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Human plasma lecithin:cholesterol acyltransferase

Preparation and use of immobilized *p*-aminophenylarsenoxide as a catalytic site-directed covalent ligand in enzyme purification

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ABSTRACT

A method is described for the preparation of *p*-aminophenylarsenoxide-linked carboxymethyl (CM) Bio-Gel A and its use as a specific, catalytic site-directed affinity chromatography ligand in the final stages of the purification of human plasma lecithin:cholesterol acyltransferase (LCAT) (EC 2.3.1.43). Previous mechanistic studies by us demonstrated that phenylarsenoxide derivatives, which are highly specific for vicinal thiols, could inhibit LCAT via a covalent interaction with the sulphydryl groups of the two catalytic cysteine residues and that this inhibition could be rapidly and completely reversed upon addition of 2,3-dimercaptopropanesulphonic acid. The ligand was covalently linked to CM Bio-Gel A activated through an N-hydroxysuccinyl ester formed by N-hydroxysuccinimide and dicyclohexylcarbodiimide in dry dimethyl sulphoxide; 87% of the added *p*-aminophenylarsenoxide was coupled to the CM Bio-Gel A in 3 h at 25°C giving 2.3 mg of *p*-aminophenylarsenoxide per ml of gel. Homogeneous LCAT free of apo A-I, apo E, apo D and albumin was obtained in an 11% yield and purified 15 013-fold overall. A 13-fold purification was obtained by chromatography upon *p*-aminophenylarsenoxide-CM Bio-Gel A. This method is a useful final step in LCAT purification and will prove valuable in the purification of other proteins and compounds containing vicinal thiols.

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INTRODUCTION

Lecithin:cholesterol acyltransferase (LCAT, EC 2.3.1.43) is a plasma enzyme, normally associated with high-density lipoprotein, which transesterifies the fatty acid from the *sn*-2 position of lecithin to the 3-hydroxyl group of cholesterol to form lysolecithin and cholesteryl ester, respectively [1,2]. The enzyme has been purified to homogeneity by a number of laboratories using a variety of techniques [3–7] and shown to be a sialoglycoprotein of relative molecular mass (M_r) 65–69 kDa [4,8]. The cDNA for the human enzyme has been cloned and sequenced resulting in the predicted amino acid sequence for the enzyme [9]. In a series of recent reports [10–13] we have defined the catalytic mechanism of this important enzyme of plasma lipoprotein metabolism. In one of those [12] we utilized organoarsenical compounds to examine the possible vicinal nature of the two catalytic cysteine residues, Cys-31 and Cys-184 [14] and were able to demonstrate that the sulphydryl groups of these two residues were 3.5–3.62 Å apart following complete inhibition of cholesteryl ester formation by LCAT due to covalent bonding of the arsenic atom to the sulphur atoms of each of the cysteine residues. Organoarsenical compounds of this type have been used to probe the spatial orientation of cysteine residues in several enzymes [15–22] and are considered highly specific for vicinal thiols such as those existing in lipoic acid. Reaction of these compounds with monothiols results in the formation of unstable compounds which rapidly decompose. Relatively stable dithioarsenites are formed with vicinal cysteine residues. These adducts are, however, decomposed by compounds such as 2,3-dimercapto-1-propanesulphonic acid, which also contains vicinal thiols, and forms a stable ring structure with the organoarsenical. Monothiols such as cysteine or 2-mercaptoethanol are ineffective in destroying dithioarsenites formed with vicinal thiols. As we had previously shown that *p*-aminophenylarsenoxide could specifically inhibit LCAT and that the inhibition was rapidly and completely reversed following addition of 2,3-dimercapto-1-propanesulphonic acid we decided to investigate the possibility that the aminophenylarsenoxide, when immobilized, could act as a specific, catalytic site-directed covalent ligand for use in the latter stages of enzyme purification. With the exception of the use of Thiopropyl-Sepharose [23] and DTNB-Sepharose [24], which interact with monothiols, the application of covalent catalytic site-direct immobilized ligands for LCAT purification has not, to our knowledge, previously been reported. Here we describe the methodology for linking *p*-aminophenylarsenoxide to the carboxymethyl side-chains of a gel matrix and the subsequent successful use of the gel as a final step in the purification of LCAT from human plasma.

EXPERIMENTAL

Preparation of plasma

Fresh, rejected whole blood was obtained from the Canadian Red Cross and cellular material removed by low-speed centrifugation. The resulting plasma was

then dialyzed extensively at 4°C against 0.025% EDTA, pH 7.4 containing 2.0 m*M* sodium azide. The *β*-lipoproteins were then precipitated from the plasma by the slow addition of 100 ml of 1% dextran sulphate (sodium salt; Pharmacia, Uppsala, Sweden) and 500 m*M* magnesium chloride for every litre of plasma. The precipitation was performed under nitrogen and with slow stirring for 15 min at room temperature. The precipitated *β*-lipoproteins were then removed by centrifugation at 10 000 *g* for 10 min at 4°C.

