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The Relation Between Lipid Mobility and the Specific Hormone Binding of Thyroid Membranes

By C. LINDSAY BASHFORD, SYLVIA J. HARRISON and GEORGE K. RADDA
Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K.
and
QUASIM MEHDI
Nuffield Department of Clinical Biochemistry, Radcliffe Infirmary, Oxford OX2 6HE, U.K.

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1. The specific binding of thyroid-stimulating hormone to isolated human thyroid membranes was examined under a variety of conditions. 2. In phosphate–saline buffer (in the presence of 0.14M-NaCl) on increasing the temperature the binding of the hormone is increased, the plots of bound/free hormone against temperature showing a distinct break around 30°C. 3. Detailed analysis showed that the increased binding is associated with an increase in the number of binding sites. 4. The motional characteristics of three membrane-bound fluorescent probes, 2-(9-anthroyl)palmitic acid, 12-(9-anthryl)stearic acid and *N*-1-naphthyl-*N*-phenylamine, were also examined as a function of temperature by measuring both fluorescence polarizations and life-times. 5. The results indicated that the 'fluidity' of membrane lipids also increased with temperature. The temperature-dependence of this property also shows a change at about 30°C. 6. Bivalent cations decreased both membrane fluidity and hormone binding. 7. Similar correlations were found between the binding of adrenocorticotrophic hormone and the fluidity of the plasma membranes obtained from adrenal-cortical cells, with the discontinuity occurring in this case at 23°C. 8. The possibility of lipid mobility being important in controlling hormone-receptor function is discussed.

The specific binding of highly purified TSH* to isolated human thyroid membranes has been studied with the aid of ^{125}I-labelled hormone (Mehdi & Nussey, 1975). Both the extent of binding and the rate of hormone uptake were found to depend on the nature of the buffer and on the other external conditions used. Such modulation of the membrane–hormone interaction might be due to the general modification of the lipid environment of the receptor site. The work described in the present paper was designed as a first step in elucidating any role that the membrane lipids might play in modulating hormone binding.

The approach used has been that previously shown to be useful in investigating the role of lipid–protein interactions in the modification of membrane-bound enzyme activities (Radda, 1974). The method relies on the correlation observed between the biological activity studied and the motional characteristics of lipid molecules in the membrane in response to a specific external perturbation. Lipid 'fluidity' can be characterized by the use of a variety of fluorescent probes and the required perturbation can be achieved by varying the solution temperature or the cation composition of the solution.

* Abbreviations: TSH, thyroid-stimulating hormone; ACTH, adrenocorticotrophic hormone.

Experimental

TSH was a gift from Professor J. G. Pierce (UCLA School of Medicine, Los Angeles, Calif. 90024, U.S.A.). ACTH was obtained from CIBA Laboratories, Horsham, Sussex, U.K., as the synthetic polypeptide comprising the first 24 *N*-terminal amino acids, which has full biological activity (Landon *et al.*, 1964). The hormones were iodinated by a modification of the method of Hunter & Greenwood (1964) as described by Mehdi & Nussey (1975). The fluorescent probes 12-(9-anthroyl)stearic acid and 2-(9-anthroyl)palmitic acid were synthesized in this laboratory (Barratt *et al.*, 1974). *N*-1-Naphthyl-*N*-phenylamine was obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and was recrystallized from 60% (v/v) ethanol before use.

Human thyroid glands and adrenal glands were obtained at the operation or *post mortem* and were kept on ice until frozen, always within 15min after removal. Plasma membranes were prepared from the thyroids and the cortices of the adrenal glands by the method described by Mehdi & Nussey (1975), which included homogenization, several centrifugations and a final discontinuous sucrose-density-gradient centrifugation. The fractions obtained from the sucrose gradient were diluted with buffer (either 0.01M-KH_2PO_4 adjusted to pH7.5 with 1M-NaOH

or the same buffer containing 0.14M-NaCl and 0.001M-$NaHCO_3$) and centrifuged for 90min at 4°C and 105000g ($r_{av.}$ 5.9cm). The pellets so obtained were homogenized in the appropriate buffer and were washed twice by centrifugation and resuspension.

