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EFFECT OF BOVINE GROWTH HORMONE ON RAT LIVER PLASMA MEMBRANES AS STUDIED BY CIRCULAR DICHROISM AND FLUORESCENCE USING THE EXTRINSIC PROBE 7,12-DIMETHYLBENZANTHRACENE

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SUMMARY

Conformational changes produced by in vitro bovine growth hormone addition to plasma membranes of hypophysectomized rat liver proteins and lipids have been studied by circular dichroism as well as intrinsic and extrinsic fluorescence. 7,12-Dimethylbenzanthracene has been used as a fluorescent probe of changes in membrane structure. The exposure of membranes to bovine growth hormone produced a change in membrane negative ellipticity. Dimethylbenzanthracene at concentrations similar to those employed in fluorescence studies had no effect on the membrane circular dichroism spectrum. Its presence did, however, prevent a response to growth hormone. There was a decrease in peak fluorescence intensity and a peak shift when bovine growth hormone ($0.5 \cdot 10^{-12}$ M) was added to liver membranes. The addition of dimethylbenzanthracene ($1.6 \cdot 10^{-6}$ M) to membranes resulted in a decrease in the intensity of the protein fluorescence peak at 335 nm and the appearance of two peaks at 430 and 407 nm, assignable to the probe. The addition of bovine growth hormone ($0.5 \cdot 10^{-12}$ M) produced a decrease in fluorescence at 335 nm and also in the peaks at 407 and 430 nm. These data are consistent with the conclusion that bovine growth hormone produces a conformational change in rat liver plasma membrane proteins and lipids.

INTRODUCTION

In a target tissue, the first step of growth hormone action is likely to be a specific binding of the hormone to a plasma membrane "receptor". This binding may lead, prior to subsequent biochemical and biological effects, to a membrane conformational change. This latter effect has been explored in this work.

In previous studies from this laboratory, changes in protein conformation have been noted in human erythrocyte membranes [1,2] and rat liver membranes after addition of growth hormone in vitro [3] and in vivo [4]. These changes were demonstrated with circular dichroism and intrinsic fluorescence. These observed changes were shown to be independent of changes in light scattering. In the present study,

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the fluorescent probe, 7,12-dimethylbenzanthrancene, was employed to monitor membrane changes. It is believed that the probe primarily samples the lipid phase.

MATERIALS AND METHODS

Liver plasma membranes of hypophysectomized rats (Charles River) were prepared as described by Neville [5]. These membranes are referred to as "fully purified membranes". For "partially purified membranes" the final sucrose density gradient centrifugation (Step 13–15 of Neville) was omitted. The preparation medium was either $NaHCO_3$ (1 mM) or $NaHCO_3$ (1 mM) and $CaCl_2$ (0.5 mM). Membrane preparations were either used immediately or stored in sucrose at 4 °C to be used within 4 days following preparation.

The membranes were characterized by electron microscopy and by assay of marker enzymes. Fully purified membranes were isolated as separate sheets which in some instances were attached by desmosomes. 5'-Nucleotidase activity in the partially purified membranes was 0.20 μmoles P_i/min per mg protein. This activity was enriched after the final purification to 0.45 μmole P_i/min per mg protein. In the same purification cytochrome oxidase activity, indicative of mitochondrial contamination, decreased from 2.5 nmoles cytochrome *c* oxidized/min per mg protein to 1.2 nmoles/min per mg protein in the fully purified membranes. Less than 3% of the total protein was estimated to be of mitochondrial origin. As assayed by glucose-6-phosphatase activity, another 3% of the total protein in the fully purified membranes was of ribosomal origin.

Bovine growth hormone was prepared in this laboratory by a modification [6] of the method of Dellacha and Sonenberg [7]. Dimethylbenzanthracene was obtained from Eastman Kodak. [^{3}H]Dimethylbenzanthracene, 17 Ci/mM, was obtained from Amersham Searle (TRK 142 Batch 23). Other reagents were of analytic grade.

