

# Platelet Hypersensitivity Induced by Cholesterol Incorporation

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**ABSTRACT** Platelets from individuals with familial hypercholesterolemia show increased sensitivity to the aggregating agents, epinephrine and ADP. Since the mechanism of this abnormal sensitivity is unknown, we examined, *in vitro*, the influence of the plasma lipid environment on the function of platelets. The composition of plasma lipids was altered by the addition of sonicated cholesterol-dipalmitoyl lecithin liposomes which were "cholesterol normal" (cholesterol-phospholipid mole ratio  $[C/P] = 1.0$ ), "cholesterol rich" ( $C/P = 2.2$ ), or "cholesterol poor" ( $C/P = 0$ ). Cholesterol-normal liposomes had no influence on platelet lipids or platelet function. In contrast, after incubation for 5 h at 37°C with cholesterol-rich liposomes, normal platelets acquired 39.2% excess cholesterol with no change in phospholipids or protein. The percent increase in platelet membrane cholesterol was three-fold that of the granule fraction. The acquisition of cholesterol by platelets was associated with a 35-fold increase in sensitivity to epinephrine-induced aggregation ( $P < 0.001$ ) and 15-fold increase to ADP aggregation ( $P < 0.01$ ), as determined both by aggregometry and by [<sup>14</sup>C]serotonin release. Response to thrombin or collagen was unchanged. Platelets incubated with cholesterol-poor liposomes underwent a selective loss of 21.4% cholesterol and this was associated with an 18-fold reduction in their sensitivity to epinephrine.

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These studies demonstrate that the cholesterol content of platelets is dependent on the lipid composition of the milieu. Cholesterol acquired by platelets may exert its effect on platelet function by a modification of the platelet membrane.

## INTRODUCTION

Hypercholesterolemia is a major predisposing factor toward atherogenesis (1, 2). Although controversy exists regarding the contribution of platelets to the early atheromatous lesion (3), platelets are operative in arterial thrombosis, and this may contribute to the progression of and morbidity from existing atheromata (4). Because of the complex relationships of platelets and cholesterol to atherosclerosis, recent studies have explored platelet function in hypercholesterolemic subjects (5, 6). These studies have demonstrated that platelets from individuals with type II hyperlipoproteinemia have an increase in both platelet factor 3 availability and phospholipid content (5) and an increased sensitivity to aggregating agents, particularly epinephrine (6). However, it is not clear whether these findings are due to an intrinsic platelet defect or are related to the platelet's lipid-rich plasma environment. Furthermore, the relevance of these observations to the *in vivo* situation is unknown.

The abnormal lipid composition of lipoproteins in certain individuals with alcoholic cirrhosis profoundly influences erythrocyte membrane lipid composition and membrane function leading to the disorder, spur cell anemia (7, 8). Employing sonicated mixtures of cholesterol and phospholipid (liposomes), we have been able to simulate the abnormal serum lipid environment of these patients and have reproduced in normal erythrocytes the structural and functional abnormalities found

in spur cells (9). Using the same approach, we undertook to examine potential mechanisms of platelet hypersensitivity in hypercholesterolemia by defining the relationship between platelet lipid composition and function and the lipid composition of the platelets' environment. These studies demonstrate that perturbations in the platelets' lipid environment influence platelet function and correlate with alterations in platelet cholesterol content.

## METHODS

**Platelet preparation.** All blood donors were fasting for 12 h, had abstained from medications for at least 2 wk before blood donation, and had normal serum lipoproteins according to standard criteria (10), except for one who conformed to type IIa. Venous blood was collected through siliconized needles into plastic syringes and anticoagulated by mixing 9 vol of blood with 1 vol citrate-phosphate-dextrose (trisodium citrate 0.0894 M, citric acid 0.0156 M, monobasic sodium phosphate 0.0161 M, dextrose 0.1418 M). All blood processing was carried out in plastic-ware at room temperature. Platelet-rich plasma was obtained by centrifugation of samples for 10 min at 100 *g*. The remaining blood was centrifuged for 15 min at 1,800 *g* to obtain platelet-poor plasma which contained less than 20,000 platelets per  $\mu$ l. Platelets were counted (11) and their volume distributions measured using a Coulter counter model Z<sub>6</sub> equipped with a 50- $\mu$ m aperture tube and a Coulter Channelyzer model C-1,000 particle size analyzer (Coulter Electronics Inc., Hialeah, Fla.).

