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Note

Flat-bed isoelectric focusing of high-density lipoproteins

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The subfractionation of high-density lipoproteins (HDL) by isoelectric focusing (IEF) has been described by several authors [1—16], who reported a remarkable heterogeneity within this lipoprotein class. Nevertheless, no reliable classification of the observed subclasses could be achieved, since these results did not correspond to each other in respect to number of subfractions, p*I* values and relative amounts. As these differences may be due in part to the methodological approach used, a new method for a rapid and reproducible subfractionation of HDL by IEF was developed.

METHODS AND MATERIALS

Normolipidemic human serum and the same serum after removal of very-low-density and low-density lipoproteins (VLDL and LDL, respectively) by polyanion precipitation were investigated. Freshly drawn fasting venous blood was allowed to clot for 2 h at room temperature. Then it was centrifuged at 900 *g* and 4°C for 20 min. Polyanion precipitation was performed using dextran sulphate (MW 500,000; Pharmacia, Uppsala, Sweden) and mag-

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nesium chloride (Merck, Darmstadt, F.R.G.) exactly as descibed by Kostner et al. [17], and the supernate (DX-HDL) was used for IEF.

IEF was carried out in an LKB multiphor chamber (LKB, Bromma, Sweden). The 1% agarose (Agarose EF; LKB) slab gel (24 × 11 cm), which was prepared according to an application note of LKB [18], was 0.5 mm thick and contained 3% Ampholine, pH 4.0–6.5. Running conditions were 10°C, 4 W, 80 min. In preliminary studies the samples were applied either near the cathode or near the anode to make sure that the subfractions really reached their isoelectric point at the end of the run. Since the region of pH 6.0 was found to be free of lipoproteins, this area was later chosen for application of samples. After the IEF run, the detection of HDL subfractions was achieved by immediate polyanion precipitation of lipoproteins in the agarose gel slab as described by Seidel [19]. pH Values were measured with a surface electrode (LKB) immediately after the IEF run.

In order to show the apoprotein content of the IEF subfractions, immunoelectrophoresis in the second dimension was carried out after IEF. For this purpose, a strip (5 × 60 mm) which contained freshly focused sample (serum or DX-HDL) was cut out of the IEF gel, placed on a 7 × 7 cm glass plate and immediately covered with 1% agarose in Tris buffer, pH 8.6, which contained 4% antiserum against apolipoprotein A-I (Behring, Marburg, F.R.G.). Running conditions for the immunoelectrophoresis were 20°C, 12 h, 30 V.

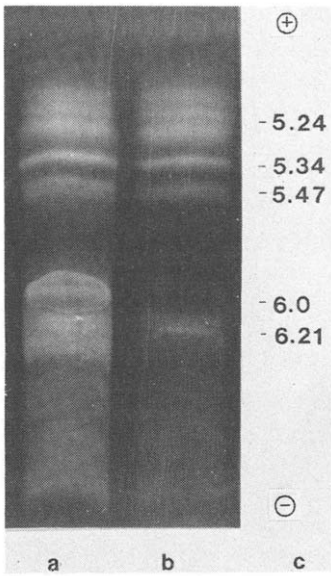

Fig. 1. Precipitation pattern of HDL after IEF. (a) Whole serum of a normolipemic male, age 37. (b) Supernatant after removal of VLDL and LDL by polyanion precipitation. (c) *pI* Values of subfractions obtained.

RESULTS

Representative patterns of lipoprotein subfractions of whole serum and DX-HDL after IEF with subsequent precipitation are shown in Fig. 1. These

patterns resulted regardless of whether the samples had been applied near the cathode or near the anode. Three groups of bands can be visualized at pH 5.47, 5.34 and around pH 5.24. It is likely that the subfractions at pH 5.47 and 5.24 consist of two and three subunits, respectively. The main difference between the pattern of whole serum and DX-HDL is the absence in the latter of bands at the application spot and near the cathode in the pH range 6.1—6.4. They contain apoprotein B (unpublished results) and are mostly removed by precipitation of VLDL and LDL by dextran sulphate. A remaining diffuse band at pH 6.21 is unidentified. According to preliminary results it contains neither apoprotein B nor apoprotein A-I or A-II. The band pattern of both samples in the range between pH 5.2 and 5.5 showed excellent agreement. It represents the subfractions of HDL. This HDL pattern has apparently not been influenced by the precipitation of VLDL and LDL with dextran sulphate.

