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Authors
Gatto, Luigini Del
Lindgren, Frank T.
Nichols, Alex V.

Publication Date
1958-09-01

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Berkeley, California

Contract No. W-7405-eng-48

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Abstract

The purpose of this work was to develop a convenient method for the ultracentrifugal analysis of all classes of serum lipoproteins that would require a minimum of time, work, and materials.

The method utilizes the principle of flotation of lipoproteins in a medium of greater density than their own hydrated density. In this procedure the isolation and the analysis of lipoproteins are done in a NaBr medium of density 1.20 g/ml. The advantages of this procedure are compared with other available methods, and its application to studies on serum lipoprotein is discussed.

AN ULTRACENTRIFUGAL METHOD
FOR THE DETERMINATION OF SERUM LIPOPROTEINS[*]

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The importance of serum lipoproteins in relation to lipid transport and metabolism has repeatedly been emphasized. Thus far the ultracentrifuge has provided one of the most useful techniques for studying the serum lipoproteins. Gofman et al. were first to apply the basic principle of ultracentrifugal flotation to the study of lipoproteins.[3] Since this early work, several improvements and modifications of this ultracentrifugal methodology have been made.[2, 5, 6] Many laboratories have adopted the methods described by deLalla for complete serum lipoprotein analysis.[2] However, until now it has not been possible to make a quantitative study of the complete human lipoprotein system in one procedure requiring only two milliliters of serum.

Materials and Experimental Methods

Two milliliters of serum, 0.94 of NaCl solution of density $\rho_{20/4}$, of 1.0060 g/ml (which corresponds to the amount of this salt solution present in 1 ml of serum) and 3 ml of NaBr solution ($\rho_{20/4} = 1.3895 \pm 0.0005$ g/ml) are pipetted into a 6-ml lusteroid preparative tube and, after capping, thoroughly mixed. The background solution density of this mixture (exclusive of serum proteins, lipoproteins, and other macromolecular constituents) is $\rho_{20/4} = 1.2052 \pm 0.0005$ g/ml.

After ultracentrifugation (Spinco Model L ultracentrifuge) at 40,000 rpm for 24 hours at a temperature between 18 and 20° C, all the known classes of serum lipoproteins are concentrated in a 1-ml volume at the top of the preparative tube. The final density of this lipoprotein fraction is $\rho_{20/4} = 1.1977 \pm 0.0005$ g/ml (representing the average value and standard deviation of six determinations on one serum sample). The corresponding background NaCl and NaBr salt mixture, consisting of 2.82 ml of NaCl solution ($\rho_{20/4} = 1.0060$ g/ml) and 3 ml of NaBr solution ($\rho_{20/4} = 1.3895$ g/ml), centrifuged under the same

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[*]Paper submitted to Analytical Chemistry.

conditions, yielded a final density in the top milliliter of $\rho_{20/4}$ = 1.1965
± 0.0003 g/ml (six determinations). This drop in density from 1.2052 g/ml
to approximately 1.1965 g/ml reflects the approach toward sedimentation
equilibrium of the salts in the preparative tube that occurs during the
centrifugal conditions used for this lipoprotein isolation.

The concentrations and flotation rates of the various lipoproteins
present in the total lipoprotein fraction are determined in a Spinco Model E
analytical ultracentrifuge operating at 26° ± 0.5° C.

If desired, a total serum-protein (albumin, globulin, etc) ultracentrifugal
analysis may be carried out simultaneously. For this analysis the 5-ml
subnatant in the preparative ultracentrifuge tube (after thorough remixing)
is diluted with distilled H_2O in the ratio of 1 part subnatant to 3 parts H_2O.
The final density of this mixture is 1.0613 ± 0.0045 g/ml (six determinations).
A detailed analysis of this type of total serum protein analytical run will be
reported by de Lalla. Figure 1 shows the schlieren patterns of a typical
lipoprotein and protein analytical run.

