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FLUOROMETRIC STUDY OF TETRACYCLINE—BOVINE SERUM ALBUMIN INTERACTION

THE TETRACYCLINES—A NEW CLASS OF FLUORESCENT PROBES

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Abstract—The interaction of four tetracyclines (tetracycline, oxytetracycline, chlortetracycline and methacycline) with bovine serum albumin is studied fluorometrically. It is demonstrated that these tetracyclines exhibit the main features of fluorescent probes; increase of their fluorescence and quantum yield both in mixtures with bovine serum albumin and in non-polar, organic solvents, accompanied by blue shift of fluorescence emission maximum.

The thermodynamic values ΔH and ΔS of the reaction of binding of tetracyclines to bovine serum albumin have been determined fluorometrically. The results reveal that the binding of tetracyclines to bovine serum albumin is due mainly to non-polar (hydrophobic) interactions.

It has been found that the divalent metal ions are not involved in the binding of tetracyclines to bovine serum albumin at least at pH 10·5.

THE INTERACTION between tetracyclines and proteins has been extensively studied, but a generalized concept is still lacking. Various assumptions have been made regarding the nature of this interaction. Chelate, ionic, covalent, adsorption and hydrophobic bindings are believed to be involved in the reaction.[1–5]

Tetracycline–protein interaction, employing fluorescent methods, has not been widely studied.[1] The method of "fluorescent probes" is based on the discovery of Weber and Laurence,[6] that certain polycyclic aromatic compounds, mainly derivatives of naphthalene, of weak fluorescence or not fluorescent in aqueous solution, emit strong fluorescence in the presence of serum albumin. It was later shown that the fluorescent probes (such as ANS and TNS) bind with hydrophobic (non-polar) sites on the protein molecule through non-covalent bonds. The enhanced fluorescence in this case may serve as a measure ("probe") of the hydrophobicity at the binding site. The sensitivity of probes to changes in the near environment provides valuable information about the conformational changes occurring at the binding site.[7,8]

As was shown in our preliminary communications,[9,10] the binding of tetracyclines to serum albumin is due mainly to hydrophobic interactions. It was also shown that tetracyclines possess the general characteristics of the fluorescent probes and represent a new class of these compounds.

Abbreviations—TC, tetracycline; OTC, oxytetracycline; CTC, chlortetracycline; MC, methacycline; BSA, bovine serum albumin ANS, 1,8-aniline-naphthalene-sulfonate; TNS, 2,6-1-oluidinyl-naphthalene-sulfonate.

MATERIALS AND METHODS

The following reagents were used; Tetracycline hydrochloride and oxytetracycline hydrochloride, were obtained from Pharmachim, Bulgaria; Chlortetracycline hydrochloride, Montecatini, Italy; Methacycline hydrochloride, Pfizer, U.S.A.; Bovine serum albumin, 100 per cent electrophoretically pure, Boehringer, Germany; Quinine sulfate, Fluka, Switzerland; Dimethylsulfoxide, Uvasol–Merck, Germany. All the other chemicals were reagent grade.

The purity of the tetracycline preparations was tested by the chromatographic method of Kelley and Buyske.[11] The calcium, magnesium and zinc content in the solutions employed was assayed with atomic absorption spectrophotometer, Unicam SP-90.[12–14] The fluorescence intensity was measured with spectrofluorometer, Opton-ZFM-4C. The data of fluorescence intensity are expressed in terms of relative units (scale units) and are related to the maximum amplification of the instrument.

One μg/ml quinine sulfate in 0·1 N sulphuric acid served as fluorescent standard. The tetracyclines were dissolved in 0·05 M glycine buffer (pH 10·5), unless otherwise stated. The concentration of the BSA solutions was checked by the biuret method.[15] The fluorescent measurements were carried out at room temperature (22°).

To determine the temperature dependence of the binding of tetracyclines to BSA, water with the desired temperature was passed through the jacket of the fluorometric accessory ZFM-4. The samples, the cuvette holder and the cuvettes were preheated. Immediately after the fluorescence measurements the temperature of the solution in the cuvette was directly checked.

