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Adenylate cyclase is inhibited upon depletion of plasma-membrane cholesterol

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A procedure has been developed that allows for the depletion of rat liver plasma membrane cholesterol by incubation with liposomes at 4°C. Upon cholesterol depletion, adenylate cyclase activity was inhibited and the membranes became more rigid, as determined by the flexibility of an incorporated fatty acid spin probe. Decreasing the cholesterol/phospholipid molar ratio elicited a pronounced drop in the net fold-stimulation of adenylate cyclase activity by glucagon. Two lipid phase separations were detected in cholesterol-depleted membranes at around 25°C and 13°C respectively. Breaks at these temperatures were observed in Arrhenius plots of both the mobility of the spin probe and the glucagon-stimulated adenylate cyclase activity for the range 2–40°C, but only the one at the lower temperature for the fluoride-stimulated activity. It is proposed that the lipid phase separation occurring at 25°C is localized in the external half of the bilayer, whereas that at 13°C is due to lipids in the inner half of the bilayer. Similar structural and functional perturbations were manifest if the cholesterol-complexing polyene antibiotic amphotericin B was added to native membranes. The mechanism of adenylate cyclase inhibition achieved by cholesterol depletion and the domain structure of the plasma membrane in relation to cholesterol distribution are discussed. Native cholesterol/phospholipid ratios appear to optimize the functioning of adenylate cyclase in liver plasma membranes.

Many hormones and neurotransmitters exert their action on target tissues by stimulating the integral plasma-membrane enzyme adenylate cyclase. They do this by binding to specific receptor proteins exposed at the cell surface, which subsequently interact with adenylate cyclase, whose active site is located at the cytosol surface of the membrane, via a guanine nucleotide regulatory protein (for reviews, see Ross & Gilman, 1980; Hebdon *et al.*, 1980; Houslay, 1981; Limbird, 1981; Houslay & Gordon, 1983). Adenylate cyclase from a number of sources is activated by raising the bilayer fluidity (see Houslay & Gordon, 1983).

Cholesterol is a major lipid component of the plasma membranes of eukaryotic cells (see Kimelberg, 1977). It interacts with phospholipids in the membrane to suppress non-specific leakage of polar substances across the bilayer (Blok *et al.*, 1977), modulates bilayer fluidity (see e.g. Kimelberg, 1977) and can create cholesterol-rich and -poor domains in the membrane (see e.g. Orci *et al.*, 1981) by virtue of its preferential interaction with specific types of phospholipids (Demel *et al.*, 1977; Van Dijck, 1979). We have recently demonstrated (Whetton *et al.*, 1983) that elevation of cholesterol concentrations in rat liver plasma membranes leads to an inhibition of adenylate cyclase activity. This is accompanied by a decrease in bilayer fluidity and the abolition of the high-temperature onset of the lipid phase separation occurring at 28°C in native membranes. In the present study, we examine the effect of cholesterol removal from rat liver plasma membranes on both the adenylate cyclase activity and the bilayer fluidity detected with an extrinsic fatty acid spin probe.

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Materials and methods

All of the experimental procedures used in the present study were as described previously by Whetton *et al.* (1983), except for those set out below. Determinations of adenylate cyclase activity were made by taking initial rates from linear time courses under all conditions (Houslay *et al.*, 1976*a*).

Arrhenius plots were analysed as described previously in some detail (Houslay & Palmer, 1978).

Preparation of (dipalmitoyl) phosphatidylcholine liposomes

(Dipalmitoyl) phosphatidylcholine (dipalmitoylglycerophosphocholine) (130 μmol) was dissolved in chloroform/methanol (2:1, v/v). In some experiments, 3 μCi of either glycerol tri[^{14}C]oleate or ^{3}H-labelled phospholipid was also added. A lipid film was prepared from this in a glass vial by evaporating the solvent under a stream of dry N_2. The glass vial was then held *in vacuo* for 2.5 h to remove residual solvent. After this, 8 ml of an N_2-saturated solution containing 240 mM-KCl, 10 mM-$MgSO_4$, 6% (w/v) sucrose in 80 mM-Tris/HCl buffer, final pH 7.2, was added to the vial. This was then sealed under an N_2 atmosphere and vigorously shaken for 20 min followed by a 10 min exposure to ultrasound, at full power on an MSE Soniprep 150 probe sonicator. Afterwards, the solution was centrifuged at 15000 *g* for 20 min. The supernatant was then used immediately.