Several different ultracentrifugal rotor speeds have been tested
(52,640; 50,740; 47,760; 44,770; 42,040; 39,460; 37,020 rpm). Successive
pictures have been taken at appropriate time intervals to allow complete
quantitative study of all the major serum lipoprotein classes, which under
these conditions have flotation rates of from 0 to 6 and 16 to 485 Swedberg
units.[8] At the recommended ultracentrifugal rotor speed of 42,040 rpm the
centrifugal field is 148,235 × g (at a radius of 6.50 cm). For this speed,
appropriate times for obtaining schlieren patterns are 122 seconds after
starting (during acceleration at approximately 22,000 rpm); 242 seconds
after starting (0 time up to speed) and at 2, 12, 14, 44, 64, and (or) 76
minutes after achieving full speed. Uniform acceleration is achieved by
maintaining a constant current (13-15 amp) with variac manipulation such
that full speed (42,040 rpm) is reached in 242 seconds. The proper amperage
value necessary will depend upon the characteristics of each individual drive
mechanism. In order to accurately measure lipoprotein concentrations in
this procedure, it is necessary to use a double-sectored analytical cell,[1]
into one sector of which is introduced a baseline solution equivalent to the
background solution ($\rho_{20/4}$ = 1.1965 g/ml). This is necessary because during
the analytic run there occur appreciable concentration-gradient changes of the
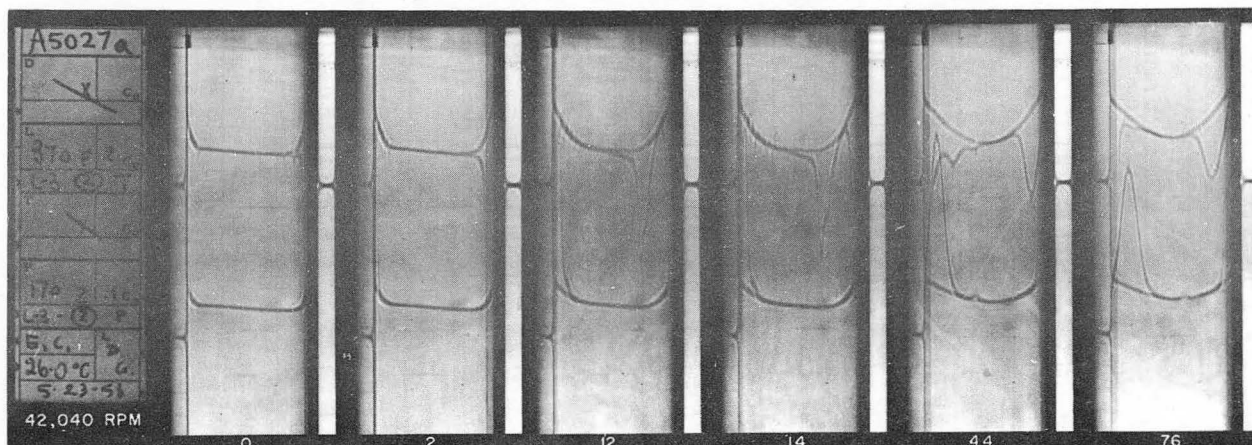
Fig. 1. A typical lipoprotein and protein ultracentrifugal run.

NaBr and NaCl salts, as well as rotor speed changes, which would frequently prevent accurate placement of the baseline. The analytical ultracentrifuge film, therefore, is a simultaneous and superimposed plot of the refractive-index gradient of the NaBr baseline solution and of the lipoprotein solution as a function of the distance from the center of rotation. Analysis of the film is made by using an enlarged (5X) tracing of each frame. Measurement of any given area under the baseline is ordinarily obtained by planimetry. The area obtained by planimetry is converted into mg/100 ml by using the relationship derived by Pickels.[7] Because of the sector shape of the analytic cell and the varying centrifugal field a radial correction is applied. This radial correction has been shown to hold for multicomponent systems,[9] and therefore it is applicable to lipoprotein analysis.

In the film analysis the total serum lipoprotein spectrum has been subdivided into five flotation-rate classes: $S_{f(1.20)}$ (185-485), (61-185), (44-61), (16-44), (0-6). The subdivision of the four faster-migrating lipoprotein classes has been made to correspond as closely as possible to the Gofman low-density lipoprotein classification. (See Table I and Fig. 2.) This correspondence was determined by analysis of seven sera by both procedures in which the ultracentrifugal background salt solution density, $\rho_{26/4}$, for the NaCl low-density lipoprotein run was 1.063 g/ml and for the NaBr total lipoprotein run was 1.198 g/ml. The flotation rates in the NaBr run were standardized to a medium of density, $\rho_{26/4}$ of 1.200 g/ml. The error in the determination of $S_{f(1.20)}$ rates as a result of the variation in density, temperature, and location on the film of the bottom of the cell is approximately 2% for all classes. However, it can increase considerably if the standard conditions are not carefully maintained.

