ORGANIC EXTRACTION AND PREPARATIVE ISOELECTRIC FOCUSSING OF RAT SERUM APOLIPOPROTEINS

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1. Introduction

The isolation and separation of serum apolipoproteins from both rats and human subjects has traditionally been achieved by delipidation followed by gel permeation chromatography in denaturing solvents and/or ion exchange chromatography [1–5]. These techniques often result in large eluate volumes of low protein concentration. In addition to characterization studies these preparative techniques have been used increasingly for the production of antigens to raise monospecific antibodies for quantitation of the native lipoprotein in serum [6–9], hepatic perfusates [10–11] and for immunochemical localization studies in tissue slices [12]. The preparative isoelectric focussing technique [13] was modified and applied to the separation of human serum apolipoproteins [14]. This technique has the distinct advantage of very clearly separating most of the apoproteins commonly found in serum lipoprotein fractions. In addition, it will separate the various isomorphic forms of apo A-I apo C and apo E in a single run. Thus this technique is both rapid and of higher resolving power than either gel permeation chromatography or ion-exchange chromatography alone.

The rat is extensively used for studies on lipoprotein biosynthesis and catabolism. These experiments often necessitate the isolation of pure apolipoproteins and the production of monospecific antibodies to them for subsequent quantitative immunochemical procedures. This paper details the application of organic extraction techniques and preparative isoelectric focussing to the separation and isolation of pure rat serum apolipoproteins and illustrates the high degree of antigen purity achieved by this method.

2. Experimental

Male hooded rats (Canadian Breeding Farms, St Constant, PQ) 250–300 g were rendered hypercholesterolemic by feeding a thrombogenic diet (ICN Biochemicals, Montreal, PQ) containing 40% butter fat, 2% cholate and 0.3% propyl thiouracil over a 6 week period. This treatment results in a very significant increment in serum apo E levels [10]. Serum lipoproteins were isolated by ultracentrifugation at the following densities: VLDL, d = 1.006 g/ml; LDL-1, d = 1.006–1.063 g/ml; and HDL, d = 1.085–1.21 g/ml [15]. All lipoprotein fractions were washed once by ultracentrifugation prior to delipidation with ethanol–ether (3:1) [16] or acetone–ethanol (1:1) [17]. Quantitative electroimmunoassay as described [19] was performed as in [6]. Gel permeation chromatographic separations of apo-VLDL were as in [19].

2.1. *Analytical and preparative electrophoresis*

Urea (7 M) polyacrylamide disc-gel electrophoresis was performed as in [10]. Analytical polyacrylamide gel isoelectric focussing was by the method in [16] using a pH 4–6 ampholine gradient (Pharmacia Fine Chemicals, Upsalla) in the presence of 7 M urea. Urea gels were stained with amido black 10B and isoelectric focussed gels by the method in [20]. Preparative flat-bed isoelectric focussing was as in [14]. All apoproteins were solubilized and fractionated in the presence of 0.1% β-mercaptoethanol or 0.1% dithiothreitol.

2.2. *Organic extraction of lipoproteins*

Acetone and isopropanol extractions of rat-serum lipoproteins were done as in [22] for human lipoproteins. The organic extracted apoproteins were further delipidated after dialysis and lyophilization [21] with

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either acetone–ethanol (1:1) or ethanol–ether (3:1) prior to electrophoretic analysis.

2.3. *Antibody production*

Antibodies against purified apoprotein fractions were raised in New Zealand white rabbits by weekly multiple site intradermal injections of 100 μg antigen/animal, emulsified in complete Freund's adjuvant (Difco Labs). After generation of high titre antibody the rabbits were exsanguinated, the γ-globulin fraction prepared from the serum [22], lyophilized, sealed in moisture-free ampoules and stored at −20°C.

