Changes in the Form of Arrhenius Plots of the Activity of Glucagon-Stimulated Adenylate Cyclase and other Hamster Liver Plasma-Membrane Enzymes Occurring on Hibernation

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1. Arrhenius plots of the glucagon-stimulated adenylate cyclase, 5'-nucleotidase, (Na⁺+K⁺)-stimulated adenosine triphosphatase and Mg²⁺-dependent adenosine triphosphatase activities of control hamster liver plasma membranes exhibited two break points at around 25 and 13°C, whereas Arrhenius plots of their activities in hibernating hamster liver plasma membranes exhibited two break points at around 25 and 4°C. 2. A single break occurring between 25 and 26°C was observed in Arrhenius plots of the activities of fluoride-stimulated adenylate cyclase, basal adenylate cyclase and cyclic AMP phosphodiesterase of liver plasma membranes from both control and hibernating animals. 3. Arrhenius plots of phosphodiesterase I activity showed a single break at 13°C for membranes from control animals, and a single break at around 4°C for liver plasma membranes from hibernating animals. 4. The temperature at which break points occurred in Arrhenius plots of glucagon- and fluoride-stimulated adenylate cyclase activity were decreased by about 7–8°C by addition of 40 mM-benzyl alcohol to the assays. 5. Discontinuities in the Arrhenius plots of 4-anilinonaphthalene-1-sulphonic acid fluorescence occurred at around 24 and 13°C for liver plasma membranes from control animals, and at around 25 and 4°C for membranes from hibernating animals. 6. We suggest that in hamster liver plasma membranes from control animals a lipid phase separation occurs at around 25°C in the inner half of the bilayer and at around 13°C in the outer half of the bilayer. On hibernation a change in bilayer asymmetry occurs, which is expressed by a decrease in the temperature at which the lipid phase separation occurs in the outer half of the bilayer to around 4°C. The assumption made is that enzymes expressing both lipid phase separations penetrate both halves of the bilayer, whereas those experiencing a single break penetrate one half of the bilayer only.

Lipid phase separations in biological membranes have been demonstrated by using a variety of physical probes (see, e.g., Overath & Trauble, 1973; Chapman, 1975; Morrisett *et al.*, 1975; Houslay *et al.*, 1976b; Sauerheber *et al.*, 1977; Tecoma *et al.*, 1977). Their existence has also been inferred from observations of breaks in the Arrhenius plots of the activity of a number of membrane-bound enzymes (see, e.g., Lee *et al.*, 1974; Morrisett *et al.*, 1975; Wunderlich *et al.*, 1975; Houslay *et al.*, 1976a,b): the temperatures at which such 'break' points occur have been correlated with lipid phase separations detected by physical methods. Manipulation of the composition of the lipid bilayer of biological membranes either directly, by using lipid fusion or substitution techniques (Houslay *et al.*, 1975, 1976b), or indirectly, by additions to culture media or diet (see, e.g., Esko *et al.*, 1977; Morrisett *et al.*, 1975; Thilo *et al.*, 1977), demonstrate that break points detected

by Arrhenius plots of the activity of membrane-bound enzymes or transport functions are related to the composition and hence the physical properties of the lipid bilayer.

In mammalian tissues, the temperatures at which lipid phase separations occur appear to vary from species to species and membrane to membrane, there being no apparent direct connection with the temperature at which the animal maintains homeothermy. Hibernating animals provide us with a striking adaptation to lower body temperature, and this work investigates changes in the Arrhenius plots of a number of enzymes associated with hamster liver plasma membranes.

Methods

Golden (Syrian) hamsters (*Mesocricetus auratus*) were encouraged to hibernate by allowing them to store food that had been directly introduced into

Abbreviation used: ATPase, adenosine triphosphatase.

their individual cages, and progressively shortening their daylight hours from 12 to 5 h over a period of 1 month. At this time they were placed in a cold-room at 4°C in the dark, and usually hibernated within 2–4 weeks. Animals were then killed some 10–14 days after achieving hibernation, when their body temperatures were in the region of 4–5°C. Control hamsters had a body temperature of around 38°C and were kept at 21°C with a 12 h light period.

Hamster liver plasma membranes were prepared by the method of Pilkis *et al.* (1974), except that the isolated plasma-membrane fraction from the 42.5%/48.2% sucrose interface was first resuspended in 1 mM-KHCO₃, pH 7.4, and pelleted at 20000 *g*_{av}. for 5 min, before resuspension in 250 mM-sucrose/10 mM-Tris/HCl buffer, pH 7.4. This suspension was centrifuged at 1000 *g*_{av}. for 10 min, and the pelleted material resuspended and washed twice in 1 mM-KHCO₃, pH 7.4, as detailed above. This washed material is designated our plasma-membrane fraction, and was usually stored in liquid N₂ at a concentration of 6–10 mg of protein/ml.

During all assays at any particular temperature, initial rates were taken from linear time courses and the pH was kept constant at pH 7.4. To clearly define the form of the Arrhenius plots it was necessary to determine enzyme activity at 35–45 individual temperatures in the range <1°C to 42°C. Plasma-membrane fractions were prepared from individual animals and three separate preparations were assayed in each instance to yield complete Arrhenius plots. Arrhenius plots of the activity of any particular enzyme activity from all three preparations showed clear, visual indication of either a single or two break points as described in the Results section. Such data were analysed by attempting to fit them to two lines, 'a' and 'b', by using a least-squares procedure with repeated iterations (see Hoare, 1972). Briefly this consisted of fitting *y* observations of initial velocity observed at progressively decreasing assay temperature to the two straight lines (a, b) in sets of [4, (*y*–4)]...[(*y*–4), 4] data points. The sum of the squares of the residuals could then be plotted against the increasing number of data points taken to constitute line 'a', to identify a minimum value.

