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Characterization of the hydrophobic interaction of steroids with endoplasmic reticulum membranes by quenching of 6,8(14)-bisdehydro-17α-hydroxyprogesterone fluorescence

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The fluorescence behaviour of 4,6,8(14)-trien-3-one steroids, which exhibit fluorescence in protic media but no fluorescence in hydrophobic environments, was used to characterize the molecular nature and temperature-sensitivity of steroid hormone–biomembrane interactions. Since 17α-hydroxyprogesterone as the key intermediate is known to accumulate in smooth endoplasmic reticulum membranes in the course of adrenal and testicular steroid hormone biosynthesis, its fluorescent analogue, 6,8(14)-bisdehydro-17α-hydroxyprogesterone (BDHP), was used as the probe molecule. With rat testis microsomal membranes and liposomes, fluorescence quenching in the presence of membranes (related to fluorescence in aqueous solution) was independent on steroid concentration but was dependent on membrane lipid concentration in terms of a hyperbolic function. Complete fluorescence loss occurred at infinite lipid concentration at 20°C, indicating complete insertion of the steroid probe into the hydrophobic portion of the membrane compartment. The partition coefficient K_P increased with increasing temperature as a consequence of increased membrane fluidity. The result that BDHP fluorescence decreased considerably with elevated temperature in both the aqueous and the membrane milieu was interpreted as the consequence of increasing molecular mobility; this effect was much more pronounced in the aqueous than in the membrane environment. On the basis of local BDHP concentrations within the membrane phase (calculated from K_P), relative fluorescence quenching was over-proportional at low temperatures; under that condition, hydrophobic interactions with rigid membrane lipid domains are obviously favoured.

Introduction

Accumulation of steroids in biological membranes gives rise to a series of relevant phenomena which are either consequences of changes in membrane structure or of steroid microcompartmentation within the cell [1]. Dependent on functional groups and overall structure of the steroid skeleton [2], steroids in biomembranes are more or less effective modulators of membrane structure and fluidity [3] with the consequence of varying ion permeability and membrane conductivity [4], enzyme-enzyme coupling [5] and lateral or rotational mobility of proteins [6], or capacity to accumulate further ligands, for instance substrates for enzymes involved in hepatic phase I biotransformation reactions [7]. On the other hand, steroid accumulation in biomembranes may generate a local substrate pool for enzymes involved in further steroid metabolism such as sterol biosynthesis in fungal and yeast cell membranes [8] or steroid hormone synthesis by enzymes localized in the inner mitochondrial and smooth endoplasmic reticulum membranes of adrenals, ovaries and testes [1,6,9].

There are several reports describing both quantitative and thermodynamic aspects of steroid association with and accumulation within biomembranes [1,10–14]. However, those studies have not yet produced an unequivocal conception as to the positioning (i.e., depth of insertion, relative interaction with membrane constituents, relation of hydrophobic and hydrophilic interactions) of steroid hormones accumulating in biological membranes, especially the endoplasmic reticulum. Exact knowledge of those interactions would be advantageous to define the structure of the steroid hormone pool which serves as the substrate pool for steroidogenic enzymes [15,16], and further to help to solve the problem whether the substrate-access channels of cer-

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tain steroidogenic enzymes are facing hydrophobic or hydrophilic domains of their respective membrane environment [1,7,12,13,17].

Fluorescent sterols have often been used to probe membrane structure [18]. In this study, we utilize a member of a new class of steroid molecules, i.e., the 4,6,8(14)-trien-3-one steroids, in order to get further insight into steroid hormone association with endoplasmic reticulum membranes. Those steroids have been characterized as being highly fluorescent in a protic but non-fluorescent in an aprotic milieu, and they can therefore be used to probe the hydrophobic nature of steroid–protein interaction as well as steroid inclusion into cyclodextrins [19,20]. The association of such a steroid, namely 6,8(14)-bisdehydro-17α-hydroxyprogesterone (BDHP), with endoplasmic reticulum membranes and liposomes prepared from rat testis is described here, emphasizing its temperature-dependence and the relation of steroid partition and fluorescence quenching. BDHP was chosen as a paradigm since its natural analogue, 17α-hydroxyprogesterone, is an important intermediate in testicular androgen biosynthesis [9,15,16,21].

