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Fluorescence of 3-keto-steroids in aqueous solution

Probes for steroid-protein interactions*

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Abstract. The physiologically important 3-keto-steroids are non-fluorescent or only weakly fluorescent in protic as well as in aprotic solvents. In contrast, the 4,6,8(14)-triene-3-one steroids are highly fluorescent in aqueous solution but they do not appreciably fluoresce in other solvents. Evidence is presented that the introduction of double bonds into the skeleton of the 3-keto-steroids leads to a decrease of the energy of the lowest $\pi-\pi^*$ state, bringing this level into the neighbourhood of the non-fluorescent $n-\pi^*$ state. As a consequence, for two states of approximately the same energy, relatively small perturbations such as those due to solvent interactions, protein binding and micelle formation, will then determine whether a system will fluoresce ($\pi-\pi^*$ state lowest) or not ($n-\pi^*$ state lowest). When the fluorescent 3-keto-steroids, having three conjugated double bonds, bind to proteins, the fluorescence intensity becomes almost zero, making these compounds useful as probes for steroid-protein interactions. This quenching of the fluorescence is explained by a decrease in energy of the $n-\pi^*$ state relative to the $\pi-\pi^*$ state of the steroids due to hydrophobic interactions with the proteins.

Key words: Fluorescent steroid probes, steroid-protein interactions, energy alternation of $n-\pi^*$ and $\pi-\pi^*$ states (level crossing)

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Dedicated to Prof. Dr. F.-W. Zilliken on the occasion of his 65th birthday

Abbreviations: 6,8-BDT, 6,8-bisdehydrotestosterone; DMSO, dimethylsulfoxide; HPLC, high pressure liquid chromatography

1. Introduction

The 3-keto-steroids occurring in nature (such as sex-hormones and corticoids) either do not fluoresce or fluoresce only very slightly (with quantum yields $\phi_F \sim 10^{-3}$ to 10^{-4}) in aqueous solution at room temperature. The fluorescence quantum yield in water is much smaller than that in other solvents such as ethanol (Schröder 1984) or *n*-hexane (Palluk 1983), where relatively low yields of the order of 0.01 are found.

Recently, we discovered a new class of steroids, the 4,6,8(14)-triene-3-one steroids (Müller et al. 1982; Palluk 1983), which are strongly fluorescent in water but virtually non-fluorescent in aprotic solvents. A number of other systems showing similar behaviour have been reported in the literature. Examples are acridine and pyrene-1-aldehyde, molecules not normally encountered in biological studies (Bredereck et al. 1962; Mataga and Kubota 1970; Dederen et al. 1979). With these molecules, which have a $n-\pi^*$ state as the lowest excited singlet state in aprotic solvents, the strong enhancement of the fluorescence intensity in water has been attributed to a blue shift of the $n-\pi^*$ state due to hydrogen bonding. This then leads to a reversal of the energies of the non-fluorescent $n-\pi^*$ and the fluorescent $\pi-\pi^*$ states.

Whereas it has already clearly been demonstrated that these fluorescent steroids are versatile and sensitive tools when investigating interactions of steroids with proteins or cyclodextrins (Palluk et al. 1982; Kempfle et al. 1984; Müller et al. 1985, 1986), the question as to the physical and/or chemical processes responsible for the phenomenon mentioned above has not yet been discussed and will be treated in this paper.

It is important to note that the biological properties (such as, for example, the enzymatic activity) of the fluorescent and the non-fluorescent steroids dif-

fer only to a minor extent. This is illustrated by the two following observations: (a) The fluorescent 3-keto-steroids are metabolized by β-hydroxysteroid-dehydrogenase (EC 1.1.1.51 from Pseudomonas testosteroni) in a manner similar to that observed with the non-fluorescent steroids. The V_{max} and K_M values of the former steroids differ only by a factor of two (Kempfle et al. 1985); (b) The binding of the 4,6,8(14)-triene-3-one steroids to antibodies that are specific for 3-keto-steroids occurs with a relative affinity of about 0.8 as compared with the normal steroids (Müller et al. 1982; Palluk 1983).

These results are seen to support our view that the structural differences introduced in the steroids by incorporating conjugated double bonds into the skeleton, lead to much smaller changes in the natural behaviour of the steroids than those caused by other modifications reported in the literature (Schröder 1984).

