Title

ANALYSIS OF LOW-DENSITY LIPOPROTEINS BY PREPARATIVE ULTRACENTRIFUGATION AND REFRACTOMETRY

Authors

Lindgren, F.T.
Nichols, A.V.
Wills, R.D.
et al.

Publication Date

1963-01-16

UCRL-10794

University of California

Ernest O. Lawrence Radiation Laboratory

ANALYSIS OF LOW-DENSITY LIPOPROTEINS BY PREPARATIVE ULTRACENTRIFUGATION AND REFRACTOMETRY

Berkeley, California

DISCLAIMER

UCRL-10794
UC-48 Biology and Medicine
TID-4500 (19th Ed.)

UNIVERSITY OF CALIFORNIA
Lawrence Radiation Laboratory
Berkeley, California

Contract No. W-7405-eng-48

ANALYSIS OF LOW-DENSITY LIPOPROTEINS BY PREPARATIVE ULTRACENTRIFUGATION AND REFRACTOMETRY

F. T. Lindgren, A. V. Nichols, R. D. Wills,
L. Wing, and J. E. Gullberg

January 16, 1963

Printed in USA. Price 50 cents. Available from the
Office of Technical Services
U. S. Department of Commerce
Washington 25, D.C.

Analysis of Low-Density Lipoproteins by Preparative Ultracentrifugation and Refractometry*

By

Lindgren, F.T., Nichols, A.V., Wills, R.D., Wing, L.+ and Gullberg, J.E.

From the
Donner Laboratory
of
Medical Physics
Lawrence Radiation Laboratory
University of California
Berkeley, California

and
Laboratory of Optics and Metrology
Department of Zoology
University of California
Berkeley, California

January 16, 1963

*The work described in this paper was supported in part by Research Grant H-1882 (C7) from the National Heart Institute, Public Health Service, Bethesda, Md., and by the U.S. Atomic Energy Commission, Washington, D.C.

+ Participant in the National Science Foundation Summer Fellowship Program for High School Science Teachers.

SUMMARY

A simplified method for the analysis of both the glyceride-rich $S_f 20\text{-}10^5$ and the cholesterol-rich $S_f 0\text{-}20$ low-density lipoproteins is presented. It consists of serum lipoprotein fractionation by preparative ultracentrifugation and subsequent quantitative analysis by refractometry. Comparison of this technique with the technically more difficult analytic ultracentrifugal methodology reveals comparable results for these two principle low-density lipoprotein groups. One of the advantages of this proceedure is that it provides a reliable and reproducible means for quantitating the principal glyceride-bearing lipoprotein group - the $S_f 20\text{-}10^5$ lipoproteins.

Analysis of Low Density Lipoproteins by Preparative Ultracentrifugation and Refractometry

INTRODUCTION

The analysis of blood lipids is an important factor in the treatment and prognosis of disease states whose principal biochemical manifestations are an elevation of particular blood lipids and lipoproteins. Because of the principal involvement of both glyceride and cholesterol in the lipid elevations associated with such a disease as coronary arteriosclerosis and its consequences, a glyceride and cholesterol measurement would provide important information to the clinician who must actually treat the patient. Usually, a glyceride determination can be made by either a glycerol analysis (1) or by means of an infrared analysis (2). A total gravimetric lipid measurement, although tedious and not routinely compatible with the usual clinical laboratory procedures, can also, in combination with a serum cholesterol measurement, provide a good estimate of the $S_f20\text{-}10^5$ lipoproteins (3) and, hence, of serum glyceride. In any event, it is not easy to simultaneously obtain both a reliable serum cholesterol and serum glyceride determination in the clinical laboratory.

A more direct picture of the native state of the blood lipids associated with coronary arteriosclerosis is provided by an ultracentrifugal analysis of the low-density lipoprotein spectra. Much of the available data (4)(5) on these low-density lipoproteins has been given for 4 lipoprotein groups; the $S_f0\text{-}12$, $S_f12\text{-}20$, $S_f20\text{-}100$, and $S_f100\text{-}400$. However, from the point of view of important metabolic responses and particularly from considerations for treatment (dietary or pharmacological), these four lipoprotein classes may be appropriately grouped into the $S_f0\text{-}20$ and $S_f20\text{-}400$. On the basis of hydrated density, these lipoproteins essentially may be described as low density (LD) (1.006-0.050 g/ml) and very low density (VLD) (0.92-1.006 g/ml) lipoproteins, respectively. Biochemically, the former correspond to the principal cholesterol-bearing lipoproteins and the latter to the major glyceride-bearing lipoproteins. Unfortunately, the complete ultracentrifugal determination of these lipoproteins, although providing intrinsically greater information than the method here presented, suffers from the fact that such analysis is both an expensive and technically

