Extraction of cholesterol from human serum lipoprotein films

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SUMMARY Serum high- and low-density lipoproteins were spread between heptane and phosphate buffer. The cholesterol from both protein films became completely soluble in the heptane phase. Other neutral lipids were also found in the heptane, but phospholipids could not be detected. Shaking of whole serum or isolated lipoproteins with heptane resulted in only slight extraction of cholesterol by the fat solvent. The addition of albumin to the lipoproteins reduced the amount of cholesterol extractable by heptane. It is postulated that when aqueous solutions of lipoproteins are shaken with nonpolar fat solvents the degree of cholesterol extraction depends on the disruption of the lipoprotein structure through contact with various interfaces, and that other proteins may protect the lipoprotein by competing for position at the interface.

FOR THE QUANTITATIVE removal of lipids from serum lipoproteins rather polar fat solvents are required. In the classical procedure of Bloor (1), for example, a mixture of ethanol and diethyl ether is employed, whereas in the newer method of Folch et al. (2), a mixture of chloroform and methanol is used. Although ethyl ether and chloroform are excellent lipid solvents, they will not extract all the lipids from serum when the latter is briefly shaken with these solvents. The addition of an alcohol converts the liquids from a biphasic to a monophasic system and facilitates the extraction. Yet this conversion is probably not the only reason why the extraction of lipids is improved. Macheboeuf (3) found, for example, that the addition of even a few per cent of alcohol to the ether promotes the extraction of neutral lipids from serum. He also found that the addition of surface-active agents such as soap, saponin, or zephirol (benzalkonium chloride) promotes extraction of lipids by relatively nonpolar solvents. These experiments have been extended by Tayeau (4) and Ayrault-Jarrier et al. (5).

The extraction of lipoproteins by relatively nonpolar solvents has received renewed interest as a method of preparing delipidated lipoproteins without denaturation of the residual protein. Avigan (6) showed that low density lipoprotein, rotated for 16 hr at 4° with ethyl ether, loses most of the cholesterol but little or no phospholipid. Grundy et al. (7) claim to have removed by a similar procedure all the cholesterol and half the phospholipid. Oncley et al. (8) froze human serum β-lipoprotein at −25° and then extracted with ethyl ether. They found that three-fourths of the cholesterol appeared in the ether phase. McFarlane (9), who had originally observed the increased extractability of lipids after freezing human serum, concluded that the structure of the lipoprotein was disrupted by removal of its bound water by freezing.

Our own interest in the extractability of lipids began with the observation that human low density lipoprotein films spread between an aqueous buffer and heptane lost nearly all their cholesterol to the heptane phase. This stands in marked contrast to the small amounts of cholesterol extracted when lipoproteins in bulk are shaken with heptane. The results of these studies are reported here.

METHODS AND MATERIALS

Lipoprotein fractions from pooled human serum samples[1] were prepared by a modified method of Bragdon et al. (10). Chylomicrons were removed by centrifugation in a No. 40 rotor of the Spinco preparative ultracentrifuge for 30 min at 12,000 rpm. Sufficient aqueous

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[1] Serum samples varied in age from 1 to 8 days after drawing of blood.

NaCl, sometimes with NaBr, was added to give a density of 1.063 of the protein-free filtrate, and low density lipoproteins (LDL) were separated at 100,000 × *g* for 24 hr at 10–15°. To some serum samples 0.03% EDTA (disodium salt of ethylenediaminetetraacetic acid neutralized to pH 7.4) was added. The LDL layer was removed by means of a tube slicer and dialyzed[2] successively against 0.8% NaCl, 0.05% EDTA, 0.05% NaF adjusted to pH 7, and phosphate–NaCl buffer of pH 7.4. In later experiments EDTA and NaF were omitted. The centrifuge tube was sliced again and the center fraction was discarded. The bottom fraction was adjusted with solid KBr to a density of 1.21 and the HDL were isolated by centrifuging for 40–48 hr. The HDL layer was dialyzed against 0.9% NaCl and then against phosphate–NaCl buffer pH 7.4. Another preparation of lipoproteins (DPL) was made by precipitation with dextran sulfate and $CaCl_2$: Dextrarine[3] was added to serum (0.04 ml/ml) and then 11% $CaCl_2$ (0.1 ml/ml). After refrigeration for 2 hr the sample was centrifuged and the supernatant solution decanted. The precipitate was redissolved in 0.9% NaCl and potassium oxalate (11).

