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In Vitro Studies on the Distribution of Probucol Among Human Plasma Lipoproteins

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SUMMARY

The role of human plasma lipoproteins as carriers in the blood transport of the cholesterol-lowering and water-insoluble drug, probucol, was investigated in *in vitro* studies. [^{14}C]Probucol was incubated in whole human blood, a serum pool, individual diluted sera, and isolated protein and lipoprotein fractions. In whole blood, about 90% partitioned in plasma. Following ultracentrifugal fractionation of the serum, it was found that less than 5% distributed in the d > 1.20 protein fraction (albumin-rich fraction) and more than 95% in the lipoprotein fractions. The distribution of probucol in the lipoprotein fractions correlated with the lipoprotein total lipid volume under saturation conditions (incubation of isolated lipoprotein fractions) as well as nonsaturation conditions (fractionation of serum exposed to [^{14}C]probucol). Incubation of the albumin-rich fraction and of apolipoproteins originating from the isolated lipoprotein fractions showed that they account for a negligible part in the interaction of probucol with blood components. The probucol uptake of individual sera was shown to be correlated to the lipid content of the serum. When probucol was incubated in erythrocyte suspensions containing variable amounts of lipoproteins, probucol partitioned less in erythrocytes as the lipoprotein concentration increased in the suspension.

INTRODUCTION

Recent attention has been focused on the interaction of exogenous compounds with plasma lipoproteins. Most of the studies on this subject have indicated that the interaction between lipoproteins and a variety of lipophilic compounds involves a partitioning phenomenon (1). These lipophilic substances are thought to be dissolved in the hydrophobic core of lipoproteins. Accordingly, recent studies have shown that a series of benzo[*a*]pyrene derivatives interacts with plasma lipoproteins in such a way that the amount of bound ligand correlates with the lipoprotein total lipid volume (2). However, additional factors may play a role in lipoprotein-ligand interactions. For example, the long-chain polyisoprenoid alcohol, dolichol, and 3-hydroxybenzo[*a*]pyrene have been shown to react primarily with the HDL[2] fractions, and to a lesser extent with the LDL and VLDL fractions (2, 3).

The aim of the present study was to examine the interactions of the cholesterol-lowering drug, probucol (Fig. 1), with plasma lipoproteins and apolipoproteins. Earlier studies have indicated that the major part of this hydrophobic drug is transported by lipoproteins in blood (4). We present here data, obtained *in vitro*, on the distribution of probucol among human plasma proteins and lipoproteins and on the maximal uptake of probucol by isolated lipoproteins and proteins.

MATERIALS AND METHODS

Chemical. [^{14}C]Probucol (7.87 mCi/mmol) was provided by Dow Chemical USA (Indianapolis, Ind.). The purity was assessed by thin-layer chromatography on silica gel in CCl_4 and was greater than 99%.

Lipoprotein preparation. Blood (collected into vacuum glass tubes) was obtained from hyperlipidemic patients. Samples were allowed to clot for several hours and were then centrifuged to yield serum. Plasma lipoproteins were isolated by sequential ultracentrifugal flotation of pooled serum at increasing density as previously described (5). Isolated fractions were dialyzed overnight before use against 0.15 M NaCl/2 mM Tris-HCl/0.3 mM EDTA, pH 7.4. The lipoprotein concentration was determined by summing chemical composition data obtained by standard methods: protein (6), phospholipid (7), cholesterol (8), and triglyc-

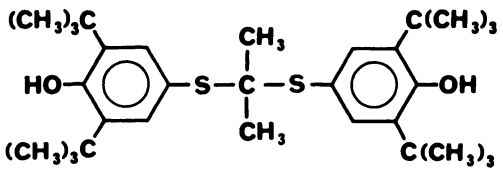

FIG. 1. *Chemical structure of probucol*

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[2] The abbreviations used are: HDL, high-density lipoproteins; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins; d, density.

