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BIOLOGY AND ULTRACENTRIFUGAL METHODOLOGY OF THE HIGH-DENSITY LIPOPROTEINS AND THE PROTEINS OF HUMAN SERUM

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BIOLOGY AND ULTRACENTRIFUGAL METHODOLOGY OF THE HIGH-DENSITY LIPOPROTEINS AND THE PROTEINS OF HUMAN SERUM

Oliver Francis deLalla

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BIOLOGY AND ULTRACENTRIFUGAL METHODOLOGY OF THE HIGH-DENSITY LIPOPROTEINS AND THE PROTEINS OF HUMAN SERUM

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November 25, 1958

ABSTRACT

A method for the ultracentrifugal analyses of the high-density serum lipoproteins and the serum proteins is described in detail.

A randomly collected population consisting of 2297 clinically healthy humans (1961 males and 336 females) constituted the subjects used in this study. Measurements were made on the concentrations of the three high-density classes of lipoproteins (HDL_1, of hydrated density 1.045 g/ml; HDL_2, 1.075 g/ml; and HDL_3, 1.145 g/ml) and of the three protein classes (P_1, a macroglobulin of S rate 19; P_2, the gamma globulin of S rate 6; and P_3, the albumin of S rate 4).

The means, the standard deviations of the distributions, and the standard errors of the means were calculated and recorded for the various age categories in both sexes.

The Pearson product-moment correlations were calculated, for each group, for each component measured with each other kind of macromolecule measured, including the low-density lipoproteins.

The results of the calculations of distributions and correlations are discussed, and some conclusions are drawn from the findings.

I. INTRODUCTION

With the development of ultracentrifugal techniques, which immediately followed the pioneering and developmental work by T. Svedberg,[1] many new fields of study have been opened to research. One of the most interesting and certainly the most fruitful of such fields is the study of macromolecules of biological systems. Of these macromolecules the lipoproteins are without doubt the current topic of most interest and of paramount importance. Although lipoproteins have been known to exist for many years,[2] it was not until ten years ago that John W. Gofman, utilizing ultracentrifuges, developed a unique method that allowed for the quantitative analysis of any lipoprotein species in its natural state.[3]

His method involves two major steps. The first step is preparative ultracentrifugation, in which the desired lipoproteins are physically separated out of a serum sample by being floated to the top of the sample tube. This is accomplished by mixing concentrated salt solution with the serum so that the resultant density of the mixture is greater than the hydrated density of the desired lipoprotein classes (sodium chloride and (or) sodium nitrate solutions were used for these studies). Under these conditions, in an ultracentrifuge operated at about 100,000 g, the desired lipoproteins float to the top in due time, while the undesired macromolecules sediment to the bottom; the time depends on the speed of rotation, the density differences between the molecules and the solvent, the temperature, etc. This effects a unique separation, which allows for quantitative removal of the top fraction containing all the desired lipoproteins.

In the second major step in the Gofman technique an aliquot of the top fraction obtained as above is run in an analytical ultracentrifuge, which is capable of furnishing a film record of the analysis and thus establishes the precise identities and quantities of the lipoprotein species present in the sample. For more detailed discussions of the technique than are given in this text, the reader is referred to other papers.[3, 4, 5]

It is not the purpose of this paper to discuss the virtues of such a method, but its importance is quite obvious to those in this

field who use such a method; without it our knowledge of lipoproteins in biology would be rather sketchy. It was through the use of his method that Gofman was able to prove conclusively the significant relationship of the low-density lipoproteins—namely the S_f^0 0–12, S_f^0 12–20, S_f^0 20–100, and S_f^0 100–400 lipoprotein classes* —to atherosclerosis and other related diseases.[6-11] The cholesterol molecule was once considered to cause atheroclerosis, but now it is thought to be only incidental to the low-density lipoproteins. Cholesterol is only a part of the lipid part of all lipoproteins, and not all lipoproteins are implicated in atherosclerosis. The major high-density lipoproteins, which are the subject of this paper, are not involved either positively or negatively.[12]

Serum lipids of healthy humans are not found in free states, but are bound in some way to protein, making up rather stable molecules known to be the lipoprotein molecules. These lipoproteins differ from one another only in their hydrated densities, molecular weights, and lipid compositions. Lindgren, et al., utilizing the Gofman differential-density separation method, were able to isolate specific lipoprotein classes, and have performed lipid analyses to determine the lipid composition of each class isolated.[13, 14] The lipoprotein concept, although still questioned by a few, is almost universally accepted. A recent paper by Thomas Hayes, also of the Donner Laboratory and a member of the Gofman staff, describes a technique which enabled him to obtain electron micrographs of the various lipoprotein molecules.[15] These photographs (Figs. 1-5) show the lipoproteins to be discrete entities, and would appear to definitely

---

*The S_f^0 unit is used (as, for example, in S_f^0 0–12) to identify the lipoprotein class on the basis of its migration rate in a NaCl solution of density 1.063 g/ml in a centrifugal-force field created by an ultracentrifuge operated at 52,640 rpm at 26°C. The subscript f denotes flotation (as opposed to sedimentation, which has positive values) in calculations of the S rate. The S unit, known as the svedberg, is defined as $S = 10^{-13}$ cm/sec/dyne/g.

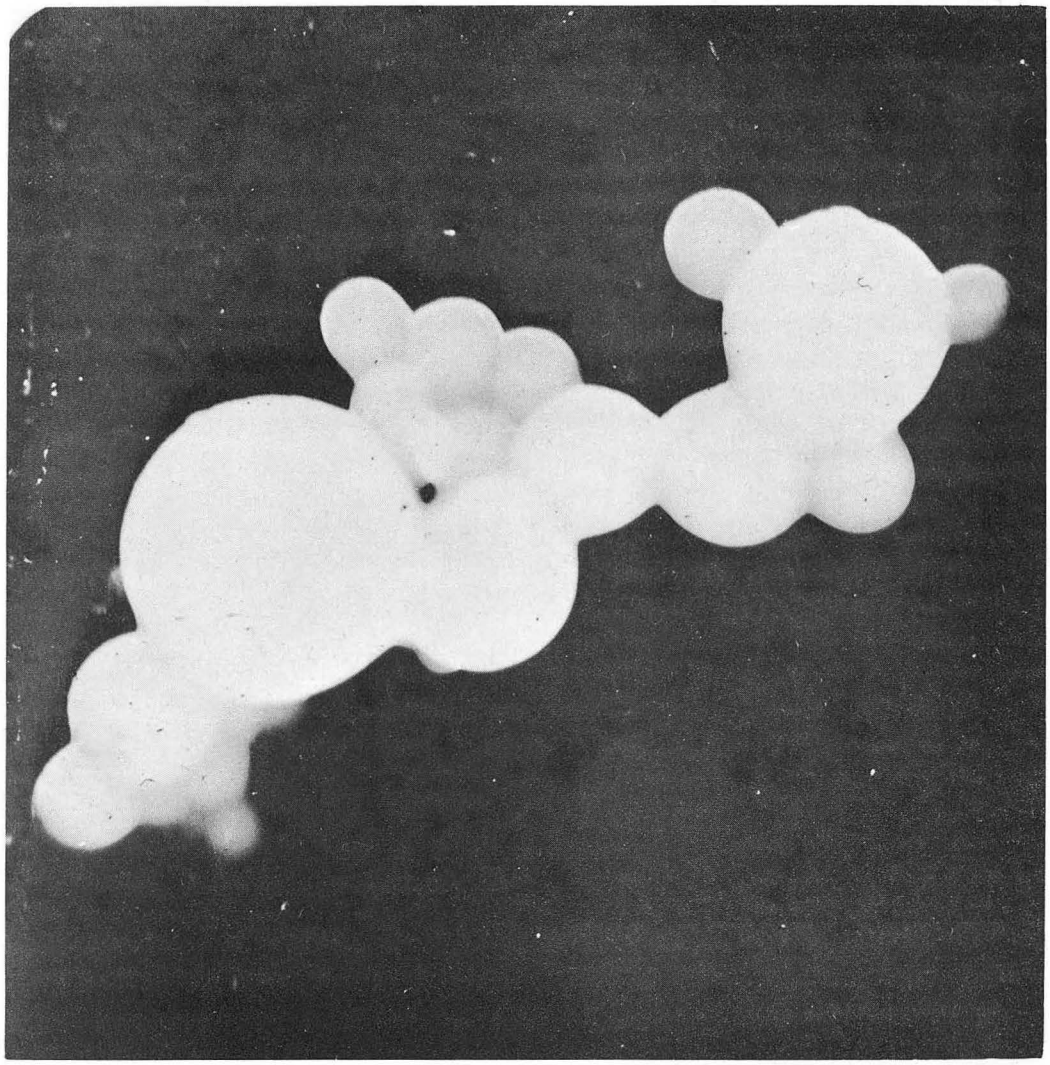

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Fig. 1. An electron micrograph of an ultracentrifugally isolated fraction of chylomicrons (X 54,230).

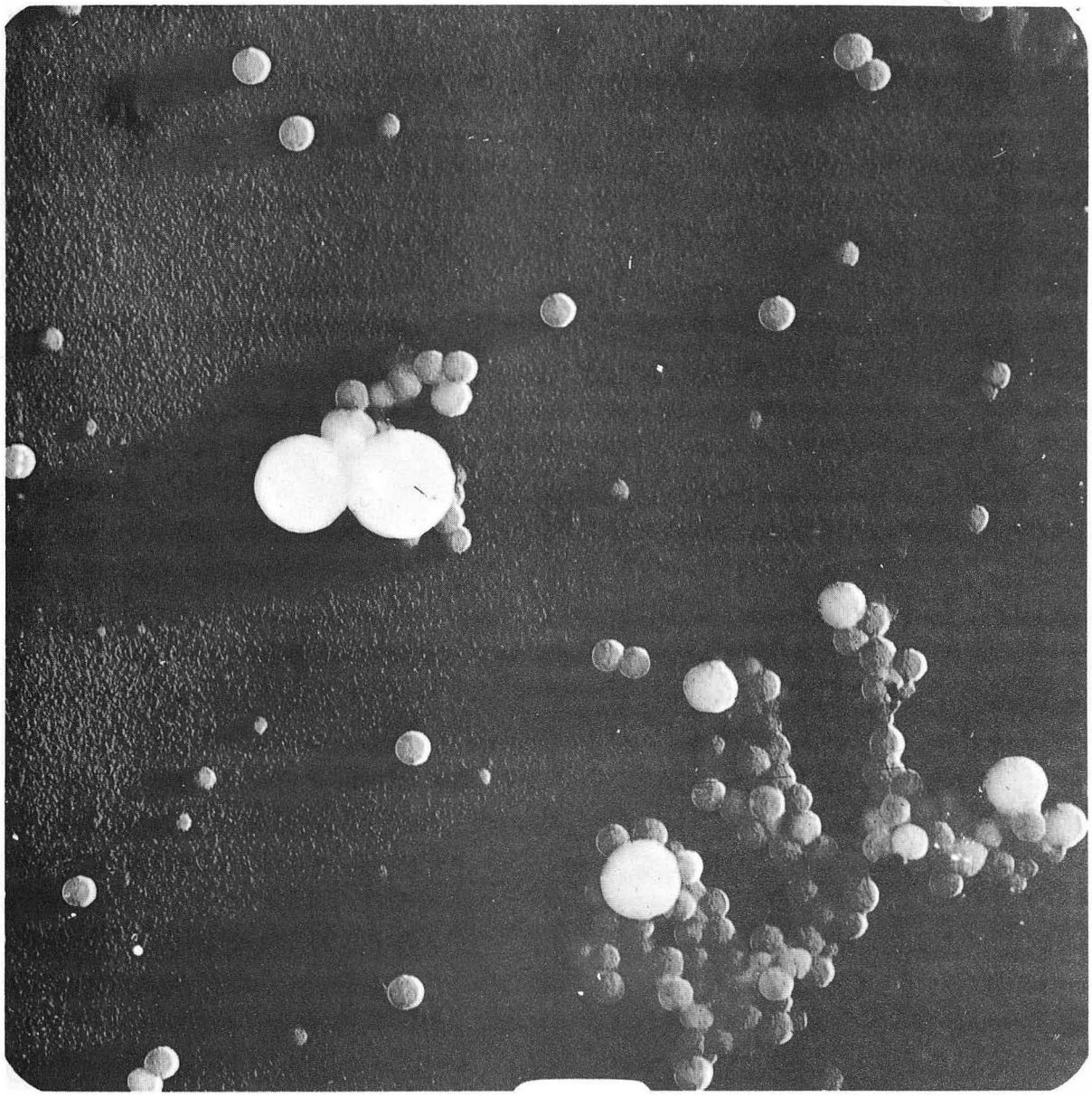

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Fig. 2. An electron micrograph of an ultracentrifugally isolated fraction of S_f^0 20-400 lipoproteins (X 54,230). The polystyrene standards (appearing as clumps of grapes) are 880 angstroms in diameter.

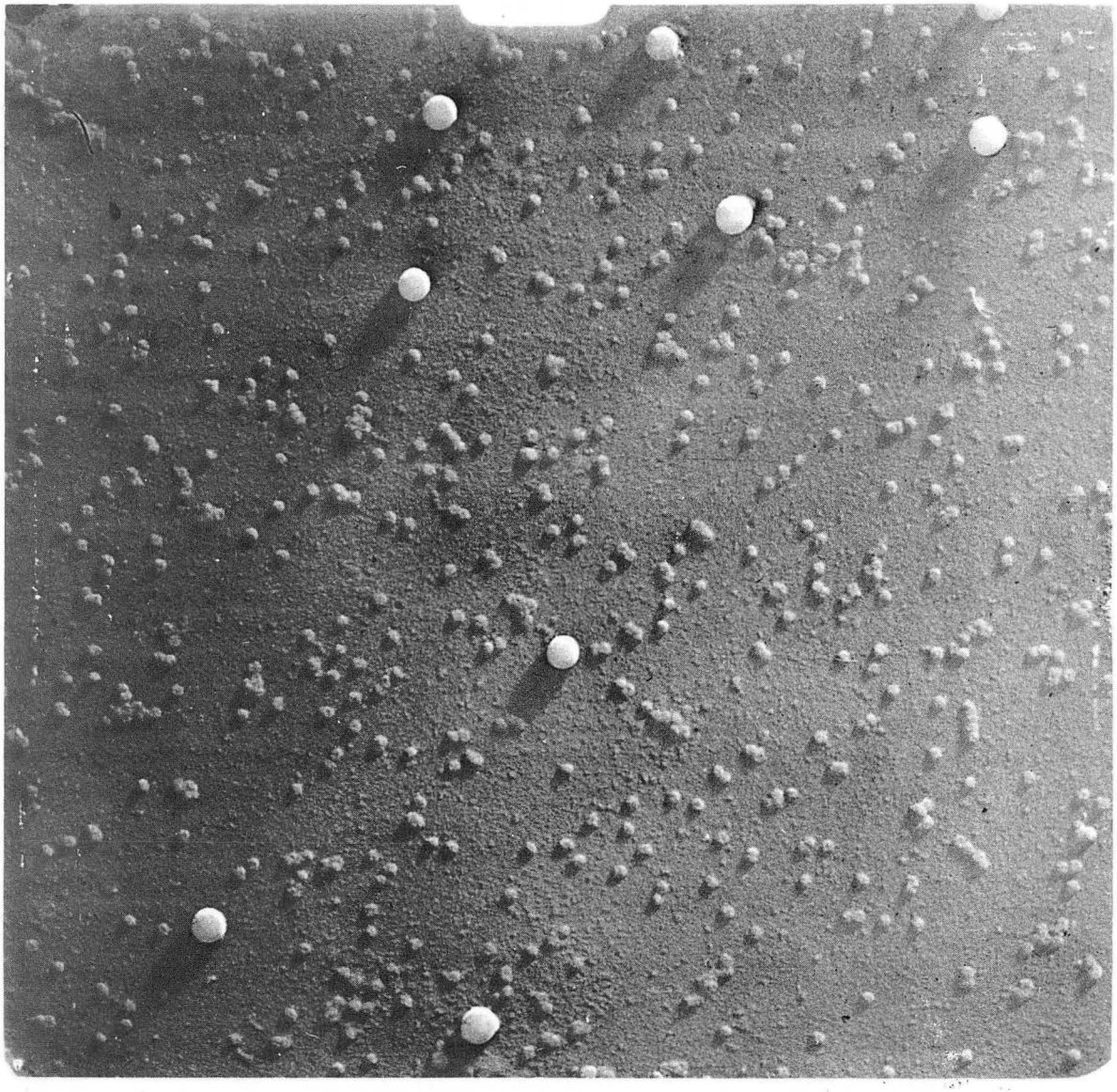

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Fig. 3. An electron micrograph of an ultracentrifugally isolated fraction of S_f^0 0–12 lipoproteins (X 54,230). The large particles are polystyrene standards 880 A in diameter.