The membrane concentrations are expressed in terms of membrane protein measured by the method of Itzhaki & Gill (1964). TSH binding to thyroid membranes was determined as described by Mehdi *et al.* (1973), the radioactivities in the pellet and the supernatant fractions being measured with a Wallac Autogamma counter. All the binding experiments were done in at least duplicate and the accuracies were ±5%.

The fluorescent probes in a concentrated methanolic solution were added to the membrane suspensions and left to equilibrate until no further fluorescence enhancement was observed. The final concentration of the probe in any experiment was in the range 1–5μmol/litre and the concentration of methanol never exceeded 0.5% (v/v). This amount of methanol had no effect on the thyroid membranes with respect to their specific binding of TSH. Fluorescence intensities and spectra were measured on a Hitachi Perkin–Elmer MPF-2A spectrofluorimeter. The temperature-dependence of fluorescence polarization was measured on an instrument designed in this laboratory (Morgan, 1974) which allowed a continuous recording of the polarization with temperature. The temperature in the sample cuvette was measured by a thermistor bead immersed in the solution above the light-path. The bead was connected to an electronic thermometer from which the output was displayed on a meter and also on the X-axis of an X–Y recorder; fluorescence polarization was displayed on the Y-axis of the recorder. Fluorescence lifetimes were determined on an ORTEC nanosecond-pulsed fluorimeter which was modified to include the nanosecond spectral source and optical system of Applied Photophysics Ltd., London W.1, U.K. The thyratron gated lamp provides a high-frequency flash of sufficient intensity to permit use of monochromators in the optical system. In our measurements a grating monochromator was used at the excitation side and a combination of filters at the emission side.

In experiments using bivalent metal ions, buffers containing phosphate are unsuitable. In these cases 0.01M-Tris adjusted to pH7.5 with 6M-HCl and containing 0.14M-NaCl and 0.001M-$NaHCO_3$ was used as the buffer.

Results

Effect of temperature and cations on TSH binding

The temperature-dependence of TSH binding to thyroid membranes is shown in Fig. 1. In phosphate–saline buffer (i.e. in the presence of 0.14M-NaCl) increasing temperature results in increased binding of the hormone, with the curve showing a distinct break at about 30°C. The 'heating' and 'cooling' curves are similar, indicating that the phenomenon causing the break is reversible. There is, in fact, a suggestion of some hysteresis. The binding experiments, however, probably lack sufficient precision to

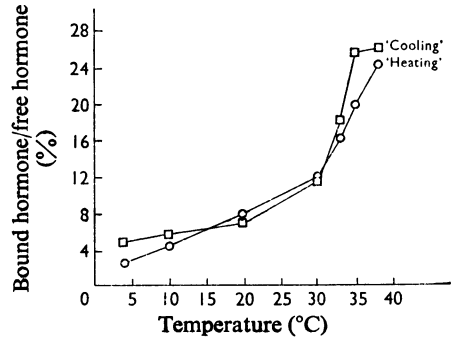

Fig. 1. *Binding of ^{125}I-labelled TSH to thyroid membranes suspended in* 0.01M-KH_2PO_4 *buffer, pH*7.5, *containing* 0.14M-*NaCl*

Thyroid membranes (0.4mg/ml) were incubated in phosphate–NaCl buffer at 37° (□) or 0°C (○) for 30min. Then 0.6ml portions were transferred to tubes incubated in water baths at the temperatures indicated. After 15min 20μl of ^{125}I-labelled TSH (approx. 20000c.p.m.) was added to each tube, which was then incubated for 4h. Binding of hormone was determined as described by Mehdi *et al.* (1973).