difficult procedure. By far the greater portion of the difficulty is in the final analytical ultracentrifugal analysis itself and not in the preparative stage of lipoprotein isolation, which is comparatively a simple procedure. The following method, a refinement of a previous procedure (6), avoids the analytical difficulties by employing a relatively simple, accurate and reproducible refractometric analysis of the low density lipoprotein fractions which have been obtained by preparative ultracentrifugation.

METHODS

All preparative runs were made at 18°C in a Spinco Model LH ultracentrifuge equipped with a "vacuum sentinel" (7) to minimize sample loss in case of vacuum failure. This seemed desireable since for this procedure 9 ml of serum is required for optimum analysis. Thus in most cases there might be insufficient serum available for a duplicate analysis. Isolation of lipoproteins was accomplished after 18 hours of centrifugation at 40,000 RPM in a 40.3 rotor. For each lipoprotein containing sample, two fractions were pipetted in depth, the top milliliter, containing quantitatively the lipoprotein fraction and the second milliliter, which provides a reference salt background for that sample. Pipetting was done in a darkened room on a fixture equipped with a focused light beam allowing visualization of the lipoproteins by their tyndall scattering. Fractions collected in 1 ml volumetric vials were transferred for storage into 9 ml air-tight screw cap vials fitted with teflon gaskets. Because of potential evaporation and condensation within each lipoprotein containing vial, it is essential for optimal accuracy that refractive index determinations be made as soon as possible (within 4-6 hours) after the fractions are collected. Storage at room temperature prior to refractometric analysis is recommended. Refractive index measurements were made with a Bausch and Lomb Precision Abbe' Refractometer (8), with a range of n_D from 1.203 to 1.508, thermostated to 26.00°C ± .02°C with a temperature controller (Precision Scientific Co).

In essence, this method consists in isolating by preparative ultracentrifugation two lipoprotein fractions from each sample. The first fraction is the glyceride-

rich very low-density lipoproteins (VLD) less dense than 1.006* gm/ml (the $S_f20\text{-}10^5$). The VLD lipoproteins are obtained ultracentrifugally without prior density manipulation of serum. For this purpose 6 ml of serum are directly centrifuged for 18 hours at 40,000 RPM. With regard to density considerations and manipulations, serum may be considered to be 94% by volume a salt solution (primarily NaCl) of density 1.0065 g/ml. If 6 ml of serum is unavailable for the VDL fractionation, the difference between the available serum and the recommended 6 ml capacity of the preparative tube is substituted with an appropriate volume of a 0.202 Molal** NaCl solution (ρ 20 = 1.0065 gm/ml).

The total low-density lipoprotein fraction ($S_f0\text{-}10^5$) or TLD fraction is obtained by centrifuging a solution consisting of 3 ml serum and 3 ml of a 3.289 Molal NaCl salt solution (ρ 20 = 1.1171 g/ml). The background 1.747 Molal NaCl solution resulting from the use of the above volumes serves as the reference background to which all TLD runs must be brought. Thus, if only 2 ml serum is available, a salt solution equivalent in density to the reference background is obtained by first adding 0.94 ml of a 0.202 Molal NaCl solution to the 2 ml of serum. Then, by adding 3 ml of the 3.289 Molal NaCl salt solution to this mixture, the final background salt solution will be the same as that resulting from mixing 3 ml serum and 3 ml 3.289 Molal NaCl. It should be pointed out that this density, 1.0652 g/ml, is the solution density before preparative ultracentrifugation and is equal to 1.0630 g/ml at 26°. However, during preparative centrifugation, appreciable salt re-distribution occurs from the top to the bottom of the preparative tube. The extent of this redistribution is dependent upon the nature and concentration of the salt as well as the time and conditions of ultracentrifugation. Under the conditions of this method, the redistribution of salt for both the VLD and TLD runs are shown in Figure 1. Thus, after the conditions of centrifugation presented here, the density of the top 1 ml of the VLD run falls from approximately 1.0065 gm/ml to 1.0061 g/ml and the top 1 ml of the TLD run is reduced from approximately 1.0652 g/ml to 1.0652 g/ml to 1.0622 g/ml. It is essential to take into account fully this salt redistribution if we are to measure lipoprotein concentrations

*Unless otherwise indicated all densities are given at a 20°C.