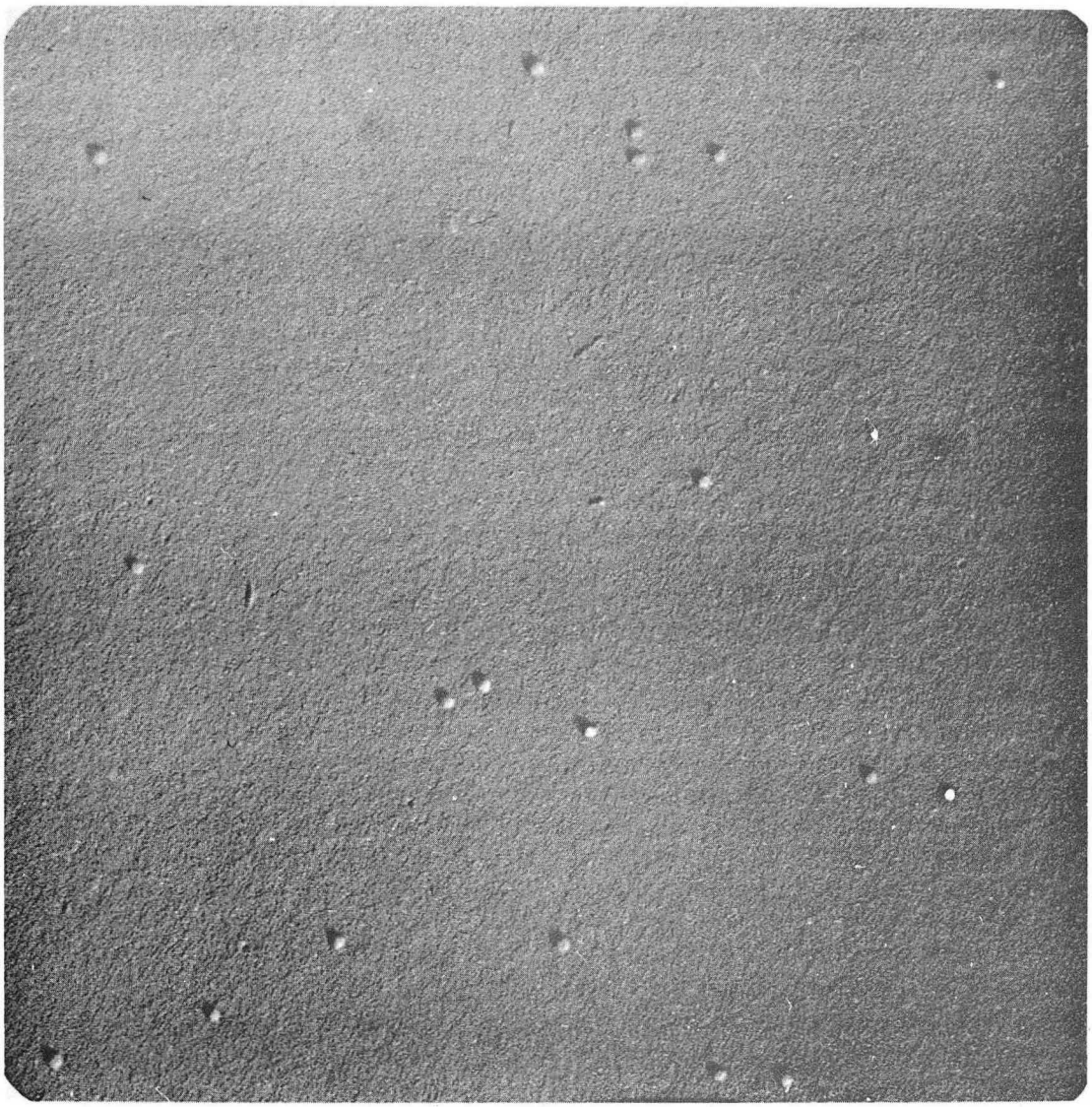

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Fig. 4. An electron micrograph of an ultracentrifugally isolated fraction of S_f^0 12-20 lipoproteins (X 54,230). There are no polystyrene standards seen in this photograph.

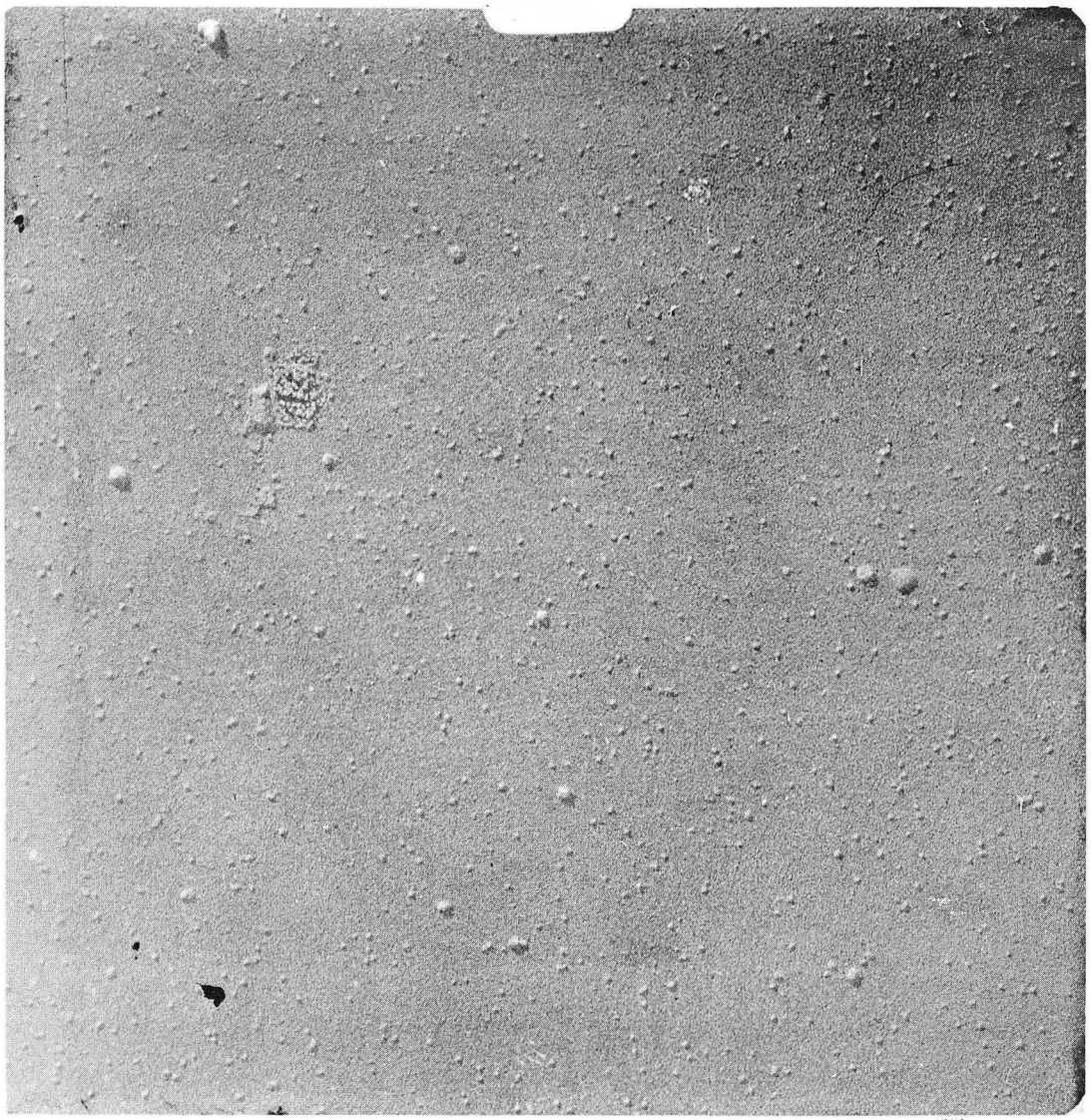

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Fig. 5. An electron micrograph of an ultracentrifugally isolated fraction of the two major high-density lipoproteins, HDL_2 and HDL_3. The mixture is predominantly HDL_3, since only about 20% of the concentration of this fraction consisted of HDL_2 (X 54,230). There are no polystyrene standards in this photograph.

verify the lipoprotein concept. (The Hayes method can be followed by others to obtain confirmatory results.) This visualization of discrete entities certainly solidifies and reinforces my thinking on this subject.

In a careful analysis following the Gofman method, one finds in the serum of healthy humans a continuum of lipoprotein classes ranging in hydrated densities from 0.93 g/ml (in the chylomicron class) to 1.04 g/ml. This range includes all the low-density lipoprotein classes, and was designated by the more useful term the S_f^0 unit by Gofman. In addition there are also present three more lipoprotein species, which have hydrated densities 1.045 g/ml, 1.075 g/ml, and 1.145 g/ml. These are known as the high-density lipoproteins, and were given the respective designations HDL_1, HDL_2, and HDL_3 by deLalla et al.[4, 16] (The cut in the lipoprotein spectrum which divides them into two major groups--the low-density and the high-density--is purely arbitrary, and was used for convenience only.) There is no existing evidence proving the presence of lipoproteins more dense than HDL_3 (hydrated density 1.145 g/ml) in healthy humans.

Prior to the Gofman technique in the study of serum lipids a somewhat ambiguous and overlapping lipid nomenclature crept into the literature. With each different method a new nomenclature was introduced, and, as a result of gross oversimplications, caused considerable confusion. To carry over any nomenclature from one method to another would be dangerous, since not only does the method of extraction differ, but there is also considerable overlapping. Until a better method is developed all serum-lipid analyses necessitate the use of the preparative ultracentrifuge and the differential-density separation method for the study of specific lipoprotein classes.

Although it is within the scope of this paper to discuss only the high-density lipoproteins, no lipoprotein study can be complete unless it includes both the low- and the high-density groups. Therefore, whenever appropriate, some low-density lipoprotein data are

included. To those undertaking a lipoprotein study it should be mentioned that unless the entire lipoprotein spectrum is obtained, only part of the story is revealed. In many disease categories the entire story may rest in either the low-density or the high-density lipoprotein groups, but in many other categories both groups are involved.[11, 12]

Study of the high-density lipoproteins by the method described herein afforded the opportunity for analysis of the serum proteins as well. The method, not published elsewhere, is described in detail under the section on Methods. The method utilizes the bottom fraction of the preparative sample after all lipoprotein material has been floated to the top and removed. The film record of the analytical run on an adjusted aliquot shows the serum of healthy humans to contain three species of proteins; we designate them P_1, a macroglobulin normally present in low concentration; P_2, which includes the gamma globulins; and P_3, the albumins. It is not very likely that P_2 is anything other than the serum gamma globulin nor that P_3 is anything other than the serum albumin, for two reasons. First: the concentrations of the two components, are within the limits described in the literature for gamma globulin as P_2 and for albumin as P_3 in normal human serum. Second: ultracentrifugally they are migrating with S rates comparable to what one would expect, considering the known molecular weight of gamma globulin and albumin. A simple experiment was conducted to show that if one mixes the appropriate proportions of isolated gamma globulin and albumin and performs the analytical ultracentrifuge analysis on this mixture, he can reproduce a pattern not detectably different from an actual protein analysis of normal human serum. Film records of this experiment are shown in Figs. 6, 7, and 8. Isolated immune serum globulin and albumin prepared from human blood for this experiment were furnished by the Cutter Laboratories of Berkeley, California. These are the fractions known electrophoretically as gamma globulin and albumin respectively. In Fig. 6 one can see a single component migrating with an S rate of 3.9. This represents a solution prepared with the Cutter human albumin

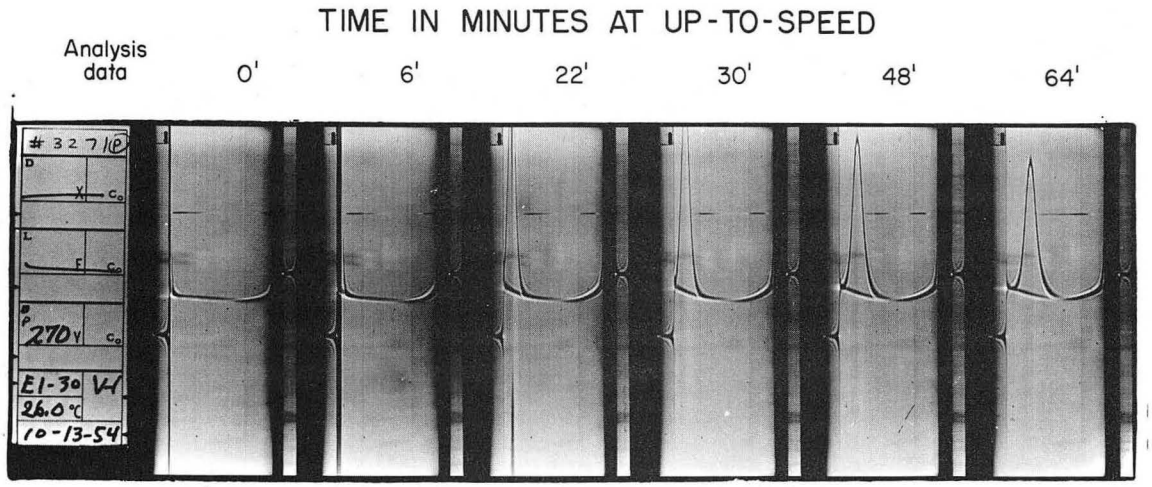

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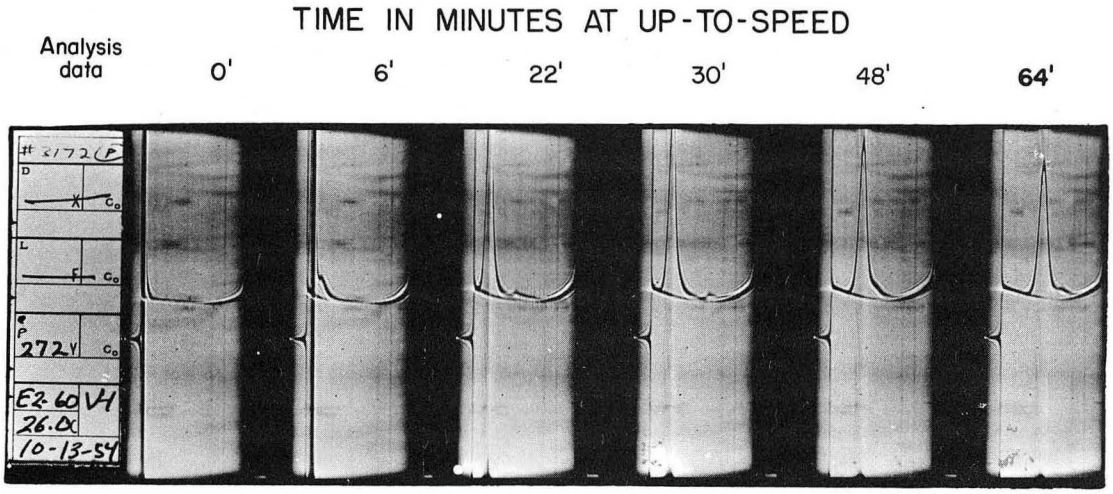
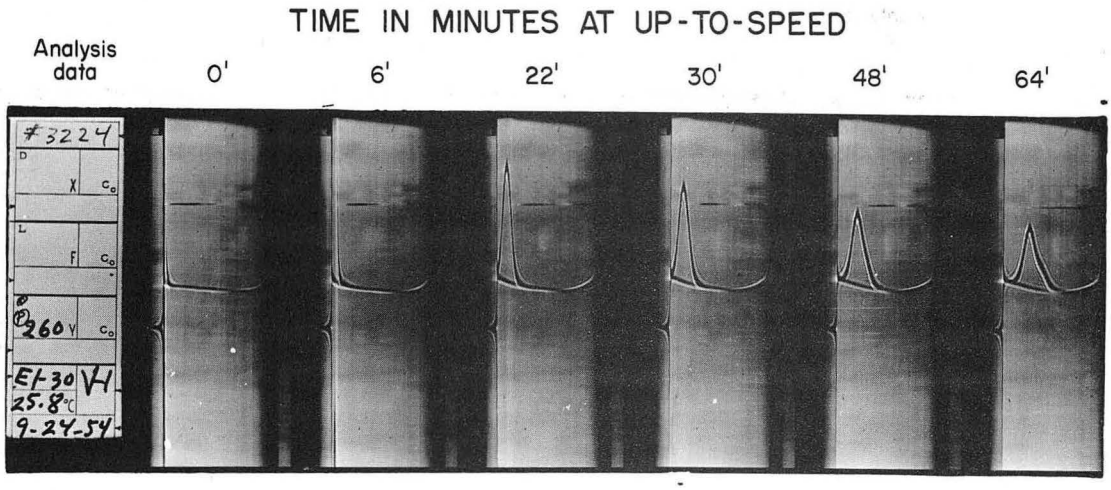
Fig. 6. An actual ultracentrifugal film record of the Cutter Laboratory human serum albumin fraction, also known electrophoretically as albumin. The measured migration rate for this component when extrapolated to infinite dilution is S = 4.



ZN-2077

Fig. 7. An actual ultracentrifugal film record of the Cutter Laboratory human immune serum globulin fraction known electrophoretically as the gamma globulin component. The measured migration rate for this component when extrapolated to infinite dilute is S = 6. There are slight contaminations of at least two macroglobulins in this sample.



ZN-2078

Fig. 8. An actual ultracentrifugal film record of a mixture of the Cutter fractions of both Fig. 6 and Fig. 7 (gamma globulin and albumin). The mixture was made to approximate the proportions of gamma globulin and albumin found in clinically healthy humans. Compare the 64-minute UTS exposure of this plate with the 64-minute exposure of Fig. 13d which is an actual pattern of the gamma globulin and albumin of a clinically healthy human. It is not possible to tell the difference.

In Fig. 7 one can see a large component migrating with an S rate of 6.2. This represents a solution prepared with the Cutter immune serum globulin. Now if these two fractions are adjusted in concentration and then mixed so that the amounts of each are representative of what they would be in the average normal human serum sample, and one then performs the analytical ultracentrifuge run on this mixture, he obtains a film record as seen in Fig. 8. Except for the absence of P_1, this pattern is not detectably different from any standard human protein pattern obtained by the method described in this text. If one compares the 64-minute UTS pattern (d) in Fig. 9 with the 64-minute UTS frame of Fig. 8, which is a comparison of the actual versus the "mock-up" patterns of the P_2 and P_3 complex (gamma-globulin and albumin respectively), he will note the remarkable similarity.

With the exception of P_1, P_2, and P_3, no other protein species were detectable in normal patients by use of the method described in the text. In certain clinical states, however, this method can reveal characteristically different protein patterns and protein species related to the clinical state involved.[21]

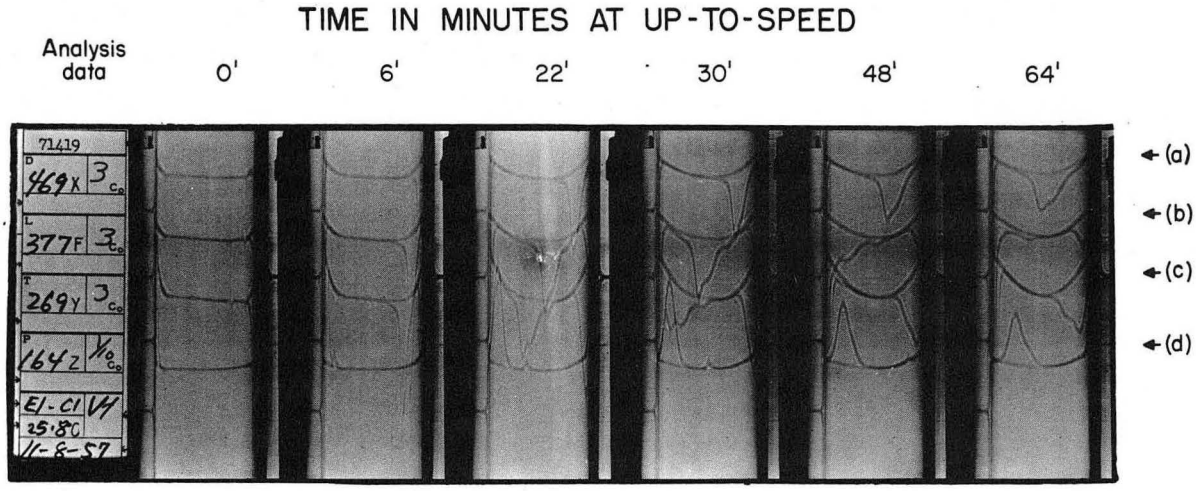


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Fig. 9. An actual ultracentrifugal film record showing the patterns obtained on a complete analysis of one subject only.
(a) represents the low-density lipoprotein run at solution density 1.063 g/ml (prep. type 1)
(b) represents the first of two high-density lipoprotein runs at solution density 1.125 g/ml (prep type 2)
(c) represents the second of two high-density lipoprotein runs at solution density 1.200 g/ml (prep. type 3)
(d) represents the protein run at solution density 1.035 g/ml.