Reading of fluorescence started seven minutes after the dissolution of tetracyclines in glycine buffer. Subsequently, in the case of CTC, MC and OTC, the fluorescence tended to increase slowly, reaching a maximum on the second hour. This slow increase was more pronounced at higher concentrations of the antibiotics. At the maximum concentrations used this increase was negligible and amounted to the following values (120 min after dissolution): for CTC, 9·8%; for MC, 19% and for OTC, 6%. The fluorescence of TC showed no alteration.

The quantum yield was calculated by the method of Parker and Rees.[16] The concentration of the tetracyclines used in this case (0·5 and 1·0 μg/ml) yielded maximum absorbancy at 390 nm below 0·020. The absolute quantum yield of quinine was taken to be 0·55.[17]

RESULTS

First we shall present the results from the experiments performed to check the suitability of the reagents (1–4).

(1) *Chromatographic control of the purity of tetracyclines*

By applying the method of Kelley and Buyske[11] the following R_f-values were obtained: TC, 0·65; CTC, 0·78; OTC, 0·54; MC, 0·86. The good agreement with the literary data for TC, CTC and OTC as well as the appearance of the spots in the u.v.-light, indicated that the preparations studied were chromatographically pure and did not contain any admixtures fluorescent at alkaline pH.

(2) *Spectral characteristics of the tetracyclines*

The data of the wave lengths of excitation, fluorescence and adsorption maxima in nm are respectively, as follows: for TC; 390, 525, 380; for CTC; 395, 525, 380; for

MC; 395, 525, 380; for OTC; 395, 525, 375. These data were in agreement with previous findings.[18]

(3) *Magnesium ions, calcium ions and zinc ions content in the solution used*

It is known that magnesium,[19] calcium and zinc[1] ions increase tetracyclines fluorescence. The estimation of this ion content by atomic-absorption spectrophotometry showed that their content in the tetracycline, BSA and glycine buffer solutions was negligible. The following data were obtained for: Mg^{2+}, below 0·05 μg/ml; Ca^{2+}, below 0·10 μg/ml; Zn^{2+}, below the sensitivity limit of the instrument.

(4) *Changes in tetracycline fluorescence in the presence of magnesium, calcium and zinc ions*

Table 1 reveals the relationship between the tetracycline fluorescence and the concentration of magnesium and calcium ions content in the solution at pH 10·5.

TABLE 1. CHANGES IN TETRACYCLINE FLOURESCENCE IN THE PRESENCE OF DIVALENT METAL IONS

Ions	Ions concentration (M)	Changes in tetracycline fluorescence in the presence of ions–ΔF (relative units)
Magnesium	2·5, 10^{-2}	185
	2·5, 10^{-3}	173
	2·5, 10^{-4}	133
	2·5, 10^{-5}	45·5
	2·5, 10^{-6}	0·0
Calcium	2·5, 10^{-2}	14·5
	2·5, 10^{-3}	60·0
	2·5, 10^{-4}	69·0
	2·5, 10^{-5}	49·0
	2·5, 10^{-6}	11·0

Tetracycline concentration, 5 μg/ml.

Under the indicated experimental conditions magnesium ions produced a larger increase in fluorescence intensity than did the calcium ions. Zinc ions did not affect the fluorescence, although a slight decrease was noted. It was found that magnesium and calcium ions influenced tetracyclines fluorescence to a different extent. For instance, magnesium at 2·5, 10^{-4} concentration yielded the following increments of fluorescence intensity (in terms of relative units): TC, 133; MC, 92; OTC, 26 and CTC, 25. At the same calcium concentration the following values were obtained: TC, 69; OTC, 28; CTC, 25; MC, 15.

The addition of an equivalent amount of EDTA (1 mol EDTA per 1 gramion calcium or magnesium) to the mixtures of tetracycline plus magnesium or calcium completely abolished the effect of these ions.