Protein concentrations were determined by the Lowry method [8] with bovine serum albumin as a standard.

Circular dichroism determinations were made in the far ultraviolet range (250–190 nm) with a Cary model 60 recording spectropolarimeter with circular dichroism attachment 6002. Cylindrical quartz tandem cells (10 mm path length) were used so that both hormone and membrane suspensions were in the light path, either separated by a quartz partition, or mixed in the same compartment. In other experiments, bovine growth hormone was added directly to the membrane suspension in a cell of 5 mm path length. Prior to dimethylbenzanthracene exposure, membranes were tested for hormone responsiveness. The concentration of dimethylbenzanthracene in the cell was $4 \cdot 10^{-6}$ M. Measurements were made either at 37 °C or at room temperature. All measurements were made in duplicate. The data are expressed in terms of mean residue ellipticity (θ) in degrees/cm^2 per dmole.

Fluorescence measurements were made with a Carry 50-026-900 differential recording spectrophotofluorometer equipped with a 450 W short-arc xenon lamp. Front surface illumination was used with a 23° angle between the exciting beam and the emitted light. Illuminating light was broken up by a chopper and passed alternately through a Rhodamine B quantum detector, located in the sample compartment, and the sample. This signal displayed on the recorder was the ratio between these two signals, thus correcting for variation of light intensity of the source. Variations in monochromator transmission and photomultiplier response with wavelength

were instrumentally compensated to give corrected spectra. All spectra are presented as obtained on the recorder. No inner filter corrections were required, inasmuch as the fluorescence intensity was linearly related to suspension concentrations over the range employed in these experiments. No corrections for Rayleigh or Raman light scattering were found necessary at the concentrations and instrumental settings used in these experiments. Fluorescence intensity is presented on an arbitrary scale. Differences considered significant are only those that exceeded instrumental noise and any differences with control procedures (e.g. solvent addition, mixing of cuvette contents, etc.). The combined instrumental and experimental error was generally 2% or less. Difference spectral measurements were done using two identical cells in the two paths of the sample compartment. All substances were added directly into the cell before the fluorometric studies. Samples remained in the thermostatted cell compartments for 10 min to equilibrate them at 37 °C. Samples were excited at 282 nm. Membrane suspensions were in 1 mM $NaHCO_3$ or Krebs–Ringer bicarbonate buffer, pH 7.4. The final concentration of GTP was $4 \cdot 10^{-6}$ M. The final concentration of $MgSO_4$ was $4 \cdot 10^{-4}$ M. The concentration of dimethylbenzanthracene was $1.6 \cdot 10^{-6}$ M.

[^{3}H]Dimethylbenzanthracene was added to a partially purified membrane suspension and membranes were centifuged at 105000 $\times g$ for 30 min. The supernatant and the pellet were separated and 5% sodium dodecylsulfate was added to both. Radioactivity in an aliquot of each phase was then determined in a liquid scintillation counter.

RESULTS

Circular dichroism

The spectra of the liver plasma membranes showed two troughs, one at 222 nm, the other one between 208 and 212 nm. When liver plasma membranes were mixed in vitro with bovine growth hormone, decreased negative ellipticity was seen

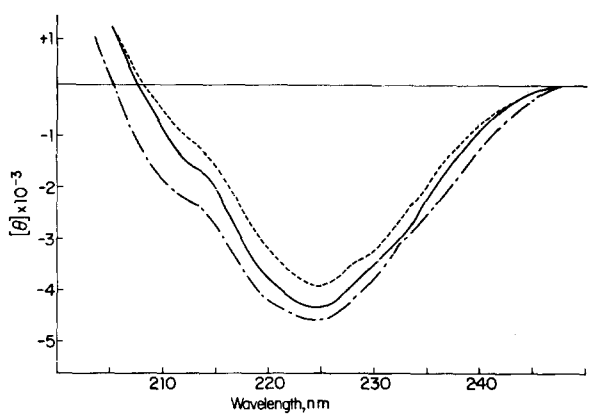

Fig. 1. Circular dichroism spectra of liver plasma membranes (in 1 mM $NaHCO_3$, pH 7.4) ———, fully purified membranes alone; - - - - - -, membranes in the presence of bovine growth hormone added in vitro at the concentration of $0.5 \cdot 10^{-12}$ M; –·——·–, membranes in the presence of bovine growth hormone added in vitro at the concentration of $0.5 \cdot 10^{-9}$ M. Signal to noise ratio, 22 at 222 nm and 7 at 210 nm.