**Preparation of liposomes.** L- $\alpha$ -Dipalmitoyl lecithin (General Biochemicals, Div. Mogul Corp., Chagrin Falls, Ohio), 40 mg, and unesterified cholesterol (Sigma Chemical Company, St. Louis, Mo.) in amounts up to 80 mg were added to 10 ml of modified Tyrode's solution (0.1369 M NaCl, 0.0027 M KCl, 0.0119 M NaHCO<sub>3</sub>, 0.00042 M NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, pH 7.4) in a fluted metal container surrounded by an ice slurry and subjected to 70 W for 60 min with a Branson sonifier using a standard tip. Thin-layer chromatography performed before and after sonication revealed the lecithin to consist of greater than 95% lecithin, while 2% was recovered in the area of phosphatidylserine and the remainder was recovered in the area of lysolecithin.

"Cholesterol-normal" liposomes were prepared from lecithin, 40 mg plus cholesterol, 23 mg, and their cholesterol-to-phospholipid mole ratio (C/P)<sup>1</sup> was 1.0. These liposomes had been shown previously not to affect erythrocyte cholesterol content during prolonged incubation (9). "Cholesterol-rich" liposomes (lecithin, 40 mg plus cholesterol, 80 mg) had a C/P of 2.2 and had been demonstrated to transfer cholesterol to erythrocytes. "Cholesterol-poor" liposomes (lecithin 40 mg) had a C/P of zero and they decreased the cholesterol content of erythrocytes. Immediately before determination of C/P and use, sonicates were centrifuged at 21,800 *g* for 30 min to sediment undispersed lipid, and glucose and albumin were added to a final concentration of glucose 0.0055 M, and albumin 0.35%. The C/P and the phospholipid composition of all liposome preparations were stable at 5°C for up to 30 days and were employed in all platelet experiments within 1 wk of preparation.

**Platelet incubation system.** Within 45 min of collection,

<sup>1</sup> Abbreviation used in this paper: C/P, cholesterol-to-phospholipid mole ratio.

platelet-rich plasma was adjusted to a platelet count of 300,000–400,000 per  $\mu$ l with platelet-poor plasma, and this mixture was incubated with an equal volume of either Tyrode's solution alone or with an equal volume of one of the liposomes in Tyrode's. Mixtures were incubated for up to 5 h in a 37°C water bath with gentle inversion of the incubation tubes hourly. Samples were removed at various times up to 5 h for analysis of platelet composition and function.

**Platelet composition studies.** Platelets were washed three times in a platelet washing buffer (12). Platelet cholesterol (13) and lipid phosphorus content (14) were measured in quadruplicate aliquots of washed platelets extracted with 80 vol of isopropanol and chloroform (15). Total phospholipid content was taken to equal lipid phosphorus times 25. After the addition of the antioxidant 2,3-ditert-butyl, 4-methylphenol, extracts were taken to dryness under nitrogen and the phospholipids were separated by thin-layer chromatography on silica gel G. Spots were visualized with iodine vapor and the gel was quantitatively recovered for measurement of phosphorus (16). Platelet protein content was measured by the method of Lowry (17). Unincubated platelets and those from platelet-rich plasma (2.5 vol) incubated with cholesterol-rich liposomes (1 vol) were washed three times (12) and subjected to subcellular fractionation by the method of Marcus, Zucker-Franklin, Safier, and Ullman (18), modified by the use of a discontinuous 30–60% sucrose density gradient consisting of stepwise 5% increments. Resultant soluble, membrane, and granule fractions were analyzed for protein and lipid. Lipid extracts were washed four times with 0.05 M KCl to remove sucrose before measurement of phospholipid (14) and cholesterol (19). Each fraction was analyzed for activity of acid phosphatase (20),  $\beta$ -glucuronidase (21), and lactic dehydrogenase (22).