The results obtained by such analysis fell into two categories. Case (i) yielded a single minimum value with a real solution for the 'break' temperature. The valleys leading to such a solution were roughly symmetrical. Case (ii) yielded two minima with real solutions for two break temperatures. Data from control animals sometimes merged these two minima so as to create clearly asymmetrical valleys. In case (ii), by restricting the range of analysis of the data as defined by temperature limits it was possible to obtain single minima as in case (i), and hence define upper and lower break points. All Arrhenius plots that on visual analysis exhibited one break yielded

a result as in case (i); all data that on visual analysis exhibited two breaks yield a result as in case (ii).

The 95% confidence limits could be obtained from such an analysis as described by Hoare (1972).

Basal and ligand-stimulated adenylate cyclase activity (EC 4.6.1.1) were assayed as described by Houslay *et al.* (1976a), except that the assay mixture was at pH 7.6 and contained 14 mM-2-mercapto-ethanol.

5'-Nucleotidase activity (EC 3.1.3.5) was followed by direct spectrophotometric assay at 265 nm of the conversion of adenosine product into inosine by adenosine deaminase (EC 3.5.4.4). The assay mixture contained 50 mM- α -glycerophosphate (sodium salt), 0.5 mM-MgSO₄, 0.1 mM-AMP and 0.4 unit of adenosine deaminase/ml in 50 mM-triethanolamine/HCl buffer, pH 7.6. One unit of activity is the amount of enzyme that converts 1 μ mol of substrate/min in all cases.

Alkaline phosphodiesterase I (EC 3.1.4.1) was assayed in the presence of 1 mM-*p*-nitrophenyl dTMP and 100 mM-triethanolamine/HCl, pH 7.6. Reactions were terminated by the addition of 2 ml of 1 M-KOH, and the *A*₄₀₀ was read against an appropriate blank (see Razzel, 1963).

Cyclic AMP phosphodiesterase (EC 3.1.4.17) activity was assayed by the two-step procedure of Thompson & Appleman (1971). All assays were carried out in 50 mM-triethanolamine/HCl buffer, pH 7.6, by using 1 mM-cyclic AMP as substrate with 5 mM-MgSO₄. Corrections were made for cyclic AMP that remained unbound to the resin, and for adenosine product that bound to the resin.

Total ATPase activity (EC 3.6.1.3) was followed by direct spectrophotometric assay in an assay volume of 1 ml containing final concentrations of 100 mM-triethanolamine/HCl, 5 mM-MgSO₄, 50 mM-KCl, 10 mM-NaCl, 0.1 mM-EDTA, 0.42 mM-phosphoenolpyruvate, 2 mM-ATP, 0.15 mM-NADH, 4 units of pyruvate kinase (EC 2.7.1.40)/ml and 10 units of lactate dehydrogenase (EC 1.1.1.27)/ml at a final pH value of 7.6. The change in *A*₃₄₀ was followed in the presence and absence of 3 mM-ouabain. The ouabain sensitivity of the preparation was tested at 37, 20 and 4°C, and no further inhibition could be obtained by raising the ouabain concentration to 15 mM. The rate obtained after addition of ouabain was designated the Mg²⁺-stimulated ATPase, and the difference between this and the total ATPase rate, i.e. the ouabain-sensitive activity, was designated as being due to (Na⁺/K⁺)-stimulated ATPase activity.

Succinate dehydrogenase (EC 1.3.99.1) was assayed as described in full by Houslay & Tipton (1973).

Glucose 6-phosphatase (EC 3.1.3.9) was assayed in the presence of 50 mM-sodium cacodylate buffer and 20 mM-glucose 6-phosphate, final pH 6.5, in a final volume of 0.75 ml. The reaction was terminated by addition of 0.5 ml of 10% (w/v) trichloroacetic

acid, and after centrifugation (60000g-min) a sample was taken for phosphate analysis by the method of Fiske & Subbarow (1925).

Lipid phase separations occurring in the membrane fraction were followed by the change in fluorescence of 4-anilinonaphthalene-1-sulphonic acid. This was carried out as described in detail by Overath & Trauble (1973), except that 50mM-potassium phosphate buffer, pH 7.6, at each temperature studied was used. The membrane fraction was suspended at a final concentration of 0.05–0.1 mg/ml with 50 μ M-4-anilinonaphthalene-1-sulphonic acid. Excitation was achieved at 366 nm and emission followed at 480 nm. Corrections were made for any changes in fluorescence in control cuvettes containing either membranes plus buffer, 4-anilinonaphthalene-1-sulphonic acid plus buffer or buffer alone. No net changes in fluorescence were observed if emission was followed at 550 nm, where 4-anilinonaphthalene-1-sulphonic acid in aqueous solution fluoresces. Phase-separation temperatures were estimated graphically as described in full by Overath & Trauble (1973).

Protein determinations were carried out by the microbiuret method of Goa (1953) with bovine serum albumin as standard. However, the membrane fractions (<1 mg) were first incubated in 10 mg of sodium cholate/ml, pH 7.2, in a total volume of 0.1 ml for 10 min. They were then treated with 1 ml of 10% (w/v) trichloroacetic acid for 5 min before centrifuging for 10 s at 14000g. To the pellet was added 1 ml of 3% (w/v) NaOH containing 1 mg of sodium cholate/ml. This mixture was then left overnight before addition of a further 1 ml of 3% (w/v) NaOH and 0.1 ml of developing reagent as described by Goa (1953). All standards were treated similarly.

Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Pyruvate kinase, lactate dehydrogenase, adenosine deaminase, ATP, NADH, triethanolamine hydrochloride and phosphoenolpyruvate were from Boehringer (U.K.), Lewes, Sussex, U.K. Glucose 6-phosphate, *p*-nitrophenyl dTMP, 4-anilinonaphthalene-1-sulphonic acid and creatine kinase were from Sigma (London) Chemical Co., Kingston upon Thames, Surrey, U.K.

