J. C., and Sarda, L. (1974), *Lipids 9,* 765.
Nano, L., and Savary, P. (1976), *Biochimie 58,* 917.
Patton, J. S., Albertsson, P. A., Erlanson, C., and Borgström, B. (1978), *J. Biol. Chem. 253,* 4195.
Rietch, J., Pattus, F., Desnuelle, P., and Verger, R. (1977), *J. Biol. Chem. 252,* 4313.
Sarda, L., and Desnuelle, P. (1958), *Biochim. Biophys. Acta 30,* 513.
Schönheyder, F., and Volquartz, K. (1945), *Acta Physiol. Scand. 9,* 57.
Scow, R. O., and Olivecrona, T. (1977), *Biochim. Biophys. Acta 487,* 472.
Spackman, D. H., Stein, W. H., and Morre, S. T. (1958), *Anal. Chem. 30,* 1190.
Ulrich, Cl., Boucrot, P., and Clément, J. (1974), *Biochimie 56,* 429.
Vandermeers, A., Vandermmers-Piret, M. C., Rathé, J., and Christophe, J. (1975), *FEBS Lett. 49,* 334
Verger, R., de Haas, G. M., Sarda, L., and Desnuelle, P. (1969), *Biochim. Biophys. Acta 188,* 272.
Verger, R., Rietch, J., and Desnuelle, P. (1977), *J. Biol. Chem. 252,* 4319.
Zilversmit, D. B. (1967), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 26,* 1599.

Relationships between Membrane Cholesterol, α-Adrenergic Receptors, and Platelet Function†

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ABSTRACT: Platelets incubated with cholesterol-rich phospholipid dispersions acquire membrane cholesterol, and this is associated with decreased membrane fluidity and increased sensitivity of these platelets to the aggregating agent epinephrine. Conversely, platelets incubated with cholesterol-free phospholipid dispersions lose membrane cholesterol, have increased membrane fluidity, and exhibit a decreased sensitivity to epinephrine. Epinephrine-stimulated platelet aggregation is initiated by the interaction of the amine with α-adrenergic receptors. Therefore, we tested whether the altered responsiveness of cholesterol-enriched or -depleted platelets to epinephrine results from a change in the number or affinity of α-adrenergic receptors or in the activity of these receptors in inhibiting adenylate cyclase. Platelet α-adrenergic receptors in intact platelets or platelet particulates were studied using [^{3}H]dihydroergocryptine ([^{3}H]DHE), an α-adrenergic antagonist. Although cholesterol-rich platelets required an 18-fold lower concentration of epinephrine for aggregation, the α-adrenergic receptors of these platelets were similar to those of normal control platelets in several respects: kinetics of association and dissociation of [^{3}H]DHE, the number of [^{3}H]-DHE binding sites, and the affinity of the receptors for [^{3}H]DHE and epinephrine. Moreover, cholesterol incorporation had little effect on basal adenylate cyclase activity or on activity stimulated by NaF or prostaglandin E_1. The maximal inhibition of adenylate cyclase activity by epinephrine was also unchanged by cholesterol incorporation, and the potency of epinephrine in producing this inhibition was only slightly (less than twofold) increased. Cholesterol-depleted platelets required eightfold more epinephrine than normal for aggregation, but, as with cholesterol-rich platelets, binding of [^{3}H]DHE resembled binding to controls. These data indicate that the increase or decrease in platelet responsiveness to epinephrine caused by cholesterol incorporation or depletion, respectively, must result from effects of cholesterol distal to the binding of epinephrine to its receptor. Furthermore, the increased platelet responsiveness caused by cholesterol incorporation is probably independent of changes in adenylate cyclase activity. Since binding to the α-adrenergic receptor appears to be unaffected by cholesterol incorporation or depletion, these results suggest that α-adrenergic receptors may exist in a lipid domain in the membrane that is inaccessible to cholesterol.

Cholesterol is a major structural component of plasma membranes and, as such, it functions to maintain membrane phospholipids in an intermediate fluid state (Chapman, 1968). In this action, cholesterol appears to be closely interposed with the phospholipids so that certain acyl carbon atoms near the membrane surface are more constrained while those deep within the hydrophobic core of the bilayer have greater freedom of motion (Rothman & Engelman, 1972). Cholesterol has been shown to affect fluidity of the lipid bilayer as assessed by rotational diffusion of hydrophobic fluorescent probes (Copper et al., 1978; Vanderkooi et al., 1974; Shinitzky & Inbar, 1976). In addition, cholesterol influences membrane transport and permeability (Wiley & Copper, 1975; Deuticke & Ruska, 1976) and the activity of certain membrane-bound enzymes (Alirisatos et al., 1977; Farras et al., 1975; Kimelberg, 1975).

