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Low density lipoproteins ultra centrifugal determination and use in predicting coronary artery disease

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LOW DENSITY LIPOPROTEINS
ULTRA CENTRIFUGAL DETERMINATION AND
USE IN PREDICTING CORONARY ARTERY DISEASE

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INTRODUCTION:

This is a discussion of the ultra centrifuge and especially its application to determination of serum lipoprotein concentrations and changes in concentration associated with various disease states. The chief interest in lipoproteins has been generated by the apparent relation between their serum concentration and development of coronary artery atherosclerosis, which leads to diminution or occlusion of coronary blood flow and to myocardial infarction. Gofman, at the University of California, has been a leader in the attempt to analyze this relationship, in coronary disease as well as in other disease states, and has interesting data to show prognostic and therapeutic application of the determination of certain lipoprotein constituents.

Historically, Machebeouf, in 1929, isolated from serum substances which contained reproducible proportions of nitrogen and lipids and therefore were called lipoproteins.[1] Oncley and his associates identified two major lipid containing fractions in serum, so-called alpha and beta lipoproteins.[2] It was shown that the alpha fraction consisted of approximately 35% lipid, and 65% protein, and that the beta fraction contained

about 75% lipid and 25% protein. In addition, it was demonstrated that essentially all of the serum cholesterol could be accounted for in one or another of these lipoprotein fractions. Since this observation, Gofman and his group have shown that after ultra-centrifugal removal of lipoproteins, essentially none of such lipids as cholesterol, phospholipid, and glycerylester remains in the serum.[3-9] Thus, a chemical determination of alpha lipoprotein or beta lipoprotein represents a combination of the lipoproteins present in serum which is greatly in excess of any two of the lipoprotein species; and, a measurement such as total serum cholesterol at best reflects the sum of the cholesterol contributions from various members of the lipoprotein spectrum and provides little information concerning which lipoproteins are present in serum, or their quantitative distribution. Similar considerations apply to other purely chemical serum liquid determinations, e.g., serum phospholipid, serum total lipid, etc.

At present, work in fractional determination of serum lipoproteins involves a quantitative separation of the lipoproteins from serum proteins by ultra-centrifugation of a serum sample in a medium, having

a density adjusted to provide for flotation of the lipoproteins of hydrated density lower than that of the medium and sedimentation of the serum proteins. This is followed by an analysis of the lipoproteins under specifically defined conditions of solution composition, density, and temperature. The determination is completed by comparing the migration rate of the boundaries which develop on exposing the solution to ultra-centrifugal forces for a given length of time, and allowing in the final numerical determination, for variations in flotation rate of the fractions which depend on variation in concentration of the separate groups.

It has been demonstrated by Svedberg[10] that a solution in an appropriately shaped cell can be subjected to high centrifugal forces to produce precisely measurable sedimentation of molecular particles. This cell is sector shaped, having walls on two sides which are flat and perpendicular to the axis of rotation, while the walls on the other two sides are flat, and if extended, would intersect along the axis of rotation. In this way, it is provided that particles originally close to any of the walls continue during sedimentation to pursue an average course,

parallel to the wall, and thus do not sediment against or away from the walls to set up convertive disturbances. Gofman's technique utilizes this physical phenomenon by making up a solution having greater density than that of the lipoproteins to be analyzed and noting the migration of these molecules toward the center of the axis of rotation.[3-12] He uses the term flotation, to describe this migration, and uses the S_f unit as measure.[5] This unit is related to the S unit of sedimentation (a Svedberg unit) which is equal to 10^{-13} cm/sec/dyne/gram of mass and describes the travel of the material toward the meniscus of the suspending solution rather than away from it.

The ultra-centrifuge most commonly used today for study of lipoprotein fractions is the Spinco Model L.[5] It is a rotor, capable of withstanding great forces, suspended on a flexible shaft, so as to be self balancing, in a chamber where vacuum and temperature control can be achieved, driven by electric power. Inserted into the rotor are cylindrical cell containers into which samples for analysis are placed between quartz windows. During the analysis, a light source is directed through the cell, is refracted and difracted by the solution, directed by a condensing

lens to a diaphragm with an inclined slit, through this to a camera lens. The use of the diaphragm with the inclined slit allows the conversion of a vertical deviation of light at the object plane, (plane of the unknown solution), into a horizontal displacement of of a point on a screen or film, without altering the vertical position of the point, which itself corresponds to a certain level in the object plane. Photographs are taken at given time periods and measurement of the areas under the peaks can be accomplished to determine the position of the boundaries, thus measuring concentrations of the various constituents of the sample.

