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Partitioning of chemically modified low-density lipoprotein in aqueous polymer two-phase systems

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Abstract

Aqueous polyethylene glycol (PEG)–dextran two-phase systems containing 10 m*M* Tris · HCl (pH 7.4) were used for the partitioning of chemically modified low-density lipoprotein (LDL). Anionic modification connected with an increase in the negative surface charge of lipoproteins favours the accumulation of modified LDL in the top phase. The partition coefficient increases depending on the extent of modification. Cationic modification yields lower values for the partition coefficient. Positively charged LDL favours a bottom-phase accumulation. With weakly charged and nearly neutral particles, the Van der Waals interaction between polymer and particle preponderates over electrostatic interactions, leading to a favoured accumulation of LDL in the PEG-rich top phase. Results of measurements of the relative electrophoretic mobility and the determination of free amino groups are in agreement with the calculated values of the partition coefficient. Because the partitioning of LDL is accompanied by aggregation at the interface, experimental techniques have to be carefully standardized. Subtle differences in the surface properties of modified LDL can be detected.

1. Introduction

Lipoproteins perform the transport function for lipids and cholesterol in the blood. A core of hydrophobic molecules (triglycerides, cholesteryl esters) is surrounded by an amphiphilic envelope of phospholipids and a mixture of specific apoproteins. All components are organized by the action of non-covalent forces to form particles of qualitatively and quantitatively different composition. The dispersive potential of such complex structures is very high [1].

In addition to the heterogeneity at the site of synthesis, lipoproteins undergo a considerable degree of metabolic alteration, including size and structure in the blood and liver and in the extrahepatic vascular system. Despite the normal heterogeneous native lipoprotein classes *in vivo*, modified particles are discussed as the main source for pathological processes in cells and tissues.

Native lipoproteins are negatively charged particles. Lipoprotein LP(X) migrating towards the cathode in an electric field has been found as an abnormal plasma constituent of patients with obstructive jaundice [2].

Chemical modification of low-density lipoprotein (LDL) connected with a strong increase in the negative surface charge of LDL has been proposed as the reason for the induction of foam cell formation [3]. Peroxidation processes [4,5], malondialdehyde reactions [6], glycosylation [7] and oxidation by endothelial cells, smooth muscle cells [8–11] and macrophages [12] have been

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suggested as sources of pathologically altered LDL *in vivo*. Reactions catalysed by myeloperoxidase probably result in the formation of hypochlorite-modified LDL [13,14]. Acetylated LDL is often used in experiments to study the receptor–lipoprotein interaction [15,16]. Modification via acetylation, malonylation and oxidation is connected with changes in the apoprotein structure and surface charge of LDL, rendering the lipoprotein highly anionic [17].

In contrast to native LDL, this modified anionic LDL cannot interact with the classical LDL receptor of tissues. Modified anionic LDL is recognized only by the scavenger receptor of macrophages [18]. The unregulated lipoprotein uptake via scavenger cell receptor-mediated endocytosis leads to lipid loading of cells and generation of atheriosclerotic plaques.

Chemically cationized LDL enters the cell membrane without receptor-mediated endocytosis. Similarly to native LDL, this modified lipoprotein effectively regulates the cholesterol metabolism in cells [19]. Chemical modification of LDL involves changes in surface charge [20] and/or conformation [21] of the particles.

On the other hand, a certain spatial organization of negative charges in modified lipoproteins or charged macromolecules is suspected to be important for the receptor–particle interaction [15]. The involvement of more than one receptor for the recognition of modified LDL species has been discussed [22–24].

Physico-chemical and physiological properties of modified HDL were studied by Salmon *et al.* [25].

A suitable method for identifying modifications on the lipoprotein surface would be a useful tool for investigating such processes *in vitro* and *in vivo*.

In previous papers, the application of two-phase systems for the characterization of the surface properties of lipoproteins was demonstrated [26,27]. The partitioning of particles and molecules in aqueous polymer two-phase systems depends mainly on the size, charge and hydrophobicity of the particle surface [28], *i.e.*, the partitioning of particles is a surface-dependent phenomenon. Because the interaction between lipoproteins and cells strongly depends on exposed groups on the surfaces, two-phase systems could be expected to provide a method for detecting surface changes of lipoproteins relevant to the lipoprotein–receptor interaction.