Human platelets have been useful in examining the effects of increasing or decreasing membrane cholesterol content. Incubation of platelets in vitro with lipid dispersions containing 2.2 mol of cholesterol/mol of phospholipid (C/PL = 2.2) re-

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sults in a selective increase of platelet membrane cholesterol (Shattil et al., 1975). Platelets that have been enriched with cholesterol in this manner are altered in several ways: they become more responsive than normal to platelet aggregating agents [e.g., (−)-epinephrine and ADP] (Shattil et al., 1975); they have decreased membrane fluidity (Shattil & Cooper, 1976), reflecting an increase in order within lipid–lipid interactions (Shinitzky & Barenholz, 1978); and they have been reported to have altered adenylate cyclase (ATP pyrophosphate-lyase [cyclizing] EC 4.6.1.1) activity (Sinha et al., 1977). In contrast, incubation of platelets with cholesterol-free phospholipid dispersions (C/PL = 0) results in a decrease in platelet membrane cholesterol, and this is associated with an increase in membrane fluidity and a decrease in responsiveness of these platelets to epinephrine (Shattil et al., 1975; Shattil & Cooper, 1976).

The mechanism by which changes in membrane cholesterol modify the sensitivity of platelets to epinephrine is unknown. One possibility is that the amount of membrane cholesterol modifies the response of platelets to epinephrine by altering the number or affinity of the membrane receptors for the amine. Alternatively, changes in cholesterol content might influence membrane events distal to these receptors. Therefore, we examined the effect of cholesterol incorporation (or depletion) on platelet membrane receptors for epinephrine (α-adrenergic receptors). We also examined the effects of cholesterol incorporation on the ability of epinephrine to inhibit platelet membrane adenylate cyclase.

Experimental Procedures

Chemicals. Adenosine 5′-tri[^{32}P]phosphate was purchased from ICN and Amersham-Searle. [^{3}H]Adenosine 3′:5′-monophosphate and di[^{3}H]hydroergocryptine (26–28 Ci/mmol) were purchased from New England Nuclear. All other chemicals were obtained from standard sources, except for those received as gifts: (−)-propranolol from Ayerst, (−)-epinephrine from Sterling-Winthrop, prostaglandin E_1 from Dr. J. Pike (Upjohn Co.), and phentolamine from Ciba-Geigy.

Platelet Preparation. All blood donors were normal volunteers who had abstained from medication for at least 2 weeks before blood donation. Venous blood was collected into plastic syringes and anticoagulated by mixing 9 volumes of blood with 1 volume of trisodium citrate (final concentration, 13 mM). All blood processing was carried out in plasticware at room temperature. Platelet-rich plasma was obtained by centrifuging samples for 25 min at 200g.

Platelet Incubation System. Cholesterol-rich and -free phospholipid dispersions (with cholesterol to phospholipid mole ratios of 2.2 and 0, respectively) were made from chromatically pure cholesterol and L-α-dipalmitoyllecithin in modified Tyrode's solution as previously described (Shattil et al., 1975). Immediately prior to use, dispersions were centrifuged at 21 000g for 30 min to sediment undissolved lipid. Lipid dispersions were then incubated with an equal volume of platelet-rich plasma for 5 h at 37 °C. Incubation of platelets with cholesterol-rich dispersions has been shown previously to increase platelet cholesterol content without changing platelet phospholipid content or the percentage of distribution of the major phospholipids (Shattil et al., 1975). In contrast, cholesterol-free dispersions deplete platelet membranes of cholesterol (Shattil et al., 1975). Control platelet-rich plasma was incubated simultaneously for 5 h at 37 °C with an equal volume of modified Tyrode's solution; this incubation does not affect platelet cholesterol or phospholipid content. Platelet cholesterol and phospholipid content were determined on platelets washed three times as described previously (Shattil et al., 1975).

Platelet Washing and Platelet Particulates. At the end of the 5-h incubations, platelets were centrifuged at 16 000g for 10 min, and the resulting pellets were resuspended and washed twice at 16 000g for 10 min in ice-cold buffer [50 mM Tris[1] (pH 7.4), 100 mM NaCl, 20 mM EDTA]. Intact platelets were used after these washes in the binding studies. Platelet particulates were prepared for use in binding assays by suspending washed platelets in hypotonic buffer (5 mM Tris-HCl, pH 7.5; 5 mM EDTA) and homogenizing them with 12 strokes of a motorized Teflon pestle (Marcus et al., 1966). For adenylate cyclase assays, platelets were disrupted by sonication at 50 W for 2 × 15 s at 4 °C with a Biosonik IV sonicator. The lysates were spun at 39 000g for 10 min, and the pellet was resuspended in 50 mM Tris (pH 7.5), 5 mM EDTA for use in binding and cyclase assays. Platelet particulates were used either immediately in the assays or were stored at −70 °C for up to 2 weeks before use.