** Solutions are given in molal concentrations (moles/1000 gm H_2O) so as to avoid temperature dependence.

accurately.

The actual calculation of lipoprotein concentrations were made using the following relationship:

$$\text{Lipoprotein concentration, mg \%} = (\Delta S_{TF} - \Delta S_{BkND}) \frac{(K_1)}{(C_o)} \frac{(1.000)}{(S.R.I.)}$$

where: ΔS_{TF} = Abbe' scale reading increment of top fraction above the water reference*.

ΔS_{BKnd} = Calculated Abbe' scale reading increment of top fraction salt background above the water reference.

K_1 = Instrument dependent conversion factor of scale reading increment to refractive index increment, out values:

$$K_1 \text{ (TLD)} = 5.30 \times 10^{-3}$$

$$K_1 \text{ (VLD)} = 5.48 \times 10^{-3}$$

C_o = Concentration factor of lipoprotein factor relative to initial serum.

S.R.I. = Specific refractive increment; 0.00171 for VLD and 0,00154 for TLD runs. As used here S.R.I. is the increase in refractive index of the indicated salt solutions resulting from the presence of 1 gm of lipoprotein per 100 ml solution, measured at 26°C.

The centrifuged background salt solution present in the centrifuged top fraction is evaluated from the second ml fractions from both the VLD and TLD runs. Refractometry on these fractions allows accurate calculations of the background refractive index of the top ml aliquots from each individual VLD and TLD run.

Experimentally, the ratio of the refractive index increment, above the water reference, of the first ml to the second ml, V^o1/V' 2 of both the TLD and VLD salt reference runs as described here is 0.985. Thus, multiplying the refractive index increment of the respective 2nd ml reference fractions by 0.985 yields, in each case, the refractive increment of the actual salt background of the lipoprotein containing fraction. Further, if pipetting difficulties occur, a pooled first and second milliliter

*A water reference reading is essential before and after each set of refractometric readings and it is further recommended that additional water readings be taken after every tenth sample reading.

lipoprotein fraction can be taken, together with a 3rd milliliter reference fraction. In this case, the ratio of V^{o}_{2}/ V^{2}_{3} for the salt background refractive increment is approximately 0.975 for both the TLD and VLD runs. In all cases, the concentration of lipoproteins is obtained from the difference in refractive index increment (above the water reference) of the lipoprotein containing fractions and the calculated refractive increment for its respective salt background.

Probably the most convenient and accurate refractometric analysis of small quantities of solution can be made with a precision Abbe' refractometer, temperature controlled to at least ± 0.1°C. The readings should be made at a constant temperature somewhat above that of room temperature, in this proceedure at 26.0°C. In this method readings should be made in a manner so as to take full advantage of the accuracy of the precision Abbe', with scale readings estimated to the nearest ± 0.005 units. Over the range of measurements, relative accuracy of refractive index measurements*, with proper care, can be made to within approximately ±0.00003 refractive index units. An ordinary Abbe' refractometer cannot give sufficient accuracy to be successfully used for this method. A Pulfrich refractometer (9), of course, can provide somewhat greater accuracy of measurement than the precision Abbe' but because of the convenience and adequate accuracy of the precision Abbe', the latter is recommended.

Refractometry can be done using only one drop of the lipoprotein fraction by taking the "reflection" reading. Thus, nearly all the fraction may be available, if desired, for other lipid analyses. The drop of lipoprotein solution should be placed slightly above the center of the outer ground glass prism and the prism closed immediately. Thereafter, a time delay of exactly one minute between application of the sample and taking the refractometric reading is recommended. This time is sufficient for approximate temperature equilibration but insufficient for any significant evaporation to occur. After each reading the surface of the opposing prisms is thoroughly washed by directing a stream of about 50cc of distilled H_2O against each prism using a polyethylene washing bottle. Thereafter, the prism surfaces are wiped

*A photoelectric sensing device can be used more effectively to set (with a servo mechanism) the quadrant of the refractometer to the critical angle characteristic of each sample. Further, with this provision a direct scale readout is possible.

