with the control in chylomicrons, reduced in VLDL and LDL, but increased in HDL. The sharp increase in protein in the composition of HDL, by contrast with the atherogenic fractions of LP was not accompanied by any proportional rise in ChS or TG. These data suggest that after administration of protamine elevation of the apoprotein A level was observed in the composition of HDL, but not an increase in the number of their particles. Since restoration of the normal structure of the plasma membranes of cells can take place under conditions of cholesterolosis as a result of removal of the excess of free ChS with the aid of apoproteins of HDL [4], this fact can be regarded as a manifestation of the antiatherogenic action of protamine.

Evidence in support of this view is given by data on recovery of the ChS concentration of the rat liver, when raised as a result of feeding the animals with stearin, in response to injection of protamine. Meanwhile, just as in rabbits, protamine did not prevent accumulation of ChS in the blood serum (Table 2).

Thus, in the early stages of HchS administration of protamine leads to changes in the composition of the blood serum LP. Compared with the control, a marked increase in the concentrations of protein components was observed in HDL, whereas in LDL the fractions of ChS and TG rose sharply.

LITERATURE CITED

1. G. Kh. Bozhko, P. V. Voloshin, V. P. Kulabukhov, et al., Zh. Éksp. Klin. Med., **28**, 466 (1988).
2. G. Kh. Bozhko, L. S. Kostyukovskaya, and V. M. Kulabukhov, 8th All-Union Congress of Neuropathologists, Psychiatrists, and Drug Addiction Specialists [in Russian], Moscow (1988), p. 322.
3. V. M. Kulabukhov, P. V. Voloshin, and L. S. Kostyukovskaya, Ukr. Biokhim. Zh., **58**, 27 (1987).
4. N. V. Perova, Kardiologiya, No. 6, 5 (1989).
5. L. B. Foster and R. C. Dunn, Clin. Chem., **19**, 1077 (1973).
6. R. J. Havel, H. A. Eder, and J. M. Bragdon, J. Clin. Invest., **34**, 1345 (1965).

AFFINITY CHROMATOGRAPHY ON HEPARIN-SEPHAROSE UNDER REDUCING CONDITIONS AS A METHOD OF SELECTIVE ENRICHMENT WITH INDIVIDUAL ISOFORMS OF APOLIPOPROTEIN E

A. D. Dergunov, L. P. Aniskovich, and V. V. Shuvaev

UDC 616.153.96:577.112.856]-074:543.544

KEY WORDS: lipoproteins; apolipoprotein E; isoforms of apo E, affinity chromatography.

One of the most important apoproteins of the plasma lipoproteins, namely apolipoprotein E (apo E), is involved in the maintenance of functional integrity of the plasma lipid transport system, by constant exchange between triglyceride-rich particles and high-density lipoproteins (HDL); this state of dynamic equilibrium, moreover, is shifted toward the latter. In the process of intravascular lipolysis of chylomicrons and very low-density lipoproteins (VLDL) apo E is transported to the heaviest HDL on the surface of which esterification of cholesterol takes place under the influence of lecithin-cholesterol acyltransferase. The reverse transport of apo E to VLDL is coupled with the transport of the cholesterol esters formed [1, 11]. The leading role of apo E in the final stages of lipoprotein metabolism consists of its ligand function during interaction of lipoproteins with specific apo E- or apo B,E-receptors of the liver and extrahepatic apo B,E-receptors [10]. The existence of multiple isoforms of human

Department of Biochemistry, Research Institute of Preventive Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow.

(Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 110, No. 7, pp. 42-45, July, 1990. Original article submitted April 22, 1988.