Preparation of membranes with lowered cholesterol contents

Plasma membranes (7–10 mg of protein) were incubated with 4–4.6 ml of freshly prepared (dipalmitoyl) phosphatidylcholine liposomes at 4°C in a solution of total volume 7 ml containing final concentrations of 160 mM-KCl, 16 mM-2-mercaptoethanol, 5.6% (w/v) sucrose, 1 mM-ATP, 21 mM-phosphocreatine, 1 mg of creatine kinase/ml and 53 mM-Tris/HCl, final pH 7.2.

After incubation for various times, samples (400 μl) were taken and washed as detailed previously (Whetton *et al.*, 1983). Samples were then either taken for cholesterol and phospholipid determinations as before or resuspended in 100 μl of 50 mM-Tris/HCl, pH 7.2, for adenylate cyclase assay.

In some instances, membranes from incubation mixtures were washed and then subjected to discontinuous-sucrose-density-gradient centrifugation to obtain plasma membranes essentially free of liposomal contamination (Whetton *et al.*, 1983). Membranes treated in this way were used for the spin-label studies.

Spin-labelling and e.s.r. analysis of rat liver plasma membranes

Methods for e.s.r. studies were essentially as described previously in some detail (Gordon *et al.*, 1978, 1980; Whetton *et al.*, 1983). Native and cholesterol-depleted liver plasma membranes were each suspended in 50 mM-triethanolamine/HCl/8% (w/v) sucrose, final pH 7.6, at 4 mg of protein/ml. These samples were added to the I(12,3) spin probe that had initially been deposited on the side of the tube by evaporation from aqueous ethanol to yield an experimentally-determined low probe concentration of 6 μg of I(12,3)/mg of membrane protein (Gordon *et al.*, 1980) at 30°C. Spectra were recorded on a Varian E-104A e.s.r. spectrometer and the cavity temperature was calibrated as set out in Gordon *et al.* (1978). The order parameters S, $S(T_{\parallel})$ were defined as in Gordon & Sauerheber (1977), being derived from the outer ($2T_{\parallel}$) and inner ($2T_{\perp}$) hyperfine splittings as Gordon *et al.* (1978).

Creatine kinase, ATP, GTP, cyclic AMP and triethanolamine/HCl were from Boehringer (U.K.) Ltd. Phosphocreatine, (dimyristoyl) phosphatidylcholine (dimyristoylglycerophosphocholine) and (dipalmitoyl) phosphatidylcholine were from Sigma (U.K.) Ltd. Glucagon was a gift from Dr. W. W. Bromer, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Benzyl alcohol and charcoal Norit GS were from Hopkin & Williams, Chadwell Heath, Essex, U.K. (Dioleoyl) phosphatidylcholine (dioleoylglycerophosphocholine) and ([^{3}H]dimyristoyl) phosphatidylcholine were gifts from Dr. G. A. Smith, University of Cambridge, Cambridge, U.K. [^{3}H]AMP, [^{3}H]cholesterol, [^{14}C]cholesterol, cyclic [^{3}H]AMP, [^{3}H]glycerol trioleate, glycerol tri[^{14}C]oleate, [^{3}H]inulin, inulin[^{14}C]carboxylic acid, ^{3}H-labelled phospholipids and ^{125}I were from The Radiochemical Centre, Amersham, Bucks., U.K. The *N*-oxyl-4,4-dimethyloxazolidine derivative of 5-oxostearic acid, I(12,3) was obtained from Syva Co., Palo Alto, CA, U.S.A.

Results

Native rat liver plasma membranes exhibit a cholesterol/phospholipid molar ratio of 0.71 (Whetton *et al.*, 1983). Over a 6 h period of incubation with (dipalmitoyl) phosphatidylcholine liposomes, the value of this ratio fell to 0.32, with the rate of cholesterol transfer, out of the membranes, dropping markedly over the same period (Fig. 1*a*). Rechallenging the washed membranes after a 6 h incubation with fresh liposomes failed to increase the rate of cholesterol removal (results not shown). The exit of cholesterol was independent of the ATP present in the incubation, and did not occur when the membranes were treated with liposomes containing cholesterol in a molar ratio with phospholipid of 0.72 (Fig. 1*a*). The washing procedure, described previously (Whetton *et al.*, 1983), removed here about 98% of the liposomes associated with the membrane sample, and these remaining liposomes did not contain sufficient lipid to alter the apparent cholesterol/phospholipid ratio of the membrane. Furthermore, the contaminating lipid was apparently in the form of intact exogenous liposomes. This was deduced from experiments