II. SUBJECTS AND MATERIALS

A. Selection of Subjects

The healthy subjects for this investigation were employees of the University of California Radiation Laboratory at Livermore, California, all of whom are periodically given routine physical examinations. Venous blood samples--30 ml from each subject--were taken between the hours of 8 a. m. and 3 p. m., without any restriction of diet and without the subjects' having any previous knowledge of this study. Thus the sampling was essentially random. There were certain restrictions, however; no individual was included in the study whose medical records showed any of the following conditions:

Acute infection at the time of sampling
Poliomyelitis with residual deformity
Surgery involving removal of part or all of any organ
Any condition requiring that the subject take medications such as thyroid, steroids, etc.
Cardiovascular disease history
Cancer history
Multiple sclerosis
Pregnant women and persons following special diets were also excluded.

The total number of subjects qualifying was 2297, of which 1961 were males and 336 were females. Their ages ranged from 17 to 65 years. The 2297 samples were collected and analyzed at a rate of about 20 per week over a 3-year period. There were no delays in the analysis of any sample. Whenever the samples were collected they were immediately put through the complete analysis, which normally takes from 7 to 10 days.

B. Materials Used

The materials used for these studies are numerous, and an attempt is made here to list all the essential items chronologically in order of their use.

Blood-collecting materials: Tourniquets; alcohol; surgical sponges; bleeding kits consisting of a bleeding set and a collecting tube (see Methods section and Fig. 10); labels; scotch tape; data sheets to record subjects' physical and medical data; and a carrying container large enough to accommodate the above supplies and one day's collected samples.

Serum-separating materials: A size # 1 or # 2 International Centrifuge or its equivalent; a balance to equal the weight of opposing buckets in the centrifuge; a data book in which to log the samples and assign identifying numbers to them; serum-storage vessels for storing serum in the refrigerator (we used rimless culture tubes 15×125 mm); labels; scotch tape; parafilm to stopper the serum sample (corks should not be used since they can not be washed and rinsed adequately--a rubber stopper is satisfactory if properly washed and rinsed); a refrigerator; and refrigerator racks in which to store the sera.

Materials used in the preparative setup step: A setup rack (which can be designed by the individual) to hold a complete "prep" (in this case 18 samples at a time--a load for a Spinco 40.3 rotor--represents a complete prep); Lusteroid tubes $\frac{1}{2}$ inch in diameter and $2\frac{1}{2}$ inches long (can be purchased from the Spinco Division of the Beckman Instruments Co., Palo Alto, California; these plastic tubes must be carefully inspected and found free of flaws of any sort and must not be less than 2-15/32 nor more than 2-17/32 inches long); a labeling pen with ink that will permanently mark the Lusteroid tube so as not to lose the identity of the sample; sodium chloride (cp), sodium nitrate (cp), distilled-water source, heavy water (D_2O 99.5% pure); "EXAX draining pipets (3 ml, 2 ml, 1 ml, 0.5 ml); data sheets for recording the information regarding the samples of a particular prep (such as preparative run conditions, including the amount of serum used in each sample, the speed and duration of the run, etc.); a Spinco Model 40.3

rotor and accessories such as Lusteroid tube caps, cap vise and wrench, a tube puller, and a Spinco Model LH preparative ultracentrifuge (all supplied by the Spinco Division of the Beckman Instruments Co.).

Materials used in the quantitative removal of the lipoprotein concentrate after preparative run is completed: A firmly secure table at least 6 feet long that will not move if accidentally jarred; a light stand as shown in Fig. 12 (not purchasable; this was designed and built by a technician on the staff); a tube puller (furnished with the rotor) for removing the samples from the tube sockets in the rotor; eye-dropper bulbs and pipets as shown in Fig. 13 (not purchasable; must be made by a glass blower according to the specifications shown); 1-ml volumetric flasks, storage vessels in which to store the lipoprotein concentrate (we used rimless culture tubes 12×75 mm); labels, scotch tape; parafilm to stopper the storage vessels; and a rack designed to store a complete prep of lipoprotein concentrates in the refrigerator.

Materials needed for analytical ultracentrifugation: A Spinco Model E Analytical Ultracentrifuge, and either a (1) Spinco Analytical A, (2) Spinco Analytical D, or (3) Spinco Analytical C rotor (depending on the number of samples to be run simultaneously); the complete assembly of the double-sector analytical cells 12 mm thick (in place of the single-sector, since the latter necessitates a separate baseline run; see text under "Methods"); a vise and torque wrench to assemble and disassemble the analytical cells (these are supplied with the purchase of the ultracentrifuge); 1-cc tuberculin syringes; blunt-tip 23-gauge needles 1 cm long for use in loading the sample into the analytical cell; sheet film which can be cut to fit the plate holder of the analytical ultracentrifuge camera (Kodak super ortho press was used by us and found to be satisfactory).

Materials for film processing and analysis: A darkroom in which to cut, load, unload, and process the film; darkroom supplies; photographic enlarger to blow up the pattern obtained on the film record for convenience in tracing this pattern; an accurate millimeter rule; a planimeter; and a slide rule and a calculating machine.

Materials needed for the setup of the protein run: "EXAX" 0.2-ml blowout pipets, "EXAX" 1-ml blowout pipets, storage tubes, labels, scotch tape and parafilm as mentioned in Section D above.

Incidentals such as general office supplies, laboratory glassware and cleaning agents, and other standard equipment and supplies needed to run a research laboratory need not be listed. It is assumed that these basic items will be found in a properly stocked and equipped laboratory.

III. METHODS

A. Collecting Whole-Blood Samples for Ultracentrifugal Analysis

Blood samples must be drawn from subjects selected at random, excluding persons who are fasting or have recently altered their diet, unless the experiment calls for such conditions.

The blood samples were collected by means of the sampling kit shown in Figs. 10 and 11. This kit was developed by us and manufactured by Becton, Dickinson Co. (who made the collecting tube) and by Cutter Laboratories (who made the bleeding set). The use of such a system allows for complete control over such problems as contamination of the sample by some foreign agent that may alter the final results. It will be noticed that the bleeding set has identical needles (20-gauge $\times 1\frac{1}{2}$ in.) at both ends, making it possible to use either one of them for the venipuncture. These sets are sterile and pyrogen-free. The collecting tube, of 30-ml capacity, is evacuated. The collecting tube can be sterile, but for these analyses it need not be. The collecting tubes are treated by some process that deposits 3 mg of merthiolate in each tube. This will give a final concentration of merthiolate, after 30 ml of blood has been drawn, of 1/10,000. The merthiolate is used as a preseravtive and is capable of preserving blood samples for at least 5 to 7 days at temperatures from 10° C to 40°C. This would be necessary in case samples of whole blood had to be shipped elsewhere.

The following procedure was found essential for collecting samples of whole blood with the above sets: Surgical sponges dampened with 70% ethyl alcohol are used to wipe clean the area around the site where the venipuncture will be made. Samples for this study were usually collected from a branch of the cephalic or cubital vein of either arm. A tourniquet is applied, the plastic protective cap is removed from one of the needles of the bleeding set, and the venipuncture is made (see Fig. 11a). If a successful venipuncture is made the blood will gradually and continuously flow towards the other end of the set (Fig. 11a). Under these conditions one can proceed by

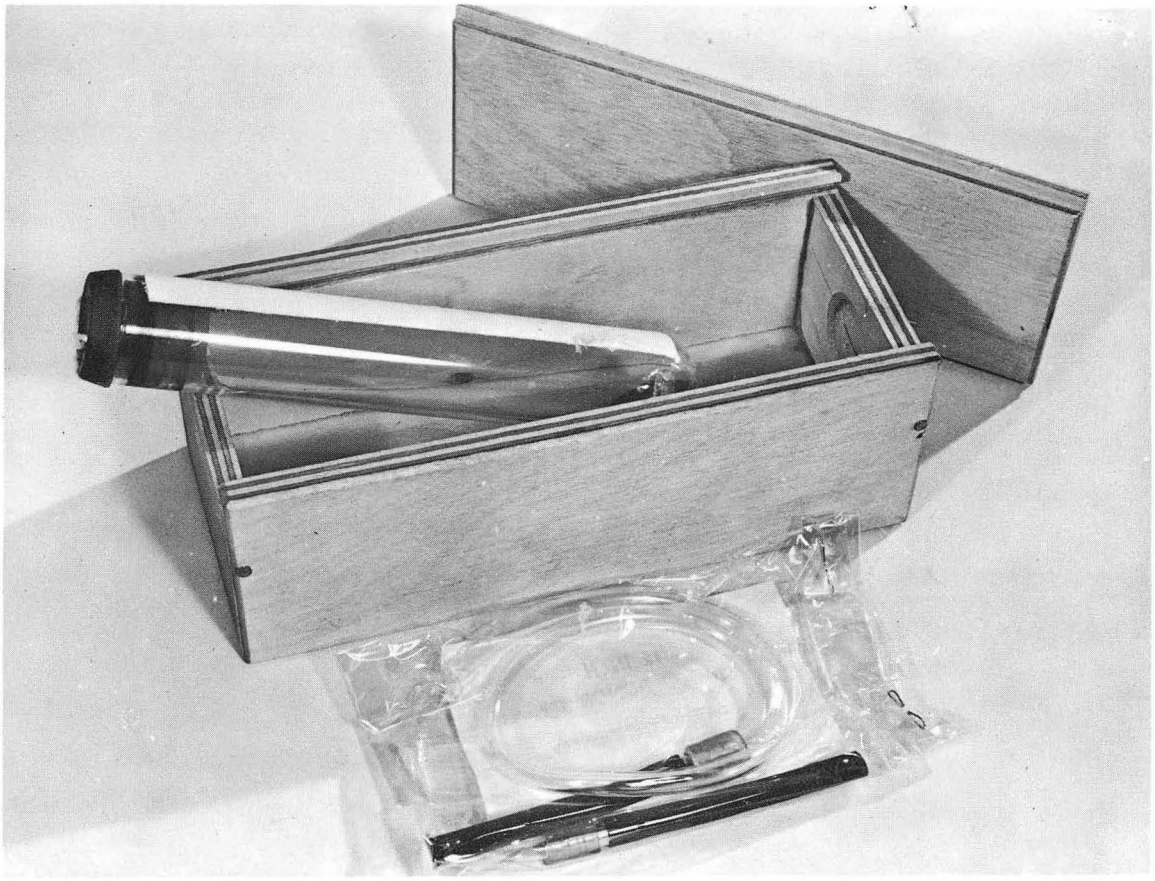

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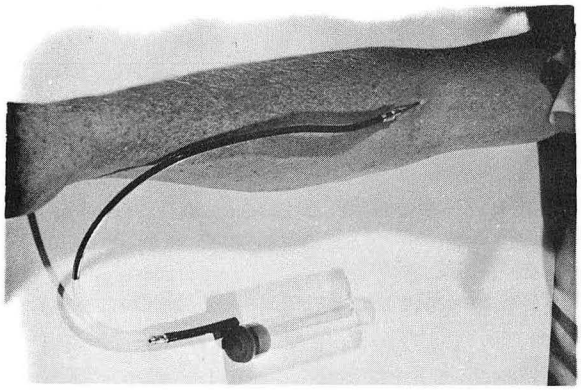
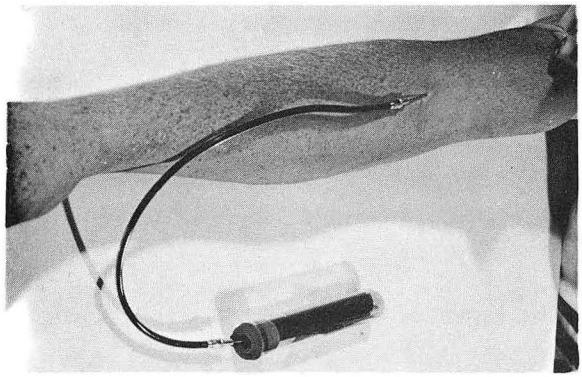
Fig. 10. A blood-sampling kit for collecting 30 ml of whole blood for ultracentrifugal analysis. It includes a collecting tube which is evacuated, a sterile bleeding set which has a 20-gauge needle at each end of a plastic tube, and a wooden protective case for use in mailing samples when necessary. The 20-gauge needles are kept sterile by a plastic jacket.

(a)



(b)



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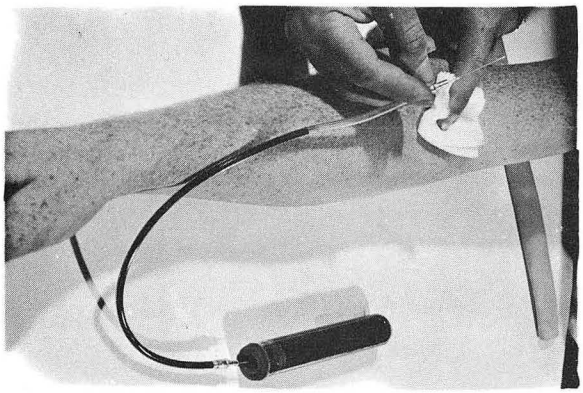

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Fig. 11. Collecting the 30 ml of whole blood needed for a complete ultracentrifugal analysis of the serum lipoproteins and proteins. This method was used for every sample represented in the data within this text.

(a) Making the venipuncture, using any one of the two 20-gauge needles at either end of the bleeding set.

(b) Allow the blood to flow down the plastic tube to the other needle before inserting it in the diaphragm of the collecting tube.

(c) After blood has stopped flowing in the collecting tube (30 ml blood), removing first the tourniquet and then the venipuncture needle.

removing the plastic protective cap from the other needle and inserting the needle through the diaphragm of the rubber stopper in the collecting tube (Fig. 11b). If the tube has not been tampered with it will have a sufficient vacuum to permit the collection of 30 ml of blood, after which the blood will stop flowing. The tourniquet is removed first, the venipuncture needle is removed second, and the other needle last (Fig. 11c). This bleeding set is now discarded; a new one is used for each sample.

If a full tube of blood is not collected, then either the collecting tube lost some vacuum or the venipuncture was not adquate. One solution to the former is to have handy an empty 30-ml syringe equipped with a needle; piercing the diaphragm with it and drawing air from the collecting tube allows more blood to be drawn. The problem of inadequate venipuncture is solved only by repeating the procedure.

If syringes and needles are used instead of the bleeding sets they must be clean and dry, and rinsed absolutely free of soaps or detergents, which are known to denature lipoproteins. If the sample is to be transferred to another vessel it also must be clean and dry and thoroughly rinsed.

The blood samples must be properly stoppered and left at room temperatures for at least 2 hours to allow for sufficient clot retraction. Whole blood collected under these conditions can be stored at temperatures ranging from 10°C to 40°C without any effects for several days. However, it is not wise to neglect samples purposely, and it is a far more reliable procedure to work on the sample, if possible, the same day it was collected. Keep whole-blood samples away from places where temperatures exceed the above range.

B. Extracting the Serum from the Whole-Blood Samples for Ultracentrifugal Analysis

An International Centrifuge, size #2, was used for this procedure. The blood samples are spun at 2500 to 3000 rpm for 15 to 20 minutes. This spinning causes the clot to pack tightly into the lower region of the collecting tube, allowing for easy decantation of the serum which constitutes the upper portion of the tube contents. The vessel used to store the serum is an ordinary clean, dry, and thoroughly rinsed rimless culture tube 15×125 mm. Parafilm cut to size is used to stopper the serum sample, which can now be stored in an ordinary refrigerator. We have found that storing samples as long as 28 days in refrigerators maintained at temperatures between 0° and 4° C did not affect the results of the analysis. A 30-ml blood sample should yield the 12 to 15 cc of serum needed for a complete analysis.

The necessity for a completely reliable labeling system can not be too strongly emphasized. In this type of analysis a new vessel is needed at each major step of the procedure. The vessel to which each successive transfer is made must not only be clean and dry and thoroughly free of detergent, cleaning agent, and soap--it must also be infallibly identified by a label that can not become lost in processing. In the absence of a foolproof method for proper labeling, there is a high chance for error that can invalidate all the work on an entire series.

C. Preparative Ultracentrifugation

The object of preparative ultracentrifugation is to separate out of the serum the desired lipoprotein classes. This is done in two phases, the setup step (which involves the alteration of the serum density), and the step for the quantitative removal of the fraction, found at the top of the tube following preparative ultracentrifugation, that contains the desired lipoprotein classes.

1. The Preparative Setup Step

(a) For preparative run (Type 2*) at solution density 1.125 g/ml. Three milliliters of serum is mixed at room temperature with 3 ml of diluent of density 1.2427 ± 0.0005 g/ml at 20° C in a Lusteroid (plastic) tube $\frac{1}{2}$ inch in diameter and $2\frac{1}{2}$ inches long. The diluent is a solution of $NaNO_3$ and D_2O (99.5% pure) adjusted to a final desity of 1.2427 ± 0.0005 g/ml at 20° C by pycnometry. The resultant density of the mixture in the plastic preparative tube is 1.125 g/ml at 26°. The preparative ultracentrifugation is performed in a Spinco Model 40.3 rotor which can hold up to 18 individual samples. The rotor is centrifuged at 40,000 rpm at 20 ± 2° C in a Spinco Model LH Utracentrifuge for a period of 23 to 26 hours. For this rotor, with a tube angle of 20°, the average centrifugal field is 114,000 × gravity at 40,000 rpm. This centrifugation insures quantitative flotation of two high-density lipoprotein classes, one of hydrated density 1.045 g/ml (HDL_1), and the other of hydrated density 1.075 g/ml (HDL_2), in addition to all the low-density lipoproteins. Since during this centrifugation those macromolecular components of hydrated densities greater than 1.125 g/ml undergo sedimentation out of the first 2 ml of tube content, a separation of the desired lipoproteins from such extraneous serum components is effected (see Fig. 14 for the distribution of lipoproteins and proteins following preparative ultracentrifugation).