Ouabain and AnalaR-grade chemicals were from BDH, Poole, Dorset, U.K. Golden (Syrian) hamsters were obtained from Roundhill Rabbit Farm, Penistone, Yorks., U.K. Glucagon was a kind gift from Dr. W. W. Bromer, Eli Lilly and Co., Indianapolis, IN, U.S.A.

Results

Membrane preparation

The procedure described yielded a purified plasma-membrane preparation that by analysis of 5'-nucleotidase activity appeared to represent over 70% of the total liver plasma membranes (Table 1). Contamination with mitochondria and endoplasmic reticulum, monitored by succinate dehydrogenase and glucose 6-phosphatase activities respectively, was low (of the order of less than 0.5% of the total; Table 1). The plasma-membrane fraction was either used at once for investigation or stored in liquid N₂ for use later. Similar results were obtained with both preparations. We obtained no evidence for any latency of effects on enzyme activity occurring after treating the membranes by subsequently freeze-thawing, homogenizing or by changing the ionic strength of the medium in which the membranes were stored. All the changes in enzyme activity occurring with the temperatures encountered during Arrhenius-plot experiments were freely reversible in either direction. The specific activities at 30°C of the various enzymes studied are shown in Table 2 for plasma membranes from both control and hibernating animals (Table 2). The only obvious effect of hibernation was the raised values for both the 5'-nucleotidase and phosphodiesterase I activities associated with the plasma membranes from hibernating animals. Clearly any small differences in activities occurring on hibernation could well be masked by small changes in the purity of the plasma-membrane fractions.

Arrhenius plots of enzyme activities from control hamster liver plasma membranes

The Arrhenius plots of the activity of glucagon-stimulated adenylate cyclase, 5'-nucleotidase, (Na⁺+

Table 1. Isolation of plasma-membrane fraction

This Table demonstrates the average results of three preparations from control hamster livers. Those from hibernating animals were essentially similar. Data are given $\pm$ s.d. ($n=3$).

Enzyme activity	Specific activity in:			
	Homogenate	Plasma membrane fraction	Purification (-fold)	Yield (%)
5'-Nucleotidase	1 $\pm$ 0.3 munits/mg	22 $\pm$ 5 munits/mg	22	>70
Glucose 6-phosphatase	1.2 $\pm$ 0.1 units/mg	0.15 $\pm$ 0.05 units/mg	0.125	<0.5
Succinate dehydrogenase	20 $\pm$ 4 munits/mg	2.2 $\pm$ 0.5 munits/mg	0.11	<0.5

Table 2. Activity of a number of liver plasma-membrane-bound enzymes from control and hibernating animals. Results were obtained at 30°C, and data are given $\pm$ s.d. The number of individual preparations tested is given in parentheses.

Enzyme activity	Specific activity of plasma-membrane fraction from:	
	Control	Hibernating
Glucagon-stimulated adenylate cyclase (μ units/mg)	876 $\pm$ 136 (5)	999 $\pm$ 226 (5)
Fluoride-stimulated adenylate cyclase (μ units/mg)	575 $\pm$ 196 (7)	550 $\pm$ 139 (7)
Basal adenylate cyclase (μ units/mg)	51 $\pm$ 12 (7)	59 $\pm$ 20 (7)
5'-Nucleotidase (munits/mg)	20.7 $\pm$ 3.6 (6)	38.3 $\pm$ 6.8 (4)
Cyclic AMP phosphodiesterase (munits/mg)	7.8 $\pm$ 3.2 (3)	6.2 $\pm$ 2.1 (3)
Phosphodiesterase I (munits/mg)	30.1 $\pm$ 5.0 (3)	76.9 $\pm$ 5.8 (3)
(Na ⁺ +K ⁺)-stimulated ATPase (munits/mg)	171.3 $\pm$ 73.0 (3)	179.0 $\pm$ 40.7 (3)
Mg ²⁺ -dependent ATPase (munits/mg)	1008 $\pm$ 184 (3)	797 $\pm$ 149 (5)

K⁺)-stimulated ATPase and Mg²⁺-dependent ATPase were clearly triphasic, exhibiting two break points at around 25 and 13°C (Figs. 1a, 1e and 1f; Table 3). The cyclic AMP phosphodiesterase and both the fluoride-stimulated and basal adenylate cyclase activities yielded Arrhenius plots with a single, well-defined break at around 25°C (Figs. 1b and 1d; Table 3), whereas the single break exhibited by the Arrhenius plots of phosphodiesterase I activity occurred at 13°C (Fig. 1c; Table 3). It is noteworthy that the activation energy of the reaction increased at temperatures below the break point, except for the activity of the cyclic AMP phosphodiesterase, a case where it decreased (Table 3). This may be because it is an extrinsic protein anchored primarily through interaction with the head-group region of the bilayer, as it can be readily shown to be released by mild sonication (see Appleman *et al.*, 1973).

If 40mM-benzyl alcohol was added to assays of ligand-stimulated adenylate cyclase activity, then a reversible increase in activity of 20–50% ensued. The Arrhenius plots of the glucagon-stimulated activity now showed two breaks occurring at around 18 and 5°C, and the single break observed with the fluoride-stimulated activity was similarly decreased to around 18°C (Figs. 1a and 1b).

Arrhenius plots of enzyme activities from hibernating-hamster liver plasma membrane

Like the Arrhenius plots of the control enzyme activities, the Arrhenius plots of the glucagon-stimulated adenylate cyclase, 5'-nucleotidase, (Na⁺+K⁺)-dependent ATPase and Mg²⁺-stimulated ATPase activities of the liver plasma membranes from hibernating hamsters exhibited two clear breaks (Figs. 2a, 2e and 2f; Table 4). The upper break at around 25°C coincided with that shown by these enzyme activities from the control animals, but the lower break was shifted dramatically down by 9°C to

about 4°C (Table 4). Furthermore, the activation energy of the reactions assayed at temperatures below the lower break point were larger than those found with the enzymes from the control liver plasma membranes. This was particularly noticeable for the glucagon-stimulated adenylate cyclase, 5'-nucleotidase and (Na⁺+K⁺)-stimulated ATPase activities (Table 4).