Materials and Methods

The fluorescent steroid probe, 6,8(14)-bisdehydro-17α-hydroxyprogesterone (BDHP; 17α-hydroxy-4,6,8(14)-pregnatriene-3,20-dione) was a generous gift from Schering, Berlin. All spectral measurements were made in 20 mM Tris + 250 mM sucrose buffer (medium A; pH 7.4). BDHP solutions were made up from a 1 mg/ml ethanolic stock solution. Absorption spectra were recorded with a Shimadzu UV-300 spectrophotometer; for routine measurements, absorption was read at 365 $\pm$ 1 nm. Fluorescence excitation and emission spectra were recorded with a Perkin-Elmer MPF-4 fluorescence spectrophotometer; for routine measurements, fluorescence was read at 465 $\pm$ 4 nm emission wavelength whereas the excitation wavelength was 365 $\pm$ 4 nm (Fig. 1). Both equipments were operated under external temperature control ($\pm$0.5 C° accuracy).

Microsomal membrane suspensions were prepared from rat testes homogenates by differential centrifugation as described previously with the only exception that, after the second ultracentrifugation step, the final pellet was resuspended in medium A [1]. Protein concentration was controlled using a modified Lowry procedure [22], and membrane lipids were determined after extraction according to Ref. 23. Liposomes (in medium A) were prepared from testicular microsomal membranes as described previously [1]. In some experiments, the partition of BDHP between the membrane phase and the buffer phase within membrane suspensions was measured by equilibration of membranes with the steroid probe, sedimentation of the membrane compartment under controlled temperature conditions in a Beckman L8-55 ultracentrifuge, and quantification of the BDHP-related absorption at 365 nm [1]. The partition coefficient K_P was defined as the ratio of steroid concentrations in the membrane versus the aqueous phases [14].

All absorption and fluorescence data were corrected for the respective background values in the absence of steroid at the corresponding temperatures; the small decrease in light scattering due to addition of steroid solutions to the membrane suspensions was also properly considered. As the key parameter within this study, the relative fluorescence (F) quenching (r.f.q.) was calculated as the ratio $(F_{\text{medium-A}}-F_{\text{membranes}})/(F_{\text{medium-A}})$ (cf. Fig. 2); its dependence on lipid concentration (cf. Fig. 3) was proved by weighted regression analysis [24]. Applicability of transformations was controlled by calculation of residuals in all cases [25].

Results

The absorption and fluorescence spectra of BDHP in the Tris-buffered sucrose solution employed in the course of this study are depicted in Fig. 1. It is evident that the absorption and excitation spectra are congruent, with absorption and fluorescence intensity maxima at $\lambda = 365$ nm (emission recorded at 465 nm). The molar absorption coefficient amounts to $\epsilon = 22000$ $l * mol^{-1} * cm^{-1}$. The emission spectrum exhibits a fluorescence intensity maximum at $\lambda = 465$ nm. Thus, all further fluorescence measurements were performed with $\lambda_{\text{excitation}} = 365$ nm and $\lambda_{\text{emission}} = 465$ nm.

Fluorescence intensity is linearly dependent on BDHP concentration (ranging from 0.05 to 4.0 μM). Irrespective of the actual BDHP concentration, there is a constant decrease of maximal BDHP fluorescence

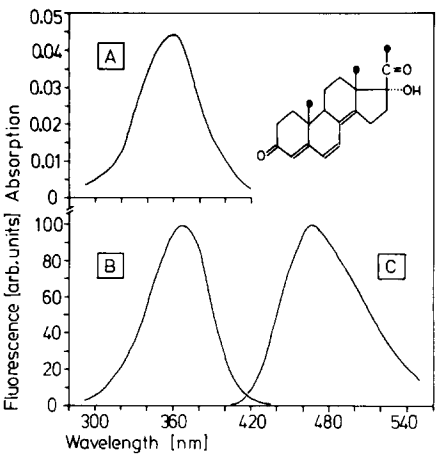

Fig. 1. Absorption spectrum (A) and fluorescence excitation (B; emission recorded at 465 nm) and emission spectra (C; excitation at 365 nm) of 6,8(14)-bisdehydro-17α-hydroxyprogesterone in medium A at 20°C; final steroid concentration was 2 μM.