(b) For preparative run (Type 3) at solution density 1.200 g/ml. Three milliliters of serum is mixed at room temperature with 3 ml of diluent of density 1.3927 ± 0.0005 g/ml at 20° C in a Lusteroid tube (as described in (a) above). The diluent is a $NaNO_3$ solution in D_2O (99.5% pure) adjusted to a final density of 1.3927 ± 0.0005 g/ml by pycnometry. The resultant density of the mixture in the plastic tube is 1.200 g/ml at 26° C. The preparative ultracentrifugation is performed as in (a) above under exactly the same conditions. In this preparative

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*Preparative Run Type 1 is reserved for the analysis of the low-density lipoproteins only. This is accomplished at a density of 1.063 g/ml.[4]

run all the lipoproteins that were obtained in (a) are present in the top milliliter, together with the third and last high-density lipoprotein class of hydrated density 1.145 g/ml (HDL_3). Under the conditions of this preparative run all the proteins have migrated down to the bottom 4 ml of the tube, again effecting a unique separation of the desired lipoproteins from the undesired macromolecular components (in this case the serum proteins). The bottom fraction of this run, after the quantitative removal of the top 1 ml (as explained below), is reserved for the protein analysis (which also is described in detail in a later section).

2. The Quantitative Removal of the Top Fractions Containing the Lipoprotein Concentrates

Care must be exercised in removing the rotor from the preparative ultracentrifuge, and also in removing the sample tube from the rotor, so as not to disturb the lipoprotein concentrate, which is found in the top milliliter of the tube contents. This top fraction should be removed immediately upon completion of the preparative run. These operations should be carried out at room temperature, since appreciable changes in temperatures may cause convection currents and result in remixing of the tube contents. Illumination of the tube contents with a light stand such as shown in Fig. 12 reveals distinctly the various layers of fractions in the tube.

The top 1 ml of either preparative run contains all the desired lipoproteins, and is quantitatively removed from the preparative tube. For this purpose a pipet is made from 7-mm o.d. glass tubing drawn out to a 1-mm capillary tip at one end and fitted with a small rubber eye-dropper bulb at the other (Fig. 13). While an even pressure is maintained on the bulb, the capillary tip of the pipet is placed 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 the bulb pressure allows aspiration of the lipoprotein --containing top fraction into the pipet with minimal disturbance. Aspiration of large quantities of air should be avoided by maintaining

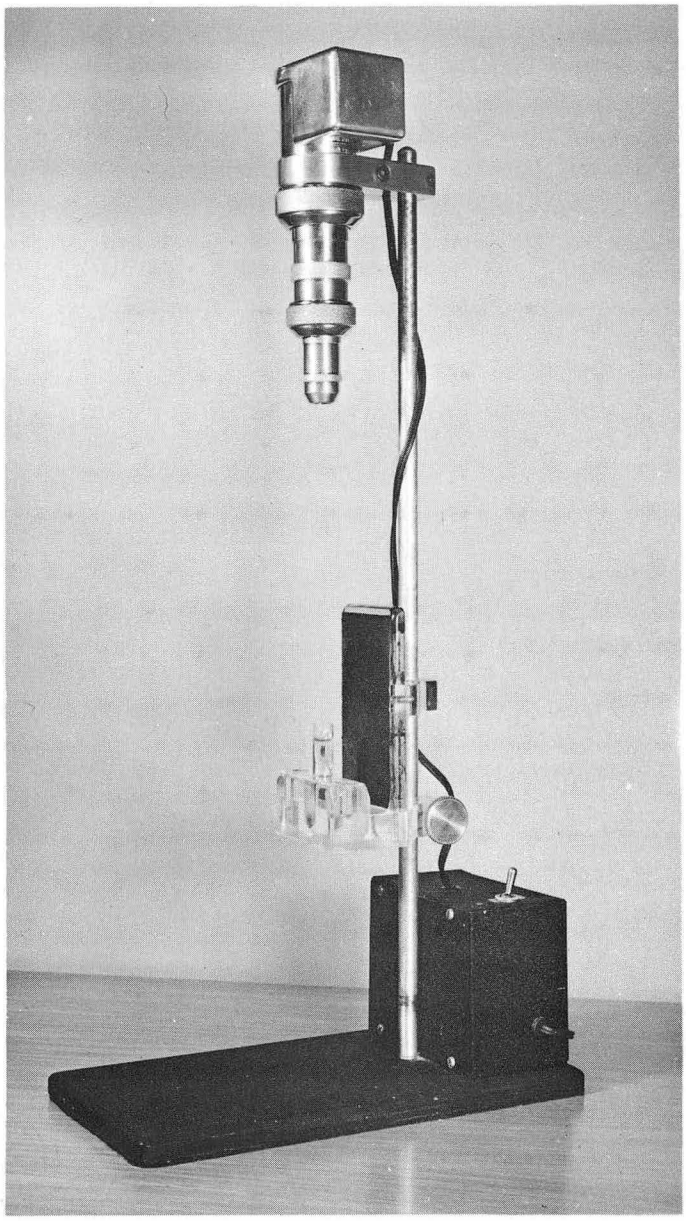

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Fig. 12. A light stand used in illuminating the top fraction of the preparative tube of Fig. 14 to aid in the aspiration of the top fraction containing the lipoprotein concentrate.

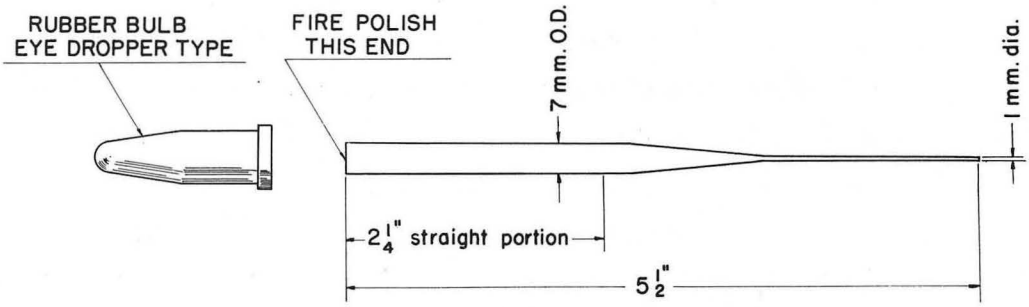

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Fig. 13. Drawing and specifications of the capillary pipe used in the aspiration of the top fraction of a lipoprotein run after preparative ultracentrifugation has been completed (see Fig. 14).

the pipet tip just below the air-liquid interface at all times during the operation. The pipetting of 0.5 to 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 ± 0.02 ml capacity. Pipetting and delivery into the volumetric flask is continued until 1.00 ± 0.02 ml of tube contents have been transferred. A small clinical centrifuge is used and is helpful in breaking bubbles, formed during the pipetting operation, which, if not broken, would interfere with volume adjustment. Since these lipoproteins, originally present in a 3.0-ml volume, have been delivered into a 1.0-ml volume, a threefold concentration has been achieved. The concentrated lipoprotein solution is then stored at 0 to 4° C, well stoppered, until analytical ultracentrifugation.

In some human sera the total lipoprotein content is very high. This may result in difficulties in pipetting the top fraction and (or) in film analysis. 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 3.0 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 and 3.0 ml is made up by the addition of a solution commonly referred to as "mock" serum. This solution is made up from sodium chloride and water pycnometered to a final density of 1.0073 ± 0.0005 g/ml. This closely approximates the density of human serum exclusive of the density contribution of proteins and lipoproteins.

3. Criteria for Preparing the Sample for the Protein Analysis

In preparing the sample for the protein analysis, the bottom fraction of a sample from the preparative run type 3 is utilized after the top 1 ml has been quantitatively removed for the high-density lipoprotein analysis. It must be kept in mind that this bottom fraction must meet the following requirements:

(a) There should be no leakage from the "prep" tube into the rotor, due to a faulty tube, during the course of preparative ultracentrifugation.

(b) The bottom fraction must contain all the solution of the preparative run minus only the top quantitative 1 ml which was removed for the high-density lipoprotein analysis. Any extra solution left in the pipette used to remove the top fraction must be returned to the prep tube.

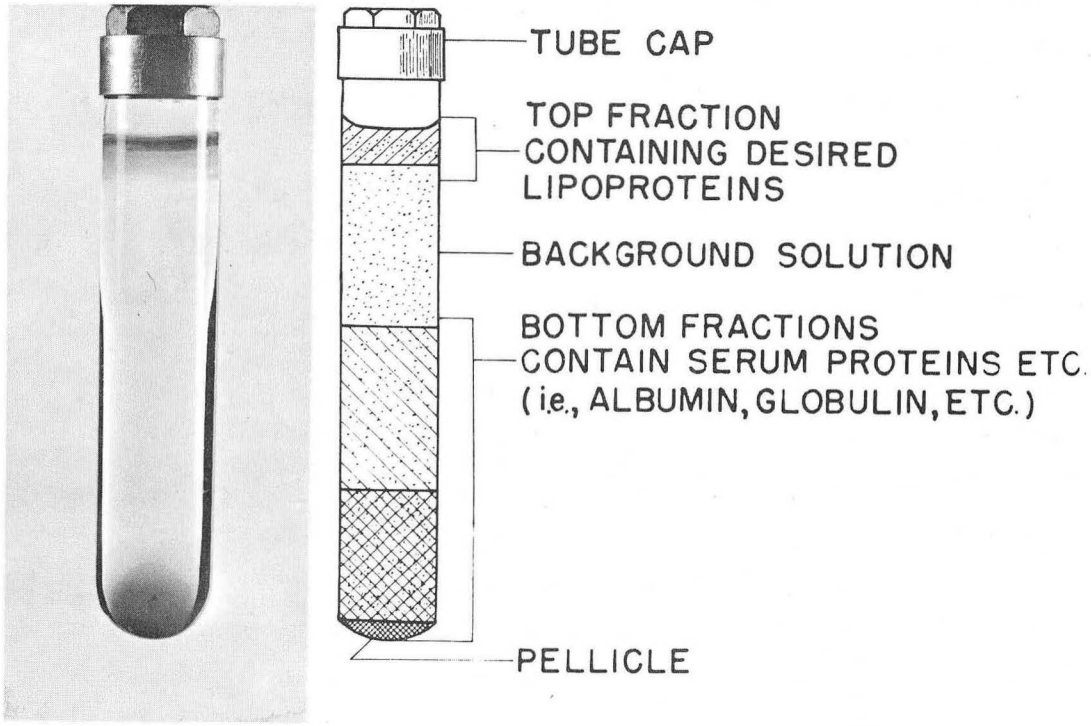
(c) Adequate mixing of this bottom fraction is absolutely essential because, owing to preparative ultracentrifugation, much of the protein material has sedimented and concentrated into a pellicle at the bottom of the tube (Fig. 14), with takes several minutes of thorough mixing to be finally redissolved.

(d) Since proteins are known to be labile, and rigorous shaking can cause denaturation, it is important that the mixing be done gently but thoroughly. The bottom fraction must be worked on as soon as possible and kept away from high temperatures that will cause denaturation.

4. The Procedure for Preparing the Protein Sample for Ultracentrifugal Analysis

(a) Adequate and thorough mixing are absolutely essential and can be accomplished by stoppering the tube and gently inverting it through 180° continuously until the pellicle at the bottom has completely dissolved (normally this would take about 2 to 3 minutes). This operation is completed only when the pellicle is dissolved.

(b) By use of a 0.2-ml "EXAX" blow-out pipette, a 0.2-ml aliquot of this bottom fraction mixture is transferred to another



ZN-2083

Fig. 14. An actual photograph of a preparative tube, showing the distribution of the various fractions after preparative ultracentrifugation has been completed. Compare this with the schematic representation of the same tube (right).

vessel which already has in it 1.00 ± 0.02 ml of distilled water delivered through the use of a 1.0 ml "EXAX" blow-out pipet. This tube should now contain a 0.2-ml aliquot of bottom fraction which has been thoroughly mixed and 1.0 ml of distilled water.

(c) This vessel is stoppered with parafilm (or a clean rubber stopper) and inverted eight times to mix the sample thoroughly. With each inversion one turns the tube on its axis through about 90° to make sure any small amount of sample that may have spattered on the vessel wall gets washed into the solution while mixing.

(d) The density of this run as a result of the above mixing (1.0 ml distilled water + 0.2 ml of bottom fraction of density 1.200 g/ml) is 1.0345 ± 0.0005 g/ml at 26° C.

This sample is now ready for analytical ultracentrifugation, and is handled exactly as are the lipoproteins.

D. Analytical Ultracentrifugation

The purpose of analytical ultracentrifugation is to quantitatively determine 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 this determination, which is performed in an analytical ultracentrifuge (Spinco Model E) equipped with a diagonal-wire phase plate, a cylindrical-lens schlieren optical system, [1, 17] and a camera. An aliquot of the lipoprotein concentrate is loaded into the analytical ultracentrifuge cell by means of a 1-ml tuberculin syringe and 23-gauge needle. This cell is placed in an analytical rotor (Spinco Model A or C; A can accommodate two cells and C can hold up to four cells. In any case the rotor must be balanced by an equal cell or a dummy cell in an opposite rotor hole). The rotor assembly is brought to 26.0 ± 0.5° C and then ultracentrifuged at 52,640 rpm. The Spinco rotors mentioned above when operated at 52,640 rpm develop a centrifugal field of 215,000 times gravity at a point approximately half-way along the fluid column in the analytical cell. Camera exposures are

taken at 0, 6, 22, 30, 48, and 64 minutes from up-to-speed time (UTS), and a film record is obtained for each analysis.

For subsequent analysis of the ultracentrifugal film record it is essential to know the equivalent UTS time represented by the period of acceleration. A close approximation utilized is that equivalent UTS time is 1/3 the acceleration time. In the Spinco Model E ultracentrifuges in Donner Laboratory at Berkeley the acceleration period is closely maintained at 5.2 minutes, which yields an equivalent UTS time of 1.73 minutes. Thus one can consider that at the time full speed is reached, the equivalent period of UTS 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 approximate reference solutions are as follows:

(a) For Prep. Type 2--a sodium nitrate-sodium chloride-heavy water mixture of density 1.1250 ± 0.0005 g/ml at 26° C.

(b) For Prep. Type 3--a sodium nitrate-sodium chloride-heavy water mixture of density 1.200 ± 0.0005 g/ml at 26° C.

(c) For the protein analysis -- a sodium nitrate-sodium chloride-heavy water mixture of density 1.0345 ± 0.0005 g/ml at 26° C (the appropriate density for protein analysis).

In our laboratory we use a double-sector cell, which consists of a centerpiece with two sectors in it. In one of these sectors is loaded the sample and in the other an equal amount of the correct above-mentioned reference solution. This provides us with a film record which has a reference line already superimposed upon the pattern, eliminating the source of error that would be involved in trying to place the correct baseline if it were run separately. Another advantage in using the double-sector cell is that about 15 to 20 minutes is cut off the time for complete film analysis. These cells are made of epon and were designed and fabricated by a member of the Gofman staff. The epon substance used in fabricating the centerpieces is a

plastic capable of withstanding the force of 215,000 times gravity with no difficulty if properly fabricated. Spinco Division of Beckman Instruments has a similar type double-sector cell available for purchase either in Dural or epon. Their epon centerpieces are rated only for speeds under 42,000 rpm. The Dural centerpiece are capable of withstanding speeds even greater than 52,640 rpm, but eventually corrode and cause effects tending to alter the quantitation. A typical centrifuge film representing a complete analysis on one subject as obtained by the analytical ultracentrifuge, is seen in Fig. 9. It shows four separate analyses--(a), (b), (c), and (d)--each with the correct reference line superimposed on the pattern. The exposure times are given for each frame. The film has an additional strip which identifies the subject and gives information on the condition of the run, such as the concentration, the patient's number, the cells used, the temperature and date of the run, the machine and rotor number, and the technician making the run. This is followed by six frames of pictures made at different times. The four patterns seen are as follows: the uppermost one, (a), represents a run on the low-density lipoproteins alone at solution density 1.063 g/ml; the second, (b) represents the first of the two high-density runs at solution density 1.125 g/ml; the third, (c) is the second of the two high-density runs at solution density 1.200 g/ml; and the bottom pattern, (d), is the protein run at solution density 1.035 g/ml. The peaks are deflected upward in the protein pattern, since the proteins are sedimenting, in contrast to the lipoproteins, which are floating and have their peaks deflected downward.

E. Ultracentrifugal Film Analysis

The purpose of film analysis is to determine the exact identity and quantity of each species of macromolecules present in the sample analyzed. This is accomplished by utilizing the film record which is provided by the analytical ultracentrifuge, as explained earlier in the text.

1. Theory

The analytical ultracentrifuge operated at very high speeds (in

this case 52,640 rpm) develops centrifugal fields on the order of 215,000 times gravity. Under these conditions macromolecules are caused to migrate either towards the cell base or towards the solution meniscus, depending on whether the solute molecules have hydrated densities greater or less than the solvent density. As migration proceeds each species in the cell establishes its own boundary region, differentiating it from others by the rate at which it migrates--the greater the difference between the solvent density and the hydrated density of the species, the faster the migration. This assumes that all other properties remain constant in a system that does not change.

The analytical ultracentrifuge makes use of a complex optical system, known as the schlieren-cylindrical-lens optical method, for analysis. (Pickels gives a good description of this method.[17]) This method utilizes a slit source of mercury light, a lower collimating lens which allows for parallel light to pass through the analytical cell (the light source is mounted so that these parallel slits are oriented parallel to any boundaries that may be formed by a migrating component), an upper condensing lens which focuses all the parallel slits on a plane at which is placed an inclined element (a bar or a wire or a wire phase plate), a camera lens which focuses the image of the cell on the film in the camera, and a cylindrical lens which magnifies effects only in the vertical direction. A simple description is not possible, and an adequate one is out of place here, since there are several thorough manuscripts on the theory of the schlieren-cylindrical lens method. The ultimate effect is to produce a pattern on the film of the refractive-index gradients of boundary regions as a function of distance from center of rotation. The automatic camera system allows for photographing the events in the analytical cell, during the course of the run, at almost any time desired. The reference run made in a given analytical ultracentrifugal cell using the reference salt solutions provides a baseline tracing which represents the refractive index gradients due to the redistribution of salt ions superimposed upon other gradients such as compressional effects and cell-assembly factors. The lipoprotein (and (or) protein) run in the same analytical cell has refractive index

gradients due to migrating lipoprotein (and (or) protein) boundaries superimposed upon those described above for the reference run. At any distance from the center of rotation, the difference between tracings for the baseline (reference) run and the macromolecules run represents refractive-index gradients resulting from macromolecules alone.