Effect of tetracycline concentration on fluorescence

Figure 1 shows that initially the fluorescence increased linearly with tetracycline concentration, reached a maximum and then decreased due to inner filter effect.

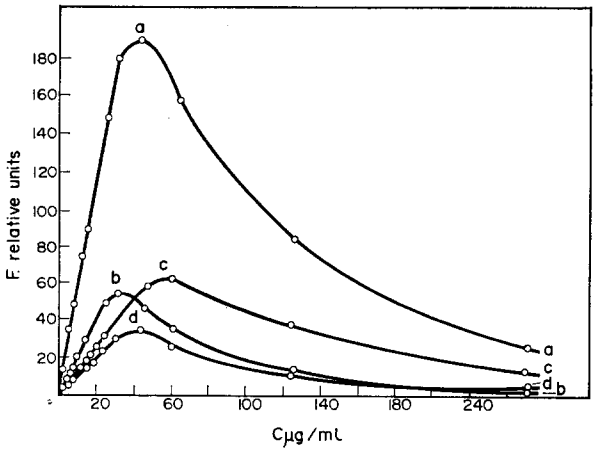

FIG. 1. Relationship between tetracycline fluorescence and their concentration. The concentration varied from 0 to 250 μg/ml. On the abscissa: tetracycline concentration (μg/ml). On the ordinate: fluorescence in relative units. Curves: a, OTC; b, TC; c, MC; d, CTC.

Effect of albumin concentration on the fluorescence of antibiotic–bovine serum albumin mixtures

Figure 2 indicates that BSA caused a manyfold increase in the fluorescence of four tetracyclines. CTC manifested the highest rise, 42-fold; TC, 27-fold; MC, 17-fold

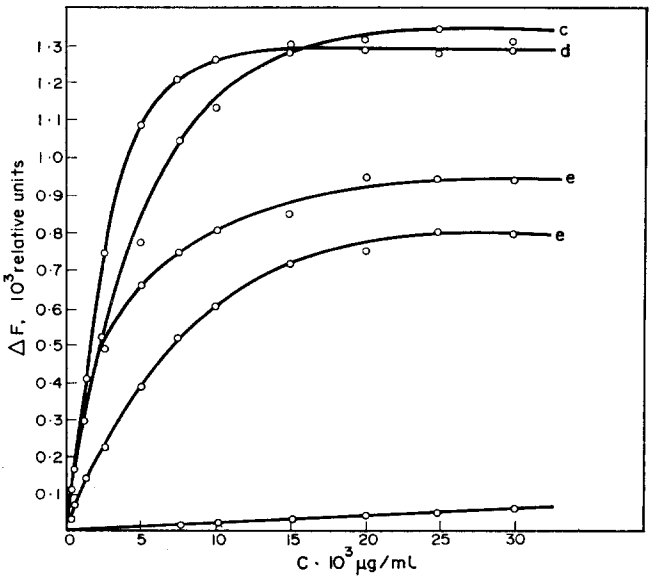

FIG. 2. Relationship between the fluorescence of tetracycline–bovine serum albumin mixture and albumin concentration at a constant tetracycline concentration. The tetracycline concentration is as follows: TC, 25 μg/ml; CTC, 31 μg/ml; OTC, 31 μg/ml and MC, 50 μg/ml. On the ordinate: $\Delta F = F_{mixture} - F_{TC} - F_{BSA}$ expressed in relative units; on the abscissa: bovine serum albumin concentration (μg. 10^3/ml). Curves: a, bovine serum albumin fluorescence; b, ΔF of BSA–TC mixture; c, ΔF of BSA–OTC mixture; d, ΔF of BSA–CTC mixture; e, ΔF of BSA–MC mixture.

and OTC, 4-fold. The corresponding curves represent curves of saturation, occurring at 2·5% (w/v) BSA concentration (molar ratio of antibiotic: BSA, 5:1).