3. Results and discussion

Studies of rat-serum apoproteins, with the exception of apo B and apo A-I, whether they be for protein chemistry or purification for the production of antibodies are, unlike human studies, hampered by the relatively low volume of serum obtainable from one rat, the low concentration of lipoproteins and thus the necessity of killing large numbers of animals to obtain enough apoprotein for preparative procedures. Fortunately there is currently good information available concerning the biosynthesis and metabolism of rat-serum lipoproteins and the modulation of these parameters by dietary regimes. Thus the elevation of serum apo E levels and the accompanying shift of this apoprotein from the HDL to the VLDL and LDL-1 density ranges, which is induced by feeding a high fat (thrombogenic) diet supplemented with propyl thiouracil, can be exploited to increase the yield of apo E/ml serum [10,23]. Similarly, feeding a high carbohydrate diet for 10 days to increase hepatic VLDL synthesis [24] followed by lipolytic blockade with Triton WR-1339 [25] will result in the accumulation of apo C-II, C-III and apo E in the VLDL density range. Fig.1 shows a print taken from a preparative isoelectric focus run of 60 mg rat apo HDL. The separation achieved is typical of the technique. The apoprotein bands were characterized by their isoelectric points and designated according to [16,26]. Of particular significance is the marked separation achieved between the apo A isomorphs and those of apo E. Gel permeation chromatography using BioGel A-1.5 M is relatively poor in this respect and apo A-I (mol. wt 27 000) and apo E (mol. wt 35 000) tend to co-chromatograph [19]. Also of note is the fact that low molecular weight rat apoproteins, apo A-II,

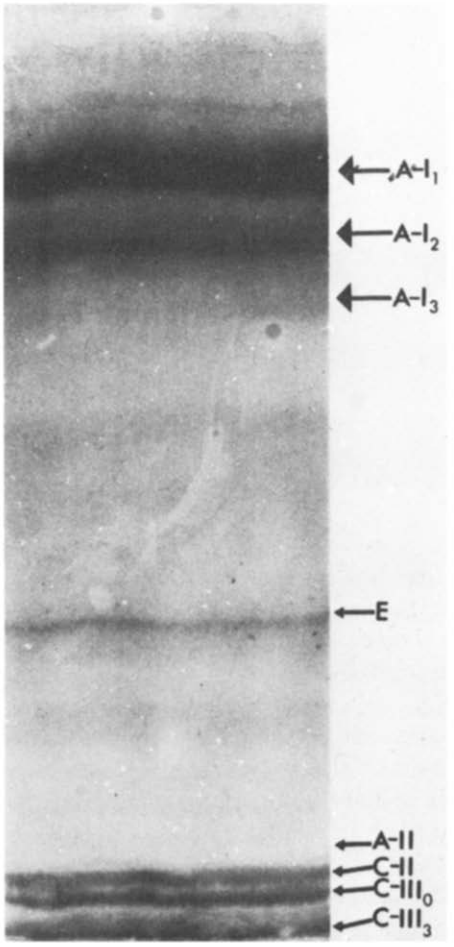

Fig.1. Paper print of a preparative isoelectric focus of rat HDL apoproteins (60 mg) in a pH 4–6 ampholine gradient according to [13]. Apoproteins were designated as in [16] and had similar pI points. The apo HDL was prepared by acetone–ethanol delipidation [17] of serum HDL.

C-II and the isomorphic forms of C-III are also resolved by the preparative isoelectric focussing technique. Following elution, dialysis and lyophilization of the apoprotein fractions from the preparative isoelectric focussing plate, they were analyzed by polyacrylamide gel isoelectric focussing to establish their degree of homogeneity. Fig.2 shows an analytical isoelectric focussing gel of apo HDL and each of the 3 apo A-I fractions isolated from the preparative run shown in fig.1. It can be clearly seen that while each fraction contains predominantly one apo A-I isomorph, it is substantially contaminated with the other two isomorphic forms. This is as yet of little consequence with respect to antibody production as the only