Lipoprotein fractions were spread between heptane and a phosphate buffer in a glass or silica trough 62 × 15 × 5.5 cm. The glass trough was made from plate glass cemented with Araldite;[4] the silica trough was purchased from American Thermal Fused Quartz Co., Montville, N. J. The heptane was purified by distillation. The phosphate buffer (ionic strength 0.15) contained 0.0157 M Na_2HPO_4, 0.0029 M KH_2PO_4, 0.1 M NaCl and had a pH of 7.4 when made with distilled water not previously freed of CO_2. The spreading of lipoproteins was accomplished by a technique commonly used to produce monomolecular films. An Agla micrometer syringe was mounted so that the bent needle approached the heptane–water interface from the underside, and so that the bevelled portion of the needle lifted the interface slightly (12). Attempts to use the spreading

[2] Dialysis tubing was boiled with distilled water three times in order to remove surface active materials.

[3] Dextrarine (l'Equilibre Biologique S. A., Commentry, Allier, France) was diluted 1:1 with saline to contain 5% dextran sulfate.

[4] Araldite type one is a one-component epoxy-based resin supplied in stick form.

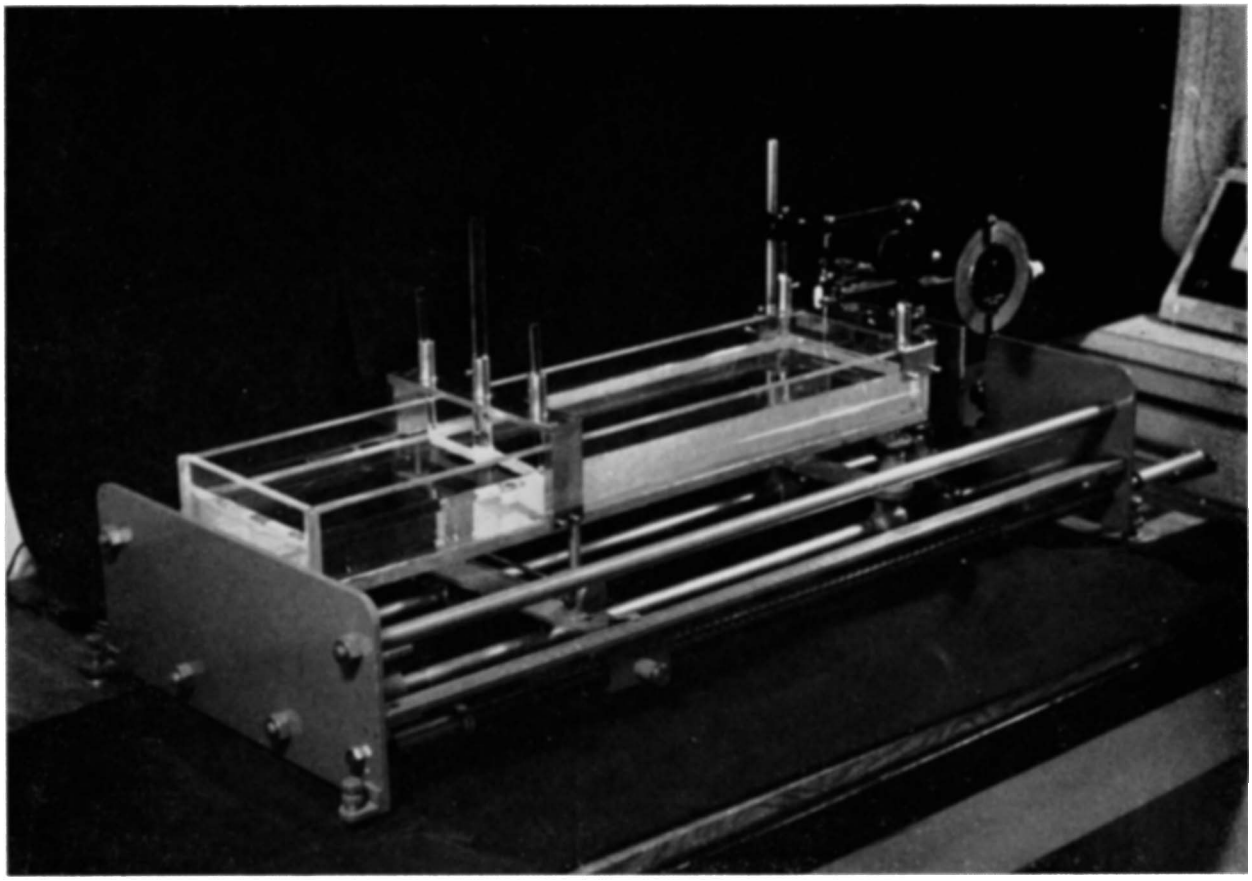

FIG. 1. Glass trough and Teflon band. Movable bridge with center rod positioned about one-third from left end of trough. On the right-hand side a du Noüy tensiometer is shown. The tensiometer was used only occasionally to check film pressures.

technique of Trurnit (13), developed for the air-water interface, were not successful.

The equipment used for spreading is shown in Figs. 1 and 2. It consists essentially of a trough with two metal bridges. The bridge at the right-hand side holds two glass rods vertically, close to each corner. The other bridge holds three glass rods and is able to slide over the edge of the trough. A Teflon band 165 × 3.5 cm is stretched around four of the glass rods, as shown in Fig. 2, and the two ends