0007-4888/90/0007-0894$12.50

apo E is explained as the result of combination of two independent variations of apo E: hereditary changes of the protein and post-translation modification of the principal isoforms with carbohydrate chains [14]. It has been suggested [14] that biosynthesis of apo E takes place under the control of four independent alleles in one genetic locus, each of them coding for one of the main components E-4, E-3, E-2, and E-2*; E-3, moreover, is the parental type of apo E. Cysteine-arginine substitutions are responsible for charge differences between the principal isoforms of apo E [14], which are manifested as 3 to 6 bands after fractionation of the proteins by isoelectric focusing. The four principal bands are called E-I, E-II, E-III, and E-IV, and their corresponding pI values are 5.50, 5.65, 5.85, and 6.1 [4]. It may be emphasized that different aspects of disturbance of lipoprotein metabolism are directly connected with predominance of one or other isoform of the apoprotein [10, 14], as a result of which the obtaining of a highly pure apo E and preparations of it rich in individual isoforms, a very urgent task.

EXPERIMENTAL METHOD

Samples of blood plasma from healthy donors were pooled. The VLDL fraction was isolated by double flotation in a density of 1.006 g/ml in a 45 TI rotor at 14°C for 24 h at 44,000 rpm. The plasma and supernatant were treated with phenylmethylsulfonyl fluoride (PMSF) up to 50 μM. After lyophilization the VLDL preparation was successively delipidized in an atmosphere of nitrogen: 1) by double extraction at 4°C with a mixture of absolute ethanol:diethyl ether (3:1) for 3 h; 2) by treatment for 1 h with a mixture of absolute ethanol:diethyl ether (2:1); 3) by extraction of the protein residue for 2 h with diethyl ether. Next, 40-50 mg of VLDL proteins, after complete solution in the course of 2 h at 4°C in 4-6 ml of a 6 M solution of guanidine chloride (GnCl)/0.01 M Tris-HCl, pH 8.0/10 mM dithiothreitol (DTT)/1 mM PMSF was applied to a column (2.6 × 80 cm) with sepharose CL-6B. The column was eluted with 4 M GnCl solution/0.01 M Tris-HCl, pH 8.0/1 mM DDT at the rate of 10 ml/h. After estimation of the distribution of proteins into fractions by electrophoresis in a polyacrylamide gel concentration gradient (5-20%) in a system with sodium dodecylsulfate (SDS) [8], the apo E-containing fractions were pooled and dialyzed against a 0.15 M solution of NaCl/1 mM Na_2-EDTA/0.02% sodium azide/0.01 M Tris-HCl, pH 7.4 (the "standard" solution). Affinity chromatography of the apo E-enrich preparation was carried out by the method in [4] with the addition of extra DDT in our experiments to the "standard" and eluting solutions. After mixing of the apo E-containing preparation, containing 5 mM DDT, with 14-16 ml of heparin-sepharose (1.5 mg heparin/1 ml gel), washed with the "standard" solution, made up as recommended in [3], the mixture was left overnight on a shaker. After separation of the unbrowned protein on a glass filter and packing into a column (1.6 ± 6.5 cm) the gel was washed with the "standard" solution with 1 mM DDT at the rate of 10 ml/h until absorption in the eluate disappeared. The apo E was successively eluted from the column by an increase in the NaCl concentration in the "standard" solution with 1 mM DDT up to 0.4 M, and later to 1.0 M. Analytical isoelectric focusing of the isolated apo E in 8.6 M urea was carried out by the method in [7], using Pharmalytes for the pH region 4-6.5. The control gels were cut into strips 1 cm wide to measure the pH profile and the Pharmalytes were extracted with 1 ml of 0.1 M KCl solution. Protein was determined by the method in [9] in the presence of 5% SDS. Glucuronic acid was determined by the method in [5] to assess the degree of "sewing" of the heparin to CNBr-activated sepharose 4B.