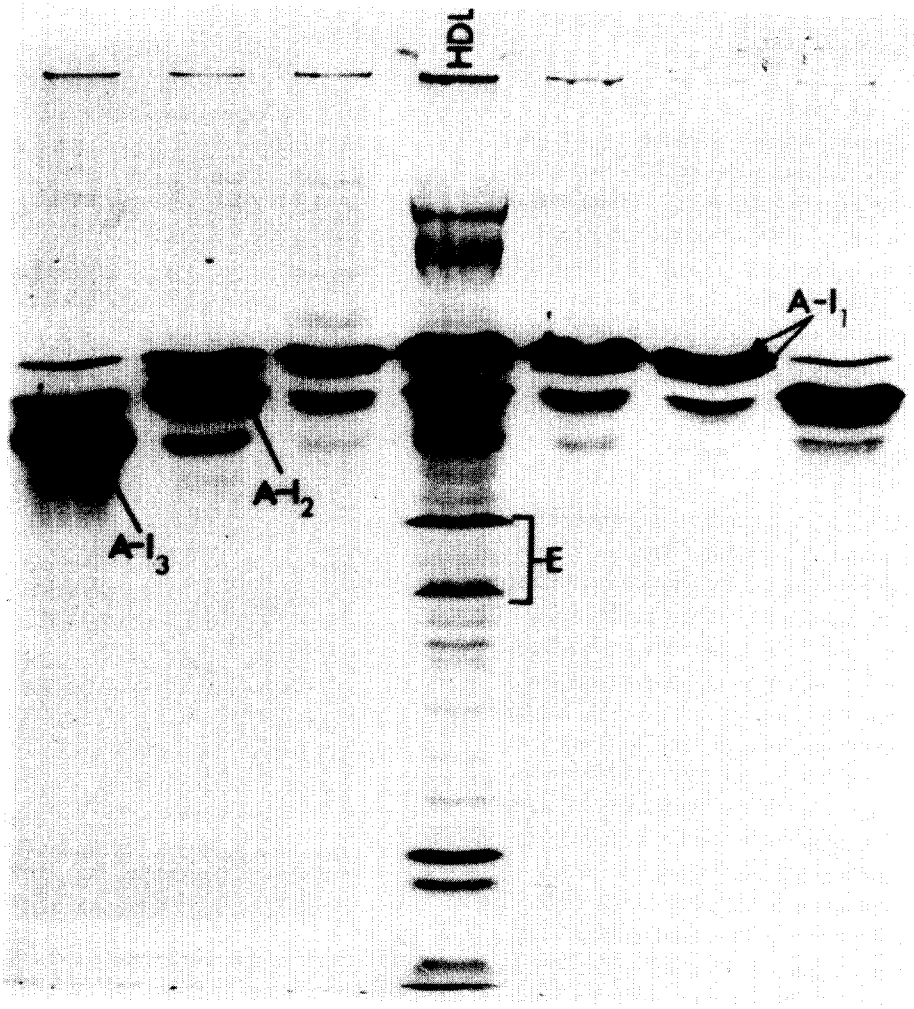

Fig.2. Analytical 7.5% polyacrylamide slab-gel isoelectric focus of rat apo HDL and each of the apo A-I isomorphs fractionated in fig.1. The pH gradient was 4–6 as described in [16]. The three bands between the origin and the apo $A\text{-}I_1$ band of HDL represent rat albumin.

reported difference between apo A-I isomorphs is their degree of sialylation [16]. Much purer preparations of individual A-I isomorphs are obtained after a second preparative isoelectric focussing run of the enriched apo A-I isomorph fractions shown in fig.2. The most significant point shown by fig.2 is the complete lack of apo E or albumin (the most cathodic bands in HDL) in the isolated apo A-I fractions. Fig.3 shows an analytical polyacrylamide gel isoelectric focus of the isolated low molecular weight proteins (A-II, C-II, C-III) together with apo E. Apo HDL is shown as a reference. Each of the isolated low molecular weight protein fractions show a small, but antigenically significant, contamination with other low molecular weight apoproteins. These minor contaminants are reduced to undetectable levels by refocussing each of the isolated low molecular weight fractions shown in fig.3. The apo E fraction analyzed in fig.3 was isolated from VLDL rather than HDL and is seen to be free from contamination by albumin and apo A-I, both of which co-chromatograph with apo E on gel permeation chromatography of apo VLDL [19]. Any or all of the apo E isomorphs can be used to generate antibodies against apo E as, like apo A-I, the basis of rat apo E isomorphism appears to be sialic acid [27].