are slipped into a groove in the center glass rod of the movable bridge. This rod is then turned, whereby the slack of the Teflon band is taken up. A glass collar between the bridge and the Teflon band and a flared bottom end of the glass rod prevent the band from slipping up or down when the rod is turned. A space of 3–4 mm between the Teflon band and the bottom of the trough allows the aqueous phase to pass below the band, while the heptane flows over it as the band itself is rolled on the central glass rod. The use of this equipment for the determination of film pressures at the heptane–water interface has been previously described (14). In the present series of experiments it was important to prevent contaminating the heptane with cholesterol. Therefore, persons operating the equipment wore rubber gloves as well as surgical masks and caps.

In a preliminary experiment the amount of lipoprotein that could be spread at any one time with minimal losses to the aqueous phase was determined. In a small trough phosphate buffer and heptane were introduced while the interface was cleaned by suction with a capillary. The interfacial tension was measured with a du Noüy tensiometer while small quantities of lipoprotein were injected into the interface. This was continued until further injection of lipoprotein did not produce much additional lowering of the interfacial tension, which occurred at a film pressure of about 15 dynes/cm. Since at the higher film pressures additional lipoprotein may fail to spread, these preliminary data were used to determine how much lipoprotein could be spread in the large trough to reach film pressures of no more than one-half the maximum and usually much less. However, we had to inject sufficient material to allow duplicate analyses of cholesterol in the heptane phase to be carried out. To achieve this we injected slowly the calculated quantity of lipoprotein with an Agla syringe. After a 2 min wait