(Fig. 1). The final concentration of bovine growth hormone was $0.5 \cdot 10^{-12}$ M and the membrane protein concentration in the cell 20 μg/ml so that the ratio of membrane protein to bovine growth hormone was $1 \cdot 10^6$ (by wt). With this hormone concentration, the decrease in negative membrane ellipticity at 222 nm was $11 \pm 2\%$. This represents the mean of six duplicate runs made with six different membrane preparations. The change appeared as a decrease in ellipticity, with pure membranes as well as partially purified membranes. Similar changes were noted when the spectra were recorded at room temperature or at 37 °C. The concentration of bovine growth hormone had an effect on the direction of the change in ellipticity. With a hormone concentration of $0.5 \cdot 10^{-9}$ M and a ratio of membrane protein to bovine growth hormone of $2.8 \cdot 10^3$ (by wt) there was greater negative ellipticity at 222 nm ($7 \pm 0.5\%$) (Fig. 1). The in vitro addition of dimethylbenzanthracene at the final concentration of $4 \cdot 10^{-6}$ M to the membrane suspension did not alter the negative ellipticity of membranes. The addition of dimethylbenzanthracene, however, prevented the response of membranes to bovine growth hormone ($0.5 \cdot 10^{-12}$ M).

The absorbance measured from 700–210 nm on triplicate determination was between 0.04 at 400 nm and 0.7 at 230 nm. There was essentially no noise in the readings of those optical densities and it was concluded that there was no change in light scattering (i.e. less than 1 %) on the addition of growth hormone to membrane suspensions.

Intrinsic fluorescence

Fully purified membrane suspensions showed a broad asymmetric emission band with a peak at 335 nm. A shoulder at 315 nm was seen and its intensity was half that of the peak. With the partially purified membranes the peak appeared at 330 nm. Replicate spectra of the membrane suspension in the buffer could be obtained over a period of 12 h.

The in vitro exposure of the membranes to bovine growth hormone at concentrations of 10^{-7}–10^{-17} M resulted in a decrease in peak intensity and a peak shift to higher wavelength (from 335 to 338 nm) (Fig. 2). Bovine growth hormone alone at a concentration of 10^{-7} M had no detectable fluorescence at the instrumental setting used. When GTP and Mg^{2+} were added to partially purified membranes there was a change in fluorescence with concentrations of bovine growth hormone as low as 10^{-17} M. No further change could be seen with additional bovine growth hormone. This change in fluorescence was observed as soon as measurements could

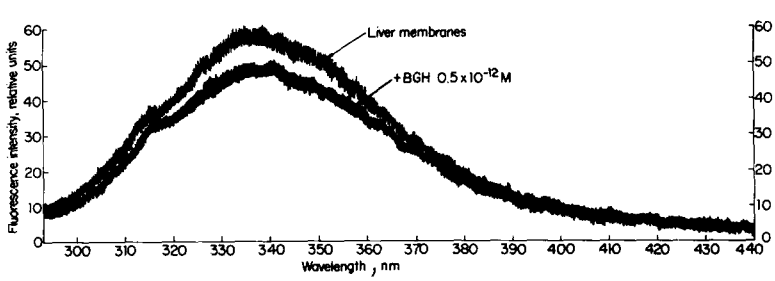

Fig. 2. Emission of fluorescence of fully purified plasma membranes in the absence and in the presence of bovine growth hormone (BGH, $0.5 \cdot 10^{-12}$ M). Excitation bandwidth, 2.1 nm. Emission bandwidth, 6.2 nm. Signal to noise ratio, 7 at 340 nm.

be made after the addition of bovine growth hormone (within 30 s) and the change remained constant for at least 12 h. The change was seen at temperatures as high as 57 °C and as low as 17 °C. Controls, such as addition of buffer or a non-specific protein, such as bovine serum albumin to the membrane suspensions did not show any effect on the spectrum. Most of the fluorescence changes occurring after the addition of hormone were studied with difference spectral measurements.