**Platelet function tests.** In preliminary experiments it was determined that, when incubated for 5 h at pH 7.4–7.6 in any of the media used, platelets demonstrated maximal sensitivity to aggregating agents after adjustment of the incubation mixture to a final concentration of 0.001 M MgCl<sub>2</sub> and to pH 7.78–7.82. Therefore, after each incubation but immediately before all platelet function testing, platelet-rich plasma was adjusted to this magnesium concentration and pH by the addition of appropriate amounts of 0.1 M MgCl<sub>2</sub> and 0.2 N NaOH. Platelet sensitivity to aggregating agents was studied by two methods. In the first, platelet aggregation was studied in an aggregometer attached to a strip chart recorder (Chrono-log Corp., Broomall, Pa.) according to a modification of the method of Born (23), as previously described (6). The sensitivity of platelets to *l*-epinephrine, ADP (Sigma Chemical Company, St. Louis, Mo.), collagen (calf skin, acid soluble, Worthington Biochemicals, Freehold, N. J.), or bovine thrombin (2,500 NIH U/mg) (24) was defined as the lowest concentration of the aggregating agent producing a complete "second wave" response. As previously determined (6), complete second wave response of normal platelets consisted of a greater than 82% increase in light transmittance of platelet-rich plasma in response to epinephrine, a greater than 71% increase in response of ADP, and a greater than 79% increase in response to collagen. Because of the apparent log-normal distribution of platelet sensitivities in control subjects (6), sensitivities are recorded as the log of the aggregating agent concentration. In the second method, platelet [<sup>14</sup>C]serotonin release was measured by the method of Jerusalem and Zucker (25). Normal platelets release more than 40% of their [<sup>14</sup>C]serotonin in response to maximal concentrations of epinephrine or collagen, and more than 30%

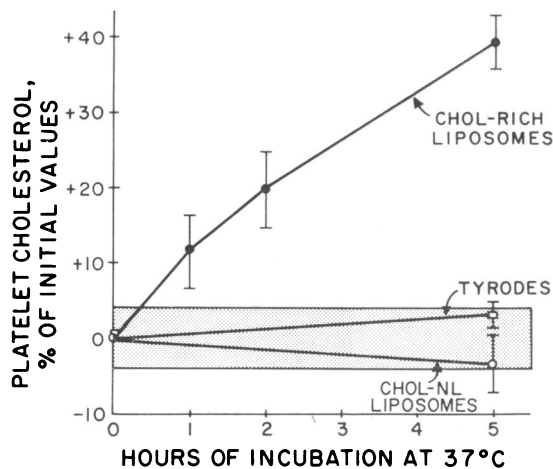


FIGURE 1 Effect of incubation of platelets with Tyrode's solution, cholesterol-normal liposomes (chol-nl), and cholesterol-rich liposomes (chol-rich) on platelet cholesterol content. The shaded area represents normal platelet cholesterol content  $\pm 2$  SEM (27 subjects). Values for mean platelet cholesterol  $\pm 1$  SE are the averages of nine experiments at zero hour, four experiments at 1 h, six at 2 h and nine experiments at 5 h of incubation.

in response to ADP. In our experiments platelet sensitivity was defined as the lowest concentration of the aggregating agent producing a normal percent [ $^{14}$ C]serotonin release.

