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THE UPTAKE OF LIPIDS BY HUMAN α-LIPOPROTEIN

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SUMMARY

1. The uptake of Celite-dispersed lipids by human α-lipoprotein has been studied.

2. The α-lipoprotein can bind many lipids but shows a considerable degree of discrimination amongst sterol and vitamin A derivatives.

3. Plant sterols can compete with cholesterol for binding sites on the molecule but there is no competition between cholesterol and palmitic acid.

4. The α-liproprotein binds a number of anionic dyes, methyl orange being bound to the greatest extent. This competes with cholesterol but not with palmitic acid.

5. β-Lipoproteins (Sf 2-10) are severely damaged by incubation with lipid-coated Celite.

INTRODUCTION

Much work has been done on the physical properties and chemical composition of human plasma lipoproteins and on their roles in lipid transport. As yet, however, very little is known of their structure or of the specificity of composition of their lipid moieties. The low density lipoproteins probably consist of a lipid core surrounded by a film of protein and phospholipid. This is suggested by the ease with which the lipid may be extracted[1] and the disruption of their structure by phosphatidase-D[2,3]. The α-lipoprotein* consists of roughly equal parts of peptide and lipid, is not attacked by phosphatidase-D[2] and is relatively resistant to ether extraction[1]. GURD[5] has therefore suggested that most, if not all, of the lipid molecules in this lipoprotein have some contact with peptide and that the structure is analogous to plasma albumin heavily laden with sodium dodecyl sulphate.

Information on the surface of macromolecules can be obtained by studying their combination with small molecules and ions. Many studies of this sort have been performed on plasma albumin but only a small number on lipoproteins[6-9]. This paper describes an investigation of lipid uptake by the α-lipoprotein using the method devised by AVIGAN[9].

MATERIALS AND METHODS

[^{14}C]Cholesterol, [^{14}C]tripalmitin, [^{14}C]cetyl alcohol and [^{14}C]hexadecane were obtained from The Radiochemical Centre, Amersham, and diluted where necessary

* ONCLEY[4] has proposed that the high density (α-)lipoproteins represent a single family of average density 1.12.

with unlabelled material. [^{14}C]Palmitic acid was prepared by saponification of the radioactive tripalmitin and its purity checked by chromatography on silicic acid. Radioactivity was associated only with the palmitic acid.

Ergosterol and 7-dehydrocholesterol were purified by recrystallization from methanol until the $E^{1\%}_{1\,cm}$ was > 290. 7-Dehydrocholesterol acetate was prepared by acetylation of the purified sterol with acetic anhydride in dry pyridine. It was purified by chromatography on silicic acid[10] and recrystallization.

Vitamin A and vitamin A acetate were obtained from Roche Products Ltd., Welwyn Garden City. Retinoic acid and vitamin A methyl ether were obtained through the courtesy of Dr. O. ISLER of Hoffman-La Roche, Basle. β-Carotene was obtained from L. Light and Co. Ltd., Colnbrook, and purified by chromatography on alumina[11]. The dyes used were all commercial products.

Analytical procedures

Nitrogen was determined by the micro-Kjeldahl method, phosphorus by the method of KING[12] and total sterol as described by GREEN, LOW AND MORTON[13]. Phospholipid was estimated by multiplying the lipid phosphorus figure by the factor 25. Radioactive compounds were determined either by plating an aliquot of the lipid from chloroform solution on to a planchette and counting in a thin-window counter or directly in a Packard Tricarb Scintillation Spectrometer. In each case samples were counted to a statistical error of less than 2 %.

The compounds listed below were estimated from their ultraviolet absorption spectra after correction for irrelevant absorption. The amount present was calculated assuming the following values for $E^{1\%}_{1\,cm}$ at the stated wavelength (sterols in alcohol, carotenoid derivatives in cyclohexane): 7-dehydrocholesterol, 281.5 mμ, 310; 7-dehydrochlolesterol acetate, 281.5 mμ, 280; ergosterol, 281.5 mμ, 298; β-carotene, 455 mμ, 2456; vitamin A acetate, 326 mμ, 1515; retinoic acid, 326 mμ, 1460; vitamin A methyl ether, 326 mμ, 1656.

Preparation of lipoproteins and albumin

Human plasma, from pooled samples of A.C.D. blood, was concentrated by ultrafiltration to a protein concentration of approx. 7 % and then fractionated by the COHN[14] Method 10. The α-lipoprotein used was Precipitate IV-1 which had been extracted twice with ice-cold distilled water to remove albumin. Samples were checked by paper electrophoresis and by protein, sterol and phospholipid analysis. The only contaminant detected was albumin but this could be present in concentrations up to 50 % that of the lipoprotein.