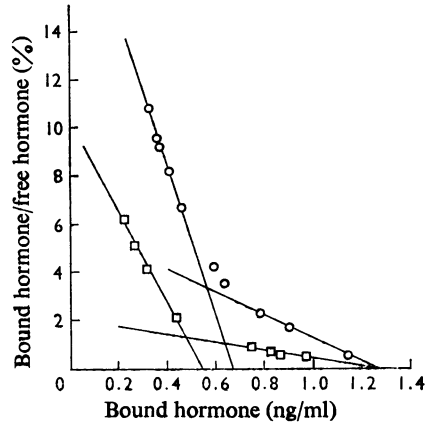

Fig. 2. *Scatchard plots of the binding of TSH to thyroid membranes*

Binding of TSH at a series of hormone concentrations was determined as described in Fig. 1 at two different temperatures, 25° (○) and 37°C (□). Dissociation constants at 25°C are 0.125nM (specific binding) and 0.843nM, at 37°C they are 0.212nM (specific binding) and 2.41nM.

study this phenomenon (but see below). The binding of TSH at a series of hormone concentrations can be analysed by the method of Scatchard (1949). The results of two such experiments are shown in Fig. 2. These plots indicate that the affinity of the specific binding site for TSH is little affected by temperature. In a series of experiments the dissociation constant for the interaction was in the range 0.1×10^{-9}–0.3×10^{-9} M at both 25° and 37°C. We may conclude therefore that the principal effect induced by temperature variation is a change in the number of available 'receptor' sites.

The binding characteristics of the thyroid membranes in phosphate buffer in the absence of NaCl are noticeably different. In particular the extent of binding is up to an order of magnitude higher. Again this can be ascribed to a fourfold increase in the available number of binding sites and to a smaller change in the affinity. The effect of temperature in this case is negligible and the binding is much more rapid. Fig. 3 gives the results of a binding experiment in the two types of buffer at three different temperatures. Addition of bivalent cations to a buffer containing NaCl resulted in a further significant decrease in the binding of the hormone (Fig. 4).

Fluorescent-probe studies on thyroid membranes

The interaction of the three probes 12-(9-anthroyl)-stearic acid, 2-(9-anthroyl)palmitic acid and *N*-1-naphthyl-*N*-phenylamine with thyroid membranes is characterized by the enhancement and blue shift of their fluorescence emissions similar to those observed with a variety of other membranes (Radda & Vanderkooi, 1972). The fluorescence parameters of the three probes in thyroid membranes at 25°C are summarized in Table 1. The time-course of probe uptake can be conveniently followed by measurements of fluorescence intensity. As observed in other membrane systems the uptake of 12-(9-anthroyl)-stearic acid is relatively slow (it is complete in about 30min); 2-(9-anthroyl)palmitic acid uptake is faster (complete in about 5min), and the binding of *N*-1-naphthyl-*N*-phenylamine is too rapid to measure by conventional methods. Since all these observations have already been studied in greater detail on other

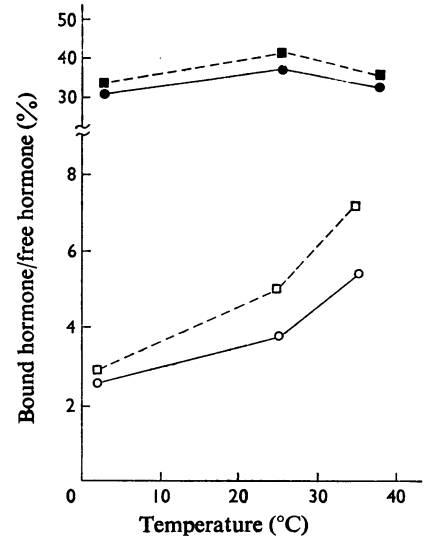

Fig. 3. *Binding of TSH to thyroid membranes in buffers of different ionic strength*

Binding of TSH was measured as described in Fig. 1 in 0.01 M-KH_2PO_4 buffer, pH7.5 (■, ●), and in the same buffer containing 0.14 M-NaCl (□, ○). Incubations were carried out for 120min (■, □) or 15min (●, ○).

