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STUDIES ON INTERACTIONS BETWEEN PORCINE APOLIPOPROTEIN A-I AND ISOLATED LIVER ENDOPLASMIC RETICULUM LIPIDS

BY

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ABSTRACT

Using a dansyl fluorescent probe attached to porcine Apolipoprotein A-I, experiments are conducted where isolated pig liver endoplasmic reticulum membrane lipids are added to the labeled protein. From fluorescence polarization and intensity change measurements, qualitative evidence of the interaction between the protein and lipid is obtained. From Perrin Plots, the particle size is seen to increase as lipid is added to labeled protein. Also, changes in λ_{max} and fluorescence quenching signify that the environment of the probe changes as lipid is added to protein, again indicating interaction.

1. INTRODUCTION

Serum lipoproteins, complexes of protein and lipid, function in the transport of water-insoluble lipids via the bloodstream to various organs throughout the body. Lipoproteins are physically distinguished by relative floatation rates in high gravitational fields such as those found in the ultracentrifuge. They are generally divided into four classes, namely chylomicrons, very low density, low density, and high density lipoproteins.

Chylomicrons and very low density lipoproteins (VLDL) are both transporters of triglycerides. Chylomicrons originate in the intestine while VLDL come from the liver. In the bloodstream both undergo a partial delipidation whereby lipoprotein lipase hydrolyses the triglyceride to glycerol and free fatty acids. In the case of chylomicrons, the remaining remnant particles are degraded by the liver. However, when the triglyceride is removed from VLDL, the result is a lipoprotein particle with a higher density and a cholesterol ester core; this is the low density lipoprotein.

The role of low density lipoproteins (LDL) has been fairly well established. It is thought that LDLs transport cholesterol via the bloodstream to the peripheral cells.

Goldstein and Brown (1) have proposed a mechanism by which the cells internalize and metabolize the LDL. Briefly, it involves the LDL binding to a high affinity receptor site on the cell's exterior, followed by internalization of the complex, protein degradation, and LDL cholesterol ester hydrolisis to free cholesterol and fatty acid. This process serves to inhibit the synthesis of endogenous cholesterol by inhibiting an enzyme involved in cholesterol biosynthesis.

The role of high density lipoproteins (HDL) is less clear. HDLs have generated interest because there is a negative correlation between HDL levels and the incidence of cardiovascular disease (2). In other words, persons with higher levels of HDL are less prone to be afflicted with cardiovascular diseases such as atherosclerosis. Glomset (3) postulated that HDLs remove cholesterol from peripheral cells and transport it to the liver for removal from the body. Another function of HDL may be to competitively inhibit the binding of LDL to cellular receptor sites, thus preventing the uptake of the LDLs and the cholesterol contained within them (4-6).

The origin of HDL is not clear, either. Mature HDL particles are spherical in shape with a diameter of about 100Å and are rich in cholesterol esters. They also contain triglycerides, free cholesterol, and phospholipids. The polar lipids are believed to associate with apolipoproteins on the exterior of the particles while the neutral lipids compose the core (7). The major apolipoprotein is Apo A-I.

It is believed that HDLs originate in the liver and intestine as precursor apolipoprotein-lipid complexes (nascent HDL) which are quite different both in structure and composition from the mature particles found in the plasma. In rat liver perfusion experiments, the perfusate was found to contain particles in the HDL density range. These nascent HDL particles were found to contain high levels of the protein Apo E and phospholipids but low levels of Apo A-I and cholesterol esters (8,9). In similar experiments where lecithin-cholesterol acyltransferase (LCAT) activity was inhibited, discoidal particles, 46 Å thick and 190 Å in diameter, were found in the HDL density range (10). In humans, liver perfusion experiments were not performed, but similar particles were discovered in blood serum in certain cases. In patients with abnormal LCAT activity, such as those with familial LCAT deficiency (11) or alcoholic hepatitis (12), discoidal HDL were found. In the alcoholic hepatitis case, the composition of the discoidal HDL was found to be similar to nascent rat HDL (13). As the patient's liver condition improved and LCAT activity was restored, the plasma HDL returned to normal composition (12). In view of this evidence, one can conclude that discoidal HDL particles are secreted by the liver in the absence of LCAT activity. Furthermore, it can be deduced that LCAT activity is required to obtain the normal HDL structure and composition. Indeed, Glomset (14) proposed some time ago that LCAT activity transforms discoidal HDL into spherical particles rich in cholesterol ester. The experimental data

seem to confirm this postulate; when LCAT was added to plasma from patients with abnormal LCAT activities, the discoidal HDL were transformed to spherical ones (15).

The intestine is also suggested as a source of nascent HDL. There is no direct evidence of the secretion of discoidal particles, but it is known that the intestine is a major source of Apo A-I in humans and rats (16,17). It is known that the Apo A-I secreted by the intestine in chylomicrons and VLDL appears rapidly in the HDL fraction (18).

Still at the crux of the matter is the manner by which protein and lipid associate to form the nascent HDL particles. HDL-density particles have been shown to exist in the Golgi apparatus of rat liver, and it has been suggested that the site of assembly may be in the smooth endoplasmic reticulum (19). The mechanism of protein-lipid complex formation, however, is unknown. It is not known whether the reaction is catalyzed by a lipid transfer factor or whether it is spontaneous.

Jonas and Krajnovich (20) have demonstrated that human and bovine Apo A-I will spontaneously form complexes with pure synthetic phospholipids under certain conditions. Knowing this, it would be interesting then to determine whether the same could be done with dispersions of liver endoplasmic reticulum (ER) membrane phospholipids.

The pig is a suitable animal to conduct such experiments on, for its lipoprotein pattern is similar to that of the human (21). Also, the structure and composition of its HDL are well known (22-24). Using fluorescent probes attached to

the Apo A-I protein in fluorescence polarization and intensity experiments, the spontaneous interaction between Apo A-I and liver ER phospholipids will be studied and discussed in view of our current knowledge.

II. EXPERIMENTAL

A. MATERIALS

Fresh pig blood and liver came from the University of Illinois Meat Science Laboratory, Urbana, Illinois. Human Apolipoproteins A-I and C-I and complexes of human Apo A-I, dimyristoyl phosphatidylcholine, and (^{14}C) cholesterol were obtained from Dr. A. Jonas. Adenosine monophosphate (5'), cytochrome c, bovine serum albumin, and dansyl chloride were obtained from Sigma Chemical Co., St. Louis, Missouri. Dialysis tubing was supplied by Spectrum Medical Instruments of Los Angeles, California, and Union Carbide Corp. of Chicago, Illinois. Eastman Silica Gel Chromatogram Sheets, without fluorescent indicator, were obtained from Eastman Kodak Co., Rochester, New York. Glass-backed Silica Gel 60F-254 thin layer chromatography sheets were purchased from Brinkmann Instruments, Des Plaines, Illinois. Sephacryl S-200 gel filtration resin came from Pharmacia Fine Chemicals of Piscataway, New Jersey. Dowex 2X-8 anion exchange resin and Biogel A-5M gel filtration resin were obtained from Bio-Rad Laboratories, Richmond, California.

B. INSTRUMENTS

High-speed centrifugation was carried out in a Beckman

L5-65 ultracentrifuge, while a Beckman J-21B preparative centrifuge was used for low-speed spins. Absorbance readings were taken using a Beckman Model 35 Spectrophotometer; a Perkin-Elmer MPF-3 Fluorescence Spectrophotometer was used to obtain fluorescence intensity measurements. Fluorescence polarization values were measured on an SLM Instruments Series 400 polarization spectrophotometer with Corning Glass 3-74 emission filters and with the temperature controlled with a Forma Scientific Masterline water bath. Radioactive scintillation counting was done on a Beckman LS-100C Liquid Scintillation Counter. Lipid vesicles were prepared by the sonication of a lipid suspension with a Model W185 Sonifier produced by Heat Systems-Ultratronics, Inc. A Gilson Micro-fractionator was used to collect fractions eluting from gel filtration columns. A Rotovapor flash evaporator was used to remove organic solvent from extracted lipid samples. Lyophilization was carried out using a Virtis Model 10-117 lyophilizer.

C. METHODS

1. Phosphorus Determination

Assays for organic phosphorus and inorganic phosphate were performed by the method of Chen et al (25). Various dilutions of an inorganic phosphate stock solution were used to construct a standard curve.

2. Protein Determination

Protein concentration was determined by the method of Lowry et al (26) as modified by Markwell et al (27). Solutions of bovine serum albumin were used as standards to generate a standard curve.

3. Apo A-I Isolation

a. Isolation of Lipoproteins

The procedure used for lipoprotein separation was based on that used for the bovine system by Jonas (28). EDTA was added to fresh pig blood at a concentration of 1 mg/ml to prevent coagulation. After refrigeration for a few hours, it was centrifuged at 3000 rpm for 15 minutes using the JA-14 rotor in the Beckman J-21B centrifuge, and the supernatant serum was removed by aspiration.

Lipoproteins were isolated from pig serum by differential centrifugation. Serum was centrifuged at 50,000 rpm at 15°C for 19 hours using a Ti 55.2 rotor in the Beckman L5-65 ultracentrifuge. This first spin floated chylomicrons and VLDLs which appeared as a white layer; they were removed by gentle suction. The remaining solution was adjusted to a density of 1.087 g/ml with solid NaCl and centrifuged as before. The yellowish solution on top was removed and the solution left in the tubes was adjusted to a density of 1.210 g/ml with solid NaBr and recentrifuged as described above. The white layer which contained HDL was then removed. The HDL and VLDL solutions were each dialyzed for 40 hours against five 1-liter changes of 0.01% EDTA at 5°C.

b. Delipidation

The dialyzed HDL and VLDL solutions were delipidated. The HDL solution was delipidated by two procedures to test the efficiency of each relative to the other.

1) Chloroform/Methanol Extraction

This procedure is a modified version of the method of Weisgraber et al (29). The VLDL and about half of the HDL solution were lyophilized. Each was placed in a separate conical centrifuge tube, suspended in 10 ml cold chloroform: methanol (2:1 v/v), and allowed to sit at 5°C for one hour. They were then centrifuged at 5,000 rpm at 5°C for 5 minutes using the JA-20 rotor in the Beckman J-21B preparative centrifuge and the supernatants decanted and discarded. The pellets were suspended and recentrifuged as described above three times in 10 ml cold ethanol and one time in cold methanol. After the last centrifugation, a small amount of methanol was left in. The samples were stored at 5°C.

2) Ethanol/Ether Extraction

The other half of the HDL solution was delipidated with cold ethanol/ether following the procedure of Scanu (30).

The extent of delipidation was estimated by the amount of phospholipid phosphorus left after the extractions using the method of Chen et al (25).

c. Apolipoprotein Identification and Separation

In order to verify the composition of the proteins obtained from the delipidation of lipoporteins, sodium dodecylsulfate polyacrylamide gel electrophoresis was performed by the procedure of Weber and Osborn (31). Standards of human Apo A-1 and Apo C-1 were also run.