If there is any significant dependence of flotation rate on concentration (S_f vs C), the concentration of any particular class of lipoprotein requires correction because of the Johnston-Ogston effect.[4] The effect results in the apparent decrease in concentration of the faster molecular class because of a negative concentration gradient of the slower component in the boundary region of the faster component. At the same time, there is a corresponding apparent increase in concentration of the slower molecular class. Johnston and Ogston have treated this phenomenon theoretically for two components. Such apparent changes in concentration are a function of the concentration and flotation rate of each component.

Table I

Correspondence between $S_{f(1.06)}$ and $S_{f(1.20)}$	
S_f in NaCl medium ($\rho_{26/4}$ 1.063 ± 0.0005 g/ml)	S_f in NaBr medium ($\rho_{26/4}$ 1.2000 ± 0.0005 g/ml)
383	467
346	457
53.2	105
46.9	106
43.3	99.5
42.0	105
15.0	48.5
14.3	47.5
6.7	30.4
6.7	30.5
5.9	28.3
5.9	28.9
5.8	27.4
5.7	28.3
5.6	27.3
5.6	28.1
5.6	27.3
5.6	26.9
5.4	27.4
5.4	26.6
4.8	26.9
4.8	26.6
4.1	25.2
1.1	15.8
0.9	16.5

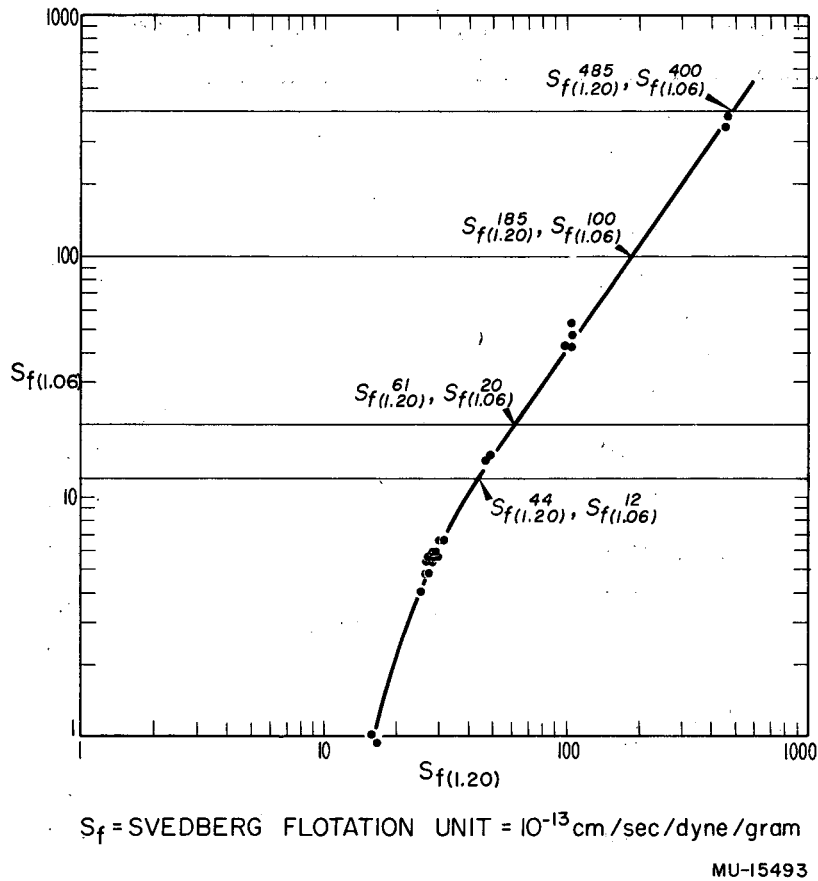S_f = SVEDBERG FLOTATION UNIT = 10^{-13} cm/sec/dyne/gram

MU-15493

Fig. 2.   The correspondence between the flotation rates in
NaCl ($\rho_{26/4}$ = 1.063 g/ml) and the flotation rates in
NaBr ($\rho_{26/4}$ = 1.200 g/ml).

