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The Activity of Glucagon-Stimulated Adenylate Cyclase from Rat Liver Plasma Membranes is Modulated by the Fluidity of its Lipid Environment

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1. The local anaesthetic benzyl alcohol progressively activated glucagon-stimulated adenylate cyclase activity up to a maximum at 50mM-benzyl alcohol. Further increases in benzyl alcohol concentration inhibited the activity. The fluoride-stimulated adenylate cyclase activity was similarly affected except for an inhibition of activity occurring at low benzyl alcohol concentrations (approx. 10mM). 2. The fluoride-stimulated adenylate cyclase activity of a solubilized enzyme preparation was unaffected by any of the benzyl alcohol concentrations tested. 3. Increases in 3-phenylpropan-1-ol and 5-phenylpentan-1-ol concentrations progressively activated both the fluoride- and glucagon-stimulated adenylate cyclase activities up to a maximum, above which further increases in alcohol concentration inhibited the activities. 4. The 'break' points in Arrhenius plots of glucagon-stimulated adenylate cyclase activity in native plasma membranes, and in plasma membranes fused with synthetic dimyristoyl phosphatidylcholine so as to constitute 60% of the total lipid pool, were decreased by approx. 6°C by addition of 40mM-benzyl alcohol. This was accompanied by a fall in the associated activation energies. 6. Arrhenius plots of fluoride-stimulated adenylate cyclase activity in the presence and absence of 40mM-benzyl alcohol were linear, although addition of benzyl alcohol caused a dramatic decrease in the associated activation energy of the reaction. 7. 5′-Nucleotidase activity was stimulated by benzyl alcohol, and the 'break' point in the Arrhenius plot of its activity was decreased by about 6°C by addition of 40mM-benzyl alcohol to the assay. 8. It is suggested that benzyl alcohol effects a fluidization of the bilayer, which is clearly demonstrated by its ability to lower the temperature of a lipid phase separation occurring at 28°C in the outer half of the bilayer to around 22°C. The increase in bilayer fluidity relieves a physical constraint on the membrane-bound adenylate cyclase, activating the enzyme. 9. The various inhibition phenomena are discussed in detail, together with the suggestion that the interaction between the uncoupled catalytic unit of adenylate cyclase and the lipids of the bilayer is altered on its physical coupling to the glucagon receptor.

Many hormones exert their effects on target cells by elevating the intracellular concentration of cyclic AMP through the activation of the enzyme adenylate cyclase. They achieve this by binding to specific receptors on the external surface of the plasma membrane, which subsequently interacts with the catalytic unit of adenylate cyclase (EC 4.6.1.1) with its active site exposed to the cytosol surface of the membrane. This system effects a vectorial transfer of information across the bilayer membrane. Convincing biochemical (Schramm *et al.*, 1977) and physical (Houslay *et al.*, 1977) evidence has been obtained to support the contention that, in the absence of hormone, the receptor and catalytic units of adenylate cyclase are discrete units able to migrate independently in the plane of the bilayer. When hormone binds to the receptor, this complex interacts with the catalytic unit of adenylate cyclase, activating it and forming a multicomponent system spanning the bilayer membrane.

We have demonstrated in Sprague–Dawley rat liver plasma membranes that a phase separation occurs in the outer half of the bilayer at around 28°C, whereas no such phase separation occurs in the inner half of the bilayer (Houslay *et al.*, 1976*a*,*b*,*c*). Arrhenius plots of fluoride-stimulated adenylate cyclase activity are linear, presumably because the uncoupled catalytic unit is exclusively localized in the inner half of the bilayer where there is no such lipid phase separation (Houselay *et al.*, 1976*c*). However, when the catalytic unit physically interacts with the glucagon receptor, forming a multicomponent complex spanning the bilayer, Arrhenius plots of adenylate cyclase activity exhibit a well-defined break at 28°C as a result of the lipid phase separation in the outer half of the bilayer modulating the activity

Abbreviation used: $(Ca^{2+} + Mg^{2+})$-ATPase, Ca^{2+}-stimulated Mg^{2+}-dependent adenosine triphosphatase.

of the catalytic unit. Such a physical interaction between the glucagon receptor and the catalytic unit of adenylate cyclase may be achieved by addition of glucagon, yielding a (functional) complex with stimulated adenylate cyclase activity, or alternatively with the competitive glucagon antagonist des-1-histidine-glucagon producing a (structural only) complex in which the catalytic unit is not activated (Houslay *et al.*, 1976*a*,*b*,*c*). When defined synthetic phosphatidylcholines are introduced into the bilayer by either lipid fusion or cholate-mediated lipid-substitution procedures (Houslay *et al.*, 1976*b*), such that they constitute some 50–60% of the total lipid pool, the break in the Arrhenius plot of glucagon-sensitive adenylate cyclase is shifted in accordance with the phase-separation temperatures of the synthetic phosphatidylcholines. However, the Arrhenius plot of the activity of the uncoupled catalytic unit situated in the inner half of the bilayer, when stimulated by fluoride, is unaffected by such gross changes in the bilayer composition. The relative insensitivity of the uncoupled catalytic unit is attributed to either poor penetration of the protein into the bilayer or the ability of the catalytic unit to select specific lipids out of the total lipid pool, which exhibits no such phase separation, to constitute its immediate shell of lipid (Houslay *et al.*, 1976*b*).

The present study defines the action of the local anaesthetic benzyl alcohol on the glucagon-stimulated adenylate cyclase of rat liver plasma membranes. The mode of action of this compound on model membrane systems has been studied in detail. When the bilayer is in the liquid crystalline phase it can partition into the bilayer, increasing its fluidity, and subsequently decreasing the phase-transition temperature of the bilayer by some 8°C, at which point it is excluded from the crystalline-gel-phase lipid (Colley & Metcalfe, 1972). Benzyl alcohol not only penetrates the bulk-phase lipid but it will also enter the immediate shell of lipids surrounding a penetrant protein, as has been demonstrated for the $(Ca^{2+}+Mg^{2+})$-ATPase from sarcoplasmic reticulum. Such penetration leads to an increase in the fluidity of this shell of lipids, termed the lipid annulus, effecting an increase in the activity of the enzyme (Hesketh *et al.*, 1976).

Benzyl alcohol thus provides us with a tool for the investigation of the effect of changes in the fluidity of the native lipid annulus of the glucagon-sensitive adenylate cyclase from rat liver plasma membranes.

Materials and Methods

Rat liver plasma membranes were prepared from male Sprague–Dawley rats, weighing 200–300g, by the method of Pilkis *et al.* (1974). The isolated membranes were thoroughly washed to ensure removal of any bound ligands that would elevate the basal activity of adenylate cyclase (Houslay *et al.*, 1976*a*). Membrane fractions were stored in 1mM-$KHCO_3$, pH7.2, at protein concentrations between 6 and 20mg/ml in liquid N_2. Similar results were obtained with fresh membrane fractions and those stored in liquid N_2.

The assay of adenylate cyclase (EC 4.6.1.1) was carried out as described in detail by Houslay *et al.* (1976*a*), and the cyclic AMP produced was determined by the method of Brown *et al.* (1972). Standard-deviation determinations for assays of adenylate cyclase activity are given with $(n-1)$ degrees of freedom.