α-Adrenergic Binding Assays. α-Adrenergic binding sites were quantitated by incubating intact platelets or platelet particulates with di[^{3}H]hydroergocryptine ([^{3}H]DHE, 4000–300 000 cpm) at 37 °C in 250-μL samples. Binding reactions were terminated by adding 4 mL of the incubation buffer containing 10 μM phentolamine and then filtering immediately over glass-fiber filters (Whatman GF/C). The α-adrenergic antagonist phentolamine was included in the buffer used to terminate the reactions in order to minimize nonspecific retention of radioactivity on the filters and, thus, to equalize samples incubated with radioligand alone or ligand plus competitors of binding. The filters were rapidly (<20 s) washed with 20 mL of the incubation buffer at 37 °C and then counted in a liquid scintillation system (^{3}H efficiency = 50%). Nonspecific binding was determined by incubating platelets or platelet lysates with 10 μM phentolamine and [^{3}H]DHE; this value was generally less than 30% of the total amount of radioactivity bound. This concentration of phentolamine was about 100-fold greater than that which competes for 50% of those [^{3}H]DHE binding sites for which phentolamine competes (data not shown). Binding assay determinations were run in duplicate or triplicate; duplicate values generally differed by less than 10%. Specific [^{3}H]DHE binding (total minus nonspecific) was linear with particulate protein from 0.7 to 2.4 mg/mL and with intact platelets from 0.8 to 2.0 × 10^9 platelets per mL.

Adenylate Cyclase Assay. Platelet particulate protein (generally 0.05 mg in 0.04 mL) was incubated in a final volume of 0.1 mL with the following (final concentrations): 50 mM Tris-HCl (pH 7.5), 0.2 mM ethylene glycol bis(β-aminoethyl ether)-N,N'-tetraacetic acid, 2 mM mercaptoethanol, 12 mM $MgCl_2$, 0.5 mM [^{32}P]ATP (0.5–2 × 10^6 cpm), 0.5 mM cAMP, 6 mM phosphocreatine, and 35 units/mL creatine phosphokinase. In some incubations, 5 μM (−)-propranolol was included in order to eliminate the stimulation of adenylate cyclase by the β-adrenergic action of epinephrine (Jakobs et al., 1976; Newman et al., 1978). Other agents used to stimulate cyclase included PGE_1 (5 μM) and NaF (10 mM). All tubes contained freshly prepared ascorbic acid (1 mM) or freshly prepared (−)-epinephrine bitartrate dissolved in ascorbic acid. Reactions were initiated by adding protein and incubating the samples for 10 to 12 minutes at 37 °C. The incubations were terminated and samples were assayed as described (Salomon et al., 1974). Basal cyclase activity or activity in response to

[1] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

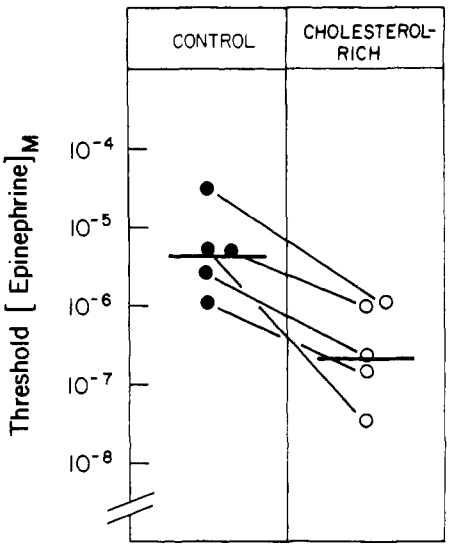

FIGURE 1: Epinephrine-induced platelet aggregation. Platelet aggregation in response to epinephrine was determined as described under Experimental Procedures in control platelets that had been incubated in modified Tyrode's solution for 5 h or in platelets incubated with cholesterol-rich phospholipid dispersions (C/PL = 2.2) in modified Tyrode's solution for 5 h. The geometric mean threshold concentrations of epinephrine (horizontal lines) under the two conditions are significantly different ($p < 0.02$).

epinephrine or PGE_1 either alone or together was linear for at least 15 min and with protein up to at least 0.1 mg. The K_m for ATP ranged from 0.04 to 0.06 mM when determined under basal conditions or with stimulation by PGE_1 alone or by PGE_1 plus epinephrine. Cyclase determinations were run in triplicate; the coefficient of variations of triplicate samples was generally less than 5%. Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin standards.

Platelet Aggregation Studies. Platelet mixtures (0.5 mL) were placed in a cylindrical cuvette 3 mm in diameter containing a silicone-coated stirring bar. Aggregation was recorded as the percent decrease in light transmittance as platelet aggregates formed in an aggregometer (Sinha et al., 1977). The sensitivity of platelets to (−)-epinephrine was defined as the lowest concentration of epinephrine producing a complete "second wave" response and this corresponded to a greater than 82% decrease in light transmittance (Shattil et al., 1975).