Phenyl-Sepharose CL-4B chromatography

Phenyl-Sepharose was obtained from Pharmacia, and a 26 × 2.6 cm I.D. column prepared and equilibrated with 10 m*M* Tris–HCl, pH 7.4 containing 150 m*M* sodium chloride and 1 m*M* EDTA at 4°C. The *β*-lipoprotein-free plasma was then adjusted to a final concentration of 1 *M* sodium chloride by the slow addition of solid salt with stirring under nitrogen. The plasma was then applied to the Phenyl-Sepharose column at a flow-rate of 30 ml/h. Typically 1 l of solution containing variable volumes of plasma was applied to the column which was then extensively washed with the equilibrating buffer at a flow-rate of 50 ml/h until the absorbance at 280 nm of the eluate was less than 0.02. This process required 3–4 l of buffer and several days of washing. The column was then eluted with Milli Q water containing 4 m*M* 2-mercaptoethanol at a flow-rate of 30 ml/h. Fractions of 10 ml were collected. Following enzyme elution residual protein and lipid was removed from the Phenyl-Sepharose by batch washing of the gel twice with isopropanol and four times with 95% ethanol. The gel was recovered by low-speed centrifugation following each washing. The gel was then washed three times with distilled water, the column repacked and equilibrated with 1 l of the Tris–HCl buffer above.

Ion-exchange chromatography on a quaternary methylamine matrix (QMA)

QMA (Waters Accell) with a particle size of 37–55 *μ*m and a pore size of 500 Å was obtained from Millipore Waters Chromatography Division (Milford, MA, USA), and an 18 × 2.5 cm I.D. column prepared and equilibrated at 4°C with 10 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES), pH 7.0 containing 25 m*M* sodium chloride, 1 m*M* EDTA and 4 m*M* 2-mercaptoethanol. Milli Q water was used throughout. Active LCAT fractions from the Phenyl-Sepharose chromatography (about 180 ml) were dialysed against the same buffer overnight and applied to the QMA column at a flow-rate of 2.0 ml/min. The column was then washed with 100 ml of the equilibrating buffer and eluted firstly with a linear 240-ml gradient of 25–200 m*M* sodium chloride in the above buffer followed by a further 170 ml of 200 m*M* buffered sodium chloride; 4.0-ml fractions were collected. The column was then regenerated with 130 ml of 1 *M* sodium chloride followed by 180 ml of the equilibrating buffer. Reproducibility of the gradient was ensured by the use of a Waters 650 advanced protein purification system.

Thiopropyl-Sepharose 6B chromatography

Thiopropyl-Sepharose 6B was obtained from Pharmacia and a 22 × 1.5 cm I.D. column prepared and equilibrated with 10 m*M* Tris–HCl buffer, pH 7.4 containing 140 m*M* sodium chloride and 1 m*M* EDTA at 4°C. Active fractions of LCAT from the QMA chromatography were pooled and dialyzed at 4°C against the equilibration buffer following addition of 2-mercaptoethanol to a final concentration of 4 m*M*. This was followed by a 3 4 h dialysis against the equilibration buffer in the absence of 2-mercaptoethanol. The LCAT preparation was then applied to the Thiopropyl-Sepharose 6B column at a flow-rate of 10 ml/h and the column washed with 100 ml of the equilibrating buffer. The column was eluted with 10 m*M* Tris–HCl, pH 8.0 containing 140 m*M* sodium chloride, 1 m*M* EDTA and 2.5 m*M* dithiothreitol at a flow-rate of 30 ml/h. Fractions of 3 ml were collected. Following enzyme elution the gel was regenerated by passage of 200 ml of 1.5 m*M* 2,2′-dipyridyl disulphide, pH 8.0 containing 50 m*M* sodium bicarbonate and 1 m*M* EDTA at a flow-rate of 30 ml/h. The gel was then washed with