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erides (9). Values for lipoprotein molecular weight and total lipoprotein volume were obtained from ref. 10. The lipid lipoprotein volume was estimated by subtracting the volume occupied by protein (molecular weight × percent protein × partial specific volume of protein) from the total lipoprotein volume. Partial specific volume of apolipoprotein was assumed to be 0.738 ml/g (11).

Preparation of apolipoproteins. The isolated lipoprotein fractions were delipidated using ethanol/diethylether (3:1, v/v) and diethyl ether at 4° as previously described (5).[3] HDL apoproteins were solubilized in 0.15 M NaCl/2 mM Tris-HCl, pH 7.4. LDL and VLDL apoproteins were solubilized in 8 M urea/0.15 M NaCl.

Uptake studies. Incorporation of high amounts of probucol in isolated lipoproteins was achieved by incubating the samples with probucol immobilized on glass beads, as previously described for benzo[*a*]pyrene (2), with some modifications. Briefly stated, appropriate volumes of [^{14}C]probucol stock solution in benzene were pipetted into glass vials, 240 glass beads were introduced (3 mm diameter), and sufficient chloroform/benzene mixture (4:1, v/v) was added to submerge the glass beads. The solvent was then eliminated under reduced pressure at 55° with slow rotation of the vials to ensure even coating of the beads. Since the amount of probucol that can be immobilized on glass beads is physically limited,[4] isolated lipoproteins were diluted to 0.1 g/liter with the dialysis buffer and sera to 5% with 1 M NaCl/2 mM Tris-HCl/0.3 mM EDTA, pH 7.4. Incubations were conducted with gentle shaking in a Brunswick water bath at 37°. Control incubations, consisting of the corresponding buffer used, were included in the experiment. Net uptake is the amount taken up by the samples minus that taken up by the control incubation. Net uptake data are used in all calculations. Sodium azide (0.02%) was added to retard bacterial growth.

Effect of lipoprotein concentration on probucol distribution between erythrocytes and lipoproteins. Lipoproteins were removed from the serum pool as previously described (12), and the total lipoprotein fraction was dialyzed against the NaCl/Tris-HCl/EDTA buffer. Washed erythrocytes were then resuspended in the buffer containing variable levels of the total lipoprotein fraction (from 0 to 100%) to a final hematocrit of 44%. The samples were then incubated for 1 hr at 37° with trace amounts of [^{14}C]probucol, and the probucol distribution between erythrocytes and the medium was determinated using counting methods and calculations described earlier (13).

Estimation of maximal uptake values. A plot of the uptake (C) versus time (t) showed typical saturation kinetics. The uptake data were subsequently ascribed to an exponential model:

$$C = C_{max} \cdot (1 - e^{-kt}) \quad (1)$$

where C_{max} is the concentration at plateau and k is a time constant. The estimation of C_{max} was then performed with an iterative nonlinear program using a Gauss-Newton algorithm.

RESULTS

Distribution of probucol in blood and serum. Whole blood samples and the serum pool were incubated for 1 hr at 37° with trace amounts of [^{14}C]probucol coated on glass vials. In blood samples, 88–91% of [^{14}C]probucol distributed in plasma and 9–12% in erythrocytes (hematocrit ranging between 37% and 42%, $n = 5$). The serum pool was separated into the different density fractions, and more than 96% of the drug appeared in the lipoprotein fractions (d < 1.20), whereas less than 4% was found in the protein-rich d > 1.20 fraction. The probucol distribution is summarized in Table 1. Obviously, the major part of the drug was distributed in VLDL and LDL. When the data were expressed as the drug to lipoprotein molar ratio,[5] we found that VLDL took up about 10 times more probucol than did LDL, and LDL took up about 20 times more probucol than did HDL. Interestingly, the ratio of the molar uptake to the lipoprotein total lipid volume was not different among the lipoprotein fractions.