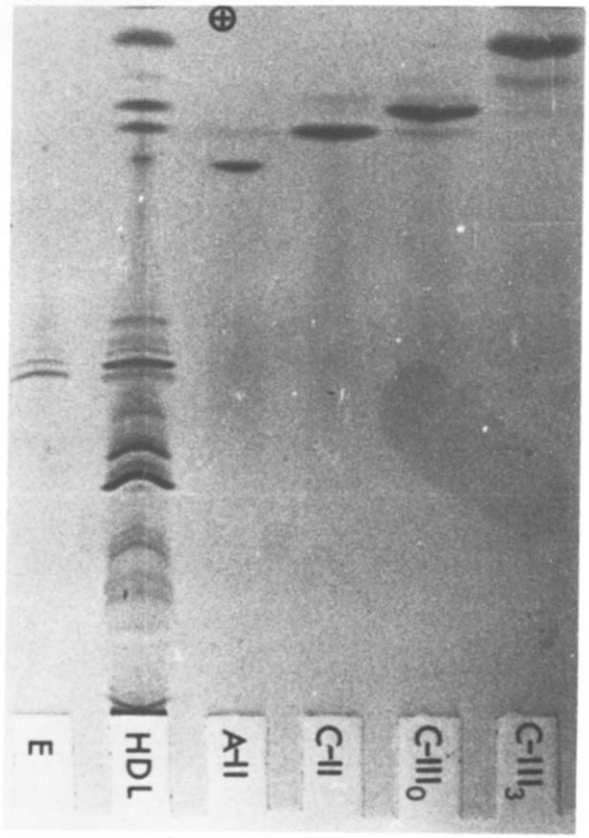

Fig.3. Analysis of rat apo C-II, C-III and A-II fractions obtained by preparative isoelectric focussing of apo HDL (fig.1) by analytical polyacrylamide slab-gel isoelectric focussing in a pH 4–6 gradient [16]. Apo E was prepared from delipidated [16] rat VLDL by preparative isoelectric focussing as in fig.1.

Of critical importance in generating reproducible isomorphic patterns for rat apo E is the method used finally to delipidate the apoproteins prior to isoelectric focussing. Rat apoproteins and their isomorphs, as separated by analytical gel isoelectric focussing, were first characterized in [16,26]. In [16,26] ethanol–ether (3:1) was the principal delipidating agent and the pattern obtained, which is both stable and reproducible for apo VLDL analyzed by the method in [16,26] is shown in fig.4A. Band E-3 corresponds in relative concentration and pI point to the pI 5.41 apo E isomorph [16]. Application of the ethanol–acetone (1:1) delipidation method [17] to rat VLDL followed by analytical gel isoelectric focussing of the apoproteins yields the apo E isomorph pattern shown in fig.4B. Again the pattern is stable and reproducible provided ethanol–acetone (1:1) is used as the delipidating solvent. There is no evident impact of the choice of delipidating solvents on the resolution of the apo C. However the divergence in

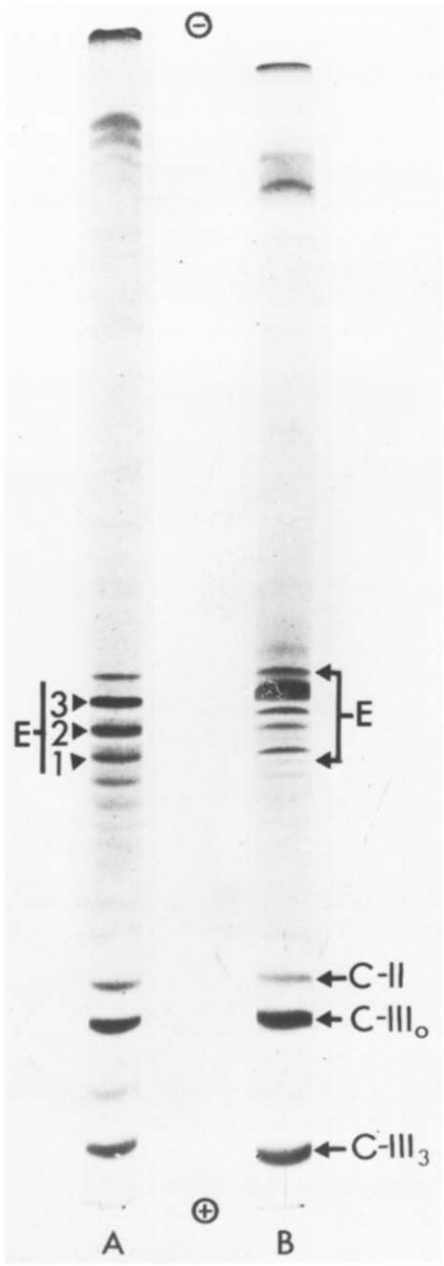

Fig.4. Analytical 7.5% polyacrylamide disc gel isoelectric focus (pH 4–6) of rat apo VLDL. (A) VLDL delipidated by ethanol-ether (3:1) [16]; (B) VLDL delipidated by acetone–ethanol (1:1) [17]. Note the differing isomorph patterns for apo E.

apo E pattern between the methods may be due to residual, low level contamination of the apoproteins with phospholipids.