intensity by 53% in the presence of testicular microsomal membranes equivalent to 1 mg lipid/ml (data not shown). This phenomenon is indicative of a BDHP concentration-independent association of a constant steroid fraction with hydrophobic membrane regions. Such a conclusion is clearly confirmed by experiments comparing the fluorescence quenching (relative to fluorescence intensities in aqueous buffer) of a series of BDHP concentrations in the presence of increasing testicular microsomal membrane concentrations. If the parameter ((relative fluorescence quenching)/(1–relative fluorescence quenching)) is plotted against the parameter (relative fluorescence quenching · BDHP concentration) in analogy to a Scatchard diagram (Fig. 2), it turns out that (at least within the BDHP concentration range employed here) no saturation of hydrophobic association of BDHP with the membrane can be observed. The ordinate intercepts increase continuously with increasing membrane concentration (Fig. 2). Further quantitative characterization of the membrane effect on relative BDHP fluorescence quenching can be derived from a double-reciprocal plot (Fig. 3). This diagram reveals a saturation function of fluorescence quenching with increasing membrane concentration: Assuming a ligand-independent relative fluorescence quenching, 104.1 ± 2.3% (mean ± S.D., $n = 120$) of initial fluorescence (in buffer) is lost at infinite

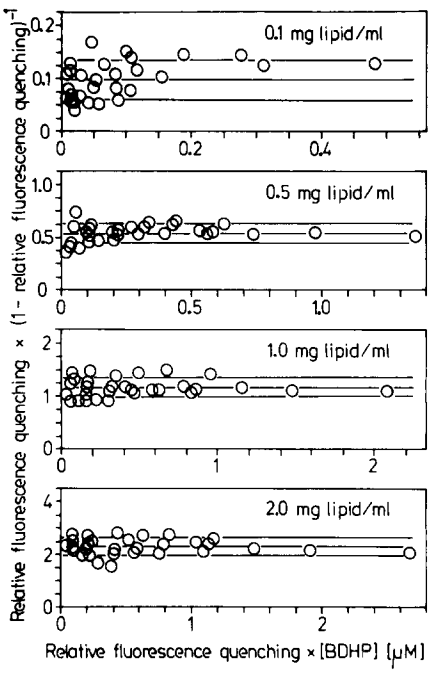

Fig. 2. Transformation of 6,8(14)-bisdehydro-17α-hydroxyprogesterone fluorescence quenching data obtained with testicular microsomal membranes of different concentrations in medium A at 20°C in analogy to the Scatchard plot. Relative fluorescence (F) quenching (r.f.q.) was calculated as the ratio $(F_{\text{medium-A}} - F_{\text{membranes}})/(F_{\text{medium-A}})$, and means ± S.D. for the ratios (r.f.q.)/(1–r.f.q.) are shown.

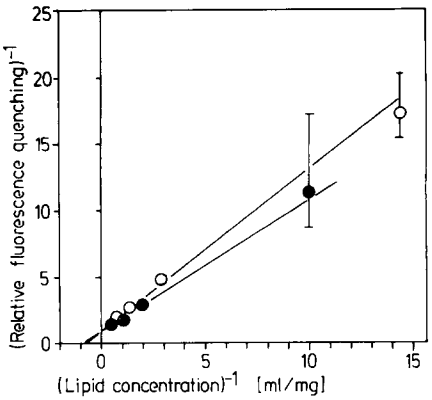

Fig. 3. Double-reciprocal plot demonstrating the dependence of relative 6,8(14)-bisdehydro-17α-hydroxyprogesterone fluorescence quenching on lipid concentration using testicular microsomal membranes (closed circles) or liposomes prepared from testicular microsomes (open circles). Means ± S.D. values are given; with high lipid concentrations, S.D. values lie within the symbols. Values obtained for microsomal membrane incubations correspond to those presented in Fig. 2. The ordinate intercept is the reciprocal of maximal fluorescence quenching in the presence of infinite lipid concentration, and the abscissa intercept is the negative reciprocal of the lipid concentration which causes half-maximal fluorescence quenching.

microsomal membrane concentration, and half-maximal fluorescence quenching occurs in the presence of 0.98 ± 0.05 mg lipid/ml (Fig. 3).