Results

Cholesterol Enrichment and Epinephrine-Induced Platelet Aggregation. In five experiments, incubation of normal platelets with cholesterol-rich phospholipid dispersions for 5 h at 37 °C produced a 44 ± 6% (mean ± SD) increase in platelet cholesterol content. This was associated with an 18-fold decrease in the threshold concentration of epinephrine required to produce complete second wave aggregation (Figure 1).

Cholesterol Enrichment and α-Adrenergic Binding Studies. Work from several laboratories has indicated that epinephrine-stimulated platelet aggregation is mediated by epinephrine's action at an α-adrenergic receptor (O'Brien, 1963; Barthel & Markwardt, 1974; Yu & Latour, 1977). Therefore, to test whether the increased sensitivity to epinephrine in cholesterol-rich platelets resulted from a modification of the receptors for epinephrine, we compared binding to α-adrenergic receptors in normal and cholesterol-rich platelets. We used the α-adrenergic antagonist [^{3}H]DHE, previously reported as a useful radioligand for this receptor in both intact platelets and platelet particulates (Newman et al., 1978; Kafka et al., 1977; Goldfien et al., 1978).

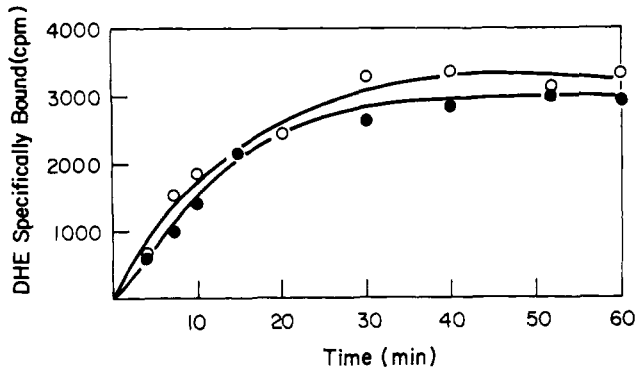

FIGURE 2: Time course of [^{3}H]DHE binding to intact platelets. Control (●) and cholesterol-rich (○) platelets were each incubated with 2.6 nM [^{3}H]DHE at a platelet concentration of 2.0×10^9 platelets/mL. Specific binding was determined at the indicated times as described under Experimental Procedures.

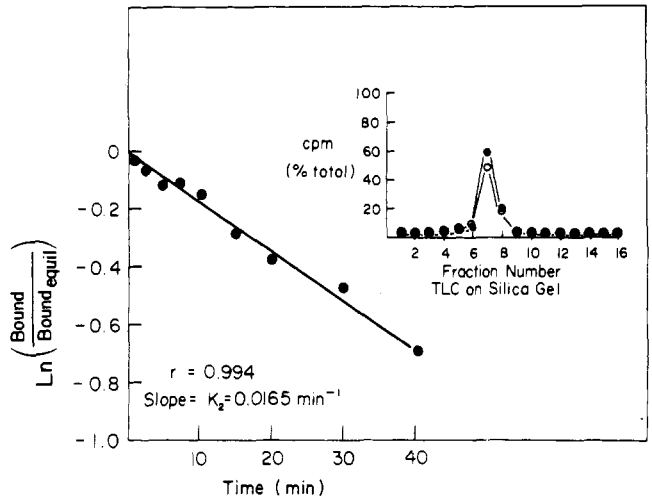

FIGURE 3: Dissociation and thin-layer chromatography of [^{3}H]DHE bound to intact platelets. [^{3}H]DHE bound to platelets for 50 min was dissociated at $t = 0$ by the addition of 10 μM phentolamine, and specific binding was determined at the indicated times. Binding is expressed as the natural log of the amount bound at time t divided by the amount bound at equilibrium, where the amount bound at equilibrium was the amount specifically bound at time $t = 0$. The inset shows migration on a thin-layer silica gel plate in chloroform/benzene/ethanol/NH_4OH (4:2:1:0.1) of [^{3}H]DHE and of radioactive material extracted with ethanol from intact platelets that had been incubated with [^{3}H]DHE for 50 min.

The time course of [^{3}H]DHE binding to intact control and cholesterol-rich platelets is shown in Figure 2. Specific binding (see Experimental Procedures) achieved equilibrium in 30–40 min, although in experiments at lower receptor or [^{3}H]DHE concentrations equilibrium was reached only after 50 min. The rate of binding to cholesterol-rich platelets was identical to control platelets. From such experiments, we calculate an association rate constant of 6×10^6 min^{-1} M^{-1}. The time course in platelet particulates was similar to that of the intact platelets (data not shown).