HDL apolipoproteins from both delipidation procedures were pooled and fractionated on Sephacryl S-200 in a buffer 3M in guanidine HCl, pH 8.2, 0.1M in Tris, and 0.005% in EDTA, using a 90 x 2.5 cm glass column kept at 5°C. Fractions of about 4 ml each were collected with a Gilson Microfractionator. Absorbances of the fractions were measured at 280 nm with a Beckman Model 35 Spectrophotometer. Pooled fractions containing Apo A-I were dialyzed against four 6-liter changes of 0.01% EDTA at 5°C and finally lyophilized and stored below 0°C until used.

4. Endoplasmic Reticulum Isolation

The procedure for isolating endoplasmic reticulum (ER) membranes from pig liver is based on the discontinuous sucrose gradient method for rat liver by Dallner (32). About 30g of fresh pig liver were homogenized with 150 ml cold 0.44M sucrose in a kitchen blender. The homogenate was filtered through three layers of cheesecloth to remove fibers and any large chunks of liver. The filtrate was centrifuged at 10,000 x g at 5°C for 20 minutes to sediment heavier particles. The supernatants were pooled and adjusted to 150 ml

with cold 0.44M sucrose. About 18 ml of the resulting suspension were placed in each of eight polycarbonate centrifuge tubes. A discontinuous sucrose gradient was created by layering 7 ml of 1.3M sucrose underneath the liver homogenate suspensions. The gradients were centrifuged in the Beckman L5-65 ultracentrifuge at 105,000 x g at 5°C for 7 hours. During the spin, the homogenate suspension separated into five fractions (Figure 1). These fractions were separated with a Pasteur pipet and stored in closed containers at 5°C. The white band in the middle of the gradient (Fraction 3) was believed to be smooth ER. The pellet at the bottom (Fraction 5) was resuspended with a small amount of the liquid above it. Seven such batches were prepared and the respective fractions pooled.

b. Enzymatic Marker Assays

NADPH-cytochrome c reductase served as a marker for ER membranes, and 5' nucleotidase indicated plasma membrane contamination. The NADPH-cytochrome c reductase assay was performed by the method of Sottocasa et al (33). Cytochrome reduction was monitored by following absorbance changes at 550 nm using a molar extinction coefficient of 27.7×10^3 $cm^2\ mol^{-1}$. The 5' nucleotidase assay was that used by Solyom and Trams (34). The inorganic phosphate released was determined by the method of Chen et al (25). Protein contents were determined by the method of Markwell et al (27) in order to compute specific activities.

6. Folch Lipid Extraction

Total lipids were extracted from the smooth ER membranes (Fraction 3) by the method of Folch et al (35). One volume of ER membrane suspension was added to 19 volumes of a 2:1 (v/v) chloroform:methanol solution with stirring. Four volumes of a 0.73% NaCl solution were then added and stirred. The resulting mixture was poured into a large graduated cylinder and allowed to equilibrate for 2-3 hours at 5°C. The bottom phase below the interfacial fluff was removed with a vacuum aspirator. The solvent was removed from this lipid-solvent solution by a Rotovapor flash evaporator.

7. TLC Methods

Thin layer chromatography (TLC) of lipids was performed using two different systems. Nonpolar lipids were chromatogrammed on Eastman Silica Gel Chromatogram Sheets using a mixture of petroleum ether : diethyl ether : acetic acid (90:10:1 v/v/v) as the solvent. Polar lipids were run on Brinkmann Instruments Silica Gel 60F-254 glass-backed plates using chloroform : methanol : acetic acid : water (70:30:10:3 v/v/v/v) as a solvent. Appropriate lipid standards were also run. Spots were made visible by exposure of the plates to iodine vapors.

8. Nonpolar Lipid Extraction

Enrichment of the ER lipid sample in polar lipids was accomplished by the extraction of the neutral lipids with

acetone. The nonpolar lipids dissolved in the acetone while the polar ones precipitated. The liquid was decanted, and the precipitate was dried, weighed, and dissolved in a small amount of chloroform:methanol (2:1 v/v). Phospholipid was assayed for in both the acetone extract and precipitate by the method of Chen et al (25).

9. Lipid Vesicle Preparation

Lipid vesicles were prepared for protein-lipid interaction experiments by suspending the dried polar lipid in a buffer containing:

0.01M Tris
0.005% EDTA
0.001M NaN_3
0.10M NaCl pH 8.0

The suspension was sonicated for 30 minutes on the Heat Systems-Ultrasonics W185 Sonifier at a power setting of 4 under a nitrogen atmosphere in the dark. The sample was centrifuged at 30,000 rpm for 30 minutes at 5°C in the Ti 50 rotor using the Beckman L5-65 ultracentrifuge in order to remove any large lipid sheets and any metal particles from the sonicator tip. The supernatant contained the lipid vesicles.

10. Dansyl Labeling of Apo A-I

The Apo A-I protein was labeled with dansyl chloride (Dns-Cl) by the method of Weber (36) as modified by Jonas (37). The protein concentration of the resultant solution was

determined by the method of Markwell et al (27).

11. Fluorescence Polarization Measurements

Changes in Dns fluorescence upon the titration of labeled Apo A-I with lipid vesicles were measured on the SLM Instruments polarization spectrophotometer using Corning Glass 3-74 emission filters. The excitation wavelength was set at 340 nm, and the temperature of the sample was maintained at 37.0°C with a Forma Scientific Masterline water bath attached to the cuvette holder. Two ml of dansylated Apo A-I (Dns-Apo A-I protein content 0.11 mg/ml) were placed in a quartz fluorescence cuvette and allowed to equilibrate at 37°C; 0.4 ml of lipid vesicles containing 4.8 mg phospholipid (PL) per ml were added in 10-50 µl aliquots. After each addition of lipid, the mixture was stirred thouroghly and allowed to equilibrate at 37°C for five minutes. Polarization measurements were then taken.

12. Perrin Plots

Data for Perrin Plots were obtained by measuring Dns fluorescence polarization while varying the viscosities of protein-lipid and free labeled protein solutions. A sample of 2.0 ml Dns-Apo A-I (0.11 mg Apo A-I/ml) plus 0.4 ml lipid vesicles (4.8 mg PL/ml) were equilibrated at 37°C to allow them to react. Also, a reference sample of 2.0 ml Dns-Apo A-I solution plus 0.4 ml Tris buffer (pg. 13) was prepared. Predetermined amounts of ethanol-recrystallized sucrose

corresponding to known viscosities were added and mixed. Fluorescence polarization measurements were taken at 23.0°C using the SLM polarization spectrophotometer with the temperature held constant by a Forma Scientific water bath.

13. λ_{max} and Fluorescence Intensity Changes

Measurements of changes in wavelength of maximum fluorescence intensity (λ_{max}) and changes in intesnity at λ_{max} (quenching) were performed on the Perkin-Elmer MPF-4 Fluorescence Spectrophotometer using an excitation wavelength of 340 nm. Two ml of Dns-Apo A-I solution (0.11 mg protein/ml) were placed in a fluorescence cuvette; a total of 0.4 ml of lipid vesicle suspension (4.8 mg PL/ml) were added in 20 µl aliquots to give a four-fold excess of phospholipid to protein, by weight. After each addition, the mixture was allowed to equilibrate for 5 minutes at room temperature, and the fluorescence intensity spectrum from 460 to 540 nm was taken. The wavelength of maximum fluorescence intensity was noted after each run.

14. Gel Filtration Sizing of Complexes

Determination of protein-lipid complex size was accomplished by passing a reaction mixture of Dns-Apo A-I and lipid vesicles through a Bio Gel A-5M column (45 x 1.8 cm). The reaction mixture was prepared by mixing lipid vesicles and Dns-Apo A-I solution in a ratio of 4:1, phospholipid:protein, by weight. It was incubated at 37°C for five hours to allow

complex formation to occur. One ml of this mixture was loaded on the column and eluted with the same Tris buffer whose composition is given on page 13, except that 0.15M NaCl was used instead of 0.10M NaCl. Fractions of approximately 2 ml each were collected using a Gilson Microfractionator. A sample of Dns-Apo A-I was passed through the column separately in the same manner. Fluorescence intensities were measured on the Perkin-Elmer MPF-3 Fluorescence Spectrophotometer at room temperature with excitation and emission wavelength settings of 340 and 500 nm, respectively. The elution peaks of the protein-lipid mixture and the free protein solution were compared. The five fractions of the eluted protein-lipid mixture containing the most fluorescent label (Fractions 39-43) were pooled for the determination of the protein:phospholipid ratio. Phospholipid phosphorus was determined by the method of Chen et al (25). Protein concentration of porcine Apo A-I was determined by absorbance at 280 nm after accounting for Dns absorbance using Dns molar extinction coefficients of 1.85×10^3 and 4.30×10^3 $M^{-1}cm^{-1}$ at 280 and 340 nm, respectively (36). The extinction coefficient used for porcine Apo A-I was taken to be the average of that of human and bovine Apo A-I, since no coefficient for porcine Apo A-I was available; this averaged value was calculated to be 12.0×10^2 $g^{-1}cm^2$ (28,38). A molecular weight of 27,000 for porcine Apo A-I was assumed.

A reference complex of human Apo A-I, dimyristoyl phosphatidylcholine, and cholesterol of molecular weight

around 200,000 (39) was also eluted through the column; in this case the elution pattern was determined by measuring the (^{14}C) cholesterol counts per minute in the fractions. This pattern was compared with that obtained from Dns-Apo A-I plus lipid and that of free Dns-Apo A-I.

III. RESULTS AND DISCUSSION

Two methods of lipoprotein delipidation were performed for comparison of completeness of lipid extraction. Both the Weisgraber (chloroform/methanol) and Scanu (ethanol/ether) methods of delipidation left less than 1% of phospholipid (PL) by weight in the apolipoprotein samples. The VLDL extracted by the Weisgraber procedure had approximately 0.75% PL by weight, assuming an average PL molecular weight of 775. The HDL delipidated by the Scanu method had about 0.14% PL by weight, while that treated by the Weisgraber procedure had essentially no phospholipid within the range of the phosphorus assay. It is difficult to determine whether the Weisgraber delipidation was more complete than the Scanu method based on the HDL extraction data, for small absolute errors here lead to large relative errors. In any case, both methods seem to remove most of the lipid, judging from the phospholipid contents.

The polyacrylamide gel electrophoresis indicated that the delipidated HDL was rich in Apo A-I; dark bands in the gels containing delipidated HDL had migrated to a position similar to that of the human Apo A-I standard (Figure 2). The human Apo C-I standard gel had no dark band, only a light one near the end. It is possible that the Apo C-I, being a

relatively low molecular weight protein, ran off the end. The pig VLDL protein gel showed a very dark band near the end, but its identity was unknown. It was concluded that Apo A-I was the major protein in the delipidated HDL protein samples, and that it could be isolated by gel filtration.

A graph of absorbance at 280 nm versus fraction number for the gel filtration of the HDL apolipoproteins is shown in Figure 3. The large peak represents Apo A-I; fractions 55-64 were used in the Apo A-I isolation. (The smaller peak to the right represents Apo C.)