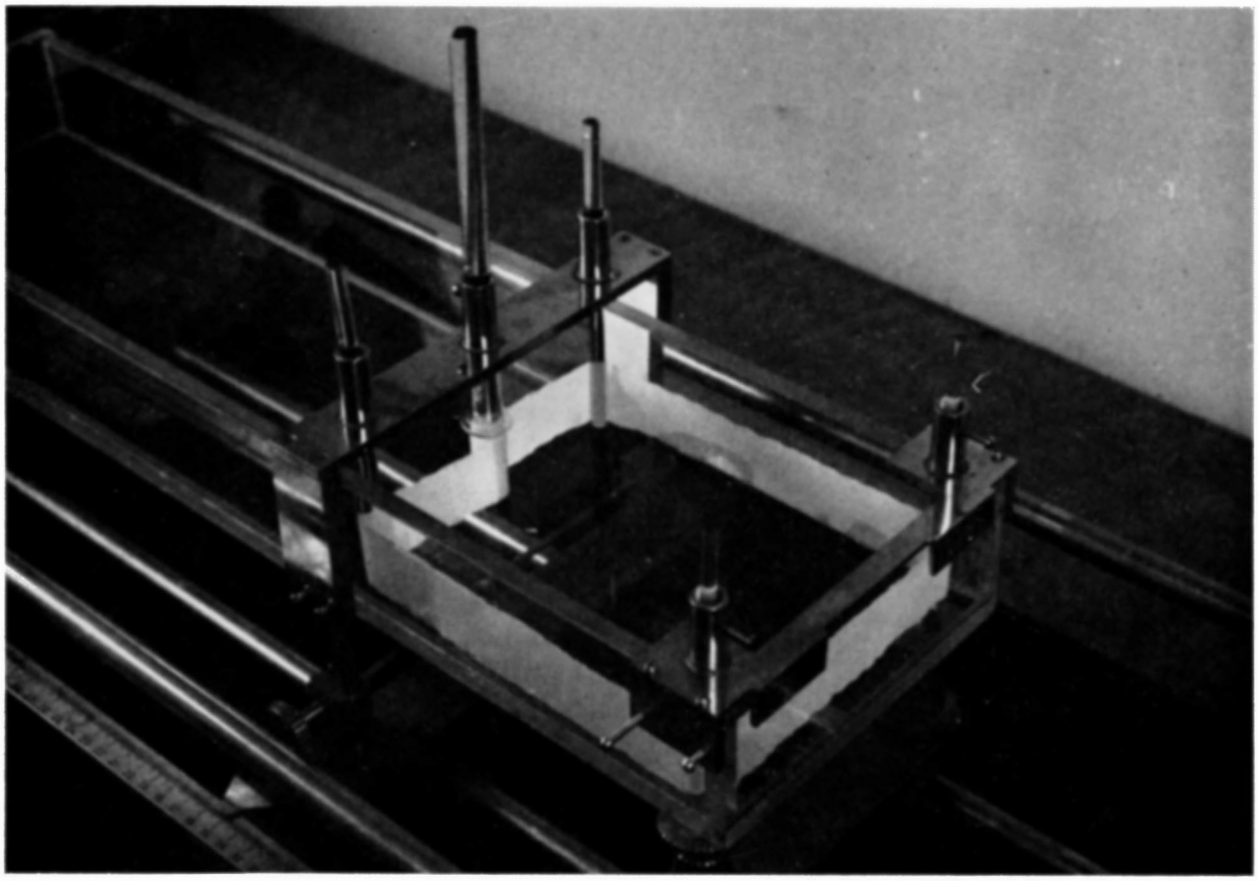

FIG. 2. **Teflon band partially rolled up during sweeping of interface.** Photograph taken without buffer or heptane in trough. A glass flared sleeve on center rod of movable bridge keeps Teflon band from sliding upward.

to allow extraction of lipid from the lipoprotein film, the center glass rod in the movable metal bridge (Fig. 2) was turned and the bridge itself was moved by hand toward the right-hand side of the trough. The Teflon band was thus kept taut while sweeping the protein film from the interface. This was continued until the movable bridge reached the far right of the trough.

A second quantity of lipoprotein was then injected into the clean interface at the left. After 2 min the movable bridge and its three glass rods were lifted out of the trough and moved to its far left side. The Teflon band thus unrolled above the trough and was lowered through the interface at the far left end. The band was then rolled up, thus sweeping the surface before the next injection. This procedure was repeated 10–15 times. At the end of the experiment, which usually lasted 2–3 hr, the Teflon band was once more rolled up so that the portion to its left contained no lipoprotein film. The heptane from that side was then removed by means of suction into a separatory funnel. Care was taken not to remove any of the interface. The volume of heptane so removed was measured in a graduated cylinder. The Teflon band was now released and the rest of the heptane removed by suction. This time much of the water phase was also sucked off. After separation in the separatory funnel the volume of the last portion of heptane was measured, but the heptane was not added to the first portion since it might contain finely dispersed droplets of water with lipoprotein film. The second portion of heptane was usually about one-third of the total amount of heptane, and the amount of cholesterol found in the first fraction of heptane was multiplied by a factor ranging from 1.4 to 1.5 depending on the exact proportion of heptane in the first and second fraction. In one experiment with LDL the cholesterol content of the entire heptane fraction was determined after it had been allowed to settle completely in a separatory funnel. The results of this experiment agreed well with that of the others.

After distillation of the first heptane fraction to a residual volume of about 10 ml, the residue was transferred to a 30 ml test tube and the evaporation was continued on a sand bath under a nitrogen atmosphere or in an evacuated rotary evaporator. The residue was then saponified according to the method of Abell et al. (15) and cholesterol determined with $FeCl_3$–H_2SO_4 color reagent (16).