The form of the Arrhenius plots of the basal (unstimulated) and fluoride-stimulated adenylate cyclase and the cyclic AMP phosphodiesterase activities were apparently unchanged by using liver plasma membranes from hibernating animals: all exhibited a single break at around 25°C (Figs. 2b and 2d; Table 4). However, the Arrhenius plot of the phosphodiesterase I activity was markedly affected; a single break at around 4°C could be clearly demonstrated (Fig. 2c; Table 4), as distinct from a single break at around 13°C exhibited by control liver plasma membranes (Fig. 1c; Table 4).

The addition of 40mM-benzyl alcohol to ligand-stimulated adenylate cyclase assays effected a reversible 20–40% increase in enzyme activity at 30°C. Arrhenius plots of glucagon-stimulated adenylate cyclase activity in the presence of 40mM-benzyl alcohol showed a single break at around 18°C, as did those of the fluoride-stimulated activity (Figs. 2a and 2b).

4-Anilino-naphthalene-1-sulphonic acid fluorescence and hamster liver plasma membranes

An Arrhenius-plot transform of the fluorescence of 4-anilino-naphthalene-1-sulphonic acid partitioning into the plasma-membrane lipid phase with change in temperature indicated two discontinuities for preparations of plasma membranes from both hibernating and control animals (Fig. 3). Treatment of these plots as described by Overath & Trauble (1973) yielded values of around 25 and 13°C for the

Fig. 1. Arrhenius plots of the activity of liver plasma-membrane enzymes from control hamsters

The log of initial velocity (v) is plotted against the reciprocal of the absolute temperature. (a) Glucagon-stimulated adenylate cyclase activity in the absence (●) or presence (■) of 40mM-benzyl alcohol (v in μ units/mg). (b) Basal adenylate cyclase activity (▲) and fluoride-stimulated adenylate cyclase activity in the absence (●) and presence (■) of 40mM-benzyl alcohol (v in μ units/mg). (c) Phosphodiesterase I activity (●) (v in munits/mg). (d) Cyclic AMP phosphodiesterase activity (●) (v in munits/mg). (e) 5'-Nucleotidase activity (●) (v in units/mg). (f) (Na^++K^+)-stimulated ATPase activity (●) and Mg^{2+} -dependent ATPase activity (▲) (v in munits/mg).

control membranes and of around 25 and 4°C for the hibernating membranes (Table 4) for the temperatures at the midpoint of such discontinuities.

Discussion

The present work demonstrates that there are clearly defined breaks in the Arrhenius plots of the activity of a number of hamster liver plasma-membrane enzymes. The occurrence of breaks or discontinuities in such Arrhenius plots may be attributed to a number of causes, such as change in rate-limiting step or kinetic reaction mechanism. In this study we have shown that a number of different enzyme activities associated with the liver plasma membranes of control animals exhibit either one or two Arrhenius-plot breaks at either 25 or 13°C. The main effect of hibernation was to lower the latter temperature to around 4°C. This phenomenon of lowering the 13°C break to 4°C on hibernation is demonstrated by five enzymes, namely glucagon-stimulated adenylate cyclase, 5'-nucleotidase, (Na^++K^+)-stimulated ATPase, Mg^{2+} -dependent ATPase and phosphodiesterase I. We consider it unlikely that enzymes with such diverse activities should display such similarities in the temperature at which breaks in the Arrhenius plots of their activity occur, and such similarity in response to hibernation, if these breaks were due to causes such as change in reaction mechanism, rate-limiting step or even in the enzyme protein occurring on hibernation.

We suggest that a simple unifying explanation of these common breaks shown by a number of very different membrane enzymes is that they represent lipid phase separations occurring in the plane of the bilayer. Consistent with such an interpretation is the observation that the fluorescent membrane probe 4-anilinnaphthalene-1-sulphonic acid exhibits discontinuities in the change in fluorescence of the species partitioning into the bilayer with change in temperature. Overath & Trauble (1973) and Wunderlich *et al.* (1975), among others, have demonstrated that Arrhenius plots of such data can be used to detect lipid phase separations. Such a transform of our data (Fig. 3) led to the identification of two phase separations occurring at around 25 and 13°C in membranes from control animals, and at around 24 and 4°C in membranes from hibernating animals (Table 3). These show a good correlation with the observed temperatures at which break points occurred in the Arrhenius plots of enzyme activities (Table 3). Furthermore it has been shown in model systems that the neutral local anaesthetic benzyl alcohol can increase the fluidity of defined lipid bilayers and lower the temperature of lipid phase transitions by 6–8°C (Colley & Metcalfe, 1972), and it is also able to decrease the temperature at which lipid phase separations occur in rat liver plasma membranes by about 6°C (Dipple & Houslay, 1978). These effects have been demonstrated to reach a plateau with concentrations of benzyl alcohol of about 40–60mM. The addition of benzyl alcohol to

Table 3. Break points and activation energies from Arrhenius plots of the enzyme activities from liver plasma membrane from control hamsters

These results are the average of data obtained by using membrane preparations from different individual animals. Altogether seven individual animals were used, but the data for a particular enzyme are given for three preparations from individual animals. Data are given with 95% confidence limits in parentheses (see the Methods section), or for 4-anilidonaphthalene-1-sulphonic acid fluorescence as $\pm$ s.d. ($n=3$). The width of the lipid phase separation (ΔT °C) is also given $\pm$ s.d.