## RESULTS

*The effect of liposomes on platelet composition.* When platelet-rich plasma was incubated with either Tyrode's solution alone or with cholesterol-normal liposomes for up to 5 h at 37°C, there was no change in cholesterol

content per platelet (Fig. 1). In contrast, platelets incubated with cholesterol-rich liposomes underwent a progressive increase in cholesterol content, amounting to 39.2% after 5 h. Acquisition of cholesterol was selective, and there was no change in the phospholipid or protein content per platelet (Fig. 2). Moreover, platelets incubated with cholesterol-rich liposomes exhibited no change in the percent distribution of the major phospholipids. There was no difference in mean cell volume of platelets incubated under these three conditions. Platelets incubated for 5 h with cholesterol-poor liposomes lost  $21.4 \pm 1.5\%$  (SD) of their cholesterol with no loss of phospholipid or protein. Thus, as has been observed with erythrocytes (7), the cholesterol content of platelets is responsive to modifications in the lipid composition of the plasma environment.

In order to localize the subcellular site of cholesterol acquisition by platelets incubated with cholesterol-rich liposomes, platelets were fractionated into membrane, soluble, and granule fractions (Fig. 3). The percent distribution of enzymes was similar in cholesterol-rich and normal platelets and similar to the results obtained by Marcus, Zucker-Franklin, Safier, and Ullman (18). The membrane fraction contained only 14.9% of the acid phosphatase and 15.7% of the  $\beta$ -glucuronidase, while the granule fraction contained 44% of the acid phosphatase and 67.9% of the  $\beta$ -glucuronidase. The soluble fraction contained 41.1% of the acid phosphatase, 16.4% of the  $\beta$ -glucuronidase, and 99.5% of the lactic dehydrogenase.

The C/P of the subcellular fractions of normal and cholesterol-rich platelets is shown in Table I. The mem-

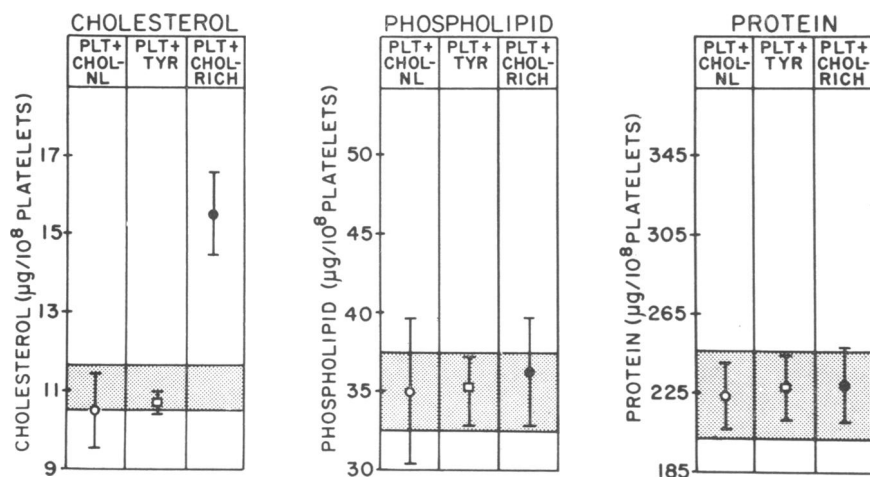


FIGURE 2 Effect of incubation for 5 h at 37°C on platelet cholesterol, phospholipid, and protein content. Platelet-rich plasma was incubated with Tyrode's solution (plt + tyr), cholesterol-normal liposomes (plt + chol-nl), and cholesterol-rich liposomes (plt + chol-rich). Shaded areas represent normal platelet values  $\pm 2$  SEM. Values for mean platelet cholesterol, phospholipid, or protein  $\pm 2$  SEM are the averages of nine experiments.

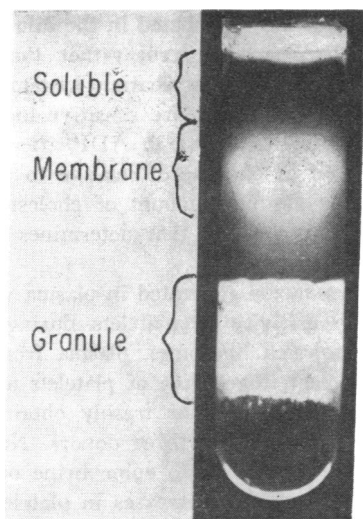


FIGURE 3 Platelet soluble, membrane and granule fractions obtained by discontinuous sucrose density gradient centrifugation of platelet homogenate.