The technique of DeLalla and Gofman[12] has been used for quantitative determination of the low density lipoprotein fractions. With this technique, 5cc of serum are mixed with 4 cc of a NaCl solution having a specific gravity of 1.1315. This mixture is spun at 30,000 rpm for 13 hours at 20 C. At the end of this time, the top 1 cc. of the solution contains all the "low density lipoproteins" and includes the chylomicrons present. This amount is removed and remaining in the tube is the suspending solution, the albumins, globulins, and high density lipoproteins.

Eight (0.8) cc's of this top layer is placed in the ultracentrifuge cell described above and studied optically while being spun at 52,640 rpm. Six photographs are taken over a period of 38 minutes - one at 0 minutes, (time of attaining a speed of 52,640 rpm), at 6", 12", 22", 30", and 38". Gofman has found that chylomicrons have an S_f 40,000 flotation rate - i.e., they float so fast that their concentration cannot be determined ultra-centrifugally. In addition, he noted the presence of other proteins with flotation rates S_f0 to S_f400. He has arbitrarily divided these into groups S_f 0-12, S_f 12-20, S_f 20-100, and S_f 100-400. The photograph at 0 minutes is used to analyze the S_f 100-400 fraction; that at 6 minutes is used to analyze the S_f 20-100 fraction - the boundary formed by the 100-400 group having passed off the field by this time. At 30 minutes the boundary of the 20-100 fraction has passed off the screen and this photograph used to measure the 0-12 and 12-20 fractions.

The calculation of the various lipoprotein concentration includes consideration of the self slowing of flotation rate with increasing concentration,[12-13] and distortion of the boundary occurring during ultracentrifugation[14]. Values obtained using these corrections

have been denoted by Gofman and his group as Standard flotation rates.[15]

Using this technique, Gofman and co-workers have fractionated serum lipoproteins in over 70,000 persons attempting to present a cross sectional representation of the population.[16] In general, their work shows a general rise in concentration of all the lipoprotein groups with advancing age.[17] This rising trend with age continues in the female into the seventh decade. In the male, all the measured classes reach a maximum mean value in the fifth or sixth decade of life and then show a decrease in mean concentration in the sixth or seventh decades. The decimation of the male population by coronary heart disease and the relationship between the occurrence of this disease and serum lipoprotein levels account, in part, for the decreasing rate of increase in concentration with increase in age; however, Glazier "et al"[17] mention in published observations indicating the existence of an additional factor of a metabolic nature which accounts for the male trend in lipoprotein levels. While there is no significant difference in lipoprotein levels of 0-12 and 12-20 groups in the sexes in the first two decades of life, between twenty and fifty years of

age, the level of those molecules included in the Standard S_f 12-20 group in makes is significantly higher than in females of the same age. Significant by greater concentration of the Standard S_f 0-12 group exists between ages 20 and 60, and through the seventh decade levels of Standard S_f 12-20 are significantly higher in women. Concerning those lipoproteins included in the Standard S_f 20-100 and 100-400 group, the sex difference found beginning in the second decade and persists until the sixth decade at which time the concentrations are about equal.

Also, it was found that in samples taken in a non-fasting state, the Standard S_f 100-400 fractions elevated while the Standard S_f 12-20 and 20-100 groups showed little change as compared with samples taken fasting. The concentration of Standard S_f 0-12 is higher in the fasting state as compared to non-fasting. Thus, it appears that the lipoproteins of high flotation rates are concerned in early metabolism of exogenous food stuffs; those of lower flotation rates are involved in a later phase of food metabolism.[17]