Another property which makes these compounds superior as probes for steroid-protein interactions, as compared with other probes that are not structurally related to steroids, should be mentioned. Upon binding to specific antibodies, to binding proteins, or to cyclodextrins (Palluk et al. 1982; Müller et al. 1985) the fluorescence of the 3-keto-steroids disappears completely. The addition of the non-fluorescent natural steroids mentioned above to the virtually non-fluorescent solutions of 3-keto-steroids bound to proteins, leads to an increase in the overall fluorescence intensity. This is because a fraction of the fluorescent 3-keto-steroids is displaced by the natural steroids – and only those probe molecules which are not bound to the protein show fluorescence, as discussed above. In this manner it is possible to study steroid-protein interactions (which result in a change in the fluorescence intensity) by titration with non-fluorescent steroids. Using this simple and efficient procedure, the detection limit of these fluorescent steroids lies in the range of 10^{-11} to 10^{-12} moles/l (or 10 pg/ml) (Müller et al. 1982).

The observations that the 4,6,8(14)-triene-3-one steroids show (a) virtually no fluorescence in aprotic solvents, (b) strong fluorescence in water and, (c) no fluorescence in aqueous solutions upon binding of the steroids to proteins, will be discussed in the present paper.

2. Materials and methods

The fluorescent 4,6,8(14)-triene-3-one steroids were generous gifts from Schering AG, Berlin, from Roussel-Uclaf, Paris and from Duphar, Amsterdam. Ergosta-4,6,8(14),22-tetraene-3-one was isolated from Fungus Laricis (Schulte et al. 1968) or was synthesized following the method of White et al. (White and Taylor 1970). The substance was purified by repeated "reversed phase" HPLC.

The solvents D_2O, dimethylsulfoxide (DMSO), ethanol, methanol (Merck); formamide, dimethylformamide, tetrahydrofuran, acetone, 1,4-dioxane, isopentane, *n*-hexane and cyclohexane (Baker) were the best commercially available qualities suitable for fluorescence studies.

Absorption spectra were recorded on a Cary 14 or a Cary 118C spectrophotometer having a thermostatted cell compartment. Fluorescence spectra were measured using a Hitachi-Perkin Elmer MPF-4 or MPF-2A spectrofluorimeter (MPI Göttingen) equipped with a temperature control unit for the cuvettes. All solutions were deoxygenated by bubbling with dry nitrogen gas for 20 min.

The fluorescence quantum yields (ϕ_F) were determined at 20 °C employing a quantum-corrected FICA 55 spectrofluorimeter, using quinine sulfate as a standard ($\phi_F = 0.55$ in $0.1\,N$ H_2SO_4). In these measurements, solution and standard had identical optical densities at the excitation wavelength. A correction for the difference in solvent refractive index (n^2) was taken into account (Demas and Crosby 1971; Birks 1977).

3. Results and discussion

The fluorescence spectrum of 6,8-bisdehydrotestosterone (6,8-BDT) in water is shown in Fig. 1. For comparative purposes, the fluorescence spectrum of this compound in methanol is also shown, as an example of the considerably lower quantum yield of 6,8-BDT in non-aqueous protic solvents (see Table 1). In aprotic solvents, such as the non-polar *n*-hexane and the polar solvent acetone, fluorescence of 6,8-BDT could not be detected (Table 1).

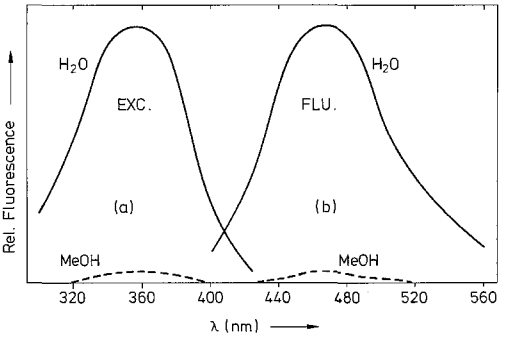

Fig. 1. Fluorescence spectra of 6,8-bisdehydrotestosterone in H_2O (*full lines*) and in methanol (*dashed lines*); concentration: 10 ng/ml; $T = 20.0$ °C. (**a**) excitation ($\lambda_{emission} = 465$ nm), (**b**) emission ($\lambda_{excitation} = 355$ nm)