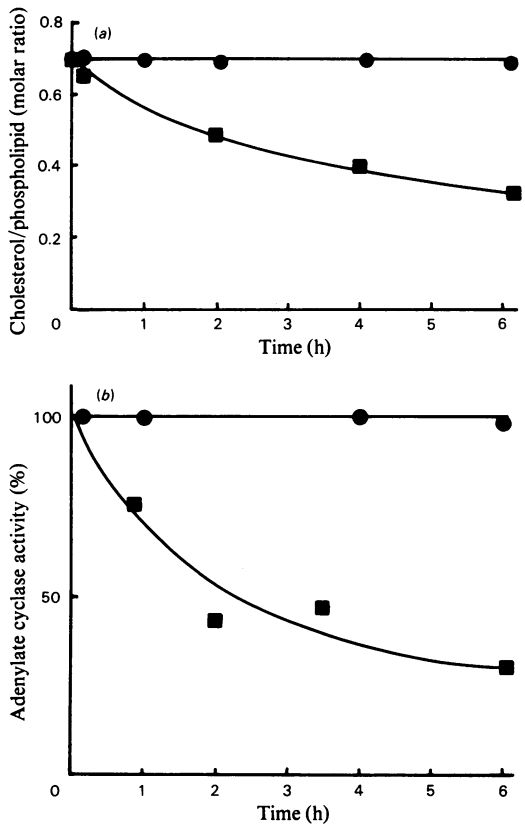

Fig. 1. *Cholesterol depletion of liver plasma membranes*
Rat liver plasma membranes were incubated at 4°C with (dipalmitoyl) phosphatidylcholine liposomes under the conditions described in the Materials and methods section. (*a*) shows the change in cholesterol/phospholipid molar ratio observed during incubation with these liposomes (■) and also with liposomes containing cholesterol (●) at a cholesterol/phospholipid molar ratio reflecting that of the native membranes (0.72). (*b*) shows the change in glucagon-stimulated adenylate cyclase activity under these two conditions.

where the exogenous liposomes were loaded with radiolabelled inulin, for after incubation and separation we found that the ratio of radiolabelled liposomal phospholipid to radiolabelled inulin was identical for both the treated membrane sample and the native liposomes. As free radiolabelled inulin did not adhere to the membranes, any label associated with the membrane was presumed, under our experimental conditions, to be entrapped in the liposomes. This residual liposomal contamination could be removed by discontinuous-sucrose-density-gradient centrifugation (see the Materials and methods section), since cholesterol-depleted membranes collected at the 20/30% (w/v) sucrose interface, whereas liposomes remained at the 4.6/10% (w/v) interface. The membranes so obtained were not detectably contaminated with liposomes (liposomal phospholipid <1% of endogenous membrane phospholipid pool).

Upon removal of cholesterol from the membranes by incubation with liposomes, there was a progressive fall in adenylate cyclase activity. This is shown in Fig. 1(*b*) for the glucagon-stimulated activity, and similar results could be obtained for the other ligand-stimulated activities. Such a change in activity was not due to denaturation over the incubation period, as in control experiments incubation of membranes with liposomes having a cholesterol/phospholipid (C/P) molar ratio of 0.72 failed to elicit alterations in enzyme activity (Fig. 1*b*). Moreover, incubation of cholesterol-depleted membranes with cholesterol-loaded liposomes (Whetton *et al.*, 1983) reversed this inhibitory effect (>90% recovery of activity at a C/P ratio of 0.7).

Decreasing the C/P ratio of rat liver plasma membranes led to a progressive inhibition of both basal and ligand-stimulated adenylate cyclase activities at 30°C (Fig. 2). The ligand-stimulated responses, however, seemed to be more sensitive to cholesterol depletion than the basal activity. This is emphasized by the sharp drop in the fold-activation (Fig. 2*e*) over the basal activity induced by glucagon that occurred upon decreasing the C/P ratio of the membrane. This was not due to any loss in specific binding of glucagon to its receptors on the plasma membrane, as the specific binding of ^{125}I-labelled glucagon to its receptor was $114 \pm 5\%$ (mean ± S.D., $n = 3$, for C/P = 0.42 ± 0.02).