brane fraction of cholesterol-rich platelets had undergone a 55% increase in C/P. In contrast, the granule fraction had gained only 19%. When the granule fraction was arbitrarily separated into upper and lower halves, it was observed that both fractions had gained an equal amount of cholesterol. Less than 15% of the total lipid recovered was present in the soluble fraction either before or after incubation with cholesterol-rich liposomes. Whole platelets gained 25% cholesterol. This is less than the 36–42% gain observed in the platelet function studies (Fig. 1) due to the greater dilution of liposomes by plasma in these subcellular fraction studies (1 vol liposomes; 2.5 vol plasma) than in the platelet function studies (1 vol liposomes; 1 vol plasma).

*The effect of liposomes on platelet aggregation.* Previous workers have commented on the difficulty of maintaining platelet function during incubation at 37°C in vitro (26). Using the system described herein, there was no change in the sensitivity to ADP, collagen, or thrombin in platelets incubated with Tyrode's solution for 5 h. However, there was a significant ( $P < 0.01$ ) decrease in epinephrine sensitivity (from 0.25  $\mu\text{M}$  before incubation to 1.58  $\mu\text{M}$  after incubation for 5 h).

No difference in platelet sensitivity to aggregating agents was observed immediately after the addition of platelet-rich plasma to either cholesterol-normal or cholesterol-rich liposomes. Moreover, platelets incubated with cholesterol-normal liposomes were the same as Tyrode's controls at 5 h. In contrast, platelets incubated with cholesterol-rich liposomes became markedly hypersensitive to epinephrine with time (Fig. 4). The effect occurred in the platelets of all subjects including

TABLE I  
Subcellular Localization of Acquired Cholesterol

	Cholesterol/Phospholipid		Percent increase cholesterol
	Normal platelets	Chol-rich platelets	
	<i>mol/mol</i>		
Whole platelets	0.64* (0.62–0.67)‡	0.80 (0.76–0.83)	25
Membrane	0.47 (0.46–0.48)	0.73 (0.72–0.73)	55
Granule	0.68 (0.66–0.71)	0.81 (0.77–0.85)	19

\* Mean of three experiments.

‡ Range.

those from the donor with type IIa hyperlipoproteinemia. The mean increase in sensitivity was 35-fold as compared with Tyrode's controls, and this was signifi-

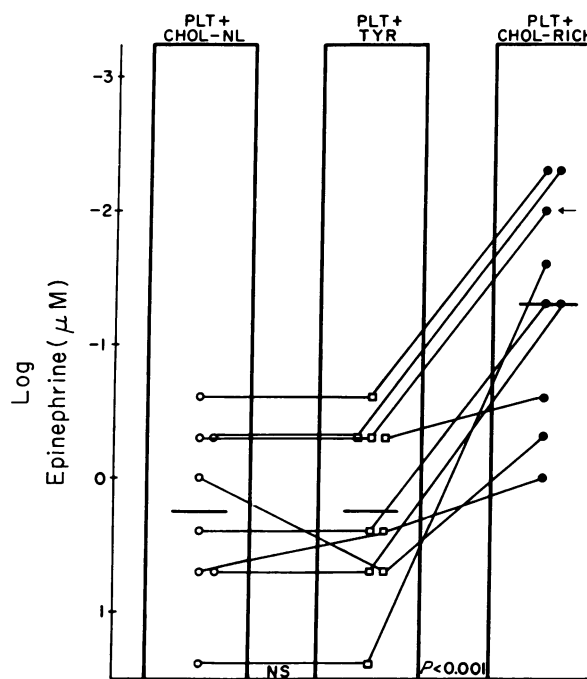


FIGURE 4 Sensitivity of platelets to epinephrine-induced aggregation after 5 h of incubation with either Tyrode's solution, cholesterol-normal, or cholesterol-rich liposomes. Platelet sensitivity is plotted as the log of the epinephrine concentration so that platelet sensitivity increases in proportion to the height along the vertical axis. Heavy horizontal bars indicate the mean sensitivity for experiments in each incubation system. Lighter lines connect the values for a given subject's platelets under the three experimental conditions. The platelets from nine subjects were studied. The response of platelets from the donor with type IIa hyperlipoproteinemia is identified by the arrow.