Table 1. Fluorescence and absorption data of 6,8-bisdehydrotestosterone in different solvents at 20 °C

Solvent	Fluorescence quantum yield ϕ_F [a]	Absorption	Fluorescence intensity maxima	
		λ_{max} [nm]	$\lambda_{excitation}$ [nm]	$\lambda_{emission}$ [nm]
H_2O, D_2O	0.27	355	355	468
Methanol	0.008	346	346	464
Ethanol	0.003	346	346	464
DMSO	–	346	–	–
Formamide	0.001	351	–	–
Dimethyl-formamide	0.001	351	–	–
Tetrahydro-furan	–	334 2 maxima	–	–
Isopentane	–	320	–	–
n-hexane	–	320	–	–
Cyclohexane	–	320	–	–
Dioxane	–	320	–	–
Acetone	–	320	–	–

Concentration: 400 ng/ml

[a] Fluorescence quantum yield ϕ_F, with quinine sulfate in 0.1 *N* H_2SO_4 as a standard ($\phi_F = 0.55$)

In the absorption spectrum of 6,8-BDT in H_2O the lowest energy maximum is observed at 355 nm (Table 1). The excitation spectrum of 6,8-BDT in water, shown in Fig. 1, is identical to the absorption spectrum (see Table 1). In methanol, as also depicted in Fig. 1, the absorption and excitation spectra are likewise identical (Table 1). These observations can be considered as proof that the fluorescence, especially in alcohols where 6,8-BDT fluoresces only weakly, indeed originates from this compound and not from an impurity. It is of interest to note, in this connection, that under some circumstances excitation spectra can be more easily obtained than normal absorption spectra, such as for instance in turbid solutions not uncommon in biochemical preparations.

Many molecules of biological interest possess $n-\pi^*$ states, due to the presence of nitrogen or oxygen atoms having lone-pair non-bonding electrons. The question of whether in compounds having $n-\pi^*$ and $\pi-\pi^*$ states, such as the 3-keto-steroids, fluorescence can be observed or not, is governed by the relative energies of these states (Becker and Kasha 1955; Mataga and Kubota 1970). It has been shown (Kasha 1949) that molecules with a $n-\pi^*$ state as the lowest excited singlet state are generally only weakly fluorescent or do not fluoresce at all.

For some molecules, as mentioned in the Introduction, the virtually non-fluorescent $n-\pi^*$ state may have the lowest energy in aprotic solvents, whereas in protic solvents such as water, the $\pi-\pi^*$ state may become the lowest state, i.e. a state reversal can take place. The fluorescence intensity of such molecules is then expected to increase strongly when going from aprotic to protic solvents such as, especially, water.

Unfortunately, the $n-\pi^*$ state of the 3-keto-steroids discussed here cannot easily be detected in the absorption spectra (Fig. 2), due to the fact that the $n-\pi^*$ absorption band ($\log\varepsilon \sim 1.4$; Bredereck et al. 1962) is hidden under the much stronger $\pi-\pi^*$ absorption bands ($\log\varepsilon \sim 4.4$; see Table 2).

3.1. 3-keto-steroids in pure solvents

In addition to 6,8-BDT, a series of other 3-keto-steroids has been investigated and, similarly, these compounds were found to be strongly fluorescent in water but virtually non-fluorescent in aprotic solvents (see Table 2). All of these fluorescent steroids

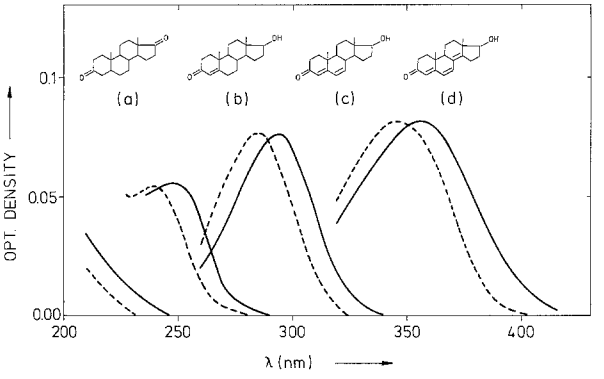

Fig. 2. Absorption spectra of the androstane-3-one series with (**a**) none –, (**b**) one –, (**c**) two – and (**d**) three double bonds in the ring system. Solvents: H_2O (*full lines*) and methanol (*dashed lines*); concentration: 400 ng/ml ($\hat{=} 1.4\times10^{-6}$ moles/l), $T = 20.0$ °C