Transfer of probucol among lipoprotein and d > 1.20 fractions. Transfer of probucol among the lipoprotein and d > 1.20 fractions was studied in order to assess the reversibility of the binding. In separate experiments, each isolated fraction containing [^{14}C]probucol was incubated overnight at 37° in the presence of the three other fractions not previously exposed to [^{14}C]probucol. The fractions were then reisolated to determine the distribution of probucol in each fraction. The results are summarized in Table 2. The distribution of probucol between the isolated fractions in the four experiments is roughly superimposable on the distribution earlier observed (Table 1), regardless of the [^{14}C]probucol source. Thus, probucol can transfer among lipoprotein and d > 1.20 fractions.

Molar uptake of probucol by individual lipoprotein fractions and proteins. The isolated lipoprotein and d > 1.20 fractions and the apolipoproteins originating from each pure lipoprotein fraction were separately incubated with [^{14}C]probucol immobilized on glass beads. During the course of the incubation, aliquots were removed for counting. Uptake versus time plots are depicted in Figs. 2 and 3. These plots show that typical saturation kinetics occurred in all cases. The results are summarized in Table 3 for lipoprotein fractions. Interestingly, the ratios between the molar uptake of the isolated lipoprotein fractions are in the same range as those previously observed for the lipoprotein fractions isolated from serum exposed to [^{14}C]probucol (see Table 1). Moreover, the uptake remained constant when expressed as molar uptake per lipoprotein total lipid volume.

Figure 3 shows the uptake of probucol by the d > 1.20 fraction and the apolipoproteins. The uptake is far lower than that of the lipoprotein fractions. The uptake by 0.1 g/liter VLDL apolipoproteins (5.62 ± 0.02 μM) was a little more than twice the uptake by the d > 1.20 fraction (2.21 ± 0.11 μM) at 1 g/liter. Evidently, the uptake by apolipoproteins is higher than that by the d > 1.20 fraction, and the uptake by the apolipoproteins of the LDL fraction is the highest.

Saturation levels of probucol in sera. Fourteen individual sera, diluted to 5%, were incubated with [^{14}C]probucol. The means, standard deviations, and ranges of the variables measured in the sera are presented in Table 4. The concentration of probucol in the samples increased with time, and reached a plateau after about 23 hr of incubation. The data are depicted in Fig. 4, which illustrates the linear relationship found between saturation levels of probucol in the samples and the corresponding

[3] The apoprotein of LDL contained 2.9% phospholipid by weight. Less than 0.5% of the delipidated proteins of HDL and VLDL were phospholipids. No other lipids could be detected.

[4] The tubes contained 101 nmol of probucol and 4 ml of macromolecule or buffer solution at the beginning of each incubation.

[5] Drug to lipoprotein molar ratio (molar uptake) is obtained by dividing the probucol concentration by the lipoprotein concentration in each isolated fraction.

TABLE 1

Distribution of probucol in serum

Sera were pooled from 15 hyperlipidemic patients and incubated for 1 hr at 37° with [^{14}C]probucol immobilized on glass beads. The pooled serum was then fractionated into individual lipoprotein fractions by sequential ultracentrifugation, and the percentage of distribution of [^{14}C] probucol in each fraction, relative to the total amount recovered, was obtained. Lipoprotein fractions obtained from serum not exposed to probucol were used in determining lipoprotein concentration.

Fraction	Macromolecule concentration		Probucol distribution (%, on a wt. basis)	Molar uptake[a]	Molar uptake/ lipoprotein lipid volume[b]
	μM	g/liter			
VLDL	0.116	2.28	38.2	112	5.9
LDL	1.50	3.45	44.4	10	5.4
HDL	9.44	1.70	13.2	0.5	5.7
d > 1.20		74	3.5	—	—

[a] Molar uptake data were obtained by dividing the probucol concentration by the lipoprotein concentration in each isolated fraction.

[b] The molar uptake per lipoprotein lipid volume (in micromoles per milliliter) is the ratio of the molar uptake to the lipoprotein total lipid volume.