In this work, the partitioning of chemically modified LDL was studied. Both anionic and cationic modified LDL were studied as a function of the degree of modification, using the fluorescamine method to detect the number of functional groups, electrophoretic measurements to describe the relative changes in surface charges and two-phase partitioning to obtain the partition coefficient and mass distribution between the two bulk phases and the interface.

2. Experimental

2.1. Materials

LDL preparation

LDL was obtained from freshly taken plasma from healthy volunteers by sequential ultracentrifugation according to Havel *et al.* [29]. After separation of VLDL the remaining plasma was adjusted with NaBr to a density of 1.072 kg/l, covered with a density solution of 1.064 kg/l and centrifuged for 24 h at 40 000 rpm (UP 65 M centrifuge, 8 × 11 rotor). LDL was recentrifuged and dialysed twice at 4°C for 25 h against 10 mmol/l phosphate buffer (pH 7.4) containing 0.137 mol/l NaCl, 0.005 mol/l KCl and $1 \cdot 10^{-4}$ mol/l Na_2EDTA. The protein concentration of LDL was determined using the Lowry procedure [30]. All samples were checked for purity by horizontal agarose gel electrophoresis or non-denaturing polyacrylamide gel electrophoresis (PAGE), placing the gels on a cooling plate (16°C) according to the instructions given in the users' information for the Multiphor II Electrophoresis System from Pharmacia–LKB, Biotechnology.

For the electrophoretic control of purity, gels of size 125 × 260 mm and thickness 2 mm were used. Up to sixteen samples can be applied simultaneously and checked under equal running

conditions. Samples were applied in 0.5-mm deep gel slots of size 7 × 1 mm. Gels with gel concentrations of 3.5% and 5.0% in PAGE and 1.2% in agarose gel electrophoresis were prepared on the hydrophilic side of a gel support by using suitable moulds. Substances for PAGE were obtained from Bio-Rad Labs. and agarose was purchased from Serva.

Lipoproteins were revealed by staining with Coomassie Brilliant Blue (Fluka) for the recognition of the apo-proteins and Sudan Black (Chemapol, Prague, Czech Republic) or Fat Red 7B (Serva) for the determination of the lipid components.

LDL diluted to 1, 2, and 4 mg/ml protein with dialysis buffer was stored at 4°C and used within 2 days after dialysis.

LDL modification

All LDL samples were thoroughly dialysed against isotonic phosphate-buffered saline (PBS) buffer solution (pH 7.4).

Acetylation

Acetylation was performed in a modified procedure according to the method of Fraenkel-Conrat [31] by mixing the LDL samples with a saturated solution of sodium acetate (1:2, v/v) at 0 and 22°C. Acetic anhydride was added slowly in small portions during continuous stirring of the mixture in the range $0–100 \cdot 10^{-3}$ mmol acetic anhydride/mg LDL.

After an incubation period of 30 min, each sample was dialysed for 24 h at 4°C against isotonic PBS buffer solution (pH 7.4).

Hypochlorite modification

Sample volumes of 1 ml containing 2 mg of lipoprotein were incubated at 22°C with 20 m*M* NaOCl solution. The amount of NaOCl in the samples was varied in the range $0–100 \cdot 10^{-5}$ mmol NaOCl/mg LDL [27]. After an incubation time of 5 min the non-dialysed samples were used for partitioning and determination of unreacted groups and for the measurement of the electrophoretic mobility.

Cationization of LDL

LDL was cationized at room temperature in a modified version of the original method [19,32]. Briefly, sample volumes of 20 ml with protein concentrations of 2 mg/ml were added to 20 ml of a 2 *M* solution of N,N-dimethyl-1,3-propanediamine (DMPA) (Fluka) previously adjusted to pH 6.5 with HCl. The amount of DMPA in the mixture was 10^{-3} mol/mg LDL.

Solid 1-ethyl-3(3-dimethylaminopropyl)carbodiimide · HCl (Sigma) was added to the mixture to a final concentration of 0.3 *M* with stirring.