EXPERIMENTAL RESULTS

Preliminary experiments to study solubilization of VLDL apoproteins in the traditional [4, 6] system (6 M urea) revealed a great loss of apo E due to the combined precipitation of this apoprotein with the extremal hydrophobic VLDL apoprotein — apo B. Conversely, the use of 6 M GnCl as solubilizing agent led to complete dissolving of the apoproteins. It will be clear from the data on fractionation of the completely solubilized VLDL apoproteins on sepharose CL-6B in the presence of GnCl and DDT, illustrated in Fig. 1, that the apoproteins separated into three peaks. The separation factor of peak I from II was 0.85 and of peak II from III 0.93. According to the results of electrophoretic analysis of the apoproteins contained in these pools, peak I consisted of apo B and apo E, whereas peak III contained apo C — a fraction of low-molecular-weight proteins of VLDL. Peak II, containing on average 17% of protein, was represented mainly by apo E, and also by unidentified proteins. These proteins probably belong to apo B and its proteolysis products (in the high-molecular-weight region) and to apo A-1 (in the low-molecular-weight region). During isoelectric focusing of apo E, isolated by anion-exchange chromatography, apo A-1 also was determined (the basic protein of HDL with mol.wt. of 28 kD) as a minor component [12].

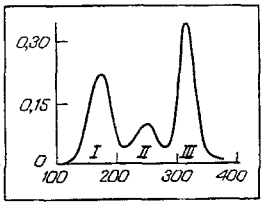

Fig. 1. Gel chromatography of apo preparation from VLDL on sepharose CL-6B. Abscissa, volume of eluate (in ml); ordinate, optical density at 280 nm. 48.6 mg of apoproteins applied to column. Distribution of apoproteins among fractions of peaks I, II, and III analyzed by electrophoresis in a system with SDS.

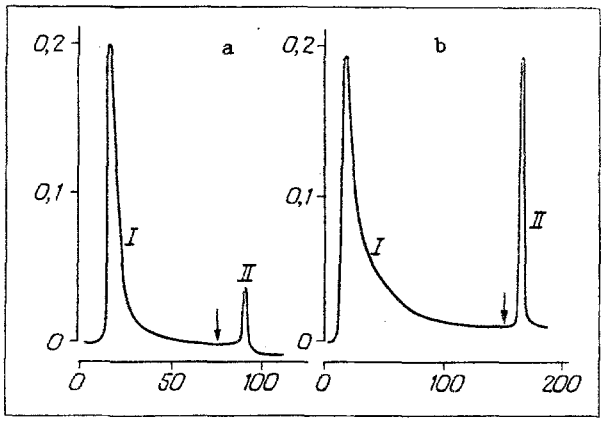

Fig. 2. Affinity chromatography of apo E-enriched preparation on heparin-sepharose 4B in absence (a) and in presence (b) of 1 mM DTT. Abscissa, volume of eluate (0.4 M NaCl solution in "standard" buffer, in ml); ordinate, optical density at 280 nm. Arrow indicates beginning of elution with 1.0 M NaCl solution in "standard" buffer. Aliquots of protein from peaks I and II analyzed by electrophoresis in a system with SDS and isoelectric focusing.

Further purification of apo E was carried out by affinity chromatography on heparin-sepharose under previously chosen conditions of equilibrium binding and absence of restrictions for the number of binding sites. To remove apo E the column was washed successively with solutions of 0.4 M NaCl (peak I) and 1.0 M NaCl (peak II, Fig. 2). In our experiments, compared with the conditions of fractionation described in [4], we added DDT as reducing agent. Addition of DDT to the "standard" and eluting solutions was a fundamental step in our work. This modification was due to the presence of high-molecular-weight protein components in both apo E pools obtained in the absence of a reducing agent, which was found after electrophoretic analysis of the proteins of peaks I and II. Probably apo E under these conditions forms complexes with different degrees of oligomerism, on account of intermolecular disulfide bonds. In the presence of DDT, high-molecular-weight components were absent in peaks I and II after electrophoresis, and the content of the stain in the apo E band exceeded 95%. Comparison of the data given in Fig. 2 shows that: 1) the ratio of the amounts of "high-affinity" (peak II) and "low-affinity" (peak I) apo E in the absence and in the presence of DDT were 0.06 and 0.13, respectively; 2) about twice the volume of 0.4 M NaCl solution was needed to remove all the "low affinity" apo E under reducing conditions. This behavior can be explained byu reduction of the degree of affinity or apo E for a carrier during the formation of protein complexes on account of charge changes, steric hindrances, and/or other factors. The molecular weight of apo E, determined from the dependence of mol.wt. on electrophoretic migration of proteins in the gel, was 33 kD. The protein content at the stages of isolation of apo E in one typical experiment was: 1) isolation of VLDL 196 mg;