The addition of as little as 0·0025 M EDTA (sufficient to bind a 100-fold larger amount of divalent cations than observed) did not significantly affect fluorescence (Fig. 3). BSA induced 10-fold increase of the quantum yield, both in the absence and presence of EDTA.

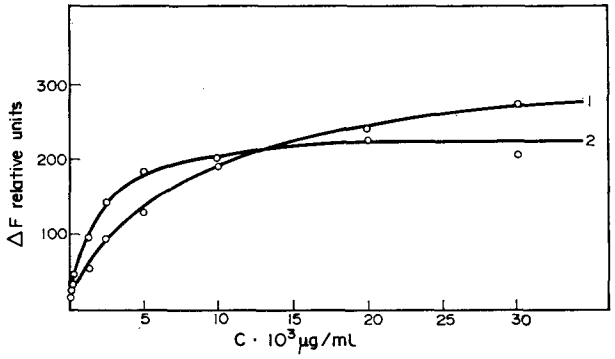

FIG. 3. Relationship between $\Delta F = F_{mixture} - F_{TC} - F_{BSA}$ and BSA concentration (BSA concentration ranging from 0 to 3 10^3 μg/ml) keeping the tetracycline concentration constant (0·5 μg/ml), in the absence of EDTA, curve 1, and in the presence of EDTA, curve 2. EDTA concentration, 0·0025 M. On the abscissa, ΔF in relative units, on the ordinate, bovine serum albumin concentration in μg. 10^3/ml.

To elucidate the nature of this effect the spectral characteristics of the BSA–tetracycline mixtures were recorded as well as those obtained with tetracycline dissolved in different organic solvents.

Tetracycline exhibited a fluorescence maximum at 525 nm, whereas the mixture of BSA (3% w/v final concentration) and tetracycline (0·5 μg/ml final concentration) had a maximum at 480 nm. Therefore, there is a shift towards the short-wave length region (blue shift).

Owing to the insolubility of tetracycline hydrochloride in most organic solvents, subsequent experiments were performed with tetracycline base, m.p. 171–172° (170–173° according to literature data).

Figure 4 demonstrates that the fluorescence of tetracycline base, dissolved in organic solvents, was much higher than that in aqueous solution increasing in the following

TABLE 2. QUANTUM YIELD OF TETRACYCLINE AND OF TETRACYCLINE–BOVINE SERUM ALBUMIN MIXTURES IN THE PRESENCE AND ABSENCE OF EDTA

Solution	Quantum yield
Tetracycline hydrochloride in water	0·001
Tetracycline hydrochloride in 0·05 M glycine buffer (pH 10·5)	0·014
Tetracycline in 0·05 M glycine buffer (pH 10·5) + EDTA	0·007
Tetracycline–bovine serum albumin mixture	0·143
Tetracycline–bovine serum albumin mixture + EDTA	0·082

Tetracycline concentration, 0·5 μg/ml; bovine serum albumin concentration, 3%; EDTA concentration, 0·0025 M.

order: methanol, *n*-octanol, dimethylformamide, dimethylsulfoxide. The enhanced fluorescence was accompanied by blue shift of the fluorescence maximum.

When tetracycline base was dissolved in water–ethanol mixtures, the higher the percentage of ethanol, the more intense the fluorescence (Fig. 5). The quantum yield of tetracycline base, dissolved in organic solvents, is given in Table 3.

TABLE 3. QUANTUM YIELD OF TETRACYCLINE BASE, DISSOLVED IN WATER–ETHANOL MIXTURES AND SOME ORGANIC SOLVENTS

Ethanol in solution (%)	Quantum yield
100	0·140
75	0·056
50	0·008
0	0·002
Tetracycline dissolved in dimethylsulfoxide	0·716
Tetracycline dissolved in dimethylformamide	0·413

Tetracycline concentration, 1 μg/ml.