In Fig. 15a is a representation of the sector-shaped analytical cell, showing a single lipoprotein species undergoing flotation from the cell base towards the center of rotation. In Fig. 15b 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, dn/dx, as a function of distance, x, from the center of rotation. In Fig. 15c is shown the plot of dn/dx vs x, which correspondes to the concentration plot of Fig. 15b. In Fig. 16a an actual ultracentrifugal analytical pattern for such a system is shown, and in Fig. 16b the corresponding salt reference pattern is represented.* The plots in Fig. 15 are representative of the situation existing in the 30-minute UTS exposure of Fig. 16. In order to measure the refractive-index gradients (dn/dx) ascribable only to macromolecules (if present), it is necessary to superimpose the salt reference pattern upon the corresponding macromolecules pattern. With the double-sector cell such a task is not necessary, since the reference pattern is obtained simultaneously with the pattern. (The details of this superimposition are considered in the section on Area Determinations.) A schematic representation of the 30-minute UTS lipoprotein pattern plus the superimposed salt reference pattern of Fig. 16 is shown in Fig. 17. The cross-hatched area in Fig. 17 is a meausre of $\int (dn/dx)\, dx$ over the boundary region of the migrating lipoprotein species. Since this area represents the increment in

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*In our standard runs, as explained earlier in the text, we use the double-sector cell, which provides for both the sample and the reference solution to be run simultaneously, yielding a film record with the baseline already superimposed on the pattern.

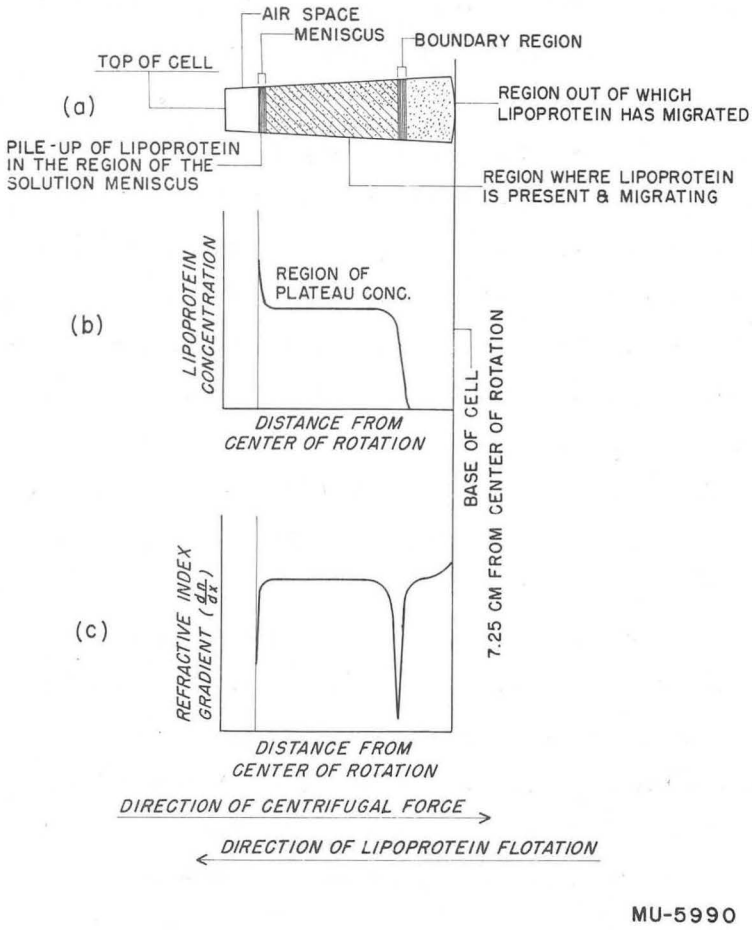

Fig. 15. A schematic representation of the events in the cell during analytical ultracentrifugation.

(a) A schematic diagram of an analytical ultracentrifuge cell containing a migrating lipoprotein species.

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

(c) A representation of the refractive index gradients as a function of distance from center of rotation. The schlieren-cylindrical-lens optical system of the Spinco Model E ultracentrifuge actually provides this type of plot on the film record.

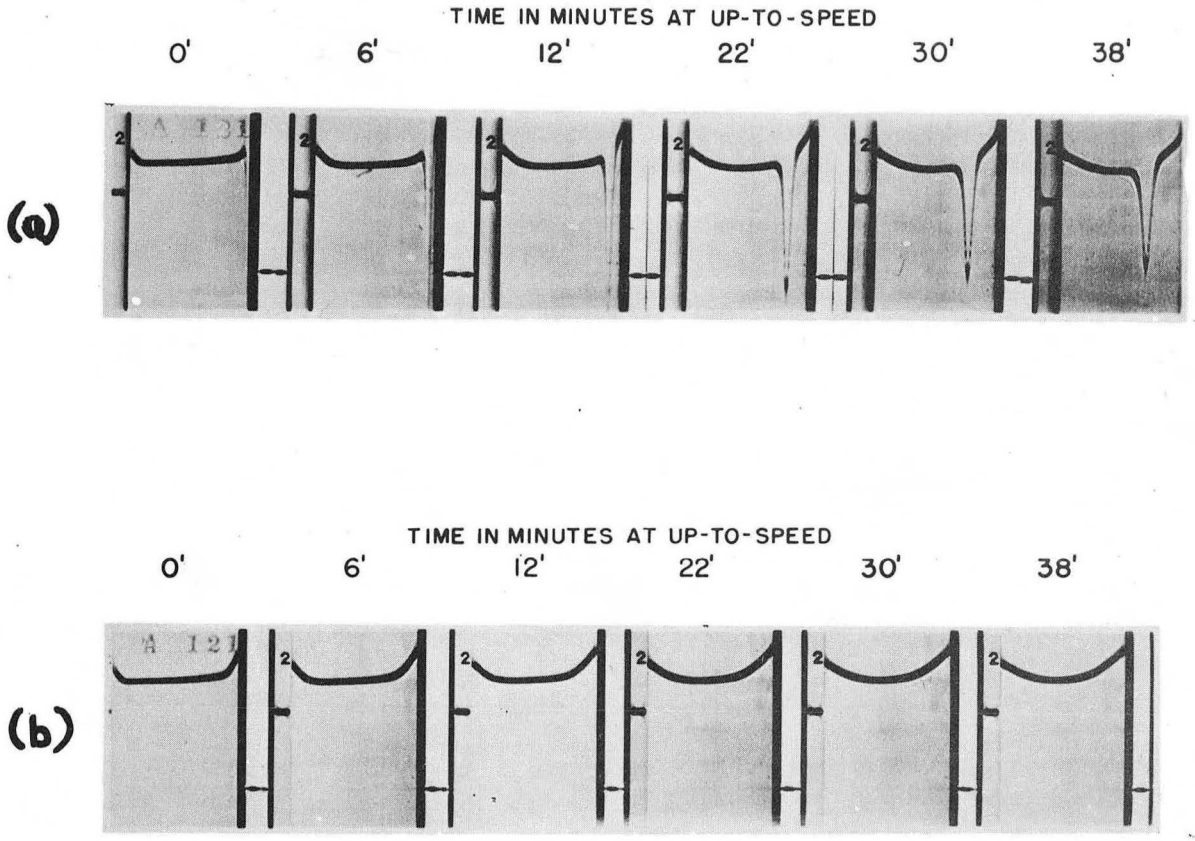

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Fig. 16. An actual ultracentrifugal film record of an ultracentrifugally isolated lipoprotein (the $S_f^0 6$ lipoprotein) at solution density 1.063g/ml:
(a) the pattern run (analytical cell contains the sample)
(b) the reference run (analytical cell contains baseline solution).

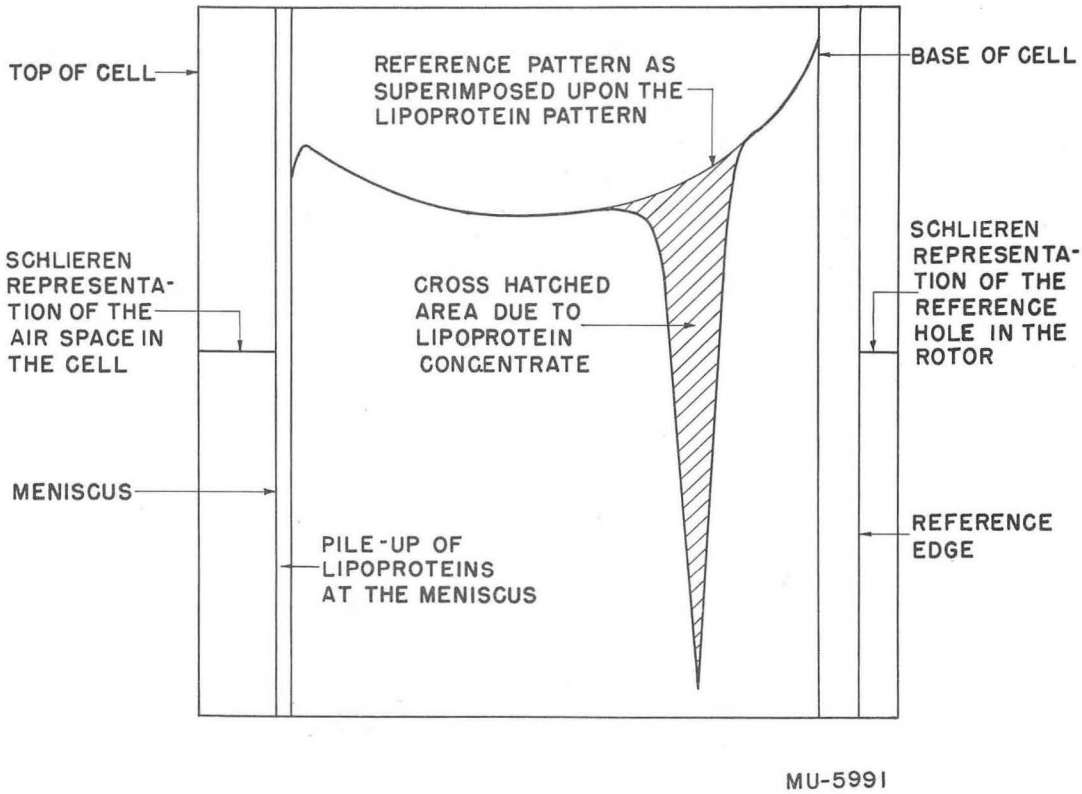


Fig. 17. An enlarged tracing of the 30-minute exposure of Fig. 16 with the baseline superimposed. The cross-hatched area represents the $\int (dn/dx)dx$ over the boundary region of the migrating $S_f^0 6$ lipoprotein. This area is proportional to the concentration of the S_f^0 6 lipoprotein.

refractive index across the boundary region, it is proportional to the increment in lipoprotein concentration across this same boundary region. This increment in lipoprotein concentration is equal to the actual lipoprotein concentration in the plateau of the cell, because the lipoprotein concentration is zero in that region of the cell centrifugally beyond the boundary region. The concentration of the lipoprotein measured thus in the plateau region must (as is usual in ultracentrifugal practice) be corrected for certain effects (as will be seen below).

It is also possible and useful to characterize lipoproteins and proteins by their migration rates, either under defined conditions or under standard conditions. The latter require such data as partial specific volume of the various lipoprotein and protein species, and the viscosity of the medium used in migration. 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/g). For sedimenting molecules such as proteins the migration rates in S units are positive, but for lipoproteins, which undergo flotation, these migration rates in S units are negative. The S_f unit, as originated by Gofman,[6] is used to provide a positive migration rate, since we have 1 S_f unit = -1S unit. The determinations of the rate of migration of lipoproteins (and (or) proteins) is based upon the application of the Svedberg identity,

$$S = \frac{(dx/dt)}{\omega^2 x} , \qquad (1)$$

where

S = migration rate per unit centrifugal field,

x = distance of lipoprotein (and(or) protein) boundary region from center of rotation,

t = time of centrifugation at full speed (52,640 rpm) with the equivalent UTS time of the acceleration period incorporated,

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

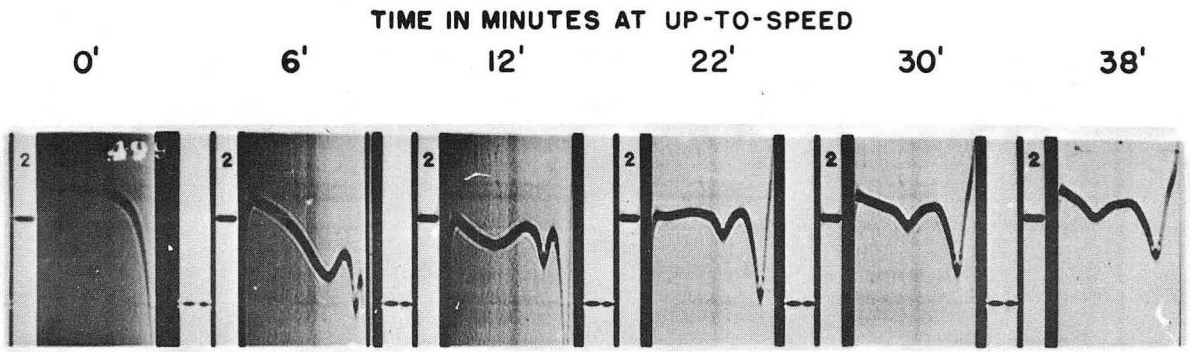
Integrating Eq. (1) yields Eq. (2),

$$\ln \frac{x}{x_0} = s\omega^2 t \Big]_0^t , \qquad (2)$$

where x_0 = distance from the origin of the boundary to the center of rotation. (For lipoproteins the cell base is the origin and for proteins the meniscus is the origin). Thus by determination of the position of a lipoprotein (and (or) protein) boundary at any time of centrifugation, and the application of Eq. (2) it is possible to determine the migration rate of that macromolecule species in S_f units (or S units in the case of proteins).

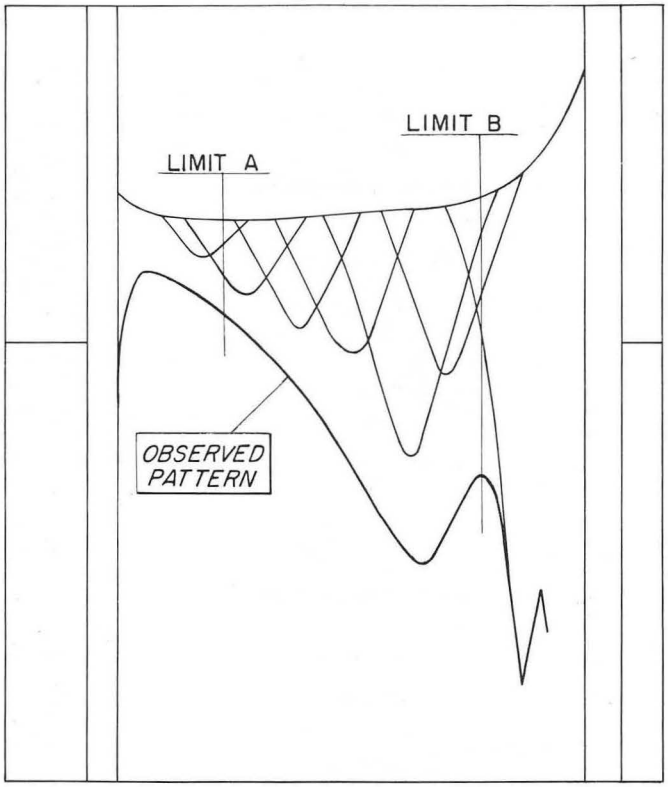
In the sera of many healthy or diseased humans there exists either a host of lipoproteins species which give rise to closely spaced boundaries or a series of lipoproteins forming an actual continuum with respect to their migration rates. This is so of the low-density lipoproteins, and the problem of determining the concentration of individual lipoprotein species is complicated. Figure 18 is a reproduction of the analytical ultracentrifuge film record of a human low-density lipoprotein concentrate (Prep Type 1 at solution density 1.063 g/ml), showing a broad deflection extending over a large region of the cell, in contrast to the sharp deflection in Fig. 16a. The broad deflection is interpreted as being the resultant of some number, possibly very large, of closely spaced boundaries. This interpretation, utilizing the 6-minute UTS exposure of Fig. 18 for illustrative purposes, is schematically represented in Fig. 19. Resolution of the area into individual lipoprotein species is difficult or even impossible. In such situations the analysis is limited to the determination of the sum of the concentrations of all lipoproteins between arbitary flotation-rate limits.

A further complication deserves consideration. The lipoproteins experience self-slowing in flotation rate as their concentration increases; further, any single lipoprotein species is increasingly slowed by a rise in the total concentration of all lipoproteins with intrinsically lower flotation rates.[8] Second, the Johnston-Ogston phenomenon



ZN-2085

Fig. 18. An actual ultracentrifugal film record of a low-density lipoprotein analysis, at solution density 1.063 g/ml (prep. type 1), showing a pattern commonly found in human serum. The reference run for this pattern would be similar to Fig. 16b.'



MU-5992

Fig. 19. An enlarged tracing of the 6-minute U. T. S. exposure of Fig. 18 with the baseline superimposed; a schematic interpretation of the broad deflection observed in the anaylsis of a continuum of lipoproteins. The observed pattern is interpreted as arising from the summation of overlapping deflections due to many individually migrating lipoprotein boundaries. Concentrations in such patterns are measured between chosen flotation-rate limits (designated here as limit A and Limit B).

operates as a result of these slowing effects.[18] The slowing effect and the Johnston-Ogston phenomenon must be considered in the quantitative analysis of analytical ultracentrifuge film records. Both effects are discussed in detail in Reference 4, but are briefly covered below.

2. Area Determinations [$\int (dn/dx)\, dx$] of the Macromolecular Species

The low-density lipoproteins pose a difficult problem in the quantitative analysis of the film record, owing to the continuum type of distribution of low-density lipoproteins usually obtained on most human sera (Fig. 18). If one desires to determine the area of the low-density lipoprotein pattern in groups such as S_f^0 0–12, S_f^0 12–20, S_f^0 20–100, and S_f^0 100–400, it becomes necessary to apply both the correction for sedimentation vs concentration and the correction for the Johnston-Ogston phenomenon to the area obtained directly from the film. Unlike the low-density lipoproteins, the high-density lipoproteins do not require such corrections, since the order of magnitude of the corrections is smaller than the uncertainty in measuring an area. The reader is advised to study References 4 and 8 carefully to appreciate and fully understand the nature and effect of such corrections.