The isolation of ER from liver homogenate by the discontinuous sucrose gradient method produced five distinc fractions (Figure 1). From the description of Dallner (29), Fraction 3, the white band near the former location of the 0.44 - 1.3M sucrose interface, was assumed to be the smooth ER, and Fraction 5 the rough ER. To test this assumption, the activity of NADPH-cytochrome c reductase, an ER marker enzyme, was assayed. The reduction of cytochrome was monitored by absorbance at 550 nm. Protein concentrations were also determined, so specific and total activities could be calculated. These assays were performed on the fractions obtained by ultracentrifugation as well as on the pellet discarded after the initial low-speed centrifugation. The specific activity gives the activity per milligram of protein, and the total activity gives the total amount of activity present. The data are presented in Table 1. The highest specific activities were in Fractions 3, 4, and 5; thus confirming the

earlier assumptions. The low-speed centrifugation pellet gave the highest total activity by far, indicating that most of the ER membranes are there, but the lower specific activity suggests that there are more contaminants there than are in Fractions 3, 4, and 5.

One possible source of contaminants in the ER fractions could be from cell plasma membranes; 5' nucleotidase is an enzymatic marker for plasma membranes. Its activity is measured by the amount of inorganic phosphate released when the enzyme cleaves off the phosphate group of 5' adenosine monophosphate in a given time. Again, the assays were performed on the ultracentrifugation fractions (1-5) and the pellet discarded after the low-speed centrifugation. The data are presented in Table 2. The specific activities are relatively close for all fractions. However, the total activity values reveal that the vast majority of the plasma membranes are in the pellet obtained from the low-speed centrifugation. In fact, this pellet contained about 82% of all the 5' nucleotidase activity. From these enzymatic assays, it was decided that the ER preparation was reasonably free from contaminating plasma membranes, and that the ER was pure enough for our purposes.

It was decided to use Fraction 3, the smooth ER, for our experiments. The total lipids were extracted with organic solvents. To test the composition of the isolated lipids, thin layer chromatograms (TLCs) were run. A solvent system for nonpolar lipids was used in which the most neutral lipids

migrate the farthest. Polar lipids, including phospholipids, tend to remain at or near the origin. After the chromatogram was developed (Figure 4), it was found that there was a relatively large amount of neutral lipid compared to polar lipid. Since it is the polar lipids, especially phospholipids, which interact with apolipoproteins to form nascent HDL, it was decided to extract the nonpolar lipids and thus enrich the lipid mixture with phospholipids. It is not certain where the neutral lipids originated. Perhaps the ER contained some triglyceride pools. Knowing that phosphatidylcholine, a phospholipid, is insoluble in acetone, it was assumed that most other phospholipids would also be, and that the triglycerides could be removed by extracting them with this organic solvent. The precipitate obtained from this process was then assumed to contain the phospholipids. In fact, it was found to contain 60 mg of phospholipid in 100 mg total lipid, assuming that the average phospholipid molecular weight is 775. This "enriched" fraction thus contains about 60% phospholipid by weight. On the other hand, before extraction the total lipid mixture contained only 11% PL by weight (132 mg PL in 1137 mg total lipid). Therefore, the acetone extraction did indeed enrich the PL concentration, but also caused a loss of some. However, the amount recovered was sufficient for our experiments. As a qualitative test, TLCs of the polar lipid precipitate and the nonpolar acetone extract were run against various lipid standards in both polar and nonpolar systems. Judging from the sizes and intensities of the spots, most of

the neutral lipids were removed by the acetone wash (Figures 5,6). On the nonpolar chromatogram, the acetone extract showed a dark spot that had migrated the same distance as the triglyceride standard. The polar lipid precipitate showed little migration. On the polar chromatogram, the polar lipid precipitate showed a dark spot at the same distance from the origin as the phosphatidylcholine standard. There were also some other spots corresponding to other polar lipids. The acetone extract showed little movement except for a small phosphatidylcholine spot. Thus it appears that most of the lipids in the polar lipid precipitate are indeed polar.

The lipid was assumed to be of an appropriate composition to perform protein-lipid interaction studies with. Lipid vesicles were produced by sonication. The interaction studies were then carried out using a fluorescent probe attached to Apo A-I. Changes in the protein structure during interaction with lipid were inferred from changes in fluorescence properties.

It would be appropriate here to mention a few words about fluorescence. Basically, an aromatic organic molecule can absorb a photon at a given wavelength, thus raising the molecule to an excited energy state. This excited molecule can later emit a photon and drop back to the ground state. The time between these events is called the fluorescence lifetime, and the emission of the photon is called fluorescence. Because the excited molecule loses energy through molecular collisions and interactions before fluorescence occurs, the

emitted photon has less energy than the absorbed one, and thus the emitted wavelength is longer than that absorbed. The fluorescent probe used in our experiments was dansyl chloride (Dns-Cl). It was covalently attached to the Apo A-I protein molecule (Dns-Apo A-I). Its excitation wavelength is near 340 nm, whereas its emission wavelength is around 500nm. Its fluorescence lifetime is about 12 nanoseconds.

When plane polarized light is used to excite fluorescent molecules, measurement of fluorescence intensity at right angles to the incident light gives two components: intensities of light parallel and perpendicular to the plane of the incident light, denoted by $I_{\parallel}$ and $I_{\perp}$, respectively. The polarization "p" is defined as:

$$p = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

In most cases, a change in polarization is indicative of a change in rotational relaxation time, which in turn signifies that the volume of the fluorescent particle has changed. However, a lack of polarization change does not necessarily rule out interaction, because local rotational motions of the probe can possibly compensate for the slower rotational motion of the larger complex particle.

A useful method for quantitating the volume and rotational relaxation time of the fluorescent particle is the use of Perrin Plots. Here, polarization is measured as solution

viscosity or temperature is changed. In our experiments, solution viscosity was varied by the addition of sucrose. A plot of 1/p versus T/η is constructed, where p is fluorescence polarization, T is the temperature, and η is the viscosity. When the curve is extrapolated to $T/\eta = 0$, the intercept on the vertical axis is $1/p_o$, the inverse of the limiting polarization. The Perrin equation (40) as modified by Weber (41) can then be applied:

$$(1/p - 1/3) = (1/p_o - 1/3)(1 + 3\tau/\rho)$$

τ is the fluorescence lifetime, and ρ is the rotational relaxation time. In the case of a spherical particle, ρ is given by the expression:

$$\rho = \frac{3V\eta}{RT}$$

V is the particle volume, and R is the gas constant. This equation shows that the rotational relaxation time is proportional to particle volume.

Another useful fluorescence method to examine protein-lipid interactions is the change of wavelength of peak fluorescence of the probe (λ_{max}). When a labeled protein molecule is in contact with aqueous solvent, there is some interaction between the dipoles of the fluorescent probe and the surrounding water molecules. Some of the energy of the

excited state is lost here. However, when the labeled protein molecule is associated with lipid, the dipole interactions in the excited state are lessened. Therefore, not as much energy is lost in this case, and the emitted photon has greater energy than in the situation where the molecule was surrounded by water. Thus, the wavelength of emitted light is shorter when the labeled protein is bound to lipid than when it is not. This is called a blue shift. In addition to λ_{max} changes, intensity changes can also occur. This is indicative of a change in the environment of the probe. In most cases, fluorescence quenching effects (i.e. changes in intensity) in a protein molecule cannot be attributed to a single intramolecular or extramolecular interaction, but do reflect environment changes around the chromophore.

The data for polarization measurements taken as Dns-Apo A-I was titrated with lipid are presented in Figure 7. The polarization remained steady around 0.193 and did not begin to rise until about 80 µl of lipid vesicles had been added. The polarization had only risen to about 0.204 by the time 400 µl of lipid vesicles had been added, corresponding to a PL:protein molar ratio of about 300:1. This shift in polarization values is small and not significant when compared to those obtained by Jonas and Krajnovich (20) for the titration of human Apo A-I with dimyristoyl phosphatidylcholine. They obtained a change of polarization value from 0.18 to 0.23. In addition, their studies show an immediate increase in polarization upon the addition of lipid. The lack

of a large polarization increase in our experiments, however, does not rule out the occurrence of protein-lipid associations. The model for fluorescence studies for this type of interaction assumes that the fluorescent probe is rigidly bound to a spherical complex. However, the labeled protein is not tightly bound to the lipid, and the protein can rotate carrying the probe with it. The rotation of the probe causes changes in the polarization. Also, the lack of a significant polarization increase could be a kinetic effect in which more time was needed to form complexes than was allowed in the experiment. Therefore, the data are inconclusive. The rise in polarization that did occur may be due to light scattering caused by turbidity induced by the relatively large amount of insoluble lipid in the solution.

The construction of Perrin Plots gave better results. A solution of Dns-Apo A-I incubated with lipid vesicles and a solution of free Dns-Apo A-I were used. Both solutions had the same concentration of protein (0.092 mg Apo A-I/ml). The lipid-protein mixture had an 8.7:1 weight excess of PL over protein, or a 300:1 molar excess, which should have been more than enough for complete complex formation (20). T/η was varied by altering viscosity by the addition of sucrose. The data are presented in Figure 8. The rotational relaxation time ρ_h of the protein-lipid mixture particles was about 270 nanoseconds while that for the free Dns-Apo A-I was about 80 ns. Since ρ_h is proportional to particle volume, the volume of the particles in the lipid-protein mixture was

greater than that of the protein alone. Therefore, the protein must have associated with the lipid vesicles in order to increase particle size. The value obtained here for rotational relaxation time agrees well with those obtained by Jonas and Krajnovich (20) for interaction between dimyristoyl phosphatidylcholine and human and bovine Apo A-I. Their complexes had average isothermal ρ_h values of 250 ± 50 and 280 ± 60 ns for human and bovine Apo A-I, respectively.

The data from the fluorescence λ_{max} and intensity change studies also indicated complex formation (Figures 9,10) Before the labeled protein was titrated with lipid, the λ_{max} value was 501 nm with a peak intensity of 37%. The intensity was measured on a relative (%) scale. Upon titration with lipid vesicles, the value of λ_{max} decreased. The rate of change decreased as more lipid was added as it approached its limiting value. If we assume that the limiting value of λ_{max} is 495 nm and extrapolate the initial slope of the λ_{max} vs. lipid added curve to this value, we obtain a "saturation" value of lipid of 70 µl, corresponding to a 55:1 molar PL: protein ratio. The saturation value is the amount of lipid required to react with all the protein in the complex-forming reaction. The λ_{max} change itself indicates that the environment of the Dns probe has changed, but the change in λ_{max} intensity found reinforces this point. Upon titration with lipid, the intensity at λ_{max} rapidly rose to about 69% and leveled off. If we again extrapolate the initial slope of the intensity vs. lipid added curve to this 69%

limiting value, we obtain a saturation value of about 40 μl lipid or a 30:1 molar PL:protein ratio. This value is not close to the ratio obtained from the λ_{max} change data, but one must bear in mind that the extrapolation methods are very rough and that all numbers given are only estimates. In addition, there is an uncertainty of ± 1 nm in the λ_{max} values.