Albumin was precipitated from Fraction V with alcohol and freed from fatty acids by the method of GOODMAN[15]. β-Lipoproteins (Sf2-10) were prepared from Fraction III-0 by centrifugation in a density gradient tube[16].

Finally, the albumin was dissolved in, and lipoproteins dialysed against, 0.16 M phosphate buffer (pH 7.65).

Incubation conditions

2.5 mg of the test lipid were dispersed on 250 mg Celite 545 (Johns-Manville) and shaken under nitrogen with 5 ml of the protein solution for 18 h at 30–35° (see ref. 9). The concentration of lipoproteins used was 2.5 mg/ml and of albumin

1.0 mg/ml. For experiments with β-carotene and vitamin A derivatives, the Celite was placed in a desiccator which was repeatedly evacuated and filled with nitrogen, and nitrogen was bubbled through the lipoprotein solution for 30 min before incubation.

After 18 h the mixture was filtered through a glass fibre filter (Whatman GF/B) and the lipid extracted from a suitable aliquot of the solution[17].

In experiments on the competition between cholesterol and other sterols, 2.5 mg [^{14}C]cholesterol and 7.5 mg test sterol were dispersed on 60 and 190 mg Celite respectively. The two portions of Celite were then bulked for incubation. This procedure was adopted to avoid the formation of mixed crystals[18]. To study competition between cholesterol and palmitic acid, the lipoprotein was first incubated as described above with the unlabelled form of one and then the uptake of the other (^{14}C-labelled) was measured.

The binding of dyes was investigated by equilibrium dialysis[19]. 10 ml of the lipoprotein solution (5 mg/ml) was dialysed against 5 ml of dye solution (1 mg/ml) for 18 h. The concentration of dye before and after dialysis was measured spectrophotometrically and corrections made for binding to the dialysis membrane. For the experiments recorded in Table III, the α-lipoprotein was dialysed against methyl orange as described, then against three changes of buffer to remove the unbound dye. Cholesterol and palmitic acid uptakes were then determined as usual.

RESULTS

The uptakes of a variety of lipids by α-lipoprotein and albumin are given in Table I. The concentration of lipoprotein was calculated from the lipid phosphorus figure after filtration, assuming the α-lipoprotein to be 20% phospholipid. Controls performed with each experiment showed that incubation with Celite for 18 h had no effect on the nitrogen, phospholipid or sterol content of the solution. There is a fairly large variation between individual results. Because of this, no correction was applied to the lipoprotein figures for uptake by contaminating albumin, even in the case of palmitic acid. Such albumin would already contain two molecules of fatty acid per molecule[20] and its uptake of palmitic acid would be even less than that of the purified protein. Hence, even when present at its maximum concentration as a contaminant, albumin would take up a relatively small proportion of the total.

The α-lipoprotein has a much greater affinity for all lipids studied than plasma albumin under these conditions. GOODMAN[20] studied the binding of fatty acids to albumin by partition analysis and reported that there were three classes of binding sites with different association constants. The technique used in the present study results in binding only to the first two of these sites; the largest class of sites with lowest association constant apparently takes up no palmitic acid or cetyl alcohol. It seems that most of the palmitic acid uptake by the α-lipoprotein results from weak van der Waals bonding by the hydrocarbon chain. Thus cetyl alcohol and hexadecane can be bound to approximately the same extent as palmitic acid. The binding forces are so weak for hexadecane that variations in the Celite can have dramatic effects. With one batch of Celite, a hexadecane uptake of less than 1 mole/mole was found whilst using another, the figure was 49 moles/mole. These sites on the α-lipoprotein

must bind more strongly than the corresponding ones on albumin since albumin uptake of palmitic acid never exceeded 7 moles/mole.

Although the α-lipoprotein can bind a wide variety of lipid molecules, the process shows considerable specificity. The two zoosterols are taken up twice as well as ergosterol, and 7-dehydrocholesterol acetate uptake is negligible. The lability of vitamin A, β-carotene and vitamin A methyl ether and their low level of uptake made it difficult, despite rigorous precautions against oxidation, to obtain definite figures for these compounds. Four experiments were performed with vitamin A but, because of the rapidity of its oxidation, no figures are included in Table I. Its uptake, if any, must be very small. On a weight basis, retinoic acid is taken up by the α-lipoprotein as well as palmitic acid, although its uptake by albumin is only 5% that of the palmitic acid. Modification of the hydrophylic end of the vitamin A molecule has a marked effect on the degree of binding.