(unidirectionally) with a non-abrasive wiper (Kimberly Clark Type 900-S). To further insure a completely dry prism surface, an unheated air stream is directed for 5 seconds onto each prism surface using a hair dryer (Oster, Model 202).

In this preliminary study for the purposes of converting refractive index measurements to lipoprotein concentrations, a specific refractive increment of 0.00171 units (10) is used here for the VLD (S_f20-10^5) fraction. This assumes, as a first approximation, equivalence in S.R.I. between B_1 lipoprotein (S_f0-20) measured in .3 Molar NaCl and S_f20-10^5 lipoproteins measured in 0.199 Molal NaCl. A specific refractive increment of 0.00154 units (11) is used for the TLD (S_f0-10^5) fraction. C lculation of the cholesterol-rich S_f0-20 lipoproteins is obtained by difference. If desired, a refractometric Atherogenic Index ($A.I._r$) approximately equivalent to the analytic ultracentrifugal A.I. (12) value may be obtained by the following relation:

$A.I._{U.C.}$ $A.I._R$ = 0.100 (S_f0-20, concentration in mg/100 ml) + 0.199 (S_f20-10^5, concentration in mg/100 ml).

RESULTS

Table I shows a comparison of 30 serum analyses by both analytic ultracentrifugation and refractometric determinations. The mean total low-density values (TLD) obtained by both methods agree very closely, which suggests that there are minimal optical dispersion effects, differences between the methods and in the serums studied there existed relatively low concentration of S_f400-10^5 lipoproteins. The elevated S_f20-10^5 lipproteins values as determined by refractometry over the S_f20-400 values determined by analytic ultracentrifugation suggest that the actual fractionation of the VLD run (S_f20-10^5) probably obtains some lipoproteins of flotation rates lower than S_f20. An estimate of the actual flotation rate of a lipoprotein of 1.006 g/ml hydrated density is approximately S_f16 (13). Further, the values of the S_f0-20 and S_f 20-400 reported here have not been corrected for $S_f$versus c and Johnson Ogston effects (14)(15). These corrections though relatively small for the two major

lipoprotein groups (5) would nonetheless tend to increase the value of S_f20-400 and decrease the value of the S_f0-20 lipoproteins. However, the primary effect under consideration is that of the self slowing (S_f versus C effect) of the lipoproteins upon each other. For the mean S_f0-20 concentrations studied here, the average S_f20 lipoprotein, present in a medium of approximately 1.4% S_f0-20 lipoproteins, would exhibit an actual flotation rate of approximately S_f17.

Table 2 compares the results of seven VLD runs analyzed by both analytic ultracentrifugation and refractometry. For such analyses, a specific refractive increment (S.R.I.) of 0.00171 is used for refractometry and a S.R.I. of 0.00154 employed for analytic ultracentrifugation. For the latter analysis, solid NaCl was added in the amount of 87.2 mg for each milliliter of VLD fraction. This amount, assuming the lipoprotein fraction consists of 1% by volume lipoprotein is sufficient to bring the small molecule background density to 1.063 gm/ml. Of course, introducing this amount of salt into each VLD fraction increases the volume and, hence, dilutes the original VLD fraction by a factor of approximately 0.973. Taking this factor into account, the actual results obtained by each method compare very favorably with one another suggesting again that optical dispersion differences and significant concentration of S_f400-10^5 lipoproteins are apparently not a problem in evaluating the VLD lipoproteins by refractometry.

A measure of the reproducibility of this method, particularly when different amounts of serum are used, is shown in Table III. Here, for a single serum sample, a reproducibility of approximately ± 5% is maintained for both the VLD and TLD runs. An additional measure of anticipated error may be calculated from duplicate analysis to give a standard error of estimate. For six duplicate analyses of the VLD and nine duplicate analyses of the TLD runs a σ SEE = 5 mg% and a σ S.E.E. = 27 mg% was obtained for the S_f20-10^5 and S_f0-10^5 lipoproteins, respectively. This accuracy and reproducibility compares favorably with that observed for lipoproteins determined by analytic ultracentrifugal analysis (5).