With fully purified membranes, not all preparations were responsive to bovine growth hormone. With partially purified membranes, the preparations were more consistently responsive. The change, more specifically the direction of the change in fluorescence, became reproducible when Ca^{2+} was added to the buffer of the membrane preparation and when GTP and Mg^{2+} were added to the membrane suspension. ATP added at a final concentration of $4 \cdot 10^{-6}$ M had the same effect as GTP. Mg^{2+} by itself, at the concentration used, had a small effect on the fluorescence emission spectrum ($3.5 \pm 0.9\%$ decrease). GTP by itself, at the concentration used, produced a small decrease in the fluorescence peak at 330 nm ($6.9 \pm 1.6\%$).

Extrinsic fluorescence

The fluorescence excitation spectrum of dimethylbenzanthracene in ethanol, with emission at 470 nm, revealed two peaks at 296 and 285 nm (Fig. 3). When incorporated into the membranes, dimethylbenzanthracene led to a decrease in the intensity of the protein fluorescence peak at 335 nm, and two peaks assignable to the probe appeared at 430 and 407 nm (Fig. 4).

The in vitro addition of bovine growth hormone ($1 \cdot 10^{-12}$ M) to a membrane

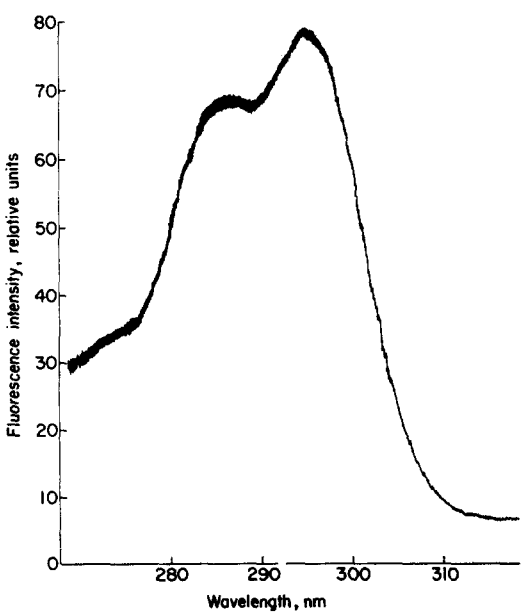

Fig. 3. Excitation spectrum of 7,12-dimethylbenzanthracene in ethanol. Emission at 407 nm. Emission bandwidth, 6.2 nm. Excitation bandwidth at 285 nm, 5.5 nm. Signal to noise ratio, 30 at 285 nm.

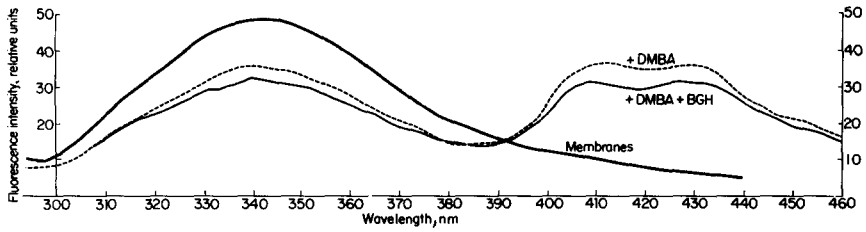

Fig. 4. Emission of fluorescence of fully purified liver plasma membranes. ———, membranes alone; -------, membranes in the presence of dimethylbenzanthracene (DMBA, $1.6 \cdot 10^{-6}$ M); ———, membranes in the presence of dimethylbenzanthracene and bovine growth hormone (BGH, $0.5 \cdot 10^{-12}$ M). Excitation bandwidth, 2.1 nm. Emission bandwidth, 6.2 nm. Signal to noise ratio, 6 at 340 nm.