Table 2. Fluorescence quantum yield (ϕ_F) of various 3-keto-steroids in H_2O at 20 °C

Compound [a]	Steroids (trivial names)	ϕ_F [b]
1 b	6,8-bisdehydrotestosterone	0.27
2 b	6,8-bisdehydrotestosterone-acetate	0.16
3 b	6,8-bisdehydroandrostenedione	0.17
5 b	17α-hydroxy-6,8-bisdehydroprogesterone	0.15
6 b	11-desoxy-6,8-bisdehydrocorticosterone	0.15
	6,8-bisdehydro-19-nor-testosterone	0.27
	9β,10α-6,8-bisdehydroprogesterone	0.15
	methyltrienolone, R1881	0.003
7 b	ergosta-4,6,8(14),22-tetraene-3-one	0.001
		0.003 (ethanol)

[a] Molar extinction coefficient, $\varepsilon = 26{,}500$ lmole^{-1} cm^{-1}, in the absorption maximum of the steroids at 355 nm. The numbers of the compounds are the same as those in the legend of Fig. 3. Concentration: 100 ng/ml

[b] Relative to quinine sulfate in 0.1 *N* H_2SO_4 ($\phi_F = 0.55$)

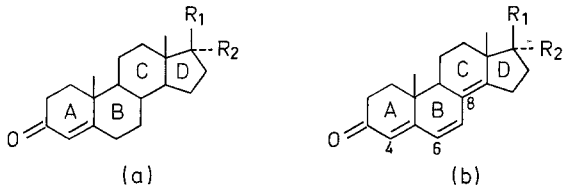

Fig. 3. General structure of natural (**a**) and fluorescent steroids (**b**) investigated here (only the trivial names are given):
1. R_1: $-OH$, R_2: $-H$; (**a**) testosterone, (**b**) 6,8-bisdehydrotestosterone
2. R_1: $-O-CO-CH_3$, R_2: $-H$; (**a**) testosterone-acetate, (**b**) 6,8-bisdehydrotestosterone-acetate
3. R_1, R_2: $=O$; (**a**) androstenedione, (**b**) 6,8-bisdehydro-androstenedione
4. R_1: $-CO-CH_3$, R_2: $-H$; (**a**) progesterone, (**b**) 6,8-bisdehydroprogesterone
5. R_1: $-CO-CH_3$, R_2: $-OH$ (**a**) 17α-hydroxyprogesterone, (**b**) 17α-hydroxy-6,8-bisdehydroprogesterone
6. R_1: $-CO-CH_2OH$, R_2: $-H$; (**a**) 11-desoxycorticosterone, (**b**) 6,8-bisdehydro-11-desoxycorticosterone
7. R_1: $-C_9H_{17}$, R_2: $-H$; **a**) ergosterone, (**b**) ergosta-4,6,8(14),22-tetraene-3-one

do have a keto-group in position 3 and possess three conjugated double-bonds in the 4-, 6- and 8(14)-positions of the steroid skeleton (Fig. 3). The 3-keto-steroids with less than three conjugated double bonds in the skeleton show the same fluorescence behaviour as the natural steroids, i.e. they are virtually non-fluorescent in water (Müller et al. 1983).

In the 3-keto-steroids having three conjugated double bonds, the occurrence of fluorescence in aqueous solution is attributed to a decrease in energy of the allowed $\pi-\pi^*$ state, shifting it below the non-fluorescent $n-\pi^*$ state. Theoretical calculations on aldehydes with conjugated double bonds (Inuzuka and Becker 1974) have shown that the introduction of double bonds into such systems decreases the energies of the $\pi-\pi^*$ states very strongly, whereas the $n-\pi^*$ states remain practically unaffected.

The presence of three or more conjugated double bonds, however, is not a sufficient condition to lead to the intensification of fluorescence, as can be deduced from the observation that nonatetraenal, an aldehyde with four conjugated double bonds, does not show any fluorescence (Kempfle M, unpublished results). It is, therefore, concluded that the rigid ring-system in the steroids plays an important role in the intensification of the fluorescence, although theoretical investigations directed to this problem in steroids have not yet been carried out.