Arrhenius plot	Break point (°C)		Activation energy (kJ/mol)		
	Upper	Lower	Above upper break	Below upper break	Below lower break
Glucagon-stimulated adenylate cyclase	24.7 (24.0–25.7)	12.3 (11.5–13.5)	39.8 (38.0–42.3)	81.2 (75.3–82.9)	106 (103–108)
Fluoride-stimulated adenylate cyclase	26.0 (25.0–27.0)	—	54.0 (52.7–57.8)	91.3 (89.2–94.2)	—
Basal adenylate cyclase	26.0 (25.4–26.4)	—	8.0 (6.3–9.2)	69.5 (67.0–77.4)	—
5'-Nucleotidase	25.1 (23.0–26.0)	13.4 (11.6–14.0)	45.6 (42.7–49.8)	82.0 (77.0–90.0)	106 (103–111)
Cyclic AMP phosphodiesterase	25.2 (24.3–26.0)	—	49.4 (46–51.9)	24.7 (20.9–29.3)	—
Phosphodiesterase I	—	13.3 (12.0–24.0)	—	33.1 (31.0–34.3)	50.7 (48.1–52.7)
(Na ⁺ +K ⁺)-stimulated ATPase	25.4 (24.5–25.8)	12.2 (11.8–12.9)	44.8 (40.2–48.6)	87.9 (86.2–92.9)	143 (139–144)
Mg ²⁺ -dependent ATPase	25.1 (24.4–26.0)	12.6 (11.0–13.1)	11.3 (9.2–13.4)	37.7 (36.4–39.3)	52.3 (50.2–54.4)
4-Anilidonaphthalene-1-sulphonic acid fluorescence	24.5 (± 0.7)	12.8 (± 1.9)	ΔT upper 3.6 (± 1.2)	ΔT lower 2.4 (± 0.6)	

assays of ligand-stimulated adenylate cyclase gave a reversible enhancement of activity of 20–50% (Figs. 1a, 1b, 2a and 2b), as demonstrated previously in some detail for the enzyme from rat liver plasma membranes (Dipple & Houslay, 1978). However, Arrhenius plots of adenylate cyclase activities in the presence of 40mM-benzyl alcohol show a marked change in form. The temperatures at which break points in the Arrhenius plots of adenylate cyclase activity occurred were decreased by some 7–8°C (Figs. 1a, 1b, 2a and 2b). This led to the apparent abolition of the lower break at 4°C occurring in Arrhenius plots of the glucagon-stimulated adenylate cyclase activity from hibernating-hamster liver plasma membranes (Fig. 2a). Such effects would be expected if these break points represented lipid phase separations whose temperatures were decreased by the addition of benzyl alcohol, the decrease in lipid phase-separation temperature being due to the energy required to extrude benzyl alcohol from regions of the bilayer where ordered phase lipid forms on cooling.

There is abundant evidence to support the concept of hormone-stimulated adenylate cyclase achieving vectorial transfer of information across the cell membrane. This is because the binding site for glucagon on the glucagon receptor is placed on the outer surface of the plasma membrane, whereas the

active site of the catalytic unit of adenylate cyclase is on the inner/cytosol surface of the membrane (see Birnbaumer, 1973; Perkins, 1973). The glucagon receptor and the catalytic unit of adenylate cyclase are distinct proteins that may be physically separated (see, e.g. Giorgio *et al.*, 1974; Welton *et al.*, 1977; Swislocki *et al.*, 1977). We have provided good physical evidence that in the glucagon-stimulated state the catalytic unit of adenylate cyclase is physically associated (coupled) with the glucagon receptor, forming a multicomponent complex that spans the bilayer membrane (Houslay *et al.*, 1977). However, in the uncoupled state when either the basal or fluoride-stimulated activity of the enzyme is monitored then the catalytic unit is free to migrate, independently in the plane of the bilayer (Houslay *et al.*, 1977). We suggest that the phase separation occurring at around 25°C and experienced by the uncoupled catalytic unit of adenylate cyclase is a property of the inner/cytosol half of the bilayer, whereas that phase separation occurring at around 13°C in the control membranes is a property of the outer half of the bilayer. Thus proteins known to span the bilayer, such as glucagon-stimulated adenylate cyclase and the (Na⁺+K⁺)-stimulated ATPase, would experience both such lipid phase separations occurring in the two halves of the bilayer. 5'-Nucleotidase and Mg²⁺-dependent ATPase are believed to be ectoenzymes,

Fig. 2. Arrhenius plots of the activity of liver plasma membranes from hibernating hamsters

The log of the initial velocity is plotted against the reciprocal of the absolute temperature. (a) Glucagon-stimulated adenylate cyclase activity in the absence (●) and presence (■) of 40mM-benzyl alcohol (v in μ units/mg). (b) Basal adenylate cyclase activity (▲) and fluoride-stimulated adenylate cyclase activity in the absence (●) and presence (■) of 40mM-benzyl alcohol (v in μ units/mg). (c) Phosphodiesterase I activity (●) (v in munits/mg). (d) Cyclic AMP phosphodiesterase activity (●) (v in munits/mg). (e) 5'-Nucleotidase activity (●) (v in units/mg). (f) (Na^+ + K^+)-stimulated ATPase activity (●) and Mg^{2+} -dependent ATPase activity (▲) (v in munits/mg).

Evans, 1974; Pommier *et al.*, 1975). Presumably as they are sensitive to both of these lipid phase separations (Tables 3 and 4) they penetrate both halves of the bilayer. However, the ectoenzyme phosphodiesterase I (Evans, 1974) only experiences a single lipid phase separation occurring at the lower temperature (Table 3), and this protein may be exclusively localized in the outer half of the bilayer. The cyclic AMP phosphodiesterase similarly shows a single break in Arrhenius plots of its activity (Table 4), but this occurs at around 25°C , corresponding to the lipid phase separation assigned to the inner half of the bilayer. A schematic representation of the proposed disposition of these enzymes in the hamster liver plasma membrane is given in Fig. 4.