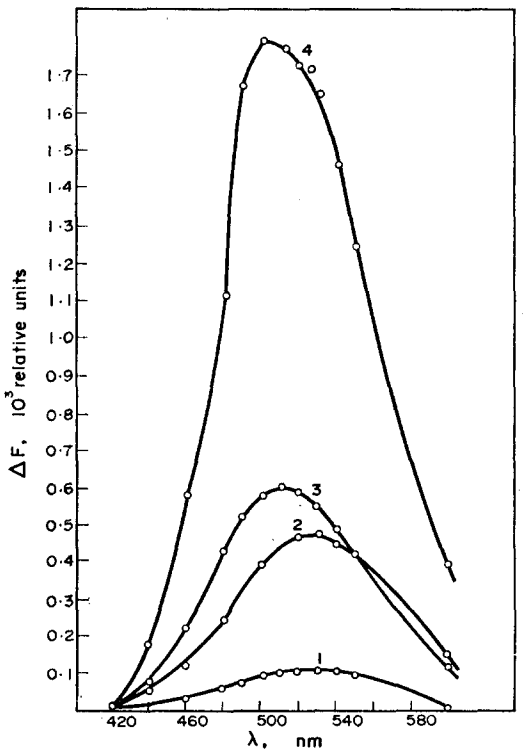

FIG. 4. Emission spectrum of fluorescence of tetracycline base, dissolved in various organic solvents. On the abscissa: wavelengths in nanometers (nm); on the ordinate: $\Delta F = F_{solution} - F_{solvent}$. Curves: 1, solution of TC base in methanol; 2, in *n*-octanol; 3, in dimethylformamide; 4, in dimethylsulfoxide.

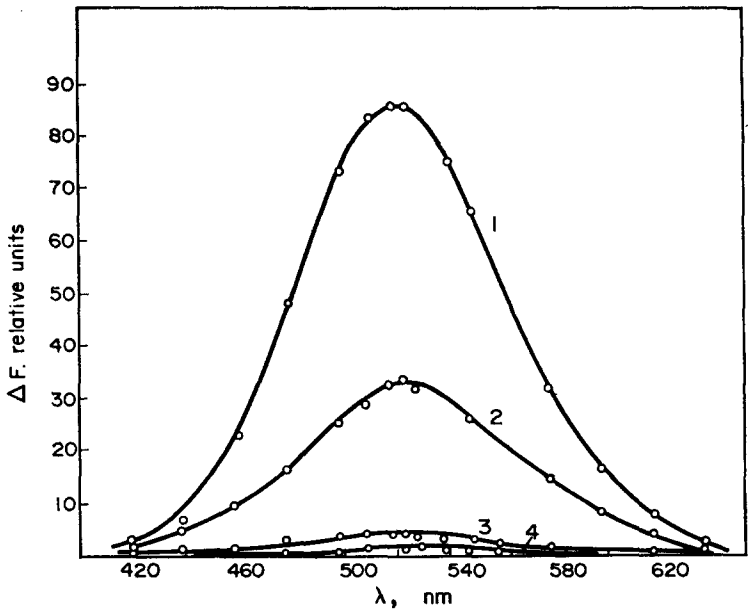

FIG. 5. Emission spectrum of fluorescence of TC base, dissolved in water–ethanol mixtures. On the abscissa: wavelengths in nm, on the ordinate: $\Delta F = F_{\text{solution}} - F_{\text{solvent}}$ in relative units. Curves: 1, 100% ethanol in the mixture; 2, 75% ethanol in the mixture; 3, 50% ethanol in the mixture; 4, 0% ethanol in the mixture.

Effect of the pH on the fluorescence of tetracyclines and their mixtures with bovine serum albumin

The pH optimum of the fluorescence of the tetracyclines–BSA mixtures lay at 10·5, i.e. the same value as that found for the solutions of the tetracyclines themselves. This coincidence gives reason to assume that the tetracyclines are responsible for the increase of fluorescence in mixtures with BSA.