Because of the relatively long period required to achieve equilibrium, we tested for possible degradation of the radioligand under the experimental conditions. The [^{3}H]DHE that was extracted from platelets after a 50-min incubation demonstrated a time course of binding to platelets similar to fresh [^{3}H]DHE (data not shown). In addition, such extracted material migrated on thin-layer chromatography as did [^{3}H]DHE (Figure 3, inset). Binding of [^{3}H]DHE was reversible (Figure 3) and demonstrated a single exponential decay with a rate constant of 0.0165 min^{-1}.

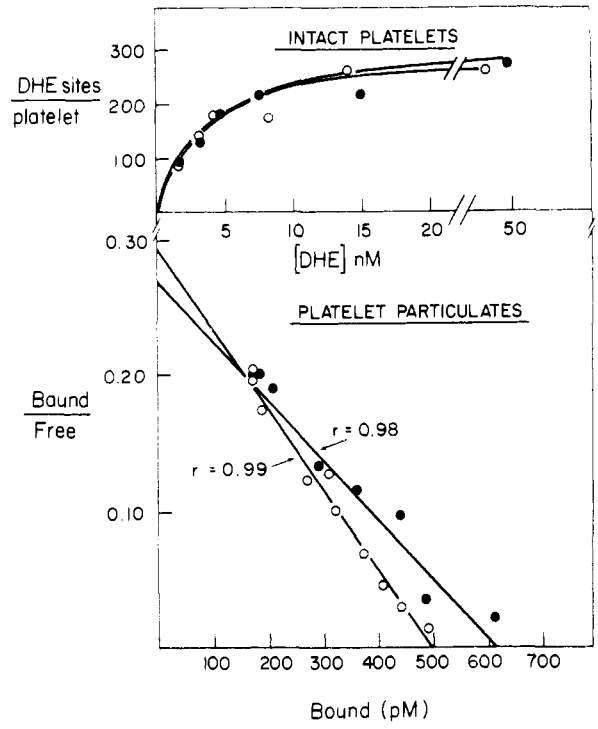

FIGURE 4: Equilibrium binding of [^{3}H]DHE to platelets. Intact control (●) and cholesterol-rich (○) platelets (upper panel) and platelet particulates (lower panel) were incubated with varying concentrations of [^{3}H]DHE for 50 min, and specific binding was determined. The concentration of intact platelets in the experiment shown was 9.6×10^8 platelets/mL and of platelet particulate membrane protein 1.5 mg/mL. The binding data for the intact platelets are plotted as the number of binding sites per platelet and were calculated from the amount of [^{3}H]DHE bound, the specific activity of the radioligand, the platelet count, and Avogadro's number. The lower panel is a Scatchard plot of the binding data for platelet particulates.

TABLE I: Equilibrium Binding of [^{3}H]DHE to Cholesterol-Rich, Cholesterol-Depleted, and Control Platelets.[a,b]

	intact platelets		platelet particulates	
platelets	dissoc const (nM)	no. of bind. sites/platelet	dissoc const (nM)	[^{3}H]DHE bound/mg of protein (fmol)
control (4)	4.4 ± 0.75	341 ± 38	3.3 ± 0.75	400 ± 58
cholesterol-rich (4)	4.8 ± 0.64	340 ± 28	3.7 ± 0.95	439 ± 88
cholesterol-depleted (4)	5.9 ± 1.2	370 ± 40		

[a] Numbers in parentheses are the number of experiments. [b] Data are expressed as mean ± SEM.

Specific binding of [^{3}H]DHE to both platelet particulates and intact platelets (Figure 4) was saturable with increasing concentrations of radioligand and yielded a single class of binding sites on Scatchard analysis (Scatchard, 1949). The dissociation constant in control platelets calculated from such experiments was 3.3 ± 0.75 nM in platelet particulates and 4.4 ± 0.75 nM in intact platelets. The number of [^{3}H]DHE binding sites was 341 ± 38 sites per intact platelet and 400 ± 58 fmol/mg particulate protein (Table I). The value of the dissociation constant derived from equilibrium binding experiments was in close agreement with that calculated from the independent experiments of association and dissociation (2.8 nM). Cholesterol-rich platelets were similar to control platelets with respect to the number of [^{3}H]DHE binding sites and the dissociation constants of these sites for [^{3}H]DHE (Table I).

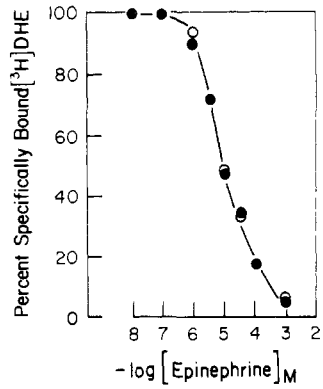

FIGURE 5: Competition for [^{3}H]DHE binding sites on intact control and cholesterol-rich platelets. Intact platelets were incubated with 2.5 nM [^{3}H]DHE and varying concentrations of (−)-epinephrine. Data are plotted as percent of specifically bound [^{3}H]DHE counts in control (●) and cholesterol-rich (○) platelets.