TABLE 2

Transfer of probucol among lipoprotein and d > 1.20 fractions

Each labeled fraction originating from the serum exposed to [^{14}C] probucol was incubated with the three other fractions not previously exposed to [^{14}C]probucol. Concentrations of the lipoproteins in the final incubation mixture were adjusted to about 80% of the starting serum. Following incubation for 16 hr at 37°, the lipoproteins were reisolated.

Probucol distribution (%)							
Before incubation				After incubation			
VLDL	LDL	HDL	d > 1.20	VLDL	LDL	HDL	d > 1.20
100	0	0	0	37	49	12	2
0	100	0	0	44	35	17	4
0	0	100	0	31	46	14	4
0	0	0	100	30	46	20	4

serum lipid concentrations. Adjusting the uptake to 100% serum showed that serum lipoproteins could take up 158 ± 40 μM (mean ± SD), corresponding to 82 ± 20 μg/ml, regardless of the uptake by other serum proteins.

Effect of lipoprotein concentration on probucol distribution between erythrocytes and lipoproteins. The results depicted in Fig. 5 show that, as the lipoprotein concentration increased in the medium, the probucol uptake by erythrocytes diminished. Interestingly, Fig. 5 shows that mean physiological plasma lipid levels (40–50% of the total lipoprotein concentration) correspond to 16–10% of probucol in erythrocytes, the remainder being in the medium containing the lipoproteins. This distribution pattern is roughly superimposable on that observed in whole blood (9–12% in erythrocytes and 88–91% in plasma).

DISCUSSION

Our *in vitro* studies demonstrate that plasma lipoproteins serve to transport the water-insoluble drug, probucol, in plasma. The major lipoprotein fractions involved are VLDL and LDL, which bind more than 80% of the total probucol in plasma. The observation that the molar uptake is positively correlated to the lipoprotein particle size, i.e., to the lipid content of the particle, strongly suggests that lipid solubility is an important factor in such interactions. Such a correlation between lipoprotein

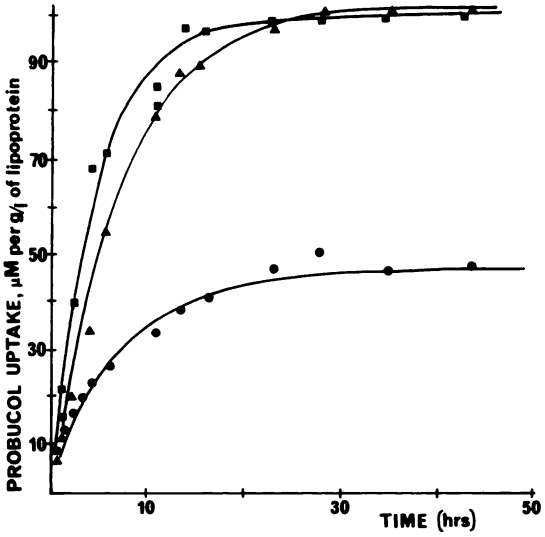

FIG. 2. *Uptake of probucol by isolated lipoproteins*

Isolated lipoproteins were separately incubated with probucol at 37° under saturation conditions as described under Materials and Methods. The lipoprotein concentrations were adjusted to 0.1 g/liter in 0.15 M NaCl/0.3 mM EDTA, pH 7.4. The control incubation was performed with the NaCl/EDTA solution. Uptake of probucol as a function of time was assessed in duplicate aliquots removed during the incubation, and the curves were drawn according to Eq. 1. The uptake of each lipoprotein fraction is the probucol concentration in the fraction minus that in the control incubation medium (1.19 ± 0.04 μM). ■, VLDL; ▲, LDL; ●, HDL.

binding capacity and particle size has also been observed for some *beta*-adrenergic receptor-blocking drugs (14, 15), antiarrhythmic drugs (16), and for some thienopyridinic derivatives (5).