The resolution of apoprotein bands on preparative flat bed isoelectric focussing is dependent upon both the pH gradient range used and the amount of apoprotein applied although in the latter case preparative isoelectric focussing has a very large theoretical capacity. Obviously if it is only required to fractionate and purify the C apoproteins or apo E, the application of a technique prior to preparative isoelectric focussing that could enrich the protein fraction which was to be applied to the preparative isoelectric focussing, with the protein or proteins in question, would be highly desirable. An organic extraction technique developed and applied [21] involved successive extractions of human VLDL first with acetone, to extract the C apoproteins and later with isopropanol to extract apo E. Fig.5A shows the results obtained when rat serum VLDL and HDL were extracted with acetone as in [21]. Acetone preferentially extracts apo C-II and C-III from the rat VLDL, there being no significant contamination with apo B or apo E. The extract does however contain a very minor band which is also present in the apo VLDL and co-migrates with apo A-II. Application of acetone extraction to rat serum HDL was also of considerable use. The acetone extract was significantly enriched in apo C-II, C-III and apo A-II and to a smaller extent apo-E when compared to normal rat serum apo-HDL, the predominant apoprotein of which is apo-A-I. Fig.5B demonstrates that isopropanol is effective in extracting apo

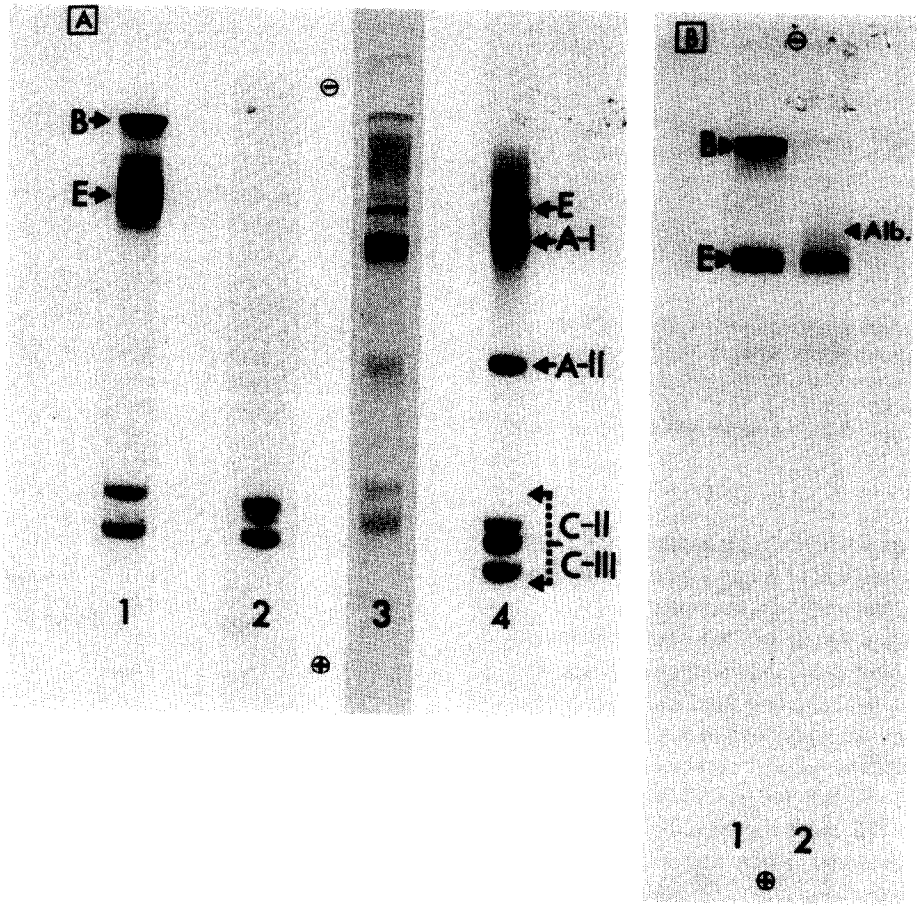

Fig.5. Urea (7 M), 10% polyacrylamide disc-gel electrophoretic analysis [10] of rat serum apo VLDL, apo LDL-I from hypercholesterolemic rats and apo HDL together with their respective acetone and isopropanol extracted apoproteins [21]. (A) Gel 1, apo VLDL; gel 2, acetone extract of VLDL; gel 3, apo HDL; gel 4, acetone extract of HDL. (B) Gel 1, apo LDL-I; gel 2, isopropanol extract of LDL-I.