TABLE II: Adenylate Cyclase Activity in Cholesterol-Rich and Control Platelet Particulates.[a]

additions	control	cholesterol-rich
none	45.4 ± 10.2	53.2 ± 7.7
(−)-epinephrine (10 μM)	29.9 ± 0.5	34.4 ± 5.2
prostaglandin E_1 (5 μM)	432 ± 72	498 ± 91
prostaglandin E_1 + (−)-epinephrine	306 ± 54	338 ± 42
NaF (10 μM)	188 ± 33	222 ± 26
NaF + (−)-epinephrine	157 ± 22	200 ± 8

[a] Activity is expressed as pmol of cAMP (mg of protein)$^{-1}$ min^{-1}. Data are mean ± SD; $n = 4$.

Studies of radioligand binding to α- and β-adrenergic receptors have indicated differences in binding properties of adrenergic agonists and antagonists (Insel & Stoolman, 1978; Maguire et al., 1976; Lefkowitz et al., 1976; U'Prichard et al., 1977). Thus, it was important to test whether competition for the [^{3}H]DHE (antagonist) binding sites by the agonist epinephrine would be altered after cholesterol incorporation. Epinephrine was equipotent in competing with [^{3}H]DHE in both cholesterol-rich and control platelets (Figure 5).

Cholesterol Enrichment and Platelet Adenylate Cyclase. Cholesterol incoporation had no significant effect upon basal adenylate cyclase activity or upon PGF_1- and NaF-stimulated activity (Table II). Moreover, the maximal inhibition of cyclase activity by epinephrine in cholesterol-rich platelet particulates was not different from inhibition in control particulates (Table II). The potency of epinephrine in inhibiting PGE_1-stimulated cyclase activity was about twofold greater in cholesterol-rich particulates than in control particulates (Figure 6).

Cholesterol-Depleted Platelets. Platelets incubated with cholesterol-free phospholipid dispersions lost 20% of their cholesterol with no change in their phospholipid content. This was associated with a mean eightfold increase in the threshold concentration of epinephrine required to produce platelet aggregation. However, such platelets were not different from controls with respect to the number of [^{3}H]DHE binding sites or the affinity of these sites for [^{3}H]DHE (Table I). The concentrations of epinephrine which competed for [^{3}H]DHE binding sites in cholesterol-depleted platelets were similar to those observed in cholesterol-rich and control platelets.

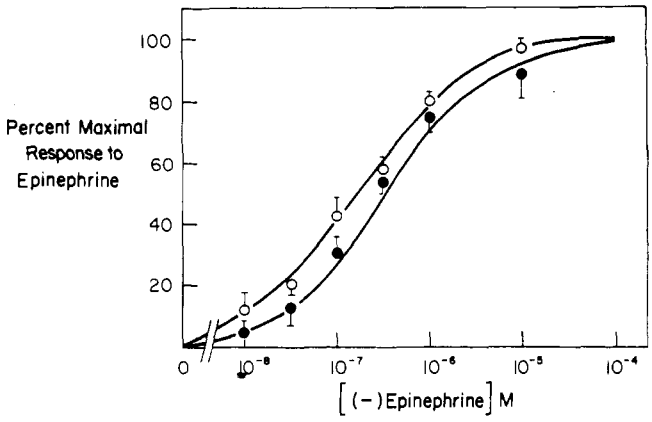

FIGURE 6: Inhibition of prostaglandin E_1 stimulated adenylate cyclase activity by (−)-epinephrine. Platelet particulate adenylate cyclase activity was determined at the indicated concentrations of (−)-epinephrine. Data are plotted as mean ± SEM for $n = 3$ or 5 separate experiments and are expressed as the percent maximal epinephrine response, where maximal response is the activity inhibited by 0.1 mM epinephrine. Activity inhibited at 0.1 mM (−)-epinephrine was 111 ± 20 and 111 ± 17 pmol min^{-1} (mg of protein)$^{-1}$ (mean ± SEM, $n = 5$) for the control (●) and cholesterol-rich (○) platelets, respectively.