DISCUSSION

It is evident that the refractometric determination of the low-density lipoproteins is not equivalent to an analytical ultracentrifugal determination. In the first place, lipoproteins above S_f400, although usually present at relatively low abundance, are measured by refractometry but are not customarily measured by analytic ultracentrifugation. On the other hand, the more subtle features of the entire low-density lipoprotein distribution, revealed in detail with an analytic ultracentrifuge determination are unavailable with refractometry. Further, certain discrepancies still exist in the determination of the low-density lipoproteins by refractometry and by analytic ultracentrifugation. Such small discrepancies may be due in part to the presence of non-migrating lipoproteins in each of the lipoprotein fractions, thus giving an apparently lower concentration of lipoproteins detected by the schlieren optical system. Further, some small dispersion effects may be anticipated in that refractive index measurements with the precision Abbe' are made with the Na D_{12} lines (5890Å and 5896Å) whereas the analytic schlieren diagram giving dn/dx (and by integration of total lipoprotein Δn) is obtained primarily with the green line of the Hg arc (5461Å). Despite these small discrepancies, however, the principal low-density lipoproteins may be measured refractometrically and compared to analytic ultracentrifuge values even in the absence of a full theoretical explanation of these differences.

REFERENCES

1. Carlson, L.A. Acta Soc. Med. Upsaliensis 64: 208, (1959).

2. Freeman, N.K., F.T. Lindgren, Y.C. Ng and A.V. Nichols. J. Biol. Chem. 227, 449 (1957).

3. Nichols, A.V., F.T. Lindgren and J.W. Gofman. Geriatrics 12, 130 (1957).

4. deLalla, O.F. and J.W. Gofman. In Blood and Other Body Fluids, edited by D.S. Dittmer, Washington, D.C , Federation of American Societies for Experimental Biology, 1961, pp. 64.

5. Lindgren, F.T., and A.V. Nichols. In the Plasma Proteins, edited by Frank W. Putnam, New York, Academic Press, Inc., 1960, Vol. 2, p.1.

6. Lindgren, F.T., and J.W. Gofman. Bull. Swiss Acad. Med. 13, 152 (1957).

7. Lindgren, F.T., and F.T. Upham. Rev. Sci. Instr. 33, 1291 (1962).

8. Bauer, N., K. Fajans, and S.Z. Lewin. In Physical Methods of Organic Chemistry, Third Edition, edited by A. Weissberger, New York, Interscience Publishers, 1960, Vol. I, part II, pp. 1239.

9. Guild, J., Proc. Phys. Soc. London, 30, 157 (1917-1918).

10. Armstrong, S.H., M.J.E. Budka, K.C. Morrison and M. Hasson. J. Am. Chem. Soc. 69, 1747 (1947).

11. Hanig, M. and J.R. Shainoff. J. Biol. Chem. 219, 479 (1956).

12. Gofman, J.W., B. Strisower, O. deLalla, A. Tamplin, H. Jones and F. Lindgren. Modern Med. 11, 119 (1953).

13. Lindgren, F.T., A.V. Nichols, F.T. Upham and R.D. Wills. J. Phys. Chem. 66, 2007 (1962).

14. Johnson, J.P. and A.G. Ogston. Trans. Faraday Soc. 42, 789 (1946).

15. deLalla, O. and J. Gofman. In "Method of Biochemical Analysis" edited by David Glick, New York, Interscience, 1954, Vol. I., p. 459.

Table 1. Comparison of Analysis Ultracentrifugal (U. C.) Data with Refractometric (Δn) Lipoprotein Determinations (Values expressed in mg/100 ml).

Serum No.	Conc.* (TLD)	S_f0-20 U.C.	S_f0-20 Δn	S_f20-400 U.C.	Conc (VLD)	S_f20-10^5 Δn	S_f0-400 (U.C.)	S_f0-10^5 (Δn)
1	3 Co	407	380	11	6 Co	59	418	439
2	3	431	365	73	6	109	504	474
3	3	405	372	39	6	56	444	428
4	3	448	443	95	6	174	543	617
5	3	505	487	95	6	182	600	669
6	3	456	416	90	6	155	546	571
7	3	482	435	101	6	171	583	606
8	3	356	352	53	6	64	409	416
9	3	283	245	21	6	45	304	290
10	3	340	356	18	6	37	358	393
11	3	388	349	6	6	27	394	376
12	3	365	344	38	6	72	403	416
13	3	432	375	54	6	59	486	434
14	3	352	311	63	6	88	415	399
15	3	552	558	61	6	93	645	651
16	3	268	271	2	6	13	270	284
17	3	344	282	48	6	37	392	319
18	2 Co	347	295	627	6	742	974	1038
19	3	320	315	12	6	38	332	353
20	3	571	535	141	6	139	712	674
21	3	277	259	5	6	8	282	267
22	3	329	328	2	6	8	331	336
23	3	498	494	109	6	152	607	646
24	3	269	247	203	6	238	472	485
25	3	447	382	216	6	264	663	646
26	3	188	181	238	6	270	426	451
27	2 Co	579	474	99	6	104	678	578
28	1.5 Co	1,283	1,118	756	6	733	2039	1,051
29	3	225	240	82	6	131	307	371
30	3	499	427	117	6	200	616	627
Mean values (mg%(		423	398	116		149	538	537