TABLE I

UPTAKE OF LIPIDS BY α-LIPOPROTEIN AND ALBUMIN

2.5 mg lipid were dispersed on 250 mg Celite and incubated at 30–35° for 18 h with 5 ml protein solution. Concentration of α-lipoprotein, 2.5 mg/ml and albumin, 1 mg/ml. Results are expressed as means ± standard error of the mean (number of observations).

Lipid tested	*Uptake by α-lipoprotein*		*Uptake by albumin*	
	(mg/100 mg)	*(moles/mole*)*	*(mg/100 mg)*	*(moles/mole**)*
Cholesterol	4.3 ± 0.3 (19)	22	< 0.13 (4)	< 1
7-Dehydrocholesterol	4.3 ± 0.5 (5)	22	*** (4)	< 1
7-Dehydrocholesterol acetate	0.3 ± 0.1 (5)	1	*** (4)	< 1
Ergosterol	2.1 ± 0.4 (5)	11	*** (4)	< 1
Palmitic acid	7.0 ± 0.6 (9)	55	2.4 ± 0.2 (4)	7
Cetyl alcohol	7.3 ± 1.8 (3)	60	2.2 ± 0.3 (4)	6
Tripalmitin	2.2 ± 0.1 (5)	5	< 0.37 (4)	< 1
β-Carotene	< 0.1 (3)	< 1		
Vitamin A acetate	3.6 ± 0.1 (3)	22		
Retinoic acid	7.1 ± 0.3 (4)	48	< 0.1 (4)	1
Vitamin A methyl ether	< 1.5 (3)	< 10		

* Assuming a molecular weight of 200000.
** Assuming a molecular weight of 69000.
*** Below limit of detection.

TABLE II

COMPETITION BETWEEN STEROLS FOR α-LIPOPROTEIN BINDING SITES

2.5 mg [^{14}C]cholesterol and 7.5 mg unlabelled test sterol were incubated as described in the text with 5 ml α-lipoprotein solution. Results are expressed as means ± standard error of the mean (number of observations).

Test sterol	*Cholesterol uptake*	
	(mg/100 mg lipoprotein)	*(moles/mole lipoprotein*)*
Ergosterol	1.7 ± 0.1 (6)	9
β-Sitosterol	2.2 ± 0.5 (3)	11
Stigmasterol	2.5 ± 0.4 (3)	13
Cholestanol	2.7 ± 0.5 (9)	14

* Assuming a molecular weight of 200000.

Of all lipids tested, the α-lipoprotein shows least affinity for β-carotene. Under the same conditions the uptake of this compound is only 2 % that of the smaller hydrocarbon hexadecane and 5 % that of the larger, non-polar compound tripalmitin.

Other sterols compete with cholesterol for the same sites on the α-lipoprotein molecule (Table II). In a 3:1 excess, the phytosterols reduce cholesterol uptake by 50 %. It is surprising that cholestanol, which should be bound more readily than the phytosterols, does not have a greater effect on cholesterol uptake. Preliminary experiments in fact suggest that its uptake is even greater than that of cholesterol. Statistical analysis shows that the reduction in cholesterol uptake in presence of ergosterol and cholestanol is significant ($P < 0.01$).

The results given in Table III indicate that cholesterol and palmitic acid are bound to different sites on the α-lipoprotein molecule since preincubation with one has no significant effect on the uptake of the other. Control experiments performed at the same time showed that preincubation with Celite 545 for 18 h had no effect on the uptake of either lipid.

To obtain further information on the binding sites, competition between lipids and a protein-binding dye was studied. A variety of dyes was investigated to find the one which bound to the α-lipoprotein in greatest amount. Neutral red was bound but it is not very soluble at pH 7.65 and, as only the maximum uptake was of interest, it was not studied further. Evans blue showed only slight binding to the α-lipoprotein,

TABLE III

LIPID UPTAKE BY α-LIPOPROTEIN PREINCUBATED WITH OTHER SUBSTANCES

For details of procedure, see text. Results are expressed as means ± standard error of the mean (number of observations).

Preincubation substance	*Cholesterol uptake*		*Palmitic acid uptake*	
	(mg/100 mg lipoprotein)	*(moles/mole lipoprotein*)*	*(mg/100 mg lipoprotein)*	*(moles/mole lipoprotein*)*
Cholesterol			6.9 ± 0.9 (7)	54
Palmitic acid	4.0 ± 0.4 (6)	21		
Methyl orange	2.1 ± 0.2 (6)	11	8.0 ± 1.0 (6)	02

* Assuming a molecular weight of 200000.

