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ULTRACENTRIFUGAL ANALYSIS OF SERUM LIPOPROTEINS

Oliver de Lalla and John W. Gofman

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## ULTRACENTRIFUGAL ANALYSIS OF SERUM LIPOPROTEINS

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### I. Introduction

The serum of humans and a variety of experimental animals contains a "spectrum of lipoproteins", i. e. a series of substances containing various lipids in combination with protein of a very wide range of molecular weights and hydrated densities. (1), (2), (3) and (4). The major factor responsible for the variation in density is the difference in lipid-protein ratio from one species to another. This series of lipoproteins represents the transport system for at least 95 percent of the major serum lipid constituents such as glyceryl ester, cholesterol, cholesterol ester, fatty acid, and phospholipid. (5), (6), (7), and (8).

Advantage is taken of the variation in density in the design of ultracentrifugal analytical methods for the determination of serum lipoproteins. These lipoproteins range in hydrated density from less than 1.0 gm/ml. up to 1.145 gms/ml. The quantitative determination of these lipoproteins is greatly facilitated if they are first separated from the serum proteins (of hydrated densities of approximately 1.3 gms/ml.) The technique of effecting the separation of the lipoproteins from the proteins depends upon which segment of the entire lipoprotein spectrum is of interest. This separation is achieved by a preparative ultracentrifugation in a solution the density of which due to low molecular weight constituents is placed at a value greater than that of the most dense lipoprotein of interest but less than that of the remaining lipoproteins and the serum proteins. Three basic types of preparative ultracentrifugal steps have been found by us to provide a scheme satisfactory for the quantitative study and resolution of the entire lipoprotein spectrum. These steps are:

A. Preparative ultracentrifugation at solution density 1.063 gms/ml.

This separates quantitatively a whole series of lipoproteins ranging in density

from less than 1.0 gms/ml up to 1.04 gms/ml. from the more dense lipoproteins and serum proteins.

B. Preparative ultracentrifugation at solution density 1.125 gms/ml.

This separates in addition two additional lipoproteins, one of hydrated density 1.05 gms/ml. and the other of hydrated density of 1.075 gms/ml.

C. Preparative ultracentrifugation at solution density 1.20 gms/ml.

This separates in addition another lipoprotein of hydrated density 1.145 gms/ml.

The lipoproteins separated in such preparative steps are then subjected to analytical ultracentrifugation, yielding a film record from which a determination can be made of the lipoproteins present and their concentrations.

## II. Experimental Methods

### A. Collection and preparation of serum.

A complete analysis of the serum lipoproteins, in which all three preparative steps are utilized, requires a total of 11 ml. of serum. This means in general that about 25 ml. of blood must be obtained. The blood must be drawn into a clean, dry syringe and delivered directly into a clean, dry vessel, without the use of an anticoagulant. A two to four hour period at room temperature is allowed for clotting. The clot and any loose red cells are centrifuged out in a clinical centrifuge operated at 1500-2000 r.p.m. for 10 - 15 minutes. The serum is then decanted into a storage vessel, stoppered efficiently, and stored at 0-4°C until further processing. Storage tests under these conditions have shown no significant alteration in lipoprotein composition at least for a period of 28 days.

### B. Preparative Ultracentrifugation

The purpose of preparative ultracentrifugation is the separation of the desired lipoprotein classes from the serum. This is achieved by mixing a specified volume of serum (the density of which is 1.0073 gms/ml. exclusive of contributions to density by lipoproteins and proteins) with a specified volume of diluent of appropriate density. Thorough mixture of those component volumes provides a medium the density of which is above that of the most dense lipoprotein to be separated in this step. An adequate period of ultracentrifugation must then be used to insure quantitative flotation of the lipoproteins and sedimentation of other macromolecular constituents. The preparative ultracentrifuge (Spinco Model L-H) effects this separation satisfactorily.

Specific directions for the three types of preparative ultracentrifugations are given below.

1. Preparative ultracentrifugation at solution density 1.063 gms/ml.  
(Preparative Type 1)

Five milliliters of serum are mixed at room temperature with four milliliters of diluent of density  $1.1315 \pm 0.0005$  gms/ml. at  $20^{\circ}$  C in a plastic (Lusteroid) tube of 10.5 ml. capacity. The diluent is a solution of sodium chloride in water, adjusted by pycnometry to a final density  $1.1315 \pm 0.0005$  gms/ml. at  $20^{\circ}$  C. The plastic preparative tube is capped (using the special cap provided with ultracentrifuge rotor) and inverted ten times to insure adequate mixing. The final mixture provides a density of  $1.0630 \pm 0.0005$  gms/ml. at  $26^{\circ}$  C. This tube is loaded into the preparative rotor (Spinco Model 30.2), which can hold up to 20 such samples. Experience has shown that the addition of about 1/2 ml. of water to the rotor hole before inserting the preparative tube has prevented leakage of tube contents. The rotor, following loading and securing of the lid, is then centrifuged at 30,000 r.p.m. at  $14^{\circ}$  -  $21^{\circ}$  C. for a period of 13-16 hours. For the Spinco Model 30.2 rotor (tube angle --  $14^{\circ}$ ) the average centrifugal field is 79,420 x gravity at 30,000 R.P.M. This centrifugation insures quantitative flotation of lipoproteins of hydrated density 1.04 gms/ml. and less into the upper part of the first milliliter of preparative tube contents. Since during this centrifugation those macromolecular components of hydrated density greater than 1.063 gms/ml. undergo sedimentation out of the first milliliter of tube contents, as separation of the desired lipoprotein from such extraneous serum components is affected (See Fig. 1 for distribution of lipoproteins and proteins following preparative ultracentrifugation.)

Care is exercised in removal of the rotor from the preparative centrifuge and in unloading the tubes from the rotor to avoid disturbing the lipoprotein concentrate. The tubes should be unloaded and the lipoprotein concentrate removed immediately upon completion of the preparative run. These operations should be carried out at room temperature, since appreciable changes in temperature may give rise to convection currents and result in re-mixing of tube contents. Illumination of the preparative tube, following its removal from the rotor, reveals distinctly the various layers shown in Fig. 1. The top fraction contains the concentrated low-density (1.04 gms/ml. and less) lipoproteins. It is then essential to remove these lipoproteins quantitatively from the preparative tube.

For this purpose a pipette is made from seven mm. O.D. glass tubing drawn out to one mm. capillary tip at one end. (See Fig. 2.) A small rubber bulb (eye dropper type) is attached to the non-capillary end of the pipette to assist in the aspiration of the lipoprotein-containing top fraction of the tube contents. This is satisfactorily accomplished by use of an even pressure on the rubber bulb and placement of the capillary tip of the pipette on the liquid surface, at the point of contact of the solution meniscus with the plastic tube wall. Slow rotation of the tube with simultaneous gradual release of bulb pressure allows aspiration of the lipoprotein fraction into the pipette with minimal disturbance. Aspiration of air should be avoided by maintaining the pipette tip just below the air-liquid interface at all times during the operation. The pipetting of 0.5 ml. - 1.0 ml. in this manner will ordinarily remove quantitatively the desired lipoprotein concentrate. Any lipoprotein concentrate adherent either to the preparative tube cap or to the tube wall must also be transferred. The material pipetted out of the preparative tube is transferred quantitatively into a volumetric flask of  $1.00 \pm 0.02$  ml. capacity. Pipetting and delivery into the volumetric flask is continued until  $1.00 \pm 0.02$  ml. of tube contents have been transferred. A small clinical centrifuge is helpful in breaking bubbles formed during the pipetting operation and which, if not broken, would interfere with volume adjustment. Since these lipoproteins, originally present in a 5.0 ml. volume have been delivered into a 1.0 ml. volume, a five fold concentration has been achieved. The concentrated lipoprotein solution is then stored at  $0 - 4^{\circ}\text{C}$ , well-stoppered, until analytical ultracentrifugation, preferably within a few days.

In some human sera the total lipoprotein content is very high. This may result in pipetting difficulties in the preparative step and/ or film analysis difficulties in the analytical ultracentrifuge step. Pipetting difficulties arise when the lipoproteins pack into a pellicle at the top of the preparative tube. Film analysis difficulties arise because the analytical optical system cannot accommodate the very large deflections which result from highly concentrated lipoprotein fractions. In either event the difficulties can be circumvented by using less than 5.0 ml. of serum for the preparative step. If the lipoprotein concentration is sufficiently high, it may be necessary to use 1.0 ml. (or even less) of serum for this step. In such cases the difference between the actual serum volume used and 5.0 ml. is made up by the addition of a solution commonly referred to as a "mock" serum. This solution is made up from sodium chloride and water, pycnometered to a final density of  $1.0073 \pm 0.0005$  gms/ml. This closely approximates the density of human serum, exclusive of the density contribution of



proteins and lipoproteins.