Discussion

Membrane lipids are important modulators of the activity of membrane-bound enzymes (Coleman, 1973). Cholesterol depletion or enrichment appears to affect several such systems (Alirisatos et al., 1977; Farras et al., 1975; Kimelberg, 1975; Klein et al., 1978). With respect to adrenergic amines, treatment of avian erythrocytes with the polyene antibiotic filipin, which interacts with membrane cholesterol, decreases β-adrenergic-stimulated adenylate cyclase activity without decreasing binding to β-adrenergic receptors (Puchwein et al., 1973; Limbird & Lefkowitz, 1976). Furthermore, the enrichment or depletion of cholesterol in turkey erythrocyte membranes decreases the activity of β-adrenergic-stimulated adenylate cyclase (assayed in the presence of 5′-guanylylimidodiphosphate) without changing the number or affinity of β-adrenergic receptors (Hanski & Levitzki, 1978).

By contrast, in platelets we find that changes in membrane cholesterol content, which alter the sensitivity of platelets to α-adrenergic aggregation, are not associated with either changes in α-adrenergic receptors or in the degree of inhibition of adenylate cyclase activity. Our findings in platelets indicating that changes in cholesterol content do not change binding to α-adrenergic receptors are similar to those observed in the avian erythrocyte systems in which perturbations of membrane cholesterol have little effect on binding to β-adrenergic receptors. Thus, it is possible that the recognition sites for catecholamines by both α- and β-adrenergic receptors are located in a domain in the membrane which is not accessible to or influenced by cholesterol. Nevertheless, in view of the lack of effect of cholesterol incorporation on maximal α-adrenergic inhibition of platelet adenylate cyclase activity, in contrast with the decrease in β-adrenergic-stimulated cyclase activity produced by perturbing membrane cholesterol in avian erythrocytes, the two adrenergic receptors may differ in their interaction with lipids within the membrane.

Although little information exists regarding the localization of α-adrenergic receptors within the platelet membrane, our data indicate that membrane fluidity (i.e., motion of fatty acid chains within the bilayer), which is altered by cholesterol incorporation or depletion (Shattil & Cooper, 1976), must play only a minor role in the interaction of epinephrine with the α-adrenergic receptor and in the inhibition of adenylate cyclase by this receptor. Our findings do not eliminate the possibility that lateral mobility of receptors within the plane of the membrane might be involved in action of the α-adrenergic receptor.

The cholesterol content of cholesterol-enriched platelets is 80% greater than that of cholesterol-depleted platelets. When membrane fluidity is expressed in terms of fluorescent anisotropy of the probe 1,6-diphenyl-1,3,5-hexatriene, each percent increase in cholesterol content is associated with a 0.12% increase in fluorescent anisotropy (Shattil & Cooper, 1976). These changes in cholesterol content and fluidity are associated with a 150-fold difference in the potency of epinephrine in aggregating platelets, yet with no difference in the binding of epinephrine to the α-adrenergic receptors of these platelets. Thus, the change in responsiveness to epinephrine must result from an effect of cholesterol acting distal to the α-adrenergic receptor binding site for the agonist. Since the events that follow receptor occupancy are poorly understood, it is not possible to specify how fluctuations in membrane cholesterol lead to altered platelet aggregation. Changes in platelet calcium distribution (Lüscher & Massini, 1975) and prostaglandin synthesis (Hamberg et al., 1975) appear to be important for platelet aggregation. Both of these processes are associated with platelet membranes and, therefore, might be influenced by cholesterol.

We initially hypothesized that changes in cAMP generation might explain the change in platelet responsiveness because it had been reported that incorporation of cholesterol into platelet membranes increases basal adenylate cyclase activity while abolishing NaF- and PGE_1-stimulated activity (Sinha et al., 1977). In that previous report, cyclase activity was assayed in platelet sonicates with both Mg^{2+} and ATP at 2 mM. We assayed adenylate cyclase activity in platelet particulates at 12 mM Mg^{2+} and 0.5 mM ATP. Under these conditions, we found increases in cyclase activity in response to both PGE_1 and NaF, and these increases were similar to those of control platelet particulates. To test whether the differences in experimental conditions might account for the differences between our results and those of the previous report, we repeated experiments under conditions essentially identical to those used by Sinha et al. (1977). As previously reported by Sinha et al. (1977), we found that incorporation of cholesterol increased basal adenylate cyclase activity. However, cholesterol incorporation failed to obliterate the stimulation of cyclase activity produced by 2 mM NaF or 1 μM PGE_1, the same concentrations used by Sinha et al. (1977). Thus, we are unable to confirm the findings of the previous report with respect to the loss in hormonal response of the enzyme.

The minimal increase in the potency of epinephrine in inhibiting adenylate cyclase and the similar extent of inhibition by epinephrine that we observed after cholesterol incorporation suggest that a decrease in cAMP is not the signal responsible for the increased aggregation of these platelets to epinephrine. Although cAMP is an important negative modulator of platelet aggregation in vitro (Salzman & Weisenberg, 1972), our data demonstrate that it is possible to dissociate changes in the ability of platelets to generate cAMP from changes in platelet aggregation in response to epinephrine.