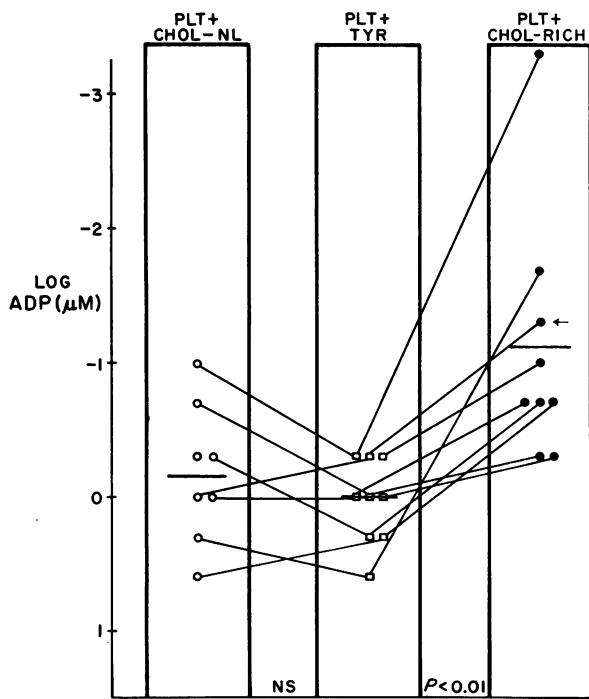


FIGURE 5 Sensitivity of platelets to ADP-induced aggregation after 5 h incubation. Platelets from nine subjects were studied. The response of platelets from the donor with type IIa hyperlipoproteinemia is identified by the arrow.

cant ( $P < 0.001$ ). Because incubated control platelets were less sensitive than unincubated controls, a lesser difference was apparent when epinephrine sensitivity after incubation was compared to control epinephrine sensitivity before incubation. However, this was also significant ( $P < 0.05$ ). Platelets incubated with cholesterol-rich liposomes were also significantly ( $P < 0.01$ ) more sensitive to ADP as compared with platelets before incubation or to platelets incubated with Tyrode's solution alone or with cholesterol-normal liposomes (Fig. 5). However, the mean increase in sensitivity (15-fold) was less striking than that observed with epinephrine. Platelet aggregation with collagen and thrombin was unaffected by incubation under any of these conditions. In contrast, platelets from two subjects incubated with cholesterol-poor liposomes became 18-fold less sensitive to epinephrine ( $P < 0.01$ ) while retaining normal sensitivity to ADP, as compared with platelets incubated with Tyrode's solution alone.

To determine whether it was the lipid composition rather than the absolute cholesterol concentration of cholesterol-rich liposomes that was responsible for the observed platelet hypersensitivity, 1.0 vol of liposomes was diluted with 2.5 vol of Tyrode's solution. Platelets

(3.5 vol) subsequently incubated in the diluted liposome system gained 25% cholesterol rather than the 39% gained in the undiluted incubation system. Nevertheless, they became 20-fold more sensitive to epinephrine and threefold more sensitive to ADP after 5 h. Thus, it is the amount of cholesterol relative to phospholipid rather than the absolute amount of cholesterol present in the incubation medium that determines platelet hypersensitivity.

To exclude a factor generated in plasma which might impart hypersensitivity to platelets during incubation with cholesterol-rich liposomes, plasma from a 5-h incubation was centrifuged free of platelets and 2 vol of this was added to 1 vol of freshly obtained platelet-rich plasma from each of three donors. No change in the sensitivity of platelets to epinephrine or ADP was observed. Thus, striking increases in platelet sensitivity to epinephrine and ADP aggregation were not due to mere incubation of platelet-rich plasma with liposomes, but correlated with the acquisition of cholesterol only by platelets incubated with cholesterol-rich liposomes.