Though the fluorescence quenching data have been referred to membrane lipid concentrations so far, the possibility can not be excluded that these phenomena may, either partly or completely, also be related to hydrophobic association of BDHP with one or more membrane-bound steroid-binding proteins or enzymes. To investigate this problem, liposomes prepared from testicular microsomal membranes by lipid extraction and ultrasonic treatment were similarly tested for their capacity to reduce BDHP fluorescence. In this system, the relative fluorescence quenching is independent of the steroid concentration as well (data not shown), 99.1 ± 4.1% of initial fluorescence in buffer (mean ± S.D., $n = 20$) is lost at infinite liposomal concentration, and half-maximal fluorescence quenching occurs with 1.22 ± 0.09 mg lipid/ml (Fig. 3). These data indicate that steroid-binding membrane proteins obviously do not contribute significantly to the reduction of BDHP fluorescence in membrane systems, but that BDHP accumulation in the lipid part of biomembranes is responsible for this effect. In line with these findings, no measurable binding of BDHP to an important membrane-bound steroidogenic enzyme, namely cytochrome P450c17 catalyzing the C17,20-lyase reaction on 17α-hydroxyprogesterone [1], could be detected in testicular microsomal membranes (data not shown).

On the basis of this systematic characterization of changes in BDHP fluorescence induced by hydrophobic association with lipid domains in microsomal mem-

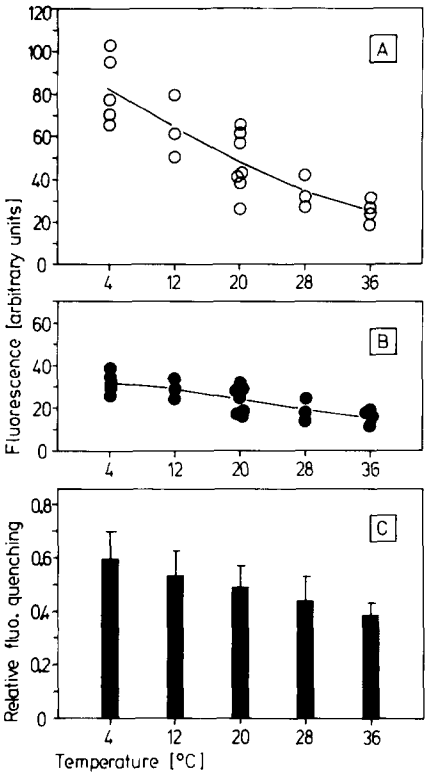

Fig. 4. Temperature-dependence of 6,8(14)-bisdehydro-17α-hydroxyprogesterone (BDHP) fluorescence in medium A (A) and in suspensions of testicular microsomal membranes (B; 1 mg/ml final lipid concentration); the respective mean values have been connected by the lines. Diagram C shows mean $\pm$ S.D. values for the relative BDHP fluorescence quenching (see legend to Fig. 2).

branes, the effect of varying temperature on these interactions was investigated. Maximal BDHP fluorescence in Tris-buffered sucrose is strongly dependent on temperature; a rise from 4°C to 36°C results in a 70% loss of fluorescence (Fig. 4A). A similar, but much less distinct temperature effect is observed in the presence of testicular microsomal membranes; in this case, a rise from 4°C to 36°C results only in a 52% reduction of BDHP fluorescence (Fig. 4B). As a result, the relative fluorescence quenching evoked by a constant membrane concentration decreases almost linearly with increasing temperature (Fig. 4C). Two different mechanisms can be conceived to explain this result; either the global accumulation of BDHP in the microsomal membranes may decrease with elevated temperature, and/or the mechanism of local molecular interaction of BDHP with membrane lipids may change with temperature. To consider the first possibility, BDHP distribution between the membrane compartment and the buffer space was quantified after separation of both fractions by ultracentrifugation using the BDHP absorption at $\lambda = 365$ nm. With increasing microsomal membrane concentrations, the membrane-bound BDHP fraction likewise increases, but the partition coefficient K_P is independent on the actual membrane

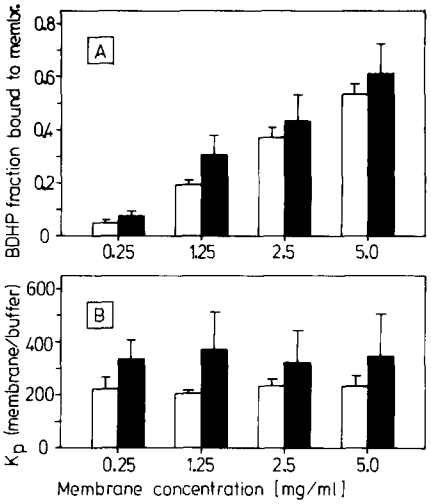

Fig. 5. Dependence of 6,8(14)-bisdehydro-17α-hydroxyprogesterone (BDHP) accumulation in microsomal membranes on membrane concentration (sum of lipid plus protein fraction) as determined from BDHP absorption (365 nm) at 4°C (white bars) and at 20°C (black bars) (A). Partition coefficients K_P (B) are the corresponding ratios of BDHP concentrations in the membrane versus buffer compartments.

concentration (Fig. 5). It is also evident that the K_P value at 20°C (343 $\pm$ 119; mean $\pm$ S.D., $n = 16$) is considerably higher than at 4°C (220 $\pm$ 35). Therefore, BDHP fluorescence quenching and BDHP accumulation in microsomal membranes exhibit an inverse temperature-dependence. As a consequence, the ratio of both parameters does not necessarily amount to unity, but decreases with elevated temperatures (Fig. 6): At low temperature, relative fluorescence quenching is much more pronounced than it should be on the basis of BDHP partition, but at high temperature, relative BDHP fluorescence quenching is slightly less pronounced than expected from partition data. Such a differentiation as established by this experimental design leads to the conclusion that temperature effects on

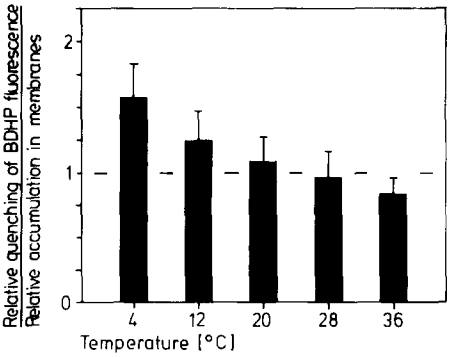

Fig. 6. Temperature-dependence of the ratios of relative 6,8(14)-bisdehydro-17α-hydroxyprogesterone (BDHP) fluorescence quenching (cf. Fig. 4C) versus relative BDHP accumulation (means $\pm$ S.D.; n = 3–7). These data are expected to be independent on either steroid probe or membrane lipid concentrations.

fluorescence quenching of BDHP are dictated by changes in molecular interaction mechanisms of this steroid probe with membrane lipids, in addition to its gross accumulation in biological membranes.

Discussion

The naturally occurring 3-oxosteroids, such as most gestagens, corticoids and androgens, show no or only slight fluorescence irrespective of the surrounding milieu, whereas the 4,6,8(14)-trien-3-one steroids (Fig. 1) are highly fluorescent in aqueous solution but non-fluorescent in aprotic solvents [19] *.

These steroids are thus most promising candidates to probe the nature of molecular interactions of steroids with their microsurroundings in proteins [19], cyclodextrins [20] or, as demonstrated with the present study, in biological membranes. It is noteworthy that the 4,6,8(14)-trien-3-one steroids closely resemble their naturally occurring 4-en-3-one counterparts; thus, enzyme kinetic parameters for turnover by 3β-hydroxysteroid oxidoreductase (EC 1.1.1.51) differ only by a factor of two, binding to antibodies that are specific for the 4-en-3-one steroids occurs with a relative affinity of 0.8, and binding affinity of 6,8(14)-bisdehydro-17α-hydroxyprogesterone (BDHP) to transcortin was 0.3 relative to that of 17α-hydroxyprogesterone [19,28].

The transfer of BDHP from an aqueous into a membrane-containing environment results in fluorescence quenching (Fig. 2), indicating the desolvation of the steroid probe upon transfer to the hydrophobic compartment and the loss of hydrogen bonds [19,26]. Quantitative analyses performed in the present study reveal that BDHP fluorescence quenching remains constant over a wide range of steroid concentration (from 0.05 up to 4.0 μM), indicating that there is no saturation effect at high ligand concentration (Fig. 2). These data implicate a simple partition process of the steroid probe into the microsomal membrane compartment and exclude specific association with membrane-bound binding sites, but they do not prove complete fluorescence quenching and thus complete hydrophobic insertion of the probe into the membrane [4,10,11]. Therefore, loss of BDHP fluorescence had further to be tested with a series of steroid concentrations against a series of membrane concentrations (Figs. 2 and 3). The results unequivocally verify that fluorescence quenching is complete at infinite membrane concentration, and that this pattern is mediated by membrane lipids and not by membrane proteins (Fig. 3).