Estimation of the thermodynamic values ΔH *(enthalpy change) and* ΔS *(entropy change) of binding of tetracyclines to bovine serum albumin*

The temperature dependence of the reaction of binding of the four tetracyclines to bovine serum albumin was examined at 4, 17, 37 and 43°. The concentration of the antibiotics was varied, keeping the albumin concentration constant (7·46, 10^{-6} M). The concentration of the tetracyclines was varied from 0 to 40 μg/ml until curves of saturation were obtained.

It is well known that the fluorescence of the molecules is strongly affected by temperature, its rise resulting in a drop of intensity. By determining $\Delta F = F_{\text{mixture}} - F_{\text{antibiotic}} - F_{\text{BSA}}$, we eliminated the effect of temperature on the fluorescence of tetracyclines and determined fluorometrically the extent of their binding by bovine serum albumin at various temperature.

The obtained curves of saturation were normalized from 0 to 1 by applying the formula

$$\bar{Y} = \frac{\Delta F - \Delta F_0}{\Delta F_\sim - \Delta F_0},$$

where $\bar{Y}$ is the relative alteration of the fluorescence of the mixture; ΔF, the fluent value of the fluorescence of the mixture; ΔF_0, the minimum value of the fluorescence of the mixture at a concentration of the antibiotic 0 μg/ml; $\Delta F_\sim$, the maximum value of fluorescence of the mixture at saturation of bovine serum albumin with antibiotics. $\lg K$ is derived from the relation $\bar{Y} = f(\lg C_{M_1}/C_{M_2})$ at $\bar{Y} = 0{\cdot}5$. The data for $\lg K$ for each isotherm allow the calculation of the changes in enthalpy and entropy of binding according to the formulae:

$$\Delta H = -4{\cdot}57 \frac{\lg K_2 - \lg K_1}{(1/T_2 - 1/T_1)} \text{ and } \Delta S = \frac{\Delta H}{T},$$

where ΔH is the enthalpy change; ΔS, the entropy change; K, equilibrium constant; T, absolute temperature; C_{M_1} and C_{M_2}, the molar concentration of the antibiotic and bovine serum albumin, respectively.

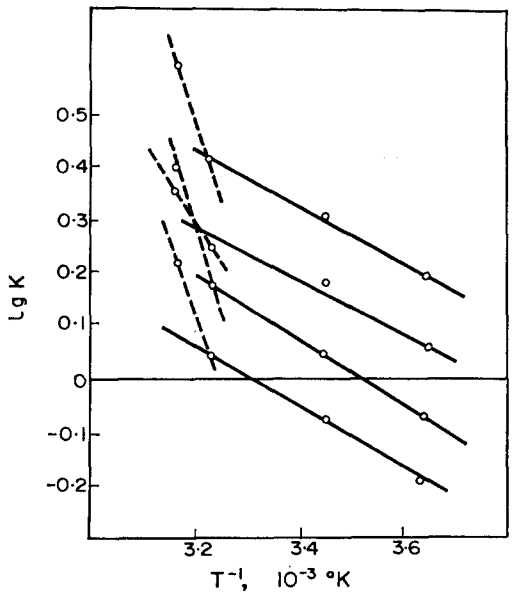

FIG. 6. Dependence of $\lg K$ on the reciprocal value of absolute temperature. On the abscissa: $1/T$; on the ordinate: $\lg K$. Curves: 1, TC–BSA mixture; 2, CTC–BSA mixture; 3, OTC–BSA mixture; 4, MC–BSA mixture.

The relationship between $\lg K$ and $1/T$ is shown in Fig. 6. It is evident that this relationship is linear between 4° and 37°, the curve changing sharply above 37°. The data for enthalpy and entropy changes, calculated for the temperature range 4–37°, are presented in Table 4. The results prove that bovine serum albumin–tetracyclines binding is accompanied by absorption of heat and the change of enthalpy is of the order 2–3 kcal/mol. The differences between ΔH for different tetracyclines are small as compared with the accuracy of the method ($\pm 0{\cdot}5$ kcal/mol).