The measurement of the film area begins with magnifying the film record 5 X (this factor is arbitrarily chosen for convenience) with the aid of a photographic enlarger or a projector. A tracing is made through the center of the enlarged pattern observed on the template, which already has the salt reference line inscribed. Proper orientation of the ultracentrifuge pattern with respect to the template involves two major steps if salt reference runs are not made simultaneously. (a) Vertical adjustment by superimposing the schlieren representation of the air space (see Fig. 17) for the reference run upon that of the analytical run; (b) horizontal adjustment by superimposing the reference edge of the salt reference run upon that of the analytical run. It is essential (as previously mentioned) that the baseline template be obtained from a salt reference run made in the same cell and rotor under identical ultracentrifugal operating conditions. If,

however, the double-sector cell is used, then a tracing is made through both the sample pattern and the reference pattern at the same time. The enlarged tracing is then subjected to analysis.

a. HDL_1--High-density lipoprotein (1.045 g/ml) obtained from preparative run Type 2 at solution density 1.125 g/ml

Figure 20 is an enlarged tracing of the 30-minute UTS exposure of Fig. 9. In determination of this lipoprotein a difficulty is encountered which arises from the failure of the 1.045-g/ml lipoprotein (HDL_1) to resolve itself from the major complex of the low-density group of lipoproteins. This complex can be seen migrating just ahead of the 1.045-g/ml lipoprotein (30-minute UTS exposure of Fig. 9b). The area representing this lipoprotein is approximated as follows, with the aid of Fig. 20.

(i) An estimate is made of the "peak" position (maximum ordinate) for the 1.045-g/ml lipoprotein.

(ii) A line is drawn vertically from this "peak" position to the salt reference line.

(iii) The dotted area to the right of this line (Fig. 20) is measured with a polar planimeter and doubled. (This assumes that the boundary is symmetrical about the vertical line.)

b. HDL_2--High-density lipoprotein (1.075 g/ml) obtained from preparative run Type 2 at solution density 1.125 g/ml

Figure 21 is an enlarged tracing of the 64-minute UTS exposure of Fig. 9b. This lipoprotein species is much more precisely measurable, since it is completely resolved as a boundary unassociated with other lipoproteins (see 64-minute UTS exposure Fig. 9b). The entire area associated with this lipoprotein boundary region is taken as representative of the concentration of the 1.075-g/ml lipoprotein (HDL_2) as shown in cross-hatching of Fig. 21. A planimeter is also used to determine this area.

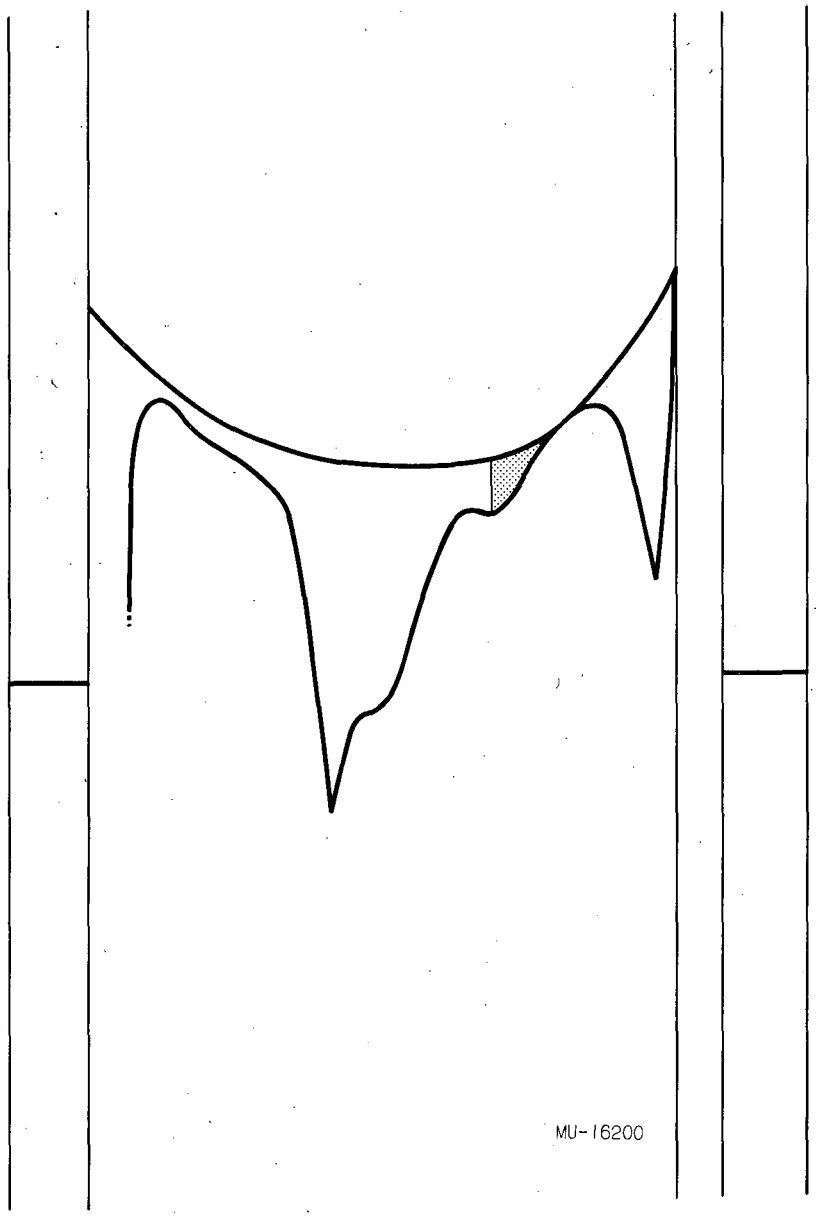
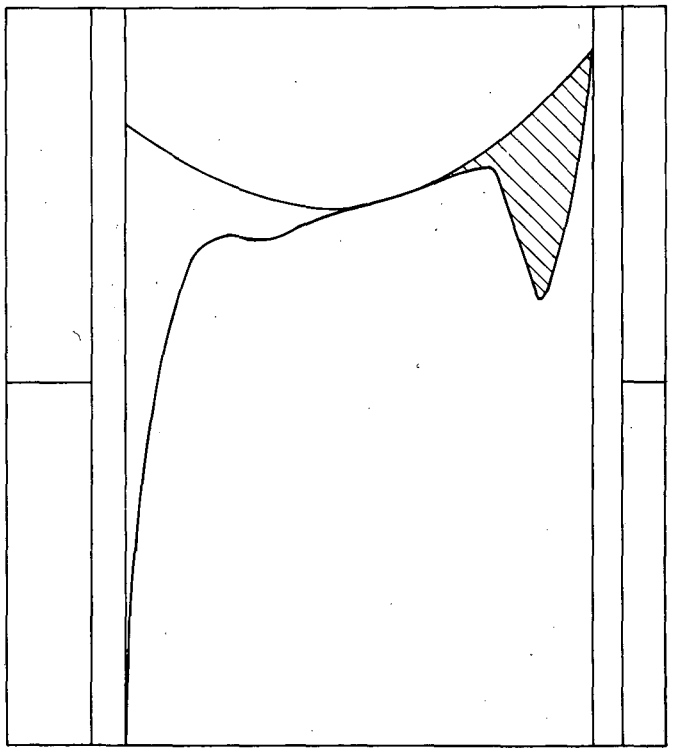


Fig. 20. An enlarged tracing of the 30-minute UTS exposure of Fig. 9b. The dotted area actually represents about half the total concentration of the high-density lipoproteins of hydrated density 1.045 g/ml (HDL_1).



MU-5996

Fig. 21. An enlarged tracing of the 64-minute UTS exposure of Fig. 9b. The cross-hatched area represents the actual concentration of the high-density lipoprotein of hydrated density 1.075 g/ml (HDL_2).

c. HDL_3--High-density lipoprotein (1.145 g/ml) obtained from Preparative run Type 3 at solution density 1.200 g/ml

Figure 22 is an enlarged tracing of the 64-minute UTS exposure Fig. 9c. This tracing involves the complex of two lipoprotein species. These are the lipoproteins with hydrated densities of 1.075 g/ml (HDL_2) and 1.145 g/ml (HDL_3). The lipoprotein with a lower density migrates faster than the one of higher density. The total area of the complex, as shown in Fig. 22 is measured with a planimeter and the concentration of the 1.145-g/ml lipoprotein (HDL_3) is obtained as follows:

(i) The total area is recorded. This area represents the concentration of both HDL_2 and HDL_3.

(ii) The area as obtained for HDL_2 in the above section from Fig. 21 is subtracted from the total area obtained in Fig. 22.

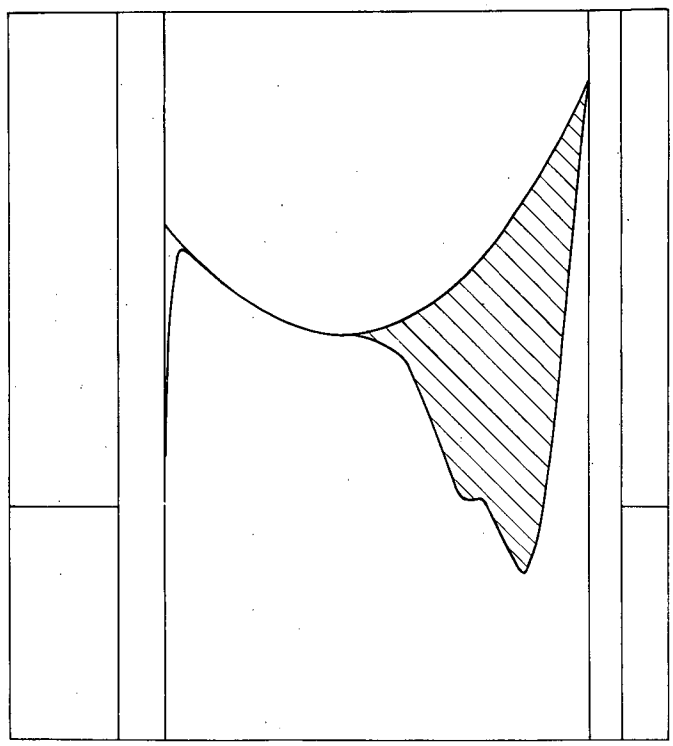
(iii) The remainder represents the concentration of HDL_3, since we have $(HDL_2 + HDL_3) - HDL_2 = HDL_3$.

d. P_1--Protein of S rate ranging from 14 to 19 S units (depending on its concentration) obtained from the bottom fraction of the preparative run Type 3

The preparation of the protein run is described in "Methods", Section III. Figure 23 is an enlarged tracing of the 30-minute UTS exposure of Fig. 9d. This protein is a macroglobulin which was first described by Pederson[1] and Mutzenbecker[19] as the S-20 component. It is quantitatively determined by planimetry of the entire dotted area of Fig. 23, which is completely resolved as a separate component unassociated with other protein species.

e. P_2 and P_3--Proteins of S rates 6 and 4 respectively, known in the literature as the gamma globulins and the albumins

Figure 24 is an enlarged tracing of the 64-minute UTS exposure of Fig. 9d. Since the two components are seen as a complex, unresolved from each other, the following method is used to determine the area of each component.



MU-5997

Fig. 22. An enlarged tracing of the 64-minute UTS exposure of Fig. 9c The cross-hatched area represents the actual concentration of the high-density lipoproteins of hydrated density 1.075 g/ml (HDL_2) and of hydrated density 1.145 g/ml (HDL_3).

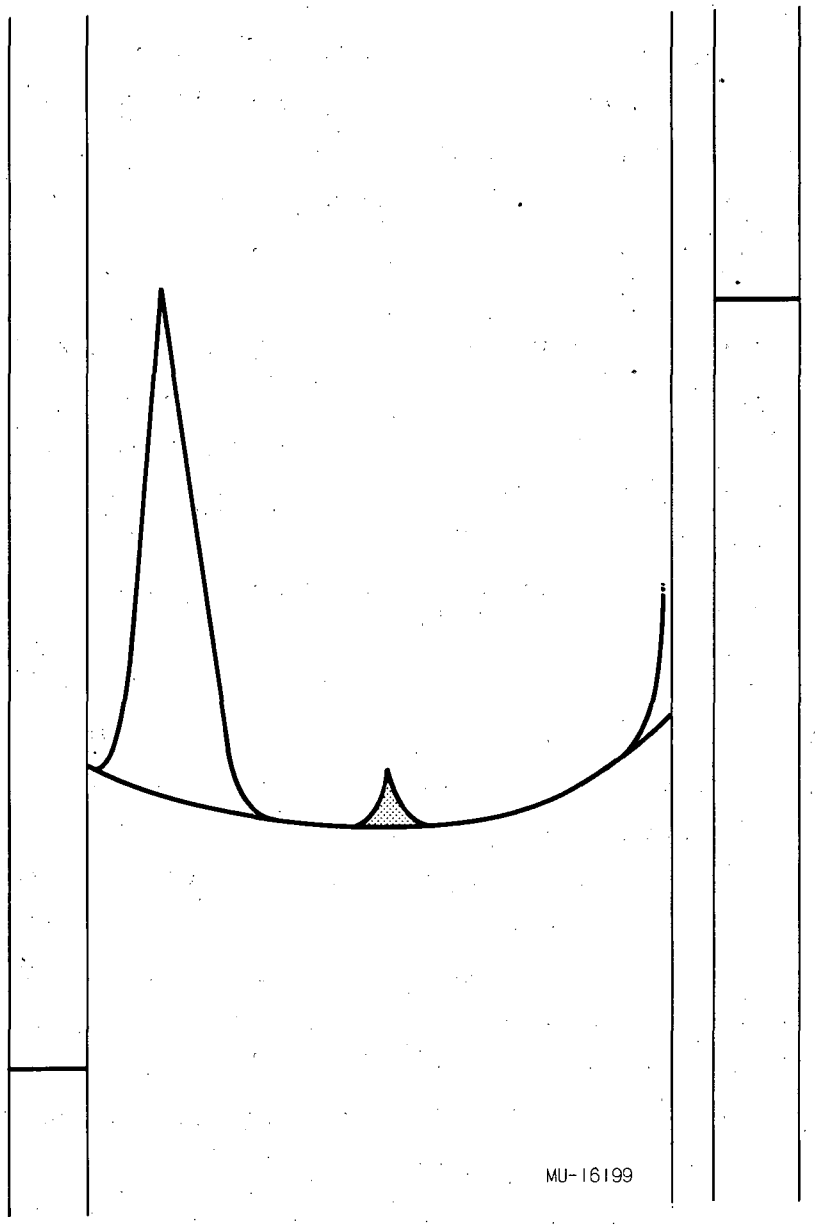


Fig. 23. An enlarged tracing of the 30-minute UTS exposure of Fig. 9d. The dotted area represents the actual concentration of P_1, a macroglobulin usually found in most clinically healthy humans.

(i) A vertical line is drawn parallel to the meniscus line from the "peak" position (maximum ordinate) to the reference line, as shown in Fig. 24.

(ii) The total area is determined by the use of a planimeter and recorded. This represents the total concentration of $P_2 + P_3$.

(iii) The dotted area to the left of the vertical line is then determined separately, and doubled. This is considered as the total area due to P_3 alone (the albumins of S = 4).

(iv) P_3 is subtracted from the total area of $P_2 + P_3$, and the remainder is considered to be P_2 (the gamma globulins of S = 6).

3. Mathematical Corrections to Be Applied to the Areas Obtained Above

There are five corrections on the $[\int (dn/dx)dx]$ determinations to be considered in work with ultracentrifugal films.

(a) Correction for sedimentation vs concentration.

(b) Correction for Johnston-Ogston phenomenon.

(c) Correction due to position in the sector-shaped cell relative to the center of rotation. Two effects are grouped into one here: the effect of the inhomogeneous force field as the distance from the center of rotation changes, and the radial dilution (or concentration) effect, as a molecule migrates towards the base of the cell (or the solution meniscus), due to the sector shape of the cell.

(d) The adjusted concentration of the serum sample during the preparative operations.

(e) The temperature of the analytical rotor during the analytical ultracentrifugation.

(a) The sedimentation-versus-concentration correction is not needed for either the high-density lipoprotein or the protein determinations, since their measurement as single species is unlike the situation in the low-density analysis, in which definite S-rate cutoff points are to be made in a continuous-type spectrum of lipoprotein

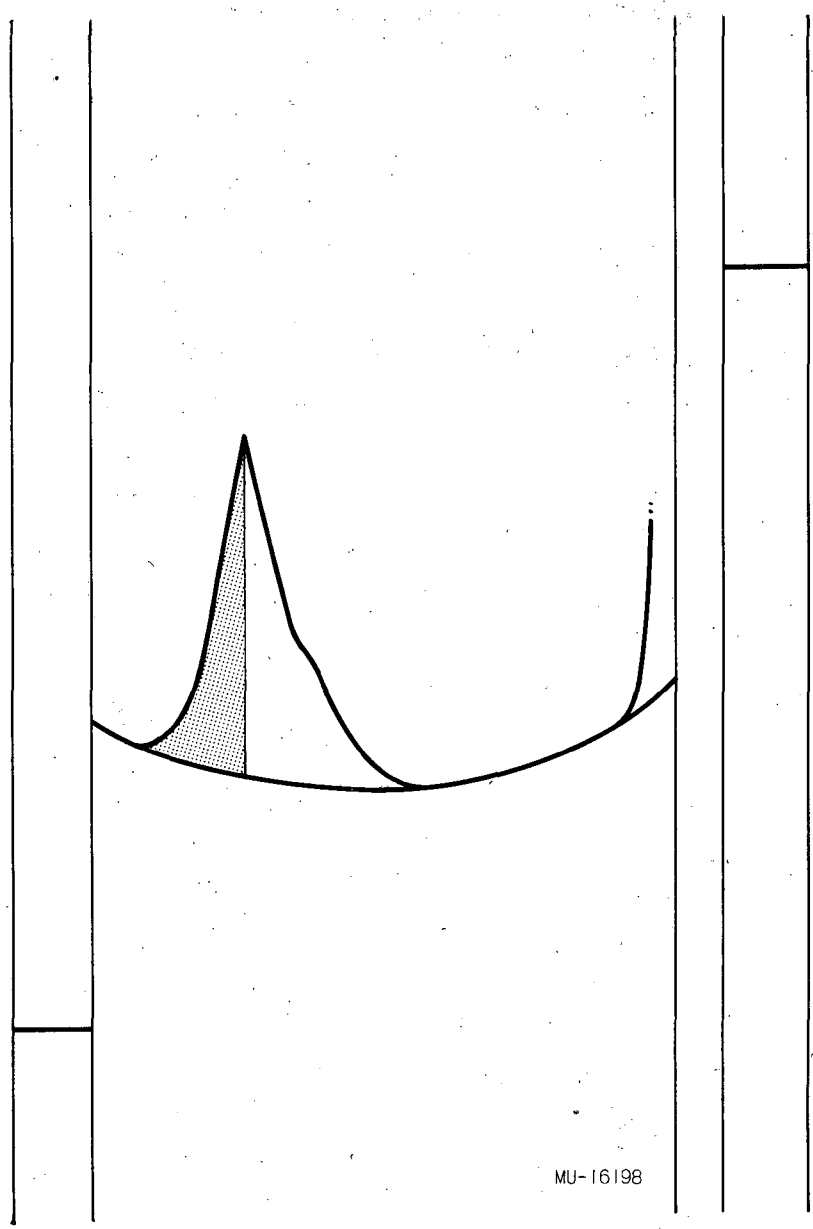


Fig. 24. An enlarged tracing of the 64-minute UTS exposure of Fig. 9d. The total area represents the actual concentration of P_2 and P_3, known electrophoretically as gamma globulin and albumin. The dotted area represents $\frac{1}{2}$ P_3.

species. However, for a more accurate determination of the S rates of the high-density lipoproteins and the proteins, one must undertake the task of isolating these molecules and perform sedimentation-versus-concentration studies on them. Such a study is currently under way, and although it may appear to be a simple procedure, it is quite difficult to isolate all the species--especially the P_1, which on the basis of unpublished evidence seems to be affected the most by such a phenomenon.