suspension containing dimethylbenzanthracene, in the absence of GTP and Mg^{2+}, produced a $12 \pm 4\%$ decrease in fluorescence at 335 nm. In the peaks at 407 and 430 nm there was also a decrease ($14 \pm 2\%$). When bovine growth hormone was added to partially purified membranes containing dimethylbenzanthracene, in the presence of GTP and Mg^{2+}, there was a decrease in fluorescence at 330 nm as determined by difference spectrofluorometry.

Membrane emission spectra of fully purified membranes and increasing concentrations ($1.6 \cdot 10^{-6}$–$2.6 \cdot 10^{-5}$ M) of dimethylbenzanthracene are shown in Fig. 5. There is a progressive decrease in the intrinsic fluorescence and increase in the extrinsic fluorescence at 407 and 430 nm.

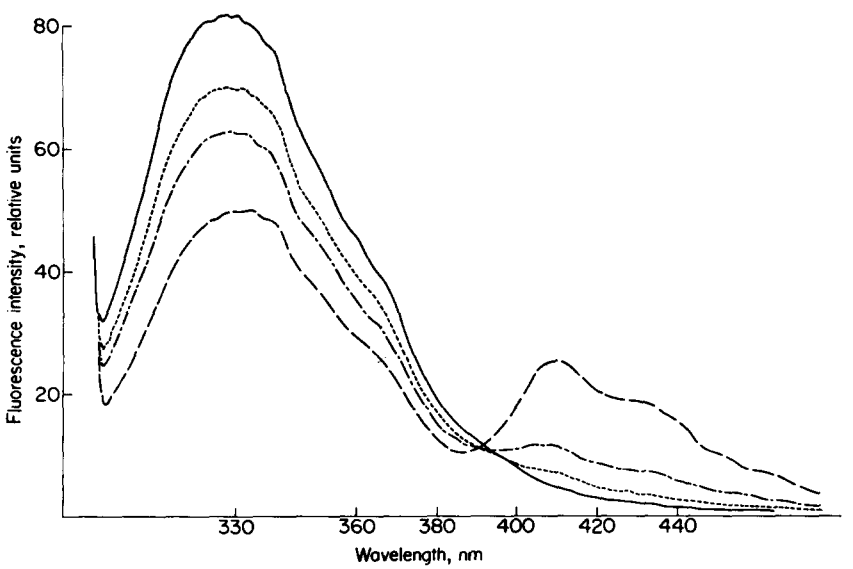

Fig. 5. Emission of fluorescence of fully purified liver plasma membranes in the absence and presence of increasing concentrations of dimethylbenzanthracene. ———, membranes alone; -------, membranes in the presence of dimethylbenzanthracene ($1.6 \cdot 10^{-6}$ M); –·—·–, membranes in the presence of dimethylbenzanthracene ($6.4 \cdot 10^{-6}$ M); – – –, membranes in the presence of dimethylbenzanthracene ($2.6 \cdot 10^{-5}$ M). Emission bandwidth, 6.2 nm. Excitation bandwidth, 7 nm. Signal to noise ratio, 40 at 330 nm.

Membrane uptake of dimethylbenzanthracene

Utilizing [^{3}H]dimethylbenzanthracene, the amount of dimethylbenzanthracene incorporated into membrane was determined as described. Results are given in Table I. As measured by $A_{280\,nm}$ about 10% of the protein was still in the supernatant after the centrifugation, so that the radioactivity in the supernatant may be associated with what could be membrane or soluble proteins. If the supernatant contained non-sedimented membrane fragments, it appeared that there was complete uptake of dimethylbenzanthracene by the membranes. When bovine growth hormone was added no displacement of the dimethylbenzanthracene was detected. The uptake of dimethylbenzanthracene by membranes was also measured fluorometrically. The same protocol was followed with unlabeled dimethylbenzanthracene added to membranes. The results (Table II) were similar to those noted (Table I) utilizing [^{3}H]dimethylbenzanthracene, i.e. the uptake of the probe by membranes was complete.