Additional information on complex formation can be obtained from gel filtration column experiments. Basically, the system employed separates particles by size. The gel beads contain pores of a certain size in which small molecules passing down the column will pass through. Large molecules, which are too big to fit in the pores, pass around the gel beads instead of through them. The particles passing through the pores are slowed down while going through them. The result is that larger particles run the length of the column faster than small ones, and therefore elute first. In our studies, free Dns-Apo A-I and a mixture of Dns-Apo A-I and lipid vesicles were passed through the column separately. If the protein and lipid have indeed formed complexes, they should elute in a fraction ahead of where free protein comes off, since complexes are larger than single protein molecules. As it turned out, the distinction was not very great. As determined by fluorescence intensity measurements, the peak concentration of labeled protein, possibly bound to lipid, was in fraction 41, while that for free Dns-Apo A-I was in fraction 42 (Figures 11,12). This is not a significant

shift, so the data must be interpreted as inconclusive. The elution pattern of the human Apo A-I - dimyristoyl phosphatidylcholine - (^{14}C) cholesterol complexes (Figure 13) of molecular weight 200,000 suggests that the protein-lipid complexes produced here are of a similar molecular weight, assuming that complexes were indeed formed. The peak concentration of the (^{14}C)-containing complexes was in fraction 41, the same fraction number in which eluted the peak concentration of labeled protein in the Dns-Apo A-I/ lipid mixture. (The smaller peak to the left of the main peak in Figure 13 may have been some contamination in the column.)

In light of the other experiments conducted, it appears as if porcine Apo A-I and ER lipids do indeed form complexes. To resolve this discrepancy with the gel filtration experiment, it should be noted that the free protein might exist in an oligomeric form. According to Jonas and Krajnovich (20), bovine Apo A-I occurs in such a form which evidently dissociates to monomeric units when interacting with lipids. If this were the case with porcine Apo A-I, it may explain why the free protein eluted in a position similar to that of the supposed lipid-protein complexes. The oligomer would be larger than the single protein molecule and thus would elute before the position expected for a monomeric protein. If these assumptions are valid, it appears then that the protein oligomers have a size similar to that of protein-lipid complexes. However, the Perrin Plots suggest that the

lipoprotein particles are much larger than the free protein particles, but the gel filtration data suggest that they are of a similar size. Another possibility is that the gel filtration elution pattern may depend on the symmetry and shape of the particle. If the lipiprotein and free protein particles have different symmetries, they may give different elution patterns which could not be compared. Therefore, the gel filtration results may be misleading.

The molar PL:protein ratio for the peak fractions of the Dns-Apo A-I / lipid reaction mixture was calculated to be 27:1 which was fairly close to the 30:1 value calculated from the fluorescence quenching data, but did not agree well with the 70:1 ratio obtained from λ_{max} shifts. When forming complexes of synthetic dimyristoyl phosphatidylcholine (DMPC) with human and bovine Apo A-I, Jonas et al (42) obtained molar ratios in the vicinity of 90:1, PL:protein. Our results do not agree with these, but one must bear in mind that our lipid vesicles were only 60% phospholipid, so our PL:protein ratios do not really reflect what the total lipid:protein ratio is. Also, Jonas (43) has shown that the molar PL:protein ratio varies depending on the type of phospholipid used; the number of PL molecules per protein molecule decreases with increasing PL hydrocarbon chain length and with increasing unsaturation. It is possible that our ratios are lower than those obtained for DMPC because the ER phospholipids may have longer hydrocarbon chains or more unsaturated bonds than DMPC.

The results obtained in these studies are fairly good qualitative indications that porcine Apo A-I does form lipiprotein complexes with liver ER lipids. One should bear in mind that the quantitative values presented here are only estimates and were computed using some very crude approximations. Further experimentation will be needed to further quantitate the process and determine the mechanism. Also, the possibility that Apo A-I may interact with whole ER membranes is worth investigating.

Figure 1. Discontinuous Sucrose Gradient Isolation of ER Membranes

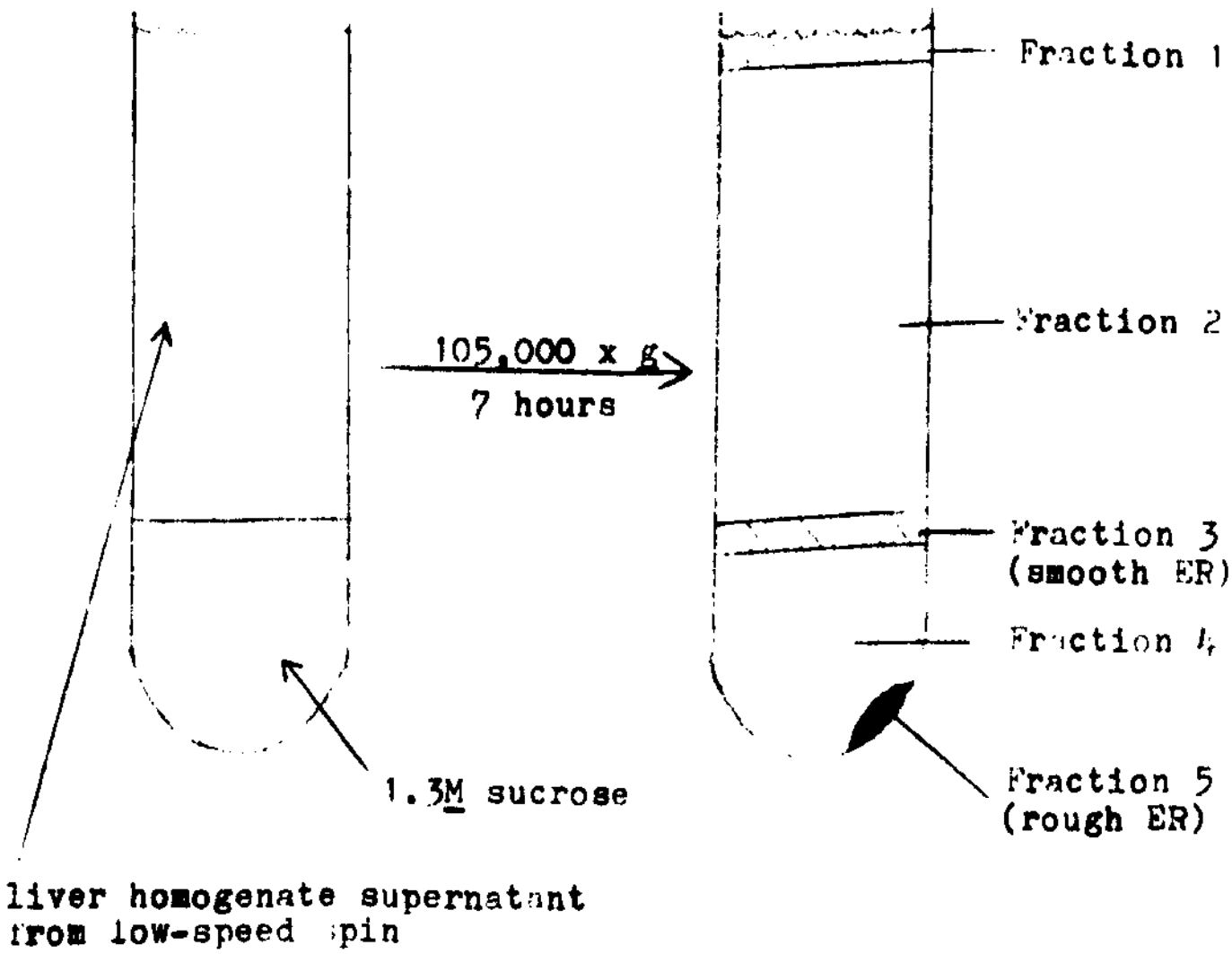
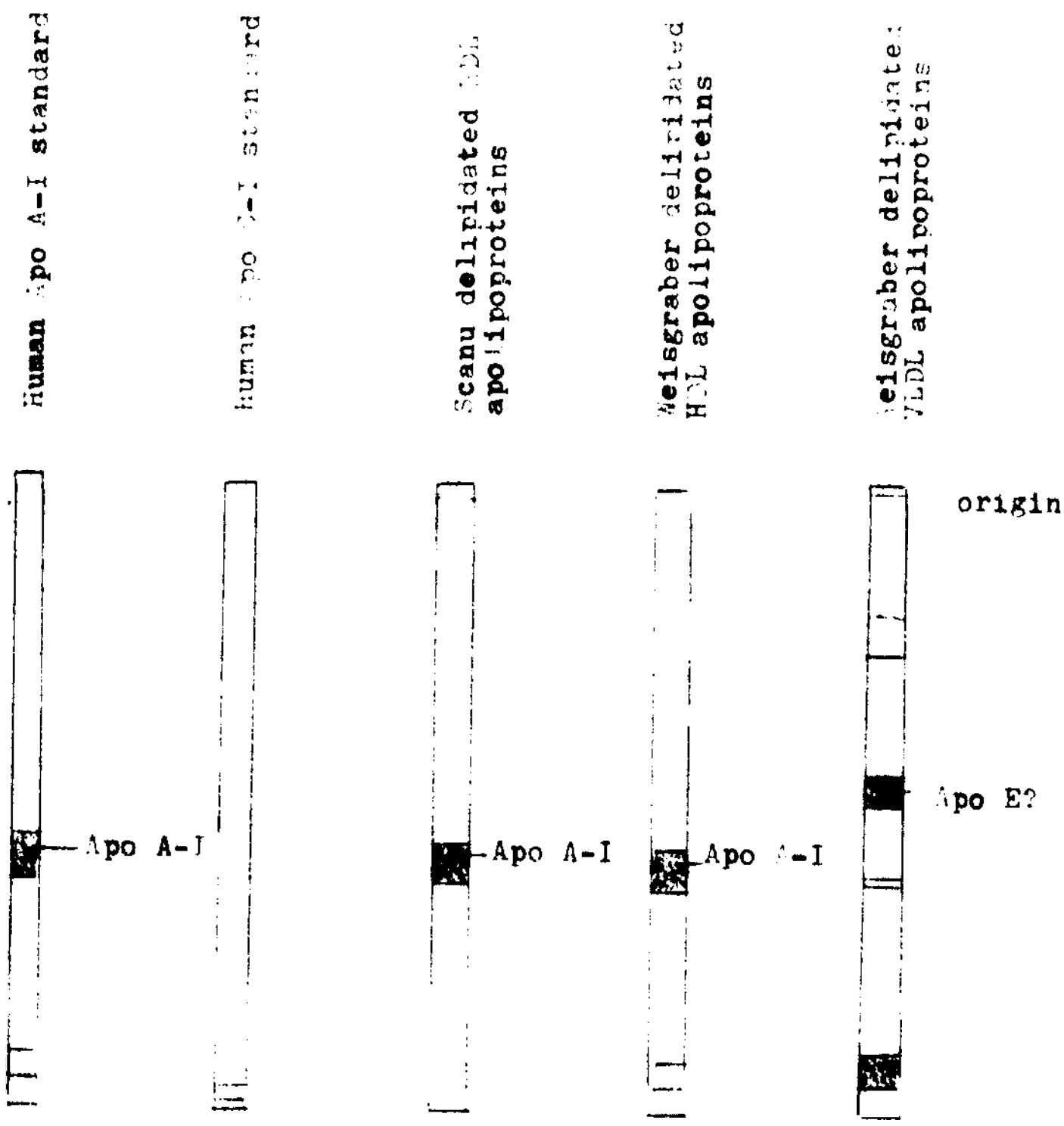


Figure 2. Polyacrylamide Gel Electrophoresis



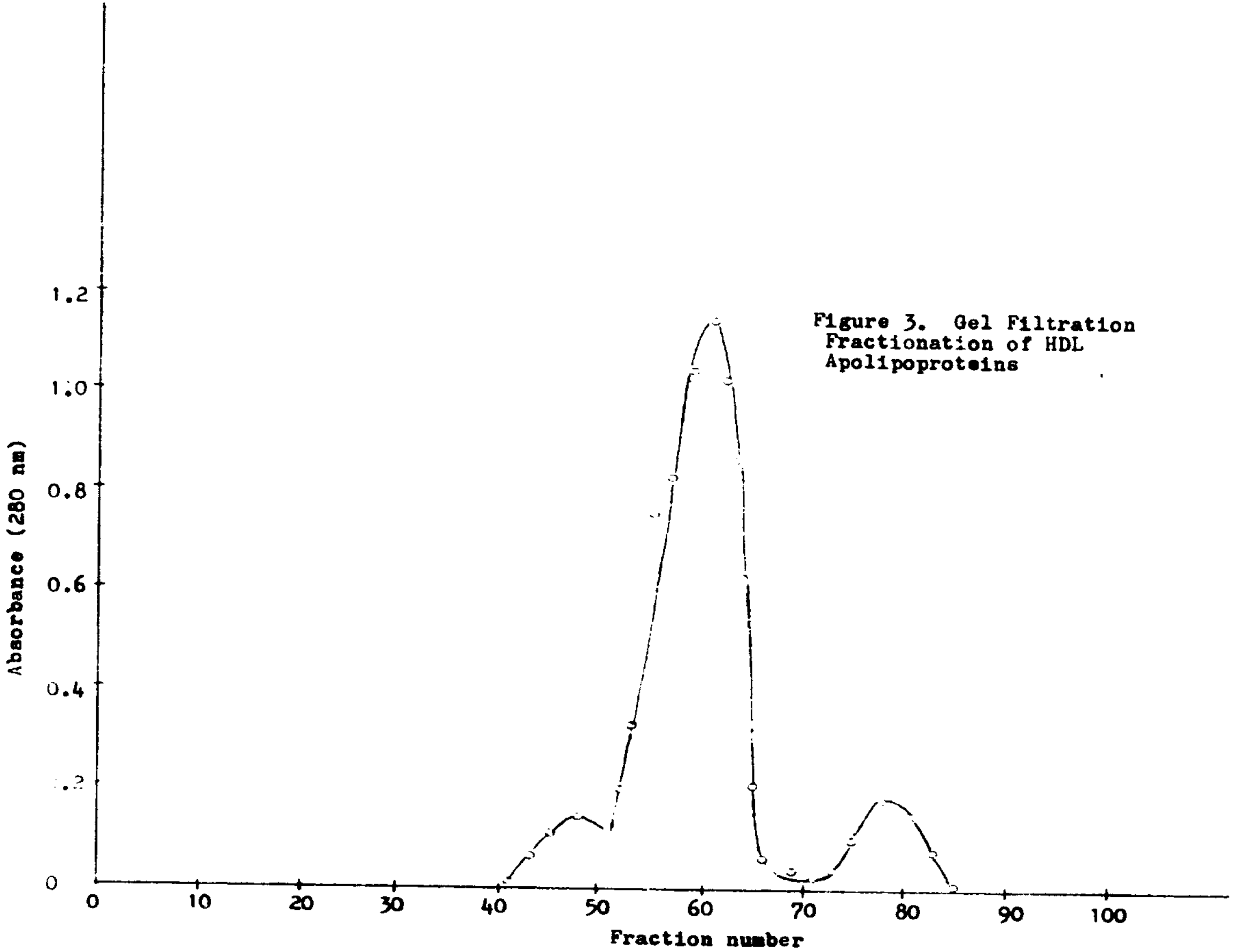


Figure 3. Gel Filtration Fractionation of HDL Apolipoproteins

Figure 4. TLC for Nonpolar Lipids
Before Acetone Extraction

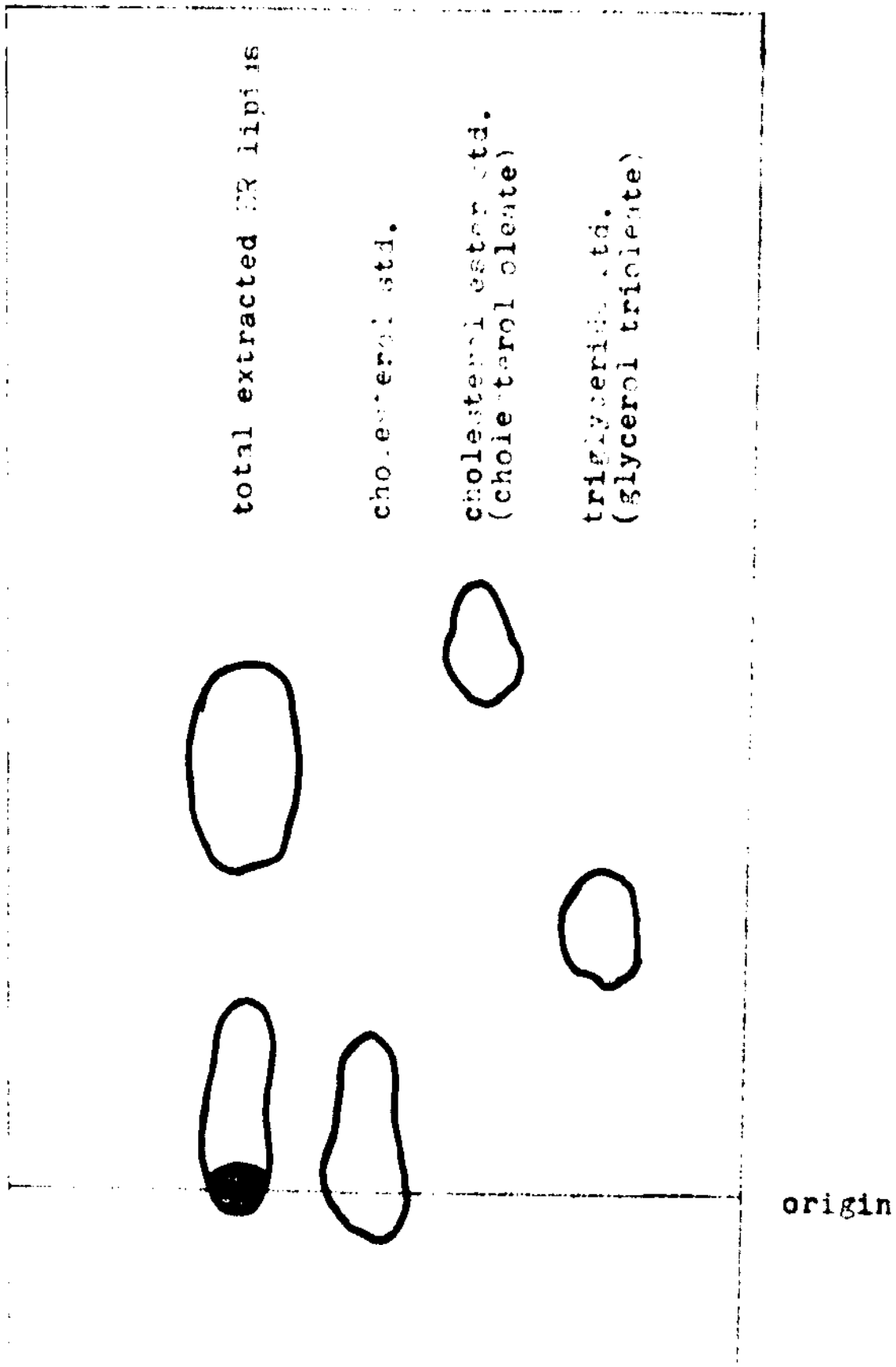


Figure 5. TLC for Polar Lipids After Acetone Extraction

