and biological properties are very similar to those of hCG. Bovine thyroid stimulating hormone also exhibits positive cooperativity in the binding of ANS and was shown to self-associate in the presence of the dye (Ingham et al., 1974). The ability of a hormone to reversibly alter its degree of self-association in response to a change in concentration of a ligand could have important consequences for biological control, either at the level of hormone storage and release or at the level of interaction with the receptor. An effort is being made to screen small physiological molecules for their ability to compete with ANS for binding to the hormones.

The contrasting behavior of hCG and hLH (Ingham et al., 1975) with respect to the binding of ANS is interesting in view of the extensive homology between the primary structures of these two hormones. The α subunits are essentially identical (Bellisario et al., 1973) while 83 of the 115 residues in hLHβ are identical with hCGβ (Carlsen et al., 1973). Furthermore, there are 11 disulfide bonds (5 in α, 6 in β) which will restrict the conformational differences between the two hormones. There are significant differences in the carbohydrate content and, in addition, hCGβ has an

extra 32 residues at the C-terminus which include 11 proline residues and three extra carbohydrate moieties. However, the finding (Aloj et al., 1973, and unpublished results) that the binding of ANS to asialo-hCG is indistinguishable from the binding to the native hormone indicates that the sialic acid residues do not influence the self-association of the hormone or the ANS binding sites and tends to diminish the possibility that differences in the ANS binding to HLH and hCG will ultimately be traced to differences in their carbohydrate moieties.

The major differences between hLH and hCG with respect to ANS binding can be summarized as follows (refer to previous paper, Ingham et al., 1975).

(1) Time Effects. Although both hormones self-associate under the influence of ANS, there are dramatic differences in the kinetics of this reaction; whereas hCG reacts instantly (within a few seconds), hLH requires as much as an hour to reach equilibrium at the lowest concentrations of ANS and hormone investigated.

(2) Stoichiometry. Whereas the self-association of hLH proceeds only to the dimer which binds a single molecule of ANS, hCG forms dimers and tetramers, both of which bind several molecules of ANS. The fact that the hLH dimer has only one site suggests that ANS may be bound at the interface between two identical subunits, analogous to the binding of a single molecule of diphosphoglycerate between the two β subunits of hemoglobin (Arnone, 1972). The additional sites on hCG could involve the above-mentioned extra residues on the C-terminus of the β subunit.

(3) Cooperativity. While hLH exhibits negative cooperativity manifested by binding curves which are less steep than statistical, hCG exhibits positive cooperativity, with binding curves steeper than statistical. This difference is due primarily to the differences in the number of ANS binding sites.

(4) Salt Effects. The binding of ANS to hCG is strongly affected by salt concentration, whereas 0.1 *M* KCl had no effect on the equilibrium with hLH, although it did accelerate the dimerization. We have not yet explored the effects of other salts with hLH.

The present finding that the enhancement of ANS fluorescence by hCG is due to self-association of the hormone does not alter the conclusions of an earlier study (Aloj et al., 1973) in which ANS was used as a probe to measure the rates of dissociation and recombination of the α and β subunits. It was shown that under the assay conditions used in those studies, the ANS fluorescence was essentially proportional to the concentration of native (intact) hormone while the subunits had little or no effect. We have since found that the small deviation from linearity in the plot of ANS fluorescence vs. hCG concentration (Aloj et al., 1973) can be eliminated by employing low ionic strength and a higher concentration of ANS (250 μM). The observation that ANS retards the rate of dissociation of the subunits of these hormones at low pH (Ingham et al., 1973) can now be rationalized in terms of dimers and/or tetramers, stabilized by ANS, which must be dissociated before the subunits can be separated. On the other hand, the fact that ANS had no effect on the rate of subunit recombination is compatible with the hypothesis that only the completely refolded native hormone self-associates in the presence of ANS. Finally, the ability of urea to diminish the hormone-enhanced ANS fluorescence (Aloj et al., 1973; Ingham et al., 1973) can now be understood in terms of the expected interference of urea with hormone association.

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Effects of Estradiol on Uterine Ribonucleic Acid Metabolism. Assessment of Transfer Ribonucleic Acid Methylation†

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ABSTRACT: Immature rats treated with estradiol for selected periods of time demonstrated both increased methylation of uterine transfer ribonucleic acid (tRNA) and methylase activities. Whereas the former parameter was assessed by incubating whole uteri with [*methyl*-^{14}C]methionine and measuring the incorporation of isotope into the tRNA, methylase activity was obtained by measuring the rate of incorporation of methyl groups from *S*-adenosyl[*methyl*-^{14}C]methionine into heterologous tRNA (*Escherichia coli* B) in the presence of uterine cytosol preparations (100,000*g* supernatants). Although increased methylation of tRNA during the estrogen response was demonstrated, additional studies indicated that these results were largely attributable to an increased rate of synthesis of tRNA rather than gross changes in either the type or amount of methylated constituents present. Evidence in this regard included the inability of estrogen treatment to alter significantly the (a) resulting patterns of *methyl*-^{14}C-methylated constituents of uterine tRNA, (b) the extent to which [2-^{14}C]guanine residues, incorporated into tRNA, become methylated, (c) the extent of methylation of precursor tRNA in the absence of tRNA synthesis, and (d) the types of methylase activities expressed in vitro.

That modification of tRNA may participate in or render some important regulatory function in cells or tissues is both intriguing and suggestive in view of the findings that the quantity and possibly quality of tRNA methyltransferases undergo significant alterations in a variety of biological systems experiencing dramatic changes in growth processes (Starr and Sells, 1969; Borek, 1971; Randerath and Randerath, 1973). Although the majority of these findings were derived from studies designed primarily to detect differences in methylase activities and, therefore, do not provide sufficient evidence for aberrant or hypermethylated tRNAs, additional support for their existence is suggested by numerous tRNA chromatographic profiles (Sharma and Borek, 1970; Kothari and Taylor, 1973) as well as altered methylation patterns of tRNAs derived from in vivo studies (Bergquist and Matthews, 1962; Craddock, 1969; Borek and Kerr, 1972).

The present investigation assesses the methylation of uterine tRNA which occurs following estrogen treatment of immature rats by examining both the in vitro activity of tRNA methyltransferases as well as by characterizing the types and amount of methylated constituents that appear on newly synthesized uterine tRNA at selected times following the administration of estradiol. The immature rat uterine system was selected for this study since earlier investigations have reported that estrogen treatment promotes increased and/or altered tRNA methylase activities (Lipshitz-Wiesner et al., 1970; Sharma et al., 1971; Baliga and Borek, 1974), altered tRNA chromatographic profiles (Sharma and Borek, 1970), and increased uterine tRNA synthesis and methylation (Billing et al., 1969; Munns and Katzman, 1971b).

Experimental Procedures

Treatment of Animals. Immature female rats, 21–22 days old and weighing 40–50 g, were purchased from National Laboratory Animal Co., Creve Coeur, Mo. After an acclimation period of 24 hr the rats were injected intraperitoneally with a single dose of 10 μg of 17β-estradiol (Sigma Chemical Co.) in 0.25 ml of aqueous 9.5% ethanol. At the prescribed times thereafter, the animals were sacrified by cervical dislocation and within 1 min the whole uterus was explanted free of adhering tissue and collected in ice-cold Eagle's minimum essential medium (MEM) for 5–10 min prior to incubation. The above MEM medium (no. 109, GIBCO) contained all of its defined ingredients except glu-

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