A blank experiment was performed by spreading ten portions of distilled water and carrying the heptane through the exact procedure described above. In the later experiments all the heptane was evaporated by distillation in a rotary evaporator under vacuum. This was found to reduce the absorbance after addition of the cholesterol color reagent to the residue from an evaporated heptane blank.

TABLE 1 EXTRACTION OF CHOLESTEROL BY HEPTANE FROM A LIPOPROTEIN FILM

Lipoprotein*	Extraction
	%
DPL-3	76
DPL-3†	74
DPL-5	54
LDL-4	81
LDL-6	81
LDL-7	70
LDL-8	100
LDL-8	99
LDL-9	99
HDL-6	80
HDL-9	100
HDL-9	92
HDL-9	91

DPL = dextran sulfate precipitable lipoprotein, LDL = low density lipoprotein ($d < 1.063$), and HDL = high density lipoprotein ($1.063 < d < 1.21$).

* The numbers of the lipoprotein fraction designate a particular batch of pooled serum.

† This sample was spread after addition of 0.5% amyl alcohol, which has been shown to improve spreading of other proteins (17).

In the first shaking experiments, 1-ml lipoprotein samples were shaken with 10 ml heptane in a 50 ml glass stoppered centrifuge tube by hand. However, because of variability in results a mechanical shaker was employed in later experiments. The stoppers of the glass tubes were secured with adhesive tape and the tube mounted in a horizontal position. Thus the rapid vibration of the "wrist action" shaker caused good dispersion of the two phases. In some experiments glass tubes were silanized by treatment with a 2% solution of dichlorodimethylsilane in carbon tetrachloride followed by thorough rinsing with solvent and with water.

RESULTS

The extraction of cholesterol from a monolayer of lipoprotein spread between phosphate buffer and heptane varied from 54 to 100% (Table 1). This variation is probably the result of incomplete spreading of the lipoprotein and loss of lipoprotein from the interface during injection. This is evidenced by the fact that the per cent extraction increased as we gained more experience with the technique. In several instances in which a drop of lipoprotein could be seen to fall into the subphase the experiment was discarded before analysis. We found it more difficult to spread the HDL fraction quantitatively than the LDL or DPL fractions. This difficulty was resolved by dialyzing the HDL against phosphate–NaCl buffer of ionic strength 0.075 instead of 0.15. It seems likely that by reducing the density of the salt solution, the drop expressed from the needle

was kept from falling immediately into the subphase and a longer time was available for completion of the spreading.

No differences were noted between the extractability of β-lipoprotein fractions isolated with dextran sulfate (DPL) and those isolated by ultracentrifuge (LDL). Nor was any difference apparent between the extraction of lipid-rich lipoprotein (DPL and LDL) and lipoprotein of higher protein content (HDL). If the lower extraction figures are the results of incomplete spreading, the data in Table 1 would suggest that once the lipoprotein is spread the cholesterol becomes completely soluble in the heptane phase. In some instances in which the apparent extractability of cholesterol was between 75 and 80%, the ratio of cholesterol ester to free cholesterol was found to be the same as that of the lipoprotein fraction within the limits of analytical error. Therefore, no evidence of differences in extractability could be found.

In contrast to the high extractability of cholesterol from lipoprotein spread in films is the low extractability of cholesterol from lipoprotein solutions shaken with heptane (Table 2). The smaller degree of extraction of cholesterol in the shaking experiment might be due to the lower accessibility of the heptane to the cholesterol. The experiments with the monolayer had shown that when the lipoprotein was spread and presumably disrupted at the interface the cholesterol dissolved readily in the nonpolar phase. It is possible, therefore, that in the shaking experiment the lipoprotein must be disrupted before extraction will occur. The contact between the lipoprotein and the air–water, heptane–water, or glass–water interfaces might cause such disruption. The following observations would support such a mechanism.