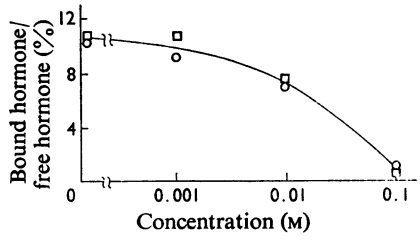

Fig. 4. *Effect of bivalent cations on the binding of TSH to thyroid membranes*

The binding of TSH to thyroid membranes suspended in 0.01 M-Tris–HCl buffer, pH7.5, containing 0.14 M-NaCl and either $MgCl_2$ (□) or $CaCl_2$ (○) at the concentration indicated was determined as described in Fig. 1.

Table 1. *Fluorescence parameters of probes in thyroid membranes*

Probes were added to a suspension of thyroid membranes (0.25mg/ml) in 0.01 M-KH_2PO_4 buffer, pH7.5, to give a final concentration of 3 μmol/litre. Emission and excitation maxima were taken from uncorrected machine spectra. The enhancement of fluorescence was calculated relative to the probe fluorescence in the buffer in the absence of thyroid membranes.

Probe	Emission maximum (nm)	Excitation maxima (nm)	Enhancement (fold)
N-1-Naphthyl-*N*-phenylamine	407	350	95
12-(9-Anthroyl)stearic acid	437	349, 368, 387	10
2-(9-Anthroyl)palmitic acid	445	349, 368, 387	15

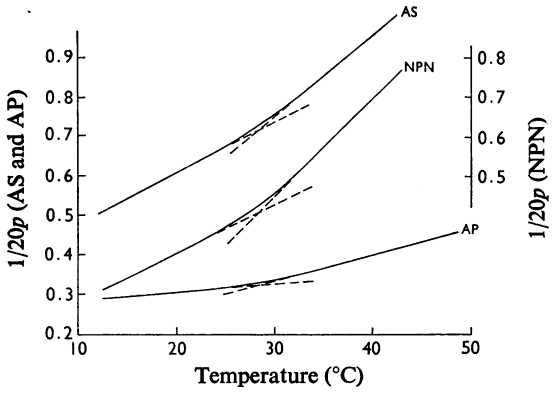

Fig. 5. *Temperature-dependence of reciprocal fluorescence polarization of fluorescence probes bound to thyroid membranes*

12-(9-Anthroyl)stearic acid (AS), 2-(9-anthroyl)palmitic acid (AP) and *N*-1-naphthyl-*N*-phenylamine (NPN) at a final concentration of 3 μmol/litre were incubated with 3ml suspensions of thyroid membranes (0.25mg/ml) in 0.01M-KH_2PO_4 buffer, pH7.5, containing 0.14M-NaCl. The temperature-dependence of fluorescence polarization was determined as described in the Experimental section. For definition of p see Table 2.

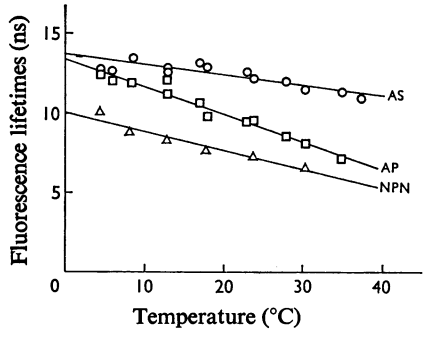

Fig. 6. *Temperature-dependence of the fluorescence lifetime of probes bound to thyroid membranes*

12-(9-Anthroyl)stearic acid (○, AS) 2-(9-anthroyl)palmitic acid (□, AP) and *N*-1-naphthyl-*N*-phenylamine (△, NPN) at a final concentration of 5 μmol/litre were incubated with 2ml suspensions of thyroid membranes (0.25mg/ml) in 0.01M-KH_2PO_4 buffer, pH7.5. Fluorescence lifetimes were measured at the temperatures indicated on the instrument described in the Experimental section. The fluorescence lifetimes exhibit linear temperature-dependence and the lines drawn represent a least-squares fit of the experimental data.

membrane systems (Radda & Vanderkooi, 1972) and showed no unusual features with thyroid membranes they will not be reported in detail here. It is sufficient to state that experiments were carried out under conditions where probe binding was complete.