For application of the Johnston-Ogston correction to a spectrum of components (as in dealing with a lipoprotein spectrum) certain simplifications are necessary. The lipoprotein spectrum is arbitrarily divided into the five component classes previously described. The effective S_f rate of each class is calculated by considering the distance traveled by that class to the mid-area position of the schlieren pattern for each of the five classes. Thus, the mid-area position of each of these classes is designated as the effective S_f rate of that class. Equation (1) expresses this mid-area S_f rate for a particular lipoprotein class as a function of the concentration:

$$S_f = S_f^0 {}_{(1.20)} \ (1 - K \sum_{i=1}^{n} C_i) \tag{1}$$

This equation also assumes equivalent slowing effects for the same concentrations of lipoproteins on a particular lipoprotein class. Thus, for any class under consideration, this equation assumes the form of a straight line for which K is the slope. The data and plots of the best straight lines for the S_f vs C for the different classes of lipoproteins are given in Table II and Fig. 3, respectively.

The values of K for the five major lipoprotein classes are as follows:

$S_{f\ (1.20)}$	$\dfrac{K}{10^{-5}\ (mg)^{-1}}$
185-485	+0.94
61-185	-6.4
44- 61	-0.47
16- 44	-0.42
0- 6	-4.8

The slopes of all these lines are negative except for the S vs C line for the $S_{f(1.20)}$ (185-485) lipoprotein class. This unexpected finding will be studied in detail later.

For all lipoprotein classes, the values of K are very small. This minimizes the Johnston-Ogston distortion present in the observed lipoprotein spectrum, and on this basis we omit the correction for the Johnston-Ogston effect in this procedure. Since the K value of each lipoprotein class has been obtained in the presence of other lipoprotein classes, the actual K

Table II

Flotation rate and concentration relationship

$S_{f(1.20)}$

	185-485		61-185		44-61		16-44		0-6	
	mg%	$S^x_{f(1.20)}$	mg%	$S^x_{f(1.20)}$	mg%	$S^x_{f(1.20)}$	mg%	$S^x_{f(1.20)}$	mg%	$S^x_{f(1.20)}$
A4882										
3 Co	2517	242	2426	101	1875	50.0	1651	26.5	565	1.80
2 Co	1787	226	1733	107	1380	52.4	1252	27.0	356	2.05
1 Co	929	221	911	113	708	55.6	629	27.5	161	2.25
A4883										
0.5 Co	475	210	455	117	319	52.5	261	28.0	61.6	2.25
0.5 Co	425	209	415	117	315	52.8	267	27.4	70.6	2.20
A4886										
2 Co	1662	226	1609	119	1193	49.5	1044	27.4	330	1.85
1 Co	781	210	768	123	584	53.0	517	27.6	133	1.80
0.5 Co	402	204	397	113	314	52.8	266	28.5	61.6	2.25
Av. Value	1122	219	1089	114	836	52.5	736	27.6	217	2.08

In the experiments A4882 and A4883 the concentrations refer to the amount of serum added to each preparative tube, whereas in experiment A4886 the concentrations refer to appropriate dilution of the top ml of preparative tube in which lipoproteins have been previously concentrated from 3 ml of serum. All S_f values have been corrected to density 1.200 g/ml at 26° C.

Mg% refers to the concentration of all lipoproteins comprehensive of the $S_{f(1.20)}$ 0-6 class to the mid-area position, x, of that class.

Table II Cont'd

Flotation rate and concentration relationship

All concentrations here have been calculated assuming a specific refractive increment of 0.00145 g/100 ml for all lipoprotein classes in a NaBr medium of density 1.20 g/ml.