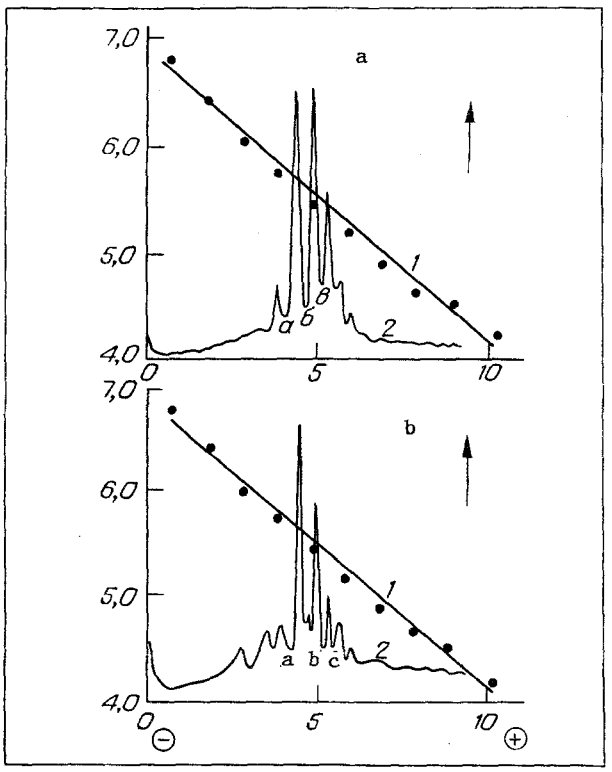

Fig. 3. Analytical isoelectric focusing of apo E from peak I (a) and peak II (b) after elution of apoprotein from heparin-sepharose. Abscissa, distance from start in CM; ordinate, pH values, vertical arrow corresponds in length to change of absorption at 633 nm by 0.4 (a) and 0.3 (b) optical density unit. 1) Profile of pH change in column of gel, 2) densitogram of proteins in gel, stained by Coomassie R-250. a, b, c) components of apo E with corresponding pI values of 5.56, 5.43, and 5.33.

2) delipidation of VLDL 162 mg; 3) gel-filtration on sepharose CL-6B (peak II) 27.8 mg; 4) affinity chromatography on heparin-sepharose 4B; peak I, 8.4 mg; peak II, 0.85 mg.

To study isoforms of apo E, analytical isoelectric focusing was carried out of aliquots of apo E from peaks I and II in urea (Fig. 3). The three main components were characterized by pI values of 5.33, 5.43, and 5.56, respectively. It can be tentatively suggested that these components correspond to the positions of E-I, E-II, and E-III. The fact that our determined values of pI are somewhat lower than those obtained by other workers [4, 13] can evidently be explained by the different temperatures at which the pH profile was measured and the actual process of focusing was carried out. During measurement of pH in solution containing urea all directly on the surface of the gel containing urea, it is also possible that the values obtained may be too high by up to 0.3 pH unit [2], whereas in our experiments the pH values were determined after the pieces of gel had been wetted in 10 volumes of 0.01 M KCl solution. When the results of isoelectric focusing of apo E from peaks I and II are compared (Fig. 3), it is clear that in the "high-affinity" apo E the protein content was considerably higher (by 1.6 times) in the hypothetical E-III position than in the hypothetical E-II and E-I positions. The relative enrichment with respect to one isoform of apo E with the greatest positive charge can evidently be explained by "removal" of isoforms with a smaller positive charge after the gel had been washed with 0.4 M NaCl solution with negatively charged heparin molecules.