The temperature-dependence of the fluorescence polarizations for the probes 12-(9-anthroyl)stearic acid, 2-(9-anthroyl)palmitic acid and *N*-1-naphthyl-*N*-phenylamine bound to thyroid membranes is shown in Fig. 5. These plots show a marked curvature that may indicate some kind of phase transition or phase separation in the membrane lipids. Extrapolation of the linear portions indicates a transition centred around 28°C. To relate fluorescence polarziation to motion of the chromophore it is necessary to know the fluorescence lifetimes for the probe molecules at each temperature. The temperature-dependence of the fluorescence lifetimes of the probes bound to thyroid membranes is shown in Fig. 6. Within the experimental error the lifetimes show a linear temperature-dependence and the lines drawn are a least-squares fit for the experimental points.

The temperature profiles reported above have all been obtained by slow heating of the sample. When they were obtained by cooling the sample from the high-temperature end of the curves a small but definite hysteretic effect was observed. In such a heating-and-cooling cycle the initial and final readings were the same and the cycle could be repeated several times with good reproducibility. These observations indicate that the temperature-dependent changes detected by the probes also have a time-dependence, particularly since the rates of heating and cooling are different. We have therefore

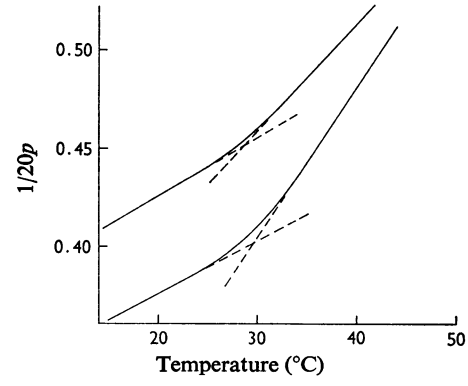

Fig. 7. *Effect of heating rate on the temperature-dependence of N-1-naphthyl-N-phenylamine reciprocal fluorescence polarization in thyroid membranes*

N-1-Naphthyl-*N*-phenylamine at a final concentration of 3 μmol/litre was incubated with 3ml of a suspension of thyroid membranes (0.25mg/ml). The temperature-dependence of fluorescence polarization was determined as described in the Experimental section.

Table 2. *Mobility of fluorescence probes in thyroid membranes*

Probes were added to a suspension of thyroid membranes (0.25mg/ml) in 0.01M-KH_2PO_4 buffer, pH7.5, or the same buffer plus 0.14M-NaCl, or 0.01M-Tris–HCl buffer, pH7.5, containing 0.14M-NaCl and 0.05M-$CaCl_2$ to give a final concentration of 3μmol/litre. All experiments were carried out at 25°C. ρ was calculated by evaluating the expression

$$\left(\frac{1}{p}-\frac{1}{3}\right)=\left(\frac{1}{p_0}-\frac{1}{3}\right)\left(1+\frac{3\tau}{\rho}\right)$$

(see the Discussion section). [τ is the fluorescence lifetime in ns and ρ the rotational relaxation-time in ns., and p the measured polarization is defined as $\frac{I_{11}-I_1}{I_{11}+I_1}$ (Weber, 1953) and is dimensionless.]

	Phosphate buffer			Phosphate–NaCl buffer			Tris–NaCl–Ca^{2+} buffer		
Probe	p	τ (ns)	ρ (ns)	p	τ (ns)	ρ (ns)	p	τ (ns)	ρ (ns)
N-1-Naphthyl-*N*-phenylamine	0.130	7.1	6.6	0.135	7.1	7.0	0.145	7.1	7.7
12-(9-Anthroyl)stearic acid	0.070	12.1	5.5	0.074	12.1	5.9	0.082	12.1	6.7
2-(9-Anthroyl)palmitic acid	0.155	9.1	11.7	0.160	9.1	12.3	0.165	9.1	12.9