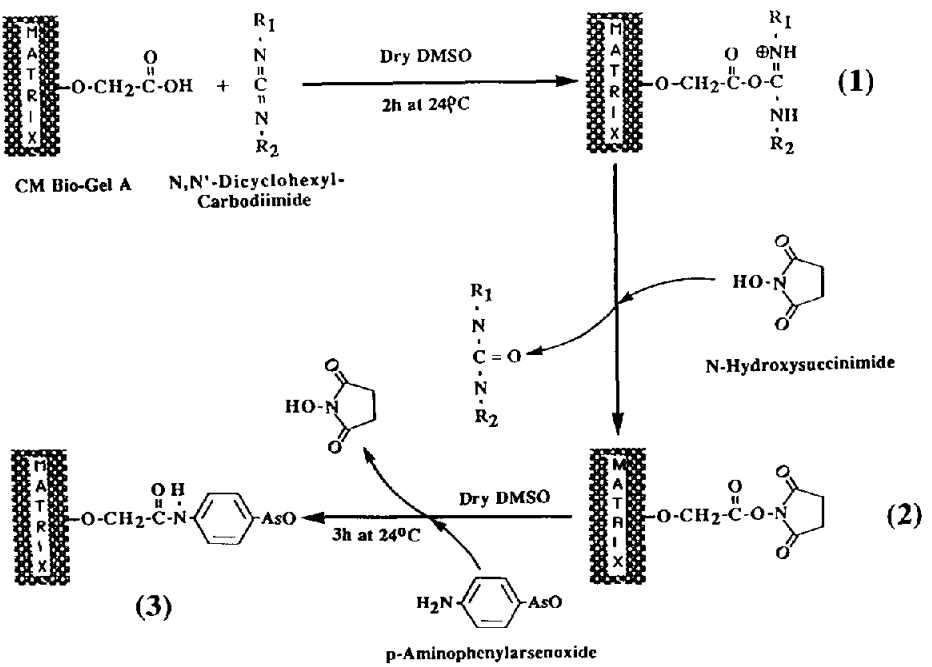

Fig. 1. Schematic representation of the reaction sequence used for the production of *p*-aminophenylarsenoxide covalently linked to CM Bio-Gel A. All reactions were performed as described in the Experimental section employing dry reagent. The product of the first reaction is the N-hydroxysuccinimidyl ester-activated form of CM Bio-Gel A (compound 2) as reaction with dicyclohexylcarbodiimide and N-hydroxysuccinamide occurs sequentially in the same flask. Following washing of the gel to remove dicyclohexylurea it was added to *p*-aminophenylarsenoxide dissolved in anhydrous dimethyl sulphoxide (DMSO). After 3 h of incubation at room temperature residual N-hydroxysuccinimidyl ester groups were reacted with an excess of glycine methyl ester, the gel washed and stored at 4°C until used.

50% aqueous acetone and 1 m*M* EDTA until the absorbance at 343 nm was less than 0.02.

Preparation of p-aminophenylarsenoxide-linked gel

p-Aminophenylarsenoxide was synthesized and purified as previously described [18,19]. Carboxymethyl (CM) Bio-Gel A was obtained from Bio Rad Labs. The reactions by which *p*-aminophenylarsenoxide was covalently coupled to the Bio-Gel A is shown in Fig. 1. All solvents were rendered anhydrous prior to use by storage over Type 3A molecular sieve (8–12 mesh) from Matheson, Coleman and Bell. CM Bio-Gel A (25 ml containing about 500 μmol of carboxylate groups) was first washed with anhydrous methanol and dimethyl sulphoxide (Aldrich) using a sintered-glass funnel. N,N'-Dicyclohexylcarbodiimide (2.9 g, Pierce) and N-hydroxysuccinimide (1.9 g, Aldrich) were dissolved in 83 ml of dry dimethyl sulphoxide in a 150-ml Quick Fit round-bottomed flask to obtain a final concentration of 150 m*M* each. The washed gel was then added with stirring, the flask sealed and the contents stirred at room temperature for 2 h to permit formation of the N-hydroxysuccinimidyl ester activated form of CM Bio-Gel A. The N-hydroxysuccinimidyl ester-activated gel was then washed with six volumes of anhydrous methanol followed by three volumes of dry dimethyl sulphoxide using a sintered-glass funnel. This procedure removed precipitated dicyclohexylurea. The activated gel (2 in Fig. 1) was slowly added with stirring to a second 150-ml round-bottomed flask containing 92 mg (500 μmol) of *p*-aminophenylarsenoxide dissolved in 115 ml of dry dimethyl sulphoxide. The molar ratio of Bio-Gel A carboxyl groups, which were assumed to be 100% activated with N-hydroxysuccinimide ester, to the arsenic compound was 1:1. Following closure of the flask the coupling reaction was allowed to proceed at room temperature for varying times. The extent to which the *p*-aminophenylarsenoxide was coupled to the gel matrix with time was determined by monitoring the decrease in ninhydrin-positive material in the gel supernatant. Fig. 2 shows that maximal coupling was achieved after 3 h and that 87% of the added arsenoxide was coupled to the gel under the above conditions. Following coupling for the requisite time (3 h) 10.4 g of glycine methyl ester (Sigma) were added to the reaction mixture (final concentration 1 *M*) which was stirred for a further 2 h at room temperature. This procedure ensured that all residual N-hydroxycuccinimidyl esters on carboxyl groups that had not reacted with the *p*-aminophenylarsenoxide were blocked by glycine methyl ester. The gel was then washed extensively with 1 m*M* phosphate-buffered saline, pH 7.0 and stored at 4°C until used.

p-Amenophenylarsenoxide-CM Bio-Gel A chromatography

Active LCAT fractions obtained from the Thiopropyl-Sepharose chromatography were pooled and dialysed against 1 m*M* phosphate-buffered saline, pH 7.0 containing 4 m*M* 2-mercaptoethanol overnight. A 1-ml volume of the phenylarsenoxide gel was then added for every 0.5 ml of the LCAT solution and the

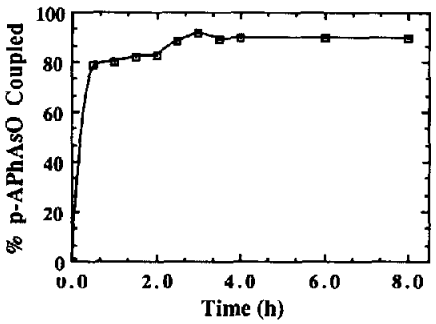

Fig. 2. Covalent coupling of *p*-aminophenylarsenoxide (p-APhAsO) to N-hydroxysuccinimidyl ester-activated form of CM Bio-Gel A with time. The activated gel was prepared and washed as described in the Experimental section. Approximately 6 ml of the gel were then transferred to a 50-ml flask containing 16 mg of *p*-aminophenylarsenoxide dissolved in 20 ml of dry dimethyl sulphoxide, the flask sealed and stirred at room temperature. Aliquots of the mixture were removed at the indicated times, the gel phase separated by centrifugation and the supernatant assayed for *p*-aminophenylarsenoxide using ninhydrin. Under the above conditions 87% of the added *p*-aminophenylarsenoxide was removed from the supernatant and coupled to the gel within 3 h.

mixture slowly shaken under nitrogen at 4°C for 2 h. Shaking was then stopped and the gel mixture allowed to stand overnight at 4°C. The gel was then placed in a 15 × 1.5 cm I.D. column and washed with the equilibrating buffer until the absorbance at 280 nm of the eluate was zero. LCAT was then eluted with 1 m*M* phosphate-buffered saline containing 1 m*M* EDTA, 2 m*M* 2-mercaptoethanol and 1.5 m*M* 2,3-dimercapto-1-propanesulphonic acid at a flow-rate of 0.7 ml/min, and 0.4-ml fractions were collected. As LCAT contains only two reduced cysteine residues which are vicinal and with which *p*-aminophenylarsenoxide forms covalent bonds the stoichiometry of the arsenoxide–LCAT interaction is 1:1 [12]. Thus the maximal binding capacity of the *p*-aminophenylarsenoxide-CM Bio-Gel A column for LCAT on a molar basis is equivalent to the number of moles of the arsenoxide immobilized. Care was taken to ensure that excess immobilized ligand was utilized thus ensuring retention of all LCAT activity present.

Other methods

The transacylase activity of LCAT was assayed in plasma and column eluates using a synthetic lecithin–cholesterol–Apo A-I proteoliposome substrate as previously described [10]. The same substrate preparation was utilized to assay all fractions from each chromatographic procedure in a given purification. Protein was measured by the method of Lowry *et al.* [25]. Sodium dodecyl sulphate (SDS) 3–15% linear polyacrylamide gradient gel electrophoresis (PAGE) was performed as described [26] as was the Ouchterlony double-diffusion analysis [27]. Rabbit polyclonal antibodies were elicited against highly purified human apo A-I, apo E and albumin as described previously by us [28]. Polyclonal rabbit anti-human LCAT was produced in a similar manner. Rabbit anti-human apo D was a kind

gift from Dr. Walter McConathy of the Oklahoma Medical Research Foundation. *p*-Aminophenylarsenoxide was determined by reaction with ninhydrin as follows. A standard solution of 1.0 mg of *p*-aminophenylarsenoxide per ml of 1 m*M* phosphate-buffered saline, pH 7.0 was prepared, and 25–400 μl aliquots (0.14–2.16 μmol) were placed in glass tubes. To this were added 1.0 ml of freshly prepared ninhydrin reagent and distilled water to make a final volume of 1.7 ml. The tubes were then heated at 100°C for 20 min, cooled, 1.0 ml of distilled water was added and the absorbance at 570 nm measured. The resulting standard curve passed through the origin and was linear up to 2.16 μmol aminophenylarsenoxide ($A_{570\ nm}$ = 0.8) with an *r* value of 0.999. Dimethyl sulphoxide did not interfere with the assay.

RESULTS AND DISCUSSION

The purification scheme here described for human plasma LCAT represents, with the exception of the QMA and *p*-aminophenylarsenoxide chromatography, a combination of individual chromatographic techniques which have been previ-