5′-Nucleotidase (EC 3.1.3.5) was assayed radiometrically with [^{3}H]AMP as substrate, by the modified method of Newby *et al.* (1975) with 1mM-AMP. In the absence of benzyl alcohol, identical results could be obtained by using the coupled assay with adenosine deaminase (EC 3.5.4.4); however, this coupling enzyme was irreversibly denatured by the addition of benzyl alcohol at all the concentrations studied.

The coupled assay for 5′-nucleotidase was carried out with 0.1mM-AMP, 50mM-α-glycerophosphate, 0.5mM-$MgSO_4$ and 0.4 unit (μmol/min) of adenosine deaminase/ml in 50mM-Tris/HCl buffer adjusted to a final pH7.6. The decrease in A_{265} corresponding to the production of inosine was followed spectrophotometrically.

The pH of all assays was kept constant at pH7.4 under all conditions. Linear assays only were used to determine the activities under various conditions of temperature and added effectors. This has been set out in detail by Houslay *et al.* (1976*a*).

Dimyristoyl phosphatidylcholine (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) was prepared by the method of Robles & Van den Berg (1969), and dimyristoyl phosphatidylcholine-fused plasma membranes were obtained as described by Houslay *et al.* (1975, 1976*b*).

Adenylate cyclase was rendered soluble with the non-ionic detergent Lubrol PX by the method of Swislocki *et al.* (1975). The upper clear fraction obtained after centrifugation for 1h at 100000*g* was used as the enzyme source. This fraction was used immediately after preparation and a check on its activity was made throughout the day. In all assays with the soluble enzyme preparation, dithiothreitol (1mM) was added to the reaction.

The phase separation shown to occur at 28°C in rat liver plasma membranes (Houslay *et al.*, 1976*a*, 1977) was followed in this study by 90° light-scattering by using the methods and analysis discussed in some detail by Trauble (1972), Sackmann & Trauble (1972) and Overath & Trauble (1973). An EEL 344 spectrofluorimeter was used for these measurements and 400nm was used for both excitation and emission. The native membranes were used as a 0.1mg/ml

suspension in the buffer system used for adenylate cyclase assay (see Houslay *et al.*, 1976*a*). Rearrangements in Lubrol PX solutions were investigated, with Bromothymol Blue as an indicator, by the procedures and analysis described by Sackmann & Trauble (1972) and Overath & Trauble (1973). Analysis was carried out with 0.1mM-Lubrol and 0.5mM-Bromothymol Blue in the buffer system used for adenylate cyclase assay (see Houslay *et al.*, 1976*a*), and changes in A_{615} were followed. Corrections were made for changes in A_{615} of control cuvettes containing Bromothymol Blue and buffer, or Lubrol PX and buffer. We showed that both these methods of analysis could detect a clearly defined lipid-phase separation at 23.5°C for dimyristoyl phosphatidylcholine, which has been well-characterized (see Kleeman & McConnell, 1976; Mabrey & Sturtevant, 1976; Lee, 1976).

Protein concentration was determined by the micro biuret method of Goa (1953) as modified by Warren *et al.* (1974*a*).

Glucagon and des-1-histidine-glucagon were gifts from Dr. William W. Bromer of Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN, U.S.A. Phosphocreatine and creatine kinase were from Sigma, Kingston upon Thames, Surrey, U.K. Cyclic AMP, ATP and triethanolamine hydrochloride were from Boehringer Corp. (U.K.), Lewes, East Sussex, U.K. Benzyl alcohol was from Hopkin and Williams, Chadwell Heath, Essex, U.K. 3-Phenylpropan-1-ol and 5-phenylpentan-1-ol were from Aldrich Chemicals, Gillingham, Dorset, U.K. Lubrol PX was a gift from ICI Pharmaceuticals, Macclesfield, Cheshire, U.K. All other chemicals were of AnalaR quality from BDH Chemicals, Poole, Dorset, U.K.

Results

The adenylate cyclase activity of the purified rat liver plasma membranes was stimulated 30–40-fold by the hormone glucagon and 6–7-fold by fluoride, as reported previously (Houslay *et al.*, 1976*b*).

The action of the local anaesthetic and membrane-fluidizing agent benzyl alcohol was examined when the membrane-bound enzyme was either in the uncoupled state stimulated by fluoride or when coupled to the glucagon receptor in the presence of hormone. The fluoride-stimulated activity appeared to be initially inhibited by benzyl alcohol at low concentrations (approx. 10mM) and then progressively activated, reaching a maximum at 50mM-alcohol. Further addition of benzyl alcohol caused this stimulated activity to decrease to a value some 50% of the original activity (Fig. 1).

When the catalytic unit was coupled to the glucagon receptor by the addition of glucagon, benzyl alcohol caused an augmentation of adenylate cyclase activity

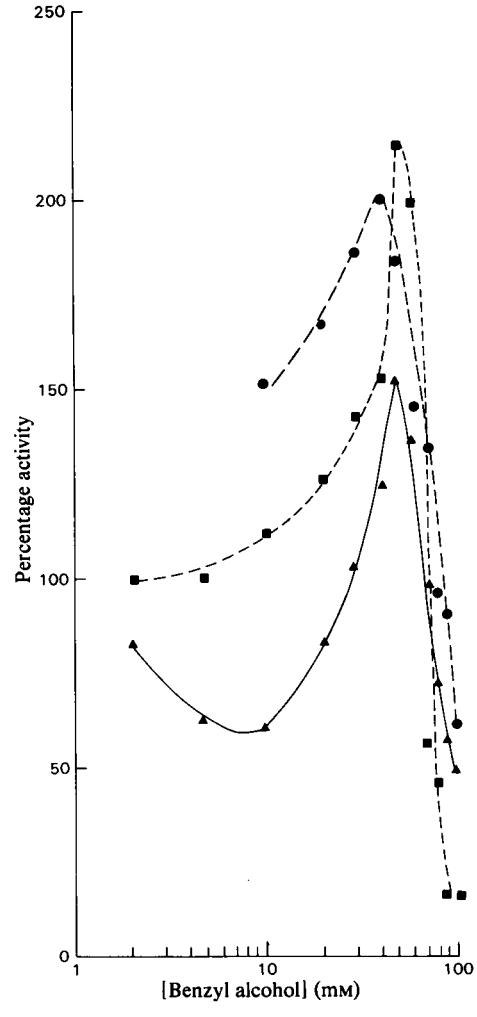

Fig. 1. *Effects of benzyl alcohol on the adenylate cyclase activity of plasma membranes*

▲, Fluoride-stimulated (uncoupled) activity; ■, glucagon-stimulated (uncoupled) activity; ●, fluoride-stimulated activity in the presence of the competitive glucagon antagonist des-histidine-glucagon (coupled). All assays were carried out at 30°C, taking initial rates from linear time courses. The number of individual determinations of adenylate cyclase activity carried out at different alcohol concentrations ranged from 3 to 16, yielding standard deviations of less than 9%. The specific activities of the responses in all these studies at 30°C were 660μunits/mg of protein in the presence of glucagon and 120μunits/mg of protein in the presence of fluoride, when no alcohol was present and with native membranes as the enzyme source. (One unit is 1μmol of substrate transformed/min.) During all of these studies, the sensitivity of at least three membrane preparations was tested in any particular experiment.