Thus, in the present investigation, in order to obtain an apo E preparation with a high degree of purity in preparative amounts, compared with the scheme described in [4], the following modifications were used: 1) the process of gel-filtration fractionation of total proteins of VLDL (in order to enrich the protein preparation relative to apo E); 2) the composition of the eluting solutions for affinity chromatagraphy (to reduce intermolecular disulfide bonds). A preparation of apo E enriched with respect to the isoform with the greatest positive charge was obtained, and this may be useful for the study of the mechanism of regulation of activity of lipoprotein-metabolizing enzymes by isoforms of apo E.

LITERATURE CITED

1. A. N. Klimov, K. A. Kozhevnikova, N. N. Klyueva, et al., Mol. Biol., **18**, 404 (1984).
2. L. A. Osterman, Investigation of Biological Macromolecules by Isoelectric Focusing, Immunoelectrophoresis, and Radioisotope Methods [in Russian], Moscow (1983).
3. Affinty Chromatography. Principles and Methods, Pharmacia Fine Chemicals (1978).
4. E. N. Avila, G. Holdsworth, N. Sasaki, et al., J. Biol. Chem., **257**, 5900 (1982).
5. T. Bitter and H. M. Muir, Analyt. Biochem., **4**, 330 (1962).
6. A. Frank, F. A. Shelburne, and S. H. Quarfordt, J. Clin. Invest., **60**, 944 (1977).
7. R. E. Greeg, G. Ghiselli, and H. B. Brewer, Jr., Biochim. Biophys. Acta, **794**, 333 (1984).
8. U. K. Laemmli, Nature, **227**, 680 (1970).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, J. Biol. Chem., **193**, 265 (1951).
10. P. W. Mahley and T. L. Innerarity, Biochim. Biophys. Acta, **737**, 197 (1983).
11. R. W. Mahley, T. L. Innerarity, S. C. Rall, Jr., and K. H. Weisgraber, J. Lipid Res., **25**, 1277 (1984).
12. F. A. Shelburne and S. H. Quarfordt, J. Biol. Chem., **249**, 1428 (1974).
13. G. Utermann, Hoppe-Seyler's Z. Physiol. Chem., **356**, 1113 (1975).
14. V. Y. Zannis and J. L. Breslow, Biochemistry, **20**, 1033 (1981).

MODEL OF DAMAGE TO CELL MEMBRANES DURING PHOTODYNAMIC THERAPY: PHOTOSENSITIZATION OF PLANAR LIPID BILAYER BY HEMATOPORPHYRIN DIMETHYL ESTER

I. N. Stozhkova and V. M. Mirskii

UDC 615.831.065.014.44:616-008.939.15-092.4

KEY WORDS: photodynamic therapy; hematoporphyrins; model membranes.

The use of photosensitizers, with the ability to accumulate selectively in the tissues of a tumor and to destroy it during illumination, is a promising method of treatment of malignant neoplasms [1, 3, 6]. Hematoporphyrin derivatives possess such properties. It has been suggested that one of the main factors in phototoxic action is damage to cell membranes [7], but a detailed investigation of these processes in vivo is limited by the narrow range of conditions within which the viability of the experimental object is not disturbed. The use of model membrane systems offers wider opportunities. Such investigations have been conducted on suspensions of liposomes [4, 5], but this system can be used to record only averaged effects. To study the mechanism of formation of single membrane defects during photodynamic therapy, it is interesting to model this process on a planar bilayer lipid membrane (BLM).

EXPERIMENTAL METHOD

A BLM was formed from 50 mg/ml of a solution of L-α-phosphatidylcholine ("Sigma," USA; type II-S) in decane (chemically pure, from Reakhim) on holes 0.8 mm in diameter in a sheet of Teflon gauze. The electrical characteristics were recorded by means of Ag-AgCl electrodes. Conductivity was measured by applying a constant voltage of 200 mV to the BLM,

Laboratory of Bioelectrochemistry of Interphase Boundaries, A. N. Frumkin Institute of Electrochemistry, Academy of Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR Yu. A. Vladimirov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 110, No. 7, pp. 45-46, July, 1990. Original article submitted July 25, 1989.

 0007-4888/90/0007-0898$12.50