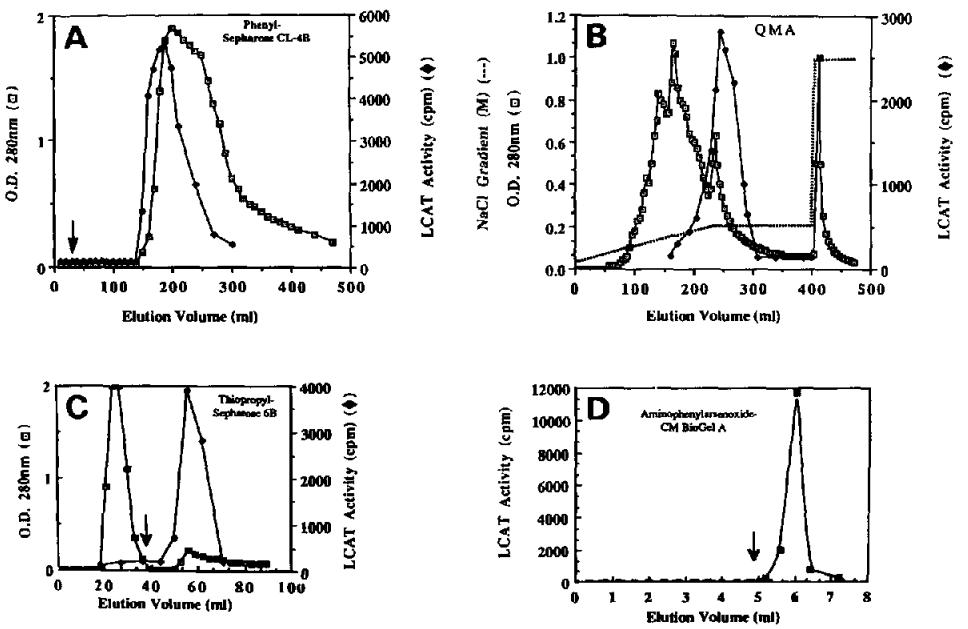

Fig. 3. Elution profiles of LCAT activity and protein from (A) Phenyl-Sepharose CL-4B, (B) Waters quaternary methylamine column, (C) Thiopropyl-Sepharose 6B and (D) *p*-aminophenylarsenoxide-CM Bio-Gel A. Column dimensions, buffers and the chromatography were as described in the Experimental section. The arrows indicate the fractions at which the washing buffer was replaced by the eluting buffer. In (B) LCAT activity eluted between 155 and 200 m*M* NaCl. Owing to the batch-type procedure used in (D) protein which did not associate with the gel is not shown. LCAT activity was assayed using a lecithin–cholesterol–apo A-I proteoliposome substrate and determination of radioactive cholesteryl ester formation as described previously [10].

TABLE I

PURIFICATION OF HUMAN PLASMA LECITHIN:CHOLESTEROL ACYLTRANSFERASE

Protein was measured by the method of Lowry *et al.* [25]. The transacylase activity of LCAT was measured using a lecithin–cholesterol–apo A-I proteoliposome substrate and the determination of radioactive cholesteryl ester formed as described previously [10]. One unit of enzyme activity is defined as that activity which can esterify 1 nmol of cholesterol per h per ml using the above substrate. The final step in the purification which employs *p*-aminophenylarsenoxide-CM Bio-Gel A was the most effective and resulted in a homogeneous preparation of enzyme with an overall yield of 10%.

Fraction	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification	
						Fold per step	Total fold
Plasma	1000	54000	61000	1.1	100	1	1
Phenyl-Sepharose	250	1200	21750	18	36	16	16
QMA	100	110	12400	113	20	6	103
HS-Propyl-Sepharose	100	10	13000	1300	21	12	1181
H_2N-PhAsO-CM BioGel A	40	0.4	6600	16515	11	13	15013

ously described by others. After the removal of cellular material from the citrated plasma obtained from the Red Cross, the plasma was extensively dialysed to remove the preservatives and anticoagulants after which EDTA and azide were added. The β-lipoproteins were then precipitated with dextran-magnesium chloride and the supernatant subjected to Phenyl-Sepharose chromatography essentially as described by Albers *et al.* [7]. The purification and yield of enzyme activity obtained in this and all subsequent procedures is shown in Table I. In our hands the yield and consequential fold purification of LCAT by Phenyl-Sepharose proved rather variable. The example shown in Table I indicates a 36% yield and 16-fold purification which represented the lower limit of recovery and purification. Upon other occasions we have obtained yields of 85% with purification values in excess of 200-fold. Albers *et al.* [7] reported values of 86% recovery with a 106-fold purification using Phenyl-Sepharose. The reason for this discrepancy and variable yield is not apparent but may reside within our use of fresh rejected whole blood rather than freshly drawn blood as a source of LCAT. The longer washing period may also result in some of the enzyme activity bleeding off the column. LCAT was eluted from the Phenyl-Sepharose column with Milli Q water and, as shown in Fig. 3A, the activity eluted immediately in the first portion of the protein peak. The active fractions were then pooled, dialysed and applied to a quaternary methylamine ion-exchange column which was washed and eluted with a 25–200 m*M* buffered sodium chloride gradient (Fig. 3B). LCAT eluted between 155 and 200 m*M* sodium chloride and showed a tendency to tail. This chromatographic procedure was highly reproducible, resulted in a good recovery of activity and a 6-fold purification over the Phenyl-Sepharose eluate to give an overall purification of 103-fold (Table I). Active fractions were pooled, dialysed and applied to Thiopropyl-Sepharose essentially as described by Holmquist [23] with the exception that no sodium taurocholate was used. As shown in Fig. 3C the majority of the applied protein did not bind to the column whereas all of the applied LCAT activity appeared to bind and elute with 2.5 m*M* dithiothreitol. This procedure resulted in a step purification of 12-fold and an overall purification of 1181-fold (Table I). This is somewhat superior to the value of 207-fold reported by Holmquist [23], however, in that case Thiopropyl-Sepharose chromatography was utilized to fractionate lipoprotein-free plasma rather than following ion-exchange chromatography as reported here.

The final step in LCAT purification was the application of catalytic site-specific covalent ligand chromatography in the form of immobilized *p*-aminophenylarsenoxide linked by a two carbon spacer to Bio-Gel A. The length of the spacer arm was chosen such that the phenylarsenoxide would be sufficiently removed from the gel matrix to permit interaction with the vicinal thiols of Cys-31 and Cys-184 within the catalytic site of the enzyme. In preparing the *p*-aminophenylarsenoxide-linked gel we elected to use the N-hydroxysuccinimidyl ester-activated gel as the condensing species rather than the gel following dicyclohexylcarbodiimide treatment (1 in Fig. 1). Formation of the N-hydroxysuccinimidyl ester

derivative is no more time-consuming and affords the advantage of yielding a highly reactive gel which is more stable than compound 1 in Fig. 1 and can thus be washed free of the initial reactants prior to interaction with the *p*-aminophenylarsenoxide. Fig. 3D shows the elution profile from the *p*-aminophenylarsenoxide-Bio-Gel A column following application of the eluate from the Thiopropyl-Sepharose column. All LCAT activity elutes following addition of 2,3-dimercaptopropanesulphonic acid to the buffer with a resultant 13-fold step purification and an overall yield of 11% of the initial enzyme activity which is very similar to other reported purification methods [7,23]. For this affinity chromatography step we elected to utilize a batch-type procedure commonly utilized in immunoaffinity chromatography in order to allow an extended time for the LCAT–aminophenylarsenoxide interaction. As both the *p*-aminophenylarsenoxide and the catalytic site of LCAT are relatively hydrophobic [14] and the inhibition of LCAT activity by this compound is quite rapid [12], extended incubations with the immobilized ligand may not be required and conventional elution chromatography could be equally successfully employed. Previous methods, as here, have based their reported recoveries upon activity measurements alone. Albers *et al.* [29], using a radioimmunoassay, have reported that normolipidemic males have a plasma LCAT concentration of 5.98 ± 1.04 μg/ml (n = 44) and females the somewhat higher value of 6.44 ± 0.81 μg/ml (n = 22). The mean value for plasma LCAT for all normolipidemic subjects studies was 6.14 ± 0.98 μg/ml (n = 66). In this study we recovered 400 μg of pure LCAT protein from 1 l of plasma which, in terms of the yield of protein, represents only 6.5% of that theoretically present within the initial plasma sample despite the recovery of 11% of the initial activity. A similar effect can be seen in the data of Albers *et al.* [7] for their purification of LCAT. These workers reported a 13% yield of pure enzyme based upon activity measurements. The total yield of LCAT protein from 1 l of plasma, however, was only 440 μg which represents 6.7% of the theoretical initial enzyme mass, a figure comparable to that in our study. There are two likely explanations for these apparent differences between the overall yield of LCAT activity and protein mass. In this report fresh rejected whole blood was utilized and in many cases the units obtained were incompletely filled and thus the ratio of the anticoagulant and preservative solution to the volume of blood was both inconsistent and usually higher than that of completely filled units. Thus although 1 l of plasma solution was used for the isolation of LCAT the actual content of plasma proteins and LCAT within that solution was somewhat lower than would be measured in human plasma drawn into tubes containing solid heparin. This is exemplified by the fact that in Table I we report that 1 l of the plasma solution applied to the Phenyl-Sepharose column contained $54 \cdot 10^3$ mg of protein whereas Albers *et al.* [7] reported a protein value of $68.7 \cdot 10^3$ mg for 1 l of plasma. Secondly, the LCAT assay is influenced by the presence of endogenous cholesterol within the sample aliquot to be assayed and this results in an underestimation of the true activity particularly when hypercholesterolemic plasma is

assayed [26]. Thus the varying cholesterol content of LCAT-containing fractions during the course of purification may influence activity determinations to varying degrees and contributes to the discrepancy between activity and mass recoveries. Furthermore, the efficiency of different batches of the artificial proteoliposome substrate used to assay LCAT activity varies, and care must be taken to utilize the same substrate preparation throughout a given purification if comparable results are to be obtained. Despite these potential problems which are common among published purification schemes for LCAT, the major advantage with the purification procedure reported here is that it involves fewer steps than other methods and yields a homogeneous enzyme preparation in similar yield from a lower initial plasma protein mass. Unfortunately, once exposed to 2,3-dimercaptopropanesulphonic acid the *p*-aminophenylarsenoxide forms a stable covalent five-membered ring structure and the column cannot be easily regenerated. However, in view of the ease with which *p*-aminophenylarsenoxide can be linked to CM Bio-Gel A this does not represent a major disadvantage to the use of this method.