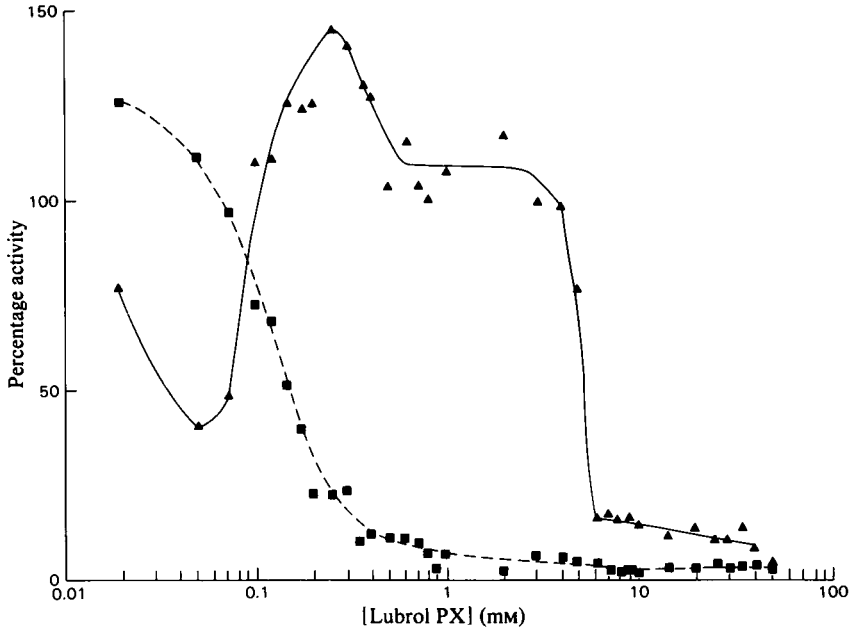

Fig. 2. *Effects of increasing concentrations of Lubrol PX on the adenylate cyclase activity of plasma membranes*
▲, Fluoride-stimulated (uncoupled) activity; ■, glucagon-stimulated (coupled) activity. All assays were carried out at 30°C and were done in triplicate. Standard deviations were less than 12%.

up to a maximum response observed at around 50–60mM-benzyl alcohol. Higher benzyl alcohol concentrations caused a progressive loss of adenylate cyclase activity until the rates exhibited were only 10–20% that of the original activity (Fig. 1).

When the effect of benzyl alcohol on the fluoride-stimulated adenylate cyclase activity was studied in the presence of the competitive glucagon antagonist des-1-histidine-glucagon, which leads to a coupling of the glucagon receptor to the catalytic unit without activating it (Houslay *et al.*, 1976*a*, 1977), then a very similar response to that observed in the presence of glucagon alone was observed (Fig. 1). There was no evidence of any inhibition of enzyme activity at low benzyl alcohol concentrations.

These experiments were carried out at 30°C; however, very similar results could be obtained at 40°C, although the concentrations at which maximum inhibition/activation could be obtained were slightly lower.

In all cases the phenomena could be completely (over 95%) reversed by diluting the membranes 10-fold into cold 1mM-$KHCO_3$, pH7.2, centrifuging at 15000rev./min for 15min at 4°C and rewashing with 1mM-$KHCO_3$, pH7.2, before assay in the presence of glucagon or fluoride. However, at concentrations of benzyl alcohol over 70mM, exposure for periods of more than 5min did lead to an irreversible loss of adenylate cyclase activity, with the response to glucagon stimulation being more sensitive to denaturation than that of fluoride stimulation.

If the enzyme was rendered soluble with the non-ionic detergent Lubrol PX, benzyl alcohol had no significant effect on the fluoride-stimulated activity at the concentrations studied (up to 100mM). This enzyme preparation has been reported not to be stimulated by glucagon, and is prepared in the presence of fluoride (Swislocki *et al.*, 1975), and therefore no examination of the effect of benzyl alcohol on the enzyme in the presence of glucagon was attempted.

The effect of increasing concentrations of Lubrol PX on the fluoride- and glucagon-stimulated adenylate cyclase activity of native plasma membranes was also investigated in some detail (Fig. 2). The results obtained showed some analogy to those observed with benzyl alcohol. Extremely low concentrations (approx. 0.025mM) of this detergent caused a fall in the fluoride-stimulated response; however, further additions of detergent caused an activation that then was rapidly decreased at higher Lubrol concentrations, where solubilization was effected. The changes in activity at low concentrations (below 1.0mM) of Lubrol could be reversed by dilution, but the inhibition at high concentrations of Lubrol appeared to be irreversible. The action of Lubrol on the glucagon-

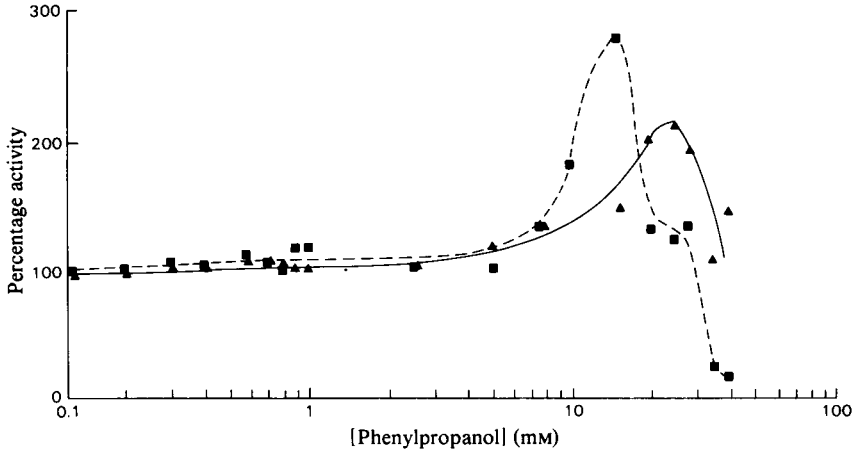

Fig. 3. *Effects of 3-phenylpropan-1-ol on the adenylate cyclase activity of plasma membranes*
▲, Fluoride-stimulated (uncoupled) activity; ■, glucagon-stimulated (coupled) activity. All assays were carried out at 30°C with between three and seven individual determinations of adenylate cyclase activity obtained at each alcohol concentration from linear time courses. Standard deviation was less than 9% at each point.