DISCUSSION

The tetracyclines as fluorescent probes

In the presence of bovine serum albumin the fluorescence of the four antibiotics examined increased manyfold (under optimal conditions: 42-fold for CTC, 27-fold for TC, 17-fold for MC and 4-fold for OTC).

TABLE 4. THERMODYNAMIC VALUES

Antibiotic	Enthalpy change ΔH (cal/mole)	Entropy change ΔS (entropy units)
Chlortetracycline	+2830	9·43
Tetracycline	+2680	8·94
Methacycline	+2530	8·43
Oxytetracycline	+2280	7·60

The coincidence of the pH optima, of both the pure tetracyclines and their mixture with bovine serum albumin, suggests that bovine serum albumin enhances the fluorescence of the tetracycline molecules themselves. The fluorescence of bovine serum albumin itself at the wavelengths of excitation and emission used is negligible.

Enhancement of tetracycline fluorescence by bovine serum albumin although in far lower degree was reported earlier (1) and attributed to the formation of chelate bridges between tetracyclines and bovine serum albumin with the aid of divalent metals.

Our studies demonstrate that, at least at pH 10·5, the enhanced fluorescence induced by bovine serum albumin is not an artefact due to the presence of divalent cations. The following lines of evidence may be presented:

(1) The quantitative assay of Mg^{2+}, Ca^{2+} and Zn^{2+} admixtures in the solutions and mixtures examined shows that the highest content of the above ions observed could yield an increase of fluorescence not higher than 100 relative units, where as the same mixtures yield ΔF of the order of 1000 and more relative units. In other words, no more than 10 per cent of the increase of fluorescence would be due to the effect of these ions.

(2) The addition of EDTA does not significantly alter the effect of enhancement of tetracycline fluorescence, induced by bovine serum albumin (in the presence of EDTA the quantum yield is increased 11·7 times by bovine serum albumin and without EDTA, 10 times). It is worth noting that the addition of an equivalent amount of EDTA results in the complete removal of the effect of divalent ions on tetracycline fluorescence in the absence of bovine serum albumin.

Consequently, a chelate bridge BSA-Me^{2+}-TC cannot be formed in the presence of EDTA, because the Me^{2+}-EDTA complex is more stable than the Me^{2+}-TC complex.

Divalent cations added to tetracycline solutions even in a very large excess (in 1000-fold higher concentrations than those assayed) did not yield an increase of fluorescence reaching that of bovine serum albumin–tetracycline mixtures.

It is obvious that the enhanced fluorescence of tetracyclines caused by bovine serum albumin cannot be accounted for by the formation of chelate metallic bridges between bovine serum albumin and tetracycline.

It has recently been assumed[5] that the bonds between tetracyclines and proteins result mainly from hydrophobic (non-polar) interactions.

It is known, however, that fluorescent probes, such as ANS and TNS increase their fluorescence greatly in the presence of some proteins, the enhancement serving as a measure for the hydrophobicity of the site of binding.

The fluorescent probes exhibit the following characteristic features:

(1) Manyfold increase of their fluorescence (quantum yield respectively) in the presence of some proteins.

(2) A blue shift of the emission (fluorescence) maximum of the "probes" in the presence of proteins.

(3) Increased fluorescence accompanied by blue shift of the "probes" dissolved in non-polar solvents as compared with their aqueous solutions.

Our experiments prove that the tetracyclines possess all the features of fluorescent probes.

Nature of binding between tetracyclines and bovine serum albumin

The reaction between tetracyclines and bovine serum albumin as evidenced from the temperature dependence is endothermic. The experimental values for ΔH were of the order 2–3 kcal/mole. The low values for the enthalpy changes show that bovine serum albumin–tetracycline interaction leads to the formation of non-covalent bonds. These data, the positive entropy changes (7–9 entropy units) as well as the fact that the tetracyclines belong to the group of fluorescent (hydrophobic) probes, give grounds to assume that tetracycline binding to proteins proceeds mainly at the expense of hydrophobic interactions.

Figure 6 shows that there is an abrupt change in the binding center (or centers) at 43°. This observation is of interest in itself.

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