During the reaction the pH was maintained at 6.5 by continuous addition of HCl. Depending on the rection time, samples were drawn from the reaction pool and immediately transferred into dialysis tubes. Dialysis at 4°C for 24 h against PBS buffer solution (pH 7.4) stops the reaction and removes unreacted DMPA and carbodiimide. The sample immediately drawn from the pool after all the reagents had been mixed (time point $t = 0$) serves as a reference (unmodified LDL).

All modification experiments were repeated three times. After dialysis all modified and unmodified LDL samples represented optically clear solutions. Lowry estimation of the apoprotein concentration and electrophoresis was repeated for each sample and control to exclude degradation events and to check the purity of the samples.

2.2. Methods

Two-phase partitioning

Two-phase systems were prepared by mixing stock solutions of dextran T 500 (20%, w/w; Pharmacia) and polyethylene glycol (PEG) 6000 (40%, w/w; Ferak) with 10 m*M* Tris · HCl buffer (NaCl-free) (pH 7.4) and water. We used 10-g systems containing 0.71 g of dextran, 0.5 g of polyethylene glycol, 2.50 g of buffer solution and 6.09 g of distilled water. These systems, carefully prepared by shaking the mixture in 10-ml graduated tubes at room temperature, were filled up with 0.2 g of lipoprotein solution or the same amount of lipoprotein-free buffer in the case of controls.

Lipoproteins or control samples were distributed in the polymer mixture by 40 inversions of the glass tubes. The turbid mixtures were allowed to stand overnight. It is not favourable to enforce the phase separation by centrifugation, because the particles themselves begin to settle, leading to irreproducible results. After phase separation and equilibration, the two immiscible polymer phases are characterized by a sharp interface.

The total volume V and the particular volumes $V(\mathrm{b})$ and $V(\mathrm{t})$ of the bottom and the top phase, respectively, were measured in control samples after phase separation and equilibration of the mixture.

After mixing the lipoprotein with the polymer solutions by shaking the tubes, a volume of 2 ml from the centre of the turbid mixture was removed for the determination of the total concentration by using a syringe. When the phase separation was finished, a further 2 ml were removed from the centre of each separation phase to determine the top and bottom phase concentrations.

Before drawing the sample from the bottom phase, the remainder of the top phase has to be removed completely to exclude the contamination of the bottom phase with top-phase material. Owing to the high viscosity of the polymeric phases, it is difficult to take accurate volumetric aliquots using a pipette. Therefore, we used only weighed aliquots of the top and bottom phases.

To determine the concentration of the partitioned lipoprotein between the top and bottom phases, the UV absorption of the protein component in the lipoprotein was measured. The samples were diluted 1:2 (w/w) with buffer solution before measuring the absorption at 280 nm. As the polymer solutions absorb UV radiation, blanks are always prepared of a system without lipoprotein.

Samples and controls for all modified and unmodified lipoproteins were prepared in triplicate.

The linearity between absorption and lipoprotein concentration for the top, bottom and mixed phases was checked by calibration. The slopes of the lines for all three phases were the same. Therefore, the measured values for the extinction are proportional to the phase concentrations.

The partition coefficient was calculated as the ratio of the top and bottom phase concentrations [33].

Lipoprotein aggregation leads to accumulation of aggregated LDL at the interface. This material has to be removed before bottom-phase material can be taken for concentration determination.

Electrophoresis of modified samples

We used agarose gel electrophoresis and PAGE to characterize the degree of modification. The procedure was the same as used to check native unmodified LDL for purity. The relative electrophoretic mobility (REM) was calculated as the ratio of the mobilities for modified and unmodified LDL.

Fluorescamine assay

The relative number of free amino groups in LDL samples was determined using fluorescamine (Serva) as described [34].

The non-fluorescent fluorescamine reacts with amino groups on the surface, yielding a highly fluorescent product. The unreacted fluorescamine hydrolyses into non-fluorescent compounds. For the fluorescence measurements 50 μl of the LDL suspension (protein concentration 2 mg/ml in experiments with hypochlorite; 2–4 mg/ml in the case of acetic anhydride modification) were mixed with 170 μl of 0.01 mol/l Na_2HPO_4 solution. To this solution 5 μl of a 2 mmol/l solution of fluorescamine in acetone were added. After 2 min the fluorescence was measured at 470 nm. The exitation wavelength was set at 396 nm.