**[ $^{14}$ C]serotonin release.** The sensitivity of platelets to aggregating agents as determined by [ $^{14}$ C]serotonin release was similar to that seen with platelet aggregation. In Fig. 6, the two methods of determining platelet sensitivity are compared, testing platelet sensitivity to epinephrine, ADP, and collagen after incubation for 5 h in either Tyrode's solution, cholesterol-normal or cholesterol-rich liposomes. The two methods correlated extremely well, with a correlation coefficient of 0.96. Thus, whether determined by aggregation or by [ $^{14}$ C]serotonin release, platelets made cholesterol-rich have an increased sensitivity to both epinephrine and ADP.

## DISCUSSION

These studies demonstrate that the cholesterol content and function of platelets are dependent upon the ratio of cholesterol to phospholipid in the platelets' milieu. The acquisition of cholesterol by platelets was associated with an increased sensitivity to aggregation by both epinephrine and ADP, whereas the loss of cholesterol was associated with a decreased sensitivity to epinephrine. This was true whether measured by aggregation or [ $^{14}$ C]serotonin release, two closely related functions (27). In contrast, platelets incubated with liposomes which caused no change in platelet cholesterol or phospholipid content underwent no change in sensitivity to aggregating agents.

The acquisition and loss of cholesterol by platelets in these experiments is similar to that which we have observed when erythrocytes are incubated with cholesterol-rich liposomes (9). Cholesterol in normal platelets is almost entirely unesterified (28). The cholesterol in erythrocyte membranes is entirely unesteri-

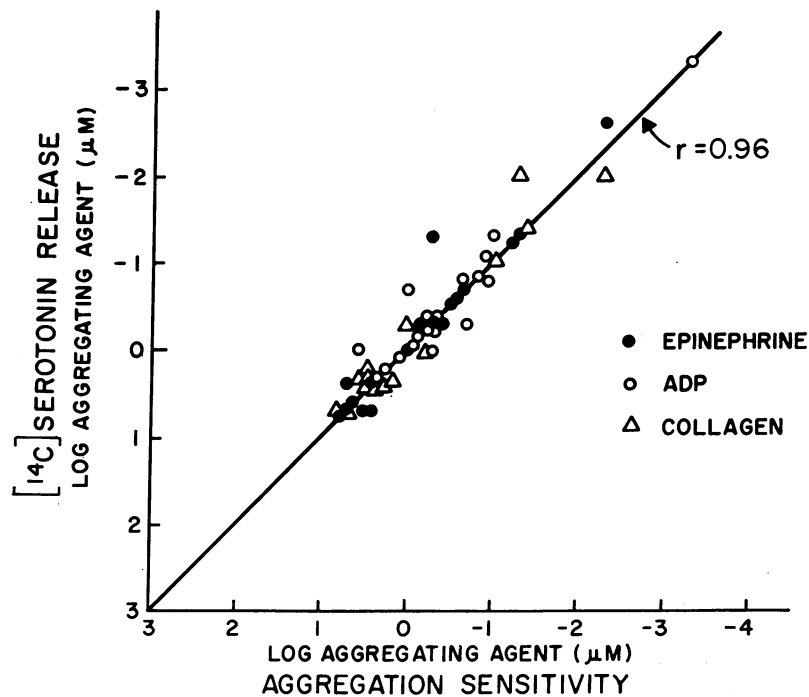


FIGURE 6 Platelet sensitivity at 5 h in all incubation media as determined by aggregating agent-induced release of [<sup>14</sup>C]serotonin compared with platelet sensitivity as determined by aggregometry. Sensitivity is plotted as the log of the concentration of the aggregating agents, epinephrine, ADP, and collagen. Collagen concentration is presented as  $\mu$ l of collagen solution (5.5 mg collagen/ml) added to 0.5 ml of platelet incubation mixture.