(b) The Johnston-Ogston correction is not needed for HDL_1, HDL_2, and HDL_3 and P_1 since the numerical factor involved is estimated to be smaller than the uncertainty in measuring the area. However, a small but significant correction is applied to the P_2 + P_3 proteins. Formula (7) in Reference 4, as derived by Gofman for use in the low-density lipoprotein analysis, was changed as follows for use in the protein analysis.

That formula was originally written as

$$\Delta C = \frac{RC_f^{meas} \; C^{\beta}_s}{N + RC_f^{meas}} \; ; \qquad (3)$$

where ΔC is the Johnston-Ogston correction, by which one finds

$$\text{true } C_f = C_f^{meas} + \Delta C,$$

$$\text{true } C_S = C_S^{meas} - \Delta C;$$

and C_f^{meas} is the measured concentration of the faster of the two components,

C_S^{β} is the measured concentration of the slower of the two components,

$R = kS_S^0$ (k is the constant of the sedimentation-versus-concentraction dependence relation and S_S^0 is the migration rate, at infinite dilution, of the slower migrating species),

and $N = S_f^{\alpha} - S_S^{\beta}$ (S_f^{α} is the measured migration rate of the faster-migrating species and S_S^{β} is the measured migration rate of the slower-migrating species).

The adjustments to Eq. (3) above for use in the protein analysis involved the following assumptions:

(i) S_f^{α} and S_S^{β} are fairly constant, therefore N is constant. S_f^{α} is taken to be 6.0 ± 0.2, and S_S 4.0 ± 0.2, therefore $N = 2.0 \pm 0.4$.

(ii) A second assumption was made on the basis of a sedimentation-versus-concentration study performed on a mixture of $P_2 + P_3$, which gave a k value of 9.3×10^{-4} mg%$^{-1}$, yielding an S_S^0 of 4.4; therefore $R = k\, S_s^0$ or 4.1×10^{-3}. And now Eq. (3) becomes

$$\Delta C = \frac{(4.1 \times 10^{-3})(C_f^{meas})(C_S^{\beta})}{2.0 + (4.1 \times 10^{-3})(C_f^{meas})}, \qquad (4)$$

where C_f^{meas} = measured P_2,

C_S^{β} = measured P_3.

Equation (4) can be reduced further (if C_f^{meas} of the denominator is considered to be constant at the mean of a normal population) to

$$\Delta C = \frac{(P_2^{meas})(P_3^{meas})}{500} \quad . \qquad (5)$$

The P_2 (or C_f^{meas}) in the protein analysis varies between 5 and 25 in more than 95% of the cases; therefore the assumption made above that it is constant is justified, since without it the formula would be reduced to

$$C = \frac{(P_2^{meas})(P_3^{meas})}{488 + P_2^{meas}} \quad , \qquad (6)$$

which will not yield a ΔC much different from that in Eq. (5).

(c) The area measured to represent a macromolecular species really represents the concentration of that macromolecular species in the plateau region (see Fig. 15b) as measured in the boundary region. The area thus measured must, as is usual in ultracentrifugal

practice, be corrected for position in the sector-shaped cell due to the radial concentration effects (as in the case of a floating boundary) or for radial dilution effects (as in the case of a sedimenting boundary) and the inhomogeneous centrifugal field, as shown in

$$C_i = C_m (R_a/R_0)^2, \qquad (7)$$

where C_i = initial concentration of macromolecules in the solution,

C_m = measured concentration, determined from $\int(dn/dx)dx$ over the boundary region,

R_0 = distance from center of rotation to the origin of the boundary (the cell base in the case of floating boundaries and the solution meniscus in the case of sedimenting boundaries), and

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

(d) The adjusted concentration of the serum sample is recorded to serve as a reminder that such an adjustment is necessary for these analyses. In standard preparative runs Type 2 and Type 3, 3 ml of serum is mixed with 3 ml of diluent and centrifuged, after which the desired lipoproteins, which have previously been in three volumes, are concentrated into one volume, and the 1 ml is quantitatively removed. A threefold concentration effect has been achieved through such an analysis, therefore the area representing the concentration in the analytical cell must be divided by 3 to represent the actual concentration in the serum of the subject; further, in the protein preparation the actual concentration is 1/10 of the original serum concentration, therefore the area as obtained from the tracing should be multiplied by 10 to yield the actual serum concentration. This correction is made in Section 4 below.

(e) Correction for temperature (which affects the viscosity of the solution) is important for S-rate consideration; the magnitude of this effect per degree must be known. We have eluded this problem by performing all our analytical ultracentrifuge analyses at $26^{\circ} \pm 0.4^{\circ}C$, and therefore have standardized all the reading methods to $26^{\circ}C$.

4. Conversion of Area Measurements from mm^2 to mg%

After the above corrections have been made, the measured areas can now be converted to concentration units by using a formula derived by Pickels,[20]

$$C = \frac{A \tan \theta \, 1000}{LTMmNE^2\Delta n}, \qquad (8)$$

where C = concentration in mg% in the original serum sample,

A = area in mm^2 of the measured macromolecular species, $[\int (dn/dx)\, dx]$ (corrected both for radial effects and for Johnston-Ogston effects if applicable),

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

L = optical-level area (distance along the optical path from the center of the condensing lens above the rotor to the diagonal wire, in mm),

M = magnification of the cell height (magnification by the camera lens system),

m = magnification by the cylindrical lens system,

N = factor by which the lipoproteins or proteins have been concentrated in the preparative procedure,

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

and Δn = specific refractive increment for the macromolecular species encountered in the particular solution medium. Table I shows the values of Δn for the various macromolecular species as obtained in our laboratory, for use to convert to mg% the areas measured in our standard analyses.

Table I

Specific refractive increments of lipoporteins and proteins in their respective preparative solutions

Macromolecular species	Specific refractive increment (Δn)	Preparative run type (see text)
All low-density lipoproteins	0.00154 $(g/100\ ml)^{-1}$	1
HDL_1 and HDL_2	0.00169	2
HDL_3	0.00154	3
P_1, P_2, and P_3	0.00185	derived from 3 (see text)

IV. DATA, CALCULATIONS, AND RESULTS

The ultimate understanding of lipid-transport systems in biology requires knowledge of the population distributions of both the low-density lipoproteins and the high-density lipoproteins. Of equal importance for protein metabolism are the population distributions of the serum proteins. Through utilization of the method described in this text it was possible to obtain the determinations leading to the data described below.

A. Biological Distributions

The mean serum concentrations, the standard deviations of the distributions, and the standard errors of the means of HDL_1, HDL_2, HDL_3, P_1, P_2, and P_3 on 2297 clinically healthy humans (1961 males and 336 females) of ages ranging from 17 to 65 years are shown in Tables II through VII. These data are grouped as follows:

Group 1	a, males b, females	17–29 yr.
Group 2	a, males b, females	30–39 yr.
Group 3	a, males b, females	40–49 yr.
Group 4	a, males b, females	50–65 yr.

On close examination of Tables II through VII, with emphasis on the groups comprised of 90 or more subjects, one finds the following interesting facts:

§ In contrast to Dr. Gofman's findings relating the age trend in the low-density lipoproteins,[11] it was not possible to prove that an age trend existed in the HDL_2 and HDL_3 levels in both sexes.

§ Although the HDL_1 levels appear to be rather constant with age in both sexes, it is possible to prove by a t test that the slight differences with age are significant.

§ A significant change in concentration with age can be demonstrated on the basis of the t test for P_1 and P_3 for both sexes

Table II

HDL_1 concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] HDL_1	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	23	7	0.3
2a	30-39	834	24	15	0.5
3a	40-49	399	25	15	0.8
4a	50-65	143	27	22	1.9
B, Females					
1b	17-29	190	21	7	0.5
2b	30-39	99	22	9	0.9
3b	40-49	37	23	5	0.9
4b	50-65	10	25	7	2.2

[a] All values in mg/100 ml

Table III

HDL_2 concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] HDL_2	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	37	28	1.1
2a	30-39	834	36	28	1.0
3a	40-49	399	37	28	1.4
4a	50-65	143	42	32	2.7
B, Females					
1b	17-29	190	80	41	3.0
2b	30-39	99	81	45	4.5
3b	40-49	37	89	53	8.8
4b	50-65	10	117	66	22.0

[a]All values in mg/100 ml

Table IV

HDL_3 concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] HDL_3	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	217	40	1.6
2a	30-39	834	219	42	1.5
3a	40-49	399	226	50	2.5
4a	50-65	143	224	51	4.3
B, Females					
1b	17-29	190	228	38	2.8
2b	30-39	99	235	38	3.9
3b	40-49	37	241	43	7.2
4b	50-65	10	270	54	18.0

[a]All values in mg/100 ml

Table V

P_1 concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] P_1	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	199	75	3.0
2a	30-39	834	169	64	2.0
3a	40-49	399	155	64	3.0
4a	50-65	143	152	61	5.0
B, Females					
1b	17-29	190	272	77	6.0
2b	30-39	99	218	79	8.0
3b	40-49	37	227	85	14.0
4b	50-65	10	198	51	17.0

[a]All values in mg/100 ml

Table VI

P_2 concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] P_2	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	1322	297	12
2a	30-39	834	1345	300	10
3a	40-49	399	1369	309	16
4a	50-65	143	1431	328	28
B, Females					
1b	17-29	190	1347	293	22
2b	30-39	99	1319	357	36
3b	40-49	37	1332	244	41
4b	50-65	10	1324	344	115

[a]All values in mg/100 ml

Table VII

P_3 concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] P_3	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	4717	480	20
2a	30-39	834	4634	459	16
3a	40-49	399	4572	488	25
4a	50-65	143	4442	507	43
B, Females					
1b	17-29	190	4618	544	40
2b	30-39	99	4629	559	57
3b	40-49	37	4465	445	74
4b	50-65	10	4348	510	170

[a]All values in mg/100 ml

and for P_2 in males only; this significance is beyond the 1% level for P_1, P_2, and P_3 in males and for P_1 in females, whereas the change with age of the P_3 concentration in females is only at the 5% level of significance. The trend in all cases is for the concentration in both P_1 and P_3 to decrease with age in both sexes, and to increase in P_2 with age in males.

§ There is a very distinct difference between the levels of HDL_2 and P_1 in males and females. The level of significance is less than 1% ($p < 0.01$). In both HDL_2 and P_1 the levels of significance for females are considerably higher than for the males.

§ There is no significant provable difference--at the 5% level of significance ($p = 0.05$), at least--between the males and females in the concentration of HDL_3.

§ The females have significantly lower levels of HDL_1 than the males (with $p < 0.01$) for only the 17–29-yr groups.

§ It is not possible to prove that the levels of P_2 and P_3 for females are any different from the levels for males, at least at the 5% level of significance ($p = 0.05$).

B. Lipoprotein and Protein Interclass Relationships

The low-density lipoprotein spectrum is completed with the data in Tables VIII through XIII. The components represented in these tables are discussed in greater detail in other papers.[8, 9, 11]

It is biologically important to know to what extent the factors involved in the control of the serum levels of lipoproteins and (or) proteins are alike and to what extent they differ. One approach to the evaluation of these relationships is through the Pearson product-moment correlation between the levels of any pair of components--either lipoproteins or proteins, or both. Such correlations are given in Tables XIV through XVII for the male groups and Tables XVIII through XIX for the female groups.

The correlation coefficients (Pearson r values) for each high-density lipoprotein and each protein on all other macromolecular components mentioned in this text for the various groups of

Table VIII

S_f^0 0–12 lipoprotein concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] S_f^0 0–12	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	322	86	3.6
2a	30-39	834	355	84	2.9
3a	40-49	399	380	84	4.2
4a	50-65	143	383	75	6.3
B, Females					
1b	17-29	190	283	68	4.9
2b	30-39	99	324	86	8.6
3b	40-49	37	346	67	11.2
4b	50-65	10	437	40	13.3

[a]All values in mg/100 ml

Table IX

S_f^0 12-20 lipoprotein concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] S_f^0 12-20	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	40	21	0.9
2a	30-39	834	51	23	0.8
3a	40-49	399	57	23	1.2
4a	50-65	143	56	24	2.0
B, Females					
1b	17-29	190	30	16	1.2
2b	30-39	99	41	22	2.3
3b	40-49	37	42	21	3.5
4b	50-65	10	93	36	11.9

[a]All values in mg/100 ml

Table X

S_f^0 20–100 lipoprotein concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] S_f^0 20–100	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	75	41	1.7
2a	30-39	834	91	54	1.9
3a	40-49	399	107	66	3.3
4a	50-65	143	103	58	4.9
B, Females					
1b	17-29	190	44	29	2.1
2b	30-39	99	51	36	3.7
3b	40-49	37	65	51	8.5
4b	50-65	10	77	48	16.1

[a]All values in mg/100 ml

Table XI

S_f^0 100–400 lipoprotein concentrations in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] S_f^0 100–400	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	37	43	1.8
2a	30-39	834	51	64	2.2
3a	40-49	399	66	91	4.6
4a	50-65	143	58	70	5.9
B, Females					
1b	17-29	190	9	14	1.0
2b	30-39	99	13	17	1.7
3b	40-49	37	18	24	4.1
4b	50-65	10	32	37	12.4

[a]All values in mg/100 ml

Table XII

Atherogenic Indices in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] A. I.	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	59	20	0.8
2a	30-39	834	69	24	0.8
3a	40-49	399	78	29	1.4
4a	50-65	143	76	26	2.2
B, Females					
1b	17-29	190	43	12	0.9
2b	30-39	99	51	18	1.8
3b	40-49	37	56	19	3.1
4b	50-65	10	79	22	7.4

[a]All values in Atherogenic Index (A. I.) units (See references 8, 9, and 11.)

Table XIII

Cholesterol levels in clinically healthy humans as a function of age

Group	Age (yr)	No. of subjects	Mean[a] cholesterol	S. D. of distribution	S. E. of means
A, Males					
1a	17-29	585	206	43	1.8
2a	30-39	834	226	44	1.5
3a	40-49	399	240	46	2.3
4a	50-65	143	239	40	3.4
B, Females					
1b	17-29	190	193	35	2.5
2b	30-39	99	213	43	4.4
3b	40-49	37	227	40	6.7
4b	50-65	10	301	29	9.6

[a]All values in mg/100 ml

Table XIV

Pearson product-moment correlations for lipoproteins and proteins of human serum for Group 1a

Males 17-29 yr of age (N = 803)
r = 0.07 for 5% level of significance
r = 0.09 for 1% level of significance

	S_f^0 0-12	S_f^0 12-20	S_f^0 20-100	S_f^0 100-400	A. I.	HDL_1	HDL_2	HDL_3	P_1	P_2	P_3	Cholesterol
HDL_1	0.21	-0.03	-0.03	0.04	0.09							0.17
HDL_2	-0.27	-0.25	-0.28	-0.21	-0.33	-0.17						-0.09
HDL_3	0.01	-0.01	-0.12	-0.07	-0.07	-0.01	0.30					0.14
P_1	-0.04	-0.08	-0.05	-0.05	-0.06	0.00	0.11	-0.07				-0.01
P_2	-0.07	-0.09	-0.03	0.00	-0.06	0.05	-0.03	-0.02	0.01			-0.04
P_3	0.03	-0.02	-0.03	-0.01	0.00	0.02	-0.03	0.00	0.21	-0.25		0.05

Table XV

Pearson product-moment correlations for lipoproteins and proteins of human serum for Group 2a

Males 30-39 yr of age (N = 562)
r = 0.08 for 5% level of significance
r = 0.11 for 1% level of significance

	S_f^0 0–12	S_f^0 12–20	S_f^0 20–100	S_f^0 100-400	A. I.	HDL_1	HDL_2	HDL_3	P_1	P_2	P_3	Cholesterol
HDL_1	0.06	0.02	0.22	0.27	0.23							0.15
HDL_2	-0.20	-0.17	-0.25	-0.21	-0.29	-0.08						-0.06
HDL_3	-0.03	0.02	-0.13	-0.14	-0.12	-0.02	0.36					0.08
P_1	0.03	0.01	-0.04	-0.04	-0.02	0.03	0.05	-0.06				0.06
P_2	-0.08	-0.02	0.03	0.05	0.01	0.06	-0.01	-0.07	0.06			-0.02
P_3	0.05	0.01	0.02	0.02	0.04	-0.01	0.06	0.04	0.19	-0.20		0.10

Table XVI

Pearson product-moment correlations for lipoproteins and proteins of human serum for Group 3a

Males 40-49 yr of age (N = 387)
r = 0.09 for 5% level of significance
r = 0.14 for 1% level of significance

	S_f^0 0-12	S_f^0 12-20	S_f^0 20-100	S_f^0 100-400	A. I.	HDL_1	HDL_2	HDL_3	P_1	P_2	P_3	Cholesterol
HDL_1	-0.01	-0.04	0.15	0.41	0.28							0.12
HDL_2	-0.25	-0.27	-0.26	-0.18	-0.31	-0.05						-0.05
HDL_3	-0.10	-0.04	-0.18	-0.15	-0.19	-0.04	0.41					0.06
P_1	-0.02	-0.04	0.00	-0.05	-0.04	-0.03	0.16	-0.05				0.02
P_2	0.02	-0.06	0.04	0.03	0.03	-0.08	-0.03	-0.02	0.02			0.08
P_3	-0.01	0.03	0.03	0.03	0.03	0.01	0.05	0.05	0.26	-0.27		0.03

Table XVII

Pearson product-moment correlations for lipoproteins and proteins of human serum for Group 4a

Males 50-65 yr of age (N = 140)
r = 0.16 for 5% level of significance
r = 0.22 for 1% level of significance