As previously reported for benzo[*a*]pyrene (2, 17), we found a correlation between saturating and unsaturating levels of probucol and the lipoprotein total-lipid volumes of VLDL, LDL, and HDL, suggesting that probucol is essentially dissolved in the lipid core of lipoprotein. In the study by Shu and Nichols (17), it was clearly shown that the distribution of benzo[*a*]pyrene among the lipoprotein fractions was independent of the final plasma concentration of benzo[*a*]pyrene taken up, including saturated plasma. The uptake of probucol by apolipopro-

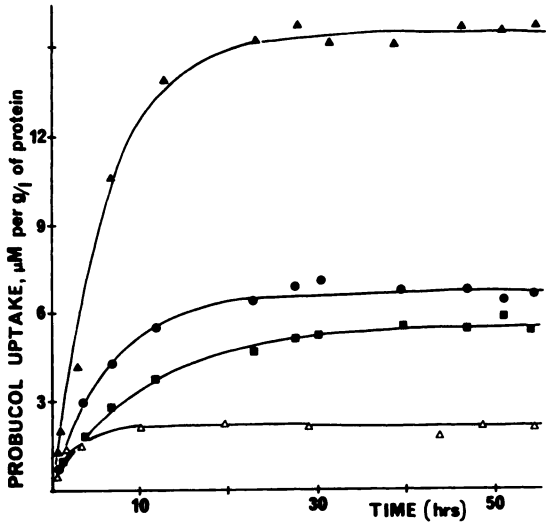

FIG. 3. *Uptake of probucol by apolipoproteins and d > 1.20 fractions*
Apolipoproteins of VLDL (■), LDL (▲), HDL (●), and the d > 1.20 fraction (△). All of the protein concentrations were 1 g/liter and dissolved in an NaCl/EDTA solution except the LDL and VLDL apoproteins, which were dissolved in 8 M urea/2 mM Tris-HCl, pH 7.4. The control incubations were performed with the corresponding buffer solutions. The uptake of each protein fraction is the probucol concentration in the fraction minus that in the corresponding control incubation (net uptake). Other details as in Fig. 1.

TABLE 3

Molar uptake of probucol by isolated plasma lipoproteins

The isolated lipopoteins were separately incubated with [^{14}C]probucol under saturation conditions as described under Materials and Methods. Molar uptake data are given to the nearest integer. Each lipoprotein concentration was 0.1 g/liter. Standard deviations are shown in parentheses. Other details as in Fig. 2.

Fraction	Concentration	Molar uptake	Molar uptake/ lipoprotein lipid volume
	μM		
VLDL	0.005	1980 (86)	104 (5)
LDL	0.043	233 (14)	125 (8)
HDL	0.555	8.5 (0.5)	102 (6)

TABLE 4

Measured variables in the plasma used for probucol uptake studies

The plasma originated from 14 drug-free volunteers. Blood samples were drawn after an overnight fast into glass collecting tubes. Lipid and protein concentrations were measured by standard methods as described under Materials and Methods. Values are means ± standard deviation; ranges are shown in parentheses.

Phospholipid	Cholesterol	Triglycerides	Protein
mM	*mM*	*mM*	*g/liter*
3.43 ± 0.72 (2.20–4.10)	5.48 ± 1.50 (2.35–7.91)	1.55 ± 1.11 (0.45–4.70)	74 ± 4 (66–80)