TABLE IV

UPTAKE OF LIPIDS BY β-LIPOPROTEIN (Sf2-10) RESIDUES

The conditions were the same as for the α-lipoprotein (Table I). Results are calculated assuming no destruction of the lipoprotein and expressed as means ± standard error of the mean (number of observations).

Lipid tested	*Uptake (mg/100 mg lipoprotein originally present)*
Cholesterol	1.2 ± 0.2 (4)
7-Dehydrocholesterol	2.3 ± 0.4 (4)
Palmitic acid	2.6 ± 0.5 (4)
Cetyl alcohol	0.4 ± 0.1 (4)
Tripalmitin	0.4 ± 0.1 (4)

phenol red and methyl red were bound to the extent of 10 moles/mole and methyl orange to the extent of 25 moles/mole. When saturated with methyl orange, the α-lipoprotein bound roughly the same amount of palmitic acid as the controls but its cholesterol uptake was halved (Table III).

The use of Celite to disperse lipids was devised by AVIGAN[9] for studies on steroid uptake by β-lipoproteins (Sf3-8). When β-lipoproteins (Sf2-10) were used in the present study, considerable amounts of lipid were lost from the complex although the residue still showed an affinity for extra lipid molecules (Table IV). The original lipoprotein solution was yellow owing to the presence of carotenoids but after incubation the solution was colourless and the Celite coloured. Phospholipid analysis showed that up to 75 % of the phospholipid had been removed from the lipoprotein. This again may be due to differences in the Celite used.

DISCUSSION

The α-lipoprotein has a large capacity for extra lipid. The results of experiments on joint cholesterol and palmitic acid uptakes (Table III) suggest that the lipid content can be increased by 25 %. SCANU AND HUGHES[21] showed that the protein of the α-lipoprotein has a very high recombining capacity toward lipid and in presence of other lipoproteins rapidly takes up lipid to reform a complex very similar to the original lipoprotein. Although they could detect no intermediates between the lipid-free protein and the intact lipoprotein, they consider that such complexes do exist. However, the magnitude of lipid uptakes reported here and the low concentration of lipid-denuded lipoproteins which must occur in plasma, make it unlikely that the present results are due to uptake by such complexes. This is supported by the lack of correlation between the relative amounts of lipids bound and the composition of the original lipoprotein. Although the intact α-lipoprotein contains more esterified than free sterol, the uptake of 7-dehydrocholesterol is fifteen times greater than that of 7-dehydrocholesterol acetate.

The relatively low uptake of ergosterol and the ability of plant sterols to lower cholesterol uptake are of interest because it has been suggested that the poor intestinal absorption of plant sterols and their blocking action on cholesterol absorption are related to their ability to enter the lipoproteins of the mucosal cell[22].

Vitamin A is not carried in the lipoproteins[23] *in vivo* and, even after absorption, only traces of β-carotene appear in the α-lipoprotein[24]. This is consistent with the low level of β-carotene uptake and the failure to find any uptake of vitamin A *in vitro*. It seems probable that β-carotene is carried in solution in the lipid core of the lipid-rich lipoproteins.

One mole of plasma albumin binds a maximum of 22 moles methyl orange[19] and β-lipoprotein, 150 moles[8,*]. Thus, per unit weight of protein, albumin, α-lipoprotein and β-lipoprotein bind roughly the same amounts of methyl orange. ROSENBERG, LEVER AND LYONS[8] found that the methyl orange was not dissolved in the lipid of the β-lipoprotein but could not decide whether it was bound to the protein or lipid parts of the surface. When incubated with a lecithin-stabilized triolein emulsion of about the same lipid concentration as in the α-lipoprotein solutions used, methyl

* Corrected to β-lipoprotein molecular weight of $3 \cdot 10^6$.

orange was not bound to the lipid particles[25]. It seems probable that methyl orange is bound to the protein and most of the palmitic acid to the lipid part of the surface. This is supported by the finding that the lipid-free protein of the α-lipoprotein does not bind palmitic acid[26] and is consistent with the fact that hexadecane uptake can equal that of palmitic acid. Table III shows that methyl orange binding has no marked effect on palmitic acid uptake, although fatty acids and similar compounds compete with organic dyes for all but two sites on plasma albumin[27,28]. It may be that unesterified sterols are bound mainly to the protein part of the surface since the process is specific for both ends of the sterol molecule, palmitic acid and cholesterol uptakes proceed independently and cholesterol uptake is greatly reduced after methyl orange binding. An investigation of the binding of sterols to the lipid-free protein should provide definite evidence on this point.

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