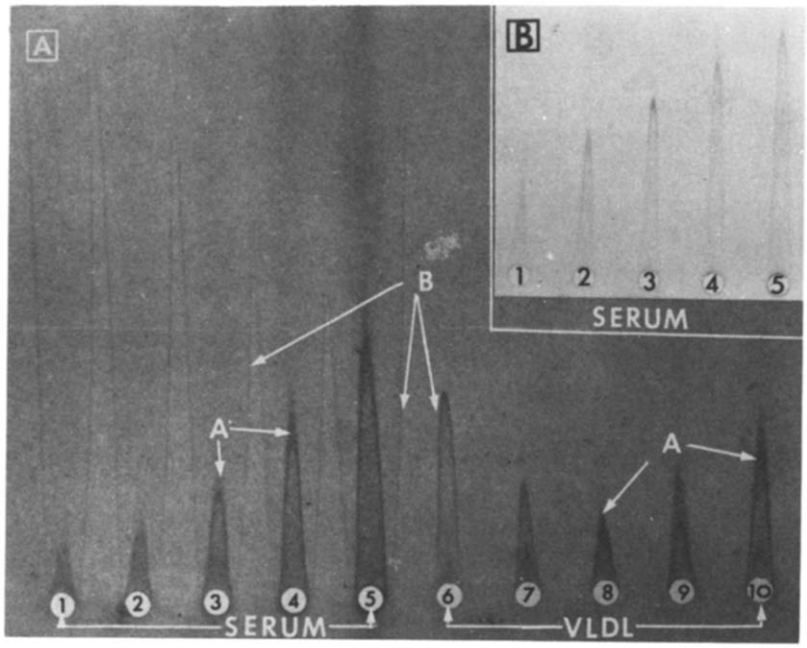

Fig.6. Rocket immunoelectrophoresis [18] of human serum and human serum VLDL using rabbit anti-human apo E. (A) Antibody prepared using apo E fractionated by gel filtration. Wells 1–5 contained 0.5–10 μl serum in 10 μl total vol. Reaction B is not due to albumin but may represent an antigen–antibody complex containing apo A-I. Wells 6–10 contained 0.3–4.8 μg VLDL protein. (B) Antibody prepared using apo E fractionated from apo VLDL by preparative isoelectric focussing. Wells 1–5 contained 0.2–2 μl serum in 10 μl total vol. The assay conditions were as in [6].

E from the LDL-1 fraction obtained from hypercholesterolemic rat serum. The use of this LDL-1 serum fraction in the isolation of apo E also provides the advantage that no prior extraction with acetone is required as little or no apo C is present. Lipoprotein fractions to be extracted with isopropanol should however be albumin-free as both human and rat albumins are relatively soluble in this solvent under the conditions employed.

Fig.6 demonstrates the superior quality of antibodies and thus antigen purity that are obtained by the use of human apo E purified by isoelectric focussing versus gel filtration-purified apo E. Both antigens were injected into rabbits at comparable levels to produce the antibodies. Fig.6A wells 1–5 contain human serum dilutions. In addition to the major 'rocket' labeled A, which is due to apo E, a broad 'flame' (labeled B) is also seen. A very similar broad flame reaction has been observed using rat serum and antibodies raised against column purified rat apo E [6]. Wells 6–10 in fig.6A contained progressively increasing concentrations of human VLDL. It can be seen that the most intense rocket (labeled A) increases in area as the VLDL concentration rises. At low VLDL concentrations however (wells 6,7, fig.6A), peak A, which is due to apo E, is barely discernible but good rockets due to reaction B, are clearly visible and could easily be confused with those due to apo E. It would thus be quite possible to obtain spuriously high values for apo E when quantitating unknown but low levels of human VLDL by electroimmunoassay using this antibody preparation. Fig.6B shows the standard curve obtained using human serum and anti-apo E raised against apo E purified by preparative isoelectric focussing. Only one rocket is obtained; there is no flame reaction equivalent to reaction B in fig.6A and there is therefore little possibility of overestimating the apo E concentration by integrating the peak area of a rocket that does not represent an antibody–antigen complex of apo E.

In summary the technique of flat-bed preparative isoelectric focussing used [14] for the separation and isolation of human serum apolipoproteins has been applied to and shown to produce high purity individual apoproteins from rat-serum lipoproteins. The organic extraction technique in [21] is equally appli-

cable to rat serum VLDL and acetone extraction of rat HDL is of considerable use in preparing apoproteins for isolation of the low molecular weight apo C and apo A-II by preparative isoelectric focussing. The precise isomorphic pattern of rat serum apo E was shown to be dependent upon the method of delipidation. The production of antibodies from apo E fractionated by preparative isoelectric focussing yields monospecific antibodies unlike those generated from apo E purified by column chromatography.

Acknowledgement

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