TABLE 2 EXTRACTION OF LIPOPROTEIN CHOLESTEROL BY SHAKING WITH HEPTANE

Lipoprotein	Time	Extraction	
		No Albumin	In presence of 5% albumin
	min	%	
DPL-2*	10	6.8	
DPL-2*	10	6.8	
DPL-3	10	10.4	
LDL-1*	10	13.0	
LDL-4*	10	10.0	
LDL-7	15	5.6	
LDL-8	15	2.2, 2.6†	1.6
LDL-9	15	15.0	4.9
LDL-10	15	6.1, 3.5†	2.3, 2.7†
HDL-7	15	55.0‡	
HDL-8	15	12.7, 8.4†	8.8, 1.5†
HDL-9	15	26.0	7.5
HDL-10	15	14.0, 10.4†	3.1, 1.9†
Whole Serum-11	15	0.59, 0.64	
Whole Serum-12	15	0.77, 0.63	
Whole Serum	50	4.0	

For explanation of abbreviations see Table 1.

* Shaken by hand, all others were shaken on mechanical shaker.

† These values were obtained from simultaneous shaking of duplicate aliquots.

‡ A very fine emulsion was produced in this instance.

When one low density lipoprotein preparation (DPL) was shaken in sequence with five different 10-ml heptane fractions, the cholesterol extracted amounted to 10.4, 6.5, 5.0, 4.3 and 3.6% respectively. Thus the cholesterol extracted initially did not represent an unbound or loosely bound fraction; the formation of additional interface in subsequent shakings apparently increased the total amount of cholesterol extractable with heptane. In one instance the shaking of a high density lipoprotein fraction with 10 ml of heptane produced a very finely dispersed emulsion (HDL-7, Table 2) which did not break immediately upon cessation of shaking as is usually the case. Fifty-five per cent of the cholesterol was extracted, which is much higher than that achieved in the other instances. This effect could have been produced by the large increase in droplet interface during shaking, although one cannot exclude the possibility that the presence of some endogenous detergent (bile acids, fatty acids?) helped to displace cholesterol from the lipoprotein (3–5).

We observed (Table 2) that when lipoproteins were shaken with heptane in the presence of 5% bovine serum albumin the per cent cholesterol extracted decreased on the average by 58% ($t_{diff} = 8.3$, $P < 0.001$). Similarly, the extraction of cholesterol from whole serum shaken with heptane for 15 min in an identical manner was less than that obtained from isolated lipoprotein, only about 1% of the total (Table 2).

The above experiments do not give much information about which interface (air–water, heptane–water, or glass–water) is the most important in disrupting the lipoprotein. Table 3 shows experiments with two additional LDL preparations which throw some light on this question. Two 1-ml samples of lipoprotein were shaken with heptane, as before. In addition two 1-ml samples were shaken without heptane, after which heptane was added and the tubes were gently inverted 10 times. A third set of lipoprotein samples was treated only by inverting 10 times gently with heptane.

The lipoprotein samples shaken with heptane lost about twice as much cholesterol to the heptane phase as those shaken in empty tubes and later rotated with heptane, or as those rotated only. Apparently the creation of additional heptane–water interface in experiment 1 as compared to experiment 3 increased the extraction of cholesterol whereas the air–water interface produced in experiment 2 seemed to have less effect. Contact with glass, on the other hand, may have contributed significantly to the solubilization of cholesterol since the extrac-

TABLE 3 Low Density Lipoprotein Cholesterol Extracted by Shaking with Heptane*

Expt.	LDL-14		LDL-15	
	Without Albumin	With Albumin	Without Albumin	With Albumin
	%			
1 Shaken with heptane 10 min	5.32, 5.22	2.84, 3.44	6.28, 5.93	3.82, 3.48
2 Shaken in empty tube + rotation with heptane†	3.84, 4.21	0.353, 0.190	3.28, 3.43	0.345, 0.363
3 Rotated† with heptane (glass tube)	2.32, 2.51	0.137	3.04, 2.90	0.151, 0.252
4 Rotated† with heptane (silanized tube)			1.25, 1.18	0.240, 0.334

* Two entries separated by commas indicate duplicate shakings.
† Rotation consisted of inverting the tube gently 10 times.

tion of cholesterol by heptane in silanized tubes was less than half that in the untreated tubes (experiments 3 and 4).

The addition of 5% albumin was surprisingly effective in diminishing the extraction of cholesterol in tubes rotated with heptane; this effect was also observed in silanized tubes. In experiment 2 the LDL samples were visibly turbid after vigorous shaking; addition of albumin prevented development of turbidity.