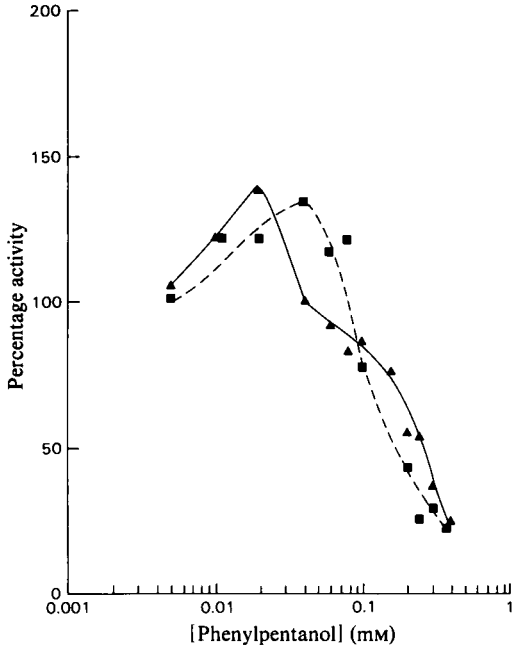

Fig. 4. *Effects of 5-phenylpentan-1-ol on the adenylate cyclase activity of plasma membranes*
▲, Fluoride-stimulated (uncoupled) activity; ■, glucagon-stimulated (coupled) activity. All assays were carried out at 30°C with between three and five individual determinations of adenylate cyclase activity obtained at each of the alcohol concentrations from linear time courses. Standard deviation was less than 10% at each point.

stimulated adenylate cyclase activity was to effect a reversible activation at low concentrations, before an irreversible loss of activity as the detergent concentration was increased.

When the activity of membrane-bound adenylate cyclase was studied in the presence of either 3-phenylpropan-1-ol (Fig. 3) or 5-phenylpentan-1-ol (Fig. 4), similar results were obtained whether the stimulating ligand was fluoride or glucagon. Increasing concentrations of the alcohols caused a rise in the adenylate cyclase activity up to a maximum, after which the activity declined to a plateau value. We obtained no evidence of inhibition of the fluoride-stimulated activity with concentrations of either 3-phenylpropan-1-ol or 5-phenylpentan-1-ol lower than those used to effect activation, as with benzyl alcohol.

Arrhenius plots of the adenylate cyclase activity in the native plasma membranes were similar in form to those described previously (Houslay *et al.*, 1976*a*), being linear in the presence of fluoride, and biphasic in the presence of glucagon, with a clearly defined break at 28°C (Table 1; Figs. 5*a* and 5*b*). The addition of benzyl alcohol, which markedly activated both the fluoride- and glucagon-stimulated activities (40mM) and ensured more than 98% reversibility over the extended incubations (up to 20min) carried out at temperatures lower than 12°C, had a marked effect on the form of the Arrhenius plots (Figs. 5*a* and 5*b* respectively). The Arrhenius plot of glucagon-stimulated adenylate cyclase activity showed a shift of the break point downwards by about 6°C, to a value of 22°C, with a decrease in the associated activation energies (Table 1, Fig. 5*b*). That for

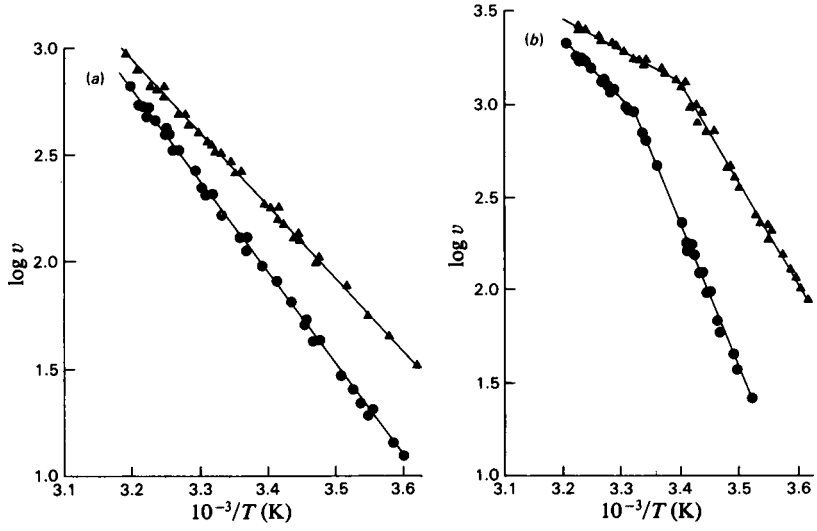

Fig. 5. *Arrhenius plots of the adenylate cyclase activity of native plasma membranes in the presence and absence of benzyl alcohol*

(*a*) With the enzyme in the uncoupled state, with fluoride alone (●) or fluoride+40mM-benzyl alcohol (▲). (*b*) With the enzyme in the coupled state, with glucagon alone (●) or glucagon+40mM-benzyl alcohol (▲). All the effects observed were reversible, and the rates plotted are initial rates (see Houslay *et al.*, 1976*a*,*b*). Reaction velocities (*v*) in all Figures are expressed as μunits/mg of protein.

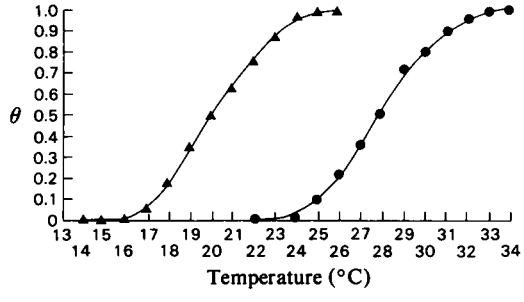

Fig. 6. *Relative 90°-light-scattering intensity of plasma membranes*

The degree of conversion θ is plotted against temperature as described by Overath & Trauble (1973), Trauble (1972) and Sackmann & Trauble (1972), for native membranes (●) and native membranes +40mM-benzyl alcohol (▲). For details see the Materials and Methods section.

fluoride-stimulated adenylate cyclase activity, although still linear, exhibited a dramatic change in slope, with the activation energy decreased from 84.1kJ/mol to only 55.3kJ/mol (Table 1, Fig. 5*a*).

We (Houslay *et al.*, 1976*b*) and others (Sauerheber *et al.*, 1977), using e.s.r. spectroscopy, have demonstrated a phase separation at 28°C in rat liver plasma membranes that is a property of the outer bilayer half (Houslay *et al.*, 1976*a*,*b*,*c*). In the present study we have used the 90° light-scattering of the native membranes as a tool for probing such a lipid-phase separation (see the Materials and Methods section). The relative intensity of this scattering showed changes in slope with temperature that were consistent with a phase separation occurring at 28°C in the native membranes, which was decreased to 20°C by the addition of 40mM-benzyl alcohol (Fig. 6).

It has previously been demonstrated that the break point at 28°C observed from Arrhenius plots of glucagon-stimulated adenylate cyclase activity in native membranes could be decreased to 22°C by incorporating synthetic dimyristoyl phosphatidylcholine into the bilayer until it constituted some 60% of the total lipid pool (Houslay *et al.*, 1976*b*). With this preparation it was found possible to lower this break point further to 16°C by the addition of 40mM-benzyl alcohol, with a decrease in the activation energy observed below this break point (Fig. 7, Table 1). The activity of the glucagon-stimulated adenylate cyclase in this fused-membrane preparation was stimulated 1.7-fold by the addition of 40mM-benzyl alcohol.