Upon cholesterol depletion of the membranes there was, initially, relatively little change in the fluidity or degree of order of the bilayer, as assessed by the flexibility of the incorporated fatty acid spin probe I(12,3) at 30°C (Fig. 2*f*). However, as the C/P ratio fell below 0.58, then a progressive increase in overall membrane order was detected by the increase in $S(T_{\perp})$. Such alterations in $S(T_{\perp})$ are of a similar magnitude to that obtained by decreasing the temperature some 4–5°C (Gordon *et al.*, 1980). These observations correlate well with the increase in bilayer order elicited by exposure of the membranes to the cholesterol-complexing polyene antibiotic amphotericin B (Fig. 3). Here, 2 μM-amphotericin B caused an increase in $S(T_{\perp})$ that is equivalent to that observed in liver membranes with a C/P ratio of around 0.47.

Arrhenius plots of adenylate cyclase activities in cholesterol-depleted membranes were quite different (Fig. 4) from those of native membranes (Houslay *et al.*, 1976*a*; Dipple & Houslay, 1978), which, in the case of the glucagon-stimulated activity, show a break at 28°C, whereas that of the fluoride-

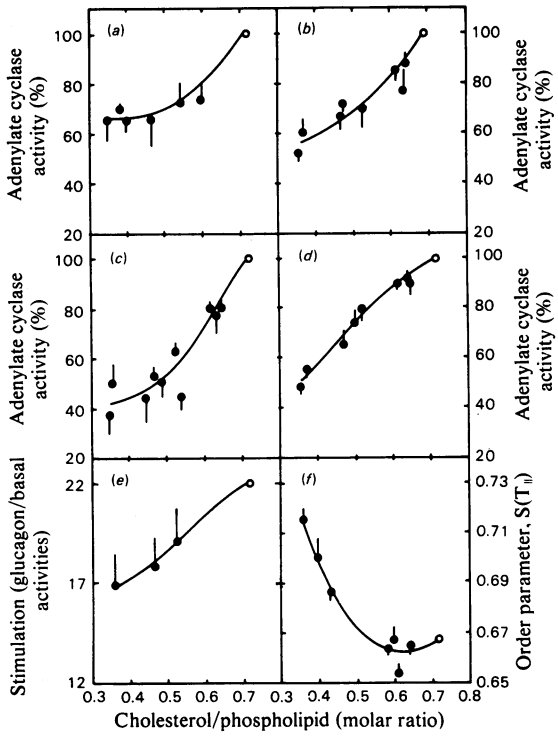

Fig. 2. *Alterations in adenylate cyclase activity and the mobility of a fatty acid spin probe, in labelled membranes, with the depletion of liver plasma-membrane cholesterol*

(*a*) Basal adenylate cyclase activity; (*b*) fluoride-stimulated adenylate cyclase activity; (*c*) glucagon + low GTP (50 nM), where the interaction obeys a Mobile Receptor Model as distinct from (*d*) glucagon + high GTP (10 μM), where interaction obeys a Collision Coupling Mechanism (see Houslay *et al.*, 1980); (*e*) an impression of the change in degree of stimulation by glucagon plus low GTP (*c*) over basal (*a*) is given here by the change in this ratio. The curve in (*e*) delineates the ratio of curves (*c*)/curve (*a*) and actual data points are given where the appropriate pairs of data (±hormone) were available; (*f*) the change in order parameter $S(T_{\parallel})$ for I(12,3)-labelled membranes. All experiments were carried out at 30°C, and points represent means ± S.D. for three determinations. The points reflect the aggregate of data collected by the manipulation of four to six different membrane preparations in each instance.

stimulated activity was linear. Here, the fluoride-stimulated activity exhibited a break at 13.2 ± 2.7°C, with activation energies of 106 ± 15.6 and 45.7 ± 9.3 kJ · mol^{-1} above and below the break point respectively. The glucagon-stimulated activity exhibited two breaks at 22.7 ± 0.9°C and 12.5 ± 4.4°C, with an activation energy of 51.0 ± 7.4 KJ · mol^{-1} above the upper break point, and activation energies of 117.9 ± 12.8 and