The analysis of a few micrograms of cholesterol in the large volumes of heptane used for the spreading experiments was somewhat difficult, and we did not have sufficient material to perform quantitative analyses on other lipid fractions. However, in one experiment with high density lipoprotein, a thin-layer chromatogram was prepared of the heptane phase recovered from the trough. The heptane, after concentration, was applied to the plate. One chromatogram was developed with heptane–diisopropylether–acetic acid 60:40:2 (v/v/v) (18) and one with chloroform–methanol–water 140:50:9. After charring with H_2SO_4, spots of cholesterol ester, triglyceride, free fatty acid, and free cholesterol were clearly visible, whereas no phospholipid could be detected.

DISCUSSION

The experiments reported here would appear to imply that the binding of cholesterol to the protein moiety of serum lipoproteins is so loose that a nonpolar solvent such as heptane can completely extract the sterol when the lipoprotein is spread at the heptane-water interface. Furthermore, the experiments in which small quantities of lipoprotein were shaken with heptane demonstrated that not only the heptane–water interface forms a suitable locus for the detachment of cholesterol from the protein moiety; contact between lipoprotein and glass also accelerates transfer of cholesterol to the nonpolar phase. One could easily imagine that at the heptane–water interface the spreading of the lipoprotein sufficiently disrupts the structure or orientation of the molecular aggregate so as to allow the less polar lipids to escape into a solvent of lower polarity than water. Similarly the adsorption of lipoprotein on glass might deform the lipoprotein structure and let some of the lipid escape. The addition of albumin to the lipoprotein solution might inhibit the escape of cholesterol from the aqueous phase by competing with the lipoprotein for a position at the various interfaces.

A different explanation is also possible. It is well known that free fatty acids promote the extraction of other lipids even under conditions in which emulsification plays only a minor role. Since albumin effectively binds fatty acids it is possible that the addition of albumin to the lipoprotein fractions prior to shaking with heptane may have exerted its inhibition of cholesterol extraction by a more complete binding of some unesterified fatty acids present in the lipoprotein. This explanation was given by Forbes et al. (19) of a similar effect of added albumin on the extractability of cholesterol from lyophilized plasma.

Some aspects of the shaking experiments are still difficult to explain. If extraction of cholesterol in the heptane–water system depends on disruption of the lipoprotein at some interface, why is the vigorous shaking with heptane no more than twice as effective as the gentle rotation? And why does albumin protect the lipoprotein so much better when lipoproteins are gently agitated with heptane than when they are shaken vigorously? It is also not entirely clear how these experiments might elucidate the role of polar solvents (ethanol, methanol) in the more conventional lipid extraction procedures applied to serum. Possibly these solvents serve to disrupt the protein structure in a manner similar to that postulated for interfaces. Alternatively the polar solvents might displace the lipids from certain binding sites, as has been suggested for some detergents (5).

The experiments of Macheboeuf and co-workers have already indicated that the phospholipids are more strongly bound to the lipoprotein than are the neutral lipids. Our findings that cholesterol, cholesterol ester, triglyceride, and free fatty acids are extracted by heptane

from a lipoprotein monolayer, but that phospholipids could not be detected in the heptane phase is consistent with this hypothesis but does not prove it. The reason that phospholipids stay at the heptane–water interface may be due in part to the tendency of these molecules to form stable monolayers at an oil–water interface.

Several investigators have proposed that the degree to which cholesterol is extracted by solvents from whole or lyophilized serum might be an indication of the strength by which cholesterol is bound to the protein (3, 4, 20–26). They have implied that cholesterol more loosely bound to serum proteins might be more easily deposited in the tissues. The present investigations with lipoprotein films appear to show that the extent to which cholesterol is extracted from serum lipoproteins depends not so much on how strongly cholesterol is bound to protein, but on the spatial arrangement of the protein with respect to the lipid. Once the protein film is disrupted the cholesterol appears to be readily soluble in even the least polar of solvents. This interpretation of the results assumes, of course, that the deformation of the lipoprotein during spreading disturbs only the bonds holding the protein molecule or molecules together and does not break a significant number of bonds between lipids and amino acid residues.

The present experiments may also have certain implications regarding the state of cholesterol and cholesterol ester in chylomicrons. If the chylomicron is pictured as an oil droplet with a film of lipoprotein on its surface and if triglyceride oil behaves with respect to cholesterol as does heptane, it seems likely that chylomicron cholesterol and cholesterol ester might be dissolved in the oil phase rather than be attached to the protein membrane.

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