Fig. 4 shows the protein composition of each of the pooled eluates from the

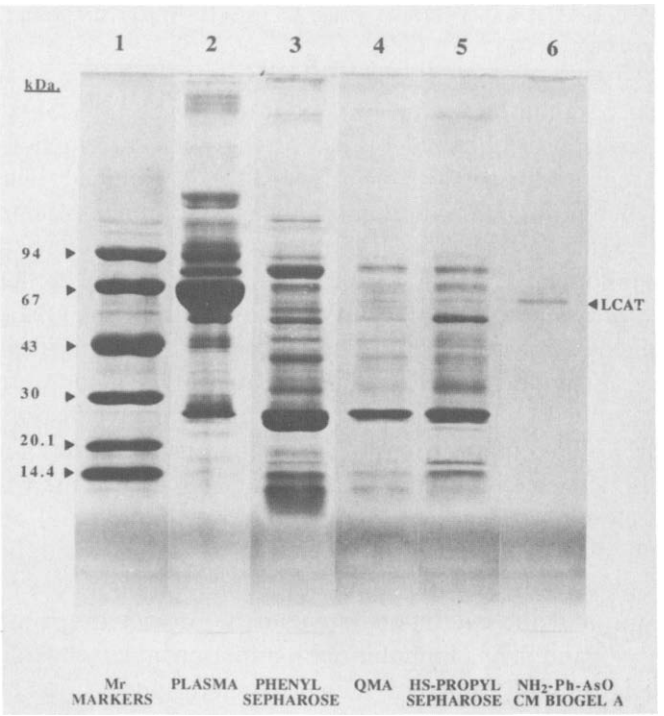

Fig. 4. SDS-PAGE (3–15% gradient) of plasma and the pooled LCAT-active fractions from each column chromatographic fractionation shown in Fig. 3. Contaminating proteins are progressively removed during purification of LCAT, however, the application of *p*-aminophenylarsenoxide-CM Bio-Gel A chromatography to the eluent from the Thiopropyl-Sepharose column clearly represents a significant purification and results in a single homogeneous band of M_r 67 kDa.

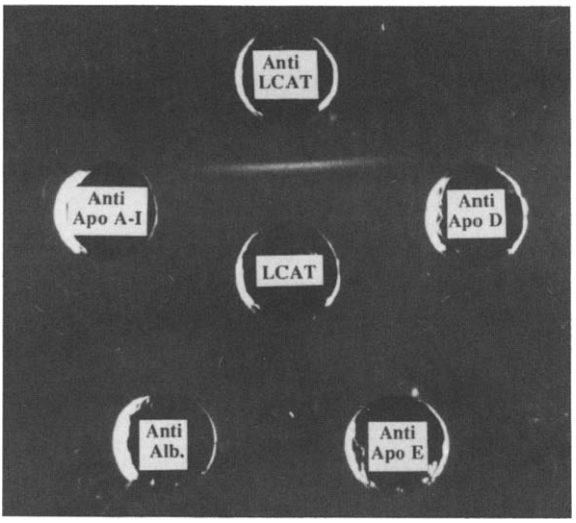

Fig. 5. Ouchterlony double-diffusion analysis of LCAT purified using *p*-aminophenylarsenoxide-CM Bio-Gel A as a final step and shown in lane 6 of Fig. 4. All antibodies were polyclonal and elicited in rabbits using highly purified antigens as described [28]. Anti-apo D was a kind gift from Dr. Walter McConathy, Oklahoma Medical Research Foundation.

sequential column chromatographic techniques as separated by 3–15% SDS-PAGE. The significant purification obtained by use of the phenylarsenoxide-linked gel is clearly apparent and a single band of M_r kDa is obtained. The purified enzyme reacted with a polyclonal rabbit antibody against human plasma LCAT and was free of detectable apo A-I, apo E, apo D and albumin as shown in Fig. 5. Application of *p*-aminophenylarsenoxide affinity chromatography earlier in the purification scheme proved unadvantageous. Fractionation of the QMA eluate upon the *p*-aminophenylarsenoxide gel resulted in only a 2-fold purification and, as shown in Fig. 6, significant levels of contaminating proteins were present in the eluate. Application of whole plasma to the *p*-aminophenylarsenoxide-CM Bio-Gel A column resulted in the retention and subsequent elution of a protein with an M_r of 68 kDa as shown in Fig. 7. No LCAT activity was associated with this protein which was immunologically identical to albumin and no enhancement of LCAT activity was observed in the elutate. This result was to be expected as LCAT is totally associated with its natural substrate, high-density lipoprotein, when in plasma and the catalytic site would be protected against interaction with the affinity ligand. Thus immobilized *p*-aminophenylarsenoxide gel chromatography is most profitably applied as a final step in the purification of human plasma LCAT and following the removal of high-density lipoprotein and albumin.