Arrhenius plots of adenylate cyclase activity in the presence of 10mM-benzyl alcohol exhibited a small lowering of the break point to 26.5°C when glucagon was the stimulating ligand. This low alcohol concentration had only small effects on the activation

Table 1. *Summary of the effect of benzyl alcohol on the form of Arrhenius plots of the adenylate cyclase and 5'-nucleotidase activities of rat liver plasma membranes*

Preparation	Enzyme	Stimulating ligand	No. of Arrhenius plots	Break point (°C)	Activation energy (kJ/mol) Above break	Below break
Native	Adenylate cyclase	Fluoride	4	Linear	84.1±3.8	
Native	Adenylate cyclase	Glucagon	5	27.8±0.5	127.3±2.9	58.6±12.6
Native+ 40mM-benzyl alcohol	Adenylate cyclase	Fluoride	3	Linear	55.3±4.2	
Native+ 40mM-benzyl alcohol	Adenylate cyclase	Glucagon	5	22.2±0.5	99.6±8.4	30.1±2.5
Native+ 10mM-benzyl alcohol	Adenylate cyclase	Fluoride	3	Linear	76.6±3.3	
Native+ 10mM-benzyl alcohol	Adenylate cyclase	Glucagon	3	26.5±0.5	89.2±2.5	33.9±12.6
Dimyristoyl phosphatidylcholine-fused	Adenylate cyclase	Glucagon	4	21.9±0.3	131.0±23.9	66.1±8.8
Dimyristoyl phosphatidylcholine-fused+40mM-benzyl alcohol	Adenylate cyclase	Glucagon	3	16.0±0.4	116.8±28.1	43.5±10.5
Lubrol-solubilized	Adenylate cyclase	Fluoride	4	16.2±0.5	67.8±2.5	93.8±3.8
Lubrol-solubilized+ 40mM-benzyl alcohol	Adenylate cyclase	Fluoride	5	16.2±0.4	64.9±2.5	95.0±7.1
Native	5'-Nucleotidase	—	5	27.5±0.7	46.5±2.5	94.6±13.4
Native+ 40mM-benzyl alcohol	5'-Nucleotidase	—	3	18.5±1.5	29.3±3.3	85.8±2.1
Lubrol-solubilized	5'-Nucleotidase	—	4	16.1±0.1	94.6±2.9	140.0±3.3

energies of both glucagon- and fluoride-stimulated adenylate cyclase activities (Table 1).

In contrast with the linear Arrhenius plots seen with the fluoride-stimulated activity in the presence or absence of benzyl alcohol (Fig. 5*a*), Arrhenius plots of the adenylate cyclase activity of the Lubrol PX-solubilized enzyme when stimulated by fluoride were clearly biphasic (Fig. 8). Furthermore, the addition of 40mM-benzyl alcohol to the assays appeared to have no effect whatsoever on the activities, and the Arrhenius plot was clearly superimposable on that obtained in the absence of alcohol (Fig. 8). In an attempt to define whether the break at 16°C seen in Arrhenius plots of the fluoride-stimulated activity of the Lubrol-solubilized enzyme was related to the physical properties of the detergent, we followed its effects on the absorbance of Bromothymol Blue solutions. Bromothymol Blue has been used by Trauble and co-workers (see Trauble, 1972; Sackmann & Trauble, 1972) to follow molecular rearrangements in the head-group region of the bilayer, and hence to indicate temperatures at which lipid-phase transitions occur. In our hands Lubrol PX had a very marked effect on the absorbance of Bromothymol Blue solutions with change in temperature (Fig. 9). This may be due to the occurrence of some kind of structural rearrangements in detergent micelles at 16°C. The form of the Bromothymol Blue absorbance profile with change in temperature in the presence of Lubrol PX was unaffected by addition of 40mM-benzyl alcohol (Fig. 9).

5'-Nucleotidase, which is considered to be an intrinsic membrane-bound ectoenzyme (Gurd & Evans, 1974; Trams & Lauter, 1974) sensitive to lipid phase separation in the plane of the bilayer (Houslay *et al.*, 1976*c*), exhibits a break at around 28°C in Arrhenius plots of its activity (Table 1, Fig. 10). It was progressively and reversibly activated by increasing concentration of benzyl alcohol, reaching a maximum at around 40mM. The activity then declined gradually with further increases in concentration of benzyl alcohol. The effect of 40mM-benzyl alcohol is to lower the break observed in the Arrhenius plot to around 21°C (Table 1, Fig. 10) in an analogous fashion to that observed with glucagon-stimulated adenylate cyclase.

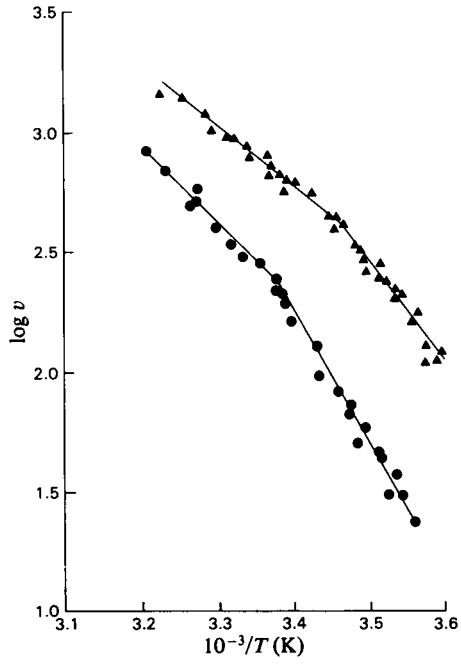

Fig. 7. *Arrhenius plots of the adenylate cyclase activity of dimyristoyl phosphatidylcholine-fused plasma membranes*
These were carried out with the enzyme in the presence of glucagon (coupled state) and in the presence (▲) and absence (●) of 40mM-benzyl alcohol.

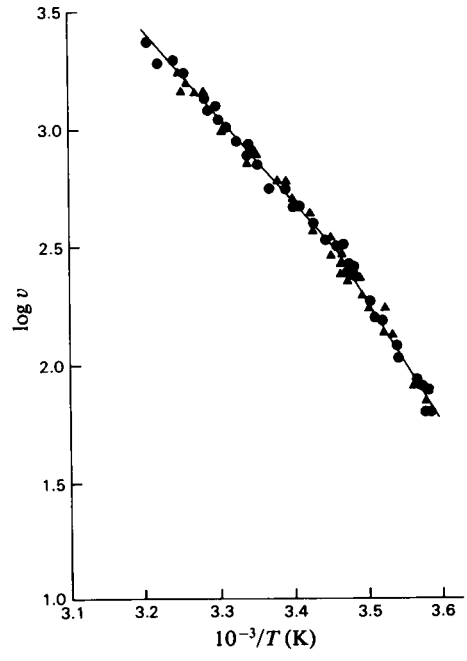

Fig. 8. *Arrhenius plots of the adenylate cyclase activity of Lubrol-PX-solubilized plasma membranes*
This enzyme was stimulated with fluoride in both cases and assayed in the presence (▲) and absence (●) of 40mM-benzyl alcohol.