	S_f^0 0–12	S_f^0 12–20	S_f^0 20–100	S_f^0 100–400	A. I.	HDL_1	HDL_2	HDL_3	P_1	P_2	P_3	Choles-terol
HDL_1	-0.01	-0.02	0.31	0.55	0.38							0.24
HDL_2	-0.26	-0.25	-0.36	-0.29	-0.39	-0.11						-0.05
HDL_3	-0.03	0.01	-0.16	-0.08	-0.11	0.04	0.42					0.24
P_1	0.21	0.18	0.11	0.02	0.14	0.08	0.02	-0.01				0.30
P_2	-0.11	-0.03	0.17	0.05	0.05	-0.10	-0.15	-0.07	0.01			0.01
P_3	0.13	0.20	0.09	0.14	0.17	0.07	0.00	0.17	0.30	-0.29		0.28

Table XVIII

Pearson product-moment correlations for lipoproteins and proteins of human serum for Group 1b

Females 17-29 yr of age (N = 188)
r = 0.14 for 5% level of significance
r = 0.19 for 1% level of significance

	S_f^0 0-12	S_f^0 12-20	S_f^0 20-100	S_f^0 100-400	A. I.	HDL_1	HDL_2	HDL_3	P_1	P_2	P_3	Cholesterol
HDL_1	0.35	0.04	0.06	0.11	0.24							0.22
HDL_2	-0.09	-0.05	-0.27	-0.22	-0.21	-0.23						0.17
HDL_3	0.15	0.18	0.05	0.14	0.17	0.12	0.25					0.37
P_1	-0.10	-0.17	-0.04	-0.05	-0.12	-0.09	0.06	-0.01				-0.06
P_2	-0.03	0.17	0.13	0.24	0.12	0.01	-0.14	-0.05	0.01			0.01
P_3	0.14	-0.05	-0.11	-0.15	-0.01	0.09	0.03	0.25	0.36	-0.32		0.13

Table XIX

Pearson product-moment correlation for lipoproteins and proteins of human serum for Groups 2b and 3b combined

Females 30-49 yr of age (N = 132)
r = 0.17 for 5% level of significance
r = 0.23 for 1% level of significance

	S_f^0 0–12	S_f^0 12–20	S_f^0 20–100	S_f^0 100–400	A. I.	HDL_1	HDL_2	HDL_3	P_1	P_2	P_3	Cholesterol
HDL_1	0.24	0.12	0.15	0.33	0.26							0.25
HDL_2	-0.17	-0.18	-0.30	-0.32	-0.29	-0.21						0.07
HDL_3	-0.02	0.03	0.08	0.10	0.05	-0.03	0.27					0.19
P_1	0.00	-0.02	-0.07	-0.04	-0.04	0.04	0.12	-0.11				0.04
P_2	-0.04	0.01	0.02	0.09	0.01	-0.06	-0.03	0.05	0.17			-0.03
P_3	0.12	0.07	0.07	0.05	0.11	0.04	0.05	0.18	0.20	-0.11		0.18

both sexes are found in Tables XIV through XIX. (It was necessary to combine Groups 2b and 3b of the female population in order to increase the number of subjects; because there were only 10 females in Group 4b, no calculations were made for the correlation coefficients for this group.

For convenience, Tables XIV through XIX include the values of the correlation coefficients for the 5% level of significance ($p = 0.05$) and the 1% level ($p = 0.01$) for the number of degrees of freedom represented by each group.

The following results are obtained from the data of the above-mentioned tables:

1. HDL_1 versus the Low-Density Lipoproteins, the Atherogenic Index, and Cholesterol

A moderate positive correlation of the HDL_1 class with S_f^0 0–12 is seen in only the young males (17–29 yr) and in females 17–49 years; the p value is less than 0.01. No significant relationships were found, at least at the $p = 0.05$ level, between HDL_1 and S_f^0 12–20. The relationship between HDL_1 and S_f^0 20–100 and S_f^0 100–400 becomes increasingly stronger with age in both sexes, but with no significant correlation in 17–29-yr-old males and females. This relationship is positive and of the order of $p \ll 0.01$. The Atherogenic Index would therefore be expected to be significantly correlated with HDL_1 in the positive direction, since the AI is directly calculated from the results of the four low-density lipoprotein classes. The correlation of HDL_1 versus the AI is seen in all groups and at the $p < 0.01$ level. Since the low-density lipoproteins carry part of the serum cholesterol as a moiety, there is thus demonstrated a positive significant correlation between HDL_1 and cholesterol in all groups.

2. HDL_1 versus the High-Density Lipoproteins

HDL_1 shows a moderate negative correlation with HDL_2 in males and females through ages 39 yr for males and 49 yr for females. In the 17–29-yr male and female groups we find $p \ll 0.01$, and in the 30–39-yr males and 30–49-yr females, $p \approx 0.05$. No significant

correlation, at least at the $p = 0.05$ level, was found between HDL_1 and HDL_3.

3. HDL_1 versus the Serum Proteins

No significant correlation, at least at the $p = 0.05$ level, was found for HDL_1 with any protein component.

4. HDL_2 versus the Low-Density Lipoproteins, the Atherogenic Index, and Cholesterol

There is seen to be a significant inverse correlation between the HDL_2 class and all the low-density lipoproteins in all groups except in 17–29-yr females. There is no correlation between HDL_2 and S_f^0 0–12 or S_f^0 12–20 lipoproteins in these young females. The inverse relationship for HDL_2 versus S_f^0 0–12 and S_f^0 12–20 is on the order of $p \ll 0.01$ in all the male and female groups, except for the older females, where $p \simeq 0.05$. The Atherogenic Index would naturally follow in the same direction, since it grows out of the low-density lipoprotein levels. HDL_2 versus cholesterol showed a significant inverse relationship in 17–29-yr males ($p < 0.01$), and a positive one in the 40–49-yr males ($p = 0.05$).

5. HDL_2 versus HDL_1 and HDL_3

HDL_2 is inversely related to HDL_1, as indicated in B2 above. There is a strong direct correlation between HDL_2 and HDL_3 in all groups of both sexes, with $p \ll 0.01$.

6. HDL_2 versus the Serum Proteins

In 17–29- and 40–49-yr males HDL_2 is positively related to P_1, with $p < 0.01$, and in 17–29-yr females HDL_2 is inversely related to P_2, with $p = 0.05$. No other significant values, at least at the 5% level, were found between HDL_2 and the serum proteins.

7. HDL_3 versus the Low-Density Lipoproteins, the Atherogenic Index, and Cholesterol

In 40–49-yr males and 17–29-yr females there is a relationship between HDL_3 and S_f^0 0–12 which is negative for males and positive for females, with $p \simeq 0.03$. Only one group showed a low-order positive relationship ($p \simeq 0.03$) between HDL_3 and S_f^0 12–20; it was the 17–29-yr

female group. In all the male groups there is a moderate inverse relationship between HDL_3 and S_f^0 20–100, with $p < 0.01$. In the 30–39-yr and 40–49-yr male groups this relationship is repeated for the HDL_3 versus S_f^0 100–400. The 17–29-yr male group shows a low-order inverse correlation between HDL_3 and S_f^0 100–400, with $p = 0.05$. However, the 17–29-yr female group had a low-order positive correlation between HDL_3 and S_f^0 100–400, with $p = 0.05$--just opposite that of the male groups. The relationship of HDL_3 versus Atherogenic Index would follow along the same direction as in the low-density findings. There is a moderate positive correlation between cholesterol and HDL_3 in the 17–29-yr males and females, and in the 50–65-yr males, with $p < 0.01$. In the 30–39-yr males and the 30–49-yr females this positive correlation is only of low order, with $p \approx 0.05$.

8. HDL_3 versus HDL_1 and HDL_2

These data are shown under B 2 and B 6 respectively.

9. HDL_3 versus the Serum Proteins

There is a low-order inverse correlation between HDL_3 and P_1 in the 17–29-yr male group with $p = 0.05$. In the 30–49-yr female group HDL_3 is directly related to P_3, with $p < 0.01$, and the positive correlation with P_3 is also seen in the 50–65-yr males and the 30–49-yr female group, but of low-order significance, where $p \approx 0.03$. There were no other significant findings, at least at the $p = 0.05$ level, between HDL_3 and the serum proteins.

10. P_1 versus the Low-Density Lipoproteins, the Atherogenic Index, and Cholesterol

There is a direct correlation between P_1 and S_f^0 0–12 lipoproteins in the male group 50–65 yr of age, with $p \approx 0.03$. In the 17–29-yr-old male and female groups a low-order inverse relationship is noticed, with $p \approx 0.03$. There were no other significant findings, at least at the $p = 0.05$ level, between P_1 and the low-density lipoproteins and Atherogenic Index. In the 50–65-yr male group there was a direct correlation between P_1 and cholesterol, with $p < 0.01$.

11. P_1 versus the High-Density Lipoproteins

These data are found above in Sections B 3, B 7, and B 10 for HDL_1, HDL_2, and HDL_3 repsectively.

12. P_1 versus P_2 and P_3

In the 30–49-yr female group there is a low-order direct correlation between P_1 and P_2, with $p = 0.05$. The 17–29-yr male and female groups and the 30–39-yr males and females show a moderate positive correlation between P_1 and P_3. The level of significance is found to be $p < 0.01$ for the groups mentioned, except for 30–49-yr females, with $p \simeq 0.03$.

13. P_2 versus the Low-Density Lipoproteins, the Atherogenic Index, and Cholesterol

For P_2 versus S_f^0 0–12 only the 17–29-yr and 30–39-yr male groups showed a low-order inverse relationship, with $p = 0.05$. In the 17–29-yr males P_2 was found to be correlated inversely with S_f^0 12–20, with $p = 0.01$; and in 17–29-yr-old females P_2 was found to be directly related, with $p \simeq 0.03$. The 50–65-yr male group showed a positive correlation between P_2 and S_f^0 20–100, with $p \simeq 0.03$. In the 17–29-yr female group there was a moderate direct relationship between P_2 and S_f^0 100–400, with $p \lesssim 0.01$. The Atherogenic Index and the cholesterol failed to show any significance with P_2, at least at the $p = 0.05$ level.

14. P_2 versus the High-Density Lipoproteins

These data are found above in Sections B 3, B 7, and B 10 for HDL_1, HDL_2, and HDL_3 respectively.

15. P_2 versus P_1 and P_3

The data for P_2 versus P_1 are found in Section B 13 above. There is a moderate inverse relationship between P_2 and P_3 for the 17–29-yr male and female groups, and for the 30–39-yr, the 40–49-yr, and the 50–65-yr male groups. All groups have levels of $p < 0.01$.

16. P_3 versus the Low-Density Lipoproteins, the Atherogenic Index, and Cholesterol

In the 17–29-yr females there is a low-order positive relationship between P_3 and S_f^0 0–12 lipoproteins, with $p = 0.05$. In the 50–65-yr

males there is a low-order positive correlation between P_3 and S_f^0 12–20 lipoproteins, with $p \approx 0.03$. A low-order negative correlation is seen between S_f^0 100–400 lipoproteins and P_3 in the 17–29-yr female group, with $p \approx 0.03$. The Atherogenic Index is positively correlated with P_3 in the 50–65-yr males, probably owing to the relationship between P_3 and S_f^0 12–20 of this group; the value for p is 0.05. In the 17–29-yr and the 50–65-yr males there is a moderate direct correlation between P_3 and cholesterol, with $p < 0.01$. This direct relation is also seen in the 30–49-yr females, but only of low-order significance, with $p \approx 0.03$.

17. P_3 versus the High-Density Lipoproteins

These data are found in sections B 3, B 7, and B 10 above for HDL_1, HDL_2, and HDL_3 respectively.

18. P_3 versus P_1 and P_2

These data are found in sections B 13 and B 16 above for P_1 and P_2 respectively.

V. DISCUSSION AND CONCLUSIONS

It is evident from the low magnitudes of the correlation coefficients that the metabolic factors involved in the regulation of the serum levels of any one of the lipoprotein or protein classes are largely independent of those involved in the regulation of the levels of either of the other lipoprotein or protein classes. There are, however, some interesting facts that have been obtained from the data in this text. They are best summarized as follows:

A. There is a remarkable constancy with age in both sexes for the ages 17 through 65, in the levels of HDL_2 and HDL_3. This important fact suggests--though with no certainty--that the so-called "alpha" lipoproteins, which are really part of the high-density lipoprotein classes (mostly HDL_2 and HDL_3), are not a protective agent against atherosclerosis. There is some literature following the school of thought that atherogenesis is a direct function of the ratio of the low-density lipoproteins to the high-density lipoproteins. This would infer that at a constant value for the low-density classes a decreasing concentration of high-density lipoprotein is representative of an increased risk of developing overt coronary disease. It is obvious from the mean levels of HDL_2 and HDL_3 that they remain rather constant throughout the major part of the human life span. (Incidentally, there is strong evidence that coronary disease can and does occur at normal levels of HDL_2 and HDL_3.[21]) Since it was not possible to prove any significant relationship between age and HDL_2 or HDL_3, in both sexes from 17 to 65 yr of age and--as has been shown by Gofman--there is a distinct and direct age relationship in both sexes in the serum levels of the low-density lipoproteins, this evidence still supports the Gofman hypothesis that atherogenesis is a direct function of low-density lipoproteins (S_f^0 0–12, S_f^0 12–20, S_f^0 20–100 and S_f^0 100–400) over a period of time.[8, 9, 11]

B. The female lipoprotein and protein patterns are characteristically different from the male patterns throughout the age span covered in this text. The females have significantly higher concentrations of HDL_2 and P_1. It is possible that the fallacious concept that

"alpha" lipoproteins are protective came from the knowledge that females have more "alpha" lipoproteins than males and less coronary disease than males. If this concept were correct then at about ages 50-55 yr--at which females start having coronary disease as frequently as males, and beyond which they have coronary disease more frequently than males--they should have lower levels of high-density lipoproteins. These data do not show such a trend; ; on the contrary there is some evidence that in the older males and females the serum levels of the high-density lipoproteins rise.[21] This will be very difficult to prove, since it is not likely that a clinically healthy population large enough in number between ages 50 and 80 is readily available and randomly collectable.

C. The levels of P_1 and P_3 decrease significantly with age in both sexes for the age span covered in this text, and it is not possible to prove at better than the 5% level of significance that such an age trend is seen in the P_2 levels of either sex. The interesting point here is that although P_2 and P_3 are significantly inversely correlated, the former remains fairly constant with age while the latter decreases with age in both sexes.

D. It is interesting to note that HDL_1 is positively correlated with S_f^0 0–12 in young males and females, since these subjects are characterized by reasonably low levels of S_f^0 20–400. As the level of S_f^0 20–400 increases as a function of age (and this is seen in Tables X and XI), then HDL_1 becomes more significantly related as the S_f^0 20–400 increases while HDL_1 itself increases slightly in concentration. This observation is related to the clinical state known as idiopathic (or essential) hyperlipemia. These subjects are characterized by a unique and characteristic lipoprotein disorder[11] in which HDL_1 is grossly increased, and so are the S_f^0 20–400 lipoprotein classes, while S_f^0 0–12 is significantly lowered. It is also interesting to note that HDL_1 is correlated inversely with HDL_2 in only the young males and females characterized by low levels of S_f^0 20–400. These groups, as seen earlier, had positive correlations between HDL_1 and S_f^0 0–12. It will

be seen below that HDL_2 is inversely correlated with S_f^0 0–12, which is in agreement with the above findings. Why the HDL_1 remains significantly and directly correlated with S_f^0 0–12 lipoproteins in females only and falls off beyond age 29 in males can not be explained at this time. It could possibly be related to the changes that occur during the idiopathic hyperlipemic-type shift mentioned above. The S_f^0 20–400 levels in males 30–39 yr has already reached 142 mg%, and this figure increases to 173 mg% in the next age decade, but in the 40–49-yr females the S_f^0 20–400 lipoprotein concentrations have only reached 83 mg%. The difference between 142 mg% and 83 mg% is statistically significant beyond the $p = 0.01$ level.

E. In general, the HDL_2 class is significantly correlated inversely with all the low-density lipoproteins in both sexes and in all groups. This relationship is at the $p \ll 0.01$ level. The only exceptions are in the 17–29-yr females, where it was not possible to prove at the $p = 0.05$ level that HDL_2 is related to either S_f^0 0–12 or S_f^0 12–20. In the 30–49-yr females, for HDL_2 versus either S_f^0 0–12 or S_f^0 12–20, the levels of significance were found to be $p = 0.05$ and $p = 0.03$ respectively. Perhaps an explanation of the lack of correlation between HDL_2 and S_f^0 0–12 or S_f^0 12–20 in 17–29-yr females only is of metabolic origin, but at present it can not be seen with these data.

F. A very interesting phenomenon is the high positive correlation between HDL_2 and HDL_3 that is seen in all age groups and both sexes. These relationships are significant at the $p \ll 0.01$ level, and are very similar to the findings by Gofman that correlations between two lipoprotein classes are highest when the two classes being tested are most closely related ultracentrifugally.[11] With the exception of some clinical states, wherever either HDL_2 or HDL_3 is elevated, it is more than likely that the other will also be elevated, and conversely, whenever either HDL_2 or HDL_3 is lowered it is more than likely that the other will also be lowered.[11, 21]

G. Of equal interest is the definite negative correlation of HDL_3 with S_f^0 20–400 in all the age groups in the males but not the females.

This is what was found, as mentioned above, when S_f^0 20–400 was correlated with HDL_1 but the direction was positive. The interesting fact here is that when HDL_1 is tested with HDL_3 no significant relationship is found. This may tend to suggest independent metabolic functions for HDL_1 and HDL_3, but a Pearson product-moment correlation is not to be taken as a test for such findings. On the contrary, when one looks at the individual values he is more likely to find that S_f^0 20–400, HDL_1, and HDL_3 are really very closely related metabolically. This sort of approach is being made now, and will certainly be more fruitful in seeking out relationships which tend to get hidden when one relies entirely upon the results of a correlation coefficient.

H. With the exception of a few scattered significant relationships between proteins and lipoproteins, there does not appear to be any reason to lean heavily in the direction that lipoproteins and proteins are relatedly involved in the same metabolic states. On the contrary, we have seen from unpublished observations that in many metabolic disorders characterized by grossly disturbed lipoprotein patterns the protein patterns are normal, and vice versa. But it is more than chance alone that P_2 and P_3 are related, since for every group except older females there has been a significant negative relationship between P_2 and P_3, at the $p << 0.01$ level. The same holds true for the P_1 and P_3 relationships. In this case even the older females show significant correlations for P_1 and P_3. The correlation coefficients for P_1 versus P_3 are all positive and are at the $p << 0.01$ level of significance in all but one group, which was at the $p = 0.03$ level. It is interesting to note that the P_1 was shown not significantly correlated with P_2 in all groups except the 30–49-yr females--which coincidentally was the only group in which P_2 versus P_3 did not show any significance, at least at the $p = 0.05$ level.

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