TABLE I

UPTAKE OF [^{3}H]DIMETHYLBENZANTHRACENE BY PARTIALLY PURIFIED LIVER MEMBRANES

Results are expressed as per cent of radioactivity in membranes (pellet). $A_{280\,nm}$ is also expressed as per cent in pellet. n.d., not determined.

Dimethylbenzanthracene concentration (M)	Cpm (per cent in pellet)	$A_{280\,nm}$ (per cent in pellet)
0	88	n.d.
$2 \cdot 10^{-6}$	89	82
$1 \cdot 10^{-5}$	90	79
$4 \cdot 10^{-5}$	90	84

TABLE II

UPTAKE OF DIMETHYLBENZANTHRACENE BY PARTIALLY PURIFIED LIVER MEMBRANES

Fluorescence at 408 nm is measured in supernatant and pellet. Results are expressed as per cent in membranes (pellet).

Dimethylbenzanthracene concentration (M)	Fluorescence at 408 nm (per cent in pellet)
$1.6 \cdot 10^{-6}$	81
$3.2 \cdot 10^{-6}$	90
$6.4 \cdot 10^{-6}$	89
$1.2 \cdot 10^{-5}$	93

DISCUSSION

In vitro exposure of liver plasma membranes from hypophysectomized rats to concentrations of bovine growth hormone as low as $1 \cdot 10^{-12}$ M led to rapid conformational change as seen with circular dichroism and fluorescence suggesting that

both secondary and tertiary structure of the membranes proteins were affected. We have shown that the membrane suspension had less optical activity in the presence of bovine growth hormone ($0.5 \cdot 10^{-12}$ M) suggesting less helical content of membrane proteins. A thousand fold increase of bovine growth hormone concentration ($0.5 \cdot 10^{-9}$ M) reproducibly altered the direction of the change. With this higher concentration, an increase in membrane ellipticity was seen. Since bovine growth hormone itself, at a concentration of $1 \cdot 10^{-9}$ M, did not show any ellipticity under comparable experimental conditions this could not represent merely increased protein concentration. This observation could have physiological significance since the circulating growth hormone level of the rat is $2 \cdot 10^{-9}$ M [9]. It is not likely that the changes observed occurred in other organelles which were present in such small amounts (3% or less) as estimated by enzyme assays.

Although the magnitude of the observed circular dichroism is small, on the basis of duplicate spectra with each sample and multiple membrane preparations, the range of values, number of experiments and signal to noise ratios, the small differences would appear to be reproducible and significant. We conclude that the differences reflect changes in secondary and not tertiary or quaternary structure. Since circular dichroism was determined in the far ultraviolet region (250–190 nm), where the contribution of side chain optical activity is off by an order of magnitude, it would be difficult to account for our 11% change (θ of approx. 400 degrees/cm^2 per dmole) in ellipticity as the consequence of change in side chain activity whose contribution in the far ultraviolet is at most several hundred degrees/cm^2 per dmole. Moreover, changes in tertiary structure, as indicated by our fluorescence results, would contribute minimally to the observed changes in ellipticity. Similarly, there was less than 1% change in absorbance between 700 and 210 nm. It was not possible to extend the spectral data below 210 nm under the same experimental conditions as in circular dichroism due to the large absorbance associated with Rayleigh scattering as well as absorption. We conclude that the changes in ellipticity are not reflections of changes in quaternary structure.