Discussion

The present study demonstrates that 40mM-benzyl alcohol can activate adenylate cyclase in both the uncoupled and coupled states. This activation can be as much as 2-fold, and is caused primarily by a decrease in the activation energy of the reaction. We consider that this activation is achieved by relieving the physical constraint of the lipid annulus on the enzyme and the receptor. This may be related to an increase in the fluidity of the lipids in the annulus and bulk lipid pool. Consistent with this interpretation are our observations that the phase separation at 28°C in rat liver plasma membranes (Houslay *et al.*, 1976*a*,*b*; Sauerheber *et al.*, 1977), and believed to occur in the outer half of the bilayer (Houslay *et al.*, 1976*b*,*c*), is decreased by about 6°C to 22°C, as indicated by Arrhenius plots of adenylate cyclase in the coupled state (Fig. 5*b*) and of the intrinsic ecto-enzyme 5′-nucleotidase (Fig. 10) in the presence of 40mM-benzyl alcohol, as well as change in relative 90°-light-scattering intensity of the plasma membranes (Fig. 6). Furthermore, if synthetic dimyristoyl phosphatidylcholine is incorporated into the bilayer until it constitutes some 60% of the total lipid pool, the break temperature for coupled adenylate cyclase activity is decreased to 22°C, and addition of benzyl alcohol further decreases this to 16°C (Fig. 7). The observed decrease in activation energies would add further support to this.

The decrease in the bilayer lipid-phase-separation temperature by about 6°C on addition of benzyl alcohol may be related to its effect in decreasing lipid-phase transition temperatures in defined lipid bilayers by about 6–8°C as indicated by e.s.r. and n.m.r. studies (Colley & Metcalfe, 1972). This can most easily be explained if the alcohol disrupts the packing of the chains into the crystalline form below the transition, and a lower temperature is required before chain interactions are sufficiently strong to expel the alcohol from the chain region of the bilayer to allow crystallization to ensue. The increase in binding of benzyl alcohol above the phase transition (Colley & Metcalfe, 1972) can be attributed to the greatly increased fluidity of the chains (Hubbell & McConnell, 1971), allowing partition of the alcohol into the hydrophobic interior of the bilayer. This amounts to a 'classical' depression of freezing point, and good agreement between theory and experiment for alcohol effects has been given by Hill (1974), as discussed in some detail by Lee (1977*a*).

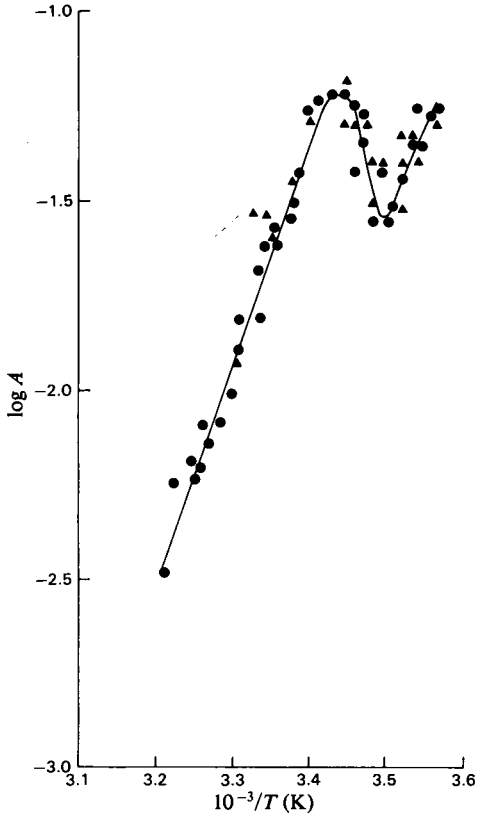

Fig. 9. *Effect of Lubrol PX on the change in absorbance of Bromothymol Blue solutions*

Experiments were carried out in the presence (▲) and absence (●) of 40mM-benzyl alcohol. These values represent a net change in absorbance (A_{615}) after correction for those of the control experiments as described in the Materials and Methods section.

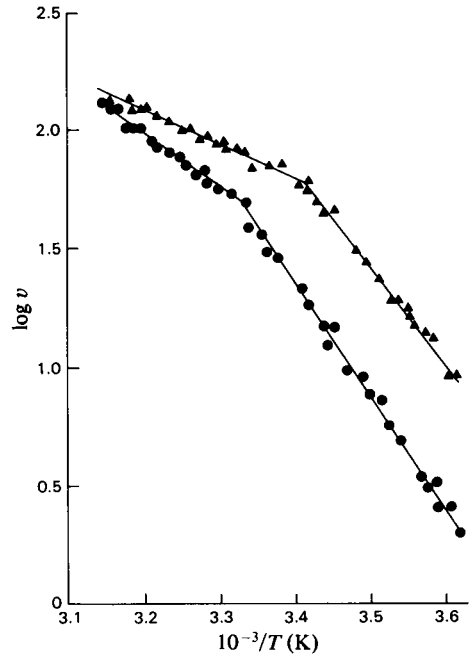

Fig. 10. *Arrhenius plot of the 5'-nucleotidase activity in plasma membranes*

Data are given for assays carried out in the absence (●) and presence (▲) of 40mM-benzyl alcohol. The initial rate (v) of the reaction is given in munits/mg of protein.

Evidence that benzyl alcohol can fluidize the ring of lipids immediately surrounding penetrant proteins (the lipid annulus) is provided from physical and biochemical studies on $(Ca^{2+}+Mg^{2+})$-ATPase from sarcoplasmic reticulum in a defined lipid environment of dipalmitoyl phosphatidylcholine. This system convincingly demonstrates that benzyl alcohol fluidizes annular phospholipids. This decrease in rigidity leads to activation of the $(Ca^{2+}+Mg^{2+})$-ATPase, presumably by affecting the conformational flexibility of the protein (Hesketh *et al.*, 1976).

If the catalytic unit of adenylate cyclase is rendered soluble by using the non-ionic detergent Lubrol PX, then benzyl alcohol has no effect on the activity at 30°C (see Fig. 8). This is presumably because the enzyme has been dispersed and the hydrophobic parts of the enzyme are masked with detergent, allowing it to come into solution rather than be embedded in and constrained by the structure of the bilayer.

Evidence consistent with change in the environment of the enzyme after solubilization with Lubrol PX is the observation that the Arrhenius plots of fluoride-stimulated solubilized adenylate cyclase activity were biphasic in form, with a single well-defined break at 16°C (Fig. 8), in contrast with the linear plots observed with the native enzyme (Fig. 5*a*). It is tempting to attribute this effect to a change in the physical nature of Lubrol PX, whose melting point is around 19°C (ICI information sheet no. D1383). Indeed we have observed that its effects on Bromothymol Blue absorbance (Fig. 9) are consistent with the occurrence of some kind of structural rearrangement in the detergent micelles at around 16°C. Such a structural change may be transmitted to the protein if it exists in intimate contact with detergent, presumably as a Lubrol–(lipo)protein complex. This structural rearrangement is apparently insensitive to the addition of 40mM-benzyl alcohol, as addition of alcohol has no effect on the Bromothymol Blue absorbance profile in the presence of Lubrol PX. This observation may be an explanation

for the apparent insensitivity of the Lubrol-solubilized enzyme to addition of benzyl alcohol and that the temperature of the break point in Arrhenius plots of the fluoride-stimulated activity of this enzyme preparation is unaffected by addition of benzyl alcohol to the assay (Fig. 8). The inability of benzyl alcohol to influence these phenomena at the concentrations tested may be due to many reasons, including a low partition coefficient and the relatively high detergent concentrations in the adenylate cyclase assays, cf. membrane lipid in the plasma membranes. Such experiments provide clear evidence that the uncoupled catalytic unit is sensitive to changes in the physical character of its environment. Furthermore the activation energies of the reaction, certainly about 16°C, are lower than those exhibited by the native enzyme (Table 1), which implies that Lubrol imposes less, or a different kind of, constraint on the protein than did the lipids of the bilayer, which would be compatible with our observation that benzyl alcohol did not affect the activity of the solubilized enzyme (Fig. 2). Indeed low concentrations of Lubrol PX actually activated both the glucagon- and fluoride-stimulated enzyme activities (Fig. 2), presumably by partitioning into the bilayer and fluidizing it in an analogous fashion to benzyl alcohol. The much diminished fluoride-stimulated activity occurring at the higher Lubrol concentrations, where solubilization takes place, may well be due to displacement of lipids essential for maximal activity to be displayed. Indeed the apparent unique ability of Lubrol PX to sustain a solubilized adenylate cyclase activity (see Swislocki *et al.*, 1975) may imply a need for hydrophobic molecules of a special character to sustain the fully active enzyme. The inhibition of fluoride- but not of glucagon-stimulated adenylate cyclase activities seen at low (0.025mM) concentrations of Lubrol PX (Fig. 2) bears a striking analogy to that seen with benzyl alcohol, and it is tempting to rationalize this by a similar interpretation (see below). Clearly the glucagon-stimulated activity will be more sensitive to inhibition at the higher detergent concentrations, where solubilization is achieved, as, not only might the detergent inhibit physical interaction between the receptor and catalytic unit (the coupling interaction), but also the diffusion of the two components would now be free to occur in three dimensions rather than being restricted to the two dimensions of the bilayer. The net effect of such increased freedom of diffusion may be a loss of some 10–100-fold in the probability of interaction. Also, specific lipids implicated in the coupling process (see, e.g., Rubaclava & Rodbell, 1973; Birnbaumer, 1973) may be displaced by the action of the detergent.

Thus changes in the fluidity of the membrane may be expected to regulate the activity of adenylate cyclase in the coupled and uncoupled states. The highly significant activation of the catalytic unit achieved by increasing bilayer fluidity would imply that it is normally embedded firmly in the membrane and constrained by the rigidity of its annular lipids. The relative insensitivity of the uncoupled catalytic unit to gross changes in the phosphatidylcholine content of the bulk lipid pool, which altered the fluidity of the substituted membranes (Houslay *et al.*, 1976*b*), is likely therefore to be due to the ability of the catalytic unit to select specific lipids from the bulk lipid pool to compose its lipid annulus and hence modulate its activity. In doing so the catalytic unit must have excluded the added phosphatidylcholines. The unchanged form of the Arrhenius plots would then be a reflection of this selected lipid annulus, as it is the physical properties of the lipid annulus that to a major extent modulate the activity of intrinsic enzymes (see Warren *et al.*, 1974*b*; Hesketh *et al.*, 1976). The ability of proteins to segregate specific lipids to constitute their lipid annulus is not unknown, e.g. the $(Ca^{2+}+Mg^{2+})$-ATPase from sarcoplasmic reticulum can specifically exclude cholesterol from its lipid annulus (Warren *et al.*, 1975), and β-hydroxybutyrate dehydrogenase will preferentially select dioleoyl phosphatidylcholine rather than mitochondrial lipids to constitute its lipid annulus (Houslay *et al.*, 1975). The proposal that adenylate cyclase may segregate specific lipids from the bulk lipid pool to constitute its lipid annulus is not altogether unreasonable, as certain lipids that are minor membrane components have been implicated as being essential for its function (see Rethy *et al.*, 1972; Rubaclava & Rodbell, 1973; Birnbaumer, 1973).

The progressive inhibition seen at high alcohol concentrations (Figs. 1, 3 and 4) may have been due to alcohol partitioning into the interior of the protein, perturbing its structure (as presumably happened to the soluble protein adenosine deaminase, which was inhibited by benzyl alcohol; see the Materials and Methods section). However, if this were true, we might well have expected to observe inhibition at high benzyl alcohol concentrations with the preparation rendered soluble with Lubrol PX; this was clearly not the case. However, it is conceivable that, at high concentrations, benzyl alcohol displaced annular lipid from adenylate cyclase, leading to a loss of enzyme activity, as has been demonstrated for the $(Ca^{2+}+Mg^{2+})$-ATPase (Hesketh *et al.*, 1976). That no inhibition was observed at high concentrations of benzyl alcohol with the preparation rendered soluble with Lubrol PX may indicate that benzyl alcohol at the concentrations studied cannot displace the detergent presumably necessary for the maintenance of enzymic activity from around the protein. This indicates that Lubrol PX is firmly associated with the protein, as seems apparent from the form of the Arrhenius plots (see above).

The greater inhibition of the glucagon-stimulated activity of the membrane-bound enzyme could be related to benzyl alcohol effecting a perturbation at the coupling interface between receptor and catalytic unit, or it may be that a certain minimum rigidity of the bilayer is essential for coupling to take place, as has been demonstrated for the complement-mediated immune attack on the univalent cardiolipin antigen embedded in an artificial lipid bilayer (Humphries & McConnell, 1975).

An intriguing observation that emphasizes the difference in the interactions of the uncoupled catalytic unit with the bilayer and the receptor-coupled catalytic unit is the inhibitory effect of benzyl alcohol, at low concentrations, on the activity of the uncoupled catalytic unit (Fig. 1). From Arrhenius plots of glucagon-stimulated adenylate cyclase activity (Table 1) and from physical data (Colley & Metcalfe, 1972), it is clear that fluidization of the bilayer had begun at this concentration (approx. 2–10mM), and indeed the enzyme in the coupled state was activated in accord with this (Fig. 1). This inhibitory effect is clearly a prerogative of the uncoupled catalytic unit and is not directly related to fluoride stimulation, because when des-histidine-glucagon was added to achieve physical coupling between receptor and catalytic unit without stimulation (Houslay *et al.*, 1976*a*, 1977), together with fluoride to activate the enzyme, the response to benzyl alcohol mimicked that demonstrated when glucagon alone was the stimulating ligand (Fig. 1). From n.m.r. studies (Colley & Metcalfe, 1972) it was concluded that benzyl alcohol was positioned in the bilayer such that its hydroxyl group aligned with polar head groups of the phospholipids, and the aromatic residue pointed towards the interior of the bilayer. However, there appeared to be an interaction between benzyl alcohol molecules and the head groups of the phospholipids positioned in the immediate vicinity that was detected by an upfield shift of the $\overset{+}{N}Me_3$ resonance attributable to the aromatic ring current of benzyl alcohol. This effect decreased as the number of methyl groups between the aromatic ring and the terminal hydroxyl group increased, presumably as the bulky aromatic ring was displaced further into the bilayer interior and away from the head-group region (Colley & Metcalfe, 1972). From studies completed with 3-phenylpropan-1-ol and 5-phenylpentan-1-ol, which at the concentrations that we tested were shown to exhibit negligible aromatic ring-current interactions with the head-group region of the bilayer in n.m.r. spectroscopy studies (Colley & Metcalfe, 1972), there was no indication of inhibition of the activity of the uncoupled catalytic unit by the alcohols at low concentrations, although they activated the fluoride-(uncoupled) and glucagon-(coupled) stimulated adenylate cyclase activities in parallel, with inhibition occurring at higher concentration, as with benzyl alcohol. Such experiments may be rationalized by assuming that the bulky aromatic nucleus of benzyl alcohol interacted with the head-group region of the phospholipids of the annulus of the catalytic unit, giving an inhibition of enzyme activity in the uncoupled state. This was overcome by increased penetration of benzyl alcohol into the bilayer due to the increased bilayer fluidity activating the enzyme. 3-Phenylpropan-1-ol and 5-phenylpentan-1-ol, both of whose aromatic ring is aligned further away from the head-group region of the bilayer, did not inhibit the enzyme. These observations strongly implicate the lipid annulus of the catalytic unit as playing a major role in determining the activity in the uncoupled state. The inhibitory phenomenon would appear to be overridden when coupling occurs between the catalytic unit and the glucagon receptor. Presumably coupling between receptor and catalytic unit causes some kind of conformational change that affects either the disposition of the catalytic unit in the bilayer or its interaction with annular lipids.

Changes in bilayer fluidity may provide a means of modulating the activity of adenylate cyclase *in vivo*. As the catalytic unit appears not to sense the lipid-phase separation in the outer half of the bilayer (Houslay *et al.*, 1976*a*,*b*,*c*), clearly asymmetric changes of the bilayer lipids may provide a means of selectively modifying the hormone-stimulated state of the enzyme, as distinct from the resting (uncoupled) state. There is evidence that changes in bilayer fluidity occur during the cell cycle and during transformation, at which time concentrations of cyclic AMP also appear to be affected [see Nicolson (1976) and Parker *et al.* (1974) for reviews], which may implicate a functional significance to our observations. Indeed it has been observed that commercial heparin preparations contain benzyl alcohol, and its addition to serum preparations used for cell culture can alter intracellular cyclic AMP concentrations (Atkinson *et al.*, 1976), presumably by activating adenylate cyclase. The effect of diet on membrane composition, and the ingestion of drugs such as chlorpromazine and propanolol, which are known to have membrane-fluidizing properties (Lee, 1977*b*), could all affect the activity of this enzyme through their action on the bilayer. Finally we point out that the concentration at which benzyl alcohol causes maximal activation of adenylate cyclase also blocks conduction of action potential in frog sciatic nerve (Seeman, 1972).

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References

Atkinson, J. P., Udey, M. C., Wedner, H. J. & Parker, C. W. (1976) *J. Cyclic Nucleotide Res.* **2**, 297–305.

Birnbaumer, L. (1973) *Biochim. Biophys. Acta* **300**, 129–158

Brown, B. L., Elkins, R. P. & Albano, J. D. M. (1972) *Adv. Cyclic Nucleotide Res.* **2**, 25–40

Colley, C. M. & Metcalfe, J. C. (1972) *FEBS Lett.* **24**, 241–246

Goa, J. (1953) *Scand. J. Clin. Lab. Invest.* **5**, 218–222

Gurd, J. W. & Evans, W. H. (1974) *Arch. Biochem. Biophys.* **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**, 4145–4151

Hill, M. W. (1974) *Biochim. Biophys. Acta* **356**, 117–124

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. (1976*a*) *Biochim. Biophys. Acta* **436**, 489–494

Houslay, M. D., Hesketh, T. R., Smith, G. A., Warren, G. B. & Metcalfe, J. C. (1976*b*) *Biochim. Biophys. Acta* **436**, 495–504

Houslay, M. D., Johansson, A., Smith, G. A., Hesketh, T. R., Warren, G. B. & Metcalfe, J. C. (1976*c*) *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

Hubbell, W. L. & McConnell, H. M. (1971) *J. Am. Chem. Soc.* **93**, 314–317

Humphries, G. M. K. & McConnell, H. M. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2483–2487

Kleeman, W. & McConnell, H. M. (1976) *Biochim. Biophys. Acta* **419**, 206–222

Lee, A. G. (1976) *FEBS Lett.* **63**, 359–363

Lee, A. G. (1977*a*) *Biochim. Biophys. Acta* **472**, 285–344

Lee, A. G. (1977*b*) *Mol. Pharmacol.* **13**, 474–487

Mabrey, S. & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3862–3866

Newby, A. C., Luzio, J. P. & Hales, N. C. (1975) *Biochem. J.* **146**, 625–633

Nicolson, G. L. (1976) *Biochim. Biophys. Acta* **458**, 1–72

Overath, P. & Trauble, H. (1973) *Biochemistry* **12**, 2625–2634

Parker, C. W., Sullivan, T. J. & Wedner, H. J. (1974) *Adv. Cyclic Nucleotide Res.* **4**, 1–79

Pilkis, S. J., Exton, J. H., Johnson, R. A. & Park, R. A. (1974) *Biochim. Biophys. Acta* **343**, 250–267

Rethy, A., Tomasi, V., Trevisani, A. & Barnabei, O. (1972) *Biochim. Biophys. Acta* **290**, 58–69

Robles, E. C. & Van den Berg, B. (1969) *Biochim. Biophys. Acta* **187**, 520–526

Rubaclava, B. & Rodbell, M. (1973) *J. Biol. Chem.* **248**, 3831–3837

Sackmann, E. & Trauble, H. (1972) *J. Am. Chem. Soc.* **94**, 4482–4488

Sauerheber, R. D., Gordon, L. M., Crossland, R. D. & Kuwahara, M. D. (1977) *J. Membr. Biol.* **31**, 131–169

Schramm, M., Orby, J., Eimerl, S. & Korner, M. (1977) *Nature (London)* **268**, 310–313

Seeman, P. (1972) *Pharmacol. Rev.* **24**, 583–655

Swislocki, N. I., Magnusson, T. & Tierney, J. (1975) *Arch. Biochem. Biophys.* **179**, 157–165

Trams, G. G. & Lauter, C. J. (1974) *Biochim. Biophys. Acta* **345**, 180–197

Trauble, H. (1972) *J. Am. Chem. Soc.* **94**, 4482–4488

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974*a*) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 622–626

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G. & Metcalfe, J. C. (1974*b*) *Biochemistry* **13**, 5501–5507

Warren, G. B., Houslay, M. D., Metcalfe, J. C. & Birdsall, N. J. M. (1975) *Nature (London)* **255**, 684–687