This appears to be an important finding within the context of the subcellular organization of steroid metabolism pathways [9,16]. Most enzymes involved in the steroid-hormone activation and inactivation processes are bound to the smooth endoplasmic reticulum membranes; examples for such membrane-associated enzymes are the cytochromes P450c21 and P450c17 which catalyze the key reactions in the conversion of 17α-hydroxyprogesterone (the physiological analogue of BDHP) into corticoids and androgens, respectively [15–17]. Up to now, the subcellular microenvironment from which such enzymes accept their substrates, has not been defined with certainty. Previous studies had already quantified the gross extent of steroid hormone accumulation in smooth endoplasmic reticulum membranes or liposomes, but did not prove whether intermediates in steroid hormone biosynthetic processes are really immersed in the hydrophobic part of biomembranes [1,2,11,14]. By confirming corresponding predictions [6,12,14], at least for the 6,8(14)-bisdehydro analogue of 17α-hydroxyprogesterone, the present study may promote further investigations of the hydrophobic structure and relative localization of substrate-access channels of a variety of steroid-metabolizing enzymes such as P450c17 in testicular microsomes.

Further insights into the nature of steroid–lipid interactions in biomembranes come from experiments testing the temperature-dependence of BDHP fluorescence quenching. With increasing temperature, BDHP fluorescence decreases continuously in the aqueous buffer environment as well as in the presence of microsomal membranes (Fig. 4). Within a defined medium, loss of BDHP fluorescence with increasing temperature can be interpreted as a relatively increasing conversion of energy into molecular motion [19]; thus, the much more pronounced loss of BDHP fluorescence in buffer than in the presence of membranes indicates that the temperature-dependent increase in the mobility of the steroid probe is much more pronounced in buffer than within the membrane (Fig. 4).

However, the exact quantitative evaluation of this general effect requires additionally its relation to

* The 3-oxosteroids generally have two different electronic systems, and both systems take up energy after irradiation and absorption of a photon, giving rise to excited electronic states. In such molecules with a (poly)ene-carbaldehyde structure, $n \rightarrow \pi^*$ transitions describe promotion of a lone-pair, non-bonding electron of the oxo-group to a vacant π antibonding orbital, whereas $\pi \rightarrow \pi^*$ transitions describe π-electron promotion from a bonding to an antibonding orbital in conjugated systems [26]. The radiation-emitting level is always the lowest excited level [26]. If the n-π^* state is the singlet excited state with the lowest energy, no fluorescence can be observed due to intramolecular quenching; if the π-π^* state is the excited state with the lowest energy, fluorescence occurs [19,26]. Introduction of two additional double bonds into the non-fluorescing 17α-hydroxyprogesterone molecule, yielding BDHP, reduces the energy of the fluorescent π-π^* state so that it becomes comparable to that of the n-π^* state [19,27]. In any such system with two excited states of only slightly differing energies, relatively small perturbations of the microenvironment (e.g., solvent interactions or protein binding comprising hydrophobic interactions) will then dictate whether the system fluoresces or not [19,26].

BDHP accumulation in the membrane. This parameter was quantified using absorption spectroscopy since it cannot be unequivocally determined solely on the basis of fluorescence quenching. As expected on the basis of previous studies [1], the partition coefficient K_P is independent on membrane concentration and increases with temperature, probably due to heat-induced increase in membrane fluidity (Fig. 5). However, the relative BDHP fluorescence quenching in microsomal membranes does not equal the relative accumulation of this probe within the membrane compartment; rather, the ratio between both parameters decreases continuously if the temperature is elevated from 4°C to above the phase transition (Fig. 6). This over-proportional fluorescence loss at low temperatures may suggest that increasing membrane rigidity favours the formation of hydrophobic interactions between a steroid probe molecule and the surrounding membrane lipids under such conditions.

In conclusion, the remarkable fluorescence behaviour of BDHP makes this representative of the class of 4,6,8(14)-trien-3-one steroids a very sensitive molecular tool to probe the hydrophobicity of its actual microenvironment and to unravel molecular relationships between steroids and membrane lipids that become important when steroids distribute into subcellular membranes in the course of steroid hormone synthesis and metabolism.

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