2. Preparative ultracentrifugation at solution density 1.125 gms/ml.  
(Preparative type 2)

Three milliliters of serum are mixed at room temperature with three milliliters of diluent of density  $1.2427 \pm 0.0005$  gms/ml. (at  $20^{\circ}\text{C}$ ) in a plastic tube of 7.5 ml. capacity. The diluent is a solution of sodium nitrate in heavy water (99.5 percent  $\text{D}_2\text{O}$ ), adjusted by pycnometry to a final density of  $1.2427 \pm 0.0005$  gms/ml. at  $20^{\circ}\text{C}$ . The resultant density of the mixture in the plastic preparative tube is 1.125 gms/ml. at  $26^{\circ}\text{C}$ . The preparative centrifugation in this case is performed in a Spinco Model 40.3 rotor, which can hold up to 18 individual sample tubes. The rotor is centrifuged at 40,000 R.P.M. at  $14-21^{\circ}\text{C}$  for a period of 23-26 hours. For the Spinco Model 40.3 rotor (tube angle -  $20^{\circ}$ ) the average centrifugal field is 114,400 x gravity at 40,000 R.P.M. This centrifugation insures quantitative flotation of two additional lipoproteins, one of hydrated density 1.05 gms/ml. and the other of hydrated density 1.075 gms/ml. Removal of the lipoprotein concentrate resulting from this centrifugation is effected in the same manner as described above. Since the lipoproteins, originally present in a three milliliter volume have been delivered into a one milliliter volume, a three-fold concentration is achieved.

3. Preparative ultracentrifugation at solution density 1.20 gms/ml.  
(Preparative type 3)

Three milliliters of serum are mixed at room temperature with three milliliters of diluent of density  $1.3927 \pm 0.0005$  gms/ml. ( $20^{\circ}\text{C}$ ) in a plastic tube of 7.5 ml. capacity. The diluent is a solution of sodium nitrate in heavy water (99.5 percent  $\text{D}_2\text{O}$ ), adjusted by pycnometry to a final density of  $1.3927 \pm 0.0005$  gms/ml. at  $20^{\circ}\text{C}$ . The resultant density of the mixture in the plastic preparative tube is 1.20 gms/ml. at  $26^{\circ}\text{C}$ . The preparative ultracentrifugation is carried out in exactly the same manner as described in (2) above.

C. Analytical Ultracentrifugation

The purpose of analytical ultracentrifugation is the determination quantitatively of the concentration and distribution of the lipoprotein species present. The lipoprotein concentrate obtained in any one of the preparative ultracentrifugal steps outlined above is immediately available without further manipulation for analytical ultracentrifugation. This step is performed in an

analytical ultracentrifuge (Spinco Model E) equipped with a diagonal wire-cylindrical lens schlieren optical system and camera. An aliquot of the lipoprotein concentrate is loaded into an analytical ultracentrifuge cell. This cell is placed in an analytical rotor (Spinco Type A), which must be balanced either by a dummy cell or its equivalent in the form of a second analytical cell. The rotor assembly is brought to  $26.0 \pm 0.5^{\circ}\text{C}$  and then ultracentrifuged at 52,640 R. P. M. The Spinco rotor (type A) at 52,640 R. P. M. develops a centrifugal field of 215,000 X gravity at approximately half-way along the fluid column in the analytical cell. Camera exposures are taken at the following intervals of centrifugation after attainment of the full speed of 52,640 R. P. M.

(a) For centrifugation (Prep Type 1) at solution  
1.063 gms/ml. :

0, 6, 12, 22, 30, 38 minutes

(b) For centrifugation (Prep Type 2 and 3 at  
solution densities 1.125 gms/ml. and 1.20  
gms/ml. respectively:

0, 16, 32, 48, 64 minutes

For the subsequent analysis of the ultracentrifugal film record it is essential to know the equivalent U. T. S. (up-to-speed) time represented by the period of acceleration. A close approximation utilized is that equivalent U. T. S. equals  $1/3$  the acceleration time. In the Spinco Model E ultracentrifuges in our laboratory at Berkeley the acceleration period is closely maintained at 5.2 minutes, which yields an equivalent U. T. S. of 1.73 minutes. Thus one can consider that at the time full speed is reached, the equivalent period of U. T. S. centrifugation is already 1.73 minutes.

For film analysis it is necessary to make reference runs in the same analytical cells under similar conditions using solutions identical with those containing the lipoprotein concentrate, but free of lipoproteins. The appropriate reference solutions are as follows:

(a) For Preparative Type (1)

A sodium chloride-water mixture of density  
 $1.0630 \pm 0.0005$  gms/ml. at  $26^{\circ}\text{C}$ .

~~(b) For Preparative Type (2)~~

~~A sodium chloride-water-sodium nitrate-heavy  
water mixture of density  $1.1250 \pm 0.0005$  gms/ml. at~~

~~26°C~~

(b) For Preparative Type (2)

A sodium chloride-water-sodium nitrate-heavy water mixture of density  $1.1250 \pm 0.0005$  gms/ml. at  $26^{\circ}$  prepared by mixing equal volumes of "mock" serum and the specific diluent for this type of preparative procedure (Type 2).

(c) For Preparative Type (3)

A sodium chloride-water-sodium nitrate-heavy water mixture of density  $1.200 \pm 0.001$  gms/ml. at  $26^{\circ}\text{C}$ . prepared by mixing equal volumes of "mock" serum and the specific diluent for this type of preparative procedure (Type 3).

D. Ultracentrifugal Film Analysis

The film record obtained provides a magnified tracing of refractive index gradients as a function of distance from the center of rotation. The reference run made in a given analytical ultracentrifuge cell using the reference salt solutions provides a baseline tracing which represents the refractive index gradients due to redistribution of salt ions superimposed upon other gradients, such as compressional effects, and cell assembly factors. The lipoprotein run in the same analytical cell has refractive gradients due to migrating lipoprotein boundaries superimposed upon those described above for the reference run. At any distance from the center of rotation, the difference between the baseline run tracing and the lipoprotein run tracing represents refractive index gradients resulting from lipoproteins alone.

In Fig. 3(a) is a representation of the sector-shaped analytical cell, showing a single lipoprotein species undergoing flotation from the cell base toward the center of rotation. In Fig. 3(b) is shown a plot of concentration of

this lipoprotein species as a function of distance from the center of rotation. The optical system actually provides a measure of refractive index gradient  $\left(\frac{dn}{dx}\right)$  as a function of distance (x) from the center of rotation. In Fig. 3(c) is shown the  $\left(\frac{dn}{dx}\right)$  versus x plot which corresponds to the concentration plot of Fig. 3(b). In Fig. 4(a) an actual ultracentrifugal analytical pattern for such a system is shown, and in Fig. 4(b) the corresponding salt reference pattern is represented. The plots in Fig. 3 are representative of the situation existing in the 30 U. T. S. minute exposure of Figs. 4(a) and (b). In order to measure the refractive index gradients  $\left(\frac{dn}{dx}\right)$  ascribable to lipoproteins alone it is necessary to superimpose the salt reference pattern upon the corresponding lipoprotein pattern. The details of this superimposition are considered in the section on area measurements. A schematic representation of the lipoprotein pattern plus the superimposed salt reference pattern is shown in Fig. 5. The cross-hatched area in Fig. 5 is a measure of  $\int \frac{dn}{dx} dx$  over the boundary region of the migrating lipoprotein species. Since this area represents the increment in refractive index across the boundary region, it is proportional to the increment in concentration of lipoprotein across this same boundary region. This increment in lipoprotein concentration is equal to the actual lipoprotein concentration in the plateau region of the cell because the lipoprotein concentration is zero in the region of the cell centrifugal to the boundary region. The concentration of lipoprotein measured thus in the plateau region must, as is usual in ultracentrifugal practice, be corrected for the radial concentration effects of the sector-shaped cell and the inhomogeneous centrifugal field as shown in equation (I). (9)

$$C_i = C_m \left(\frac{Ra}{Ro}\right)^2, \text{ where} \quad (I)$$

$C_i$  = initial concentration of lipoprotein in the solution

$C_m$  = measured concentration, determined from the  $\int \frac{dn}{dx} dx$  over the boundary region

$Ro$  = distance from center of rotation to cell base

$Ra$  = distance from center of rotation to the "peak" position (maximum ordinate) of the boundary.