2.3. Instrumentation

Measurements of the fluorescence were carried out using a Perkin-Elmer LS 50 spectrofluorimeter. Both slits were installed at 5 mm. The READ regime of the Fluorescence Data Manager Software (Perkin-Elmer) was used.

Measurements of the UV absorption at 280 nm

were performed with a U-2000 spectrophotometer (Hitachi).

All measurements were repeated four times with stirring the samples.

If not indicated otherwise, all reagents were of analytical-reagent grade from Laborchemie (Apolda, Germany).

3. Results and discussion

Cationization of LDL with the nucleophilic N,N-dimethyl-1,3-propanediamine via carbodiimide activation of the apo-protein carboxyl groups converts a negatively charged free carboxyl group of the apo-protein into a positively charged tertiary amine derivative. In other words, the modification of protein carboxyls effects a change of two unit charges per modified carboxyl group [32].

Owing to the complexity of the reaction, it is difficult to maintain identical reaction conditions for each sample in a series of different samples. Therefore, instead of the usually used variation of the pH or the reaction temperature and the concentration of the modifying agent to modify the extent of modification, we preferred to vary the reaction time as a possibility of changing the degree of modification.

Fig. 1 shows the electrophoretic mobilities of LDL samples with increasing degree of cationization. Native LDL (1) with a negative surface charge corresponding to its negative

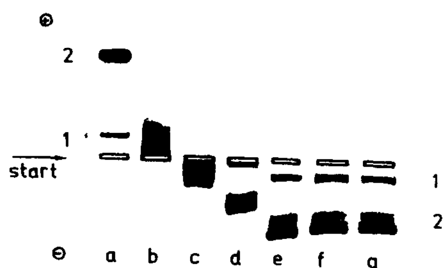

Fig. 1. Influence of reaction time on the degree of cationization (results of PAGE). 1 = LDL; 2 = albumin; reaction time: (a) 0; (b) 1; (c) 15; (d) 30; (e) 120; (f) 360 min; (g) sample after standing overnight.

mobility is changed into a form of LDL with reduced and neutral surface charge. The increase in reaction time effects an increase in positive surface charge. The LDL migrates towards the cathode in the electric field. The electrophoretic bands estimated by Coomassie Brilliant Blue staining of the polyacrylamide gel are identical with those detectable by Sudan Black staining, showing that a separation of the apo-protein from the lipoprotein does not occur during the modification process (data not shown). In contrast to LDL, the albumin shows higher negative and positive mobility.

The electrophoretically checked LDL samples with different degrees of cationization were used to study the LDL partitioning between the top and bottom phases of a PEG–dextran two-phase system. After phase separation for all the samples studied optically clear bulk phases were obtained. Only the bottom phase was slightly turbid for unmodified LDL (reaction time $t = 0$), but the phase became completely clear after dilution 1:2 with buffer solution. In all instances aggregated LDL was accumulated at the interface.

The partition coefficient K and the changes in the relative electrophoretic mobility (REM) as a function of the degree of modification are shown in Table 1. The greatest changes in electrophoretic mobility take place in the first few minutes after the reaction has started. In the course of a very short time the REM decreases from the value -1 to zero (neutral state of LDL) but only after several hours is the value $+1$ reached (cationized form of LDL).

Two-phase systems for controls and samples were prepared in triplicate. The calculated partition coefficients in Table 1 are mean values representing the ratio of LDL concentrations in the top and bottom phases.

The initial phase of LDL cationization seems to be connected with drastic changes in the surface properties of lipoprotein particles. The partition coefficient increases from $K = 0.202$ to a maximum of $K = 1.205$ with decreasing negative surface charge of the LDL particles. The results obtained show that subtle differences in the surface properties of modified LDL can be

Table 1
Partition coefficient (K) and relative electrophoretic mobility (REM) of LDL with increasing degree of cationization

Reaction time (min)	REM	K	Remarks
		0.22 ± 0.05 ($n = 9$)	Native LDL
0	−1.00	0.202	Unmodified LDL
1	−0.05	1.205	Electrically neutral LDL
15	+0.16	0.917	LDL with increasing degree of cationization
30	+0.15	0.306	
120	+0.66	0.268	
360	+0.89	0.277	

Experiments carried out in triplicate. The mean error is of the order of 20%.

detected by phase partitioning with high sensitivity.