For the only weakly fluorescing steroids with less than three conjugated double bonds (see above), it is therefore concluded that the energy of the $\pi-\pi^*$ state is higher than that of the $n-\pi^*$ state. This influence on the energy of the $\pi-\pi^*$ state of the number of conjugated double bonds is illustrated by the absorption spectra shown in Fig. 2. Introduction of double bonds into the 3-keto-steroid skeleton, progressively shifts the maximum of the lowest-energy $\pi-\pi^*$ absorption band to longer wavelengths, from around 250 nm for a single double bond to approximately 350 nm for three conjugated double bonds (see Fig. 2). In water the absorption band of all compounds is shifted more to the red than in methanol.

The red shift of the lowest-energy absorption bands ($\pi-\pi^*$) in the spectra with increasing number of double bonds clearly demonstrates a decrease in the energy of the $\pi-\pi^*$ state (Fig. 2). Furthermore, it can be seen from this figure that the energy shift in water (full lines) goes parallel to that in methanol (dashed lines, Fig. 2). From the fact that the spectra are more strongly red shifted in water than in methanol (see above) it is concluded that the $\pi-\pi^*$ state in water is of lower energy than in methanol. Further, it is deduced that the energies of the $\pi-\pi^*$ and $n-\pi^*$ states are close to each other and small energy shifts can then have large effects. This explains that the 3-keto-steroids fluoresce more strongly in water than in methanol.

The quantum yields of a number of these fluorescent steroids are listed in Table 2. When only the residues in the C17 position are varied (for example, compounds 1 and 2 or 3), the quantum yields are observed to differ only by a factor of two, whereas the absorption spectra remain unchanged.

It is of interest to point out here that the 3-keto-steroid methyltrienolone (Table 2), although having three conjugated double bonds, shows only weak fluorescence. The behaviour of this compound is currently under investigation. The other compound listed in Table 2 that is not strongly fluorescent, ergosta-4,6,8(14),22-tetraene-3-one, will be discussed in a subsequent section.

3.2. Temperature dependence of the fluorescence of 6,8-bisdehydrotestosterone

The temperature dependence of the fluorescence intensity of 6,8-bisdehydrotestosterone (6,8-BDT) in methanol and in water is shown in Fig. 4 (cf. previous section). The fluorescence intensity of 6,8-BDT increases strongly upon cooling, in water as well as in methanol. In methanol an increase of more than a factor of 10 is found upon lowering the temperature from 20° to −90 °C. In connection with these observations, the maxima of the fluorescence band and of the lowest-energy absorption band shifts to the red when lowering the temperature (see Fig. 5). This means that decreasing the temperature has a similar effect, although to a much smaller extent, as introducing double bonds into the steroid

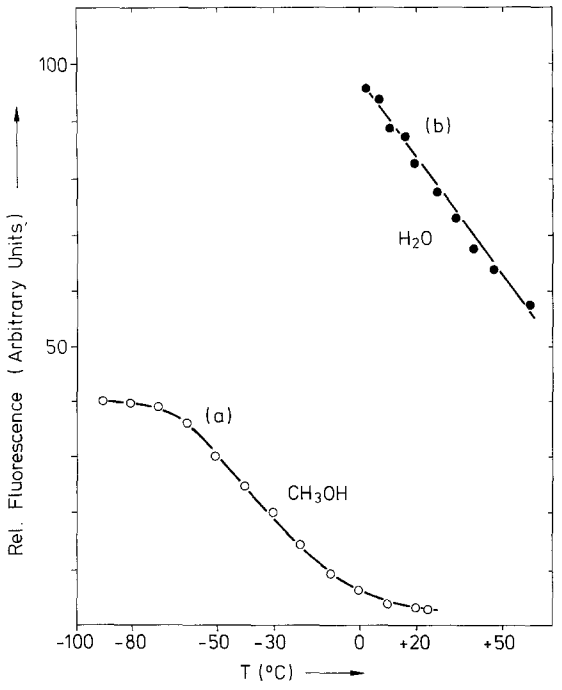

Fig. 4. Temperature dependence of the fluorescence intensity of 6,8-bisdehydrotestosterone (**a**) in methanol, (**b**) in H_2O; concentration: 400 ng/ml, $\lambda_{excitation} = 355$ nm