It is also possible and useful to characterize lipoproteins by their migration rates either under defined conditions or under standard conditions. The latter requires such data as partial specific volume of the various lipoprotein species and the viscosity of the medium used in flotation. At present these data are not available, so that it is necessary and satisfactory to describe

migration rates under arbitrarily defined conditions. Customarily ultracentrifugal migration rates are expressed in terms of the Svedberg, or S, unit (1 S unit =  $10^{-13}$  cm/ sec/ dyne/ gram) for lipoproteins undergoing flotation, the migration rates in S units would be negative. The  $S_f$  unit is used to provide a positive migration rate, since 1  $S_f$  unit = -1 S unit. The determination of the rate of flotation of a lipoprotein is based upon the application of the Svedberg identity (Equation II)

$$S = \frac{dx/dt}{\omega^2 x} \quad (II)$$

S = migration rate per unit centrifugal field.

x = distance of lipoprotein from center of rotation

t = time of centrifugation at full speed (52,640 R. P. M.) with the equivalent U. T. S. time of the acceleration period incorporated.

$\omega$  = angular velocity. ( $2\pi f$ , where f is frequency of rotation.)

Integrating <sup>(II)</sup> yields Equation III.

$$\ln \frac{x}{x_0} = S \omega^2 t \int_0^t t, \text{ where} \quad (III)$$

$x_0$  = distance from cell base to the center of rotation.

Thus by determination of the position of a lipoprotein boundary at any time of centrifugation and the application of Equation III, it is possible to determine the flotation rate of that lipoprotein species in  $S_f$  units.

In many sera there exists either a host of lipoprotein species which give rise to closely spaced boundaries or an actual continuum of lipoproteins with respect to flotation rate. This complicates the problem of determination of the concentration of individual lipoprotein species. Fig. 6 is a reproduction of the analytical ultracentrifuge film record of a human lipoprotein concentrate (Prep Type 1) showing a broad deflection extending over a large region of the cell, in contrast to the sharp deflection of Fig. 4(a). The broad deflection is interpreted as being the resultant of some number, possibly very large, of closely spaced boundaries. This interpretation, utilizing the second exposure (6 minutes U. T. S.) of Fig. 6 for illustrative purposes, is schematically represented in Fig. 7. Resolution of area to each individual lipoprotein species is difficult or even impossible. In such situations the analysis is

limited to the determination of the sum of concentrations of all lipoproteins between arbitrary flotation rate limits. A further complication deserves consideration. The lipoproteins experience self-slowng in flotation rate as their concentration increases, and further any single lipoprotein species is increasingly slowed by rise in concentration of all lipoproteins of intrinsically lower flotation rates. (10) Secondly, the Johnston-Ogston phenomenon operates as a result of these slowing effects. (11) The slowing effect and the Johnston-Ogston phenomenon must be considered in the quantitative analysis of analytical ultracentrifuge film records.

1. Film analysis for ultracentrifuge runs at solution density 1.063 gms/ml.

In this ultracentrifugal run essentially the continuum type of pattern (as shown in Fig. 6) is generally obtained. Analysis of such film, including the corrections for slowing with concentration and for the Johnston-Ogston phenomenon, requires knowledge of the alteration of flotation rate as a function of concentration. It would be ideal to measure the slowing effect over the expected ranges of concentration for lipoproteins of the entire range of flotation rates encountered. Such extensive data are not yet available. Hence certain reasonable approximations are being assumed. First, any lipoprotein species is assumed to slow other lipoprotein species to the same extent as they slow themselves with increasing concentration. Second, the flotation rate vs. concentration function is taken to be linear over the range of concentrations acceptable for film analysis. Thus, equation IV may be written for a single lipoprotein species.

$$F = F^{\circ} (1 - kc) \tag{IV}$$

where F = flotation rate ( in S<sub>f</sub> units) at concentration c

F<sup>°</sup> = Standard flotation rate, or rate of flotation at infinite dilution.

k = a constant which determines the magnitude of the slowing effect.

Incorporating the first assumption into the consideration allows the writing of the more general equation (V).

$$F_n = F_n^{\circ} \left[ 1 - k (c_1 + c_2 + \dots + c_n) \right] \tag{V}$$

where F<sub>n</sub> = flotation rate of the nth species of lipoprotein in the presence of a concentration c<sub>1</sub> of lipoprotein 1, c<sub>2</sub> of lipoprotein 2, . . . , and c<sub>n</sub> of the nth species.

F<sub>n</sub><sup>°</sup> = Standard flotation rate of the nth lipoprotein species.

k = the same constant as in Equation IV

Experimental data thus far accumulated indicate that within the range 0-4000 mg percent of lipoprotein flotation rates  $S_f^0$  3-10, the value of  $k$  is 3105 (mg percent)<sup>-1</sup>. This value was obtained by experimental measurement of flotation rate as a function of concentration. It is evident that the assumption of linear variation in flotation rate with concentration is not valid as the lipoprotein concentration becomes extremely high. For a more critical analysis it would be necessary to use the actual  $F$  vs.  $C$  function over the entire range of lipoprotein concentrations involved.

The choice of flotation rate limits between which the sum of lipoprotein concentrations is measured at the discretion of the investigator. The principles involved are identical independent of what limits of  $S_f^0$  values are chosen. For illustrative purposes the measurement of two bands of lipoproteins, the  $S_f^0$  0-12 and  $S_f^0$  12-20, are given below. For these lipoprotein bands the measurements are conveniently made in the 30 minute U. T. S. exposure for the analytical run under consideration. The specific steps in the analysis are as follows:

a. An enlarger or projector is used to magnify the ultracentrifugal film five times. (The factor five is arbitrarily chosen for convenience.) A tracing is then made through the center of the enlarged pattern observed on a template which already has the salt reference line inscribed. (See Fig. 8(a).) Proper orientation of the ultracentrifugal pattern with respect to the template involves two major points. First, the schlieren representation of the air space (see Fig. 5) for the reference run must be superimposed upon that of the analytical run. This adjusts vertical positioning. Second, the horizontal adjustment is made by superimposing the reference edge of the salt reference run upon that of the analytical run. As previously mentioned it is essential that the baseline template is obtained from a salt reference run made in the same cell and rotor under identical ultracentrifugal operating conditions.

b. The enlarged tracing is then subjected to analysis. The first task is the determination of the area due to lipoproteins of the band  $S_f^0$  0-12. This necessitates the determination of the position on the tracing which corresponds to that of the  $S_f^0$  12 lipoprotein boundary. This position is of course variable from one ultracentrifugal analysis to another because of the variability in concentration of  $S_f^0$  0-12 lipoproteins encountered from one serum sample to another. One convenient approach to the

placement of the  $S_f^0$  rate limits involves the conversion of enlarged  $\frac{dn}{dx}$  vs.  $x$  tracing into the corresponding integral curve on a template which has inscribed upon it the  $x$  vs  $c$  plots for  $S_f^0 12$  and  $S_f^0 20$ .... (Fig. 8b) The  $x$  vs  $c$  plots are obtained by substitution into Equation V and then the  $F$  values are converted to  $x$  values by use of the integrated Svedberg identity, Equation III. There exists a unique solution for the position  $x_{12}$  on the tracing which represents the position of the  $S_f^0 12$  lipoprotein boundary. This solution is the position of the  $x$ -axis which corresponds to the intersection of the integral curve with the  $x$  vs  $c$  plot for  $S_f^0 12$ . The total area represented on the integral curve up to this intersection point is a measure of the sum of concentrations of lipoproteins between the flotation limits of  $S_f^0 0$  and  $S_f^0 12$ . This sum is commonly referred to as the concentration of the  $S_f^0 0-12$  lipoprotein class, or Standard  $S_f^* 0-12$  lipoproteins. Similarly there exists a unique solution for the position  $x_{20}$  on the tracing which represents the position of the  $S_f^0 20$  lipoprotein boundary. This solution is the intersection of the integral curve with the  $x$  vs  $c$  plot for  $S_f^0 20$ . The area represented on the integral curve up to this intersection point measures the concentration of  $S_f^0 0-20$  lipoproteins. It is evident that  $S_f^0 12-20$  concentration equals  $S_f^0 0-20$  concentration minus the  $S_f^0 0-12$  concentration. In an entirely analogous fashion the sum of the concentrations of lipoproteins between any two standard flotation rate limits is determined, using an appropriate exposure for the analysis. For example, the  $S_f^0 20-100$  lipoproteins are measured in the film exposure representing six minutes of U. T. S. centrifugation time. (Actual equivalent U. T. S. time in minutes =  $6 + 1/3$  acceleration time in minutes.)