With weakly charged and nearly neutral particles the Van der Waals interaction between polymer and particle preponderates over electrostatic interactions. LDL in its neutral state accumulates in the more PEG-rich top phase, leading to high values of K.

Increasing surface charge as the result of a greater degree of modification increases the hydrophilic character of the lipoprotein. Both the increase in the positive surface charge and the higher hydrophilicity of the cationized LDL now favour accumulation in the dextran-rich hydrophilic bottom phase. The calculated values for the partition coefficient decrease as the degree of cationic modification increases. According to our earlier findings [27] using the same composition of the two-phase system as described above, the results obtained with cationized LDL are in agreement with the existence of an electrically positive potential difference between the top and bottom phases.

Anionic modification of LDL connected with an increase in the negative surface potential of the modified particles is based on the reaction of the ε-amino groups in lysine and the sulphydryl groups of the apo-B protein with the modifying agent. The derivatization of an amino group causes the transformation of a positively charged group into a neutral group. In contrast to cationization, the anionic modification results in a change of only one unit charge per modified group.

We used the acetylation and hypochlorite modification as reactions to increase the anionic character of LDL. The former leads to acetyl derivatives and the latter to chloramines. In both instances the degree of modification was varied by the ratio of the amount of modifying agent per mg of LDL.

The modified LDL samples checked for purity by agarose gel electrophoresis and PAGE were characterized by single bands.

Fig. 2 shows the relative electrophoretic mobility of modified anionic LDL as a function of the concentration of added modifying reagent.

In Fig. 2a, results of acetylation carried out at 0 and 22°C are shown. As expected, the mild reaction conditions at 0°C effect only a slow increase in the relative electrophoretic mobility, in contrast to the high slope of the corresponding curves observed for samples prepared under conditions of a strong interaction between the reagent and lipoprotein.

Fig. 2b shows the corresponding changes observed with hypochlorite modification of LDL. The curves represent results obtained from two independent LDL preparations. In both instances the modification was carried out under identical conditions. Both curves are in qualitative agreement. The difference in the slopes reflects a different sensitivity of the two LDL preparations to hypochlorite and appears to be connected with donor specificity.

In Fig. 3 the content of functional groups is shown as a function of the concentration of added modifying reagent. In agreement with the