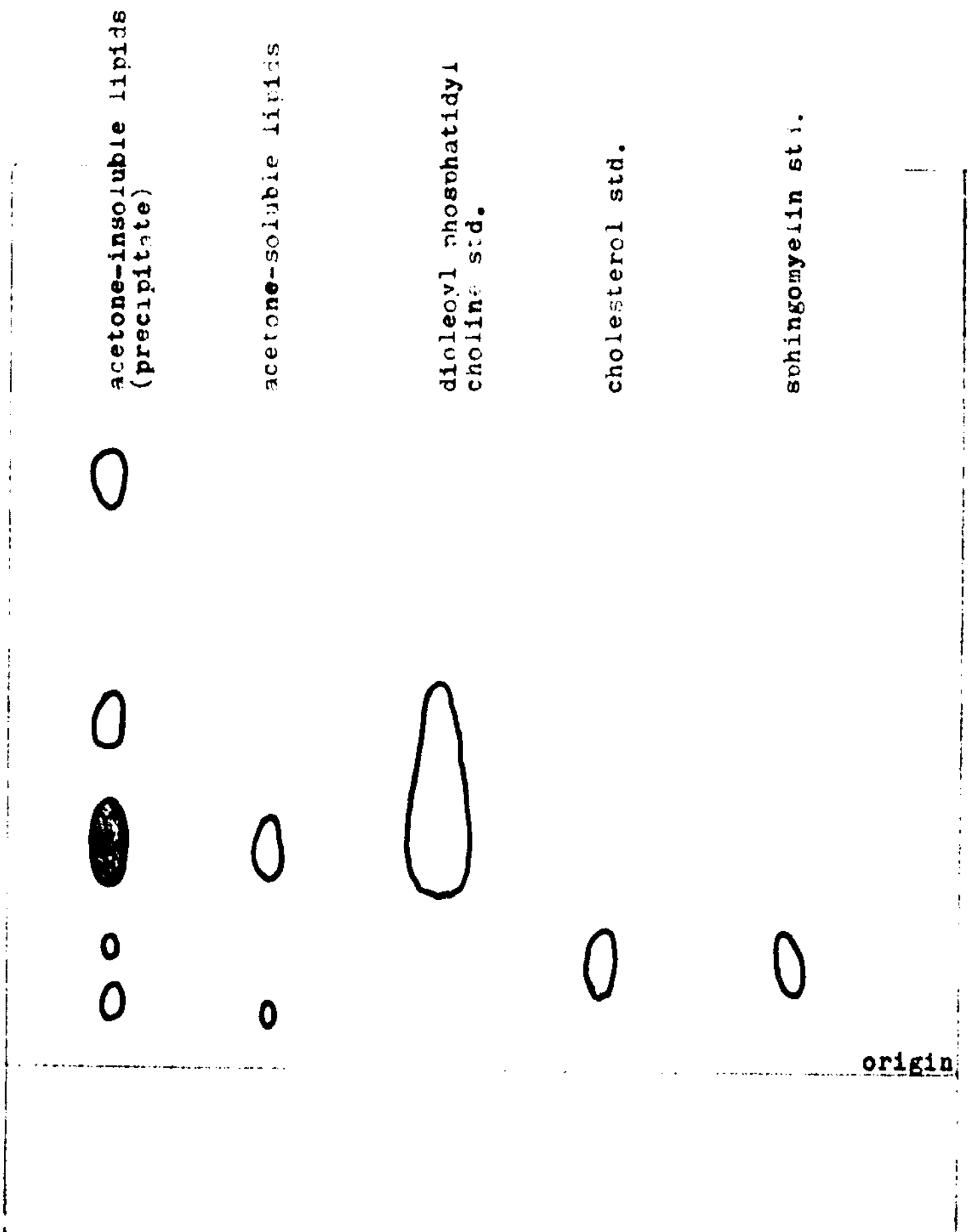


Figure 6. TLC for Nonpolar Lipids
After Acetone Extraction

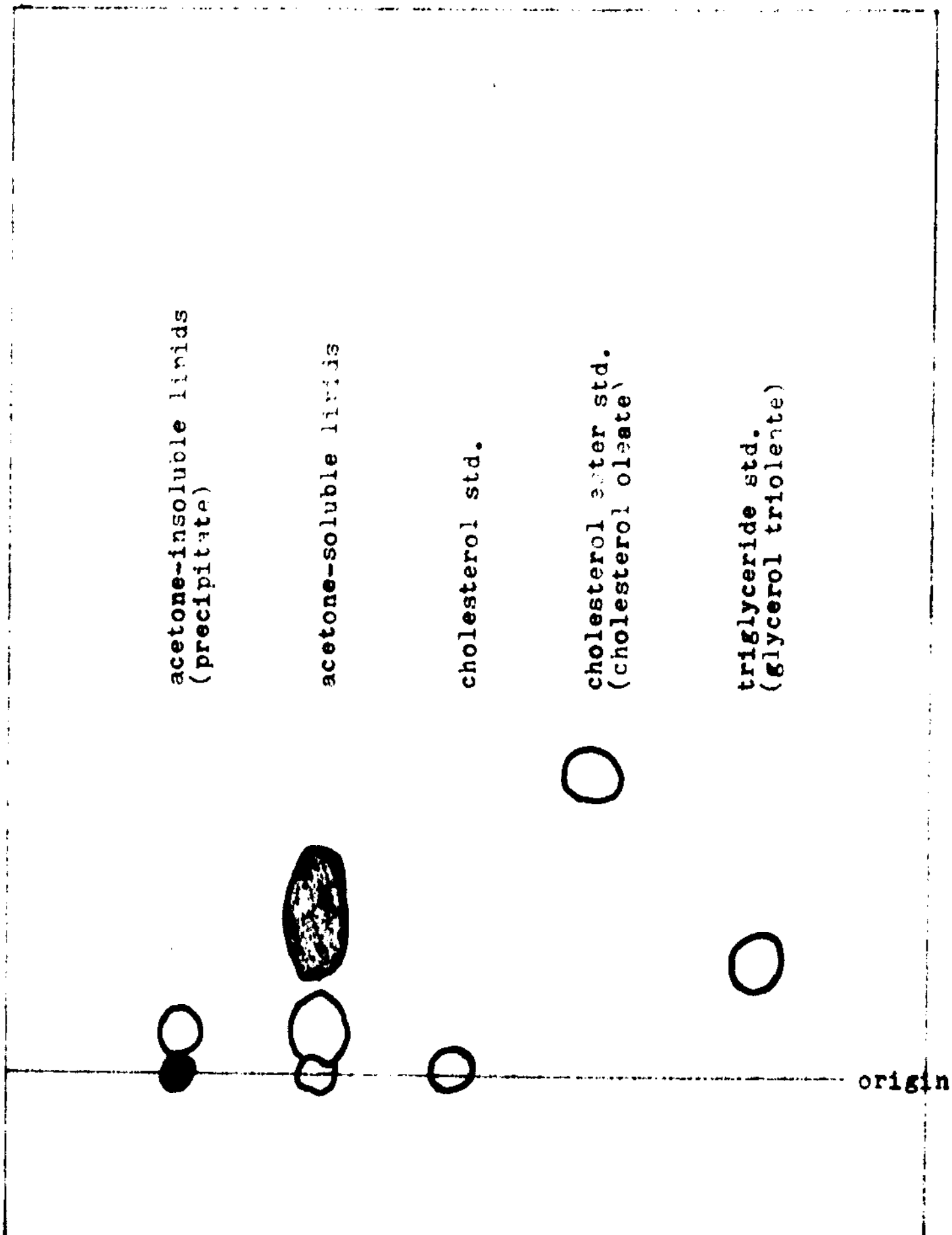


Figure 7. Fluorescence Polarization

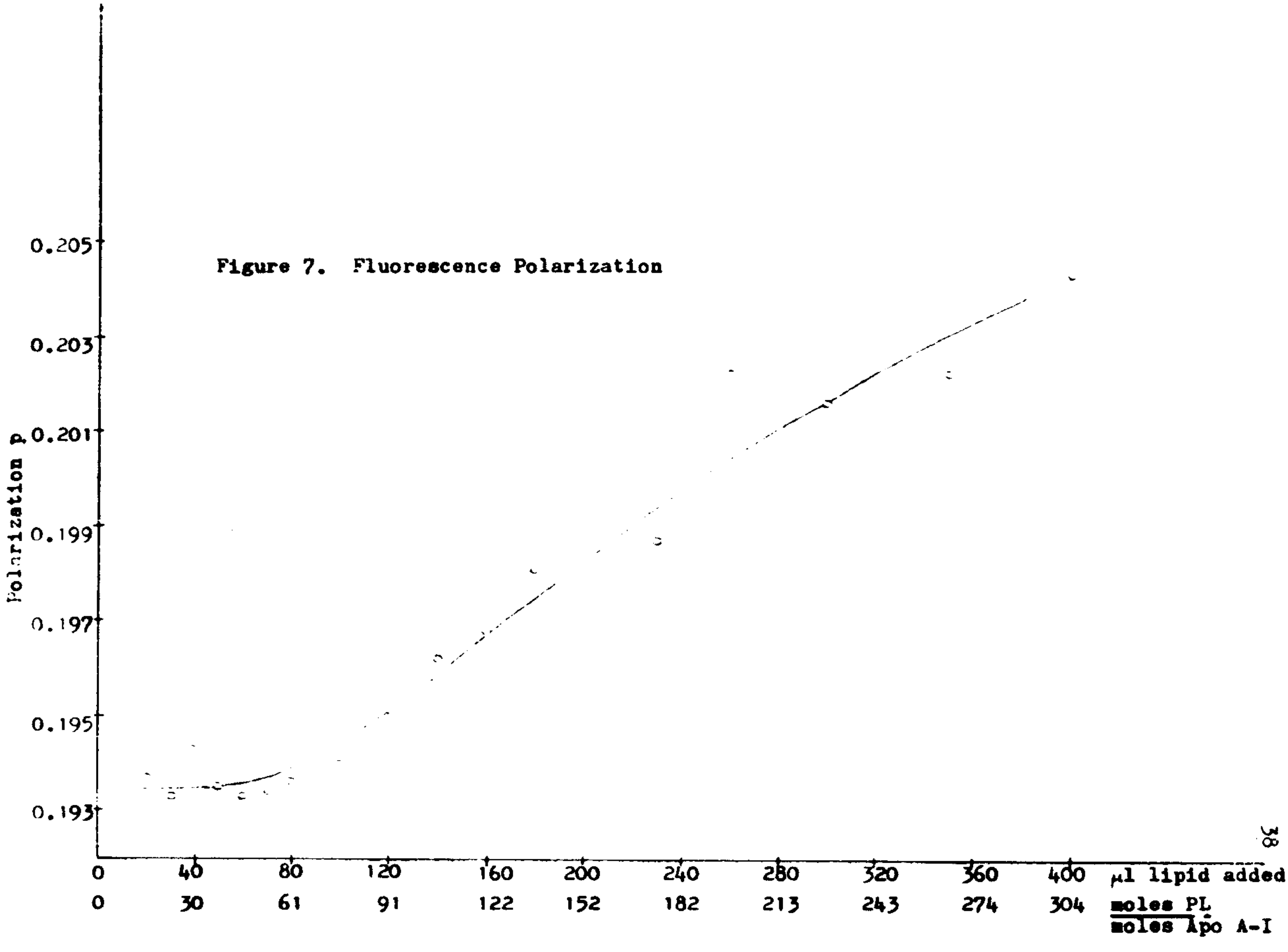
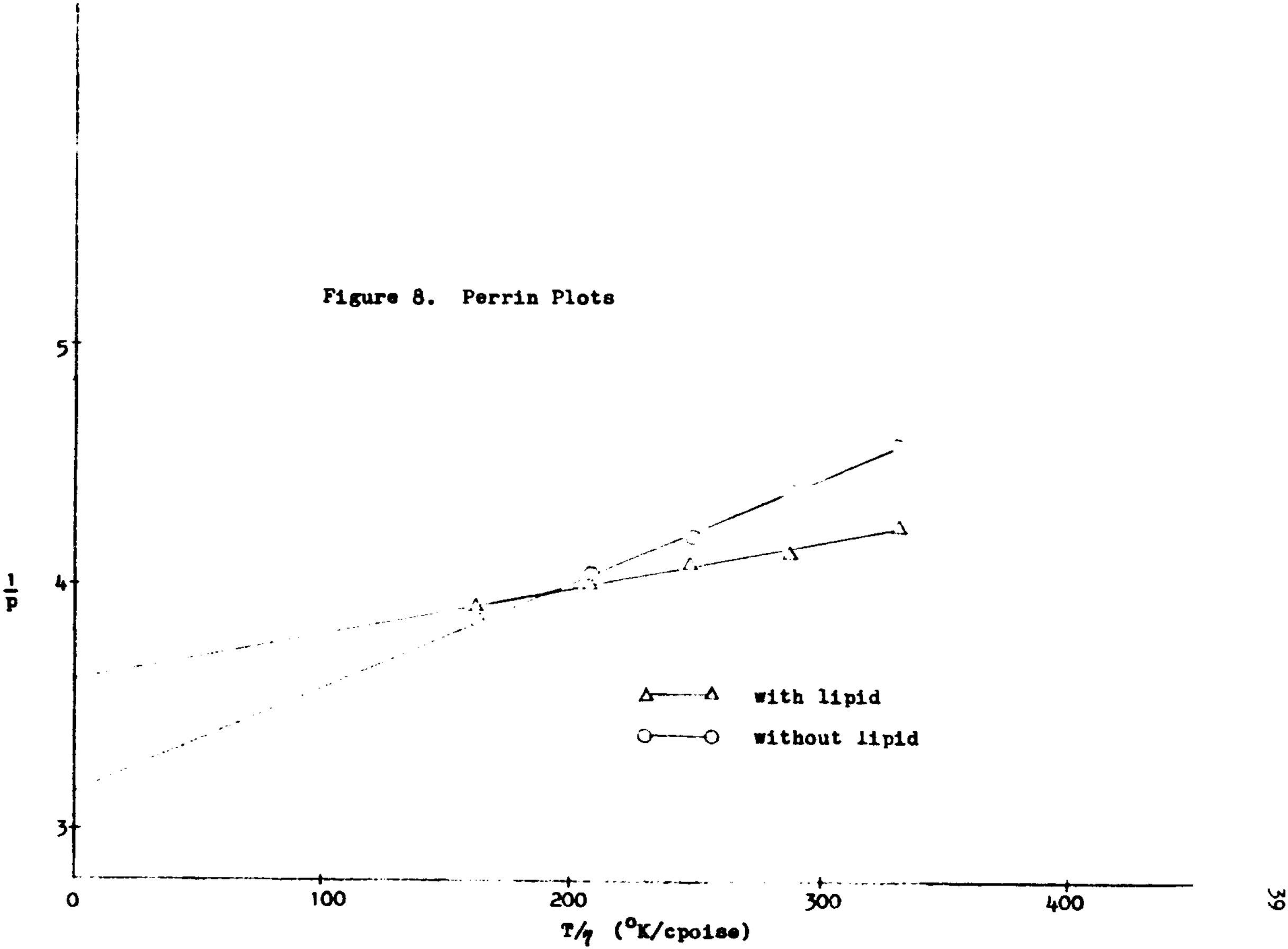


Figure 8. Perrin Plots



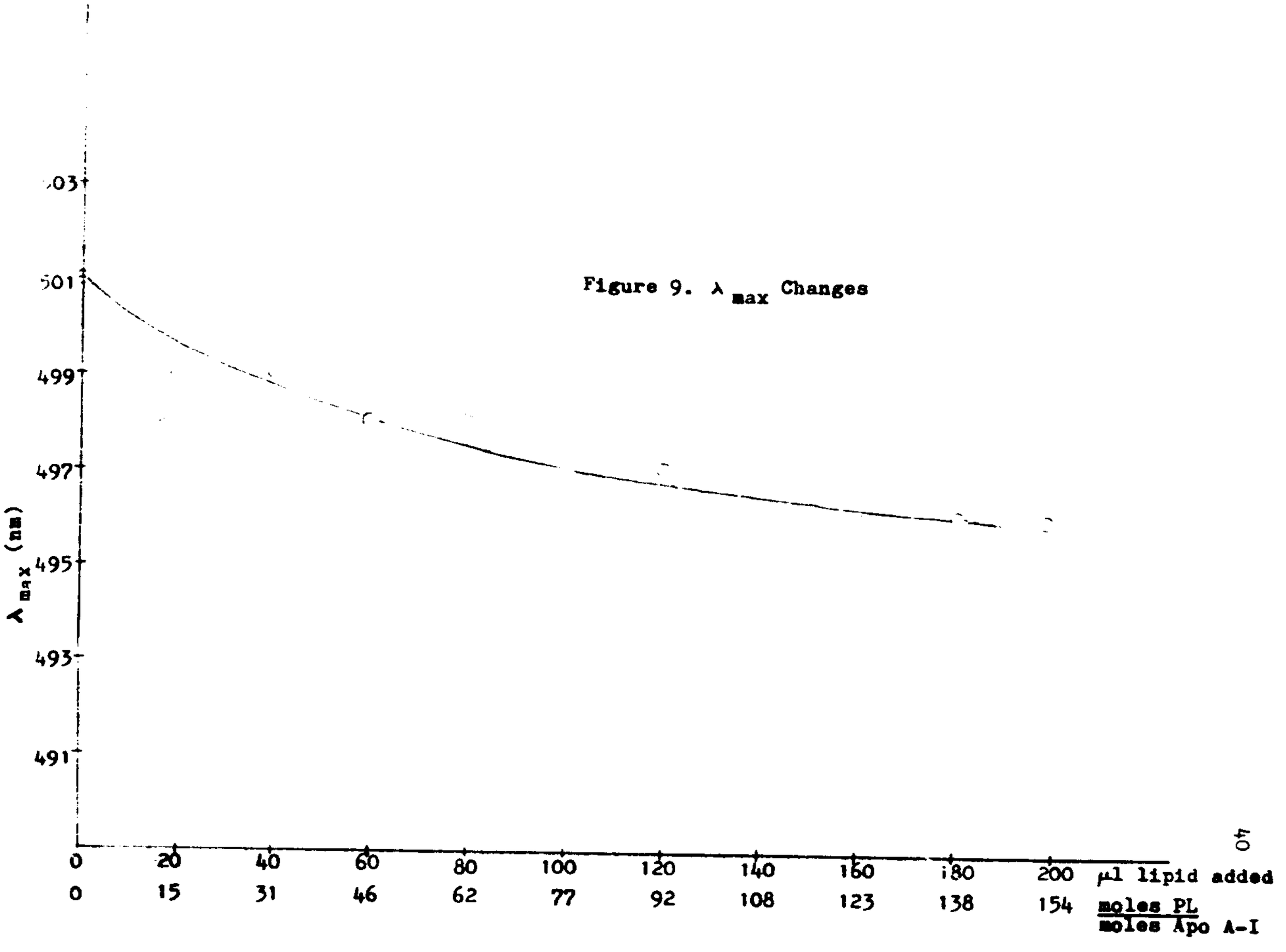


Figure 9. λ_{max} Changes

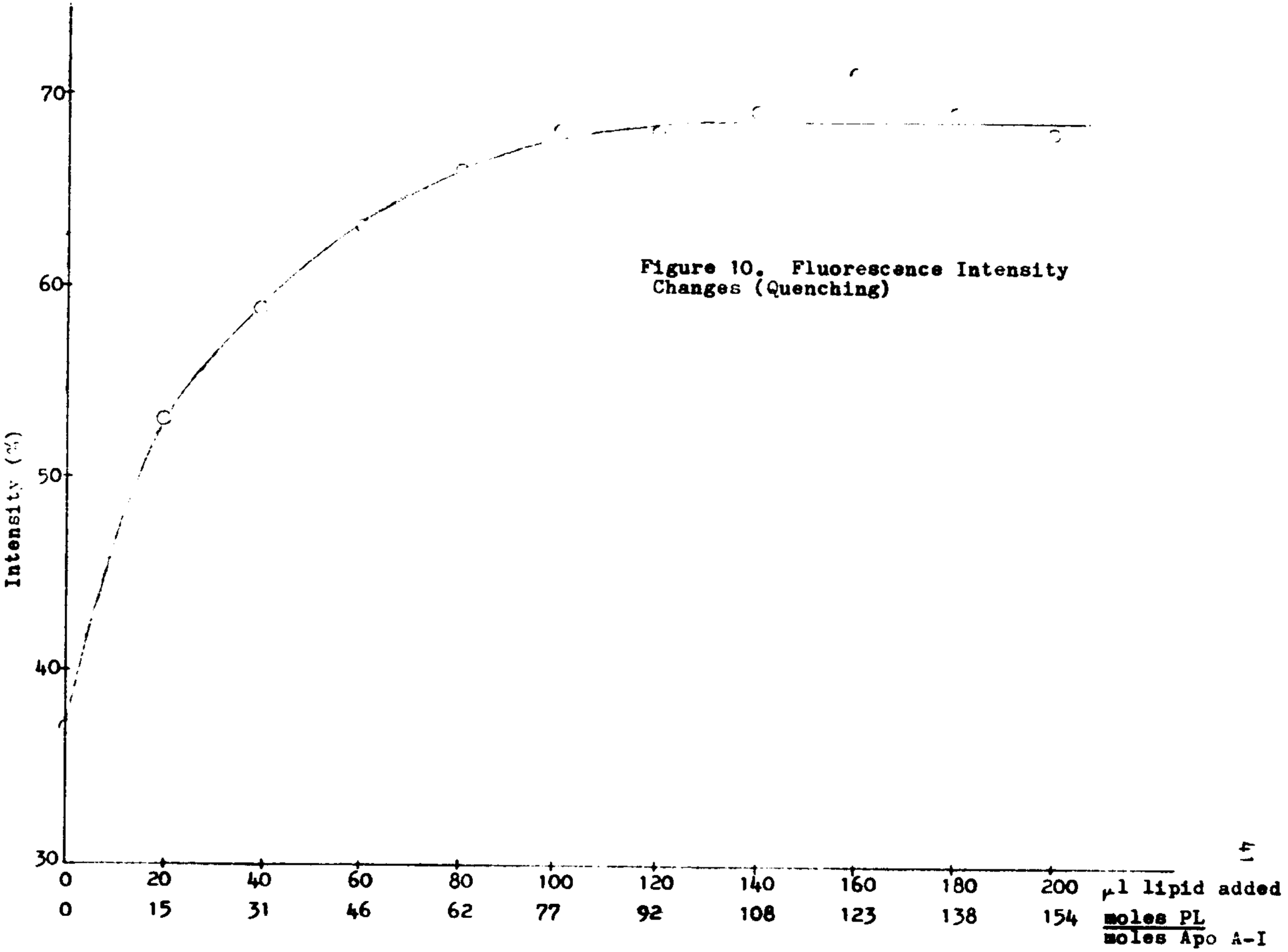


Figure 10. Fluorescence Intensity Changes (Quenching)

Figure 11. Gel Filtration Elution of Dns-Apo A-I plus Lipid

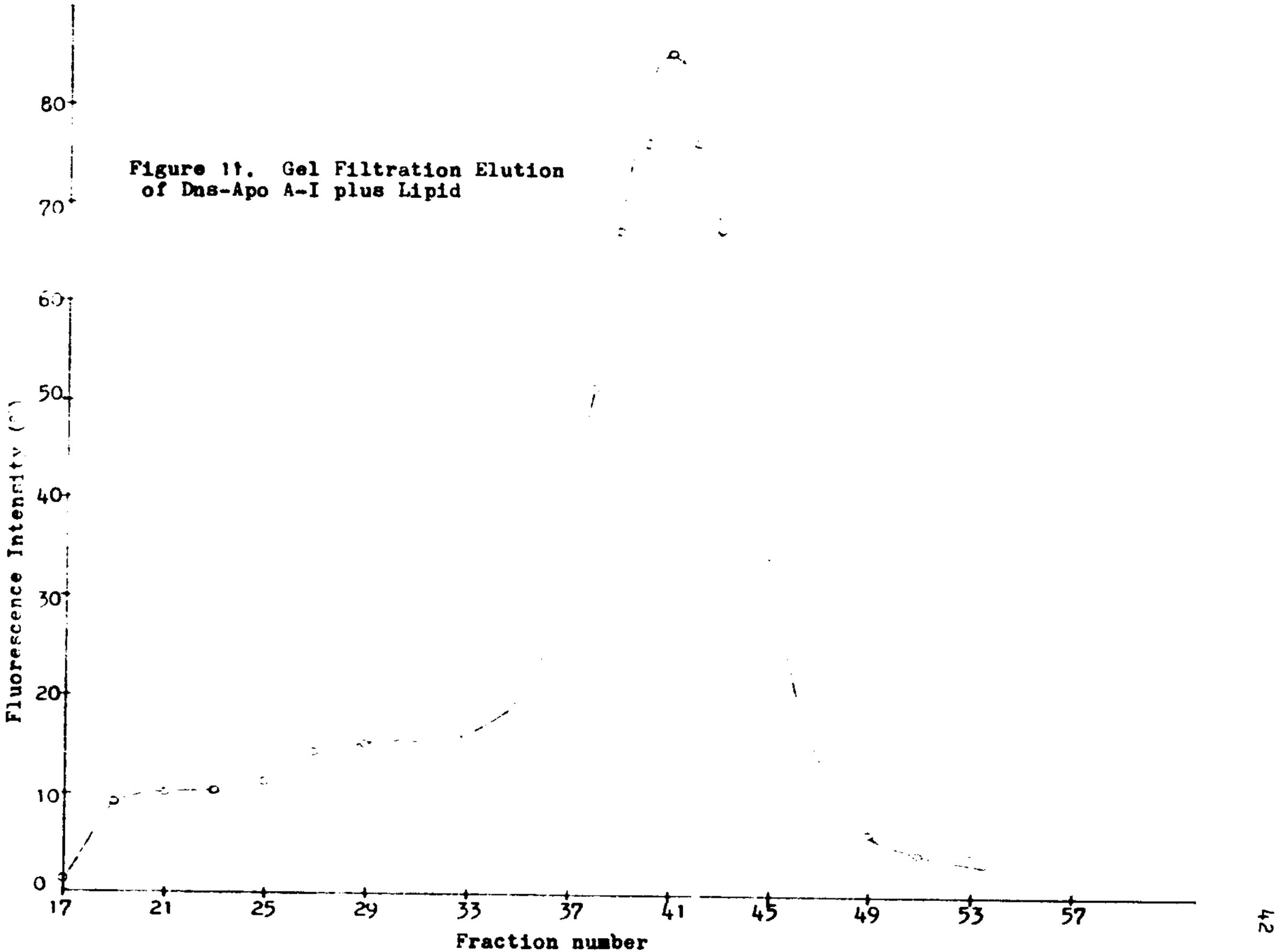


Figure 12. Gel Filtration Elution of Free Dns-Apo A-I

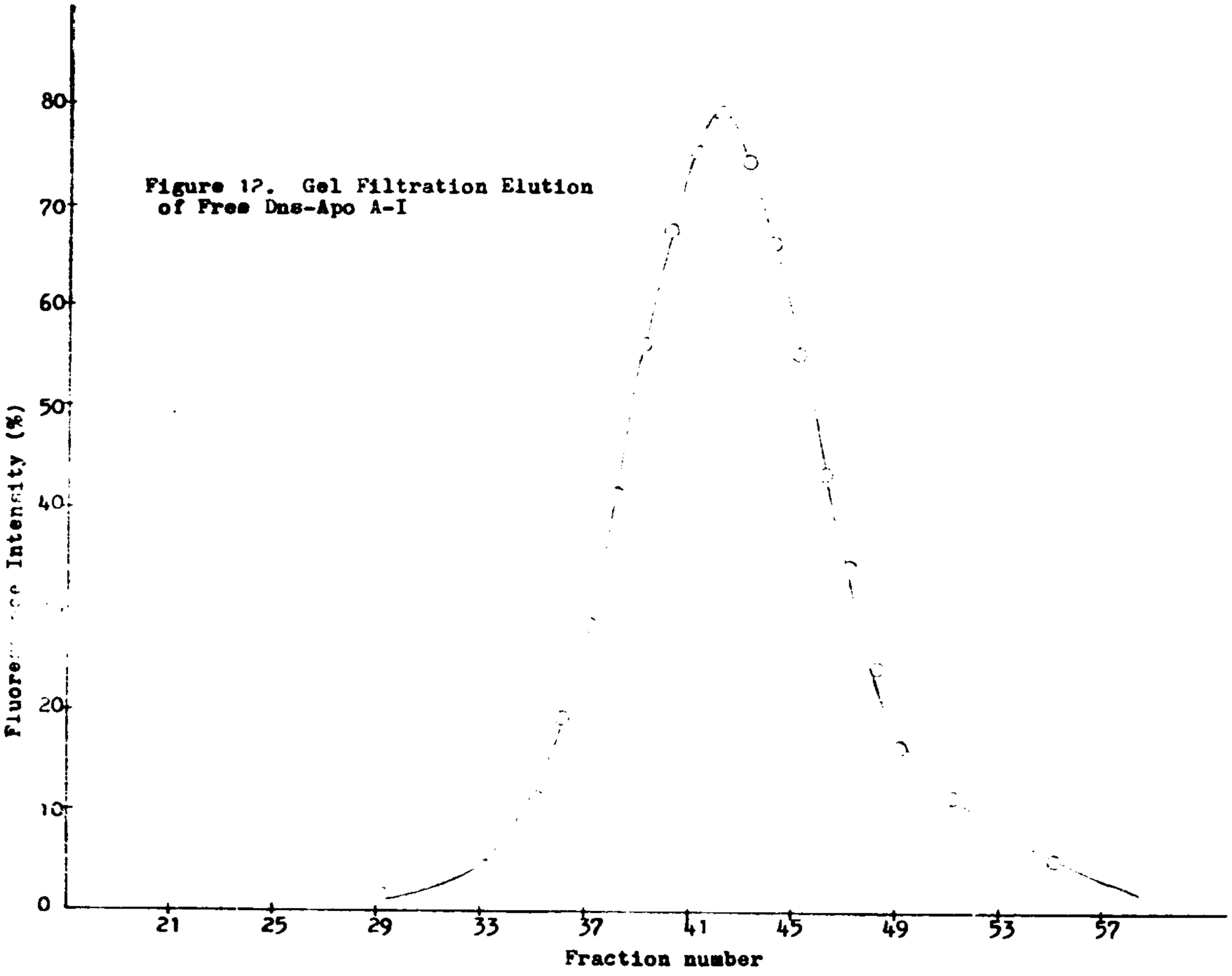


Figure 13. Gel Filtration Elution of (^{14}C)-Labeled Reference Complexes

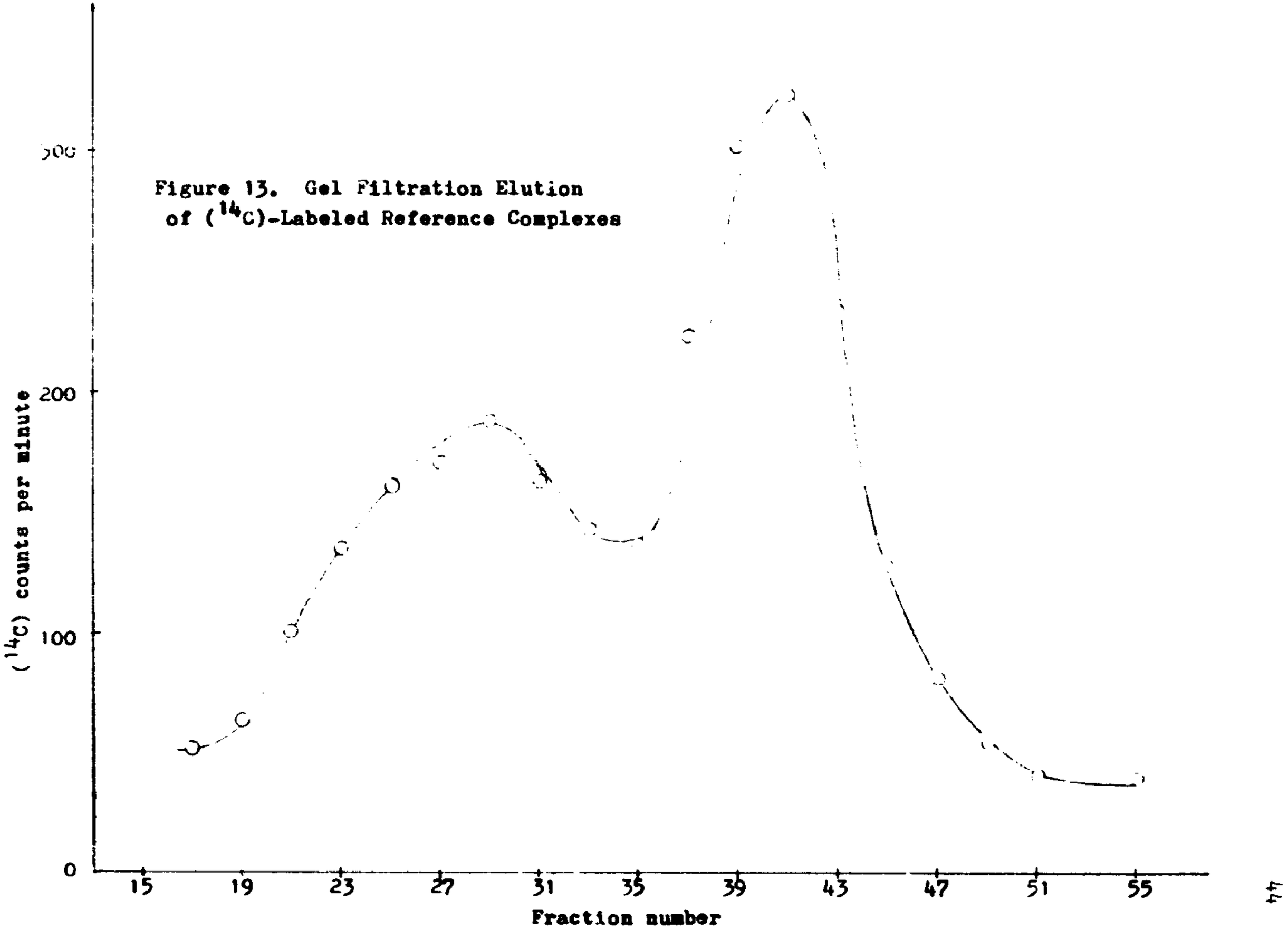


Table 1
NADPH - Cytochrome c Reductase Activity

Fraction	Specific Activity ($\frac{\text{nmoles cyt-c reduced}}{\text{mg. prot - min}}$)	Total Activity ($\frac{\text{nmoles cyt-c reduced}}{\text{min}}$)
1	1.80×10^{-2}	1.58
2	1.02×10^{-2}	12.1
3	2.34×10^{-1}	39.8
4	4.07×10^{-1}	31.1
5	2.66×10^{-1}	25.9
Low-speed centrifugation pellet	1.62×10^{-1}	400

Table 2
5' Nucleotidase Activity

Fraction	15 Minute Specific Activity $\left(\frac{\text{mg P released}}{\text{mg prot} - 15 \text{ min}}\right)$	15 Minute Total Activity $\left(\frac{\text{mg P released}}{15 \text{ min}}\right)$
1	5.09×10^{-3}	0.45
2	5.66×10^{-3}	6.72
3	3.29×10^{-2}	5.59
4	3.70×10^{-2}	2.83
5	1.21×10^{-2}	1.18
Low-speed centrifugation pellet	3.00×10^{-2}	75

LIST OF ABBREVIATIONS

DMPC	L-α-dimyristoyl phosphatidylcholine
Dns	Dansyl
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
HDL	High density lipoprotein
LCAT	Lecithin-cholesterol acyltransferase
LDL	Low density lipoprotein
ns	Nanosecond
PL	Phospholipid
TLC	Thin layer chromatogram (chromatography)
VLDL	Very-low density lipoprotein

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