This interpretation allows us to explain the selective effects of hibernation on the forms of the Arrhenius plots. For if we assume that there is little or no change in the lipid phase-separation temperature occurring in the inner half of the bilayer at around 25°C , then enzymes spanning the bilayer or located in the inner half of the bilayer will still yield Arrhenius plots with an upper break point at around 25°C (see Tables 3 and 4 and Fig. 4). However, the lowering of the lipid-phase-separation temperature in the outer half of the bilayer to around 4°C will only be experienced by those proteins spanning the bilayer, and by the phosphodiesterase I located in the outer half of the bilayer (Tables 3 and 4 and Fig. 4). Thus it would appear that on hibernation there is a change in the asymmetry of the lipid bilayer that is expressed by a decrease of the lipid phase-separation temperature occurring at around 13°C in membranes from control animals to around 4°C in membranes from hibernating animals; such a decrease may indicate an increase in the fluidity of this half of the bilayer. Such an effect may be achieved by subtle means such as alterations in the proportions of various phospholipid species or in the fatty acyl side chains associated with them. This may be difficult to detect experimentally, being localized to one bilayer half and perhaps even to laterally segregated lipid within that half of the bilayer.

There is evidence that certain enzymes can segregate specific lipids out of the bulk lipid pool to constitute their lipid annulus (see, e.g., Houslay *et al.*, 1975; Warren *et al.*, 1975). The physical properties of such a lipid annulus may then determine to some extent the activity of the enzyme, and such properties need not reflect those of the bulk lipid pool. It remains to be seen how widespread a feature of membrane proteins this is, but looked at in such a context it might appear at first sight to be rather surprising to observe such a similarity in break temperatures for the enzymes studied. This could imply that the annular composition of all the enzymes localized in one half of the bilayer would be basically

namely membrane-bound enzymes with their active sites exposed to the external surface of the intact cell (see Evans & Gurd, 1973; Evans *et al.*, 1973; Gurd &

Table 4. Break points and activation energies from Arrhenius plots of the enzyme activities from liver plasma membranes from hibernating hamsters

These results are the average of data obtained by using membrane preparations from different individual animals. Altogether seven individual animals were used, but the data for a particular enzyme activity are given for three preparations from individual animals. Data are given with 95% confidence limits in parentheses (see the Methods section) or for 4-anilinonaphthalene-1-sulphonic acid fluorescence as $\pm$ s.d. ($n=3$). The width of the lipid phase separation ($\Delta T^\circ\text{C}$) is also given $\pm$ s.d.

Arrhenius plot	Break points ($^\circ\text{C}$)		Activation energy (kJ/mol)		
	Upper	Lower	Above upper break	Below upper break	Below lower break
Glucagon-stimulated adenylate cyclase	25.9 (24.5–26.3)	4.0 (3.6–4.3)	26.8 (26.0–30.1)	76.6 (74.9–80.8)	377 (364–398)
Fluoride-stimulated adenylate cyclase	25.5 (23.6–26.7)	—	43.5 (39.8–46.0)	87.1 (81.6–92.5)	—
Basal adenylate cyclase	25.9 (25.0–26.5)	—	15.5 (14.7–20.1)	69.1 (65.7–70.3)	—
5'-Nucleotidase	25.7 (24.6–26.8)	4.4 (3.2–5.6)	46.5 (42.7–49.0)	93.3 (90.4–108)	377 (335–460)
Cyclic AMP phosphodiesterase	24.5 (23.0–26.7)	—	47.7 (45.2–51.1)	37.7 (33.5–39.8)	—
Phosphodiesterase I	—	4.1 (3.5–4.6)	—	27.2 (26.8–28.1)	64.9 (52.3–66.1)
($\text{Na}^+ + \text{K}^+$)-stimulated ATPase	25.7 (24.4–26.7)	3.3 (2.9–4.0)	41.0 (38.1–46.0)	64.0 (61.1–75.3)	425 (398–456)
Mg^{2+} -dependent ATPase	24.1 (23.3–24.9)	4.3 (3.4–4.5)	7.5 (6.7–9.2)	37.3 (34.7–38.1)	68.2 (46.0–69.5)
4-Anilinonaphthalene-1-sulphonic acid fluorescence	24.2 (± 0.5)	3.8 (± 0.4)	ΔT upper 3.4(± 1.1)	ΔT lower 2.8(± 0.2)	

the same, as would those spanning the bilayer. However, this need not necessarily be the case, as it has been demonstrated that the Ca^{2+} -stimulated Mg^{2+} -dependent ATPase from rabbit muscle sarcoplasmic reticulum, when inserted in a defined bilayer of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, exhibits two breaks in the Arrhenius plot of its activity. One of these occurs around 39°C , corresponding to the phase transition occurring in the bulk lipid phase, and one at about 29°C , corresponding to a change in the physical state of annular phospholipid (Hesketh *et al.*, 1976). Such experiments showed that this protein could sense changes in the physical state of both the bulk lipid phase as well as that of its lipid annulus, and also that perturbations of the bilayer by this protein were essentially restricted to the phospholipid annulus (see also Marcelja, 1976). The localization of such perturbation to the lipid annulus of the protein would ensure that the physical properties of the bulk lipid phase would be relatively unaffected by protein insertion (up to a certain lipid/protein ratio) and hence a variety of different proteins may respond similarly to changes in the state of the bulk lipid phase. This could provide a basis for our observation that a number of very different proteins associated with hamster liver plasma membranes exhibit remarkably similar breaks in Arrhenius plots

of their activities. Similar observations have been obtained for different enzyme activities and transport processes associated with other membranes (see, e.g., Lenaz *et al.*, 1972; Houslay *et al.*, 1976c; Thilo *et al.*, 1977). Thus a protein with a lipid annulus not reflecting the composition of the bulk lipid pool may still sense lipid phase separations occurring in the bulk lipid pool, but will have superimposed on this any changes in the physical nature of its lipid annulus. Our results imply that if there were differences in composition or physical properties of these annular lipids from those of the bulk lipid pool in hamster liver plasma membranes, then presumably no change in their physical characteristics occurred that could lead to the appearance of breaks in the Arrhenius plots of enzyme activity over the temperature range studied, other than those due to the lipid phase separations occurring in the bulk lipid pool. In theoretical calculations performed by Marcelja (1976), the phase transition temperature for annular lipid may be broadened or broadened and shifted, dependent on the strength of the Van der Waals interactions between the lipid and protein. However, if these interactions are very strong then the phase transition of annular lipid may be so broadened as to be almost smeared out. It may be that in our studies any changes in state of annular lipid either