Gofman [18] investigated in one series 808 non-coronary patients and 204 post-coronary patients - selected with definite criteria to omit those with other overt disease

associated with their atherosclerosis (e.g., xanthomatosis), or metabolic upset secondary to myocardial damage (a lapse of two months was required.) He found significant elevation in concentration of Standard S_f 0-12 and Standard S_f 12-400 concentration in the post-coronary patients of those from 40-50 and 50-60 years of age as compared with the non-coronaries, but was unable to demonstrate this in those persons 60-70 years. In another article[19] Gofman revealed a method of analysis of lipoprotein concentration based on a method of linear discriminant analysis[20] showing relative importance of the Standard S_f groups in development of atherosclerosis. He calls this result the Atherogenic Index or A.I. He found the concentration of Standard S_f 12-400 fractious, intrinsically 1.75 times as important as the concentration of Standard S_f 0-12 fractions, and his formula is A.I. equals - (mg/100ml Standard S_f 0-12) + (1.75 x mg/100ml Std. S_f 12-400)

The denominator 10 is used to yield more wieldy numbers. A comparison of the curve of A.I. of a group of individuals of various ages graphed against age and the curve of coronary atherosclerosis graphed against age shows a striking qualitative similarity in both sexes. Also, the ratio of male A.I. to female A.I. is

consistent with the ratio of degree of coronary atherosclerosis of males to females. Too, the finding of A.I. values in different individuals of the same age which vary widely in magnitude corresponds to the pathologic observation that severe atherosclerosis does not inevitably accompany chronologic agings, but may show wide variation from individual to individual at a single age. From this original work, Gofman made further refinements of his mathematical analysis using a large series[22-24] by which his A.I. formula was changed to Alpha = $\frac{(\text{Standard } S_f \text{ 0-12}) + (1.6 \text{ Standard } S_f \text{12-400})}{10}$ He has changed the value designation to alpha since it has significance whether or not it relates to atherosclerosis.

In search for a manner of interpretation of a comparison of alpha values at different ages which explains the increased incidence of coronary heart disease at advanced ages over what can be shown by mere comparison of mean lipoprotein levels Gofman determined what he calls the accumulated "coronary disease" of an individual, or A.C.D. In this determination an attempt is made to consider the time period over which a given alpha value has been operative. Since it has been found that a given person retains his relative position to

others of his age as concerns alpha value, a determination at a given age can be compared with the determined mean for his age and it can be assumed that he has had the same position from the mean in standard deviation units throughout his life. Thus the alpha value is convertible to A.C.D. value.

From studies of lipoprotein concentrations in new coronary patients, Gofman feels that this A.C.D. is convertible into an estimate of the probability or incidence of occurrence of new clinical coronary disease. This is done by comparing A.C.D. values of post-coronary persons with A.C.D. values of a representative cross-section of the population. The incidence of occurrence of a given A.C.D. in the cross section is compared to the incidence of coronary in those with the incidence of clinical coronary heart disease in persons having the given A.C.D. - the incidence rate of coronary disease for each A.C.D. can be estimated. There is good evidence that lipoprotein elevation in coronary disease precedes the inception of clinical disease.[23] Also in various metabolic disturbances such as the xanthomatoses, it is known that the lipoprotein elevation precedes the onset of clinical coronary disease.

By constructing a curve from A.C.D.'s of a given

population and incidence of coronary artery disease in that population the relative coronary disease incidence rate is plotted as a function of A.C.D. values. Then by assuming that mortality from coronary artery disease is proportional to the incidence rate of coronary disease this curve shows relative mortality rates from coronary disease in relation to A.C.D. value, and can be used to preduct occurrence of coronary disease in a given population. A comparison of predicted occurrence to the occurrence observed in Vital Statistics records was done and showed good agreement.[24] This test indicates that the A.C.D. values derived from serum lipoprotein estimation can be used to predict probability of coronary disease occurring in an individual.

With this basis Gofman has approached the problem of coronary disease from a preventive angle. He determined that ninety percent of the mortality from coronary disease is contributed by forty percent of the total population.[22] Measures which would decrease the lipoprotein concentration, therefore, could sig-nicantly lower coronary disease incidence most effectively if applied to this group and at an early age. The greater the reduction in alpha value, and the earlier it is achieved, the greater will be the corresponding decrease in mortality rate.