### c. The Johnston-Ogston consideration

Johnston and Ogston have shown that when a mixture of two macromolecular species is ultracentrifuged, the measured concentration of the faster migrating species is falsely low in an amount by which the slower migrating species is falsely high. For two species in a homogeneous force field and a rectangular cell Johnston and Ogston have derived the following equation (VI)

$$\frac{C_s^\beta}{C_s^\alpha} = \frac{S_f^\alpha - S_s^\alpha}{S_f^\alpha - S_s^\beta} \quad \text{(VI)}$$

where  $C_s^\beta$  = concentration of slower migrating species in the phase where faster migrating species is absent.



$C_s^a$  = concentration of slower-migrating species in the phase where faster-migrating species is present.

$S_f^a$  = migration rate of the faster-migrating species in the phase containing both components.

$S_s^a$  = migration rate of the slower-migrating species in the phase where both are present.

$S_s^\beta$  = migration rate of slower migrating species in the phase where it is present alone.

Utilizing the  $F$  vs  $C$  dependence (Equation V) their equation can be transformed to Equation VII:

$$\Delta C = \frac{RC_f^{\text{meas.}} C_s^\beta}{N + R C_f^{\text{meas.}}} \quad (\text{VII})$$

where  $\Delta C$  = the amount by which the fast component is erroneously low or by which the slow component is erroneously high.

$$R = kS_s^0$$

where  $k$  is the constant of the  $F$  vs  $C$  dependence relation

$S_s^0$  is the flotation rate at infinite dilution of the slower migrating species.

$C_f^{\text{meas.}}$  = measured concentration of the faster-migrating species.

$C_s^\beta$  = measured concentration of the slower-migrating species (in the solution phase where the faster-migrating species is absent.)

$$N = S_f^a - S_s^\beta$$

where  $S_f^a$  = measured flotation rate of the faster-migrating species.

$S_s^\beta$  = measured flotation rate of the slower-migrating species.

Therefore,

$$\text{True } C_f = C_f^{\text{meas.}} + \Delta C$$

$$\text{True } C_s = C_s^{\text{meas.}} - \Delta C$$

In a multicomponent system these considerations are applied by making a Johnston-Ogston correction for each component upon every other component.

In the lipoprotein analysis of the continuum type we have made the approximation that a particular lipoprotein band, e. g. the  $S_f^0$  0-12 lipoprotein band can be considered as a discrete component and assigned a flotation rate, which is determined by the position on the  $x$  axis of the integral curve that divides the total concentration for that band in half. This position is referred to as the mid-area position. The flotation rates corresponding to such mid-area positions (by use of the Svedberg identity) are used in application of the Johnston-

Ogston correction.

d. Correction for position in the cell of measured area

As indicated previously the areas measured at any boundary position differ from the true area representing the lipoprotein concentration because of radial concentration effects. The measured areas can be converted to true areas by use of Equation I. The boundary positions utilized in applying Equation I are the mid-area positions described in (c) above. Our practice has been to apply this radial concentration correction after application of the Johnston-Ogston correction. The radial concentration effects actually give rise to a changing Johnston-Ogston correction during the ultracentrifugal run. A more refined treatment of the Johnston-Ogston phenomenon takes this into account. (12).

e. Conversion of Area to Mg percent

The following equation, (VII), derived by Pickels, (13), (14), for the schlieren optical system of the Spinco analytical ultracentrifuge is used to convert area to concentration in mg percent.

$$C = \frac{A \tan \theta 1000}{L T M m N E^2 \Delta n} \quad \text{(VIII)}$$

where C = concentration of lipoprotein in the original serum in mg percent

A = area in square millimeters of the measured lipoprotein band (corrected both for radial concentration and Johnston-Ogston effects.)

$\tan \theta$  = tangent of the angle of the diagonal wire element with respect to slit image.

L = optical lever arm (distance along the optical path from the center of the condensing lens above the rotor to the diagonal wire in millimeters).

M = magnification of cell height (magnification of camera lens system).

m = magnification of cylindrical lens system.

N = factor by which the lipoproteins have been concentrated in the preparative procedure.

E = linear magnification of the enlarger used in preparing the tracing (5X in this text).

$\Delta n$  = specific refractive increment for the lipoproteins encountered, in the particular solution medium. For lipoproteins of hydrated density 1.04 gms/ml. and less,  $\Delta n$  is being taken as 0.00154 in a sodium chloride solution of density 1.063 gms/ml.

## 2. Film analysis of ultracentrifugal runs at solution density 1.125 gms/ml.

A typical analytical run on a human serum at density 1.125 gms/ml. is shown in Fig. 9. Two additional components are determined in this analytical run, a lipoprotein of hydrated density 1.05 gms/ml. and a lipoprotein of hydrated density 1.075 gms/ml. The 1.05 gms/ml. lipoprotein is measured by making an enlarged tracing (as described in (1) above) utilizing the 32 minutes U. T. S. exposure. In determining this lipoprotein a difficulty is encountered, which arises from the failure of the 1.05 gms/ml. lipoprotein to resolve itself from the major complex of the low-density group of lipoproteins. This complex can be seen migrating just ahead of the 1.05 gms/ml. lipoprotein. (See Fig. 10). The area representing this lipoprotein is approximated as follows, with the aid of Fig. 10:

a. An estimate is made of the "peak" position (maximum ordinate) for the 1.05 gms/ml. lipoprotein.

b. A line is drawn vertically from this "peak" position to the salt reference line.

c. The cross-hatched area is measured and then doubled. (This assumes the boundary is symmetrical about the vertical line.)

The lipoprotein of hydrated density 1.075 gms/ml. is much more precisely measurable, since it is completely resolved as a boundary unassociated with other lipoproteins. (See Fig. 9(a); 64 minute U. T. S. exposure.) The entire area associated with this lipoprotein boundary region is taken as representative of the concentration of the 1.075 gms/ml. lipoprotein, as shown in cross-hatching in Fig. 11.

A small Johnston-Ogston correction, estimated to be of the order of the uncertainty in measurement, is neglected in the analysis of both the 1.05 and 1.075 gms/ml. lipoproteins. The correction for radial concentration is made in the manner previously described (Equation I) for both lipoproteins. The conversion of the corrected areas to concentrations in mg percent is made by applying Equation VIII as above, except that the value for  $n$  in this type of run is taken as 0.00169 for both lipoprotein species involved.

## 3. Film analysis of ultracentrifugal runs at solution density 1.20 gms/ml.

A typical analytical run on human serum at density 1.20 gms/ml. is shown in Fig. 12 (a). In this run the lipoprotein of hydrated density 1.145 gms/ml. is determined. In the 64 minute U. T. S. exposure the complex of two lipoprotein species is seen. These are the lipoprotein of hydrated density 1.075

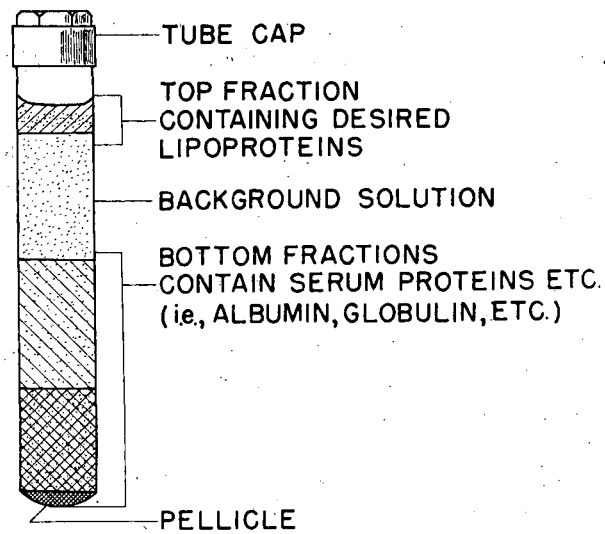
gms/ml. and that of 1.145 gms/ml. The lipoprotein of lower density migrates faster than that of higher density. The total area of this complex is measured as shown in cross-hatching in Fig. 13. The concentration of the 1.145 gms/ml lipoprotein is obtained as follows:

The composite area is corrected for radial concentration (Equation I) and then converted to mg percent by application of Equation VII. The  $\Delta n$  for these lipoproteins in this solution is taken as 0.00154. The concentration obtained in mg percent represents the sum of the concentration of the 1.075 gms/ml. lipoprotein plus that of the 1.145 gms/ml. lipoprotein. However, the 1.075 gms/ml. lipoprotein concentration was determined in (2). Therefore the concentration of the lipoprotein of 1.145 gms/ml. density is directly obtainable by difference.

Acknowledgement : The ultracentrifugal analytic method described here evolved from the combined efforts of many members of the biophysical chemistry research group at the Donner Laboratory.