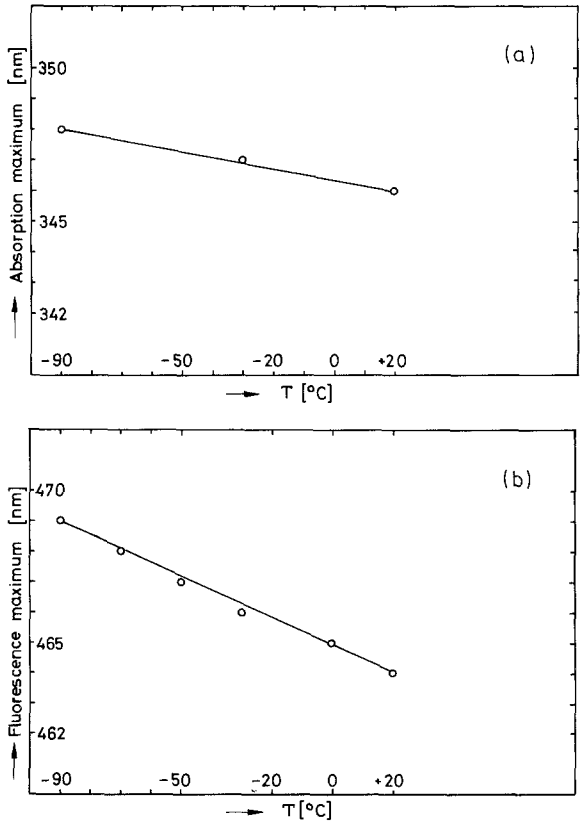

Fig. 5. The wavelength dependence of absorption (**a**) and fluorescence (**b**) spectra of 6,8-bisdehydrotestosterone (concentration: 400 ng/ml) in methanol as a function of temperature

skeleton, i.e. it leads to a further lowering of the energy of the $\pi-\pi^*$ state relative to the $n-\pi^*$ state (see Fig. 2).

As Bredereck et al. (1962) have already pointed out, the energy of the non-fluorescent $n-\pi^*$ state remains practically unchanged upon decreasing the temperature, whereas, as discussed above, the $\pi-\pi^*$ state shifts to the red (Fig. 5). The fact that this procedure leads to a reversal of the energies of the $n-\pi^*$ and $\pi-\pi^*$ states, results in a large increase in fluorescence intensity.

3.3. Decrease of fluorescence intensity upon binding of the steroids to proteins

As was reported recently (Palluk et al. 1982; Palluk 1983) the fluorescence of the 3-keto-steroids vanishes almost completely when they are bound to a specific protein. This observation cannot be explained by an energy shift of the $\pi-\pi^*$ state alone, bringing it above the $n-\pi^*$ state, as a shift in the absorption spectrum after saturation of the protein with steroid is not observed. It has therefore to be concluded that it is the $n-\pi^*$ state that changes its energy, making it the state with the lowest energy in the system. It should be noted here that such a red shift of the $n-\pi^*$ state is exactly the opposite of the blue shift caused by hydrogen bonding in aqueous solution. Since it is generally assumed that protein-steroid interactions are hydrophobic in nature (Westphal 1978), the red shift of the non-fluorescent $n-\pi^*$ state postulated here is thought to be caused by these hydrophobic interactions. The steroids are thereby brought out of contact with the water and hence become non-fluorescent. As already mentioned above, the $n-\pi^*$ absorption is supposed to be hidden under the $\pi-\pi^*$ absorption in these steroid (as well as in other aldehydes). Consequently, the postulated red shift of the $n-\pi^*$ state cannot be observed by inspection of the absorption spectra.

The decrease of the fluorescence intensity upon binding of the steroids to proteins, as described here, is of considerable interest in the investigation of biochemical systems. Displacing the bound, and therefore quenched, fluorescent steroid by a non-fluorescent natural steroid leads to an increase in the overall fluorescence intensity which is due to the free fluorescing 3-keto-steroid. These protein-steroid interactions can, in this manner, easily be investigated (Müller et al. 1985).

3.4. Ergosta-4,6,8(14),22-tetraene-3-one. Fluorescence quenching due to micelle formation

Another system of biological interest is the naturally occurring ergostatetraene-3-one (Schulte et al. 1968).