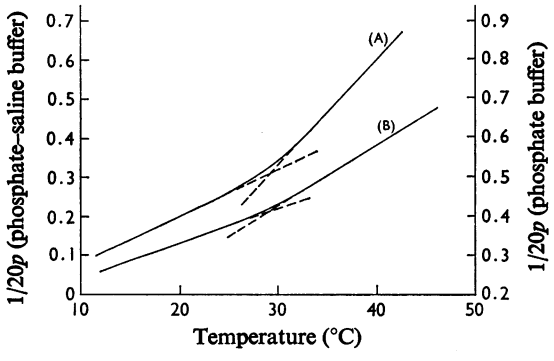

Fig. 8. *Effect of ionic strength on the temperature-dependence of N-1-naphthyl-N-phenylamine fluorescence polarization in thyroid membranes*

N-1-Naphthyl-*N*-phenylamine at a final concentration of 3μmol/litre was incubated with 3ml of a suspension of thyroid membranes (0.25mg/ml) in either (A) 0.01M-KH_2PO_4 buffer, pH7.5, or (B) the same buffer containing 0.14M-NaCl. The temperature-dependence of the reciprocal fluorescence polarization was determined as described in the text.

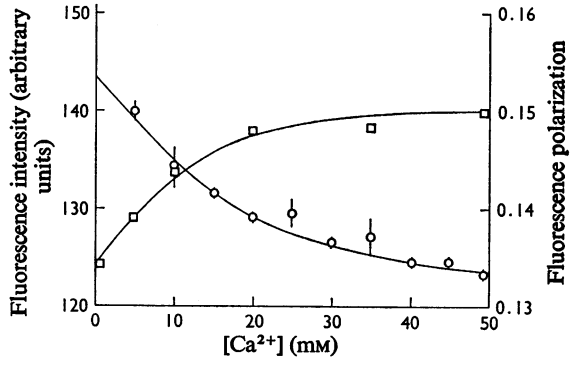

Fig. 9. *Effect of Ca^{2+} on the fluorescence of N-1-naphthyl-N-phenylamine bound to thyroid membranes*

N-1-Naphthyl-*N*-phenylamine at a concentration of 3μmol/litre was incubated with 2ml of a suspension of thyroid membranes (0.25mg/ml) in 0.01M-Tris–HCl buffer, pH7.5, containing 0.14M-NaCl. $CaCl_2$ was added as a concentrated aqueous solution to give the concentrations indicated. The fluorescence intensity (○) and the fluorescence polarization (□) were recorded. Bars represent S.E.M. of four different experiments.

studied the temperature-dependence of the fluorescence polarization of *N*-1-naphthyl-*N*-phenylamine in thyroid membranes at different rates of heating. Two such experiments are shown in Fig. 7. Although it is clear that the curvature of the plot is more marked when the heating is rapid, such time-dependence was not observed when the temperature-versus-polarization profile of *N*-1-naphthyl-*N*-phenylamine in sonicated dispersions of pure dipalmitoyl phosphatidylcholine was studied.

The effect of NaCl on the binding of TSH was to enhance the temperature-dependence compared with the lower-ionic-strength buffer. The fluorescence polarization of the three probes bound to thyroid membranes suspended in the lower-ionic-strength buffer were lower than in the presence of NaCl (Table 2), and addition of Ca^{2+} led to an increase in fluorescence polarization.

The temperature-dependence of fluorescence polarization shows significantly less curvature of the plot in the absence of NaCl (Fig. 8). Titration of Ca^{2+} with thyroid membranes in the presence of *N*-1-naphthyl-*N*-phenylamine causes a decrease in the fluorescence intensity and an increase in the polarization (Fig. 9). With the probes 12-(9-anthroyl)-

stearic acid and 2-(9-anthroyl)palmitic acid, however, titration with Ca^{2+} has no apparent effect on fluorescence intensity. It was noted that in the presence of Ca^{2+} the rate of binding of these probes was much diminished and that at concentrations greater than 50mmol/litre the binding was completely abolished. Addition of Ca^{2+} to systems where the probe was fully bound had no significant effect on the fluorescence lifetimes of the probes.