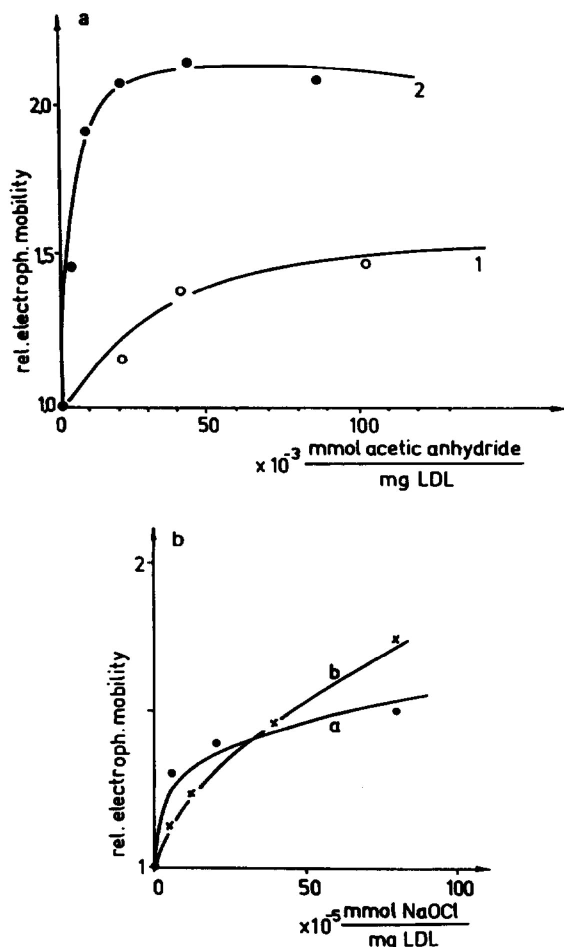

Fig. 2. Relative electrophoretic mobility for modified anionic LDL (results of agarose electrophoresis) (a) Acetylated LDL. Acetylation carried out at (1) 0 and (2) 22°C. (b) Results for NaOCl-modified LDL. Modification carried out under identical conditions for two separate LDL preparations (curves a and b).

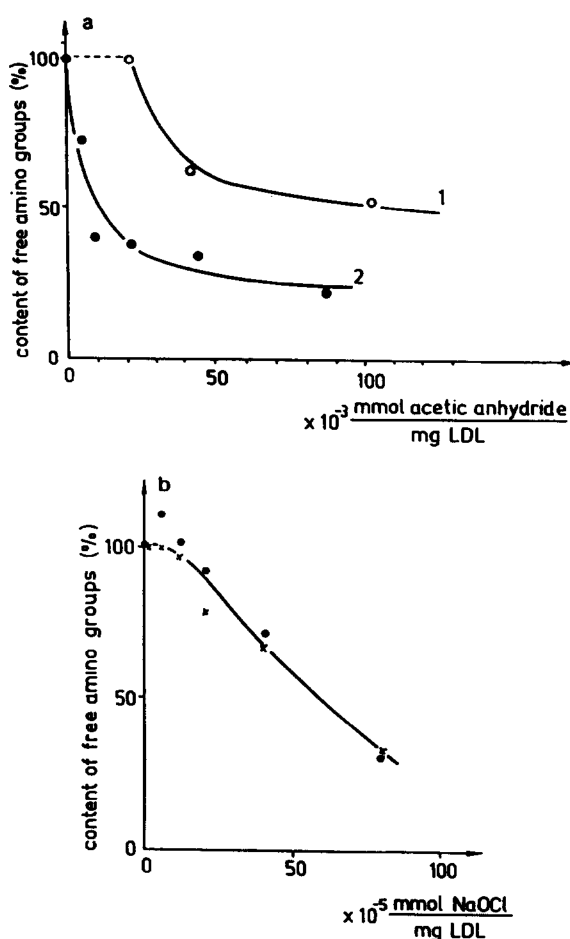

Fig. 3. Content of functional groups in modified LDL as a function of the concentration of added modifying reagent. (a) Acetylation carried out at (1) 0 and (2) 22°C. (b) NaOCl modification of two separate LDL preparations.

results given by measurements of the electrophoretic mobility (Fig. 2a), it can be seen that the level of unreacted groups remains higher with mild modification of LDL than with stronger modification (Fig. 3a). Only for ratios higher than $20 \cdot 10^{-3}$ mmol of acetic anhydride per mg of LDL could a decrease in the content of functional groups be detected, if the modification took place at 0°C.

Chloramine derivatives of the NH_2 groups produced by hypochlorite modification cause a nearly linear decrease in the content of free groups (Fig. 3b).

In Fig. 4 the calculated partition coefficient is shown as a function of the degree of modification.

The degree of modification was obtained from measurements of the amount of free functional amino groups using the fluram method. The absolute number of free groups determines the reactivity of the LDL particle. This number for the unmodified state is always set at 100% (degree of modification = 0). We assume that this number and the attainable maximum degree of