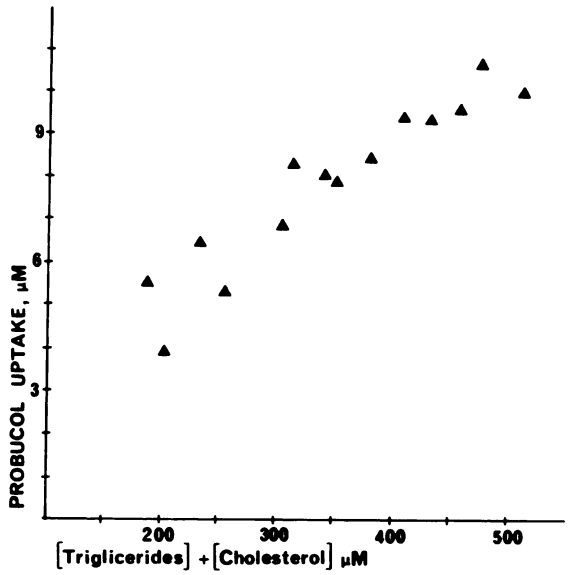

FIG. 4. *Probucol serum uptake and serum lipid concentration*
The sera of 14 subjects were diluted to 5% with 1 M NaCl/2 mM Tris-HCl/0.3 mM EDTA, pH 7.4, and were separately incubated with [^{14}C]probucol at 37° under saturation conditions as described under Materials and Methods. The control incubation was performed with the diluting solution containing the d > 1.20 fraction (3.7 g/liter), which corresponded to the mean serum protein concentration. The ordinate intercept was not found significant for the uptake.

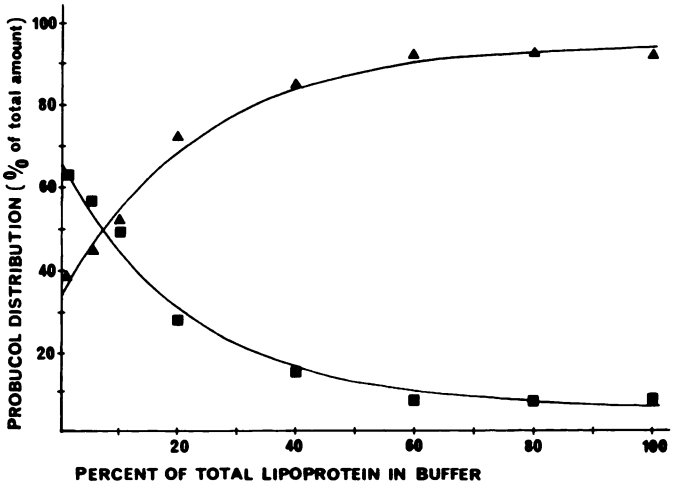

FIG. 5. *Influence of lipoprotein concentration on the distribution of probucol in erythrocytes*
Washed erythrocytes were resuspended (hematocrit = 44%) in a buffer containing 0–100% of the total lipoprotein fraction of a serum pool and incubated with trace amounts of [^{14}C]probucol at 37° for 1 hr. Cholesterol and triglyceride concentrations in the 100% sample were 11 mM and 1.7 mM, respectively. ■, Fractional amount of probucol in erythrocytes; ▲, in the buffer containing lipoproteins. Data were fitted to a monoexponential model.

teins was studied using weight concentration of apolipoproteins 10 times higher (1 g/liter) than that used for lipoproteins (0.1 g/liter), since using 0.1 g of apolipoprotein per liter in the incubation studies did not result in a sufficiently high uptake relative to the control incubation, and thus could not give reliable data for calculation of the maximal uptake. In preliminary experiments, we found that the uptake by the lipoprotein fractions was roughly proportional to the lipoprotein concentration if the total amount of probucol immobilized on the glass beads was not totally taken up at the end of the incubation (saturating conditions). Thus, to compare the uptakes of lipoproteins and apolipoproteins, we normalized the lipoprotein uptake data to 1 g/liter. Accordingly, the uptake of probucol by apolipoproteins was far lower than that of isolated lipoproteins, considering the relative

concentrations used. Consequently, the protein moiety of lipoprotein is suggested to play a minor role in the uptake of probucol by lipoproteins.

The transport of probucol by plasma lipoproteins *in vivo* has been examined in humans and in laboratory animals. Eder (4)[6] showed that probucol was essentially transported by LDL in the rhesus monkey and that the distribution of probucol between the different lipoprotein classes corresponded to the amount of lipid in each lipoprotein fraction. In man, following oral administration of probucol with a high fat meal, the majority of the drug was in VLDL at the peak of hyperlipemia, whereas, under fasting conditions, 66% of the drug was in LDL and 20% in VLDL. Thus, following oral administration, probucol is transported by chylomicrons and VLDL via the lymphatics and reaches the circulation, where it can be transferred to the different lipoprotein classes; these findings are consistent with our *in vitro* observations. The distribution of probucol among the lipoprotein classes depends on the relative magnitudes of the rates of probucol transfer among lipoproteins and the rates of turnover of these lipoproteins.