ACTH and adrenal-cortical cell membranes

If our observations have any general validity one might expect other hormone receptors to exhibit similar properties. We have therefore compared the temperature-dependence of ACTH binding with that of the fluorescence polarization of the probes in a plasma-membrane preparation obtained from human adrenal-cortical cells. The results are summarized in Fig. 10. Again the temperature at which hormone binding shows a 'transition' is detected by the fluorescence probe. In adrenal-cortical membranes this temperature is lower than that seen in the thyroid membranes and is about 23–24°C.

Discussion

Studies on lipid-bilayer vesicles by using the three fluorescent molecules employed in this work have suggested that different regions of the bilayer are sampled by the probes. The anthracene group of

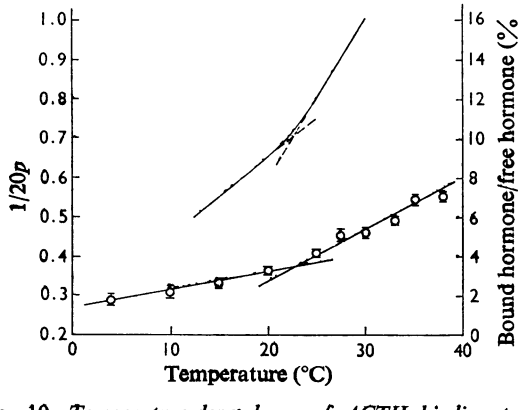

Fig. 10. *Temperature-dependence of ACTH binding to adrenal-cortical cell membranes and of the reciprocal fluorescence polarization of N-1-naphthyl-N-phenylamine bound to these membranes*

The binding of ACTH to the adrenal-cell membranes (○) was measured by the same method as TSH binding to thyroid-cell membranes. The mean of five different experiments is presented and the bars represent the S.E.M. *N*-1-Naphthyl-*N*-phenylamine at a concentration of 3μmol/litre was incubated with 3ml of a suspension of adrenal-cortical membranes (0.2mg/ml) and the temperature-dependence (——) of the reciprocal fluorescence polarization determined as described in the text.

12-(9-anthroyl)stearic acid occupies a region about 1.5nm inside from the head-group region, whereas that of 2-(9-anthroyl)palmitic acid is likely to be close to the polar/non-polar interface of the phospholipid bilayer (Radda & Vanderkooi, 1972). It has been proposed that *N*-1-naphthyl-*N*-phenylamine largely occupies the hydrophobic interior of membranes (Radda, 1971). The motional characteristics of the anthracene-containing probes in lipids reflect the hydrocarbon-chain flexibility gradient detected by other techniques (McConnell & McFarland, 1972; Radda, 1973). All three probes can be used to follow lipid-phase transitions in phospholipid bilayers and in membranes (Radda, 1974; Radda & Vanderkooi, 1972). The shifts in the fluorescence-emission maxima of these probes on binding to thyroid-cell plasma membranes and their relatively small fluorescence polarizations suggest that they occupy regions of the lipid phase in the membrane fragments. The observed fluorescence parameters correspond well to those of the probes in phospholipid model systems; binding to membrane proteins would certainly lead to increased fluorescence polarization, and is a reflexion of their mobility in the membrane lipid phase. The observed temperature profiles may then indicate changes in the lipid phase occurring at about 28°C. Indeed if we combine the polarization data with the results of the fluorescence lifetime measurements according to the Perrin equation (Perrin, 1926; Weber, 1953):

$$\left(\frac{1}{p}-\frac{1}{3}\right)=\left(\frac{1}{p_0}-\frac{1}{3}\right)\cdot\left(1+\frac{3\tau}{\rho}\right)$$

(where p is the measured polarization, p_0 is the limiting polarization produced by a randomly oriented but rigidly held set of chromophores and τ is the fluorescence lifetime) we may calculate ρ, the rotational relaxation-time (expressed as the period of rotation in s). The rate of rotation of the chromophores is then reciprocal of this relaxation-time. An Arrhenius plot of this rate permits the calculation of the activation energy for rotation. The secondary plots derived from the probe studies in thyroid membranes are shown in Fig. 11. These plots are not linear and display a break point at about 28°C, indicating that around this temperature the activation energy for probe rotation changes. The simplest interpretation of this finding is that there is a change in the phase behaviour of the membrane lipids at this temperature.