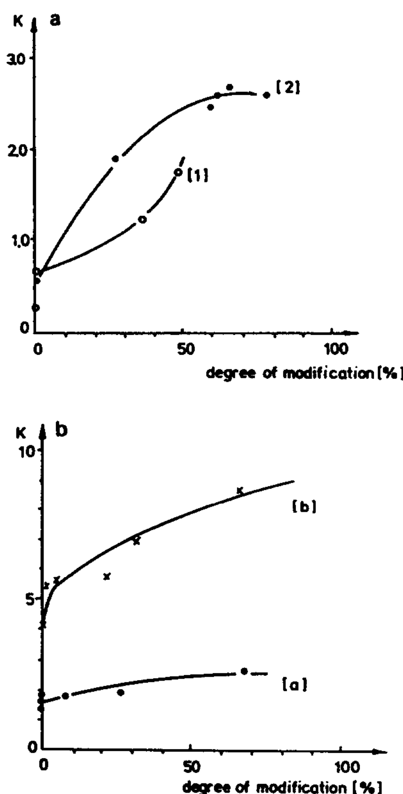

Fig. 4. Partition coefficient as a function of the degree of modification for modified anionic LDL. The degree of modification was determined from the data in Fig. 3a and b. Composition of the two-phase system: 7.1% dextran T 500–5% PEG 6000–10 m*M* Tris·HCl (pH 7.4). (a) Partition coefficient of acetylated LDL with acetylation carried out at (1) 0 and (2) 22°C. (b) Partition coefficient of NaOCl-modified LDL for two separate LDL preparations (curves a and b).

modification depend on the molecular structure of the particle surface, *i.e.*, on its steric conformation. It represents a specific property of the particle. Therefore, a special degree of modification may have different quantitative effects on the electrophoretic mobility and the partitioning of LDL particles.

The curves are characterized by a strong increase in *K* at low degrees of modification. The *K* values for acetylated LDL at 22°C are considerably higher than the values calculated for material modified at 0°C (Fig. 4a). For hypochlorite-modified LDL, differences between the LDL samples of two different donors are detectable (Fig. 4b).

Changes in partitioning and electrophoretic mobility of hypochlorite-modified LDL are clearly visible at low NaOCl concentrations, where the relative number of amino groups remains nearly constant. In this range surface properties of LDL other than those based on direct interaction between hypochlorite and amino groups could contribute to changes in surface charge [26]. The possibility of lipid oxidation by hypochlorite has to be taken into account, but experimental results given by different workers are conflicting [35,36]. An influence of OCl^- ions on the electric potential of the top phase similar to that of Cl^- ions can be excluded, because the lifetime of the OCl^- ion is very short [37]. Within a few seconds the HOCl–OCl^- system reacts completely under conditions of an excess of target groups.

A general result in the experiments dealing with anionic modification of LDL is the increased top-phase accumulation of LDL with increasing negative surface charge of the particles. These results are in agreement with the observed decrease in partitioning for cationic-modified LDL with increasing degree of modification.

Especially small changes in surface charge representing low degrees of modification are detectable with high sensitivity. Small changes in surface charge effect a strong increase in the *K* value.

As expected, samples with a high negative electrophoretic mobility are characterized by high values of the partition coefficient.

In all experiments, measurements on controls and samples were performed in triplicate. The partition coefficient is always given as a mean value. The error connected with the calculation of the partition coefficient or with the determination of the relative mass distribution between the two bulk phases and the interface is a maximum of 20% using the experimental techniques described above.

When LDL was modified in a special manner, the same LDL preparation was used to obtain spectrofluorimetric, electrophoretic and spectro-

photometric data. The results in Figs. 2–4 are representative examples.

To be certain that all experiments were performed with LDL in its native state, for the acetylation at 4 and 22°C and for the NaOCl modification freshly prepared LDL from different donors was always used. A statistical study of the physiological variation of measured values was not the aim of this investigation.

The obtained changes in electrophoretic mobility and the content of free functional groups for modified LDL as a function of the degree of modification are in agreement with the calculated values of the partition coefficient.

4. Conclusions

The two-phase method is a suitable technique for detecting alterations in the surface properties of lipoproteins. Nevertheless, the partitioning of particles is always accompanied by an accumulation of aggregated particles at the interface [38]. This property of particles often leads to errors connected with the application of this method. Moreover, after a well defined macroscopic interface has formed, clearing of the top phase can be observed [39].

One can retain the native state of LDL samples for only a limited time. An increased storage time of the LDL samples leads to a strong accumulation of aggregated LDL in the interface during phase partitioning. The larger the particle size as a result of aggregation and denaturation, the stronger is the interphase accumulation.

When using this method to characterize lipoproteins, it is necessary carefully to standardize the experimental techniques.

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