The significance, from a physiological standpoint, of the saturation kinetic studies is not evident. Since equilibrium is attained very slowly, it may be assumed that the rates of association-dissociation of the probucol-lipoprotein complex are relatively weak. So, once probucol has entered the circulation from the gut, the free form in plasma water quickly distributes in the tissues when equilibrium must be reached very rapidly. However, the amount of probucol that can enter the circulation is rapidly limited because of its low solubility in water (1.19 ± 0.04 μM). By contrast, probucol associated with digestive lipid micelles—that is, the major part of resorbed probucol—will enter the circulation via the lymphatics and reach the blood circulation in the form of a probucol-lipoprotein complex. Accordingly, *in vivo* studies in humans showed that absorption of probucol with a fat meal increased to about 10 times the plasma levels of probucol (18). Subsequently, because of its low rate of dissociation, the probucol-lipoprotein complex is not expected to dissociate rapidly and generate probucol in the free state. In this case, there is a plasma retention of the drug by lipoproteins.

To examine further the importance of lipoprotein in the transport of probucol in human plasma, we measured the probucol uptake of different individual sera according to their cholesterol and triglyceride concentrations. An increase in the serum lipid concentration resulted in a corresponding linear increase in the serum capacity for probucol. In subjects treated with probucol, the probucol plasma concentrations ranged from 6.8 to 70.4 μg/ml after 8 months of treatment (18). We suggest that the wide interindividual differences observed may be due, in part, to differences in plasma lipoprotein composition.

Since probucol is transported in plasma by lipoproteins, they may be considered as vehicles for the delivery of this drug to the tissues (Fig. 6). Probucol may enter

[6] A preliminary account of this work was presented at the Seventh International Symposium on Drugs Affecting Lipid Metabolism, Milan, May 28–31, 1981 (abstract no. RT 3).

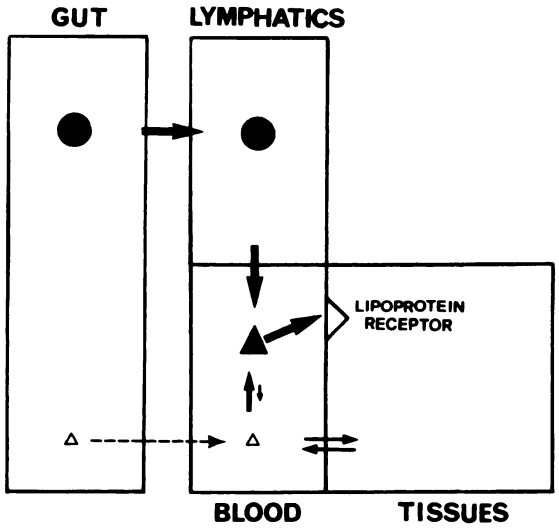

FIG. 6. *Possible mechanisms of probucol distribution within the body* △, Probucol free in water; ●, probucol-lipid micelle complex; ▲, probucol-lipoprotein complex.

the cells by an LDL receptor-mediated process or by partitioning between the lipid phase of cell membranes and the plasma lipoproteins. Such a partitioning is consistent with the observation that probucol may distribute in erythrocytes in whole blood. Moreover, the relative amount that can distribute in erythrocytes is dependent on the lipoprotein concentration. As the lipoprotein concentration increases in the medium, the fractional amount of probucol that distributes in erythrocytes diminishes and reaches an asymptote at about 5%, indicating that the access of probucol to the target tissues or to the excretion sites is likely to be influenced by the close interactions of probucol with plasma lipoproteins. The extent to which probucol turnover may be linked to the turnover of some plasma lipoprotein fraction remains to be investigated.

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