fied and exists in exchange equilibrium with the unesterified cholesterol of plasma lipoproteins (29) or cholesterol-phospholipid liposomes (9). Erythrocyte membranes incubated with cholesterol-normal liposomes exchange cholesterol with these liposomes with a half-equilibration time of approximately 2 h. However, as in the present study, no bulk transfer of cholesterol occurs. However, over the course of 20 h, erythrocytes double their cholesterol content when incubated with cholesterol-rich liposomes and lose more than 50% with cholesterol-poor liposomes. Equilibrium exchange and not platelet ingestion of liposomes also appears to be responsible for the cholesterol acquired by platelets in the present studies. First, cholesterol-normal liposomes did not alter either the cholesterol or phospholipid content of platelets. Secondly, the acquisition of cholesterol from cholesterol-rich liposomes was selective, and there was no change in platelet phospholipid content. Finally, the rate and extent of cholesterol acquisition was similar to that seen with erythrocytes at 5 h.

The largest percent increase in cholesterol during incubation of platelets with cholesterol-rich liposomes was observed in the platelet membrane (55%) rather than the granule fraction (19%). Indeed, it is uncertain whether there was any change in granule lipids,

since the granule fraction may be contaminated with some membrane material. Zilversmit has demonstrated the exchange of lipid between the plasma membrane and internal membranes (30) and this might be anticipated in platelets. However, the time-course of granule lipid acquisition would be slower, dependent not only on the exchange between liposomes and surface membrane, but also on a second exchange between surface membrane and organelle membrane.

Cholesterol appears normally to maintain the membrane lipid bilayer in an "intermediate fluid condition," permitting greater fluidity of phospholipid fatty acid hydrocarbon chains in the gel phase while restricting molecular motion in the liquid crystal phase (31). However, abnormally large amounts of membrane cholesterol apparently restrict the motion of fatty acid hydrocarbon chains nearest the membrane surface, whereas motion is increased in membranes made cholesterol poor. This has been observed in both erythrocytes (32) and lymphocytes (33) made cholesterol rich and cholesterol poor, and it would be anticipated in platelets as well.

It has been reported that ADP-induced alterations in platelet electrophoretic mobility occur at lower concentrations of ADP in patients with familial hypercholesterolemia than in normal controls (34, 35). Plasma

lysolecithin was incriminated as a causal factor. Although small amounts of lysolecithin might have resulted from the action of the enzyme lecithin-cholesterol acyl transferase in our incubation system, it would appear not to explain our results, since plasma previously incubated with cholesterol-rich liposomes did not induce platelet hypersensitivity when added to freshly obtained platelets.

Although it appears that the functional changes observed in the present studies relate directly to platelet cholesterol content, the mechanism of cholesterol action is unknown. The platelet functions examined, second wave aggregation and [<sup>14</sup>C]serotonin release, are late events in the sequence of reactions of platelets to stimuli (27), and cholesterol might exert its effect at any point in the sequence. However, the localization of acquired cholesterol predominantly to platelet membranes and the known effect of cholesterol on membrane function (9) suggest platelet membranes as a likely site for cholesterol action. Since the platelet "membrane fraction" apparently consists of both surface and internalized membranes (36), these studies do not permit more precise localization in this regard.

The functional abnormalities of platelets made cholesterol rich are similar to those observed in type IIa hyperlipoproteinemia (6). The sensitivity of platelets from the single type IIa donor in the present study increased further after incubation with cholesterol-rich liposomes. When compared to preincubation, these platelets gained 40% cholesterol after 5 h and became 25-fold more sensitive to epinephrine and 10-fold more sensitive to ADP. We have demonstrated recently that hypersensitive IIa platelets exhibit a C/P 8% greater ( $P < 0.01$ ) than normal platelets (37). In addition, an elevated free cholesterol content in IIa platelets has been suggested by others (38). Therefore, the changes in platelet lipid composition and function which occur in the in vitro, controlled environment described herein may serve as a useful model to study the mechanisms of platelet hypersensitivity in type IIa hyperlipoproteinemia.

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