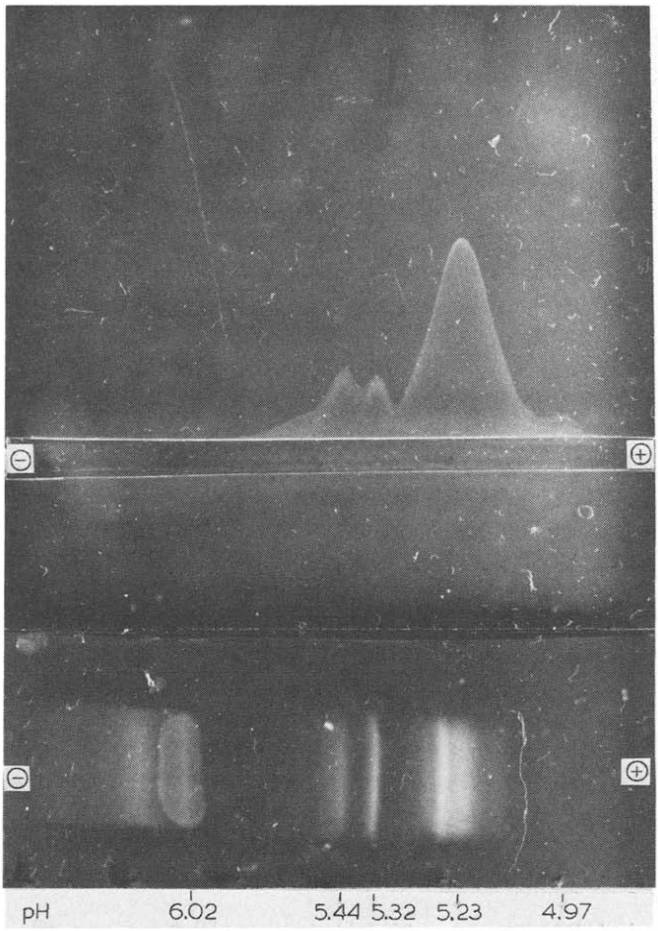

Fig. 2. Above: crossed immunoelectrophoresis after IEF of the supernatant of DX-HDL of VLDL/LDL precipitation using antilipoprotein A-I. Below: precipitation pattern of the same sample (normolipemic female, age 25).

The aim of the immunoelectrophoresis in the second dimension was to show that the bands which can be precipitated in the agarose slab after IEF indeed contain lipoproteins. For this purpose we employed antiserum against apoprotein A-I, the major apolipoprotein of HDL. Fig. 2 shows the precipitation pattern of the subfractions of DX-HDL. It was photographed together with a parallel agarose strip from the same IEF run in which HDL subfractions had been precipitated as described below. Again, one major and two minor peaks were observed, which correspond in location to the regions of the HDL bands precipitated in the original IEF plate. A fourth little peak is found at the anodic side (pH 4.97) of the large peak, and corresponds to a very weak band in the precipitation pattern. It is likely that the major peak observed is composed of more than one reaction peak; the lack of shoulders corresponding to the precipitation pattern may be explained by diffusion of the narrow bands during the time which passed between IEF and second-dimension electrophoresis (about 30 min).

DISCUSSION

IEF has rarely been used as an analytical tool for the subfractionation of HDL. A reason for this may have been the inconsistent and discrepant results in the literature which in our view may be due to the method applied by the majority of previous investigators, i.e. vertical IEF in a sucrose gradient. This procedure is time-consuming, may cause problems in reproducibility and does not allow measurement of the pH in the medium itself. Flat-bed IEF, in contrast, overcomes the disadvantages and furthermore offers the possibility of application of samples near the cathode as well as near the anode, which allows the exclusion of focusing artifacts which result in false apparent p*I* values.

Our results show that IEF in flat-bed agarose can clearly and rapidly separate HDL into at least three subfractions whose p*I* values are well reproducible. For rapid visual detection, these subfractions can be precipitated in the IEF agarose. The precipitated bands correspond well to the peak pattern produced by crossed immunoelectrophoresis with antiserum against apolipoprotein A-I which proves that the observed bands indeed represent HDL subfractions.

Number and p*I* values of the subfractions obtained do not agree with the results of other investigators. The main difference is the narrow pH range (pH 4.9—5.5) in which the HDL subfractions were focusing, while most authors found ranges between pH 4.0 and 6.0 [2, 9, 14—17]. Only Kostner et al. [6] and Eggena et al. [3] describe ranges somewhat similar to ours (pH 4.6—5.1 and 4.7—5.4, respectively) but their results differ from ours in the number of subfractions (Eggena et al.) as well as in the p*I* values of individual subfractions (Kostner et al., Eggena et al.). A possible reason for these discrepancies may be that most authors measured the pH values at room temperature and not at the temperature at which the IEF had been carried out [2, 5, 8, 9, 11—14]. Furthermore, it remained unexplored to what extent the sucrose content of the focusing solutions affected the determination of pH values. Since increasing temperatures decrease pH values, and sucrose

or glycerol also influence pH measurements, both parameters must be taken into account [20]. Our method, in contrast, allows measurement of the pH right on the surface of the cooled agarose and is not influenced by sucrose. Since the subfractions are found with identical p*I* values, regardless of whether the sample was applied near the anode or near the cathode, this focusing technique appears to be more reliable. It is of interest to note that the pH range in which we observed focusing of the intact HDL subfractions closely matches the p*I* range of delipidized aproprotein A-I (p*I* = 5.60) and apoprotein A-II (p*I* = 4.88) [21].

Of the two kinds of sample which we investigated (whole serum and DX-HDL) the latter is, in our view, the more appropriate for further studies, since LDL and VLDL are removed and the remaining HDL particles are apparently not altered by the addition of dextran sulphate. This advantage of DX-HDL over whole serum gains relevance especially in hypertriglyceridemia where one may find a considerable distortion of the IEF pattern of whole serum by interfering triglyceride-rich lipoproteins (unpublished observation).

Since HDL has several metabolic origins and functions, the present method may be a helpful tool for further studies of lipid metabolism and of lipoprotein disorders. It seems to be specially useful for the subfractionation of HDL in situations where the number of samples to be analyzed is very large, such as for clinical screening and epidemiological studies, or where the available amount of sample is very limited (e.g. studies in infants and small laboratory animals).

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