The intrinsic fluorescence of the fully purified rat liver plasma membranes revealed a spectrum with a major peak at 335 nm attributable to tryptophan residues and a shoulder seen at 315 nm originating in tyrosine residues. The addition of GTP ($4 \cdot 10^{-6}$ M) to the membrane suspension appeared to stabilize the membranes and led to more reproducible responses. It is known that GTP at the concentration of $1 \cdot 10^{-6}$ M increases NAD^+ nucleosidase (Swislocki, N. I., Sonenberg, M., Tierney, J. and Aizono, Y., personal communication), adenyl cyclase and ATPase [10] activities of partially purified liver membranes. In addition, GTP produces changes in intrinsic fluorescence suggestive of a cooperative effect on membrane protein conformation (Swislocki, N. I., Sonenberg, M., Tierney, J. and Aizono, Y., personal communication). Partially purified membranes appeared to respond more consistently to growth hormone than fully purified membranes. This difference could be result of the elimination, during the last step of the preparation, of a factor necessary to the hormone–membrane interaction, Pohl et al. [11] noted similarly that partially purified membranes were more responsive to glucagon than more purified membranes.

The decrease in peak intensity and the peak shift shown when membranes were exposed to bovine growth hormone are consistent with a change in protein

conformation and an exposure of tryptophan to less hydrophobic environments. Moreover, the peak shift, in addition to the decrease in intensity, favors a protein conformational change rather than simple quenching of fluorescence. A decrease alone, and not a peak shift could be the effect of a simple quencher.

The previously discussed experiments reflect overall changes in membrane conformation in response to the hormone, bovine growth hormone in this case. In order to attribute such a biophysical change to a specific substance in the membrane, it would be necessary to have a probe with specific affinity for that substance. In this study we used 7,12-dimethylbenzanthracene, which is not known to have affinity for a specific component. Studies are in progress to determine where dimethylbenzanthracene is inserted into the membranes, i.e. in the hydrophobic portion of the proteins, the lipid phase, or both.

Dimethylbenzanthracene was chosen as a membrane probe because its marked hydrophobicity favored membrane uptake of the probe from aqueous buffers. In addition excitation of membranes and dimethylbenzanthracene at 282 nm, close to absorption peaks of tryptophan and dimethylbenzanthracene at 282 and 285 nm, respectively, yielded emission spectra with easily identifiable peaks assignable to the intrinsic and extrinsic fluorophores.

The insertion of the probe into fully purified membranes led to a decrease in fluorescence of the protein peak (335 nm) and to the appearance of two peaks at 407 nm and 430 nm. The latter two peaks are assignable to dimethylbenzanthracene. In Fig. 5 is shown the importance of the decrease in fluorescence of the protein peak related to the probe concentration. The relation is non-linear and a peak shift is observed. An energy transfer mechanism could not explain the peak shift and moreover, the overlap between the absorption spectrum of dimethylbenzanthracene (maximum at 285 and 296 nm) and the emission spectrum of the membranes is very minimal. The decrease in fluorescence at 335 nm can be attributed partly to a screening or inner filter effect. The peak shift may be more apparent than real and is consistent with preferential quenching of tyrosine over tryptophan fluorescence. Alternatively the peak shift may reflect a conformational change of the membrane due to the addition of the probe.

Contrary to the fluorescence results, dimethylbenzanthracene, at the concentration used ($4 \cdot 10^{-6}$ M) did not affect the negative ellipticity of membranes. The circular dichroism results showed, however, that dimethylbenzanthracene interfered with the response of the membrane to hormone. It is interesting to note that the change in ellipticity obtained by the addition of hormone seemed to be reversible. When the probe was added to the membrane suspension after the hormone, the spectrum was altered, appearing similar to the one obtained with membranes alone. Dimethylbenzanthracene seems to alter the tertiary structure but not the secondary structure of membrane proteins and dimethylbenzanthracene had a different effect on the hormone–membrane interaction when studied by circular dichroism or fluorescence. As noted above, the fluorescence characteristics of the membrane probe complex were altered by the addition of bovine growth hormone. Discrepancies have previously been noted between circular dichroism and fluorescence responses [3,12].

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