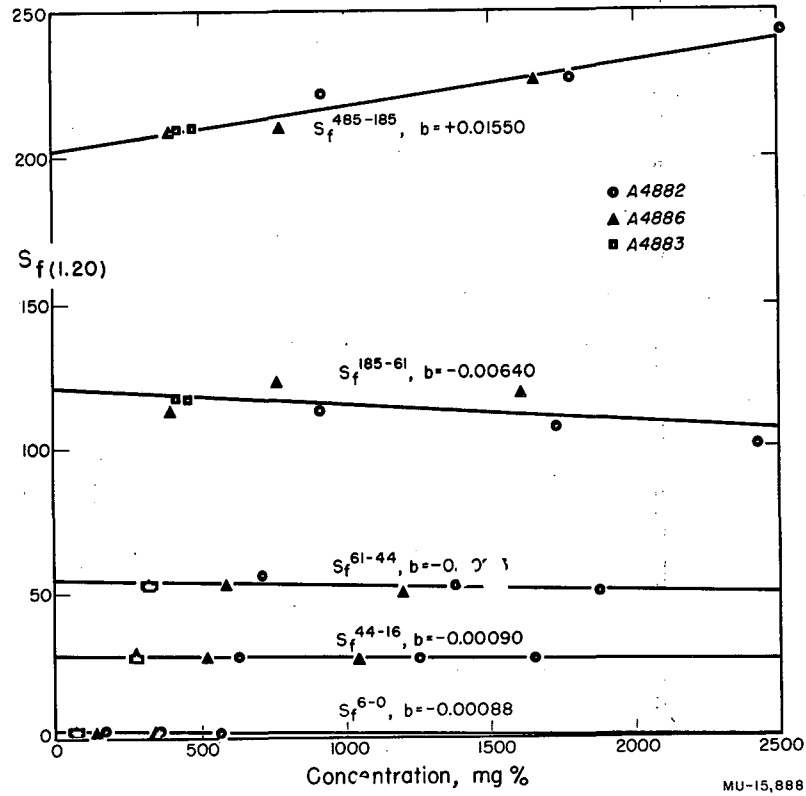$S_{f\ (1.20)}^{x}$ represents the flotation rate at point x.

Fig. 3. Plot of S_f vs C. The fitting of each of the straight lines has been made by the method of least squares. The b values are the slopes of the respective lines.

values for isolated lipoprotein classes may be different from those given here. However, the given values correspond more closely to the actual experimental conditions of the procedure than the K values for isolated lipoprotein classes.

Discussion and Conclusions

This method provides an ultracentrifugal analysis of all major classes of serum lipoproteins isolated from two milliliters of serum. The preparative ultracentrifugal isolation at 40,000 rpm for 24 hours assures a quantitative isolation. Analyses of the second milliliter reveal the presence of neither lipoproteins nor serum proteins.

The ultracentrifugal procedure used by Lewis et al.[5] employing KBr-NaCl (1.21 g/ml), and which involves ultracentrifuging at 30,000 rpm for 13 to 17 hours at temperatures between 16 and 18° C, does not quantitatively recover all the serum lipoproteins. The lower viscosity of KBr-NaCl solution (as compared with that of NaBr solution at a density of 1.20 g/ml) does not sufficiently compensate for the shortness of their preparative ultracentrifugal run, which also is made at a lower centrifugal field. Because of viscosity differences, lipoproteins float at a rate about 25% faster in KBr-NaCl (1.21 g/ml) than in NaBr (1.20 g/ml). Considering this factor in KBr-NaCl (1.21 g/ml), it is somewhat more difficult to study lipoproteins with flotation rates in the neighborhood of $S_{f(1.20)}$ 485 in NaBr (1.20 g/ml) in the analytical ultracentrifuge, without using an accurately determined acceleration picture taken considerably below 52,640 rpm. In choice of salts for increasing solution densities, it is worthy of note that NaBr has a much higher solubility than KBr. Saturated aqueous NaBr at 20°C has a density of 1.543 g/ml, whereas saturated KBr has a density of only 1.371 g/ml. Compared to the use of the D_2O-$NaNO_3$ system of deLalla[2] the use of NaBr represents a simplification as well as a further advantage of not interfering with the Kjeldahl nitrogen analysis, which may be desired.

The analytical ultracentrifuge field at 42,040 rpm represents a 36% reduction in centrifugal force with respect to that of 52,640 rpm--the speed at which lipoprotein runs have been customarily made. This change will certainly reduce breakage of analytical cells.

Since flotation rates remain nearly constant within the concentration ranges normally encountered, the analysis is simplified~because~the Johnston-Ogston correction becomes negligible and therefore unnecessary. Moreover, it is possible to follow the previously established flotation classification of the low-density serum lipoproteins.[2] In this procedure the lipoproteins of the class $S_{f(1.20)}$ 0-6 correspond to the total of the two principal high-density lipoproteins (HDL-2 and HDL-3).

The usefulness of this method is that it provides a simplified procedure for complete ultracentrifugal lipoprotein analyses is essentially a one-salt system (NaBr) using only 2 ml of serum.

Acknowledgments

The authors wish to express their appreciation to Dr. John W. Gofman for his many helpful criticisms and suggestions given during the course of this investigation.

This work was done under the auspices of the U. S. Atomic Energy Commission.

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