It must be emphasized that ρ may not have its true meaning for membrane-bound probes, as we cannot say with certainty that the probe molecules rotate (Wahl *et al.*, 1971). A large-amplitude vibration will also lead to fluorescence depolarization. The 'apparent rotational relaxation-time' nevertheless is still an averaged measure of the motional characteristics of the probe. Thus we may conclude from the

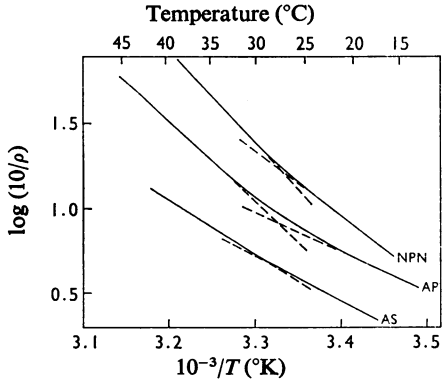

Fig. 11. *Arrhenius plots for the rate of rotation of fluorescence probes bound to thyroid membranes*

The rate of rotation of the fluorescence probes was calculated as described in the text. The plots represent the temperature-dependence of rotation rate assuming the linear relationship of fluorescence lifetime and temperature shown in Fig. 6. Abbreviations: AS, 12-(9-anthroyl)-stearic acid; AP, 2-(9-anthroyl)palmitic acid; NPN, *N*-1-naphthyl-*N*-phenylamine.

data in Table 2 that 12-(9-anthroyl)stearic acid is located in a more mobile region of the membrane lipids than 2-(9-anthroyl)palmitic acid (as would be expected) and that *N*-1-naphthyl-*N*-phenylamine shows a mobility intermediate between that of 12-(9-anthroyl)stearic acid and 2-(9-anthroyl)palmitic acid. This again is to be expected if *N*-1-naphthyl-*N*-phenylamine is in the hydrocarbon region of the membrane.

The marked change in the binding of both TSH and ACTH to their respective membranes at a temperature that corresponds to those signified by the fluorescence measurements strongly indicates that the membrane lipids have an important role in determining the constraints around the hormone-receptor site. In particular it appears that changes in lipid mobility modulate the receptor function. A comparison of the fluorescence polarization of the probes bound to membranes suspended in low- and high-ionic-strength buffers shows that there is more motional freedom in the former (where more TSH is bound) than the latter conditions (less hormone binding). The effect of bivalent metal ions can be similarly rationalized. Increasing the cation concentration results in progressive 'constraint' of the lipid phase, resulting in higher fluorescence polarization of the probes and decreased binding of the hormone. The cation also has the effect of excluding the probes from the lipid phase.

Naturally this interpretation is highly simplified, as it is likely that in the membrane lipid segregation takes place with some lipids having specific interactions with the receptor protein. In fact one interpretation of the time-dependence in the polarization-versus-temperature profiles is that after the initial sharp 'melting' of some of the segregated lipid regions a slower lateral mixing takes place. To test these ideas and the role of specific lipids in the hormone–receptor interaction it will be necessary to isolate the receptor protein and substitute well-defined phospholipids into the protein–lipid complex. We have made some preliminary observations of membrane preparations that have been 'solubilized' with detergents. In this case, where the protein–lipid interactions are disrupted, the binding of TSH to the receptor no longer shows a break in its temperature-dependence, a finding that corresponds with the linear polarization-versus-temperature profiles observed for the fluorescence probes bound to this material. These observations are consistent with our interpretation that lipid–protein interactions are important modulators of hormone–receptor interaction.

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