References

Alirisatos, S. G. A., Papstavrou, C., Crouka-Liapari, E., Molyvdas, A. P., & Nikitopoulou, G. (1977) *Biochem. Biophys. Res. Commun. 79,* 677–683.

Barthel, W., & Markwardt, F. (1974) *Biochem. Pharmacol. 23,* 37–45.

Chapman, D. (1968) *Biological Membranes: Physical fact and*

function (Chapman, D., Ed.) pp 125–202, Academic Press, New York, N.Y.
Coleman, R. (1973) *Biochim. Biophys. Acta 300,* 1–30.
Cooper, R. A., Arner, E. C., Wiley, J. S., & Shattil, S. J. (1975) *J. Clin. Invest. 55,* 115–126.
Cooper, R. A., Leslie, M. H., Fischkoff, S., Shinitzky, M., & Shattil, S. J. (1978) *Biochemistry 17,* 327–331.
Deuticke, B., & Ruska, C. (1976) *Biochim. Biophys. Acta 433,* 638–653.
Faras, R. N., Bloj, B., Morero, R. D., Sineriz, F., & Trucco, R. E. (1975) *Biochim. Biophys. Acta 415,* 231–251.
Goldfien, R., Insel, P. A., Goldfien, A., & Roberts, J. M. (1978) *Clin. Res. 26,* 106A.
Hamberg, M., Svensson, J., & Samuelsson, B. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 2994–2998.
Hanski, E., & Levitzki, A. (1978) *Life Sci. 22,* 53–60.
Insel, P. A., & Stoolman, L. M. (1978) *Mol. Pharmacol. 14,* 549–561.
Jakobs, K. H., Saur, W., & Shültz, G. (1976) *J. Cyclic Nucleotide Res. 2,* 381–392.
Kafka, M. S., Tallman, J. F., Smith, C. C., & Costa, J. L. (1977) *Life Sci. 21,* 1429–1438.
Kimelberg, H. K. (1975) *Biochim. Biophys. Acta 413,* 143–156.
Klein, I., Moore, L., & Pastan, I. (1978) *Biochim. Biophys. Acta 506,* 42–53.
Lefkowitz, R. J., Millikin, D., & Caron, M. G. (1976) *J. Biol. Chem. 251,* 4686–4692.
Limbird, L., & Lefkowitz, R. J. (1976) *Mol. Pharmacol. 12,* 559–567.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193,* 265–275.
Lüscher, E. G., & Massini, P. (1975) *Ciba Found. Symp. 35,* 5–15.
Maguire, M. E., Van Arsdale, P. M., & Gilman, A. G. (1976) *Mol. Pharmacol. 12,* 335–339.
Marcus, A. J., Zucker-Franklin, D., Safier, L. B., & Ullman, H. L. (1966) *J. Clin. Invest. 45,* 14–28.
Newman, K. D., Williams, L. T., Bishopric, N. H., & Lefkowitz, R. J. (1978) *J. Clin. Invest. 61,* 395–402.
O'Brien, J. R. (1963) *Nature (London) 200,* 763–764.
Puchwein, A., Pfeuffer, T., & Helmreich, E. J. M. (1974) *J. Biol. Chem. 249,* 3232–3240.
Rothman, J. E., & Engelman, D. M. (1972) *Nature (London), New Biol. 237,* 42–44.
Salomon, Y., Londos, C., & Rodbell, M. (1974) *Anal. Biochem. 58,* 541–548.
Salzman, E. W., & Weisenberger, H. (1972) *Adv. Cyclic Nucleotide Res. 1,* 231–247.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51,* 660–672.
Shattil, S. J., Anaya-Galindo, R., Bennett, J., Colman, R. W., & Cooper, R. A. (1975) *J. Clin. Invest. 55,* 636–643.
Shattil, S. J., & Cooper, R. A. (1976) *Biochemistry 15,* 4832–4837.
Shinitzky, M., & Barenholz, Y. (1978) *Biochim. Biophys. Acta* (in press).
Shinitzky, M., & Ingbar, M. (1974) *J. Mol. Biol. 85,* 603–615.
Shinitzky, M., & Ingbar, M. (1976) *Biochim. Biophys. Acta 433,* 133–149.
Sinha, A. K., Shattil, S. J., & Colman, R. W. (1977) *J. Biol. Chem. 252,* 3310–3314.
U'Prichard, D. C., Greenberg, D. A., & Snyder, S. H. (1977) *Mol. Pharmacol. 13,* 454–473.
Vanderkooi, J., Fischkoff, S., Chance, B., & Cooper, R. A. (1974) *Biochemistry 13,* 1509–1595.
Wiley, J. S., & Cooper, R. A. (1975) *Biochim. Biophys. Acta 413,* 425–431.
Yu, S. K., & Latour, J. G. (1977) *Thromb. Haemostasis 37,* 413–422.