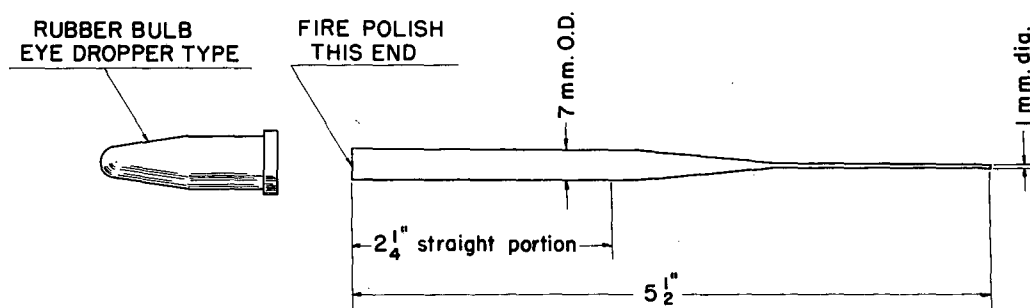
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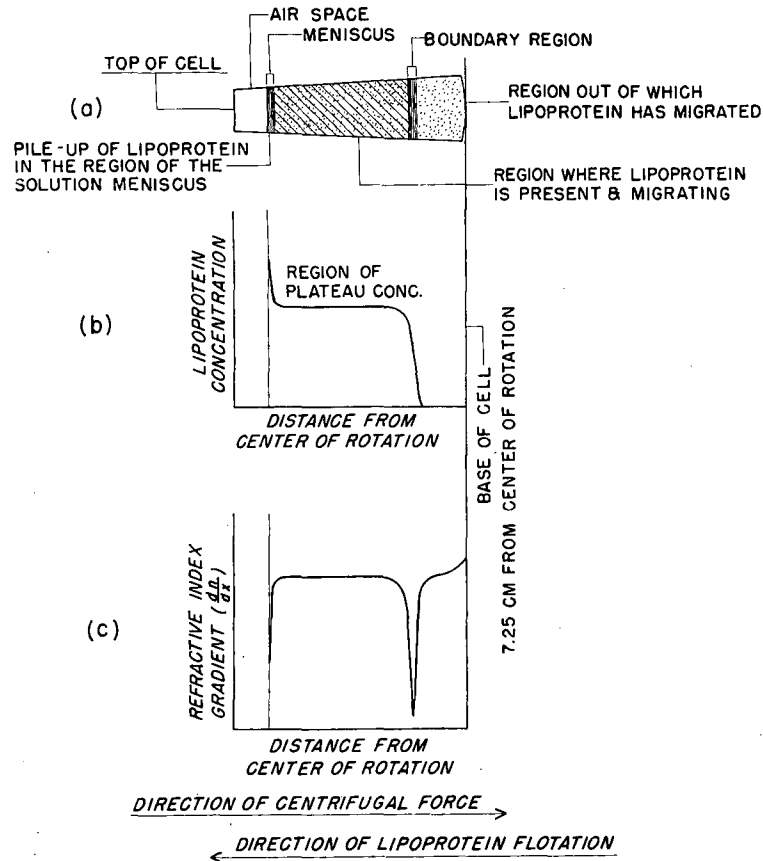
MU-5988

Fig. 1 shows the distribution of serum components in the preparative tube upon completion of the preparative ultracentrifugal run.



MU-5989

Fig. 2 shows the pipette utilized in the removal and transfer of the lipoprotein-containing top fraction from the preparative tube.



MU-5990

Fig. 3 (a) is a schematic diagram of the analytical ultracentrifuge cell containing a migrating lipoprotein species.

Fig. 3 (b) is a representation of the concentration of this lipoprotein as a function of distance from the center of rotation.

Fig. 3(c) is a representation of refractive index gradient as a function of distance from the center of rotation. The schlieren optical system of the Spinco Model E ultracentrifuge actually provides this type of plot.



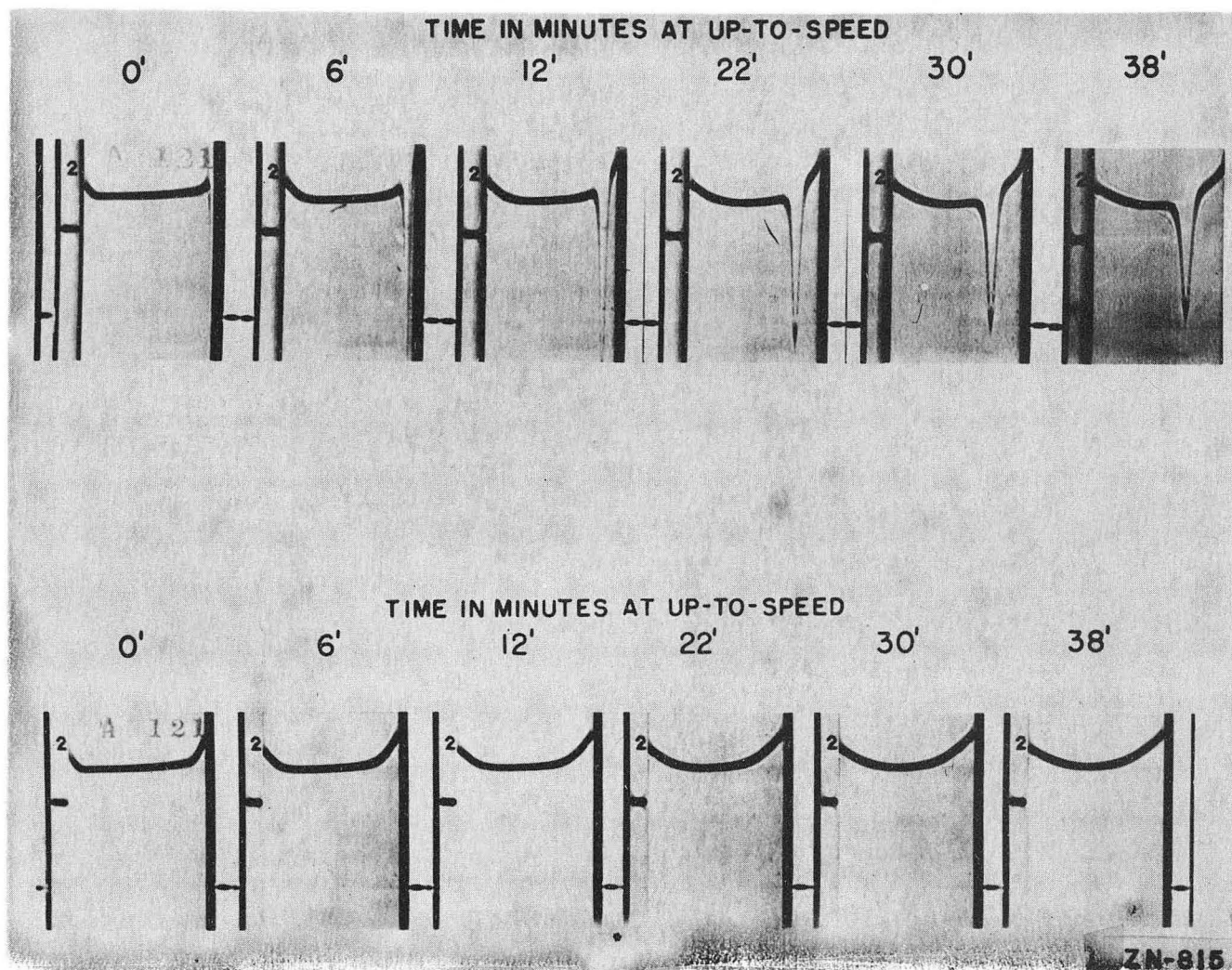
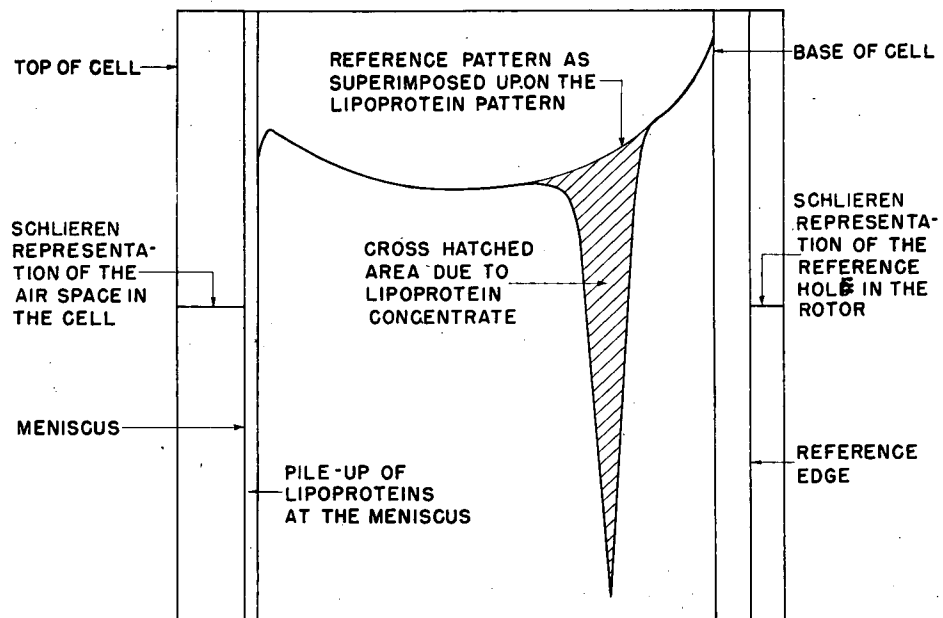