*Concentration factor of lipoprotein fraction relative to serum (6 Co indicates 6 ml of serum yielded a 1 ml lipoprotein fraction).

Table II. Comparison of Analytic Ultracentrifugal (U. C.) Data with Refractometric (Δn) Data.

Serum No.	Conc.	$S_f 20\text{-}400$ (UC)	$S_f 20\text{-}10^5(\Delta n)$
23	6 Co	150	152
24	6 Co	211	238
25	6 Co	260	264
26	6 Co	258	270
27	3 Co	105	104
28	6 Co	836	733
30	6 Co	176	200
	-	285 (Mean)	280 (Mean

Table III

TLD and VLD Lipoprotein Reproducibility on a Single Serum Sample (Refractometry)

Lipoprotein Fraction	$S_f 0\text{-}10^5$	Lipoprotein Fraction	$S_f 20\text{-}10^5$
TLD 3 Co	945	VLD 6 Co	489
" 3	928	" 6 Co	473
" 3	934	" 5 Co	471
" 2	917	" 4 Co	457
" 1	888	" 3 Co	439
—	—	" 2 Co	450

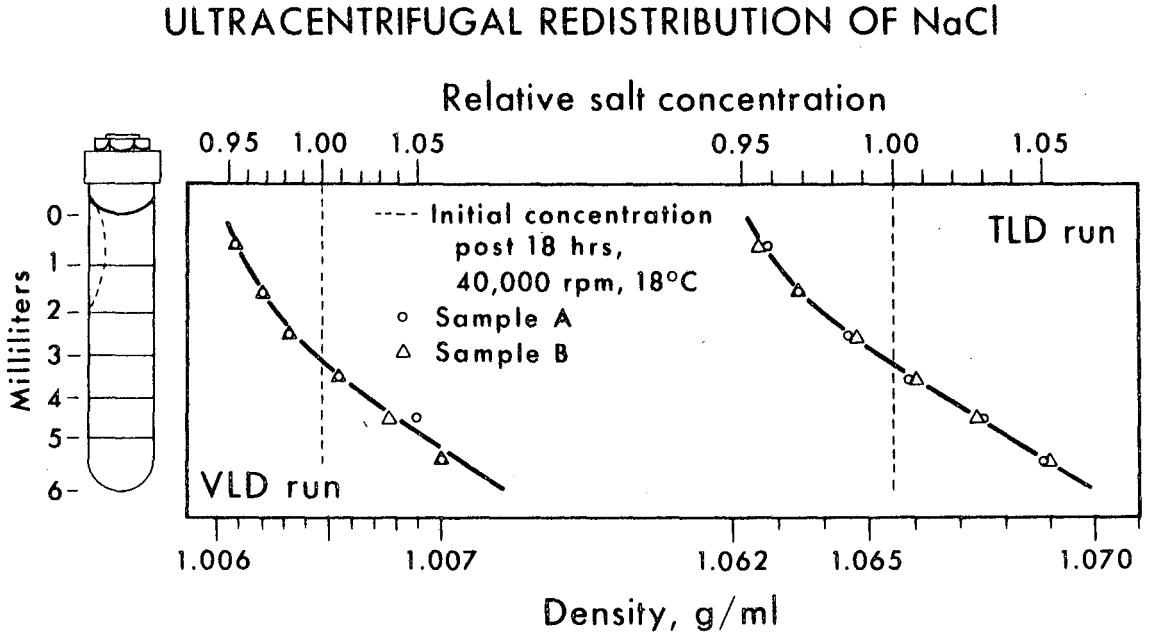

Fig. 1. The ultracentrifugal redistribution of NaCl in both the Very Low Density (VLD) and Total Low Density (TLD) preparative runs.