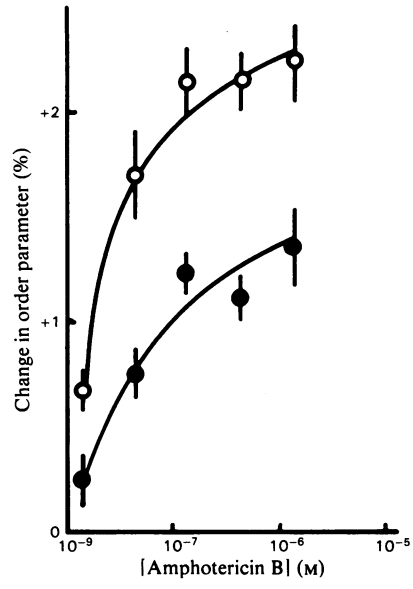

Fig. 3. *Amphotericin B treatment of spin-labelled liver plasma membranes*

The effect of increasing amphotericin B concentrations on the order parameters S (●) and $S(T_{\parallel})$ (○) of I(12,3)-labelled membranes is shown. Experiments were carried out at 30°C in duplicate using three different membrane preparations. Points represent means ± S.E.M.

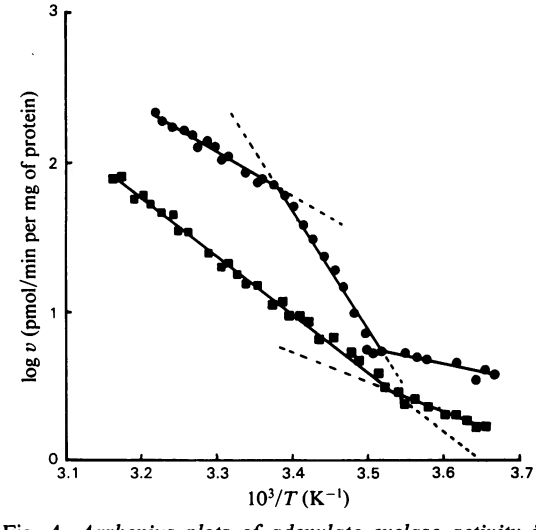

Fig. 4. *Arrhenius plots of adenylate cyclase activity in cholesterol-depleted liver plasma membranes*

The activity of fluoride- (■) and glucagon (●)-stimulated adenylate cyclase activity was followed in cholesterol-depleted liver plasma membranes (cholesterol/phospholipid molar ratio = 0.37).

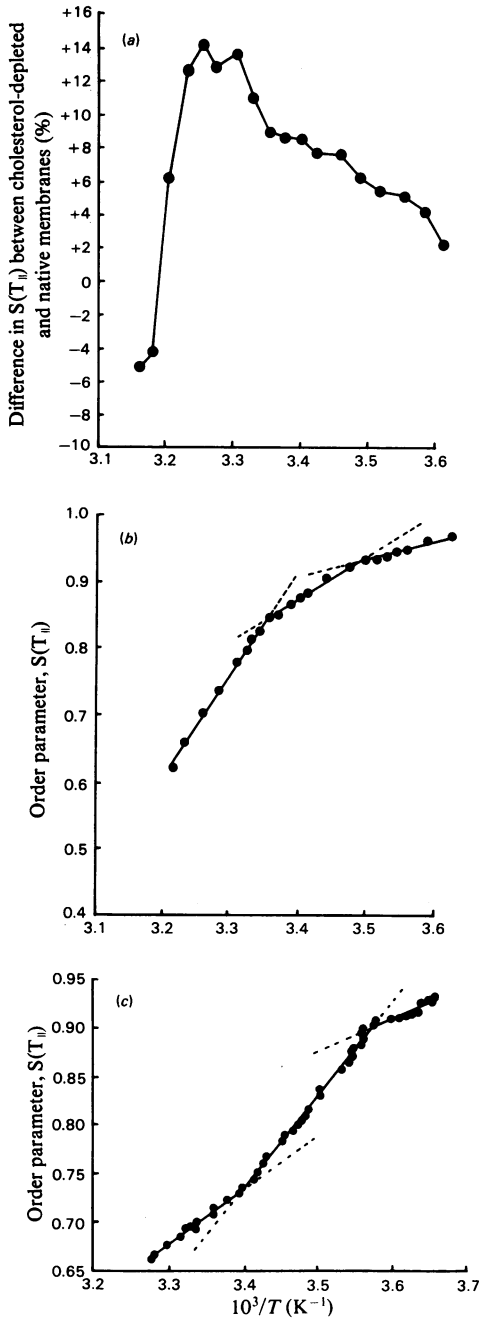

Fig. 5. *Temperature-dependence of the order parameter $S(T_{\parallel})$ of spin-labelled cholesterol-depleted plasma membranes*

(*a*) $S(T_{\parallel})$ versus $1/T$ plot, where $S(T_{\parallel})$ is shown as the percentage difference between the order parameter $S(T_{\parallel})$ values of cholesterol-depleted (C/P = 0.36) and native (C/P = 0.72) rat liver plasma membranes. Membranes were labelled with 6 μg of I(12,3)/mg of protein. Order parameters are as defined in Gordon & Sauerheber (1977). (*b*) Arrhenius plot of the order parameter $S(T_{\parallel})$ for cholesterol-depleted (C/P = 0.36) plasma membranes labelled with I(12,3). (*c*) Arrhenius plot of the order parameter $S(T_{\parallel})$ for native membranes labelled with I(12,3) in the presence of 1 μM-amphotericin B.

22.4 ± 4.1 KJ·mol^{-1} above and below the lower break point (Fig. 4). (The data were obtained from three preparations with C/P ratios of 0.38 ± 0.05; values are all means $\pm$ S.D.).

The effects of cholesterol depletion on the fluidity of I(12,3)-labelled membranes were also investigated over a wide temperature range. Fig. 5(*a*) shows that cholesterol-depleted membranes were substantially less fluid than native membranes for temperatures less than 40°C, with the maximum difference occurring between 26 and 38°C. On the other hand, native and cholesterol-depleted membranes shared the same fluidity at around 40°C (Fig. 5*a*). Using I(12,3)-labelled cholesterol-depleted rat liver plasma membranes, Arrhenius-type plots (Fig. 5*b*) of the order parameter $S(T_{\parallel})$ indicated breaks occurring at around 25.7 ± 0.5°C and 12.0 ± 1.0°C over the temperature range examined (values are means $\pm$ S.D. for three preparations with C/P ratios of 0.36).

Arrhenius-type plots of the order parameter $S(T_{\parallel})$ for I(12,3)-labelled native plasma membranes treated with 2 μM-amphotericin B (Fig. 5*c*) exhibited two breaks occurring at 22.4 ± 1.4°C and 8.6 ± 0.5°C (means $\pm$ S.D. for three preparations). Discontinuities or breaks occurring at similar temperatures were also noted in Arrhenius-type plots of S, $2T_{\parallel}$, $2T_{\perp}$ or $S(T_{\perp})$ [results not shown; see Gordon & Sauerheber (1977) for definition of $S(T_{\perp})$].

When adenylate cyclase in cholesterol-depleted membranes was stimulated by either fluoride or glucagon, then the addition of the local anaesthetic and bilayer fluidizing agent benzyl alcohol achieved degrees of activation that were very similar to those observed for the native membranes (Table 1). Upon maximal stimulation of the enzyme in cholesterol-depleted membranes by benzyl alcohol, the specific activities for both fluoride- and glucagon-stimulated adenylate cyclase were only 65% and 56% respectively of those of the native enzyme when maximally stimulated by benzyl alcohol. Similarly, the response of the fluoride-stimulated activity in cholesterol-depleted membranes to thermal denaturation was very similar to that observed in native membranes (results not shown). The half-life for inactivation at 40°C was 3.3 ± 0.2 min in cholesterol-depleted membranes, whereas that noted in native membranes was 3.4 ± 0.2 min (Whetton *et al.*, 1983; means $\pm$ S.E.M.; $n = 3$).

Table 1. *Effect of benzyl alcohol on both ligand-stimulated adenylate cyclase activity and the order parameter $S(T_{\|})$ of an incorporated fatty acid spin probe in native liver plasma membranes and plasma membranes with depleted cholesterol concentrations*

All assays were carried out at 30°C. Results are means ± S.D. ($n = 4$). The native data (i.e. for liver plasma membranes) are taken from Whetton *et al.* (1983), using membranes with a cholesterol/phospholipid (C/P) molar ratio of 0.72. The cholesterol-depleted membrane preparations had a C/P ratio of 0.47. The specific activity attained in the presence of optimal benzyl alcohol concentrations was 65% and 56% for the fluoride- and glucagon-stimulated activities respectively of that observed for the activities of native plasma membranes in the presence of benzyl alcohol.

	Increase in activity (%)		
Membrane sample	Fluoride-stimulated adenylate cyclase	Glucagon-stimulated adenylate cyclase	$\Delta S(T_{\|})$ (%)
Liver plasma membranes	160 ± 5	155 ± 14	−5.7 ± 1.0
Cholesterol-depleted liver plasma membranes	173 ± 13	173 ± 13	−5.7 ± 2.0

Discussion

Incubation of liver plasma membranes at 4°C with (dipalmitoyl) phosphatidylcholine liposomes led to a decrease in the C/P molar ratio from approx. 0.72 to approx. 0.33. This occurred without fusion with the exogenous phospholipid vesicles. Upon cholesterol depletion, we noted a reversible inhibition of adenylate cyclase activity (Fig. 2), which was more pronounced for the glucagon-stimulated activity than for the basal enzyme activity. As both increases (Whetton *et al.*, 1983) and decreases in cholesterol content lead to a net decrease in the degree of stimulation of adenylate cyclase by glucagon, it would appear that the activity of this enzyme is optimized by the cholesterol content of native membranes.

Interestingly cholesterol depletion of plasma membranes actually led to a decrease in bilayer fluidity at temperatures less than 40°C (Fig. 5*a*). This was substantiated by similar observations (Fig. 3) found using the soluble polyene amphotericin B. This drug interacts avidly with cholesterol, decreasing cholesterol–phospholipid interactions (De Kruijff *et al.*, 1974). Similarly, amphotericin B leads to a reversible inhibition of adenylate cyclase activity over the range where the decrease in bilayer fluidity occurs (Dipple & Houslay, 1979). As a variety of agents that increase bilayer fluidity also activate adenylate cyclase (see Houslay *et al.*, 1981; Houslay & Gordon, 1983), it is possible that the decreased bilayer fluidity occurring upon cholesterol depletion is responsible for the inhibition of this enzyme. However, it is clear (Fig. 2) that a decrease in the C/P ratio to approx. 0.55 has exerted a negligible effect on bilayer fluidity, yet significant inhibition of adenylate cyclase has occurred. Indeed, basal adenylate cyclase is relatively insensitive to changes in membrane fluidity (see Whetton *et al.*, 1983), yet it is highly sensitive to inhibition by cholesterol depletion. This suggests that manipulation of cholesterol concentrations is exerting effects on this enzyme other than through alterations in membrane fluidity. It has been suggested that both 'cholesterol-rich' and 'cholesterol-poor' lipid domains exist in liver plasma membranes, where adenylate cyclase and other integral membrane proteins are located in the 'cholesterol-poor' domains (Houslay & Palmer, 1978; Whetton *et al.*, 1982; Houslay & Gordon, 1983). Such a domain structure may be stabilized by the preferential interaction of cholesterol with specific phospholipids, e.g., sphingomyelin and acidic phospholipids (see Van Dijck, 1979). Cholesterol removal would release these phospholipids species into the pool of phospholipids monitored by the I(12,3) spin probe and where adenylate cyclase is presumed to reside. In these membranes, 20–30% of the total phospholipid is sphingomyelin (see Van Hoeven & Emmelot, 1972), which, unlike the other phospholipids present, has associated acyl chains that are almost entirely saturated and contains a large fraction of unusually long acyl chains (Van Hoeven *et al.*, 1975). The release of such particularly rigid lipid into the 'cholesterol-poor' pool might well account for our observations. Indeed the release of specific phospholipids from 'cholesterol-rich' domains could exert a direct inhibitory effect on the enzyme. In this respect it has been noted by Schimmel *et al.* (1980) that the acidic phospholipid phosphatidic acid inhibits adenylate cyclase activity.

We have shown that elevations in the fluidity of native membranes achieved by benzyl alcohol decreased the thermostability of fluoride-stimulated adenylate cyclase (Needham *et al.*, 1982), whereas cholesterol loading served to increase both the bilayer rigidity and thermostability of the fluoride-stimulated activity at 40°C (Whetton *et al.*, 1983). Our present findings that the fluidities of native and cholesterol-depleted membranes are similar at around 40°C (Fig. 5*a*) and that the thermal denaturation of the fluoride-stimulated activity occurs at similar rates in these two membranes would

be consistent with the hypothesis that the thermostability of this enzyme is influenced by the membrane fluidity.