The relation of overweight to heart disease was shown by Dublin and Marks.[25] Gofman and his group compared elevation of alpha values to degree of overweight, i.e., ratio of actual weight to ideal weight in clinically well individuals and showed a positive correlation.[13] Then, from the mean alpha value of the group, the probability of coronary disease developing was found to be 1.7 times the probability for mean alpha value of those at ideal weight. The actuarial data showed an incidence increase of 1.5 times in persons 40% above ideal weight compared to persons of ideal weight.[25] Also Gofman "et al" showed that for certain individuals the reduction of dietary fat intake will definitely result in maintained lowering of alpha value as long as the individual adheres to the diet.[19]

The known association of myxedema, high serum, cholesterol, and early atherosclerosis led to investigation of the administration of thyroid extract and serial determination of serum lipoprotein concentration and fractional distribution.[26-27] These papers indicate an initial decrease in Standard S_f 0-12 and 12-20 fractions, in physically healthy persons on three grains of dessicated thyroid daily, followed by a gradual upward trend of Standard S_f 0-12 lipoprotein levels, beginning at about three weeks of the test to pre-thyroid level at about 24 weeks of the test, but persistence of a markedly lower level of Standard S_f 12-20

lipoprotein level at the end of 33 weeks. In another group placed on 10 grains per day, thyroid no significant trend upward was noted after nine weeks. In neither test was a lowering of the Standard S_f 20-100 fraction or the Standard S_f 100-400 fraction shown. When the serum levels of Standard S_f 0-12 lipoproteins had stabilized at pre-thyroid levels, increasing the dose to four grains per day, again reduced significantly the concentration of this fraction and the reduction reached its peak at about six weeks, after beginning increased dosage in contrast to the three week interval noted on beginning the test, no similar response of the 12-20 fraction was seen after the increased dose. The administration of the thyroid extract had its greatest effect in those in whom the serum lipoprotein levels of Standard S_f 0-20 fractions were highest before beginning the test.

In another series of hypothyroid and myxedematous patients, the Standard S_f 0-12 and 12-20 lipoproteins were found to be elevated and were strikingly reduced by thyroid administration.[28] From these data, it is concluded that in persons ostensibly euthyroid with elevated Standard S_f 0-12 serum, lipoproteins therapeutic trial with 3to 5 grains per day of thyroid extract may show significant response.[16]

Anti-coagulant therapy has been considered as possible prophylactic treatment for coronary disease. The work of Graham, "et al"[29] has shown an almost immediate response in that a decreased amount of the Standard S_f 12-400 lipoproteins is seen with an increase of Standard S_f 0-12 fraction. The shift is gradual with initial disappearance of Standard S_f 20-100 group and increase in Standard S_f 10-20 levels and increase in Standard S_f 0-12. Gradually over 24 hours this trend is reversed, but the level of Standard S_f 12-20 remains lower than initially. Graham and his co-workers also found an " active principle" in the globulin fraction of the serum in post-heparin plasma which cleared lipemia of plasma <u>in vitro</u> which heparin itself does not. He offers the possibility that a lack of heparin or some similar substance may be responsible for lack of normal inter-conversion of lipoproteins and a piling up of S_f 12-20 and S_f 20-100 lipoproteins results in such persons. It is pointed out that pre-selection of patients via lipoprotein determinations must be made, choosing persons with great probability of early development of coronary disease, and that if this is done the small risk of long term anti-coagulant therapy can be tolerated.[22] Disadvantages to this type of therapy are

expense and lack of orally effective heparin.

In addition to the value of serum lipoprotein determinations as a predictive tool in evaluation of coronary disease, the fractional analysis has been demonstrated to show characteristic patterns in other diseases of lipid metabolism.[28-30] These include diabetes mellitros, hepatitis, xanthoma tuberosum, xanthoma tendinosum, xanthelasma, nephrosis, biliary obstruction, myxedema, and "essential hyperlipemia". Although series concerned with these diseases are small at present, indications are that ultracentrifugal methods of serum lipoprotein concentration will have diagnostic import and in addition, can aid in understanding lipid metabolism.[28]

SUMMARY

This is a discussion of the ultracentrifuge and its application to low density serum lipoprotein determinations by the method of Donner Laboratories, University of California. The application of this technique to prediction of pre-clinical coronary disease (atherogenesis) is discussed, and a resume is given of methods now known to cause decrease in the atherogenic fractions.

The characteristic serum lipoprotein patterns associated with other disorders of lipid metabolism is noted.

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