Fig. 4(a) is an actual analytical ultracentrifugal film record demonstrating a single lipoprotein species undergoing flotation in a medium of solution density 1.063 gms/ml at 26° C (Prep Type 1)(52,640 RPM)

Fig. 4(b) is the corresponding analytical film record for the salt reference run. The salt solution has a density of 1.063 gms/ml at 26° C (52,640 RPM)



MU-5991

Fig. 5 is a schematic representation of the 30 minute UTS exposure of Fig. 4(a) with the reference baseline of the corresponding exposure of Fig. 4(b) superimposed.

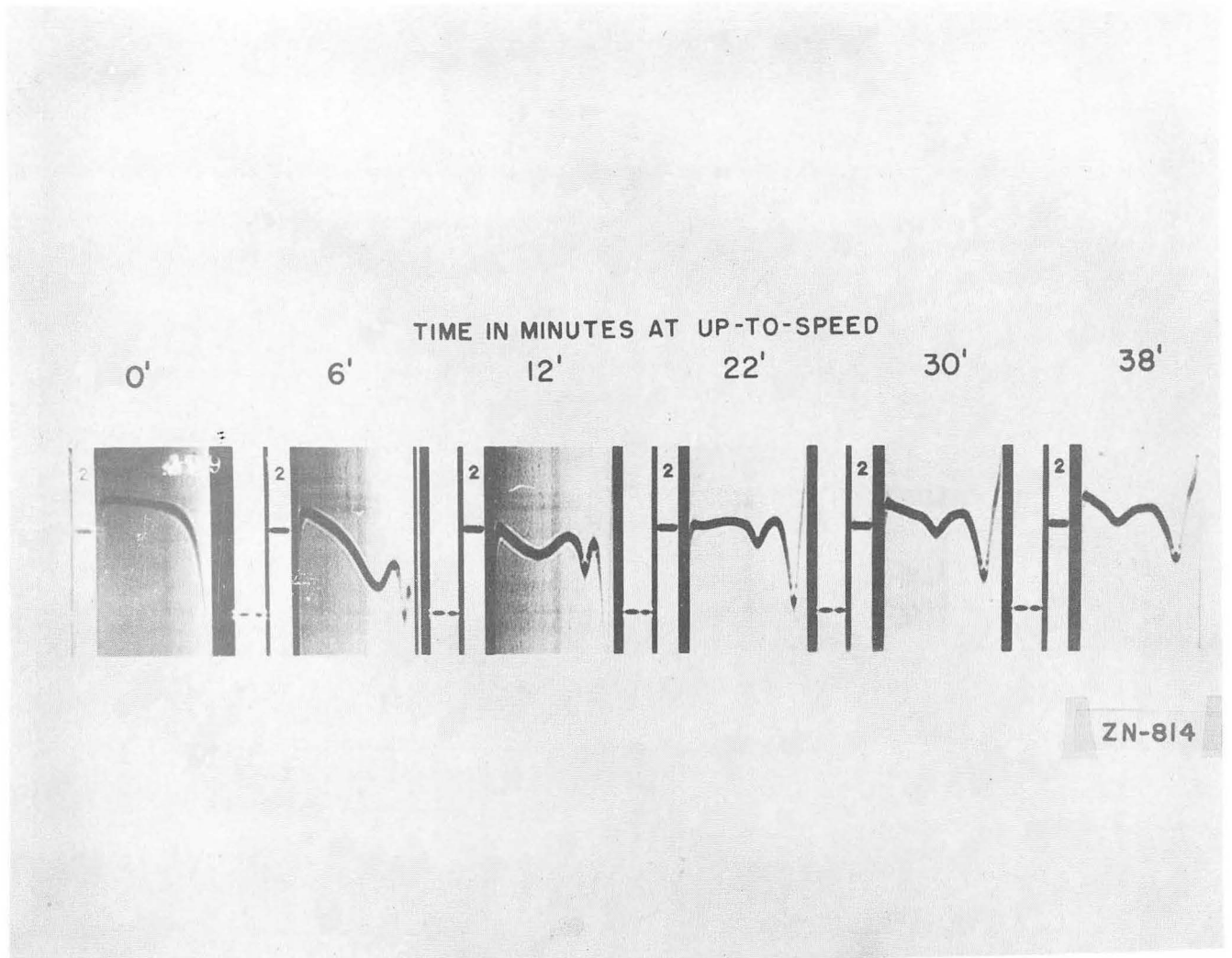
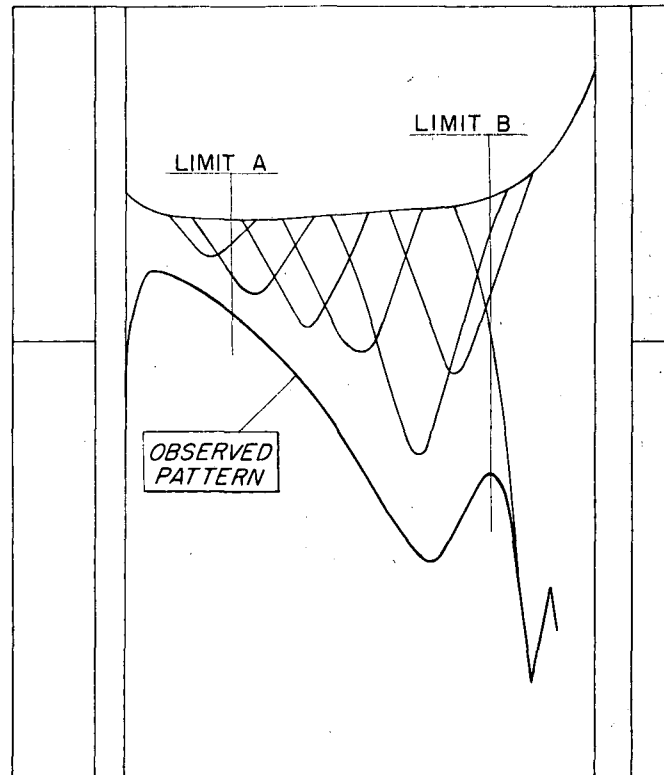
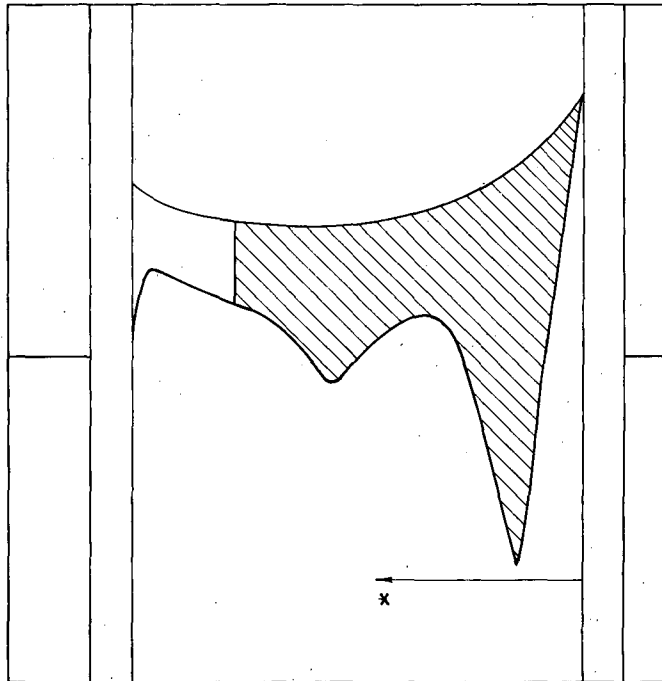