This compound has an aliphatic side chain attached to the C17-position, a chain which is structurally related to the cholesterol side chain.

The ergostatetraene-3-one fluoresces only weakly in ethanol (see Table 2). Surprisingly, this compound fluoresces even more weakly in water. This is thought to be due to the fact that this molecule forms micelles in aqueous solution, similar to what has been observed with bile salts, which are derivatives of cholic acid having a rigid cholesterol-like ring structure (Chen et al. 1975). Upon addition of ethanol to an aqueous solution of the ergostatetraene-3-one, the fluorescence intensity increases. A maximum in the fluorescence intensity, approximately 300 times higher than in pure water, is reached in a mixture of 30% ethanol and 70% H_2O. Addition of ethanol to the aqueous solution is thought to destroy the micelles until pure ethanol only weakly fluorescing (Table 2) monomers will be present. It is concluded that upon micelle formation the ergostatetraene-3-one is solubilized in an environment with a considerably reduced water content. In such a medium, as discussed above, the $n-\pi^*$ state of the system will have the lowest energy, explaining the low fluorescence intensity.

The properties described here make this compound useful as an intrinsic fluorescence label in micellar systems. The critical micellar concentration ($cmc = 5 \times 10^{-9}$ moles/l) and the kinetics of micelle formation are presently being studied by T-jump and stopped-flow relaxation techniques (Kempfle M and Winkler H, in preparation).

4. Conclusion

It has been shown in this paper that fluorescence can be a very sensitive tool for determining protein-ligand interactions, when there occurs, such as with the 4,6,8(14)-triene-3-one steroids, an important change in the fluorescence yield between free steroids and those bound to proteins. The ability of a system to fluoresce, i.e. its fluorescence quantum yield, is determined by the nature of its lowest excited singlet state. Systems having only $\pi-\pi^*$ states, such as for example aromatic hydrocarbons, will normally fluoresce with comparable quantum yield in protic as well as in aprotic solvents. On the other hand, in systems possessing $n-\pi^*$ as well as $\pi-\pi^*$ singlet states, the separation and the relative order of the $n-\pi^*$ and the $\pi-\pi^*$ state energies determines the ability of those systems to exhibit fluorescence. For the 4,6,8(14)-triene-3-one steroids it has been demonstrated here that this separation in energy is sufficiently small to make these compounds non-fluorescent in aprotic solvents but fluorescent in water.

The change in relative order of the energies of the $n-\pi^*$ and $\pi-\pi^*$ states can occur in two different ways, depending on the situation whether the $n-\pi^*$ state or the $\pi-\pi^*$ state has the lowest energy: (a) by decreasing the energy of the $\pi-\pi^*$ state, as shown by the red shift of the lowest energy band in the absorption spectrum of the 3-keto-steroid upon introduction of double bonds and a simultaneous increase in the $n-\pi^*$ state energy due to hydrogen bonding, or (b) by decreasing the energy of the $n-\pi^*$ state leading to a reversal in energy with the $\pi-\pi^*$ state, as given as a possible explanation for the fluorescence quenching of the 3-keto-steroids after binding to a specific protein.

It can now be understood that in systems where the $n-\pi^*$ and $\pi-\pi^*$ state energies are sufficiently close together, relatively small changes in energy lead to drastic effects in fluorescence behaviour. This phenomenon is the basis of a method developed here to study protein-steroid-interactions quantitatively, even in a concentration range (about 10 pg/ml) (Müller et al. 1982) which previously was only accessible with radioactively-labelled compounds. In addition, the pronounced changes in fluorescence intensity upon binding of the steroid to proteins and upon micelle formation, make it possible to perform kinetic experiments, down to the microsecond time range. It is expected that the observations presented here will lead to further applications of this technique, such as for example in the field of bioluminescence.

It might be possible, based on the present results, to synthesize other molecules having $\pi-\pi^*$ and $n-\pi^*$ states of approximately equal energy, to act as better probes for protein-steroid interactions. Furthermore, we are led to the assumption that steroids with four conjugated double bonds in their ring system will fluoresce equally well in both aqueous and non-aqueous solvents. The reasoning behind this assumption is that the $\pi-\pi^*$ state energy in such molecules is expected to be invariably the lowest one irrespective of the nature of the solvent.

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