The present exploitation of our previous finding that *p*-aminophenylarsenoxide interacts with the vicinal catalytic cysteine residues of LCAT to form a stable covalent adduct in order to purify the enzyme can be contrasted with our previ-

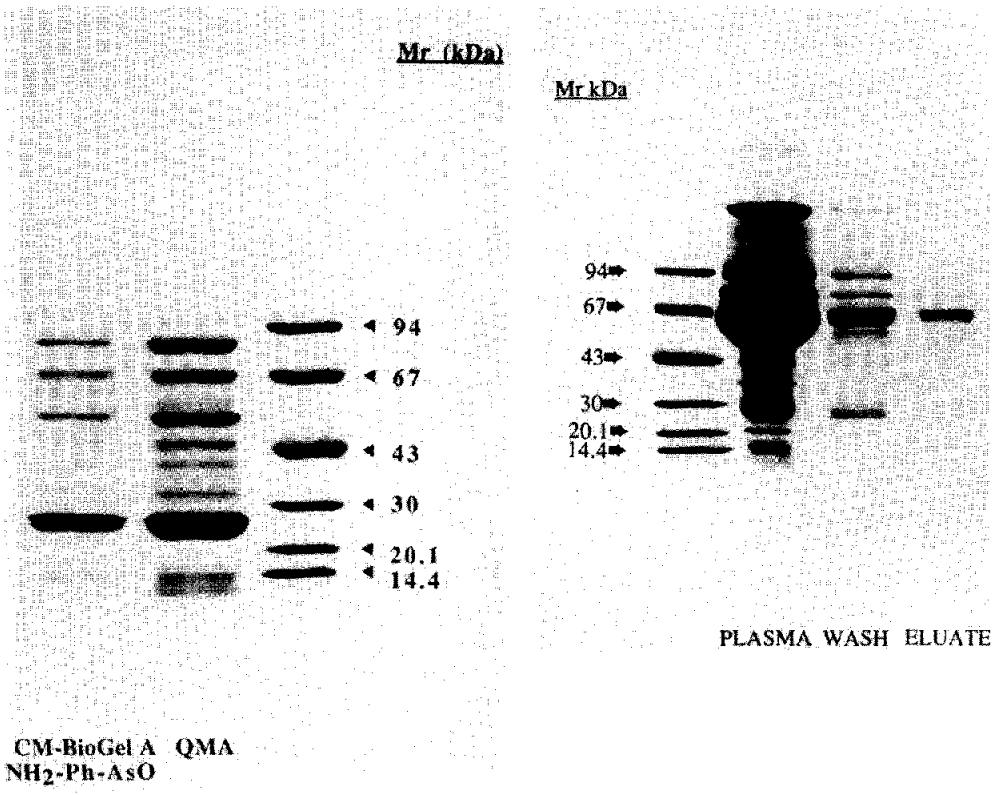

Fig. 6. SDS-PAGE (3–15% gradient) of a pooled, LCAT-active fraction from QMA chromatography and the eluate from its subsequent chromatography upon *p*-aminophenylarsenoxide-CM Bio-Gel A. A 2-fold purification and 71% yield was obtained following the latter chromatography, however, significant mass of contaminating proteins remains in the *p*-aminophenylarsenoxide-CM Bio-Gel A eluate indicating that this procedure is most profitably utilized as a final step in LCAT purification.

Fig. 7. SDS-PAGE (3–15% gradient) plasma and the retained and unretained fractions following the application of plasma onto a *p*-aminophenylarsenoxide-CM Bio-Gel A column. The retained 68-kDa protein was immunologically identical to albumin and had no LCAT activity. The retained fraction was not enhanced in LCAT activity when compared to the applied plasma.

ous use of immobilized phenylboronic acid [11]. The aromatic boronic acids inhibit serine-histidine esterases and proteases via formation of a co-ordinate covalent bond between the trigonal boron atom and the oxygen atom of the catalytic serine residue [30–34]. This interaction is stabilized by glycerol and enzymes such as Subtilisin, and other serine-histidine proteases and esterases, including LCAT, are retarded but not firmly adsorbed to these columns in the presence of glycerol but not in its absence. Owing to the relatively weak interaction between this immobilized ligand and LCAT [11] the use of immobilized phenylboronic acid as a catalytic site-directed affinity ligand for enzyme purification proved to be of rather limited value. Such is not the case with immobi-

lized *p*-aminophenylarsenoxide which represents a valuable addition to the currently available methodology for the purification of this important enzyme of plasma lipoprotein metabolism. This method is also generally applicable to those other proteins and chemical compounds which contain vicinal thiols.

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