Fig. 6 is an analytical ultracentrifugal film record, demonstrating the various lipoprotein species commonly present in human serum. The lipoprotein concentrate for this run was obtained by preparative procedure Type 1. (Solution density 1.063 gms/ml at 26° C) Rotor speed = 52,640 RPM



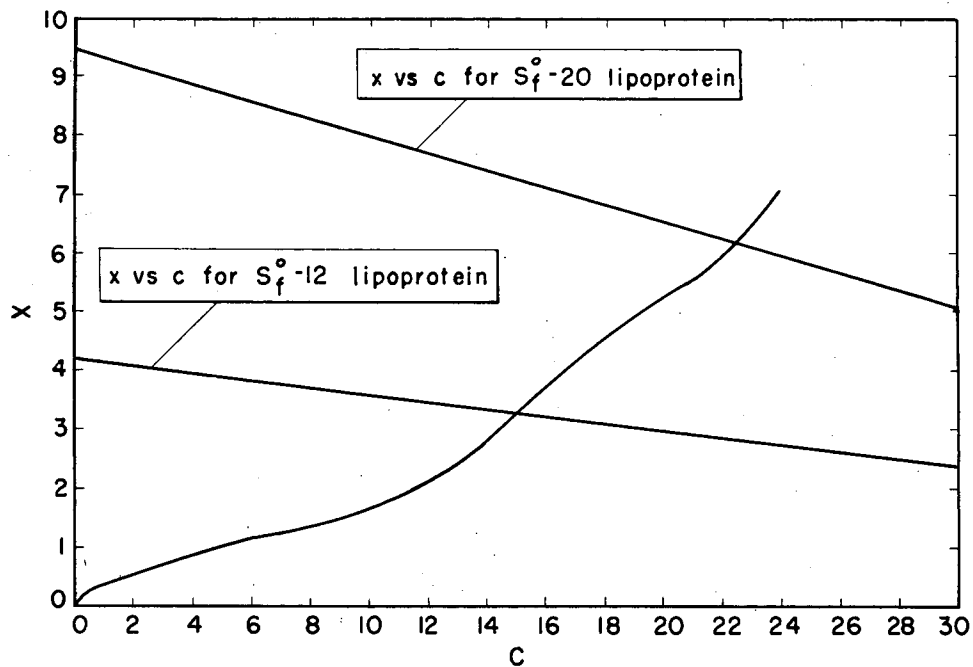
MU-5992

Fig. 7 is a schematic interpretation of the broad deflection observed in the analysis of a continuum of lipoproteins (see text). The observed pattern is interpreted as arising from the summation of overlapping deflections due to many individually migrating lipoprotein boundaries. Concentration in such patterns is measured between chosen flotation rate limits (designated here as limit A and limit B).



MU-5993

Fig. 8(a) is a representation of the enlarged tracing used in film analysis by the method described in the text. This tracing corresponds to the 30 minute UTS exposure for the analytical run shown in Fig. 6.



MU-5994

Fig. 8(b) shows the integral curve (representing the cross hatched area of Fig. 8(a) )superimposed on a template inscribed with the  $s$  versus  $o$  dependence plot for the  $S_f^{o12}$  and  $S_f^{o20}$  lipoproteins. The plotted points of the integral curve represent cumulative sums of the ordinates of Fig. 8(a) at successive equal intervals along the  $x$  axis. An ordinate in Fig. 8(a) equals the vertical distance between the lipoprotein pattern tracing and that of the salt reference tracing.

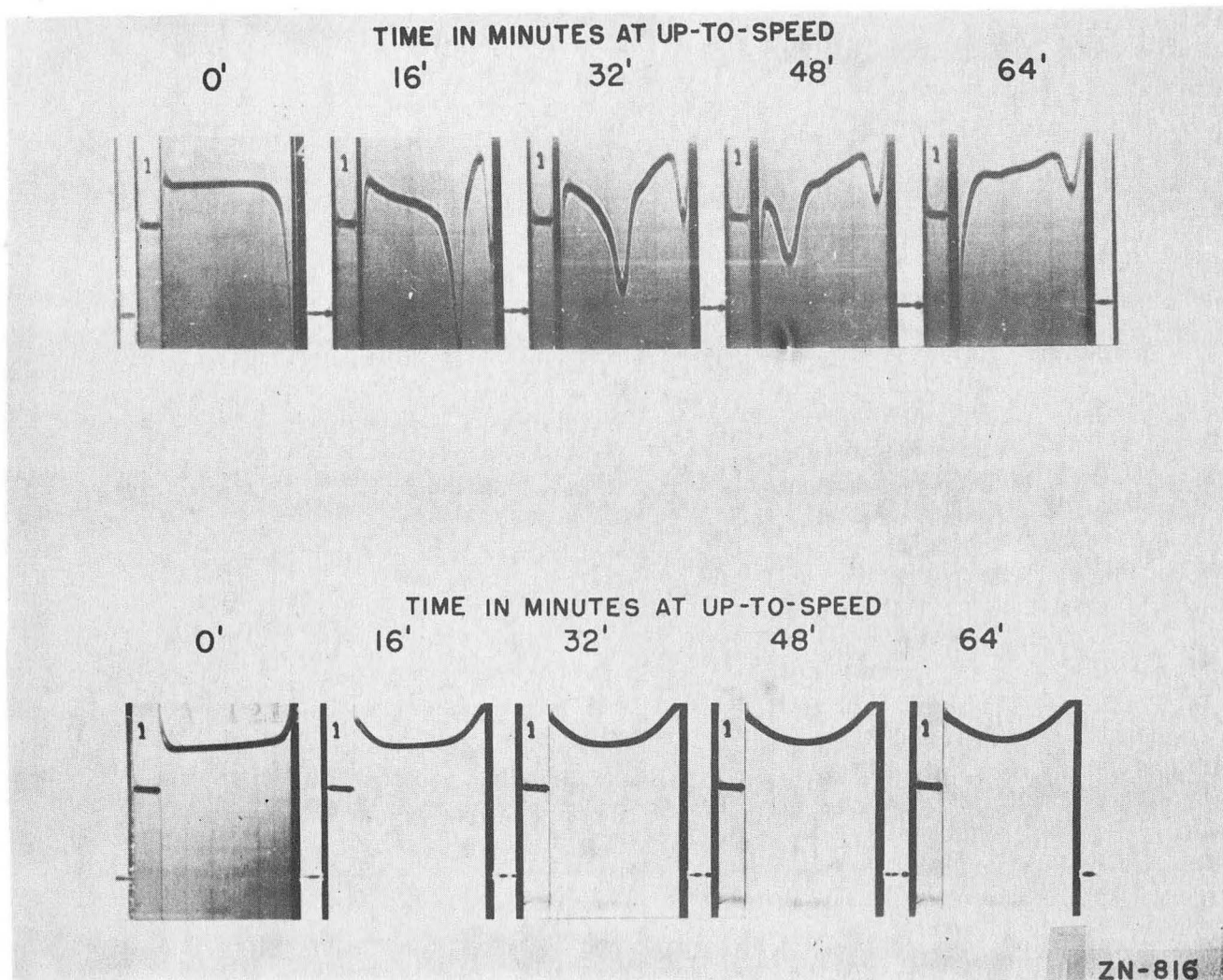
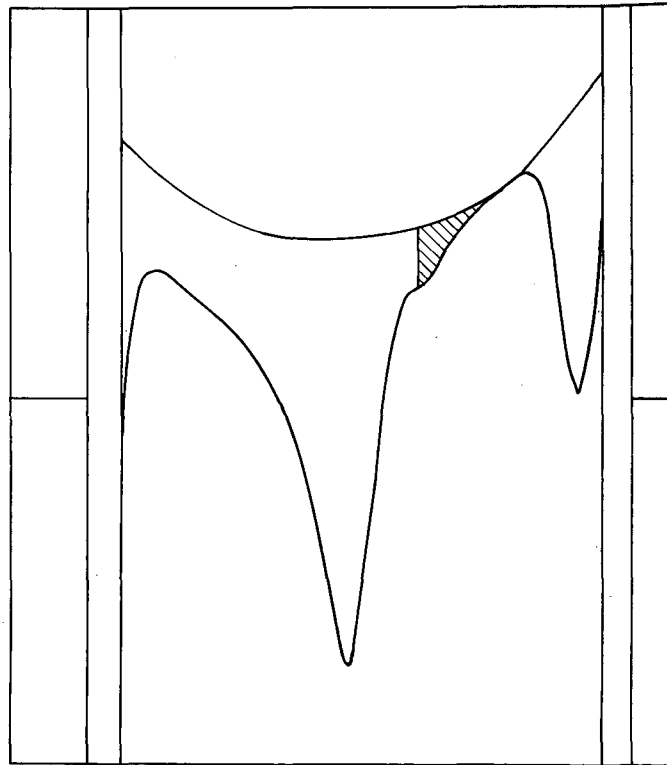


Fig. 9(a) is an analytical ultracentrifugal film record showing the lipoproteins observed in a human serum specimen at solution density 1.125 gms/ml (prep Type 2) Rotor speed is 52,640 RPM.