Fig. 3. Arrhenius plots of 4-anilino-naphthalene-1-sulphonic acid fluorescence for liver plasma membranes from control animals (▲) and from hibernating animals (●). For details see the Methods section.

reflected that of the bulk lipid pool or occurred at too high or too low a temperature to be experimentally accessible, or were so broadened in form as to be undetectable by our analysis. On this basis it would appear reasonable to assign the sharp breaks in Arrhenius plots of enzyme activity to the occurrence of lipid phase separations occurring either in the bulk lipid phase or the lipid annulus.

A difference in the physical properties of annular lipid from that of the bulk lipid pool may lead to a damping of the effect of changes in the physical state of the bulk lipid pool upon the conformation of the protein. This may be offered as an explanation for the less striking changes in activation energy occurring through the break point temperatures for the activity of the Mg^{2+} -dependent ATPase. Although an equally plausible explanation of such an effect would be a relative insensitivity of the protein to changes in the rigidity or fluidity of its environment. However, the Mg^{2+} -dependent ATPase clearly senses the lipid phase separations occurring in the bilayer, as is evident from the very distinct breaks in Arrhenius plots of its activity that can be distinguished graphically (Figs. 1*f* and 2*f*) or by line-fitting analysis

Fig. 4. Disposition of membrane proteins in hamster liver plasma membranes

A schematic representation of the localization of the proteins investigated in hamster liver plasma membranes. The temperature at which lipid phase separations occur are given ($T_s, ^{\circ}C$) and the active site of enzymes or binding site on receptors is indicated by (////). Abbreviations: GR, glucagon receptor; H, glucagon, hormone; A, catalytic unit of adenylate cyclase; SP, ($Na^{+}+K^{+}$ -stimulated ATPase; M, Mg^{2+} -dependent ATPase; PDI, phosphodiesterase I; CPD, cyclic AMP phosphodiesterase.

demonstrating a relatively narrow spread in the 95% confidence limits for the various activation energies (Tables 3 and 4).

It could be argued that the two breaks observed in the various Arrhenius plots of enzyme activities and of 4-anilino-naphthalene-1-sulphonic acid fluorescence do not define two separate and rather narrow lipid phase separations, but in fact delineate the upper and lower ends of a single rather broad lipid phase separation occurring over some $12^{\circ}C$ in membranes from control animals and some $21^{\circ}C$ in hibernating animals. Arrhenius plots of enzyme activities exhibiting two breaks would on such a model indicate that the enzyme experienced both the onset and completion of this lipid phase separation. In the cases where only a single break in the Arrhenius plot occurred, then the enzyme may either have been insensitive to this change, or the other break may have been at too high or too low a temperature to be experimentally accessible. This abolition or shift of a particular break temperature would presumably be due to the interaction between a particular enzyme and its phospholipid annulus. Such an interaction could lead to a modification of the expression of onset or completion of the lipid phase separation in the bulk lipid pool. Alternatively it may be achieved by the enzyme segregating specific lipids from the bulk lipid pool and exhibiting an appropriate response to changes in their physical state that masked those occurring in the bulk lipid pool (see, e.g., Houslay *et al.*, 1976*b*; Dipple & Houslay, 1978). However, as we have stated above, the coincidence of both the known location of the enzymes and the break pattern observed, the change in break pattern achieved upon coupling the catalytic unit of adenylate cyclase to the glucagon receptor, and that such effects were precisely repeated in membranes from both hibernating and control animals lead us to conclude

that our argument for an asymmetric lipid bilayer is the simplest model fitting the available data.

Cholesterol occurs in plasma membranes at concentrations that in model systems smear out lipid phase transitions (see Lee, 1975; Demel *et al.*, 1977) and clearly for lipid phase separations to arise there must be areas of the bilayer that are relatively cholesterol free. This may arise either by exclusion of cholesterol from the phospholipid surrounding penetrant proteins, as demonstrated by a Ca^{2+} -dependent Mg^{2+} -stimulated ATPase and other proteins (see Warren *et al.*, 1975), or by a marked difference in affinity for interaction with cholesterol shown by various phospholipid species, leading to its lateral segregation in the plane of the bilayer (see Demel *et al.*, 1977).

We note that the only significant changes in enzyme activity (at 30°C) occurring on hibernation were achieved by ectoenzymes, namely phosphodiesterase I and 5'-nucleotidase (Table 2). This may have been due to physicochemical effects related to changes in the lipid species available in the outer half of the bilayer, or to an increase in bilayer fluidity, indicated by the decrease in the lipid phase-separation temperature. The other enzymes spanning the bilayer may indeed have been affected, but to an extent that was masked by either small changes in purity of our membrane fractions or the individual variation between the activities exhibited by particular animals. The lowering of a lipid phase-separation temperature to around 4°C to coincide approximately with the temperature at which hibernating animals maintained homoeothermy (4–5°C) may have significance, for in model systems there are marked changes in the permeability of bilayers and in membrane-protein aggregation at the temperature at which lipid phase transitions occur (see, e.g., Grant & McConnell, 1974; Kleeman & McConnell, 1976; Papahadjopoulos *et al.*, 1973). The localization of this effect to the outer half of the bilayer may be to achieve a selective effect on the activity or mobility of proteins situated in this bilayer half or penetrating through it.

We suggest that Arrhenius plots of the activity of membrane-bound enzymes may yield useful information as to both the localization of the protein in the bilayer and also of bilayer asymmetry. Our experiments with liver plasma membranes from hibernating and control hamsters indicate that a change in bilayer asymmetry occurs on hibernation. This is achieved by the independent manipulation of the lipid phase-separation temperature in the outer half of the bilayer.

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References

- Appleman, M. M., Thompson, W. J. & Russell, T. R. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 65–98
- Birnbaumer, L. (1973) *Biochim. Biophys. Acta* **300**, 129–158
- Chapman, D. (1975) *Q. Rev. Biophys.* **8**, 185–204
- Colley, C. M. & Metcalfe, J. C. (1972) *FEBS Lett.* **24**, 241–246
- Demel, R. A., Javen, J. W., Van Dijk, P. W. M. & Van Deenen, L. L. M. (1977) *Biochim. Biophys. Acta* **465**, 1–10
- Dipple, I. & Houslay, M. D. (1978) *Biochem. J.* **174**, 179–190
- Esko, J. D., Gilmore, J. R. & Glaser, M. (1977) *Biochemistry* **16**, 1881–1890
- Evans, W. H. (1974) *Nature (London)* **250**, 391–394
- Evans, W. H. & Gurd, J. W. (1973) *Biochem. J.* **133**, 189–199
- Evans, W. H., Hood, D. O. & Gurd, J. W. (1973) *Biochem. J.* **135**, 819–826
- Fiske, C. H. & Subbarow, Y. (1925) *J. Biol. Chem.* **66**, 375–400
- Giorgio, N. A., Johnson, C. B. & Blecher, M. (1974) *J. Biol. Chem.* **249**, 428–435
- Goa, J. (1953) *Scand. J. Clin. Invest.* **5**, 218–222
- Grant, C. W. M. & McConnell, H. M. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 4653–4657
- Gurd, J. W. & Evans, W. H. (1974) *Biochim. Biophys. Acta* **164**, 305–311
- Hesketh, T. R., Smith, G. A., Houslay, M. D., McGill, K. A., Birdsall, N. J. M., Metcalfe, J. C. & Warren, G. B. (1976) *Biochemistry* **15**, 311–316
- Hoare, D. (1972) *Anal. Biochem.* **46**, 604–615
- Houslay, M. D. & Tipton, K. F. (1973) *Biochem. J.* **135**, 735–750
- Houslay, M. D., Warren, G. B., Birdsall, N. J. M. & Metcalfe, J. C. (1975) *FEBS Lett.* **51**, 146–151
- Houslay, M. D., Metcalfe, J. C., Warren, G. B., Hesketh, T. R. & Smith, G. A. (1976a) *Biochim. Biophys. Acta* **436**, 489–494
- Houslay, M. D., Hesketh, T. R., Smith, G. A., Warren, G. B. & Metcalfe, J. C. (1976b) *Biochim. Biophys. Acta* **436**, 495–504
- Houslay, M. D., Johansson, A., Smith, G. A., Hesketh, T. R., Warren, G. B. & Metcalfe, J. C. (1976c) *Nobel Found. Symp.* **34**, 331–344
- Houslay, M. D., Ellory, J. C., Smith, G. A., Hesketh, T. R., Stein, J. M., Warren, G. B. & Metcalfe, J. C. (1977) *Biochim. Biophys. Acta* **467**, 208–219
- Kleeman, W. & McConnell, H. M. (1976) *Biochim. Biophys. Acta* **419**, 206–222
- Lee, A. G. (1975) *Biochim. Biophys. Acta* **413**, 11–23
- Lee, A. G., Birdsall, N. J. M., Metcalfe, J. C., Toon, P. A. & Warren, G. B. (1974) *Biochemistry* **13**, 3699–3705
- Lenaz, G., Sechi, A. M., Paventi-Castelli, G., Landi, L. & Bertoli, E. (1972) *Biochem. Biophys. Res. Commun.* **49**, 536–542
- Marcelja, S. (1976) *Biochim. Biophys. Acta* **455**, 1–7
- Morrisett, J. D., Pownall, H. J., Plumlee, R. T., Smith, H. C., Zehner, Z. E., Esfoni, M. & Wakil, S. J. (1975) *J. Biol. Chem.* **250**, 6969–6976
- Overath, P. & Trauble, H. (1973) *Biochemistry* **12**, 2625–2634

- Papahadjopoulos, D., Jacobson, K., Nir, S. & Isac, T. (1973) *Biochim. Biophys. Acta* **311**, 330–348
- Perkins, J. P. (1973) *Adv. Cyclic Nucleotide Res.* **3**, 1–64
- Pilkis, S. J., Exton, J. H., Johnson, R. A. & Park, C. R. (1974) *Biochim. Biophys. Acta* **343**, 250–267
- Pommier, G., Ripert, G., Azoulay, E. & Depieds, R. (1975) *Biochim. Biophys. Acta* **389**, 483–494
- Razzel, W. E. (1963) *Methods Enzymol.* **6**, 236–238
- Sauerheber, R. D., Gordon, L. M., Crosland, R. D. & Kuwara, M. D. (1977) *J. Membr. Biol.* **31**, 131–169
- Swislocki, N. A., Johnson, C. B. & Tierney, J. (1977) *Arch. Biochem. Biophys.* **179**, 157–165
- Tecoma, E. S., Sklar, L. A., Simoni, R. D. & Hudson, B. S. (1977) *Biochemistry* **16**, 829–835
- Thilo, L., Trauble, H. & Overath, P. (1977) *Biochemistry* **16**, 1283–1290
- Thompson, W. J. & Appleman, M. M. (1971) *Biochemistry* **10**, 311–316
- Warren, G. B., Houslay, M. D., Metcalfe, J. C. & Birdsall, N. J. M. (1975) *Nature (London)* **255**, 684–687
- Welton, A. F., Lad, P. M., Newby, A. C., Yamamura, H., Nicosia, S. & Rodbell, M. (1977) *J. Biol. Chem.* **252**, 5947–5950
- Wunderlich, F., Ronai, A., Speth, V., Seelig, J. & Blume, A. (1975) *Biochemistry* **14**, 3730–3735