In marked contrast with our observations on adenylate cyclase in cholesterol-enriched membranes (Whetton *et al.*, 1983), the inhibition of activity caused by cholesterol depletion is not fully reversed by benzyl alcohol (Table 1). Instead it activates the enzyme to a similar degree seen with the native enzyme, which is in accord with its ability to increase bilayer fluidity to similar extents in both native and cholesterol-depleted membranes (Table 1). This supports our proposal (Whetton *et al.*, 1983) that the enhanced response of adenylate cyclase to benzyl alcohol in cholesterol-enriched membranes, even though the degree of fluidization achieved is identical with that seen with native membranes, actually reflects the ability of benzyl alcohol to break up either adducts of cholesterol/phospholipid or clusters of enzyme proteins exhibiting low activity states, or both.

In native plasma membranes there is a well-defined lipid phase separation with a high-temperature onset at around 28°C that has been identified by a number of investigators by using a variety of techniques (for review, see Whetton *et al.*, 1982; Houslay & Gordon, 1983). We have presented considerable evidence, which is summarized elsewhere (Houslay *et al.*, 1980, 1981; Whetton *et al.*, 1982), indicating that the lipid phase separation is localized to the external half of the bilayer and that no lipid phase separation occurs in the inner half of the bilayer. This model proposes that in the fluoride-stimulated state the activity of the enzyme is sensitive to the lipid environment of the inner half of the bilayer only. However, in the glucagon-stimulated state its activity is modulated by both halves of the bilayer. Thus in native membranes, Arrhenius plots of glucagon-stimulated adenylate cyclase activity exhibit a well-defined break at around 28°C, whereas those of fluoride-stimulated activity are linear (Houslay *et al.*, 1976*a*). Upon cholesterol depletion of the plasma membranes there is a marked change in the form of these plots, with that of the fluoride-stimulated activity showing a single break at around 13°C, whereas that of the glucagon-stimulated activity exhibited two distinct breaks at around 23° and 13°C (Fig. 4). We do not consider that this could be due to any fusion of (dipalmitoyl) phosphatidylcholine with the membranes. Indeed substitution of this lipid into rat liver plasma membranes elevates the break-point temperature seen in Arrhenius plots of the glucagon-stimulated activity from 28°C, whereas the corresponding plots of the fluoride-stimulated activity remain linear (Houslay *et al.*, 1976*b*). Instead, the break points observed in Arrhenius plots of ligand-stimulated activities (Fig. 4) appear to correlate well with the characteristic temperatures identified with the I(12,3) spin probe (Fig. 5*b*). We propose that cholesterol depletion of liver plasma membranes results in the occurrence of a lipid phase separation at around 23–25°C in the extracellular-facing half of the bilayer and the appearance of a new lipid phase separation at around 13°C in the inner half of the bilayer.

The actions of cholesterol depletion on adenylate cyclase activity and membrane fluidity are mimicked by amphotericin B, which induces lipid phase separations occurring at around 22 and 9°C in native membranes (Fig. 5*c*). These, by virtue of Arrhenius plots of glucagon-stimulated adenylate cyclase exhibiting both breaks and those of fluoride-stimulated adenylate cyclase showing only the lower one (Dipple & Houslay, 1979), may be attributed to lipid phase separations occurring in the outer and inner halves of the bilayer respectively. Although this situation is closely analogous with that observed in cholesterol-depleted membranes (Fig. 5*b*), there is one major distinction. The activation energies of the adenylate cyclase reaction decreased at temperatures below the lower break point in cholesterol-depleted membranes (Fig. 4), whereas they actually increased below the lower break point in amphotericin B-treated membranes (Dipple & Houslay, 1979). This effect appears to be lipid-mediated, rather than a direct action of amphotericin B on the enzyme, as differences in the direction of slope above the upper break point were observed between cholesterol-depleted and amphotericin B-treated membranes in spin-label studies (Figs. 5*b* and 5*c*). Cholesterol depletion thus causes major changes in the lipid organization of liver plasma membranes, which can be expected to affect the activity of a variety of integral membrane enzymes. In the case of adenylate cyclase, it would appear that its activity is optimal at the cholesterol/phospholipid molar ratios observed in native membranes. Any slight changes elicited by diet, drugs or disease (see Kimelberg, 1977) might be expected to cause marked effects on its degree of stimulation by glucagon. Our studies also imply that the manipulation of membrane cholesterol concentrations is not a useful tool to investigate the effect of membrane fluidity changes on membrane protein functioning. Not only is one unable to predict what sort of fluidity changes will be elicited but cholesterol manipulation appears to have a marked effect on the domain structure of the membrane both as regards the distribution and interaction of lipid and protein species.

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