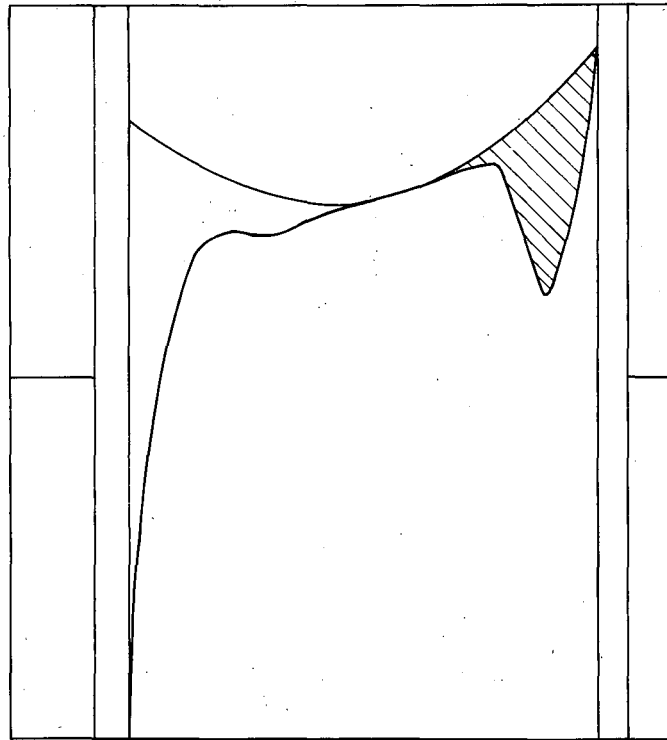
Fig. 9(b) is the corresponding salt reference run for Prep Type 2.



MU-5995

Fig. 10 shows the enlarged tracing used and the area actually measured in determination of the lipoprotein of hydrated density 1.05 gms/ml. The tracing represents the 32 minute UTS exposure of Fig. 9(a) with its superimposed salt reference tracing.





MU-5996

Fig. 11 shows the enlarged tracing used and the area actually measured in determination of the lipoprotein, of hydrated density 1.075 gms/ml. The tracing represents the 64 minute UTS exposure of Fig. 9(a) with its superimposed salt reference tracing.

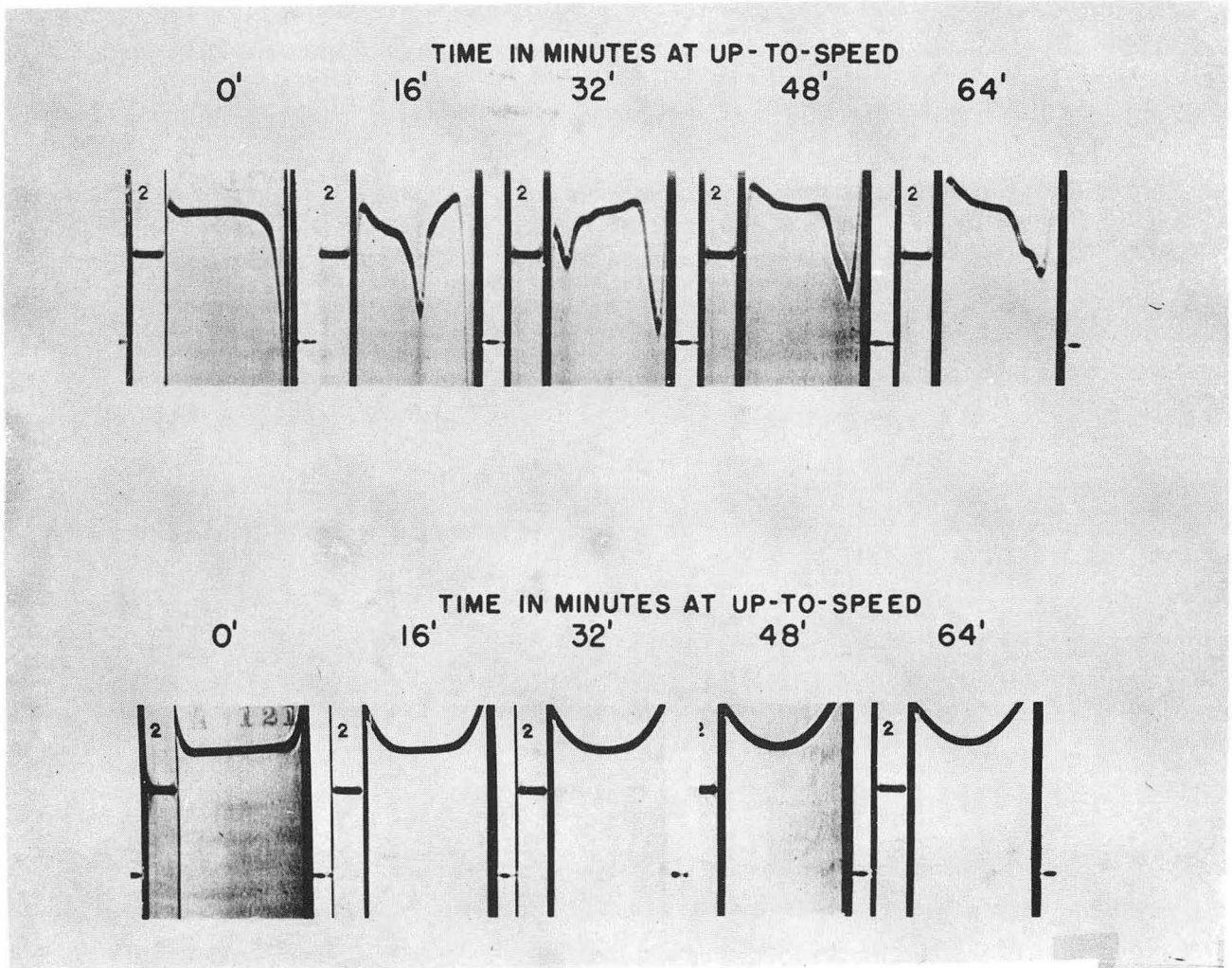
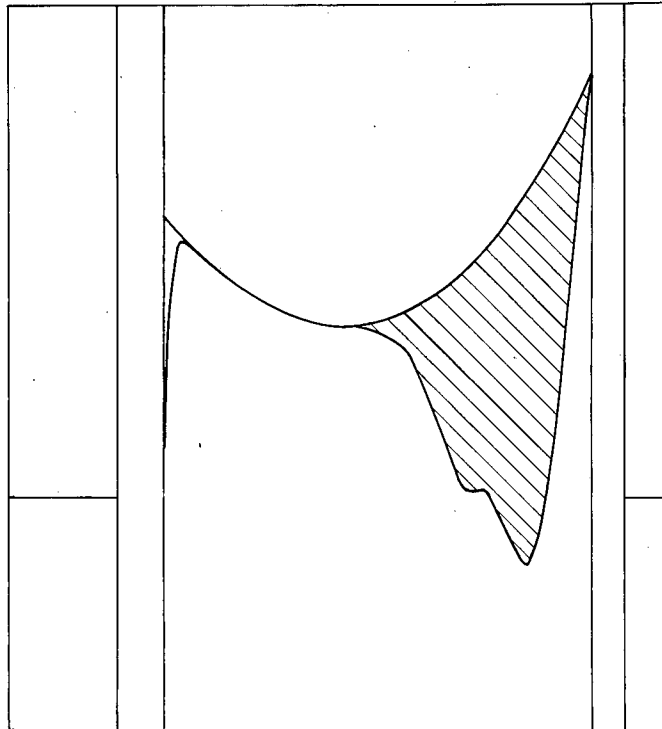


Fig. 12(a) is an analytical ultracentrifugal film record showing the lipoproteins observed in a human specimen at solution density 1.20 gms/ml (Prep Type 3) Rotor speed = 52,640 RPM

Fig. 12(b) is the corresponding salt reference run for Prep Type 3.



MU-5997

Fig. 13 shows the enlarged tracing used and the area measured in the determination of the sum of the concentration of the lipoprotein of 1.075 gms/ml and 1.145 gms/ml. The tracing represents the 64 minute UTS exposure of Fig. 12 (a) with its superimposed salt reference tracing: