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THE ISOLATION AND QUANTITATIVE ANALYSIS OF SERUM LIPOPROTEINS

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I. INTRODUCTION

During the past two decades, there has been a steadily increasing interest and literature involving human serum lipoproteins and their relationship to states of health and disease. This followed the early pioneering work of Macheboeuf (1), McFarlane (2), the Cohn group (3), Pedersen (4), Gofman *et al.* (5) and others. In addition to a great variety of experimental studies with humans and animals, there has simultaneously developed a very substantial technology involving lipid and lipoprotein analysis. Considering the limitations of space, we will not attempt to thoroughly review all this technology and its application, since several such comprehensive reviews are available (6-9).

Our main purpose, therefore, shall be to detail the present state of ultracentrifugal lipoprotein methodology as practiced at Donner Laboratory. With recent availability of high-speed large capacity computers, this technology has rapidly developed over the past few years. Essentially all procedures now involve at least one or more computer programs, many of which are constantly in a state of revision and improvement. In addition, computer programs are also used with each instrument involved in lipoprotein fractionation and analysis. For example, it would have been difficult to develop lipoprotein subfractionation techniques on non-linear density gradients without the aid of computer programs. Similarly, analysis of these subfractions by CHN elemental analysis is greatly facilitated with several computer programs.

After presenting the preparative and analytical ultracentrifugal methodology we shall consider some normal and clinical data illustrating the ultracentrifugal features of lipid and lipoprotein abnormalities. Later, density gradient procedures are presented, with data, for the isolation of chylomicron-containing fractions and for the subfractionation of all major lipoprotein classes. Finally, we will consider the more widely used and latest techniques of paper and agarose electrophoresis and compare the results of these techniques with those obtained by ultracentrifugal methodology.

II. PREPARATIVE ULTRACENTRIFUGAL TECHNIQUES FOR ISOLATION OF THE MAJOR LIPOPROTEIN CLASSES.

The early lipoprotein isolation procedures (10) and particularly those of deLalla and Gofman (11) were designed to obtain serum (or plasma) lipoprotein fractions for use in the analytic ultracentrifuge. However, with minor modifications (12-14) they are widely used for other purposes. Many laboratories interested in lipoprotein metabolism now have a growing need to study physical and chemical properties of certain lipoprotein classes in normal and clinically abnormal states. The most important requirement in the preparative isolation of lipoproteins is the control and monitoring of density. This is most conveniently achieved using precision refractometry (15) with absolute calibration by pycnometry (16).

A. Density Monitoring, Manipulation, and Calculation.

Although D_2O and several salts are frequently employed for lipoprotein isolation, we will consider here only two monovalent salt systems; NaCl for low-density applications and NaCl-NaBr for high-density lipoprotein work. With the exception of D_2O (whose refractive index, $n_D26 = 1.32782$, differs little from that of H_2O, $n_D26 = 1.33240$) the techniques for refractometry would of course apply to the other salt systems. For the preparation of accurate and reproducible salt solutions, it is recommended that only dry salts be used. For this purpose, a large batch of salt should be calcinated at 550°C overnight and stored in a Teflon sealed jar in a desiccator.

If very limited applications are involved, manual calculations using data from the International Critical Tables (17) or the Chemical Rubber Handbook (18) are adequate. However, if lipoprotein fractionation involves several steps, density gradient work, or analytical ultracentrifugal analysis, computer derived tables are recommended. These ideally should contain ρ_{20}, η_{20}, ρ_{26}, η_{26}, g salt/liter 20°C, g H_2O/liter 20°C, g salt/g H_2O, molality, molarity and wt% values. In addition, at various calibration points n_D26°C and refractometer ΔS values (characteristic of the instrument used) should be included. A complete table quadratically interpolated between each set of data points taken 3 at a time is easily constructed with a computer. The most convenient interpolation is to increment ρ_{20} 1 part in the 4th place for the complete range of salt parameters. Since preparation of salt solutions and density manipulations may occur over a temperature range, all solutions here are expressed in molal concentrations (moles/1000 g H_2O) so as to avoid temperature dependence.

Although a salt solution of a given density (and volume) is easily prepared and monitored from the above computer derived tables, density manipulation is a somewhat different consideration. For example, one complication is that adding large amounts of salt significantly changes the initial solution volume. The simplest procedure, where precise density manipulations are needed, is to utilize the interpolated ratio of mass salt/mass H_2O. Since this ratio is available for the initial and final density (and the initial mass of salt and H_2O are known) the exact increment of salt needed to bring the initial solution

to the final density can be calculated. Also, given the initial masses of water and salt, the added mass of salt and the final density of the solution, the volume of the final solution (and hence the volume change) is obtainable. Where a dual salt system is used, such as a low but constant 0.195 molal NaCl content and a varying NaBr content, the 11.42 g/l NaCl content may be considered equivalent to 10.46 g/l NaBr and the interpolated tables for NaBr alone may be used. Of course, for a given solution density, refractometer values would be different and somewhat lower for the pure NaBr system contrasted with the NaCl-NaBr system. Maintaining a constant 0.195 molal NaCl content (approximately equivalent to the small molecule serum background) simplifies the calculation and the monitoring of densities in the NaCl-NaBr system.

B. Preparation of Total Lipoprotein Fractions.

Exact manipulation of plasma or serum density is difficult because of variations in amount of total macromolecules and in the composition of the small molecule background. For most purposes, however, serum may be considered 94% by volume a solution of ρ^{1}_{20} = 1.0063 g/ml (0.195 M NaCl). The examples presented here are based on this simplifying assumption.

The usual fractions prepared for use in the analytical ultracentrifuge are total lipoprotein fractions of density less than 1.006, 1.063 and 1.203 g/ml (after preparative centrifugation). Each of these is conveniently prepared by mixing 2 ml serum aliquots with 4 ml of the following solutions:

(a) 0.195 M NaCl (ρ = 1.0063 g/ml, $n_D 26$ = 1.33435),

(b) 2.505 M NaCl (ρ = 1.0915 g/ml, n_D26 = 1.35451) or

(c) 0.195 M NaCl - 4.471 M NaBr (ρ 1.3104 g/ml, n_D26 = 1.38596).

All these solutions contain 10 mg/100 ml EDTA. The six ml serum mixtures are put into specially soaked and washed $\frac{1}{2}$ x $2\frac{1}{2}$" heavy walled (0.014") cellulose nitrate tubes and capped with an assembly using stainless steel stems. Preparative tubes should be carefully sized with a gauge for proper length and diameter. Over sized tubes may be shrunk by immersing in hot water for several days. All tubes should be soaked thoroughly in warm water overnight, rinsed 10 times and then soaked overnight in distilled H_2O. After rinsing 3 times with distilled H_2O, the tubes are placed in a basket to drain and dried for 2 days at 37°C in an explosion-proof dust-free oven. Depending on rotor speed desired, preparative centrifugation is done in a 40.3 or a 50.3 rotor. For the two lower density fractions, 18°C runs at 40,000 rpm for 18 hrs are recommended. For the total HDL fraction, 24 - 26 hrs is needed at the same temperature and rotor speed. Although rotor speeds above 40,000 rpm may be desirable in reducing running time, it should be balanced against the increased hazard of occasional tube collapse with sample loss. Nearly filling the preparative tube with additional background solutions will tend to minimize this loss, but this will greatly increase the difficulty in quantitative removal of the lipoproteins. Unless unusual circumstances require higher speeds, adding exactly 6.0 ml solution to each tube and running at 40,000 rpm are recommended.

C. Sequential Preparation of Serum VLDL, LDL and HDL Lipoproteins.

Frequently it is necessary to isolate total VLDL, LDL and HDL lipoprotein

fractions from plasma or serum. However, one difficulty involves the separation of exogenous chylomicra and, for the most part endogenous VLDL. Perhaps at the present time the best approach to the problem is by an operational definition (19), namely that lipoproteins of $S_f > 400$ be classed as chylomicra and S_f 20 - 400 be defined as VLDL. It should be understood that in a given serum there may be exogenous lipoproteins smaller than S_f 400 and endogenous lipoprotein larger than S_f 400 ($\sim$757 Å diameter). Indeed, there is evidence that these two classes may have substantially overlapping distributions (20,21).

Isolation of contamination-free total $S_f > 400$ lipoprotein fractions require a special density gradient procedure which is described in a later section. However, if large amounts of $S_f > 400$ lipoproteins are present in serum, they can be conveniently and quantitatively removed. Using a swinging bucket rotor, such as the SW 25.3 (or SW 27) fitted with buckets to handle $\frac{1}{2}$" x $2\frac{1}{2}$" preparative tubes, the procedure is simply to ultracentrifuge 6 ml of whole serum in each bucket for an appropriate time. This centrifugation is that calculated to quantitatively float S_f 400 molecules from the bottom of the preparative tube into the top 0.5 ml fraction. Using serum values of $\eta = 1.755$ and $\rho = 1.0259$ g/ml at 23°C (22), the needed centrifugation is 4.27×10^6 g.min.^{2} This requires a total of 59.2 minutes of full speed centrifugation at 12,000 rpm (including 1/3 of the acceleration and deceleration times) using the 25.3 rotor (mean r =10.44 cm). Following centrifugation the preparative tubes are carefully removed from each bucket and firmly held at a 45° angle. The $S_f < 400$ fraction is collected by puncturing the bottom of each tube at the lowest point with a sharp #22 needle and collecting the 5.5 ml undernatant. Before

puncturing the preparative tube, the bottom is wiped with clean gauze dampened with distilled H_2O and then dried with a lint-free wiper such as a Kimwipe. Although $S_f > 400$ molecules are removed from the undernatant fraction, this procedure also partially removes some S_f 20 - 400 VLDL. Thus, the $S_f < 400$ undernatant contains a somewhat distorted and reduced VLDL distribution compared to that present in the original serum sample (see section VI E). In preparing VLDL fractions, the advantage of $S_f > 400$ removal as well as the disadvantage of recovering a distorted VLDL distribution should be considered. Generally, when $S_f > 400$ lipoproteins are present in relatively large amounts the above procedure is recommended.

After collecting the $S_f < 400$ serum undernatant, sequential isolation of the VLDL, LDL and HDL fractions utilizes ½" x 2½" cellulose nitrate tubes and either 40.3 or 50.3 Beckman rotors (Beckman Instruments, Palo Alto, Ca.)[3]. All runs are made at approximately 18°C in a Beckman preparative ultracentrifuge equipped with a diffusion pump. Six ml of a serum solution containing 2 -6 ml of serum $S_f < 400$ undernatant (the balance supplied by 0.195 M NaCl, $\rho = 1.0063$ g/ml, $n_D 26 = 1.33435$) is ultracentrifuged for 18 hr at 40,000 rpm. All solutions used contain 10 mg EDTA/100 ml. The S_f 20 - 400 VLDL fraction is quantitatively removed in the first ml and a second ml taken as a reference. Then the 4 ml bottom fraction is mixed with 2 ml of a 0.195 molal NaCl - 2.398 M NaBr solution, $n_D 26$ 1.36446, $\rho = 1.1816$ g/ml, giving a resultant density before centrifugation of 1.065 g/ml. After again centrifuging for 18 hrs (in new preparative tubes) the S_f 0 - 20 LDL lipoproteins are quantitatively recovered in the top ml of background density $\rho = 1.063$ g/ml and a second ml taken as a reference.

The 4 ml bottom fraction is mixed with 2 ml of a 0.195 M NaCl - 7.620 M NaBr solution n_D^{26} 1.41267, ρ = 1.4744 g/ml, this time resulting in a solution of density before centrifugation of 1.215 g/ml. After a final centrifugation of from 24 - 26 hours, the total HDL fraction is collected in the top 1 ml of background density ρ = 1.203 g/ml and a second ½ ml taken as reference. It is recommended that each fraction, and particularly the reference fractions, be measured by refractometry to verify background densities. If salt redistribution is considered, the 2nd ml (or ½ ml) may be extrapolated to give the appropriate background refractive index and density of the lipoprotein fraction. Precision refractometry as described later can provide convenient quantification of each lipoprotein fraction.

D. Quantitative Removal of Preparative Lipoprotein Fractions.

Although the use of a tube slicing device may have advantages in special applications, it is not recommended for lipoprotein work. Pipetting provides the best quantitative removal of lipoprotein fractions with visual evaluation of completeness and with minimal disturbance to the remainder of the preparative tube. A special thin-walled Pasteur pipette with an inside bore of 0.4 - 0.6 mm is available commercially (Microchemical Specialties, Berkeley, Ca.).[3] Pipetting is done in a darkened room on a fixture equipped with a focused light beam allowing visualization of the lipoproteins by their Tyndall scattering.

After the rotor has stopped great care should be exercised to avoid any abrupt movement of the rotor or of each individual preparative tube during

manipulation prior to pipetting. Normally, for each lipoprotein containing sample two fractions are pipetted in depth, the top milliliter containing quantitatively the lipoprotein fraction and the second milliliter (or half milliliter in the 1.20 g/ml HDL run) providing (for refractometry) a reference salt background for that sample. Where unexpectedly large amounts of lipoproteins are present preventing quantitative removal in 1 ml, the fractions may be removed in 2 ml (or 1.5 ml in the HDL run) with a third ml taken as a salt reference for the 1.006 and 1.063 g/ml runs. The fractions collected in calibrated 1 ml, 2 ml (or 0.5 ml) volumetric vials are transferred for storage into 9 ml air-tight screw cap vials (No. 60910, Owens-Illinois, Co., Toledo, Ohio)[3] fitted with washed plain pulp liners and Teflon gaskets. On the same day as pipetting and before storage at 4°C, refractive index measurements are made with a Bausch & Lomb precision Abbe refractometer (23) with a range of n_D from 1.203 to 1.508. Ideally, room temperature should be approximately 23°C and the refractometer thermostated to 26 ± .03°C with a temperature controller (Precision Scientific Co., Chicago, Ill.).[3]

E. Ultracentrifugal Redistribution of Salts and Background Density Calculation.

During the type of preparative ultracentrifugal runs described for isolating all lipoprotein fractions, appreciable salt redistribution occurs from the top to the bottom of the preparative tube. The extent of this redistribution is dependent on the nature and concentration of the salt as well as the time and conditions of ultracentrifugation. Evaluation of this salt redistribution

and its reproducibility for each type of run is easily achieved by running appropriate salt background solutions and measuring each fraction in depth by refractometry. Fig. 1 shows the salt redistribution for typical 1.006 g/ml and 1.063 NaCl runs as well as for the 1.213 g/ml NaBr run. The differences between the second fraction and first fraction in precision Abbe ΔS units and corresponding $\Delta\rho$ values are reproducible and known; therefore, the background for each lipoprotein fraction can be determined from the second ml fraction (or the second 1/2 ml fraction in the HDL run). These differences are $\Delta S = 0.005$ and $\Delta\rho = 0.0001$ g/ml for the 1.0063 g/ml run; $\Delta S = 0.043$ and $\Delta\rho = 0.0011$ g/ml for the 1.065 g/ml run; and $\Delta S = 0.115$ and $\Delta\rho = 0.0035$ g/ml for the 1.213 g/ml NaBr run.

III. EVALUATION OF LIPOPROTEIN CONCENTRATIONS BY REFRACTOMETRY

By measuring the refractive index of both the lipoprotein top fraction and the undernatant background and correcting the latter to the 1st milliliter fraction, lipoprotein concentrations are obtained using the following relationships:

$$\text{Lipoprotein concentration, mg\%} = (\Delta S_{TF} - \Delta S_{BG}) \frac{K \times 10^3}{C_o \times S.R.I.}$$

where ΔS_{TF} = Abbe scale increment of top fraction salt background

ΔS_{BG} = calculated Abbe scale increment of top fraction salt background above water reference.

K = instrument-dependent conversion factor of scale increment to refractive index increment. Our values are: $K_{TLDL} = 5.30 \times 10^{-3}$ $K_{VLDL} = 5.48 \times 10^{-3}$, $K_{HDL} = 4.97 \times 10^{-3}$

Co = lipoprotein concentration factor (serum volume used/lipoprotein fraction volume).

S.R.I. = specific refractive increment as used here is the increase in refractive index $n_D 26°C$ (of the indicated background (anhydrous) salt solutions) resulting from the presence of 1 g/lipoprotein/100 ml solution. Values used are 0.00158 for VLDL, 0.00154 for TLDL, and 0.00149 for HDL in 1.006 g/ml NaCl, 1.063 g/ml NaCl and 1.203 g/ml NaBr, respectively (24).

Low density lipoprotein measurements by refractometry are useful for several purposes. Such measurements on a minimum of material provide an invarient physical method for VLDL and LDL measurement (by difference), without an analytic ultracentrifuge. If analytic centrifugation is performed, refractometry easily monitor lipoprotein concentrations insuring that only optimal concentrations of lipoproteins are run. If the LDL concentration in the isolated fraction is over 1300 mg/ml, the sample should be appropriately diluted, avoiding unnecessary and expensive re-runs. Secondly, evaluation of lipoprotein background density in both the low and high density runs as well as in subfractionation work is essential for the complete ultracentrifugal analysis described later. Lastly, if refractometry of whole serum is performed and the total lipoprotein content of serum is known, an accurate and reproducible measurement of the total serum proteins can be calculated (24).

A. Details of Refractometry Measurement

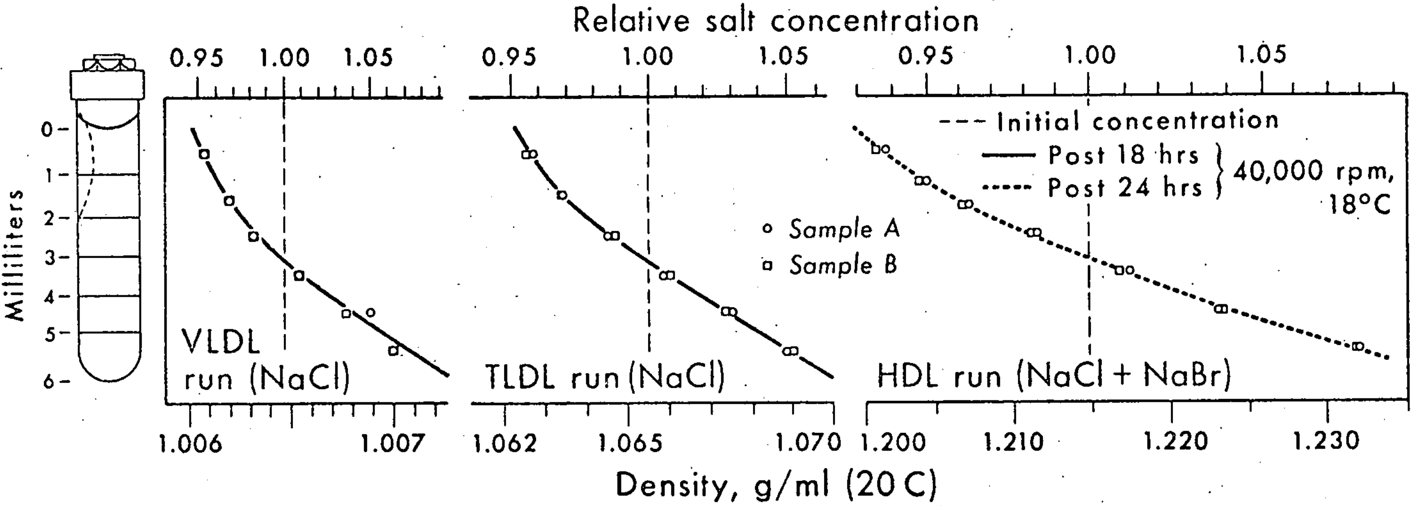
The most convenient and accurate refractometric analysis of small quantities of solution, equilibrated to room temperature, are made with the precision Abbe refractometer, temperature controlled to at least 0.1°C. Reading should be made above room temperature (in this procedure at 26°C) with scale

reading estimated to the nearest ± 0.005 units. Over the range of measurements, this accuracy corresponds to approximately ± 0.00003 in Δn (1-2 parts in the 4th place for density measurements). An ordinary Abbe, although useful in monitoring densities, is not sufficiently accurate (± 0.0002 in Δn) to give satisfactory lipoprotein results using this method.

Refractometry is done with only one drop of the lipoprotein fraction (or salt solution) by taking the "reflection" reading. Thus, nearly all the fraction is available for analytic ultracentrifugation or for lipid and protein analysis. 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 is sufficient for approximate temperature equilibration but insufficient for any significant evaporation. After each reading the surface of the opposing prisms is thoroughly washed by directing against each prism a stream of about 25 cc of distilled H_2O from a polyethylene washing bottle. Thereafter, the prism surfaces are wiped (unidirectionally) with a non-abrasive wiper (Kimberly Clark type 900-S).[3] To insure a dry prism surface, an unheated air stream is directed for 5 sec onto each prism surface from a hair dryer (Oster, Model 202).[3]

IV. ANALYTIC ULTRACENTRIFUGAL ANALYSIS OF SERUM LIPOPROTEIN FRACTIONS

Because ultracentrifugal lipoprotein studies usually involve flotation analysis in salt solutions and because stability of preparations may be essential, special precautions are needed. For example, whenever salt solutions greater than a few tenths molal concentration are used at high rotor speed,



DBL 701-5545

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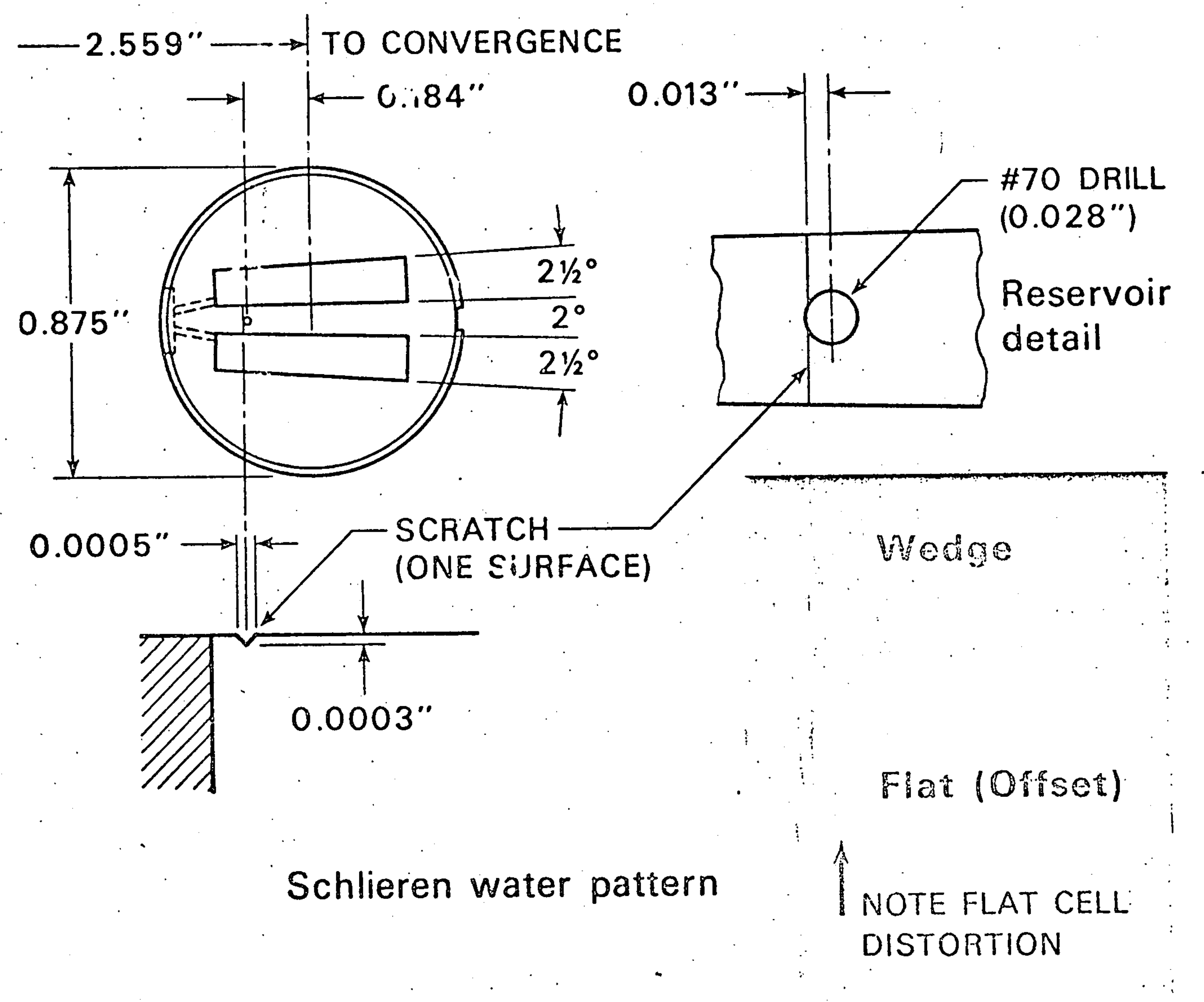
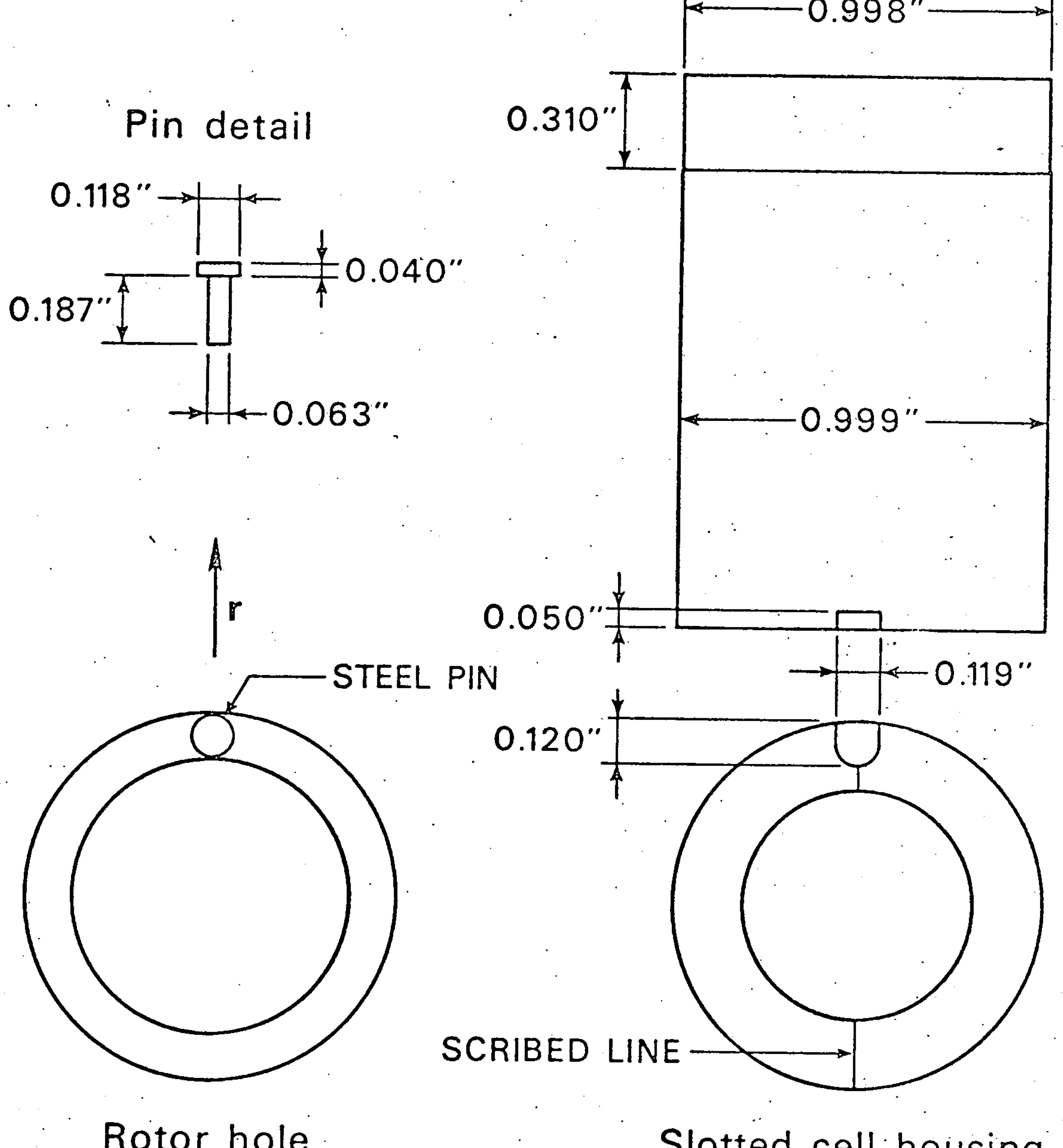


Schlieren water pattern



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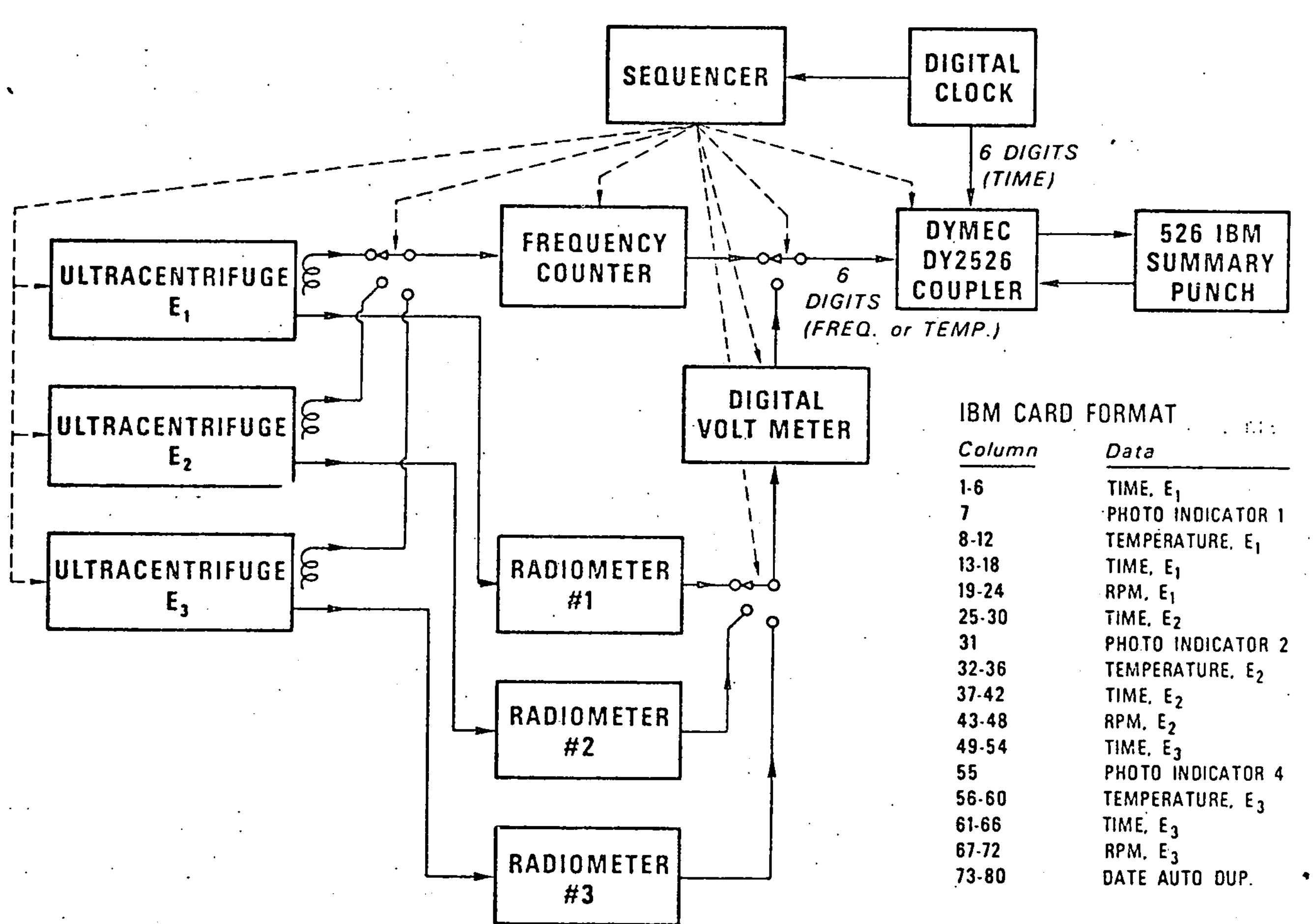

IBM CARD FORMAT

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8-12	TEMPERATURE, E_1
13-18	TIME, E_1
19-24	RPM, E_1
25-30	TIME, E_2
31	PHOTO INDICATOR 2
32-36	TEMPERATURE, E_2
37-42	TIME, E_2
43-48	RPM, E_2
49-54	TIME, E_3
55	PHOTO INDICATOR 4
56-60	TEMPERATURE, E_3
61-66	TIME, E_3
67-72	RPM, E_3
73-80	DATE AUTO DUP.

DBL 695-4698

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2	14H42M30S	0.	52687	25.00	1.9670
3	14H44M30S	2.00	52639	24.91	3.9674
4	14H48M30S	6.00	52639	24.91	7.9673
5	14H50M30S	8.00	52640	24.93	9.9672
6	14H56M30S	14.00	52640	24.95	15.9671
7	15H 4M30S	22.00	52639	24.99	23.9667
8	15H12M30S	30.00	52639	25.02	31.9664
9	15H30M30S	48.00	52639	25.06	49.9655
10	15H46M30S	64.00	52639	25.13	65.9648

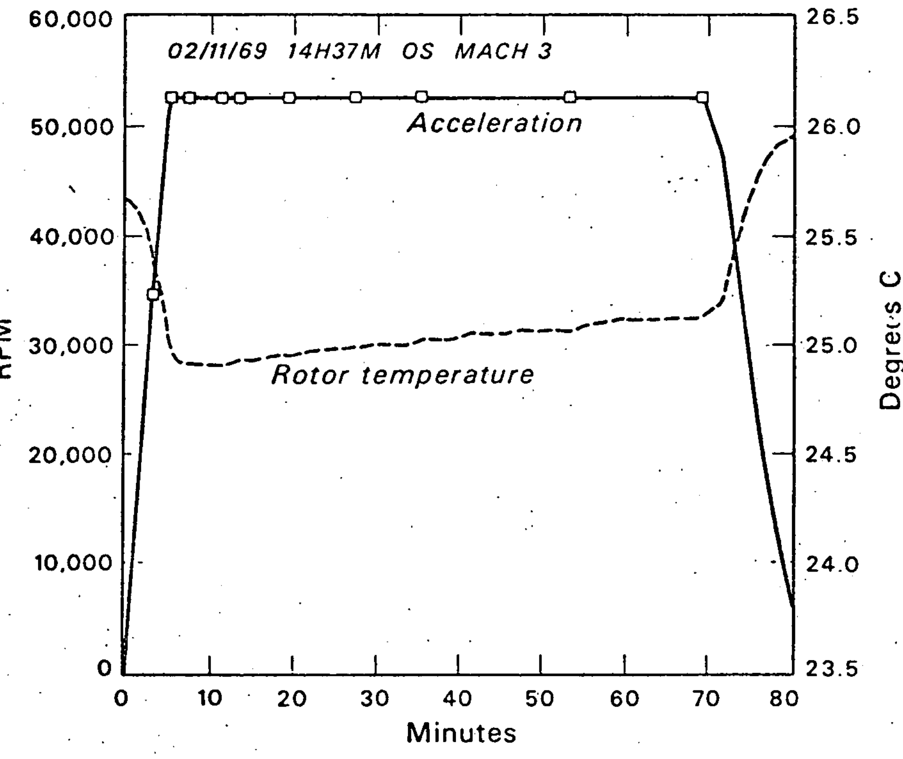
UP-TO-SPEED RPM = 52638.9 (+- .63) N = 32

M-C TEMPERATURE AVERAGES..

D-RUN, FRAMES 5-10 25.04

G-RUN, FRAMES 2- 7 24.95

DBL 695-4700



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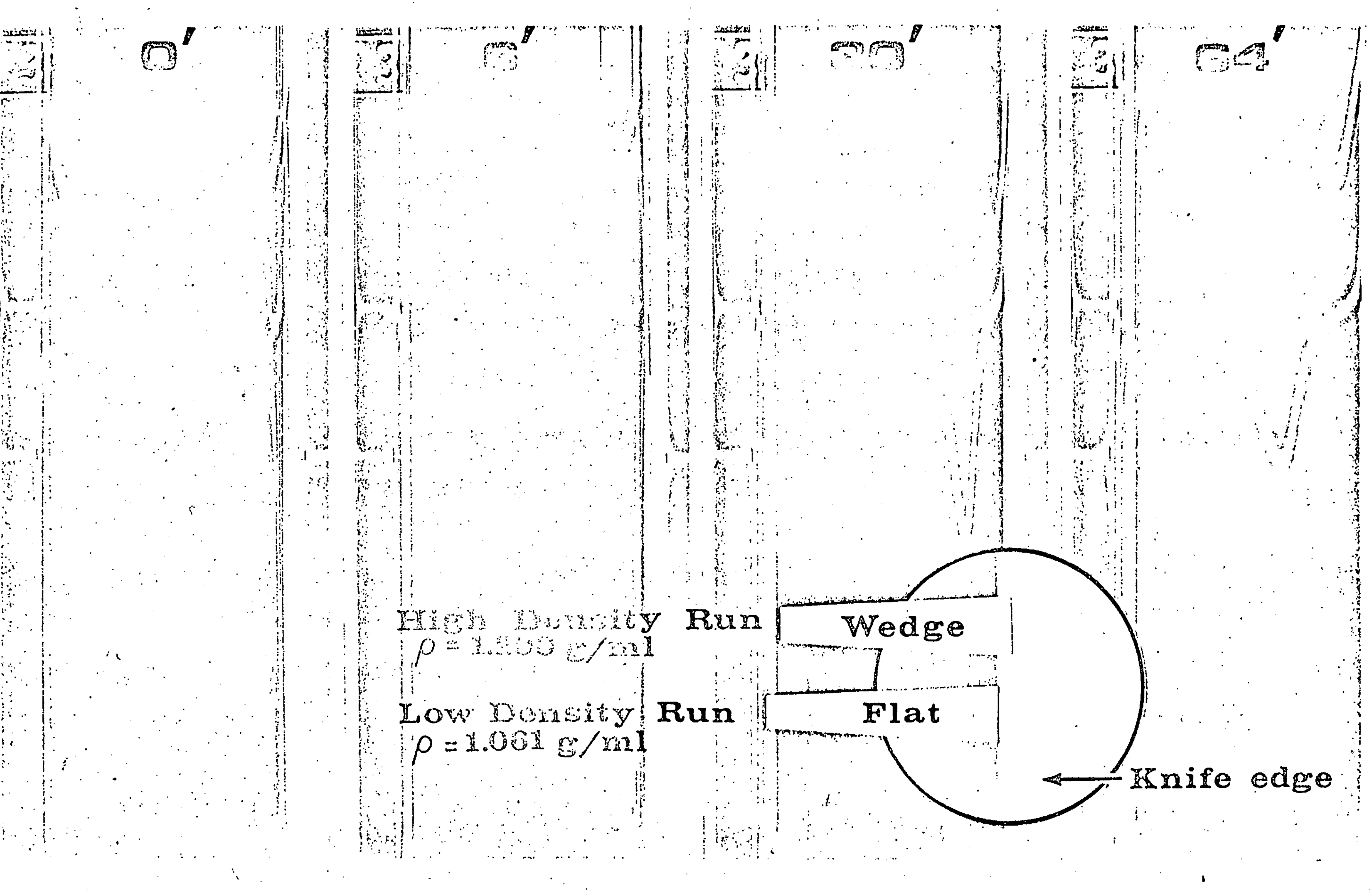
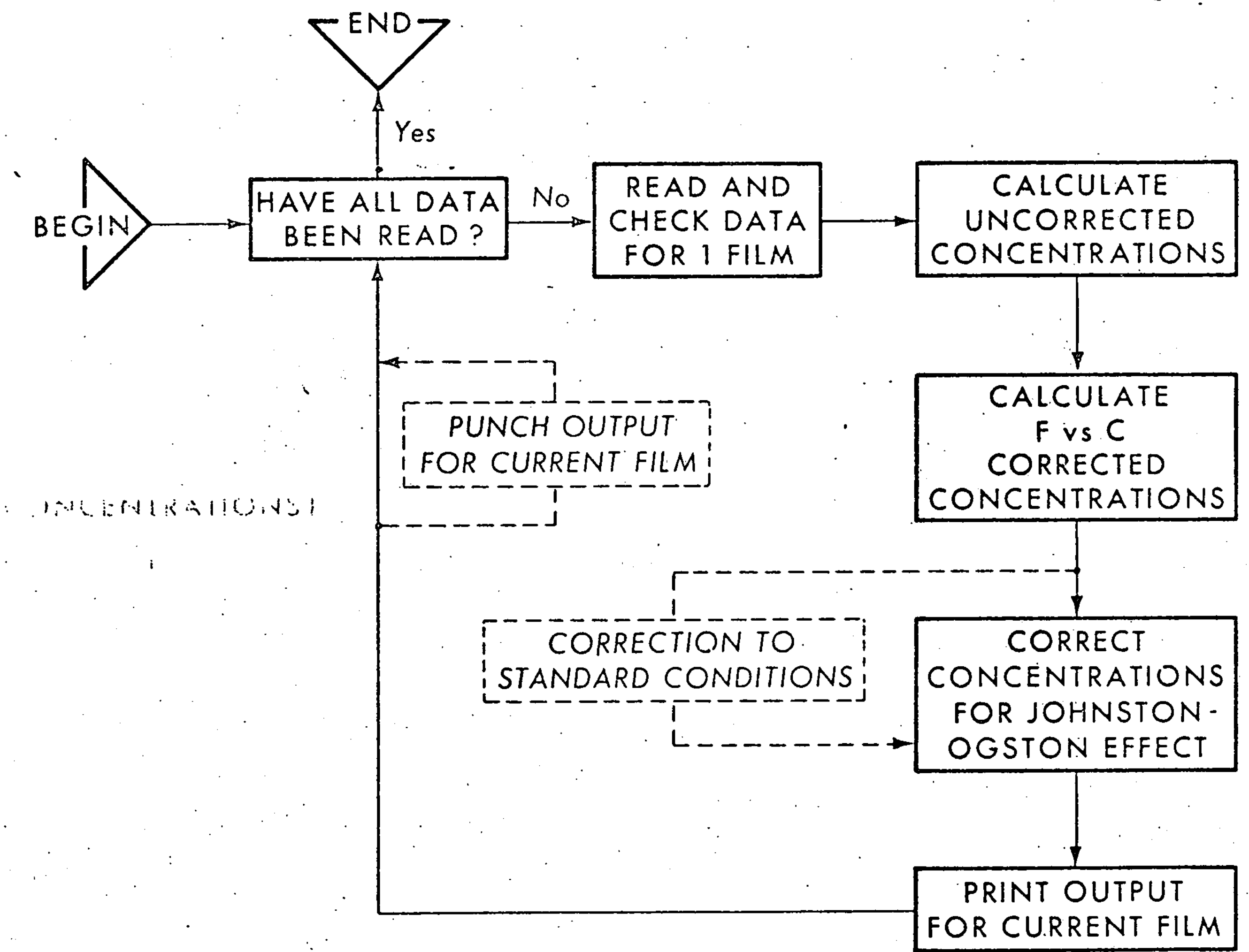
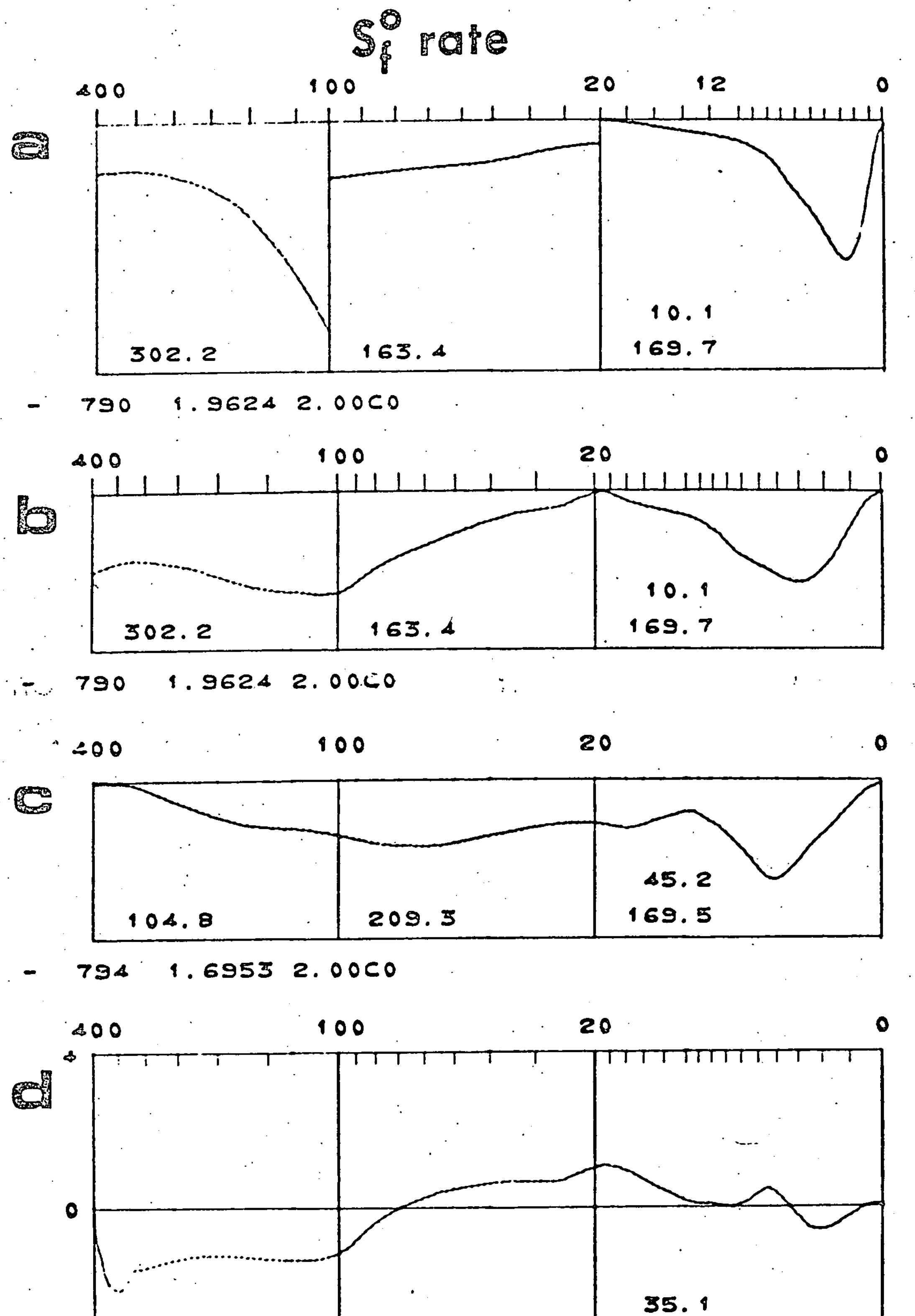


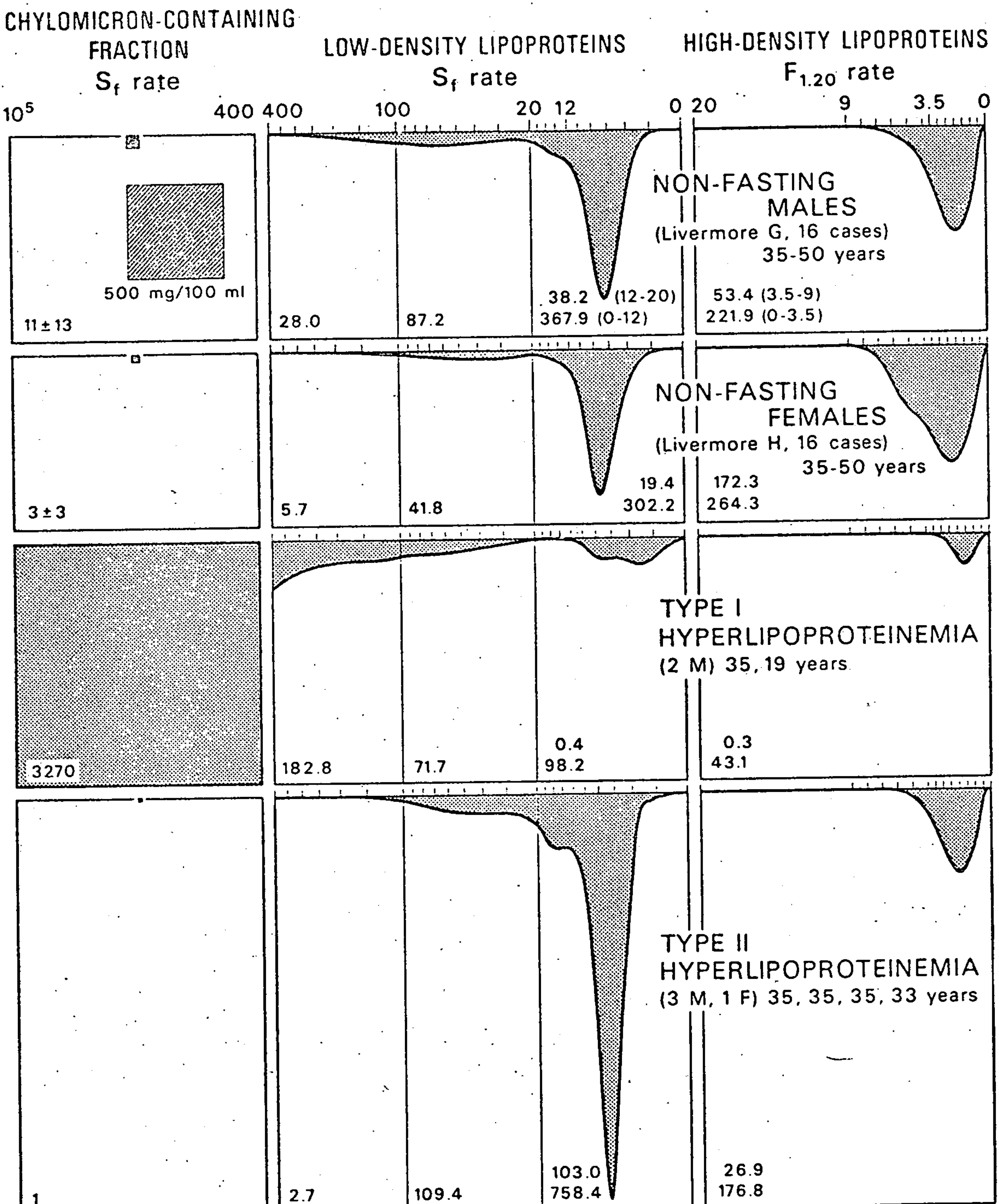
0'
High Density Run
Wedge
Low Density Run
Flat
ρ = 1.061 g/ml
Knife edge



MUB-3854



S_f^o rate
400
100
20
12
0
a
302.2
163.4
10.1
169.7
- 790 1.9624 2.00C0
b
302.2
163.4
10.1
169.7
- 790 1.9624 2.00C0
c
104.8
209.3
45.2
169.5
- 794 1.6953 2.00C0
d
-197.4
45.9
35.1
-.2
MINUS 790
HIGHLAND 1 2.00C0



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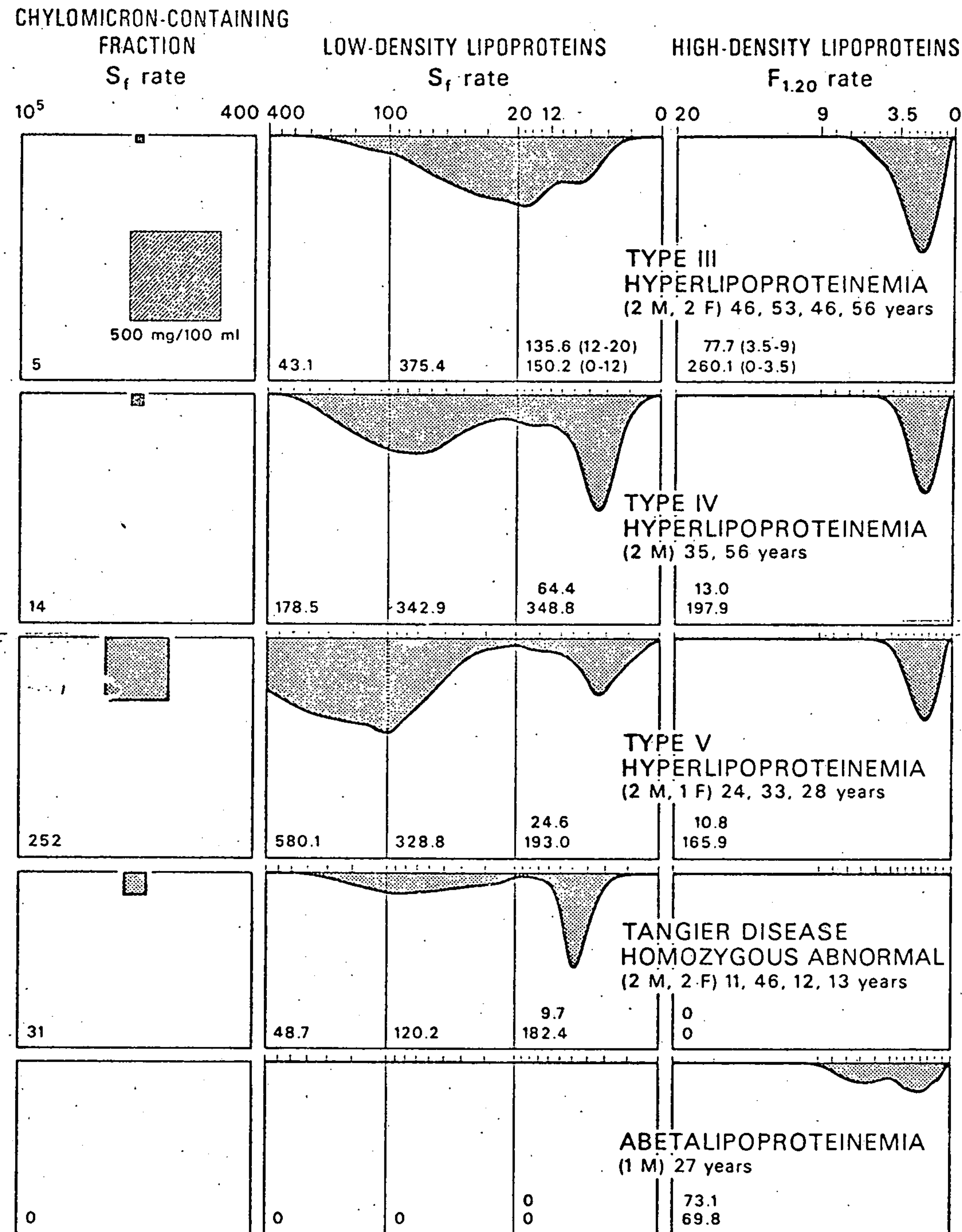

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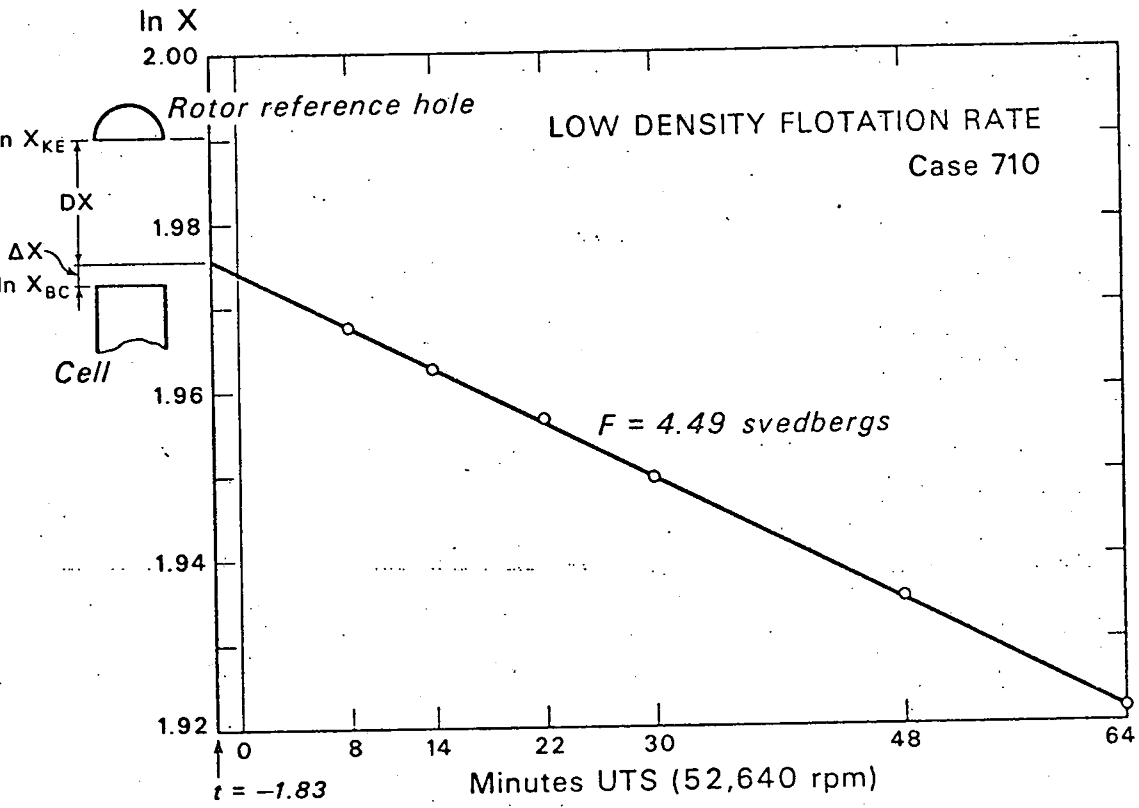


DBL 6912-5191

FILM 710D. E3 FACTOR = 11.51 JULY-20-67 YES-M 25 NOV 1969

Calculation 1

TIME FRAME	DX(CM)	T*W**2	LN(X)	D LN(X)
5	1.90	.001459	1.96766	-.00007
6	2.32	.002552	1.96255	-.00027
7	2.80	.004011	1.95667	.00041 ←
8	3.37	.005470	1.94965	-.00005
9	4.54	.008751	1.93507	.00013
10	5.60	.011668	1.92169	-.00014

BEST FIT FLOTATION RATE = -4.496

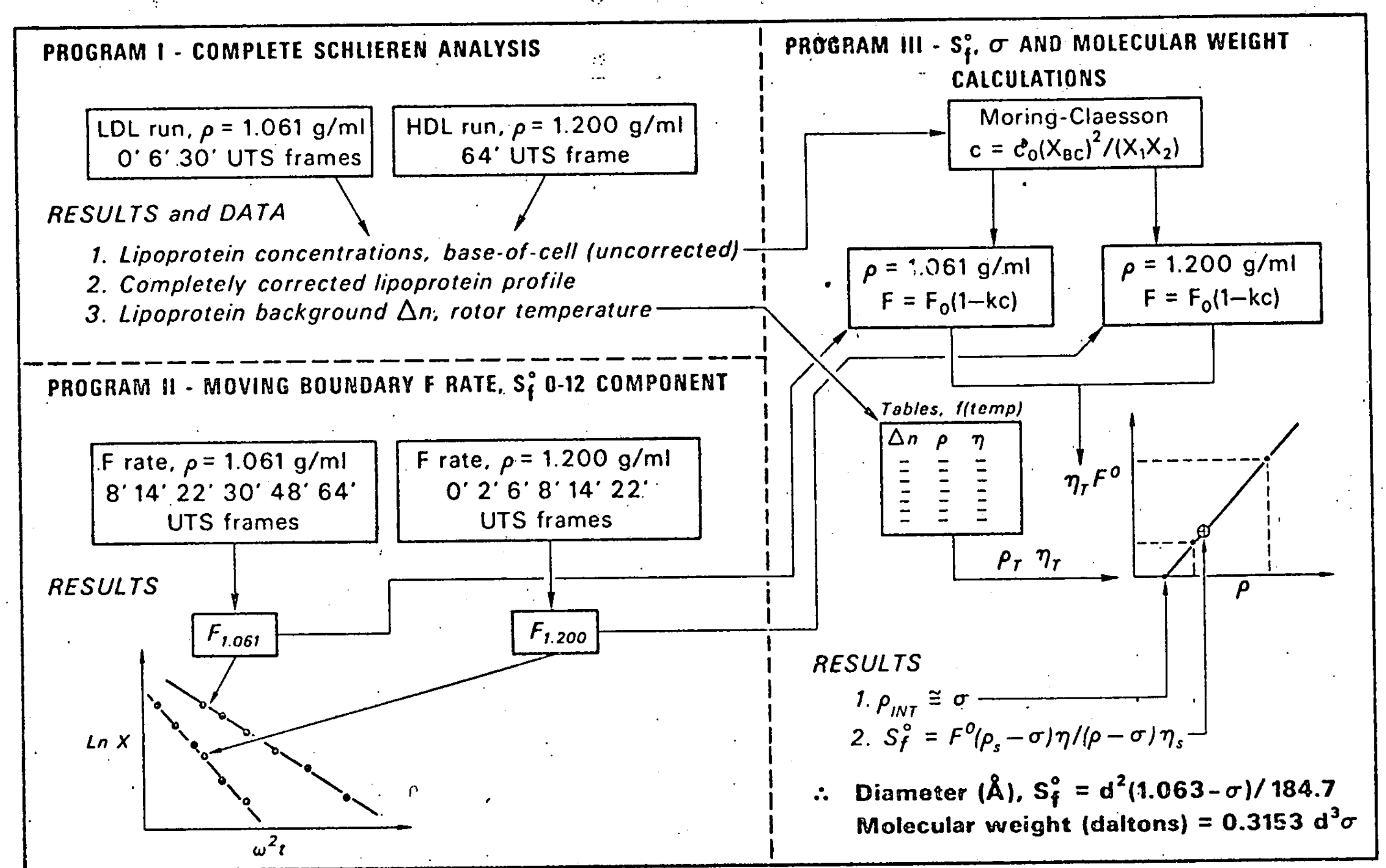
BASE OF CELL/	UTS	DX(CM)	ABS R
	1.733	1.23	7.2118
	1.833	1.23	7.2124

Calculation 2

TIME FRAME	DX(CM)	T*W**2	LN(X)	D LN(X)
5	1.90	.001459	1.96766	.00006
6	2.32	.002552	1.96255	-.00015
8	3.37	.005470	1.94965	.00003
9	4.54	.008751	1.93507	.00018
10	5.60	.011668	1.92169	-.00013

BEST FIT FLOTATION RATE = -4.485

BASE OF CELL/	UTS	DX(CM)	ABS R
	1.733	1.25	7.2107
	1.833	1.24	7.2112



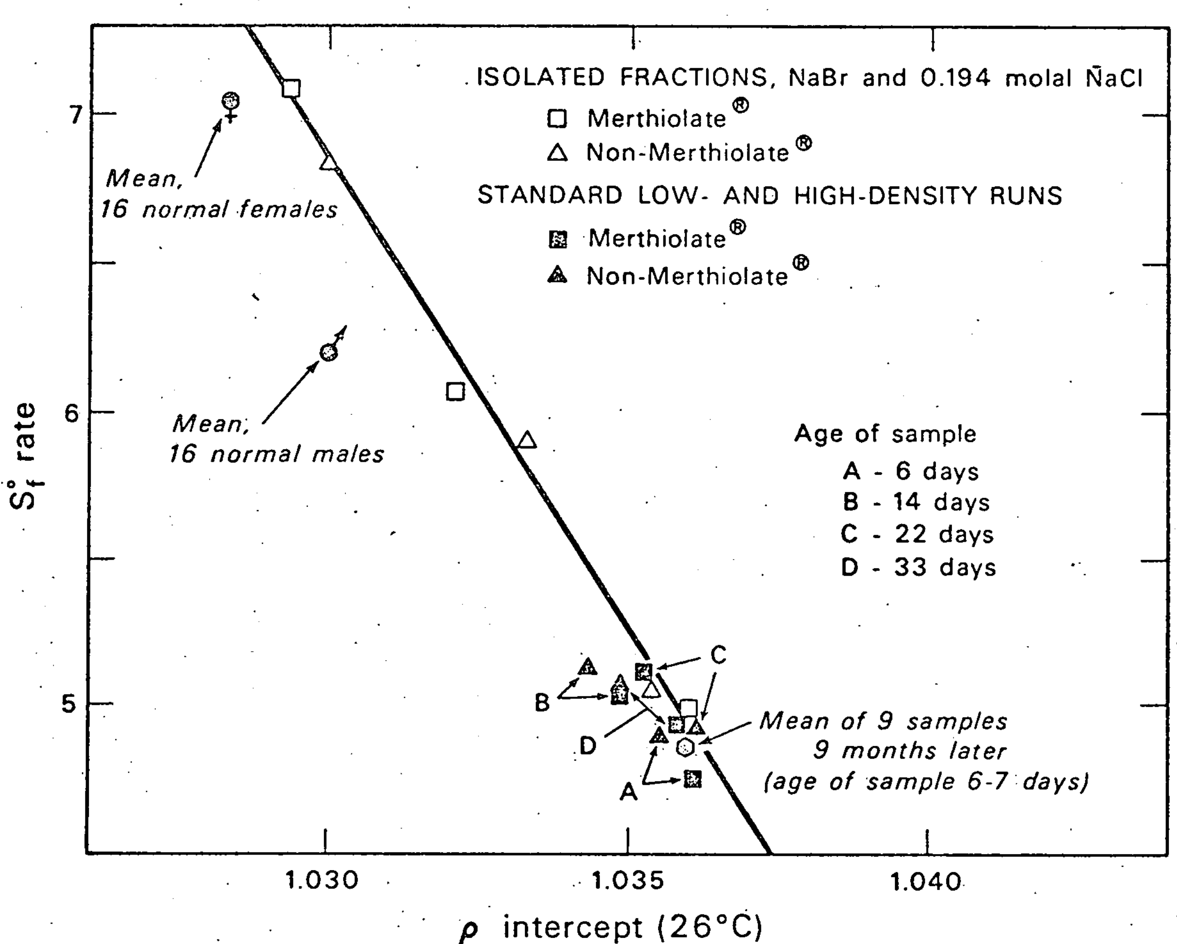
DBL 680-5476

6′

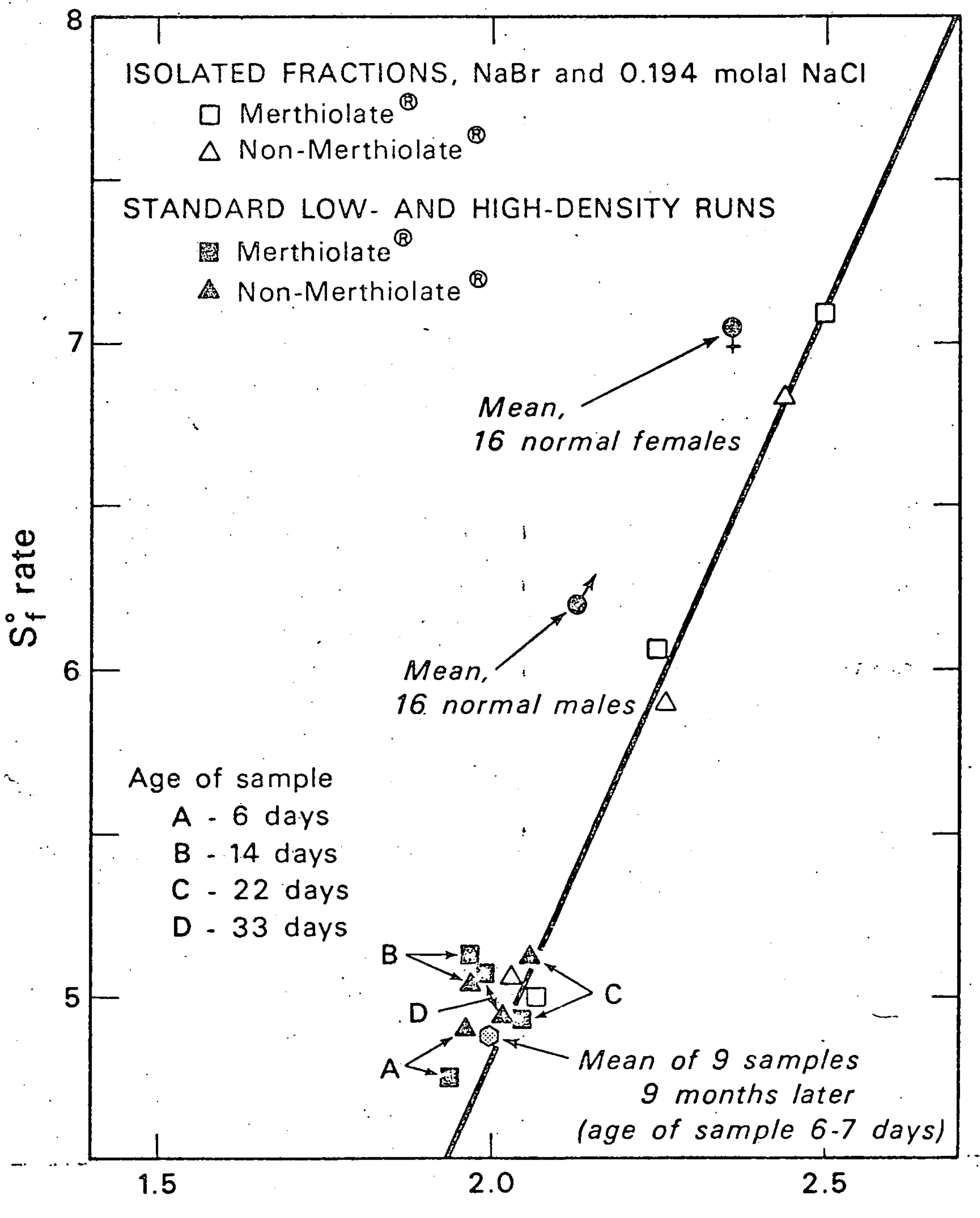
8′

14′

22′



DBL 683-4626



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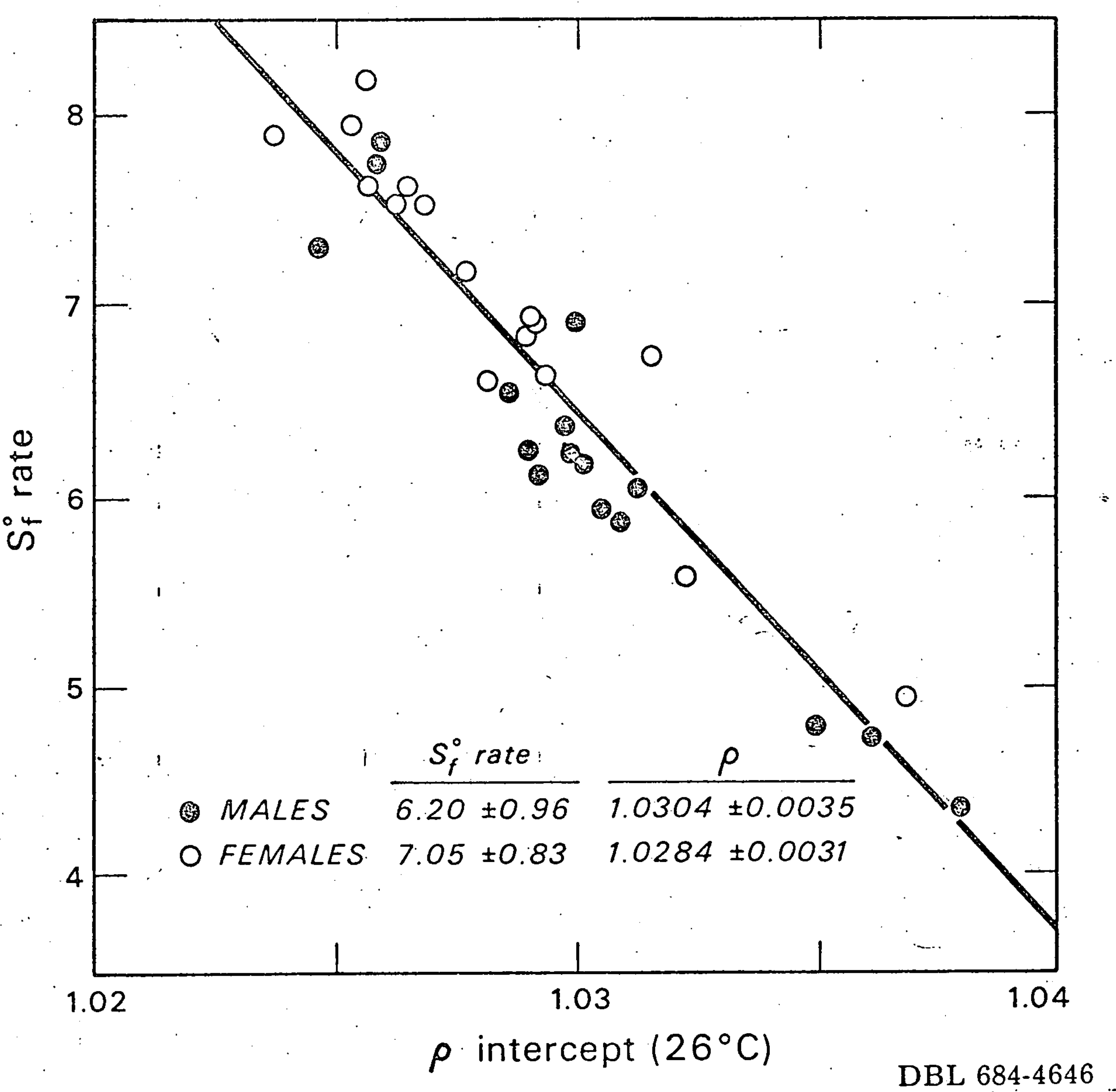

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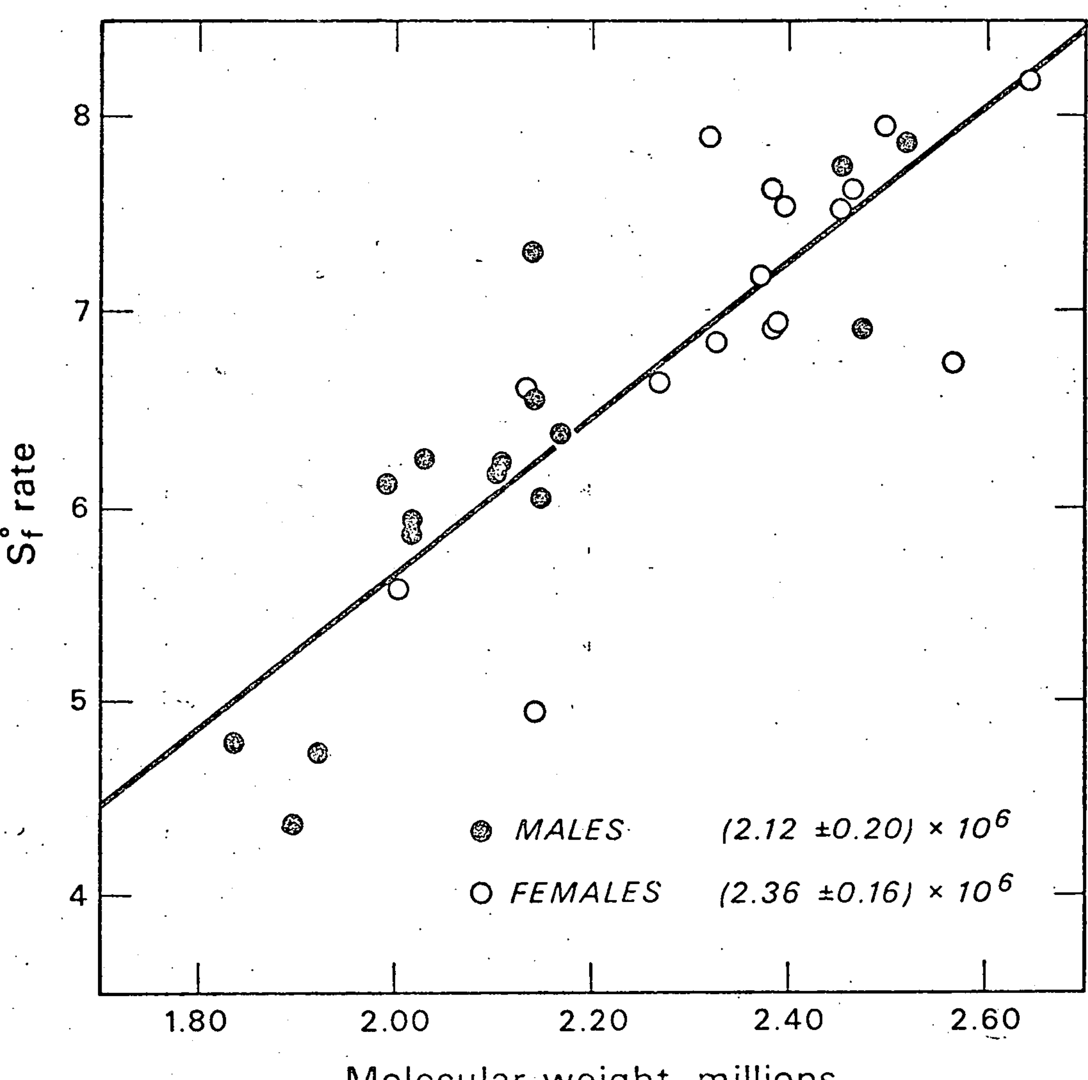
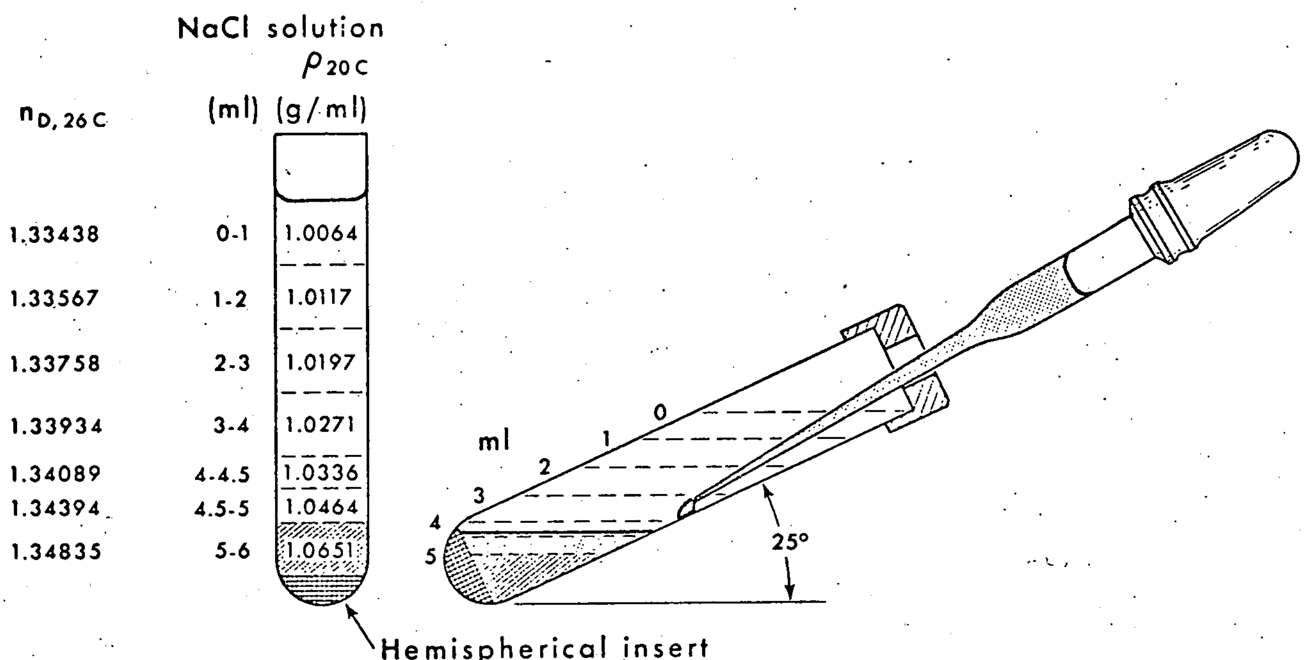
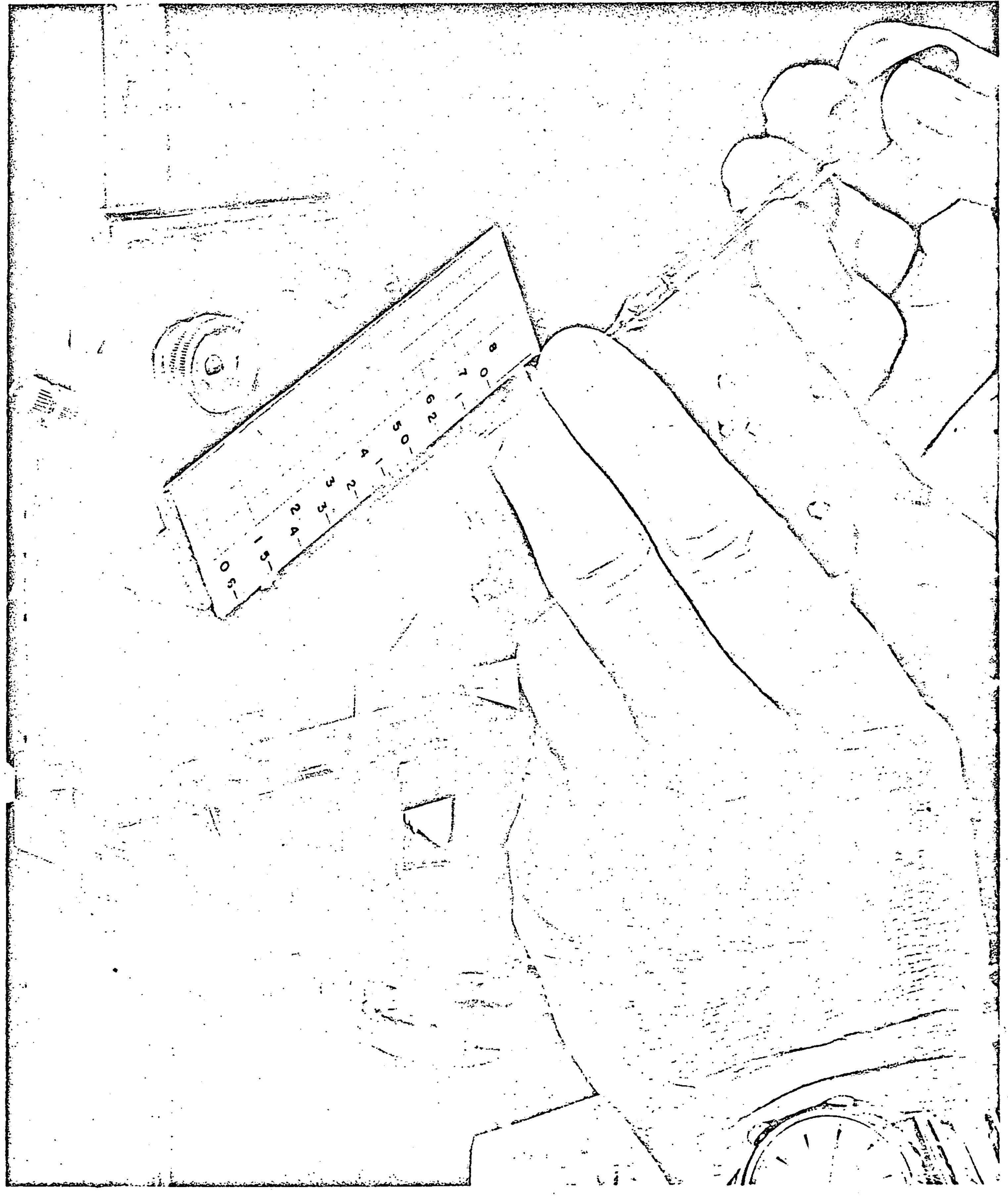
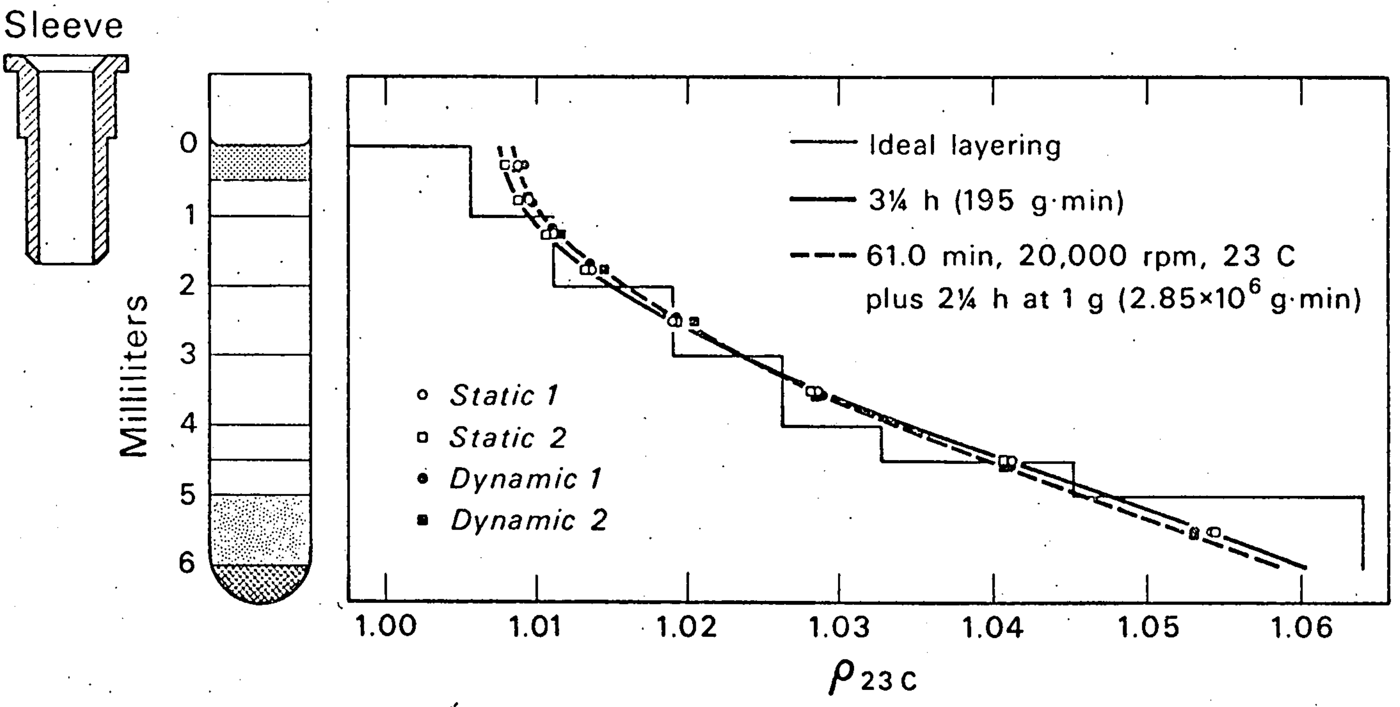


DBL 684-4651

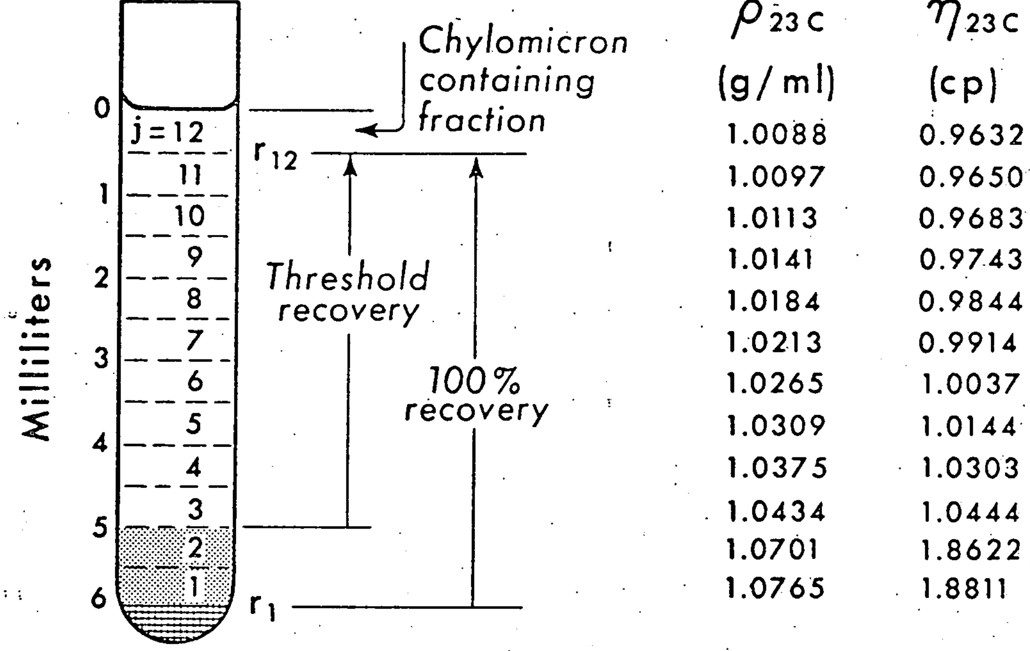

NaCl GRADIENT PREPARATION, 6 ml preparative tube
(Successive 0.5 and 1 ml layerings, drop by drop over 1 ml salt solution or serum)
NaCl solution
ρ_{20C}
$n_{D, 26C}$
(ml)
(g/ml)
1.33438
0-1
1.0064
1.33567
1-2
1.0117
1.33758
2-3
1.0197
1.33934
3-4
1.0271
1.34089
4-4.5
1.0336
1.34394
4.5-5
1.0464
1.34835
5-6
1.0651
Hemispherical insert
ml
0
1
2
3
4
5
25°
MUB-12754



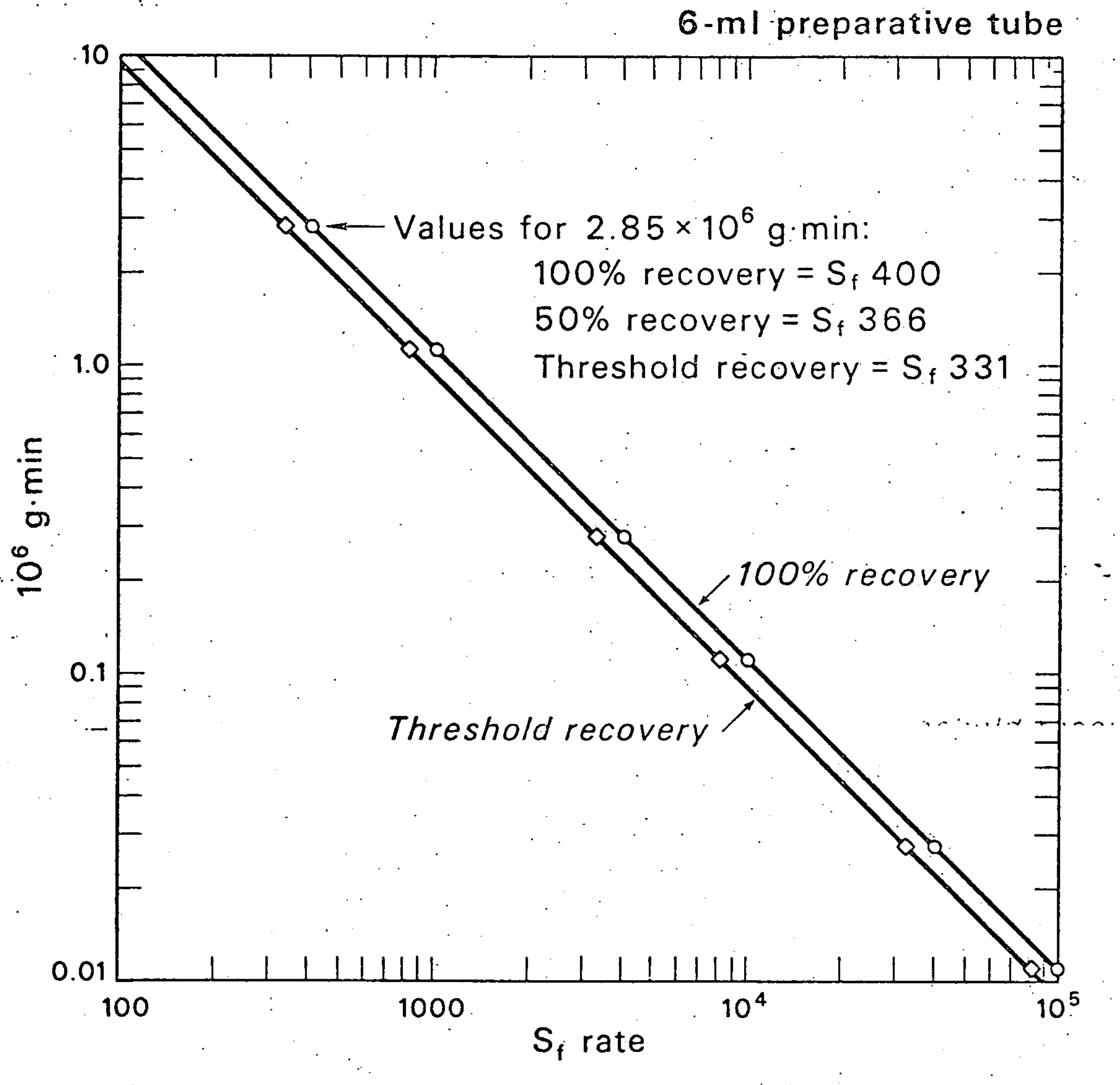


DBL 702-5575

LIPOPROTEIN RECOVERY, S_f 400-10^5 chylomicron isolation

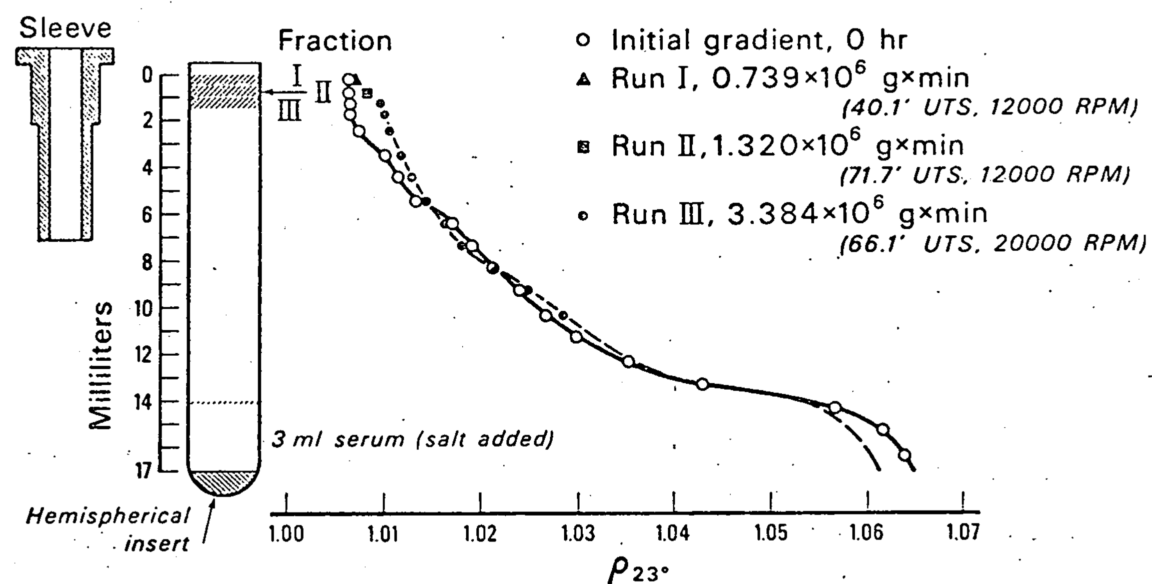


MUB-12758

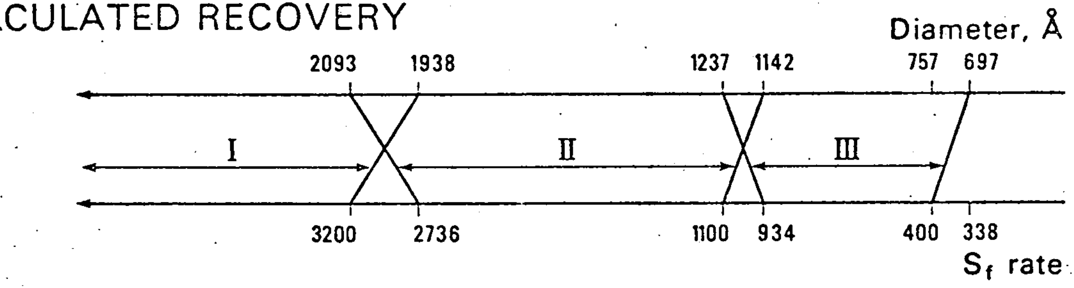


DBL 702-5551

SERUM CHYLOMICRON-CONTAINING FRACTION, SUBFRACTIONATION, S_f 400-10^5



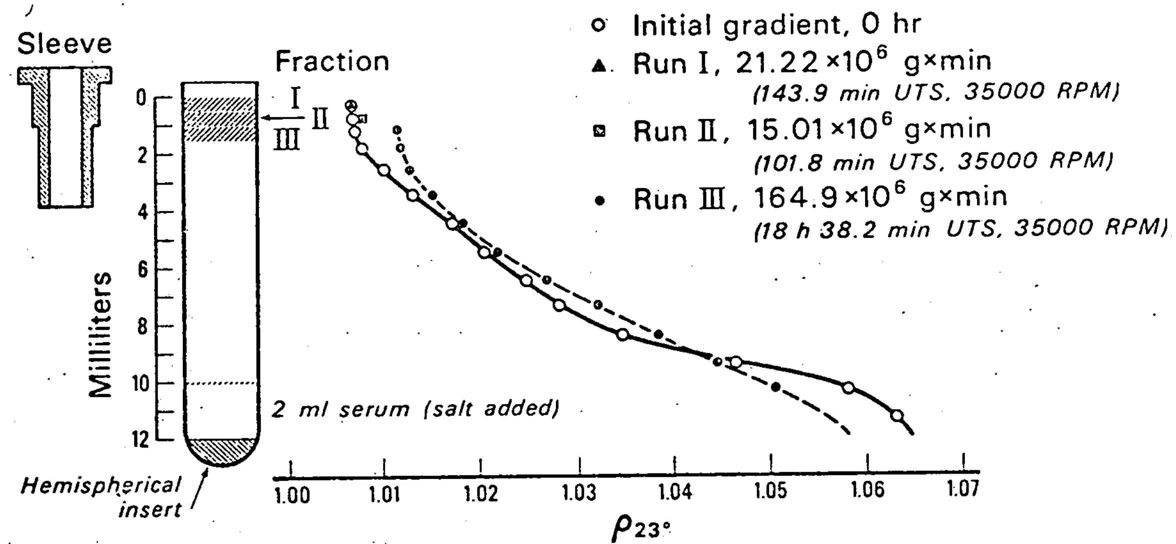
CALCULATED RECOVERY



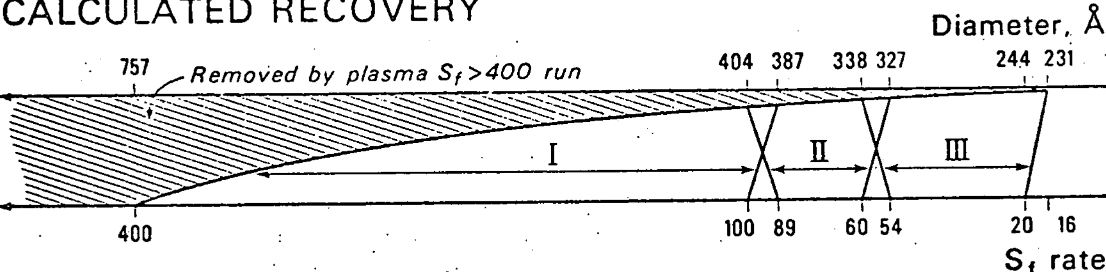
DBL 685-4739

SERUM VLDL, SUBFRACTIONATION, S_f 20-400

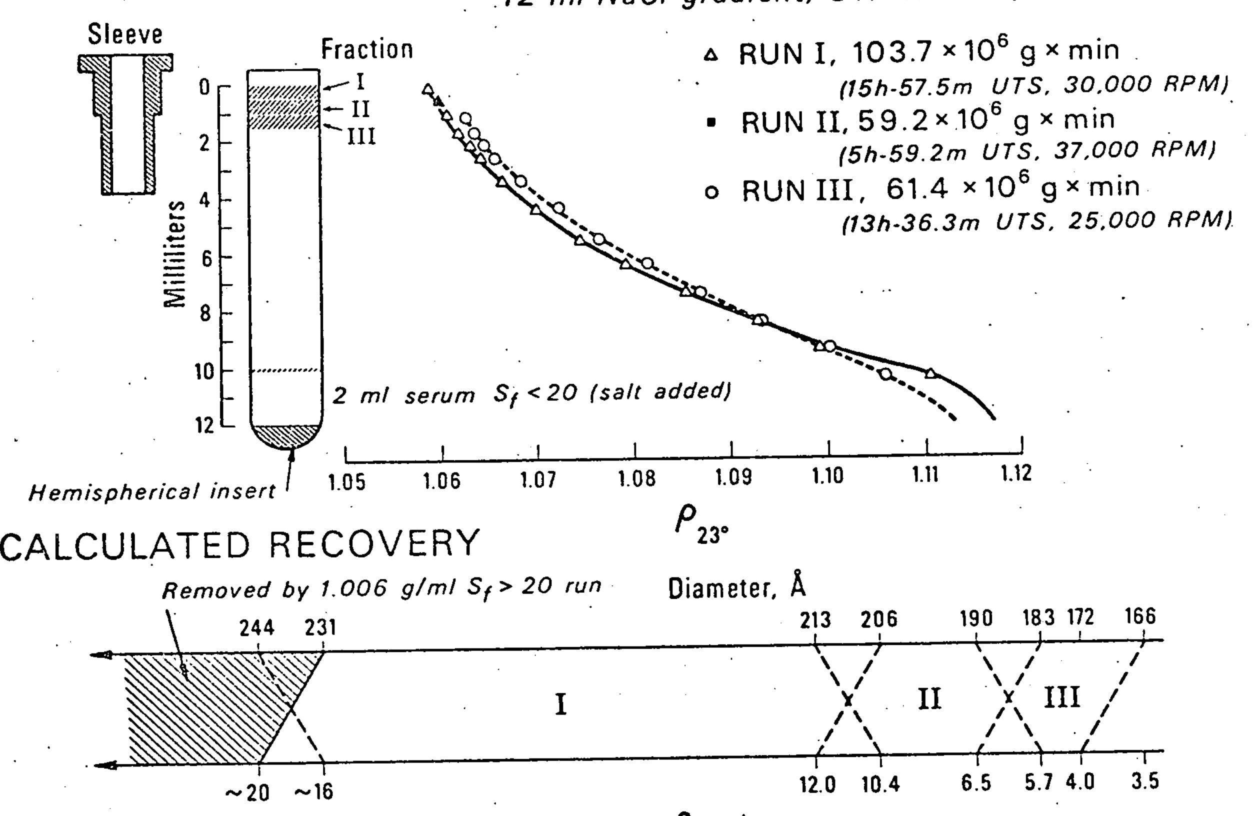
12 ml gradient, SW 41 rotor, 23 °C



CALCULATED RECOVERY

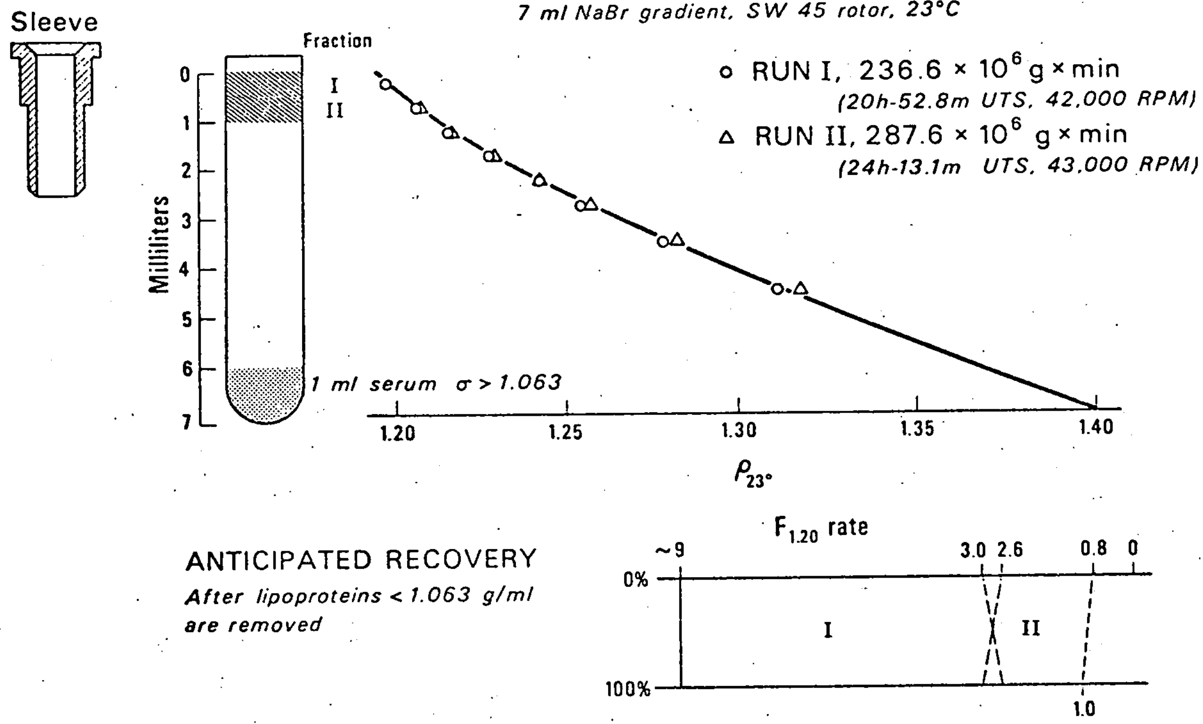


DBL 685-4740

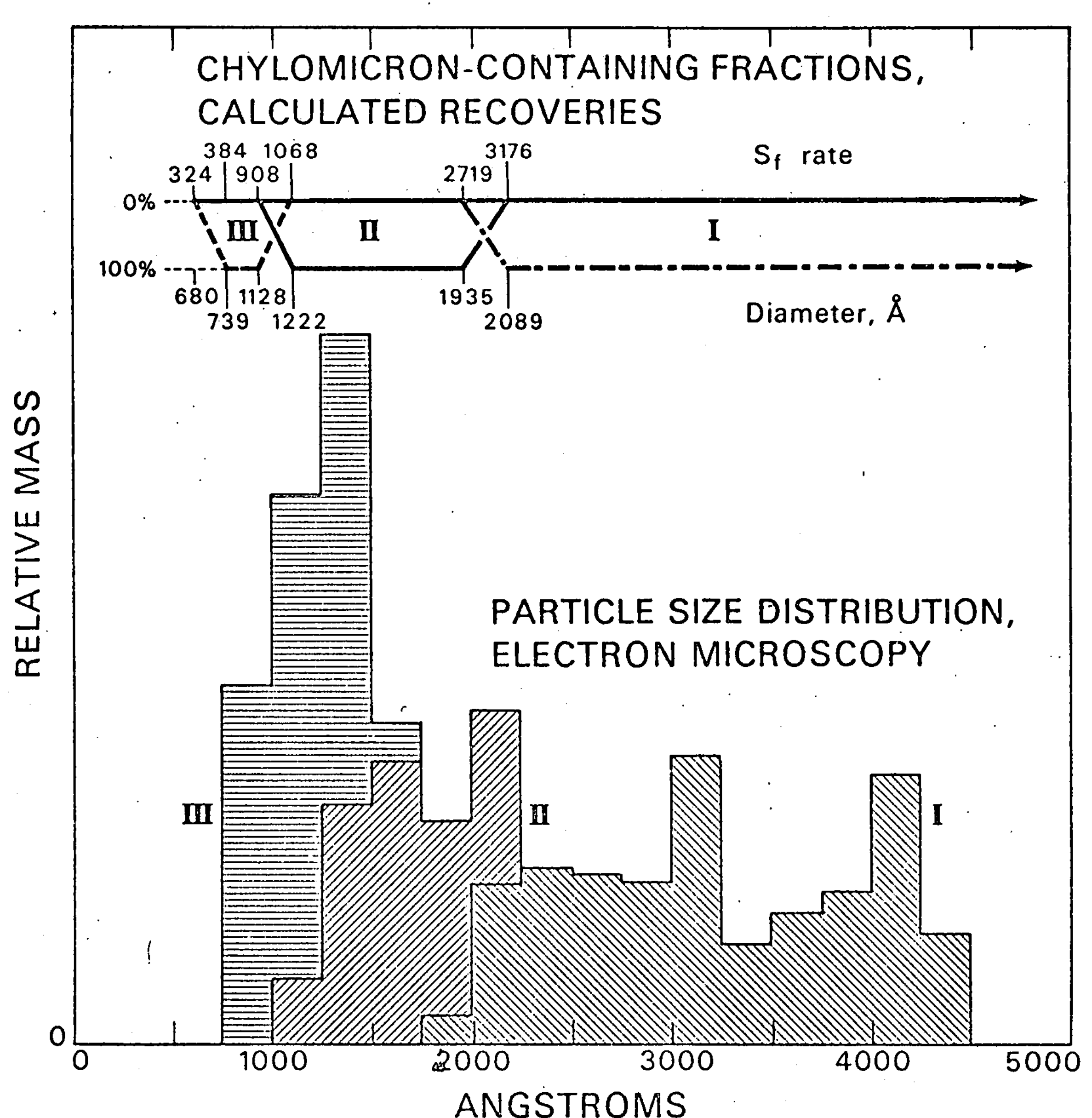


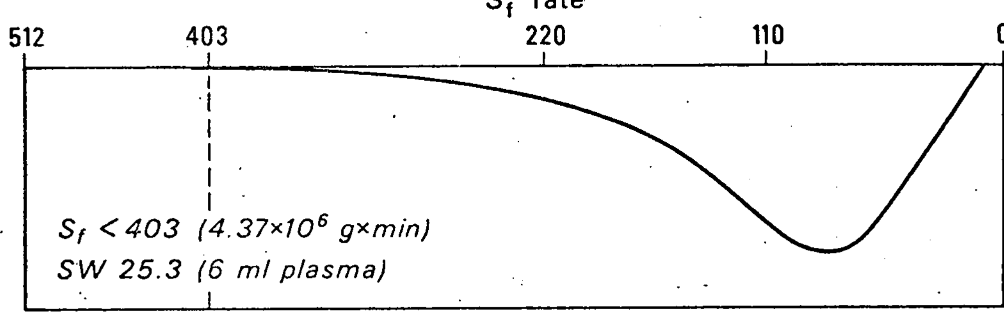
DBL 6910-5091

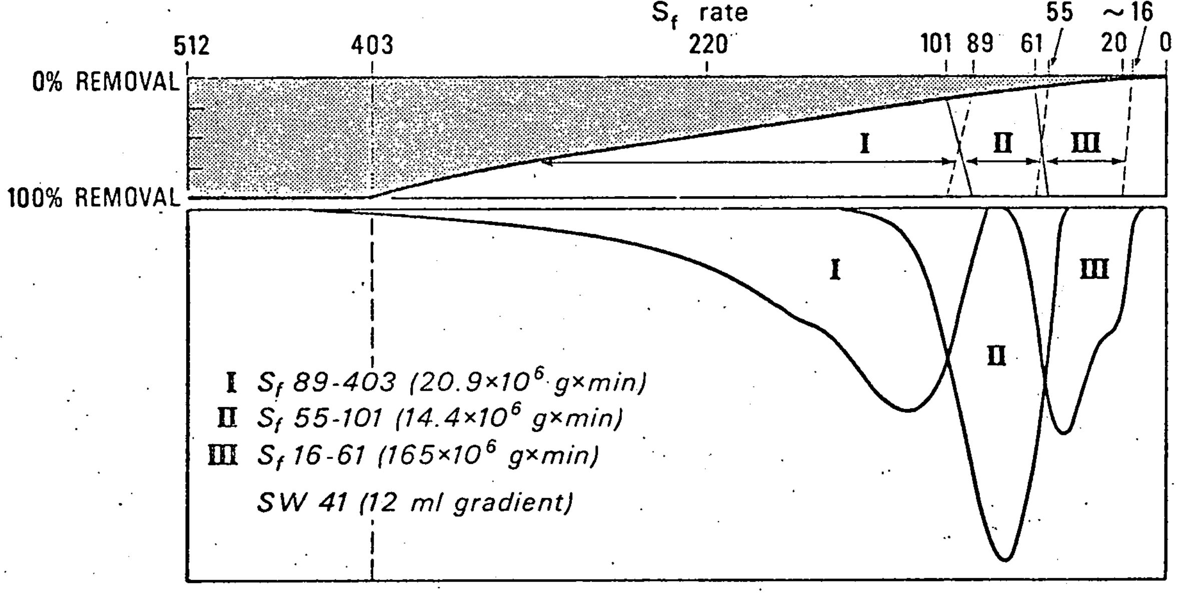
SERUM HDL SUBFRACTIONATION, $F_{1.20}$ 0-9



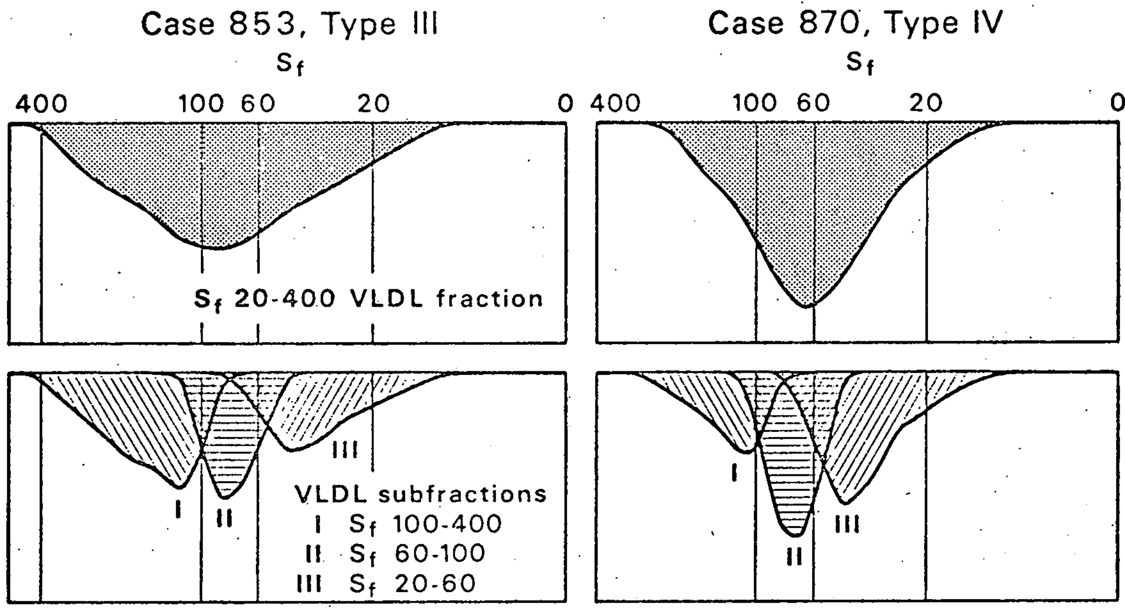
DBL 6911 5133


CHYLOMICRON-CONTAINING FRACTIONS,
CALCULATED RECOVERIES
384
1068
3176
324
908
2719
S_f rate
0%
100%
III
II
I
680
1128
1935
739
1222
2089
Diameter, Å
RELATIVE MASS
PARTICLE SIZE DISTRIBUTION,
ELECTRON MICROSCOPY
III
II
I
0
0
1000
2000
3000
4000
5000
ANGSTROMS
DBL 682-4590

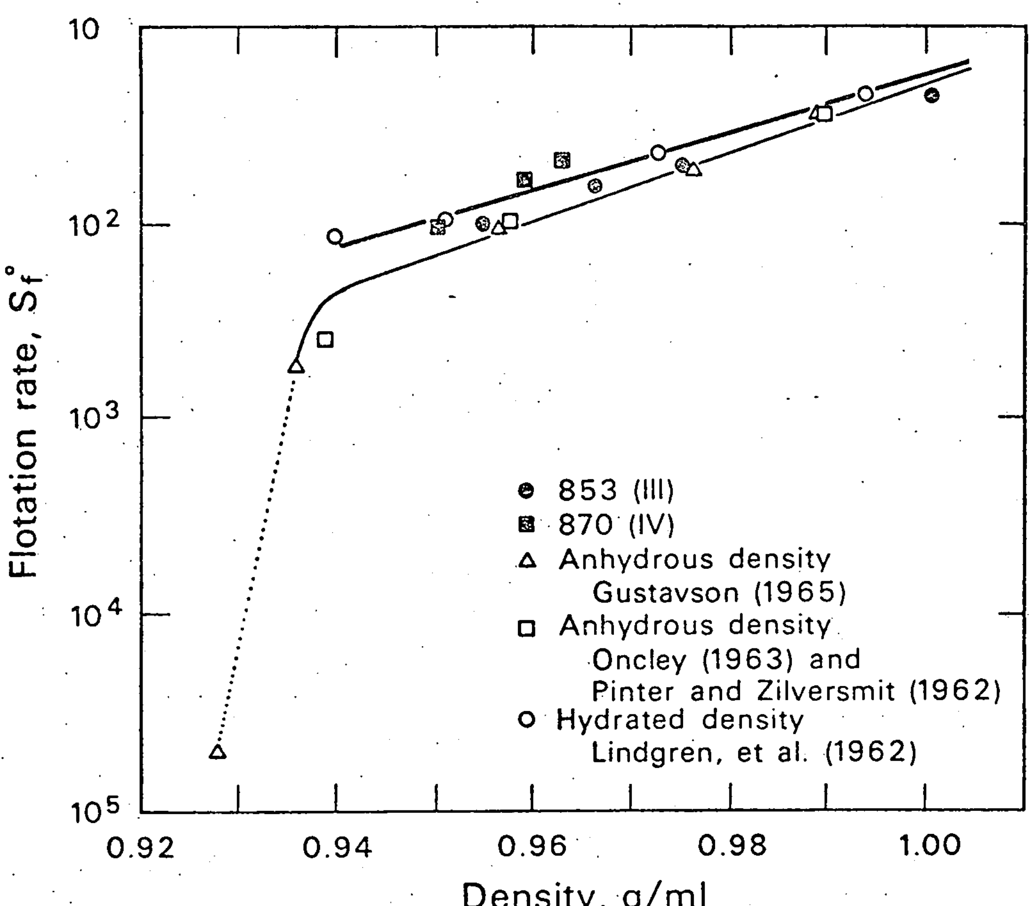




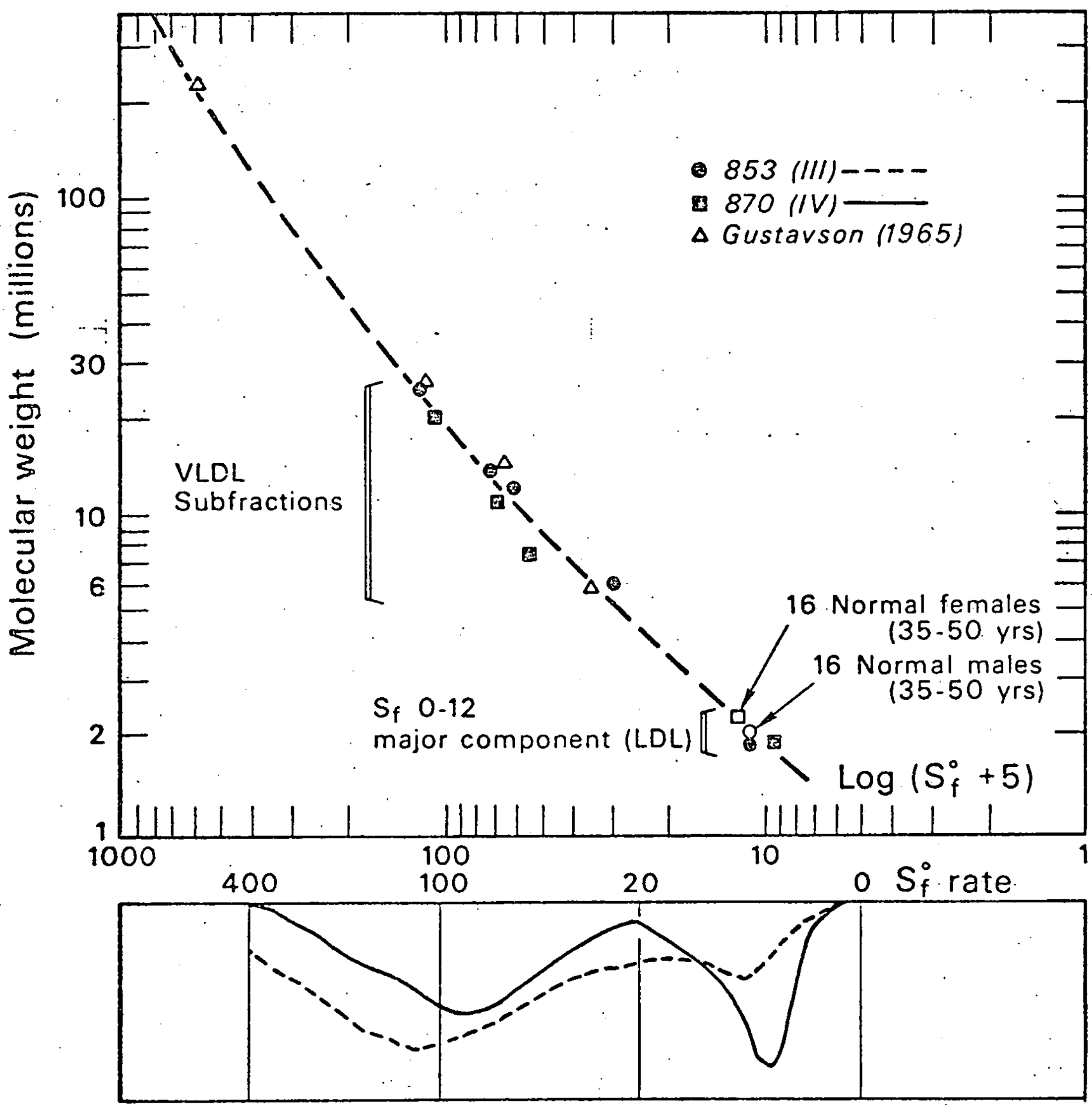
DBL 685-4742



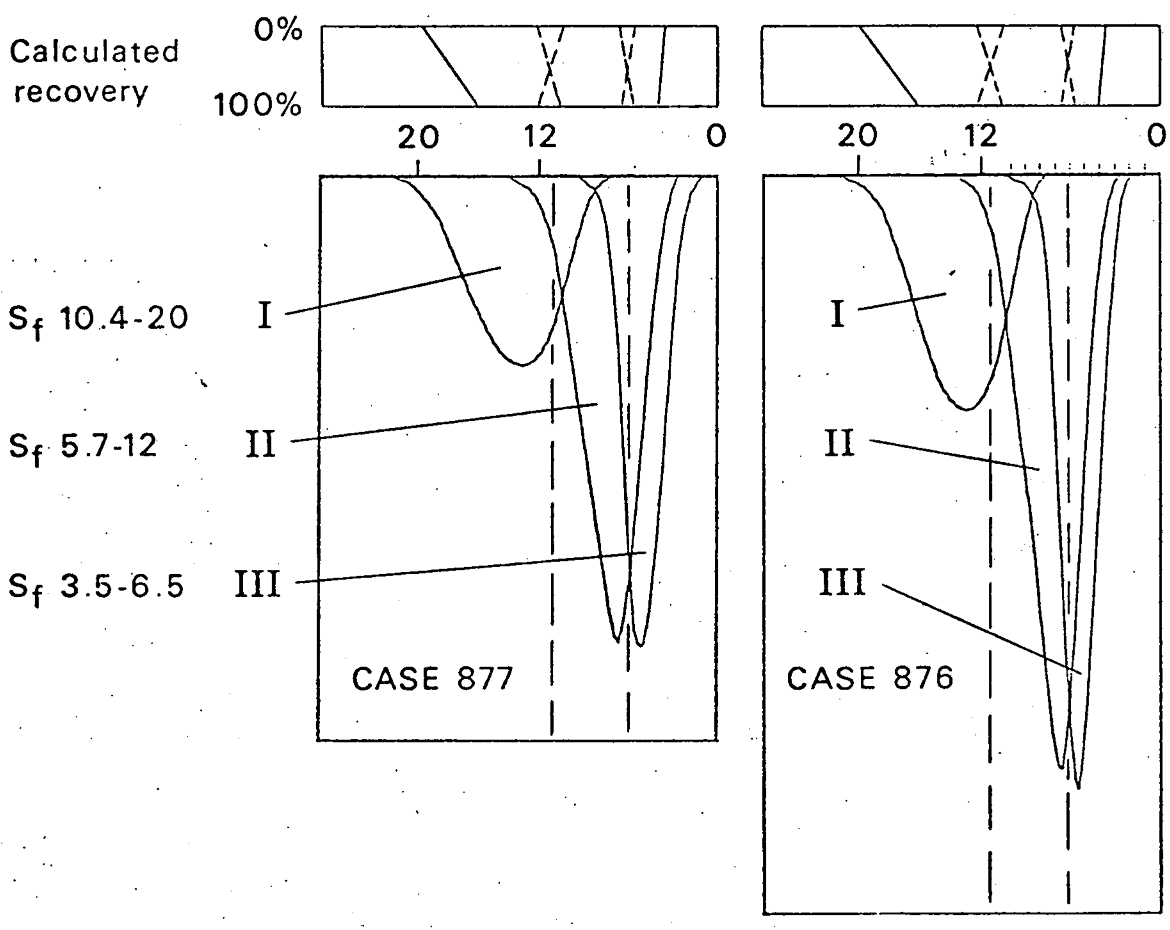
DBL 698-5005



DBL 694-4649

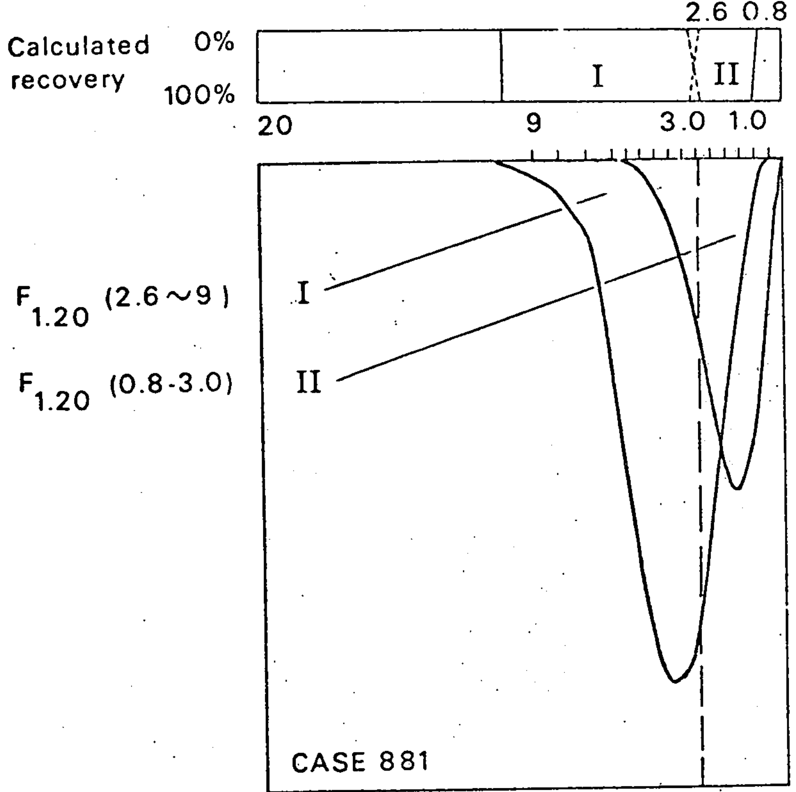


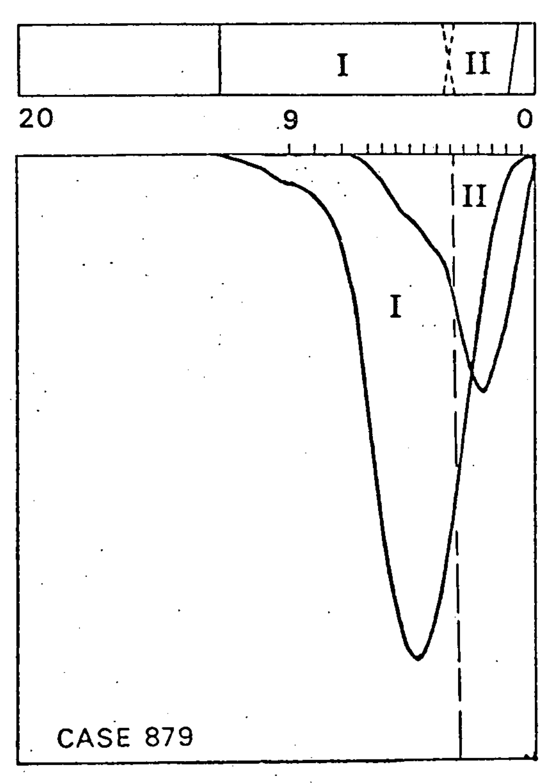
DBL 694-4650



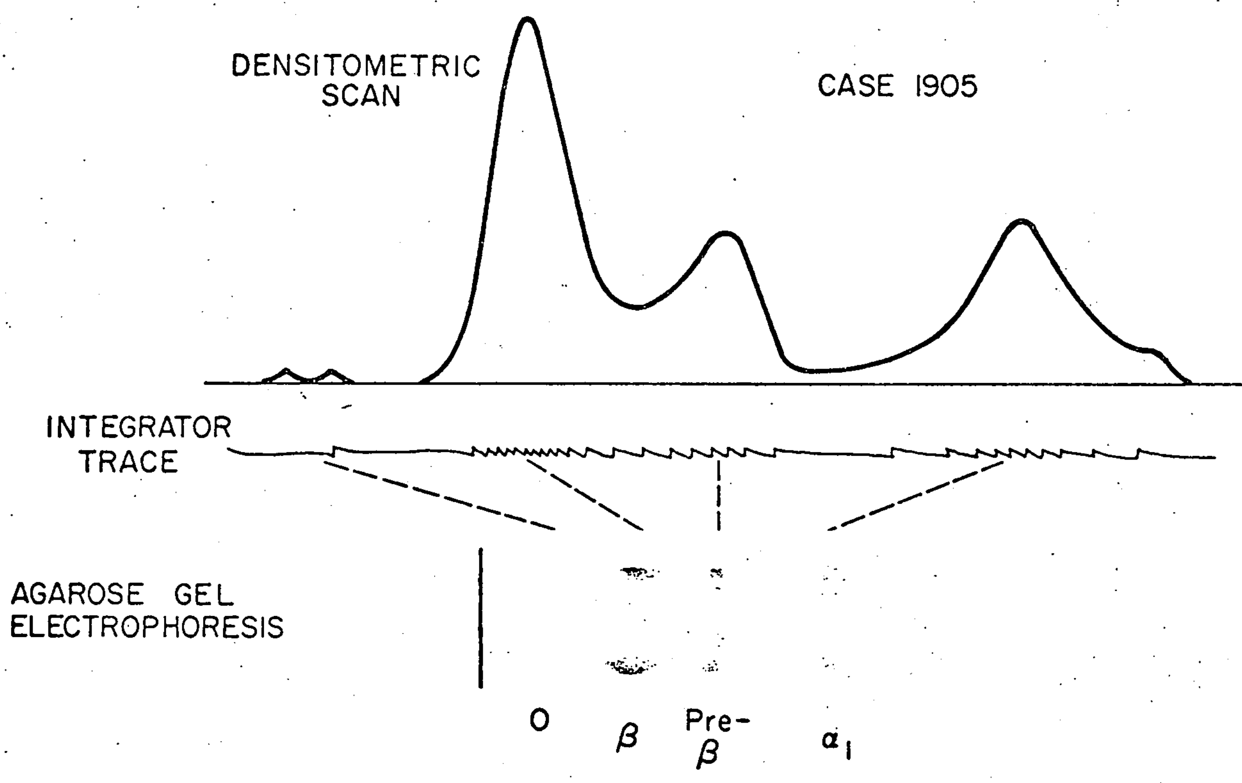
XBL 6911-6560

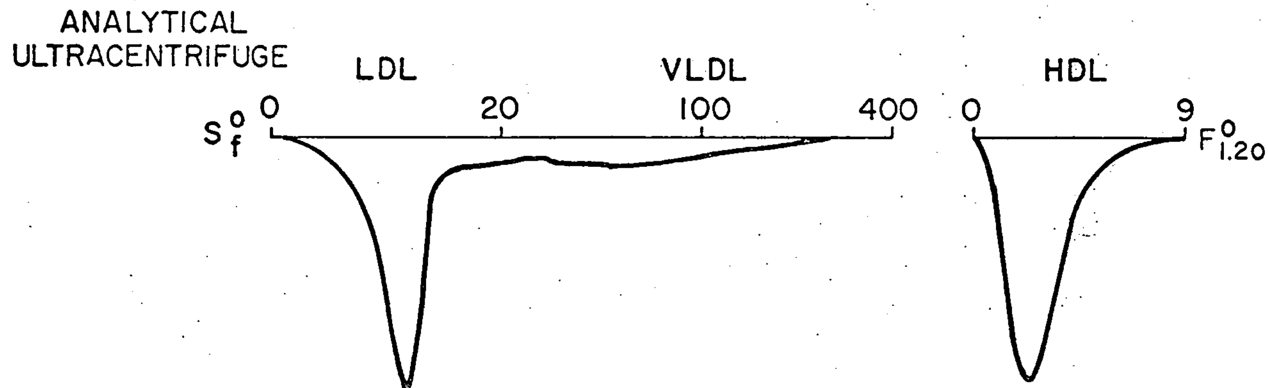
HDL SUBFRACTIONATION, Normal subjects

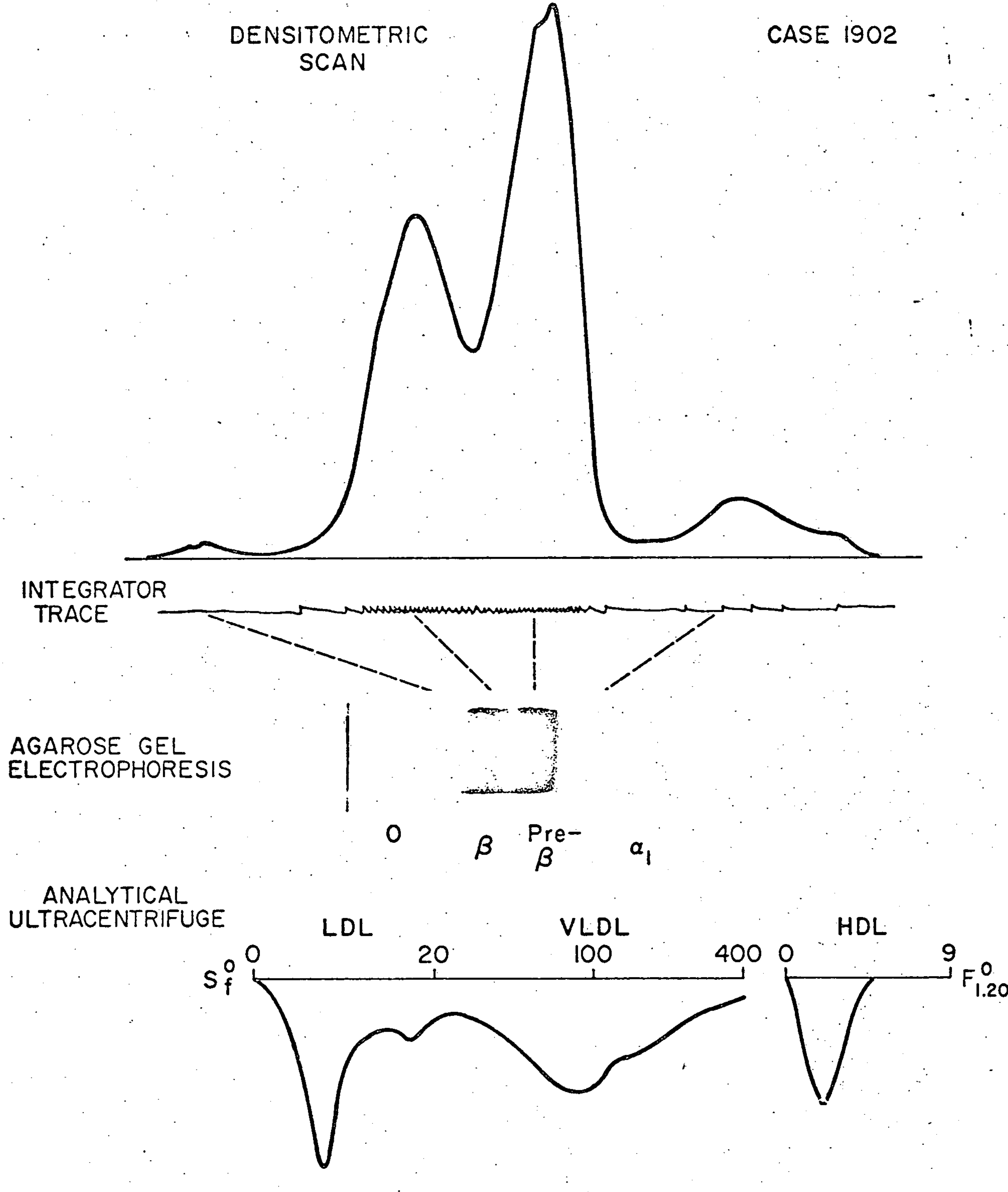




XBL 6911-6561

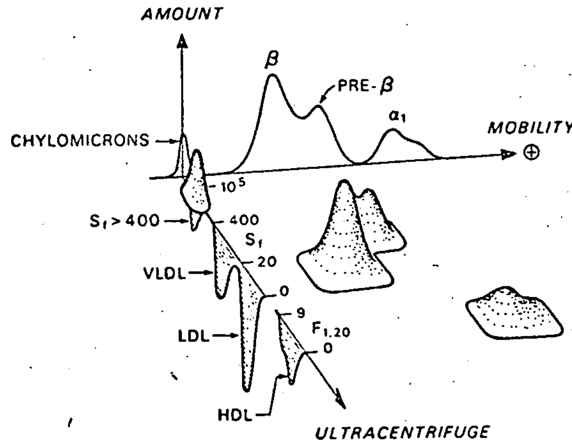

DENSITOMETRIC
SCAN
CASE 1905
INTEGRATOR
TRACE
AGAROSE GEL
ELECTROPHORESIS
O
β
Pre-
β
α1


ANALYTICAL
ULTRACENTRIFUGE
LDL
VLDL
HDL
S_f^o
0
20
100
400
0
9
$F^o_{1.20}$

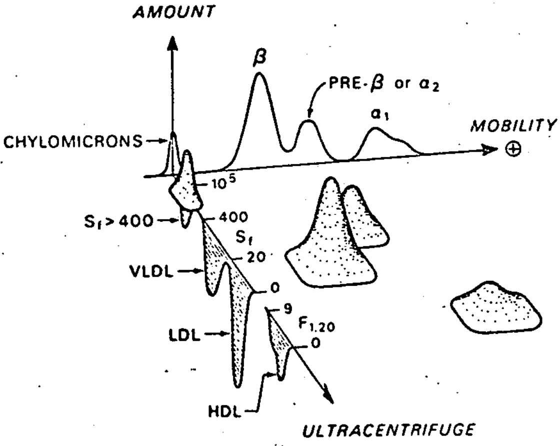


DENSITOMETRIC SCAN
CASE 1902
INTEGRATOR TRACE
AGAROSE GEL ELECTROPHORESIS
O
β
Pre-β
α1
ANALYTICAL ULTRACENTRIFUGE
LDL
VLDL
HDL
0
20
100
400
0
9
S_f^0
$F_{1.20}^0$

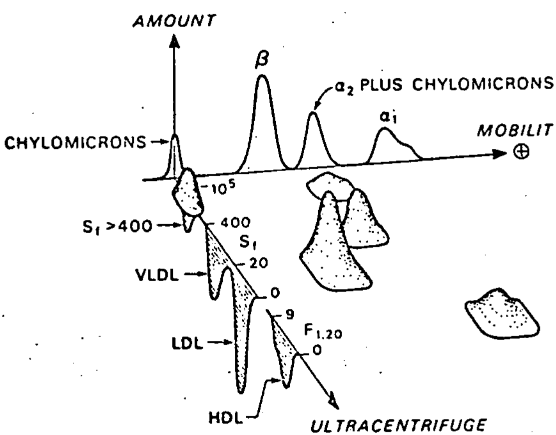
A. PAPER ELECTROPHORESIS



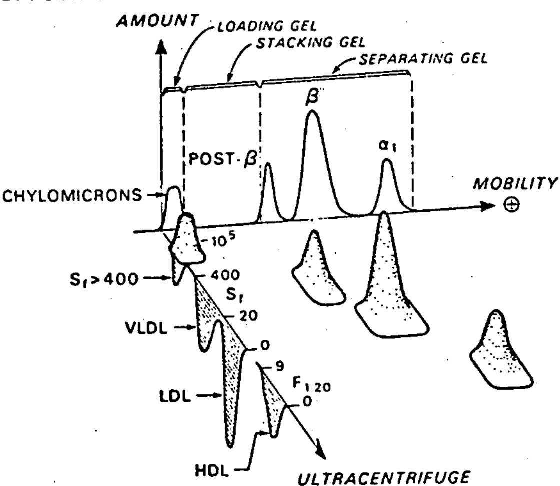
B. AGAROSE GEL ELECTROPHORESIS



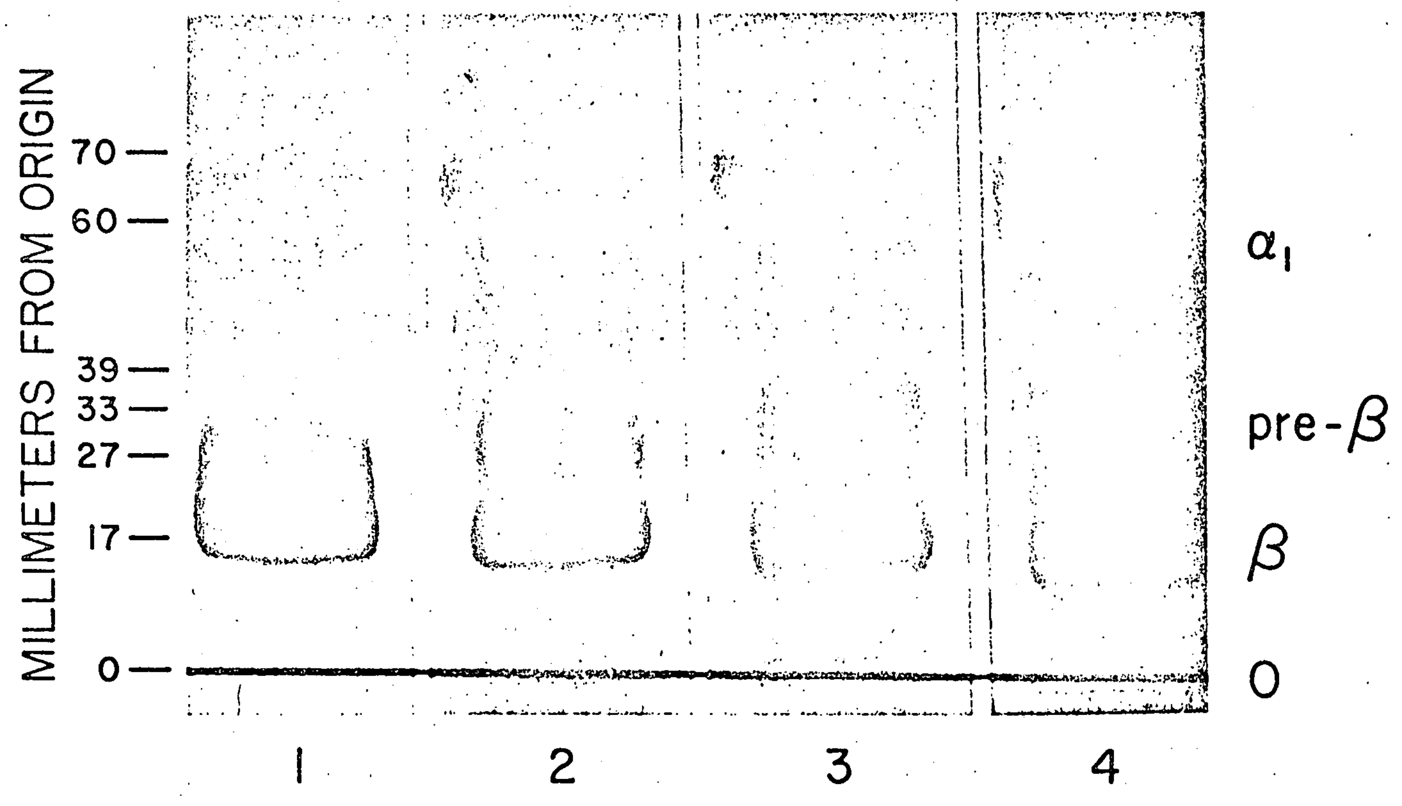
C. CELLULOSE ACETATE ELECTROPHORESIS



D. POLYACRYLAMIDE GEL ELECTROPHORESIS



DBL 704-5662


MILLIMETERS FROM ORIGIN
70
60
39
33
27
17
0
α1
pre-β
β
O
1
2
3
4

SERUM PROTEINS
(BROMPHENOL BLUE)

NORMAL LIPOPROTEIN PATTERN
(OIL RED O)

TYPE I
HYPERCHYLOMICRONEMIA

TYPE II
HYERBETA-LIPOPROTEINEMIA
(NORMAL SERUM TRIGLYCERIDES)

(ELEVATED SERUM TRIGLYCERIDES)

TYPE III
"BROAD BETA"
HYPERBETA — LIPOPROTEINEMIA

TYPE IV
HYPERPREBETA — LIPOPROTEINEMIA
(MILD)

(MODERATE)

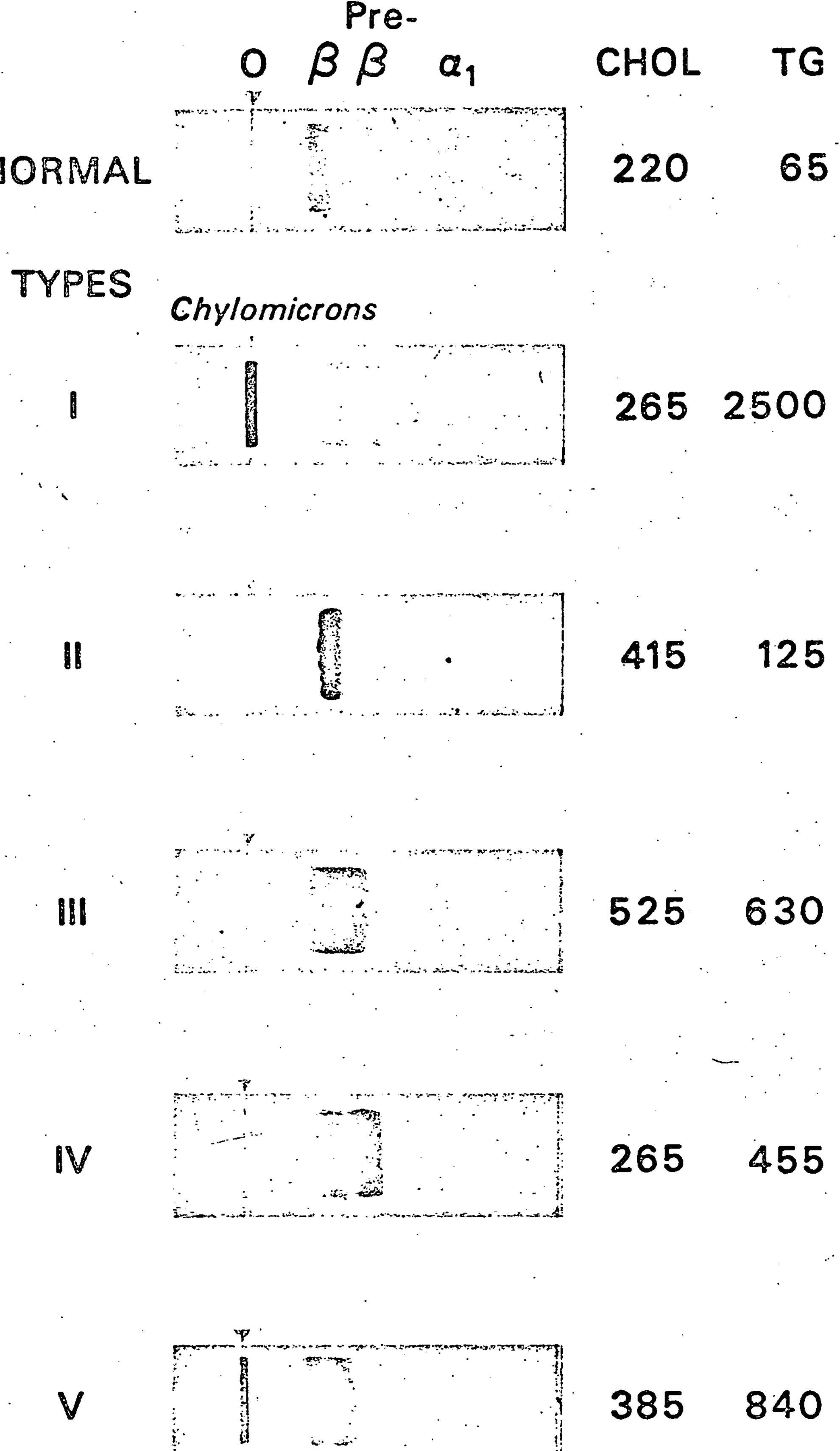
(SEVERE)

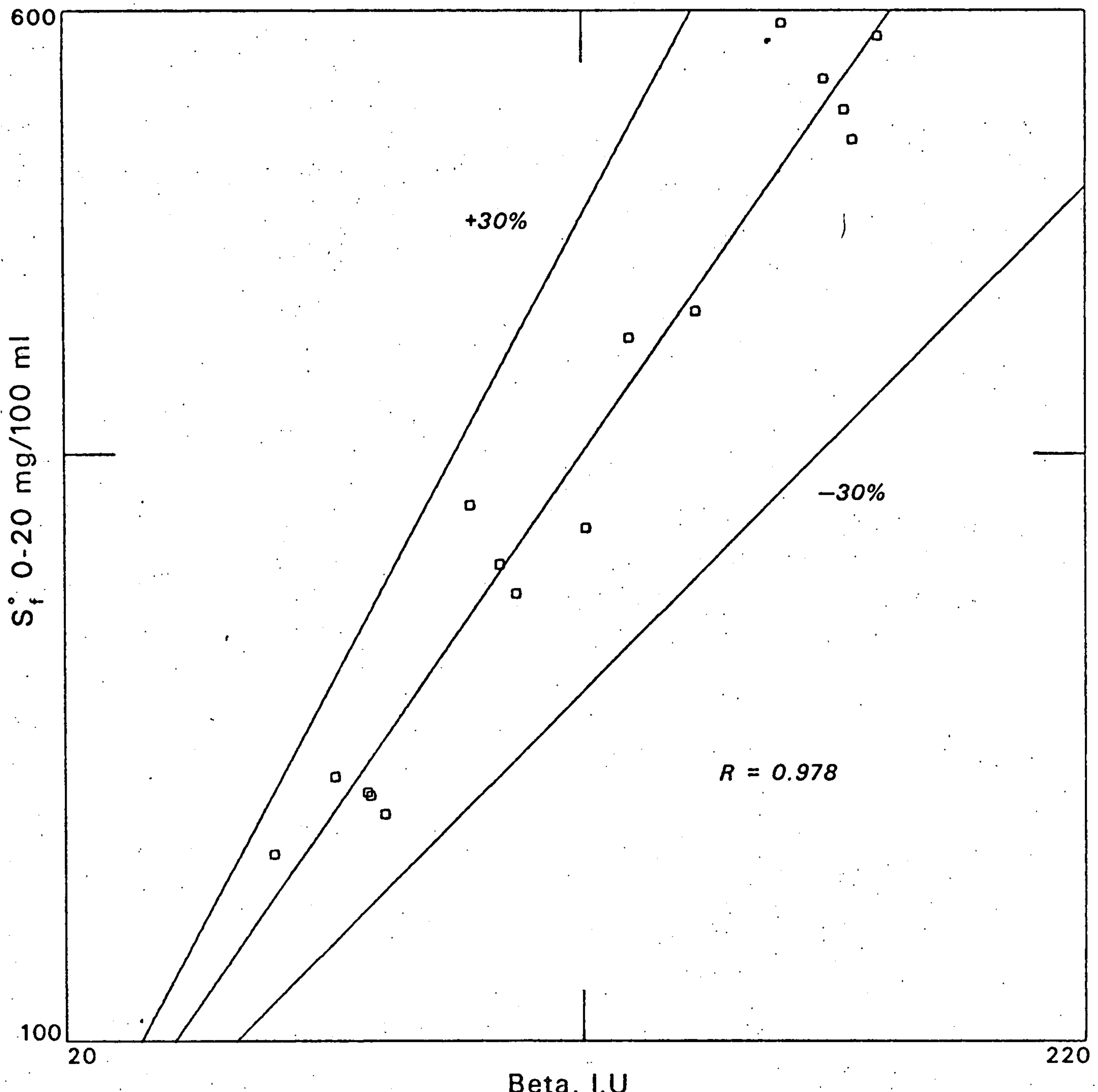
TYPE V
HYPERPREBETA — LIPOPROTEINEMIA
WITH
HYPERCHYLOMICRONEMIA

FAMILIAL ALPHA — LIPOPROTEIN DEFICIENCY
(TANGIER DISEASE)

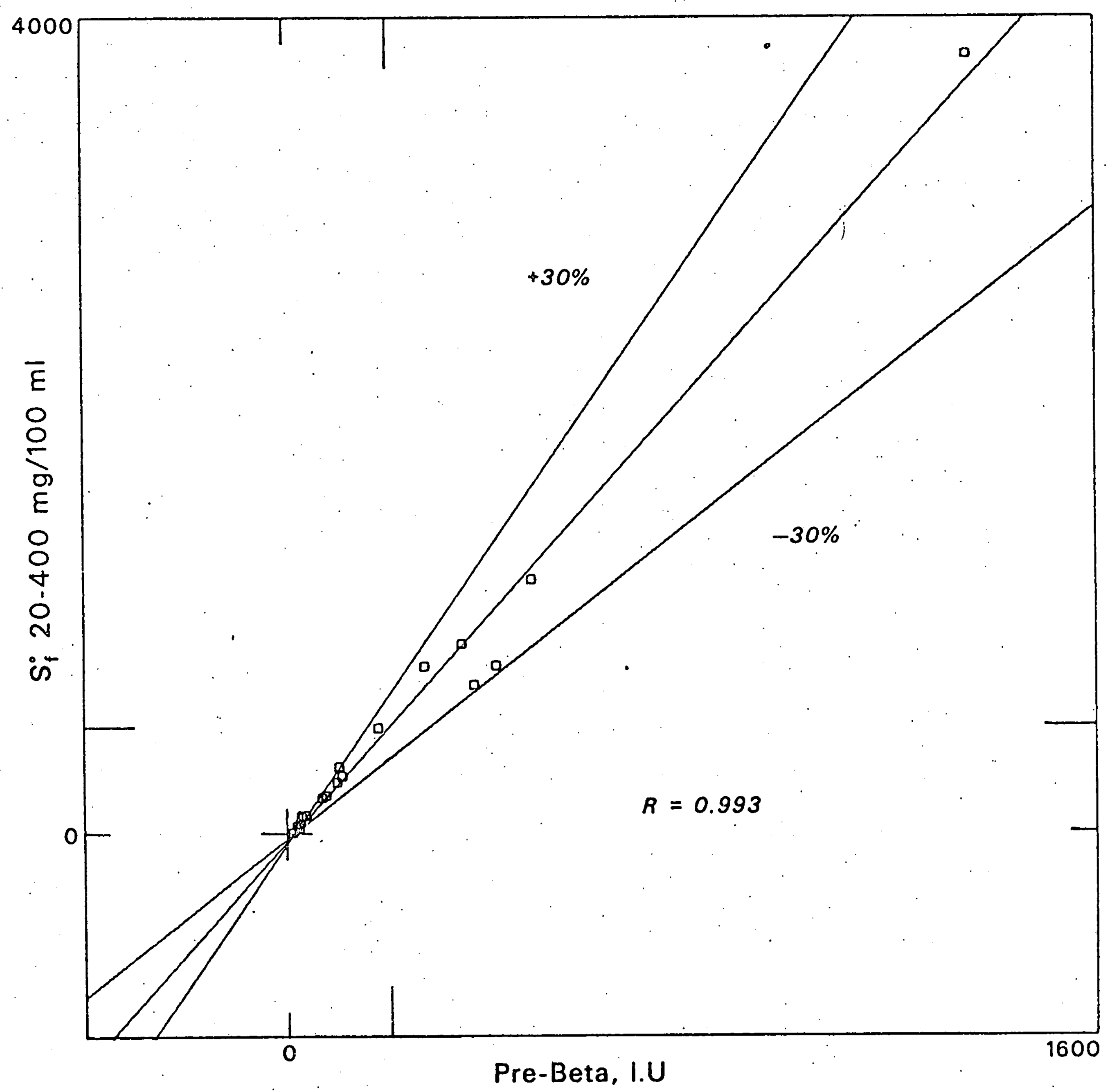
CONGENITAL ABSENCE OF
BETA-LIPOPROTEIN

TYPES OF LIPOPROTEIN PATTERNS

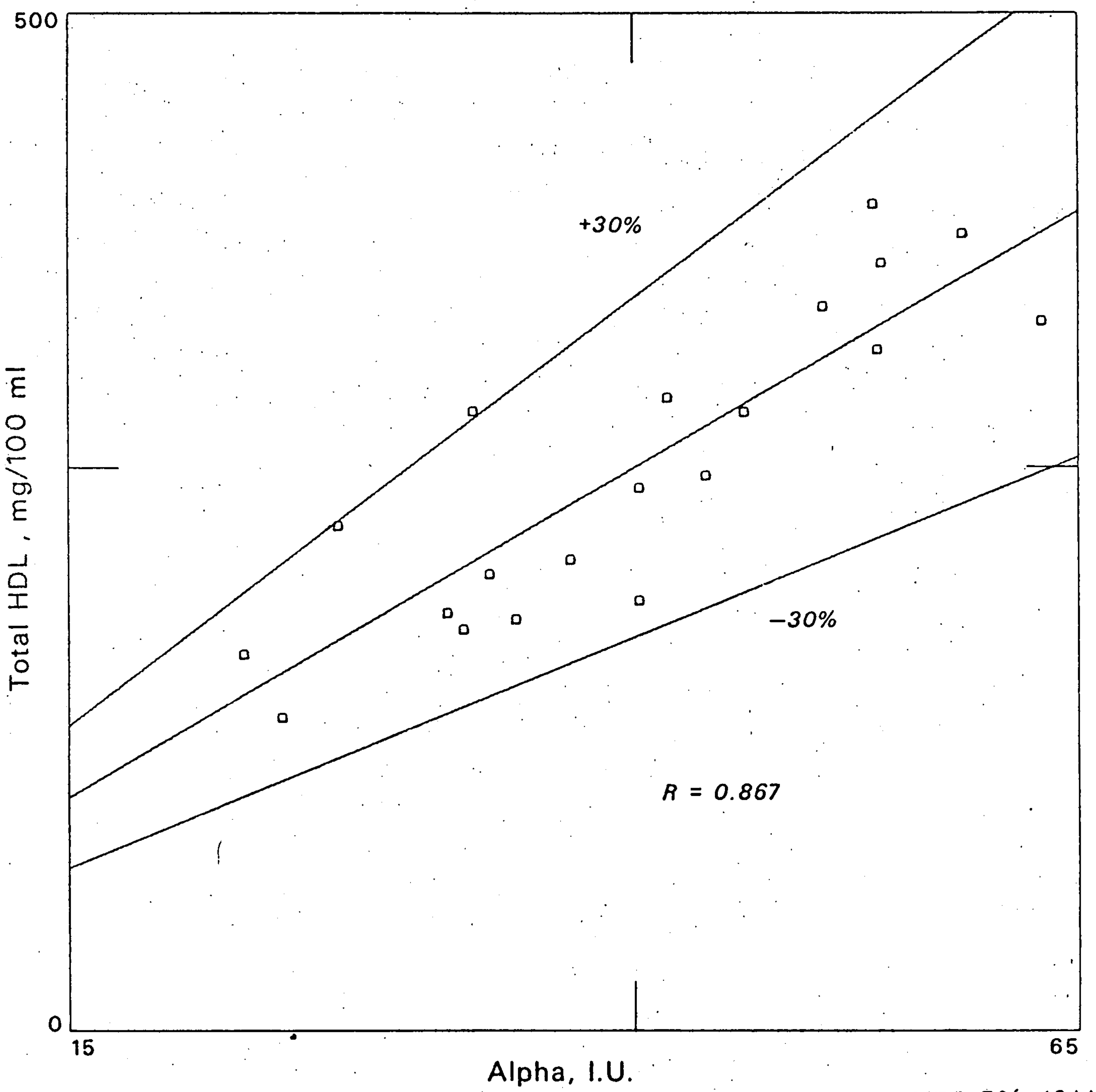




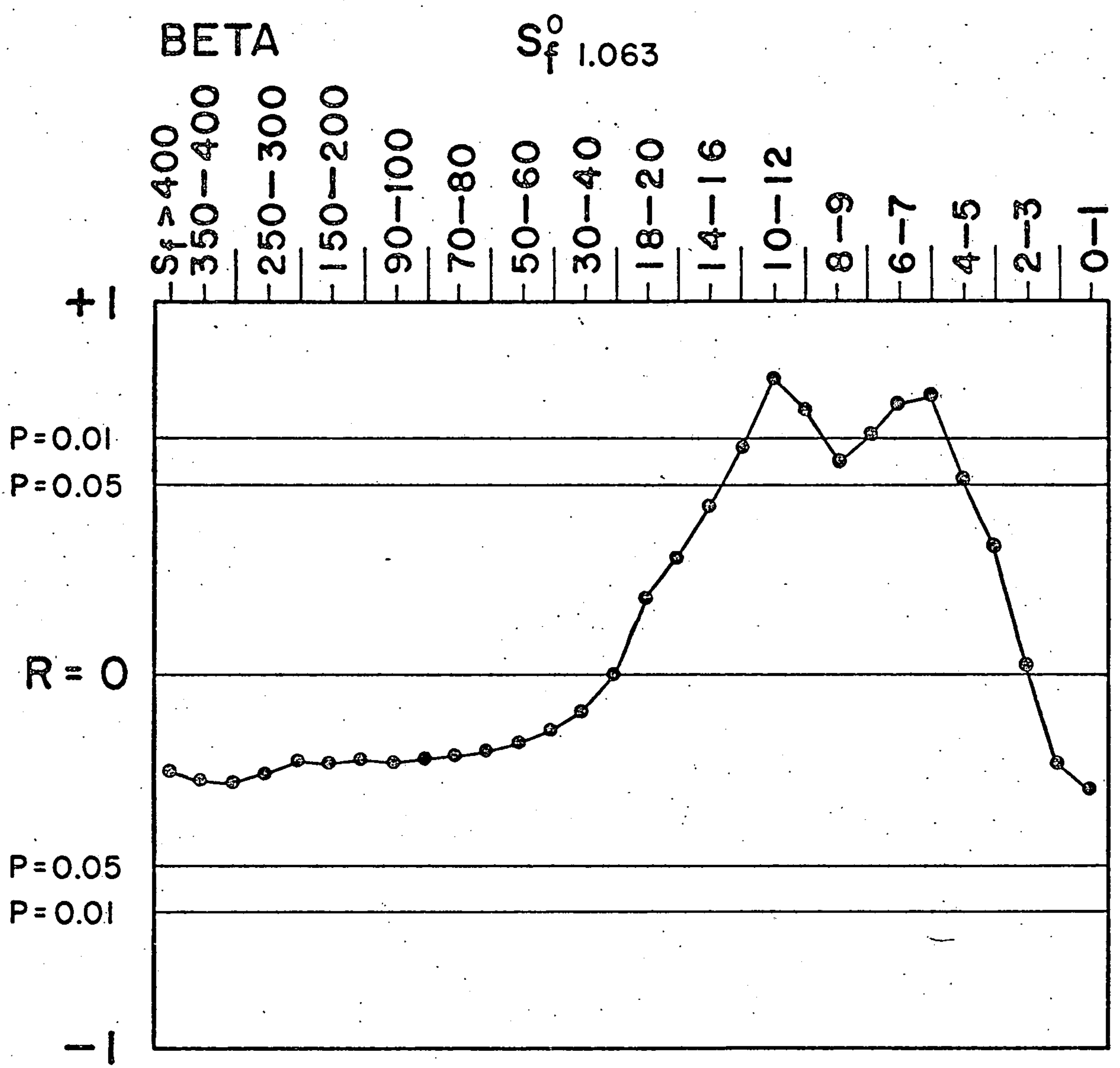
XBL 706-1213

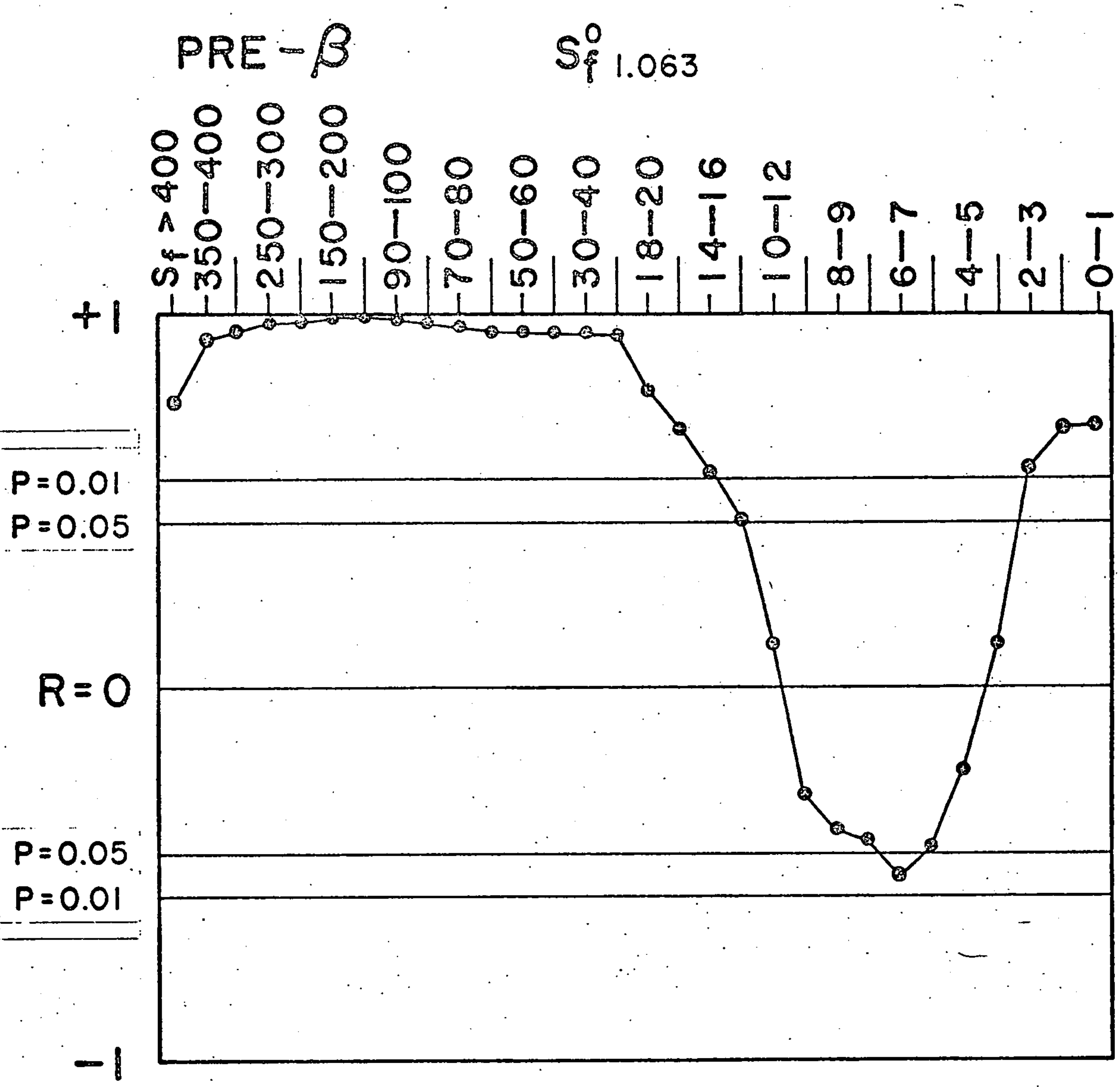


XBL 706-1212

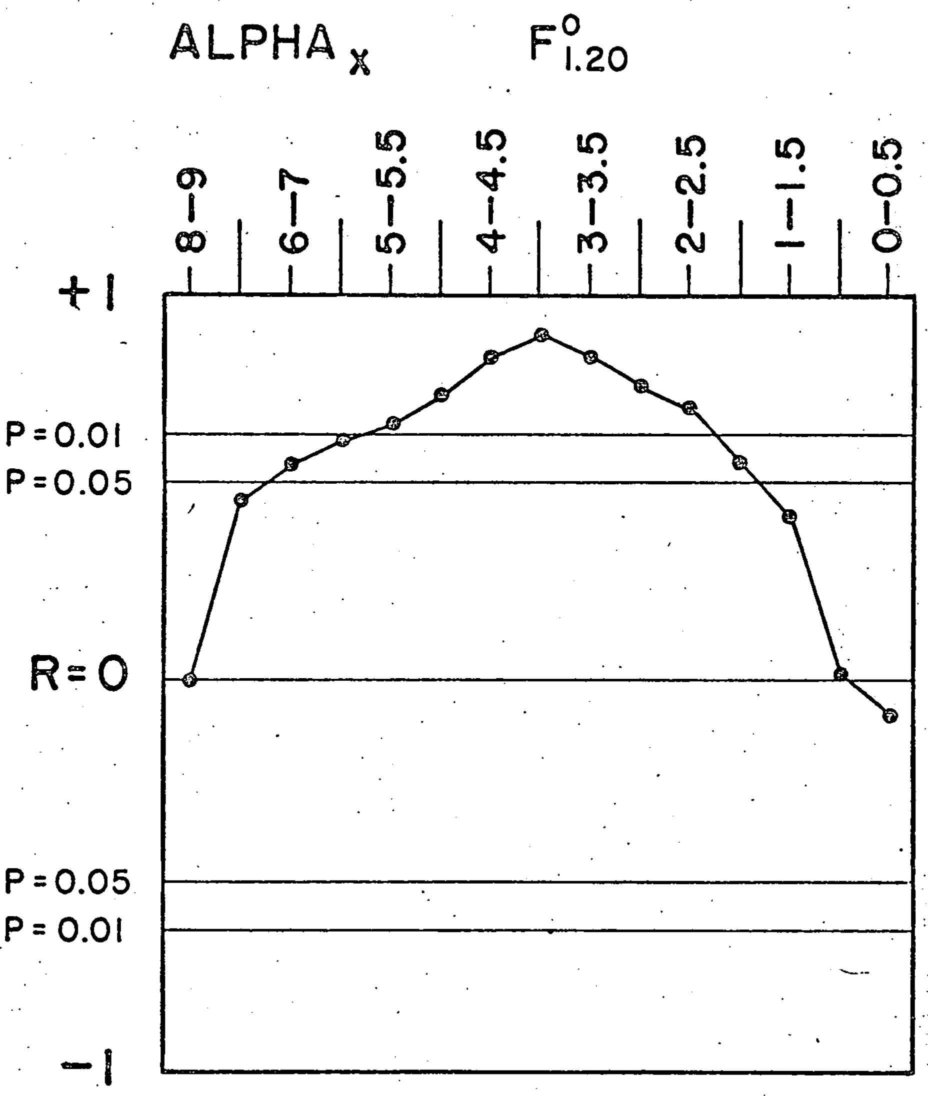


XBL 706-1211

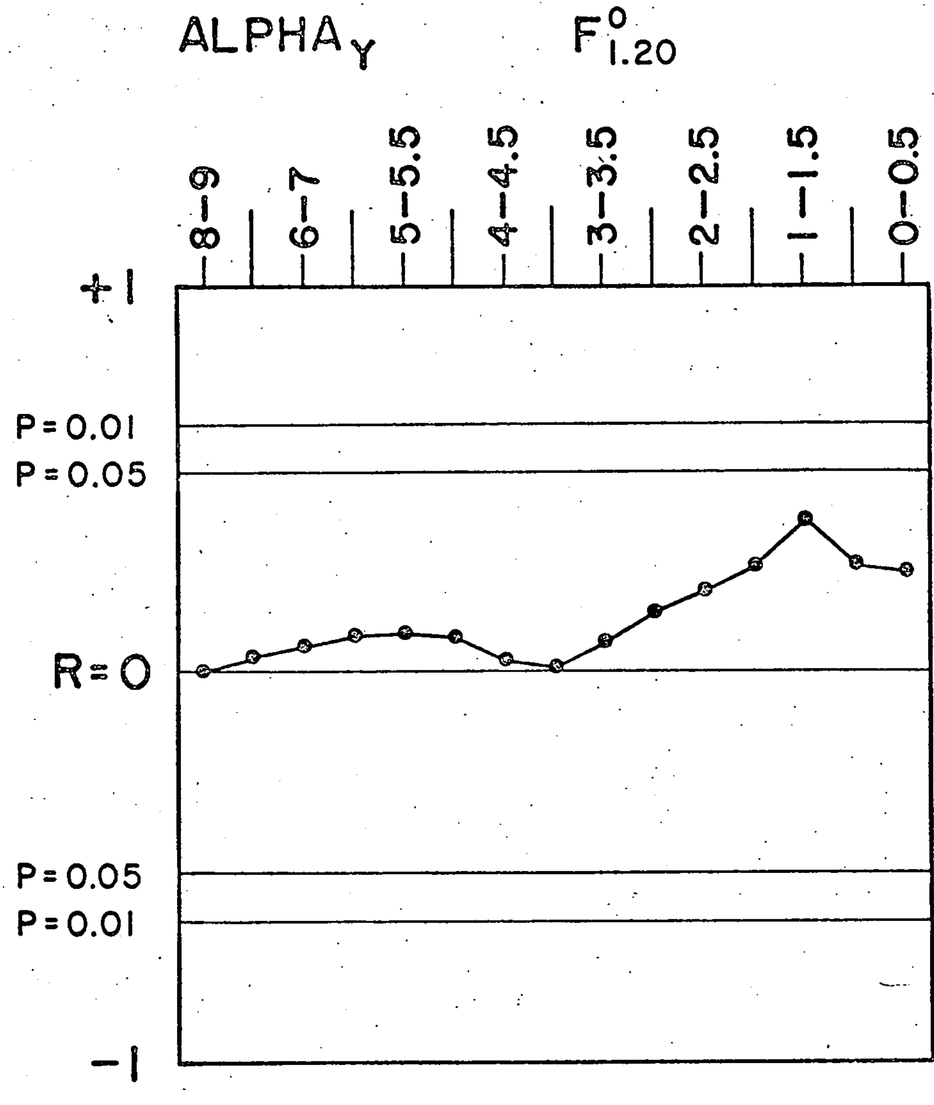

BETA
S_f^0 1.063
S_f >400
350–400
250–300
150–200
90–100
70–80
50–60
30–40
18–20
14–16
10–12
8–9
6–7
4–5
2–3
0–1
+1
P=0.01
P=0.05
R = 0
P=0.05
P=0.01
–1



PRE-β
S_f^0 1.063
S_f >400
350—400
250—300
150—200
90—100
70—80
50—60
30—40
18—20
14—16
10—12
8—9
6—7
4—5
2—3
0—1
+1
P=0.01
P=0.05
R=0
P=0.05
P=0.01
−1



ALPHA x
$F^{0}_{1.20}$
8—9
6—7
5—5.5
4—4.5
3—3.5
2—2.5
1—1.5
0—0.5
+1
P = 0.01
P = 0.05
R = 0
P = 0.05
P = 0.01
−1



ALPHA $_{Y}$
$F^{0}_{1.20}$
8-9
6-7
5-5.5
4-4.5
3-3.5
2-2.5
1-1.5
0-0.5
+1
P=0.01
P=0.05
R=0
P=0.05
P=0.01
-1

FIGURE LEGENDS

Fig. 1. Salt redistribution after typical runs: 1.006 g/ml NaCl, 1.063 g/ml NaCl total low-density lipoproteins (TLDL) and after 1.213 g/ml NaBr.

Fig. 2. Modified centerpiece showing special reservoir. Note precise meniscus equalization in the flat (offset) and wedge cell schlieren water patterns.

Fig. 3. Details of pinned analytic rotor holes and special slotted cell housings.

Fig. 4. Overall block diagram for an analytic ultracentrifuge data acquisition and control system.

Fig. 5. Typical output showing computer evaluation of a successful ultracentrifuge run.

Fig. 6. Cathode ray tube (CRT) plot (retraced) of rotor speed and rotor temperature of the same run evaluated in Fig. 5. Squares indicate times that photographs were taken.

Fig. 7. Selected schlieren photographs from a typical standard low and high density lipoprotein run (Case 710).

Fig. 8. Flow chart of ultracentrifuge schlieren pattern analysis program. Optional procedures are shown with dashed lines.

Fig. 9. CRT plots of low-density lipoprotein spectra. Traditional discontinuous plot (9a) and a continuous log plot (9b) of a fasting subject and the same subject 24 hr after a fat tolerance test (9c). Fig. 9d shows 9b subtracted from 9c. Although fully corrected, note significantly different up-to-speed equivalent (1.9624' and 1.6953') of the S_f 100-400 time frame for run 790 and 794, respectively.

Fig. 10. Low and high-density lipoprotein spectra for the indicated normal male and female populations, and for type I and II fasting plasma pools. Where total S_f 20-400 concentrations are below 150 mg/100 ml (and in the type I pool), the S_f 20-100 lipoproteins are measured in the 2' UTS schlieren photograph. Chylomicron quantification is as described in Section VI A and B.

Fig. 11. Low and high-density lipoprotein spectra for type III, IV, V and Tangier Disease fasting plasma pools and a single case of abetalipoproteinemia.

Fig. 12. Uncorrected flotation rate calculated from the 8-, 14-, 30-, 48- and 64- min photographs after reaching full speed (52,640 rpm). The acceleration time of 5.2 min is approximately equivalent to 1.83 min centrifugation at full speed.

Fig. 13. Computer output for flotation rate calculations (Case 710) showing complete imput data and best-fit flotation rate and base-of-cell output data.

Fig. 14. Schematic diagram showing relationship and utilization of the results and data from three computer programs.

Fig. 15. Schlieren flotation photographs of isolated narrow band S_f° 6.06 lipoproteins, Case 710. Conditions are 52,640 rpm, 26C; upper wedge cell is ρ_{26} = 1.200 g/ml (2.755 M NaBr-0.195 NaCl), flat cell is ρ_{26} = 1.061 g/ml (0.742 M NaBr-0.195 NaCl). Lipoprotein concentration is 465 mg/100 ml.

Fig. 16. Relationship between S_f° rate and ρ intercept obtained from isolated narrow-band LDL fractions and from results of standard low and high-density runs.

Fig. 17. Relationship between S_f° rate and molecular weight obtained from isolated narrow-band LDL fractions and from standard low and high-density runs.

Fig. 18. Relationship between S_f° rate and ρ intercept, normal nonfasting males and females.

Fig. 19. Relationship between S_f° rate and molecular weight, normal and non-fasting males and females.

Fig. 20. Overlayering procedure for preparing non-linear salt gradient for $S_f > 400$ fractionation. Each successive 0.5 and 1.0 ml solutions are added drop by drop over the bottom 1 ml salt solution or serum.

Fig. 21. Detail of lipoprotein pipetting fixture, showing rotating preparative tube holder and adjustable scale.

Fig. 22. Stability of static and dynamic 6 ml NaCl gradients used for chylomicron isolation.

Fig. 23. Dynamic gradient values of ρ and η used in calculating lipoprotein flotation recovery.

Fig. 24. Lipoprotein recovery (6 ml gradient) over the range of 0.01-10 x 10^6 g min.

Fig. 25. NaCl density gradient used for the subfractionation of the $S_f > 400$ lipoproteins. The g min values and needed equivalent full speed centrifugation (including acceleration and deceleration) at the indicated conditions are give for each successive centrifugation. Diagrammed below the gradient are the calculated ranges of S_f rates (and diameters assuming spheres) for lipoproteins recovered under ideal conditions for each run.

Fig. 26. NaCl density gradient used for isolating three fractions of the S_f 20-400 lipoproteins. Below is shown the partial removal of S_f 20-400 lipoproteins by the serum $S_f > 400$ run, as well as the calculated range of S_f rates (and diameters) ideally recovered for each run.

Fig. 27. NaCl density gradient used for LDL subfractionation with calculated recovery given below.

Fig. 28. NaBr density gradient used for subfractionation of the HDL lipoproteins with anticipated recovery given below. All NaBr solutions have a constant 0.195 M NaCl content.

Fig. 29. Computer-constructed plot of lipoprotein mass vs particle diameter (Sample 735) evaluated by electron microscopy. Each fraction is normalized to its total mass determined by elemental analysis. Above histogram is given the anticipated recovery for each of the three subfractions, calculated from the actual centrifugal conditions of each run.

Fig. 30. Schlieren patterns for the three VLDL subfractions and the total VLDL fraction from Sample 795. The VLDL distribution in all fractions has been distorted from that originally present in the parent serum by the continuously changing partial removal of the S_f 20-400 lipoproteins (shaded). The total VLDL fraction was isolated from a single stage density gradient run (200.4×10^6 g min). Recoveries are calculated from centrifugal conditions of each run.

Fig. 31. Schlieren patterns, plotted logarithmically, of total VLDL and three VLDL subfractions from Cases 853 and 870.

Fig. 32. Relationship between flotation rate and VLDL density showing data for subfractions from Cases 853 and 870 and previous literature data.

Fig. 33. Relationship between molecular weight and S_f° rate showing data for VLDL subfractions from Cases 853 and 870, as well as the data of Gustafson et al. (68). Also shown are data for the major LDL component for Cases 853 and 870 and the mean values for a normal non-fasting population (Livermore G and H). Logarithmic plots of the low density lipoprotein patterns for Cases 853 and 870 are given below.

Fig. 34. Corrected schlieren patterns for LDL subfractions from Subjects 876 and 877. Calculated recovery is shown above.

Fig. 35. Corrected schlieren patterns of HDL subfractions for Subjects 879 and 881. Anticipated recovery is given above.

Fig. 36. Relationships between agarose gel electrophoresis and its densitometric scan and the corrected schlieren patterns obtained by analytical ultracentrifugation. Left, a normal serum with slight elevation of pre-β and VLDL fractions. Right, a serum with a characteristic pattern of Type IV hyperlipoproteinemia, showing markedly elevated pre-β and VLDL fractions and decreased α_1 and HDL fractions.

Fig. 37. Schematic representation of the relationships (in a typical hyperlipoproteinemic serum) between electrophoresis in four commonly-used media and analytical ultracentrifugation. The diagrams are adapted from Fig. 9 in a study of starch block electrophoresis by H. G. Kunkel and R. Trautman (J. Clin. Invest. 35, 641 (1956)).

Fig. 38. Variations in the relative mobility of pre-β lipoprotein in agarose gel electrophoresis. Strip No. 4 illustrates splitting of the pre-β fraction which is observed occasionally in uncontrolled diabetes and in subjects taking clofibrate or contraceptive drugs. Spots in upper left of strips Nos. 2, 3 and 4 are bromphenol blue-albumin complex added before electrophoresis to mark the total migration distance. Relative mobilities of lipoprotein fractions can be determined in relation to the total migration. Reprinted with permission of author and publisher from R.P. Noble, J. Lipid Res. 9, 693 (1968) (94).

Fig. 39. Typical patterns observed in paper electrophoresis. Reprinted with permission of the publisher from F. T. Hatch and R.S. Lees, Advances in Lipid Research 6, 1 (1968), Academic Press, N.Y.

Fig. 40. Typical patterns observed in agarose gel electrophoresis.
Chol = serum total cholesterol, mg/dl.
TG = serum triglycerides, mg/dl.
Types correspond to those of Fredrickson, Levy and Lees (34).
Kindly provided by R. Noble.

Fig. 41. Scatter diagrams relating data of agarose gel electrophoresis to that of analytical ultracentrifugation by methods described in the text. (See footnote b, Table XII.) R = correlation coefficient; I. U. = integration units measured with the Beckman Analytrol densitometer[3] and standardized as explained in the text. The central line is the regression line calculated by the method of least squares. The divergent lines are plotted 30 percent of the ultracentrifugal values above and below the regression line.

Fig. 42. Correlation profiles of the amounts of the electrophoretic fractions indicated at upper left of each diagram across increments of the low (S_f° 1.063) or high ($F_{1.20}^\circ$) density ultracentrifugal schlieren patterns. The vertical axis ranges from correlation coefficients of -1 to +1. Transverse lines indicate the correlations required for probabilities of chance occurrence less than 0.05 and 0.01. $Alpha_x$ is the major band of α_1 lipoprotein; $Alpha_y$ is the faster-migrating minor component observed anodally to the major band. (See text.)

significant and changing redistribution of salt occurs during the run. This requires that double sectored cells be employed allowing accurate baseline evaluation in any frame. Since such baselines are a function of fluid column height, the baseline and lipoprotein fraction meniscus should be as closely matched to one another as possible. Thus, each sector is filled with 0.415 ml using calibrated syringes. Also, it is highly desirable to use inert Al-filled epoxy centerpieces. Such cells in our hands are routinely capable of sustained operation at 52,640 rpm. Centerpieces are washed first with water using a folded pipe cleaner and then in an ultrasonic cleaning bath; they are thoroughly rinsed with distilled H_2O and dried under a warm air blower. No detergents or solvents are used.

A. Special Features of Cell Construction and Cell Alignment

We have found that with slight modifications in design, the usual $2\frac{1}{2}°$ double sectored cells employing 12 mm thick centerpieces are desirable for lipoprotein analysis. These features include fabrication of both a standard and a 0.020" offset centerpiece (25) which allows accurate base-of-cell identification when two samples are run simultaneously, such as in the standard low and high density run. Normally the offset centerpiece is run in a cell with a 48', 30" positive wedge quartz disc, allowing clearer baseline resolution near the base-of-cell in the high density run. We have found this arrangement superior to that shown in Section IV E. Another feature, shown in Fig. 2 , is a reservoir in each centerpiece to equalize the meniscus in both the baseline and sample sectors. The dimensions of the scratch are important to allow

meniscus equalization without exchange of fluid from one sector to another. Since under repeated stress the epoxy flows somewhat, after about 30 runs a centerpiece should be carefully rescribed (with a sharp razor blade).

Precise cell alignment is very important in lipoprotein flotation analysis. Although scribe line alignment of the cell in a rotor checked with an optical device (such as the Beckman Microscope Cell Aligner) is usually adequate, occasionally cells may rotate slightly after proper alignment, giving striations in the schlieren pattern. Although this may be minimized by using a small amount of heavy silicone grease, this latter procedure prevents accurate weighing of the analytical cells. Our procedure to achieve reproducible cell alignment is to use pinned rotors and notched cell housings as shown in Fig. 3 . Each cell housing must be matched with an appropriate centerpiece such that the combined fully torqued assembly positioned in the rotor results in proper radial alignment. Our tolerance is that the top or bottom of the cell wall should be within ± 0.002" of perfect radial alignment, as checked on a special optical fixture. Whenever striations suddenly appear in a pattern, the cell alignment should be checked with the optical device.

Another useful design modification of the cell housing, shown in Fig. 3 , involves relieving the diameter 0.001" a distance of 0.310" from the screw ring end. This minimizes scratch and wear within rotor holes and on the periphery of this specially stressed region of the cell housing.

Using epoxy-filled double-sectored centerpieces at high speed involves not only the occasional risk of gross cell leakage, but also of subtle micro-leakage. Because

the seal between the epoxy centerpiece and the quartz windows is not perfect, there is nearly always some slight leakage. Our cells routinely leak approximately 0.5 - 2.5 μl from one or both sectors during a standard low and high density run. Although this amount of leakage (0.5 - 2.5 mgs) involves only an imperceptible change in meniscus position, large losses can introduce appreciable and unacceptable inaccuracies, particularly when precision moving boundary S_f rates are needed. Thus, it is important to weigh each cell to ± 0.2 mg before and after each run. A new cell or a cell that has had one or more parts replaced should be run first with distilled water to identify any unusual cell distortion and to minimize lipoprotein sample loss (see Fig. 2).

A critical manipulation to minimize cell leakage and avoid breakage is the proper torquing of the cells. Using only a dry lubricant, MS-122 Fluorocarbon (Miller-Stephenson Chem. Co., Chicago, Ill)[3] cells are torqued 50 - 70 inch-pounds, depending on their age and history. Despite precautions cell leaks (greater than 5 mg) occur approximately 1 in 60 runs.

B. Some Useful Modifications of the Analytic Ultracentrifuge for Lipoprotein Work.

Since lipoprotein analysis frequently involves macromolecules that significantly migrate during rotor acceleration to full speed (FS) it is important to reproducibly define the acceleration phase of the run. This is particularly important in our standard procedure where we take two schlieren photographs during this period; ideally the value of $\omega_{FS}^{-2}\int\omega^2(t)dt$ is needed for appropriate analysis. Several modifications of the Beckman Model E Ultracentrifuge and additional instrumentation fulfill this and other useful purposes. An automatic accelerator (26) actuated by a push-button raises the motor current from 0 - 14A in exactly 0.50 min and thereafter maintains this current until the full speed control loop comes into range. At full speed (52640 rpm) using a modified Beckman electronic speed control, an accuracy of 2 parts per million is achieved using a crystal controlled oscillator (26). In addition to information about

the acceleration phase it is useful and frequently necessary to obtain data about full speed stability, rotor temperature and the exact time when schlieren photographs are taken. An automatic data acquisition and control system for up to three analytic ultracentrifuges fulfills this purpose (27).

C. An Analytical Ultracentrifuge Data Acquisition and Control System.

When data are collected from several instruments and channeled into one recorder, a method of coding and collating is necessary. We use a master digital 24 hour clock (Parabam Model DA24, Hawthorne, Ca.)[3] to control the timing, to synchronize the start of each machine and to code the data (by time) when measurements are taken. A time sequencer switches from machine to machine collecting data in a one-two-three sequence. An interface coupler (Dymec Model 2526, Hewlett-Packard, Palo Alto, Ca.)[3] operates with an IBM 526 summary punch machine to store and record the data. Mercury reed relays select signals from gear tooth counters (Electro Model 3010-A, Chicago, Ill.)[3] for rotor speed information and from radiometers for rotor temperature information. The radiometer (Beckman Instruments, Palo Alto, Ca.)[3] is the total-radiation heat balance type used in the model L2-65B preparative ultracentrifuge. The bottom of each analytic rotor is recessed 0.040" and painted flat black to optimize radiation exchange between the rotor and the radiometer; the latter is offset 0.75" from the rotor axis and spaced 0.25" from the rotor bottom. Reed relays select radiometer outputs to channel the corresponding ultracentrifuge temperature analog voltage to a digital voltmeter (Model 3460B, Hewlett-Packard, Palo Alto, Ca.)[3]. The overall block diagram of this system is shown in Fig. 4.

Each ultracentrifuge is designed to operate independently of the system (if desired) when the connector to the central system is replaced by an appropriate jumper plug. A timed cycle of rotor acceleration and photography is determined by a program card in each centrifuge; the cycle is initiated by a push button on each centrifuge. The Beckman commutator timer is replaced by an integrated circuit, giving flexibility of 99 possible photograph times taken at 2 min intervals over a period of 200 min.

A Fortran IV computer program processes the punched card data yielding graphs of rotor speed and temperature as functions of time as well as giving the cumulative value of $\omega_{FS}^{-2} \int \omega^2(t)dt$ for the mean time of each schlieren photograph. Typical computer output of a successful ultracentrifugal run is shown in Fig. 5 . Had there been any error or failure, an error message (or messages) would describe the difficulty. An additional feature of the computer analysis, is desired, is shown in Fig. 6 . Here a cathode ray tube (CRT) plot gives the visual profile of acceleration and rotor temperature as well as the times at which photographs were taken.

We have chosen to obtain data directly in the form of IBM punched cards which allow convenient and direct submission of data for computer analysis. Such data permit quick evaluation of each day's runs, machine performance and error-detection capability. The equivalent up-to-speed (UTS) centrifugation of all the pictures is available with great accuracy; these data, particularly the 0' UTS value are essential for accurate very low-density lipoprotein analysis where significant migration of molecules occurs during the acceleration phase of the run. Also, this data acquisition system permits great flexibility

in the actual run itself, for example, non-linear acceleration or a run consisting entirely of programmed acceleration. The latter capability may be useful in minimizing effects of adiabatic temperature changes during acceleration to full speed. Also, such a system should increase the accuracy of measured sedimentation and flotation rates, particularly at low rotor speeds.

Other modifications include a camera system using 4" roll film (25) of high dimensional stability (Tri-X Ortho Film Estar Thick Base, Eastman Kodak Co., Rochester, N.Y.)[3]. This capability permits recording of greater vertical schlieren deflections, and is particularly advantageous when two patterns are routinely recorded. Also, manipulation of films present no hazard of potential breakage and film storage is greatly simplified. Another useful device is a vacuum sentinel (28) which automatically shuts off the drive mechanism and applies the full brake in the event of a cell leak and subsequent vacuum loss. Such a device provides the capability of significantly decreasing analytical-cell breakage as well as providing automatic monitoring throughout the duration of each run.

D. Calibration of Analytic Ultracentrifuge for Lipoprotein Analysis.

Calibration of the analytic ultracentrifuge is conveniently done using an appropriate Beckman calibration cell. We routinely calibrate at approximately 4 month intervals, immediately after any manipulation of the optical system or after a drive exchange. Although film of high dimensional stability is employed, we also calibrate when a new lot of film is used. For a given calibration cell, area on the schlieren film corresponds to a particular total Δn for a given centerpiece thickness. Since all patterns are analyzed using

an enlarger (Omega, Type B4, Simmon Bros., Inc., L.I.C., N.Y.)[3] this instrument also becomes part of the calibration procedure. Before and after any film analysis, including calibration patterns, the enlarger is precisely adjusted (or checked) to a 5-fold enlargement. This is conveniently done using a piece of film on which is a lightly scribed 2 cm radius circle. Thus, calibration data involve an enlarged area on the tracing corresponding to an equivalent Δn appropriate for the centerpiece thickness. In addition, total linear magnification from the cell to the enlarged tracing is measured. The above calibration factors for each machine are necessary for all computer analysis of lipoprotein fractions and in the measurement of moving boundary sedimentation and flotation rates.

Although lipoprotein concentrations may be given in the invariant physical measurement of total refractive increment, lipoprotein concentrations are routinely expressed in mass/unit volume. Our ultracentrifugal data are presented assuming a constant specific refractive increment (SRI) of 0.00154 Δn/g/100 ml for the total S_f 0 - 400 low density class, measured in 1.063 g/ml NaCl. High density lipoprotein concentrations are calculated assuming an SRI of 0.00149 Δn/g/100 ml, measured in 1.203 g/ml NaBr. All schlieren runs are made between 25 and 26°C using a phaseplate-wire combination (set at approximately 53°) and a Wratten #16 filter so total lipoprotein Δn is obtained primarily with the green line of the Hg arc (5461A). Dispersion effects are probably less than the SRI errors, so these values are also used for refractometry at 5893A. It is anticipated that more accurate SRI data will be available soon on subfractions of the VLDL, LDL and HDL classes.

E. Computer Analysis of Schlieren Lipoprotein Distributions.

Computer analysis of the analytical low density run (which includes the VLDL and LDL classes) and the high density lipoprotein spectra is essentially as described in detail earlier (6). A total of 10 schlieren photographs are taken, one during acceleration and 9 during the 64 minute up-to-speed (UTS) run at 52,640 rpm. Fig. 7 shows selected schlieren photographs of a typical low and high density run. Note the use of the 0.020" offset centerpiece in the flat cell providing positive identification of the base-of-cell position in each run. Normally the 0', 6' and 30' photographs are used for the low density analysis for the S_f 100-400, S_f 20-100 and S_f 0 - 20 lipoproteins, respectively, and the 64' frame for high density analysis. However, when serum concentrations of S_f 20 - 400 lipoprotein are below 150 mg/100 ml it is recommended that the S_f 200-100 lipoproteins be measured on the 2' UTS frame. A close estimate of this lipoprotein concentration is obtained by refractometry of the total VLDL fraction (Section II 4). Using a 5X enlarger in a darkroom, the appropriate frames are traced on precision photo-offset templates of proper linear magnification for each ultracentrifuge. Tic marks at selected S_f and F rate intervals on the template are connected using a sharp 7H pencil and a drafting machine, after which each ordinate height is manually measured. A total of 29 and 15 ordinate heights are measured in the standard low and high density patterns, respectively. All such data for each run are transcribed on to special keypunch forms, giving also the density of each fraction, concentration relative to serum and the conditions of the analytic run, for example, temperature, the up-to-speed equivalent of the 0' UTS picture, machine calibration data and useful alphanumeric coding information about the lipoprotein fraction. Our Fortran IV program of approximately 50,000 field length, runs on a Control Data Corp. 6600 computer, however with minor modifications it may be used with most high-speed large capacity computers. Figure 8 shows schematically the operation of

the program including all needed corrections such as flotation versus concentration dependence, Ogston-Johnston corrections and corrections to standard condition of density and temperature. Thus, this computer program yields numerical output fully corrected in terms of lipoprotein concentrations of the original sample, allowing valid comparisons to be made from sample to sample. It should be emphasized that the data acquisition system described earlier provides the accumulated value of $\omega_{FS}^{-2}\int\omega^2(t)dt$ for all frames. Using this O' UTS data, which may vary as much as 10% from one run to the next, permits much more accurate schlieren analysis of the very low density spectra. When unusually high concentrations of chylomicra are present, $S_f > 1000$ lipoproteins are removed from the serum (Section II-C). An additional program uses the preparative recovery data and appropriately corrects the lipoprotein spectra for partial removal of the S_f 0 - 400 lipoproteins.

F. Graphic Presentation of Schlieren Data.

In order to extend and improve the computer analysis of lipoprotein distributions, another program presents these data in graphic form, allowing rapid visual evaluation, comparison of samples and population means, as well as providing for error detection (29). Although the program routinely is used for graphical presentation of the low and high density spectra, other applications are available. For example, schlieren flotation (or sedimentation) patterns from any single frame may be analyzed at any time, including an acceleration frame. The results from any frame may be plotted at any desired up-to-speed time value for ease of comparing data. This plotting program converts S_f° rates, $F_{1.20}^\circ$ rates[4] or (s rates) into appropriate linear dimensions and lipoprotein concentrations into areas equivalent to those of the corrected schlieren patterns, routinely presented at 3 times the concentration of serum. It then calculates points along a best fit curve through the resulting histogram from which the graph is plotted. We normally use a cathode ray tube (CRT) plotter, but when greater

dimensional accuracy is required a California Computer Corp. plotter[3] is used. Since the Cal-Comp plotter is accurate to 0.01", it is also useful in drawing master templates for tracing schlieren patterns from any analytic ultracentrifuge, given the proper magnification factor and the conditions of the analytic run.

In addition to the actual plotting, the program sums lipoproteins of the total pattern and of the specified low density subfractions (S_f° 0 - 12, S_f° 12 - 20, S_f° 20 - 100 and S_f° 100 - 400). The single frame high density analysis sums the $F_{1.20}^\circ$ 3.5 - 9 and $F_{1.20}^\circ$ 0 - 3.5 subfractions corresponding roughly to the older HDL_2 and HDL_3 nomenclature (11). Final steps in the program are to draw a rectangle around each frame, with tic marks and frame boundaries which normally correspond to the templates on which the schlieren pattern was initially traced. The plots are labelled and all standard interval lipoprotein values are placed in the appropriate frames. The program is flexible allowing one to plot several patterns on one frame for comparison, or to plot the mean and/or standard deviation of a population.

In the low density lipoprotein plots we normally use an S_f scale identical to that of the 3 frame template used for tracing the enlarged schlieren patterns (see Fig. 9). However, the montage of three frames (taken at different times to include the total S_f° 0 - 400 lipoprotein distribution) results in a discontinuous curve (Fig. 9a) representing what is essentially a continuous spectrum. In Fig. 9b, a logarithmic scale has been chosen to achieve continuity while preserving the relative widths represented by the individual schlieren frames. To accomplish this, a variable K log (S_f° + 5) is used to avoid negative values. The usefulness of a similar logarithmic scale, particularly for the S_f 0 - 12

(LDL) spectra, has been discussed earlier (30). Another potentially useful scale, using the square root of S_f rate would yield a scale nearly linear in particle diameter.

Although patterns may be compared visually or by plotting them on a single graph, small but significant differences still may be hard to detect. Another version of the program subtracts the first pattern from one or more patterns yielding difference plots at any specified magnification (see Fig. 9d).

For standard low and high density lipoprotein analysis, the plotting program now runs as part of the same job so that plots are routinely obtained, usually on an overnight basis. Since our data reduction from schlieren film to punch cards involves two technicians and two keypunch operators, occasional errors are made. However, nearly all such errors result in bumps or irregularities on an otherwise smooth and familiar schlieren curve and are now easily detected by visual examination of the CRT plots.

G. Normal and Abnormal Lipoprotein Spectra.

Although it is not our purpose here to present in detail the nature of normal and abnormal lipoprotein spectra, it is nonetheless worthwhile to consider certain features of such ultracentrifugal lipoprotein profiles. An extensive earlier study by Gofman et al. (31) defined many of these broad features in both normal and certain abnormal metabolic states. More recent detailed and comparative studies utilizing current methodology are presented elsewhere (32,33). Fig. 10 and 11 show an adult normal male and female non-fasting population (Livermore G and Livermore H) as well as the main clinical types as described by Fredrickson et al. (34). The latter profiles are from a previous study (32) and are fasting plasma pools. A principal reason for re-presenting these data is to familiarize the reader with the comparative features of these normal and abnormal

patterns, using the new continuous logarithmic low density lipoprotein profiles. It is our opinion that in the future such profiles will replace the older discontinuous schlieren presentations.

Most abnormal lipoprotein patterns may be classified by paper (or agarose) electrophoresis and/or analytic ultracentrifugation as one of the above designated types. However, there are occasional patterns which seem to represent variations or combinations of these basic types. For example, certain type II patients (characterized by elevated S_f° 0 - 20) may also exhibit elevated S_f° 20 - 400 (32) and this may reflect a sensitivity to carbohydrate ingestion (Blankenhorn, D. B., A. B. Chin, L. C. Jensen, and F. T. Lindgren, unpublished observations). Also, type II patients exhibit a wide range of S_f° 0 - 12 elevations and those having concentrations above 700 mg/ml usually exhibit tendonous lesions (33).

Type III patients are characterized by the presence of substantial amounts of electrophoretic β-lipoproteins that float at 1.006 g/ml (34) and by S_f° 20 - 400 lipoproteins with an elevated cholesteryl ester/glyceride ratio (35). Although the type III pool shown in Fig. 11 shows both low S_f° 100 - 400 and $S_f > 400$, when plasma glycerides are elevated, type III patients exhibit grossly elevated S_f° 20 - 400 and possibly $S_f > 400$ lipoproteins. Thus, ultracentrifugal patterns of these type III patients may resemble type IV or V patterns.

The type IV profile in Fig. 11 shows approximately a normal S_f° 0 - 20 concentration, however, there are instances where both S_f° 0 - 20 and S_f° 20 - 400 are grossly elevated (33) and such patterns may be difficult to distinguish ultracentrifugally from type II patients with moderate glyceride elevation (32).

Type V fasting patients characteristically have markedly elevated S_f° 20-400

VLDL and elevated $S_f > 400$ lipoproteins. However, there are patterns that indeed may be a combination of, or some intermediate gradation between, the type I and type V patterns shown in Figs. 10 and 11. It becomes clear that some difficulty in placing all lipoprotein abnormalities in a limited primary category of types is to be expected. A given patient, for example, may exhibit a combination of metabolic lipoprotein abnormalities, one or more of which may be sensitive to such factors as caloric imbalance or other acute nutritional changes, certain drug therapy, and even some of the unknown environmental factors of hospitalization.

Finally, Fig. 11 shows the two distinct lipoprotein deficiencies, Tangier disease and abeta-lipoproteinemia. Each pattern shows unusual features - the former an abnormal VLDL distribution and the latter an unusually broad HDL pattern.

V. MEASUREMENT AND APPLICATION OF LIPOPROTEIN FLOTATION RATES.

The computer analysis of the low and high density lipoprotein spectra described earlier provides the S_f° or $F_{1.20}^\circ$ rate of the peak position. However, this determination is based on a single measurement of peak position and is sensitive to the correct base-of-cell position which includes the problem of cell tilt. Therefore, wherever accurate flotation rates are needed, such as the characterization of the major S_f 0 - 12 component, we use a computer program (24) to analyze peak flotation rates by the moving boundary method (36) (37). In appropriate frames, using a 5-fold projected enlargement, the distance of the maximum ordinate of the peak position is measured from the standard radial reference position of the inner knife edge. Using these measured

distances and the times of the associated frame times t_i, the program computes the best fit (least squares) straight line for the points ($\omega^2 t_i$, ln x_i). Fig. 12 shows the plot of this line, the slope of which is the uncorrected flotation rate of the peak. In the low density run the S_f rate of the major component of the S_f 0 - 12 class is normally measured in the 8, 14, 22, 30, 48 and 64 min. UTS frames. Factors such as increased lipoprotein concentration and salt redistribution with increasing time tend to change slightly the observed slope of the $\omega^2 t$, ln x_i plot. Since corrections for these changing conditions are not easily incorporated in the calculations, they are omitted from our current program. Other factors such as differential compressibility of the lipoprotein and its small molecule background present additional considerations, although for the S_f 0 - 12 class these effects may be negligible (38).

Given the UTS equivalent of the acceleration phase, the program can solve the base-of-cell position (Fig. 13). This calculated base-of-cell can be significantly displaced outward (as much as 0.2 mm in the cell) from the observed base-of-cell position, especially in the late UTS frames. A part of this displacement may be the result of cell tilt (39), estimated to be the order of 0.1 mm. However, the presence of an inconspicuously small amount of sedimenting lipoprotein or protein can give rise to a false base-of-cell that moves out slightly as a function of time. As a consequence of these two factors, the more accurate uncorrected flotation rates computed by the best-fit moving boundary method may be significantly faster than those computed by S_f rate measurement using the peak position and the base-of-cell in the 30' UTS frame. It is obvious that small errors in the lipoprotein profile result from the above mentioned

uncertainties in aligning the base-of-cell position on the photo-offset template with the assumed (or calculated) base-of-cell position of the schlieren projection.

A typical flotation rate calculation shown in Fig.13 utilizes data from 3 to 6 schlieren frames. The program also computes the deviation D LN(X) of each point from the best fit straight line and then recalculates the slope by successively omitting the most deviant value until only two points remain or until no deviation is above 0.00030. Such presentation of the data allows detection of both reading errors and occasional but subtle cell leaks (if the cells are not weighed before and after the run). The program is flexible and allows measurement of flotation or sedimentation rates in any number of frames at any time, including frames taken during acceleration. In the latter case, appropriate values of $\omega_{FS}^{-2}\int\omega^2(t)dt$ are obtained from the data acquisition system. Special application of this technique to S_f 0 - 12 flotation rates in both low and high density media follows in the next section.

A. Low Density S_f° Rates, Hydrated Densities and Molecular Weights.

Studies have indicated that the principal low density S_f 0 - 12 lipoprotein class represents a distribution in molecular weight, hydrated density and chemical composition (40-42). Values given for molecular weights of this class have ranged from 1.3 - 3 million (10,43-45) and depend, in part, on the techniques employed and the portion of the lipoprotein distribution studied. Protein content appears to be the major factor determining hydrated density of this class, which ranges from approximately 1.02 - 1.05 g/ml. Because of this distribution of values, for specific physical characterization of a given LDL spectrum it is necessary to focus on the most abundant S_f 0 - 12 component as measured in the

analytic ultracentrifuge. The flotation rate of this major low density component varies from individual to individual and has a range of about S_f 4 - 8 svedberg units. Normal females have S_f° rates approximately 1 S_f° unit faster than normal males (24, 46). Also, there appear to be unusual flotation rates of this component associated with specific lipid and lipoprotein abnormalities (32).

B. Utilization of the Standard Low and High Density Lipoprotein Run.

In previous sections we have considered computer methods for quantifying the total low and high density lipoprotein profile as well as a flexible method for computer analysis of moving boundary flotation (or sedimentation) rates, in particular, the flotation rate of the S_f 0 - 12 major component. Theoretically, there is sufficient information available from these standard low and high density runs to calculate more precise S_f° rates as well as to calculate hydrated densities (σ's) and molecular weights. Our next method utilizes the results and data from these two programs as input to a third separate computer program, which calculates S_f° rates, σ's and molecular weights. The relationship between these programs are shown in Fig. 14.

Program II calculates classical moving boundary flotation rates of the major S_f 0 - 12 component as measured in both 1.063 g/ml NaCl and 1.203 g/ml NaBr. Measurements in 1.063 g/ml are as described earlier and measurements in 1.203 g/ml are usually measured in the 0, 2, 6, 8, 14 and 22 min - UTS frames.

Program I, involving complete schlieren analysis, provides all needed lipoprotein concentrations and data such as rotor temperature which determine background density and viscosity. The background refractive indexes of each lipoprotein

infranatant are measured by precision refractometry. These values are extrapolated to the corresponding supernatant fraction. Thus, from the computer generated salt tables described earlier values of η and ρ are available corresponding to each measured flotation rate, corrected to the mean temperature of the analytic run.

A third program using the above data performs all remaining calculations. Flotation rates are corrected for concentration dependence by the relationship $F = F°(1-KC)$ where $K = 0.89 \times 10^{-4}(\text{mg}/100\ \text{ml})^{-1}$ and C is the lipoprotein concentration in the cell integrated up to the low density S_f 0 - 12 peak position, averaged over the time interval used in the moving boundary flotation rate measurement. This average is approximated by a Moring-Claesson type (47) correction: $C = Co(X_{BC})^2/(X_1X_2)$ where Co is the initial base-of-cell concentration as determined in program I, X_{BC} is the base-of-cell radial distance, and X_1 and X_2 are the first and last radial peak positions measured. In the 1.203 g/ml run, C is the sum of both the above LDL concentration and the total HDL concentration, similarly corrected. If the low and high-density fractions are run at different concentrations, the program makes the appropriate corrections. From these data an η F° versus ρ plot is made and a ρ intercept calculated. S_f° rates corrected to standard conditions are made from the relationship:
$S_f{}^\circ = F°(\rho_s-\sigma)\eta/(\rho-\sigma)\eta_s$ where standard values at 26C for 1.745 molal NaCl are $\rho_s = 1.0630$ g/ml and $\eta_s = 1.0260$ cp.

Assuming Stokes' frictional factor for spheres, a molecular diameter may be

calculated from the relationship $S_f^\circ = d^2(\rho_s-\sigma)/18\eta = d^2(1.0630-\sigma)/184.7$; here d is converted to Angstrom units in the final expression. Lastly, a minimum hydrated molecular weight is calculated, assuming spheres, from the molecular volume, Avagadro's number and the hydrated density (the latter is closely approximated by the density of zero migration or ρ intercept:

$$\text{Mol wt (daltons)} = \pi d^3/6(10^{-8}\text{cm/A})^3\, N_\circ \sigma = 0.3153\, d^3\sigma.$$

C. η F versus ρ Techniques using Isolated Lipoprotein Components.

Classical η F versus ρ methodology involves the analysis of isolated homogeneous components at two or more densities. Since the three programs just described are applicable for the analysis of single components, it is appropriate to consider such application to LDL subfractions. This application allows comparison and verification of the previous techniques; also this general method can be applied to any VLDL, LDL and HDL subfractions, such as those obtained by cumulative flotation techniques described later.

Narrow-band LDL lipoproteins may be prepared on an approach-to-equilibrium NaBr density gradient (41). After ultracentrifugation at 50,000 rpm for 48 hrs at 18C, in a 50.3 rotor, fractions are isolated in the 2nd, 3rd and 4th ml, corresponding to densities of approximately 1.031, 1.034 and 1.037 g/ml, respectively. Densities of such isolated lipoprotein fractions are manipulated to approximately 1.061 g/ml and 1.200 g/ml by appropriate solid NaBr addition and the final fraction monitored by precision refractometry. For precise density calculations the refractive increment of these lipoproteins was assumed to be 0.00154 and 0.00140 Δn/g/100 ml in the two media, respectively. Since lipoprotein refractive increment is measured by the schlieren analysis program, precise background

refractive index and hence density of each fraction in each of the media is known. As in the previous method, flotation rates are calculated and corrected to zero concentration. However, for this calculation the time dependent total component concentration is used. Similarly, ηF° versus ρ plots are made and S_f° rates, hydrated densities and molecular weights calculated. In these studies using isolated components the problem of correction for concentration dependence of the LDL component in the presence of a time dependent HDL concentration (which is different for each individual) is avoided. It is of interest that in this calculation ρ intercept values are essentially independent of the concentration dependent K factor, although S_f° rates and molecular weights are affected.

D. Application of Techniques to Small Populations.

A series of normal male and female subjects were clinically healthy employees of Lawrence Radiation Laboratory at Livermore and Berkeley, Ca. Serum was prepared from mid-morning non-fasting blood specimens. A complementary series of fasting male and female clinical referrals were obtained from Kaiser Hospital, Oakland, Ca. Unless otherwise indicated all serum or plasma samples studied contain 1/10,000 Merthiolate®. Each population set was matched for age, and grossly overweight or underweight subjects were excluded.

In the normal non-fasting series studied, total LDL fractions $1.006 < \sigma < 1.063$ g/ml, (S_f 0 - 20) were isolated as described in Section IV. Total lipids were extracted as previously described (6) and cholesteryl ester, glyceride and phospholipid composition were determined by infrared spectrometry (48).

Technical reproducibility was evaluated using 9 serum aliquots from a single non-fasting normal male subject. A measure of errors in our procedure employing total low and high density lipoprotein fractions is the standard deviation of the results:

S_f° rate of major component = 4.87 ± 0.08 Svedbergs (10^{-13}cm/sec/dyne/gm).

σ (hydrated) = ρ intercept = 1.0360 ± 0.0003 g/ml (26°C)

Molecular weight $\cong$ 1.984 ± 0.021 (millions).

For appropriate comparison using non-fasting serum obtained earlier from this same male subject, η F° versus ρ studies were made on isolated narrow-band LDL components and the results compared with our standard low and high density technique. Fig. 15 shows schlieren photographs of both the 1.063 g/ml NaBr and 1.203 g/ml NaBr runs of one of the narrow-band lipoprotein fractions prepared from the 1.034 g/ml density gradient fraction (Merthiolate® series). All density gradient samples were analyzed at total component concentrations in the range of 261 - 704 mg/100 ml. Almost identical linear regression relationship between S_f° rate and ρ intercept were obtained for the Merthiolate® and non-Merthiolate® fractions (Fig.16). Also plotted are the results (from the same serum sample) of our procedure utilizing 8 standard low and high density lipoprotein analyses obtained over a period of 33 days. Nine months later 9 additional analyses by our method were performed on serum from the same non-fasting subject and the mean value plotted, suggesting minimal biological variation in this person. Fig.17 shows a similar linear regression relationship of S_f° rate and molecular weight. Again, values obtained from simultaneous low and high density lipoprotein analyses indicate comparable results in the region of

the major component compared with the same S_f° rate on the regression line obtained with isolated lipoprotein components. These results suggest our total low and high density lipoprotein analyses give results closely similar to an ideal procedure. However, such a technically difficult method applied to each individual serum would require the identification and narrow-band isolation of the most abundant S_f 0 - 12 lipoprotein species followed by a classical η F° versus ρ analysis.

Preliminary application of this method involved nonfasting serum from a small, normal adult, male and female population, ages 35 - 40 years. Figs. 18 and 19 show the relationship observed between S_f° rate and the two calculated parameters, ρ intercept and molecular weight, respectively. Similar regression is observed with this population as compared with the components isolated from a single normal male individual. For comparison with the isolated component study the mean male and female values are also plotted on Figs. 16 and 17. Results indicate that at a given S_f° rate there is variability from person to person both in ρ intercept and in molecular weight. Although the females have significantly higher S_f° rates than the males, there appear to be very similar regression relationships for each population. Table I presents the low density lipoprotein results for this series of normals, including the correlations among the four variables, S_f° rate, ρ intercept, molecular weight of the major S_f° 0 - 12 component and VLDL concentration. The females (compared with the males) have faster S_f° rates, as has been observed before (24,46); they also have approximately a 235,000 higher mean molecular weight and a slightly lower mean hydrated density.

Total LDL of the S_f 0 - 20 class were isolated from each of the above normal nonfasting males and females. Cholesteryl ester, triglyceride and phospholipid content of the total lipid moiety is presented in Table II. The females have a higher phospholipid and lower cholesteryl ester content in those molecules than the males. However, the differences are small and there is not much variability in lipid composition in these two populations. Although some relationships were observed in the females between these lipid values and the three physical parameters of the most abundant S_f 0 - 12 component (S_f° rate, σ and molecular weight) no significant relationships were observed in the male population (see Table III). The calculated mean density of the lipid moieties of each population was almost identical. Although a high negative correlation between σ intercept and phospholipid content was observed in the females, considering the small standard deviation of σ (lipid) and phospholipid content, variations in the latter would not be expected to give the observed variations in hydrated density. Also, in the females there was a moderately positive correlation of glyceride content with ρ intercept (and a low order positive correlation in the males). These results suggest that the major factor contributing to changes in hydrated density within this class is not glyceride wt% content but is the wt% protein content of the lipoprotein as shown in Section VII C. In a much earlier density gradient study, this relationship between protein content and lipoprotein density was also observed on subfractions of S_f° 3 - 10 from pooled human plasma (40). In a recent study, however, (49) no significant correlation was found between S_f° rate of the major LDL component and protein content of the total LDL fraction. Since protein content increases with decreasing S_f° rate of LDL subfractions, the protein content of the total LDL is determined by the lipoprotein profile within intervals of the S_f 0 - 20 class and not by the S_f° rate of the major component (6).

Another preliminary application of this method was to a small series of 16 males and 19 female clinical referrals from Kaiser Hospital, Oakland, Ca. These patients were of a wider age range; many had high blood lipids, and in contrast with the normals studied, they were fasting. Table IV presents the low density lipoprotein results for these populations, including the statistical relationships. As in the normal populations, similar values, differences and correlations were observed, although the somewhat lower S_f° rates observed probably reflect a clinical population with higher levels of VLDL (see Tables I and IV). Lower S_f° rates would be expected, since in both normal male and female populations there is a significant negative correlation between S_f° rate and VLDL concentration in this study and in a previous one (6).

From theoretical considerations molecular weights obtained by our technique might be expected to be low by as much as 10%, although differences between males and females as well as the relationship observed between S_f° rate, σ and molecular weight would appear to be valid. These results, of course, apply to the small populations studied. However, it would seem unlikely, for example, that the shape factor for S_f° 4 - 8 lipoproteins would be different in males and females or would vary significantly from, say, S_f° 4 to S_f° 8. There are difficulties in applying corrections for concentration dependence in the flotation measurements, but there is no evidence that any of the observed relationships and relative differences are K factor dependent. Recalculating all data using K factors of 0.44 or 1.78 x 10^{-4}(mg/100 ml)$^{-1}$ did not significantly alter any of the observed relationships. A 20% error in the K factor used here, 0.89 x 10^{-4}(mg/100 ml)$^{-1}$, would involve approximately a 5% error in molecular weight. However, it is anticipated that as more concentration dependence data and shape factors become available, more meaningful and accurate molecular

weight data may be obtained using this procedure. The main advantage of this technique is that all the needed information is derived from a single routine simultaneous low and high density lipoprotein analysis.

E. Comparison of Ultracentrifugal Techniques for Obtaining LDL Molecular Weight.

Although our LDL molecular weights are compared with the results of others in some detail elsewhere (50), it is worthwhile to mention a few recent comparisons. For example, from somewhat similar ultracentrifugal data of the major low density component (and assuming spheres) Oncley (44) obtained a molecular weight of 2.3 million and a hydrated density of 1.032 g/ml. In their most recent hydrodynamic study, Adams and Schumaker (51) obtained a mean and standard deviation for nine LDL samples (fasting males, ages 20 - 40) of 2.38 ± 0.13 millions. These values assumed a shape factor of $f/f_o = 1.05$ and 10% hydration. Had they assumed spheres and no hydration, the value would have been approximately 2.16 ± 0.13 millions. From equilibrium data Scanu et al. (52) have given a range of 2.2 - 2.3 millions for the major low density component, $1.019 < \sigma < 1.063$ g/ml. Our data on both normals and clinical referrals are approximately of this magnitude.

VI. ISOLATION OF SERUM LIPOPROTEIN FRACTIONS USING SWINGING-BUCKET ROTORS AND DENSITY GRADIENTS.

A. Elemental Analysis of Lipoprotein Fractions.

Although routine biochemical and physical methods are available for quantifying total lipoprotein lipid, constituent lipids (8,53) and lipoprotein protein (54,55), these procedures have certain limitations and disadvantages. For

example, if limited lipoprotein sample is available and one is primarily interested in total lipoprotein mass, protein and phospholipid content, a more convenient methodology is available, namely elemental CHN and P analysis. Since such data are presented in this section, which describes density gradient preparation of lipoprotein fractions, it is appropriate to consider briefly these techniques now.

For elemental CHN analysis we use a modified CHN analyzer (Model 185, Hewlett-Packard, Palo Alto, Ca.)[3]. Aliquots of from 0.004 ml to 0.1 ml of lipoprotein solutions are pipetted into contamination-free aluminum boats (29 - 410M, Perkin-Elmer, Maywood, Ill.[3]) and weighed on a semi-micro balance. Readings are taken 20 seconds after pipetting and corrections for evaporation may be made by adding the weight increment lost during an additional 20 second period. Ideally, total lipoprotein mass should be in the range of 200-400 μg. Triplicate samples are dried at 55° overnight and at 110°C for an hour just before analysis. Other details of procedure, precautions to avoid contamination and absolute mass calibration of the instrument, including the computer program, are described in detail elsewhere (56,57). The results of agreeable duplicates of the CHN analysis provide precise total NCH composition as well as N/NCH ratios for each lipoprotein fraction.

Since total lipoprotein N is derived almost exclusively[5] from both protein and phospholipid, the latter also must be determined. The Bartlett method (58) provides such a precise semi-micro phosphorus determination. As before, appropriate lipoprotein aliquots are accurately weighed such that estimated PL content will be in the approximate range of the standard sample, namely, 1 μg phosphorus.

Contamination, especially from soaps and detergent residues on glassware and from smokers (igniting matches) should be scrupulously avoided. Cuvettes should be carefully matched and absorbance readings obtained from a stable instrument such as a Cary Model 14 recording spectrometer (Cary Instruments, Monrovia, Ca.)[3]. If no inorganic phosphorus is present in the lipoprotein fractions, we may assume all elemental phosphorus is derived from lipoprotein phospholipid. Thus, we can combine the results of the two elemental methods using computer derived tables. Knowing the total N + C + H and the approximate chemical composition of the lipoproteins, which yield an accurate ratio of N + C + H/total mass, we obtain the total lipoprotein mass. From the N/NCH ratio corrected for the phospholipid N (assuming 1 atom of N per atom P) we obtain a precise protein determination. In the calculations we assume a protein N/NCH ratio of 0.2000 for S_f 0 - 10^5 lipoproteins and 0.2056 for HDL lipoproteins; these values are derived from the amino acid composition of the LDL protein and HDL protein (7), respectively.

Under favorable operating conditions and optimum sample mass, this elemental procedure routinely yields data with standard errors of measurement of approximately 0.5% for lipoprotein mass, 0.1 - 0.3% wt% lipoprotein protein and (0.5 - 1.0%) for wt% phospholipid. In the future such data may be helpful in the detailed comparison of lipoprotein subfractions obtained from different patients exhibiting unusual lipoprotein abnormalities. Also, the accurate measurement of lipoprotein mass, independent of the presence of large amounts of inorganic salts, should permit very accurate evaluation of specific refractive increments for all low and high density subfractions.

B. Isolation of Total $S_f > 400$ Chylomicron-containing Fractions.

As mentioned before, a rigorous definition of the chylomicra is difficult, particularly if fractions are isolated from plasma or serum instead of from chyle or lymph. Since their discovery by Gage (59) over 50 years ago, procedures to isolate and quantify the chylomicrons have included the original method of dark field counting of particles (60), estimation by turbidimetric procedures (61,62), flocculation by toluidine blue (63) and polyvinyl-pyrrolidone (64) and various combinations of ultracentrifugal isolation (65-68) and mass assay. Although these techniques are useful for specific experimental work their limitation is the difficulty in reproducing and comparing results from one laboratory to another. The two basic problems of chylomicron analysis are to define unambiguously the isolation procedure and to provide an accurate and reproducible mass assay. As recommended by Dole and Hamlin (67) the fractionation should be expressed in terms of the product of mean relative centrifugal force and time (g min) as well as to define completely the conditions of preparative ultracentrifugation.

Our procedure defines a chylomicron-containing fraction isolated ultracentrifugally only in terms of its included S_f range of recovery. The method is essentially that described earlier (57) and the data given in Section IV B are from the original procedure. However, results using our slightly modified and improved procedure would be very similar, except that the included S_f range is slightly larger with lower 100% recovery as defined below. With modifications only in the centrifugal conditions, larger samples may be used in the 12 ml and 17 ml buckets and fractions may be obtained with any desired lower S_f range of recovery.

Since our overlayering procedure for preparing a non-linear salt gradient is used for all lipoprotein subfractionation work, we present it in detail now. First, 84.4 mg of dry NaCl is weighed into a screw cap vial and 1.00 ml of serum added. The capped vial is swirled and allowed to stand for about 30 minutes. This bubble free serum solution (of background density 1.065 g/ml) is transferred to the bottom of a $\frac{1}{2}$" x $2\frac{1}{2}$" cellulose nitrate tube. Before transferring, 10 drops of 1.065 g/ml NaCl solution is placed in the bottom and an epoxy hemispherical insert is positioned as shown in Fig. 20 . This essentially eliminates the curvature in the lower boundary of the sample. The inserts for each tube size are made by casting plain epoxy or Al-filled epoxy in the bottom of appropriate preparative tubes. After hardening, the hemispheres are machined and lapped to final weights (which are within 20 mg for each type).

The plastic holder is now tilted to a position 25° from horizontal and during overlayering it is illuminated by a beam of light from the side in a darkened room (see Fig. 21). As shown in Fig.20 precise volumes of the indicated sodium chloride solutions are added dropwise on the tube wall above the serum layer. All salt solutions used in the density gradient procedures contain 3 mg/100 ml EDTA. This amount, rather than the 10 mg/100 ml normally used, is necessary to minimize the NCH elemental background for both mass and protein determinations. After the gradient is finished the holder is slowly rotated to the vertical position and the layered preparative tube carefully placed into the appropriate 6 ml bucket and the bucket capped. Although we recommend a Beckman 25.3 or 27 rotor with special 6 ml buckets (mean r = 10.44 cm), a 25.1, 25.3 or 27 rotor equipped with 6 ml tube adapters may be used.

ient work. All our swinging bucket centrifugations are carried out at room temperature (~23°C) in the Beckman L2-65 (or 65B) ultracentrifuge (modified to achieve temperature stability to ± 0.2°C, rotor speed stability to ± 30 rpm and equipped with a variable overspeed safety device). Precise lipoprotein recovery depends on the density and viscosity of all regions in the gradient, therefore, the initial rotor temperature (approximately equal to room temperature and the gradient temperature) of a particular run determines the equivalent up-to-speed centrifugation (full speed time plus 1/3 of the sum of acceleration and deceleration). This value is obtained from computer tables (see Section VI C) calculated for proper recovery over the range of 22 - 26°C in $\frac{1}{2}$ degree increments. Although we recommend collection of the chylomicron-containing fraction in only 0.5 ml, there may be circumstances when this is not possible. In these instances the initial serum solution may be diluted or a full 1 ml fraction taken. If the latter is done there will be a slightly different threshold recovery. At 23°C the total g x min required for 100% $S_f > 400$ recovery is 2.85×10^6 and 2.50×10^6 for a 0.5 ml and 1.0 ml fraction, respectively.

Care should be exercised to start the centrifuge slowly; after reaching about 1000 rpm the speed control pot may be set at full speed. We do not use the brake. Following centrifugation the $\frac{1}{2}$ ml $S_f > 400$ fraction is quantitatively removed with a carefully selected capillary pipette (Section II D) and two additional $\frac{1}{2}$ ml fractions taken as reference undernatants. Ideally, the preparation of the gradient and removal of the fractions, in this and all other

density gradient procedures, should be done as close to the centrifuge as laboratory conditions permit.

The stability of the salt gradient is shown in Fig. 22. Very little difference is observed between a gradient pipetted immediately and one allowed to stand at 1 g for 2.5 - 3.5 hr. More importantly, there is good reproducibility and very little difference between the static and dynamic gradients (61.0' UTS at 20,000 rpm). Accordingly, we use a mean of the dynamic gradient values for calculating lipoprotein recoveries in our chylomicron-containing fractions.

C. Calculation of Lipoprotein Recovery

Fig. 23 shows a 6-ml preparative tube containing a dynamic salt gradient divided into 12 regions; to simplify the calculations, each of these 12 regions may be considered a homogeneous region (j) of ρ_j and η_j, with a lower boundary at radial distance r_j determined by the rotor and rotor tube.

Lipoprotein recovery for a specified top fraction may be defined in terms of 100% and threshold S_f rates. Migration up to the lower boundary of the fraction from the bottom and the top, respectively, of the layered sample is defined as 100% recovery and threshold recovery (0%). Ideally, all lipoproteins of S_f value greater than the 100% value should be recovered in the top fraction, while none lower than the threshold value should be included. Using a computer program written in Fortran IV, tables are constructed of centrifuge run times for 100% recovery of specified S_f values at various temperature and rpm values. If a given S_f rate of hydrated density σ is to be recovered in fraction 12,

the relation:

$$S_f = F_j(\sigma_s-\sigma)\eta_j/\eta_s(\rho_j-\sigma)$$

yields the flotation rates F_j which determine the times of migration Δt_j through each region:

$$F_j = (\ln r_{j+1} - \ln r_j)/\omega^2\Delta t_j$$

Then summing, $\omega^2 T = \sum_{j=1}\omega^2\Delta t_i$,

where T is the total equivalent up-to-speed time. The program then converts this ω^2T into shut-off times at specified rpm values, taking into account the acceleration and deceleration period. Using our 6 ml density gradient (23C), the conditions required to achieve 100% lipoprotein recovery over a whole range of S_f values are shown in Fig. 24. Times for a multi-stage run, such as the cumulative subfractionation of lipoprotein classes, in which successive layers are removed, are also calculated from the increment of ω^2T over the previous stage (for each successive run).

Calculating corresponding threshold recoveries, as well as 100% and threshold S_f values of a run that has been made under particular conditions of time, temperature, rpm, rotor and gradient is accomplished by reversing the calculations. This involves iteration, because the hydrated density σ must be approximated for the unknown S_f value. For example, the 100% recovery

calculation considers as a first approximation the flotation rate:

$$F = (\ln r_{12} - \ln r_1)/\omega^2 T$$

Then assuming a mean ρ, η and $\sigma_o = 0.93$ g/ml,

$$S_{f_1} = F \times 1.44 \text{ and } \sigma_1 = f(\ln S_{f_1}),$$

from literature values (68,69). Starting an interative calculation with $i = 1$, F_j is calculated over each region j from:

$$S_{f_i} \cong F_j(\rho_s - \sigma_i)\eta_j/\eta_s(\rho_j - \sigma_i)$$

Then Δt_j and hence $(\Sigma_1^{11} \Delta t_j)_i$ may be calculated from

$$F_j = \Delta \ln r_j/\omega^2 \Delta t_j$$

A new approximation is now made setting

$$S_{f(i+1)} = S_{f_i}(\Sigma_1^{11} \Delta t_j/T)$$

and one continues to iterate for convergence until

$$\left|(\Sigma_1^{11} \Delta t_j)_\eta - T\right| /T < 0.001$$

D. <u>Subfractionation of the $S_f > 400$ Lipoproteins</u>

The following procedure scaled for subfractionation of 3 - 18 ml serum represents a modification and improvement of an earlier (69) cumulative flotation procedure. Its application to the $S_f > 400$, VLDL, LDL and HDL classes

has been made possible by the development and availability of high performance, long radial-path swinging bucket rotors. The subfractionation of each class involves overlayering of serum or a serum containing fraction with a particular non-linear salt gradient, similar in many respects to the procedure just described for total $S_f > 400$ lipoprotein fractionation.

In the first step of the procedure, 327 mg of solid sodium choloride is added to 3 ml of plasma raising the background density to 1.065 g/ml. Before the 3 ml sample is placed in a 5/8" x 4" heavy walled preparative tube, a few drops of 1.065 g/ml NaCl solution are added in the bottom. Then a solid epoxy hemispherical insert (which fits snugly) is positioned in the bottom of the tube as shown in Fig. 25. A long Lucite sleeve is inserted in the top of the tube to avoid wall contamination and the serum sample is carefully introduced into the bottom, avoiding any bubbles. Except for scaling up volumes, the gradient formation is similar to the $S_f > 400$ fractionation. After rotating the tube to 25° from the horizontal, the sample is overlayered with a 14 ml NaCl density gradient. Solutions are added (from highest to lowest density) in volumes of 1 ml each for the first two and 3 ml each for the remaining four. The densities are 1.0464, 1.0336, 1.0271, 1.0197, 1.0117 and 1.0064 g/ml and the resulting gradient is shown in Fig. 25. Corresponding $n_D 26$ values are given in Fig. 20.

The next steps consist of removing 0.5 ml layers from the tops of the tubes after three successive centrifugations of 0.739×10^6 g x min, 1.320×10^6g x min and 3.384×10^6g x min. These runs are made in an SW 25.3 rotor (mean r = 11.43 cm) under conditions such that 100% recovery is achieved

for S_f 3200, $S_f > 1100$ and $S_f > 400$, respectively, after the first, second and third runs. There are several reasons for fractionation in this manner rather than centrifuging once and removing several layers. Removal of thin layers at the top of the tubes concentrates each lipoprotein fraction and the cumulative flotation procedure minimizes the time lipoproteins are exposed to an aqueous-air interface. Also, this procedure avoids the need to either isolate the total $S_f > 400$ lipoproteins free of contamination from other serum lipoproteins and proteins or to employ tedious washing procedures for each fraction. Further, increasing the radial distance the lipoproteins in each fraction must travel by bringing them into a thin top 0.5 ml layer reduces the calculated differences in S_f rate between 100% and 0% (threshold) recovery. This also reduces overlap among the fractions collected. Another reason favoring this procedure is that both small and large molecule contamination by diffusion and convection is essentially eliminated by collecting the fractions a maximum radial distance inward from the initially layered serum sample. Fig. 25 shows the stability of this gradient as well as the centrifugal conditions required for each successive run. The calculated recovery including overlap of the subfractions is given in terms of both S_f rate and particle diameter. Although cuts at any desired S_f rate may be made, the suggested S_f intervals generally yield three $S_f > 400$ subfractions, concentrated approximately six-fold, that divide the total $S_f > 400$ lipoprotein mass into roughly equal parts. This consideration is useful in all lipoprotein subfractionation studies where detailed lipid and lipoprotein studies are involved.

E. Subfractionation of the S_f 20 - 400 Lipoproteins

The following procedure is scaled to permit VLDL subfractionation of from 2 - 12 ml of serum. As suggested in Section II C if significant amounts of $S_f > 400$ lipoproteins are present they normally should be removed prior to VLDL subfractionation. After removal, 2 ml of $S_f < 400$ subnatant serum is added to 168 mg of solid NaCl bringing the background density to 1.065 g/ml. Using hemispherical inserts and the same procedure of manipulation as in the $S_f > 400$ subfractionation, the sample is placed in a heavy walled 9/16" x 3½" cellulose nitrate preparative tube. The two ml serum sample is overlayered with a 10 ml NaCl salt gradient, using the same six solutions as in the $S_f > 400$ fractionation. The following volumes (from highest to lowest density) are 1 ml for the first two and 2 ml for the next four. The gradient and its stability are shown in Fig. 26. As before, 0.5 ml layers are removed after each of three successive centrifugations in an SW 41-Ti rotor calculated to bring up first the $S_f > 100$, the $S_f > 60$ and finally the $S_f > 20$ lipoproteins into each successive 0.5 ml top fraction. At 35,000 rpm and at 23°C the necessary centrifugal conditions for each stage are given in Fig. 26. At a mean radius of 10.76 cm, the equivalent g-min for these three centrifugations are 21.2×10^6 for the first, 15.0×10^6 for the second, and 164.7×10^6 for the third. After collection of the third fraction an additional 0.5 ml is taken for measurement of the background salt density and evaluation of possible protein contamination.

F. Subfractionation of the Low Density S_f 0 - 20 Lipoproteins

The following is a convenient subfractionation procedure scaled to permit the recovery of three LDL subfractionations, each in 0.5 - 3 ml fractions,

obtained from 2 - 12 ml of serum, respectively. Before LDL subfractionation, $S_f > 20$ lipoproteins must first be removed from the serum. To achieve this, two ml aliquots of serum are each mixed with 4 ml of 0.195M NaCl, $\rho = 1.0063$ g/ml (containing 10 mg/100 EDTA). Normally six of these 6 ml serum mixtures are centrifuged at 40,000 rpm at 18°C for 18 hr in a 40.3 rotor. The $S_f > 20$ lipoproteins are removed in the top ml as well as three other one ml fractions. After cutting off the top 1" of the preparative tube with a pair of surgical scissors, the bottom 2 ml of each preparative tube is mixed with a heavy glass stirring rod (fire polished at each end) and all bottom fractions pooled. The small molecule salt background density is raised to a density of 1.118 g/ml ($n_D26 = 1.36068$) by adding 2080 mg of dry NaCl to 12 ml of this $S_f < 20$ serum fraction and thoroughly mixing. Two ml aliquots of this bubble free $S_f < 20$ serum are over-layered, each with a 10 ml NaCl gradient in six 9/16" x 3½" heavy walled preparative tubes. The solutions are added in volumes (from highest to lowest density) of 1 ml for the first two and 2 ml for the remainder. The densities are 1.0988, 1.0860, 1.0790, 1.0722, 1.0641 and 1.0588 g/ml. Corresponding n_D26 values are 1.35619, 1.35324, 1.35161, 1.35001, 1.34811 and 1.34686, respectively. Fractions containing 0.5 ml layers are removed from the top of the tubes after three successive centrifugations of $103.7 \times 10^6 g \times$ min, $59.2 \times 10^6 g \times$ min and $61.4 \times 10^6 g \times$ min, respectively, at 23°C in an SW 41-Ti rotor (mean r = 10.76 cm). Running times at the indicated rpms, as well as the gradient and its stability are shown in Fig. 27. Below the gradient are shown the anticipated range of recovery, both in terms of S_f rate and lipoprotein diameter (calculated as Stokes' spheres). As before, a 4th (and 5th) 0.5 ml fraction may be taken after the third centrifugation to evaluate

potential contamination. The recommended rotor speeds are to allow a convenient work schedule and to minimize the risk of tube collapse, particularly during the second and third centrifugation.

G. Subfractionation of the High Density Lipoproteins

Because of their relatively low molecular weight, subfractionation of the HDL spectra is more difficult and requires a very high performance rotor. In addition there are several special complications in the fractionation of HDL on density gradients in swinging bucket rotors. For example, a rotor with long radial path buckets, such as the SW 41 Ti, would involve several days of centrifugation at maximum rotor speeds. Of necessity, an equilibrium type salt gradient must be considered. However, since at a given rotor speed the radial path length determines both the extent of this equilibrium gradient and the time required to recover HDL subfractions, some reduction in path length and increase in performance is indicated. We have achieved a workable compromise with the use of a special SW 45-Ti rotor. Our procedure prepares two 1.0 - 3.0 ml HDL subfractions from approximately 4 - 12 ml of serum.

First, the total low density lipoproteins $\sigma < 1.063$ g/ml must be removed. For this purpose and to achieve concentration of the HDL, three 4 ml aliquots of serum are mixed with 2 ml of a 0.195M NaCl and 2.43M NaBr solution ($\rho = 1.1815$ g/ml, $n_D 26 = 1.36445$). After centrifugation for 24 hrs at 18°C in a 40.3 rotor, the concentrated HDL are nearly quantitatively removed in the 3.5 - 5.5 ml layer. When remixed, this fraction has approximately a 5 - 8% serum protein background, desirable to stabilize the layering procedure and providing the approximate viscosity and density increments of whole serum. If the serum protein background determined by refractometry is significantly

different, two versions of our recovery program, one considering the bottom 1 ml fraction a salt solution only, permit appropriate (but minor) corrections in the centrifugal conditions needed for fractionation. To 6 ml of this HDL containing fraction is added 3022 mg of solid NaBr, bringing the background density to approximately 1.395 g/ml, $n_D 26$ = 1.39971. One ml aliquots of this fraction are placed in ½" x 2½" heavy walled cellulose nitrate tubes and overlayered with an approximate equilibrium NaBr gradient. No hemispherical inserts are used allowing a maximum radial path to be used. Two 0.5 ml solutions of 1.3622 and 1.3424 g/ml are added first, then two 1 ml solutions of 1.3161 and 1.2856 g/ml, and finally, two 1.5 ml solutions of 1.2521 and 1.1973 g/ml complete the 7 ml density gradient. The corresponding $n_D 26$ values are 1.39446, 1.39123, 1.38690, 1.38183, 1.37629 and 1.36711, respectively. The details of this gradient, its stability and the anticipated recovery for the two runs at 236.5 x 10^6g x min and 287.5 x 10^6g x min are shown in Fig. 28. Although the SW 45-Ti rotor (mean r = 9.75 cm) is rated to perform at 45,000 rpm, two factors require the lower rotor speeds. First, the gradient is of very high density and each tube is filled the maximum amount (7 ml). Thus, our first run at 42,000 rpm is actually close to the maximum safe speed, and after 0.5 ml removal, the speed can safely be increased to 43,000 rpm. The other practical reason for limiting speed at this time is the strength and dimensional stability of the available preparative tubes. Above 43,000 rpm the tubes tend to collapse, and even if they do not, they expand and become increasingly difficult to remove from the buckets. Other suggested precautions in using this rotor include rigorous inspection and replacement (when necessary) of the bucket cap gaskets and the use of a vacuum sentinel on the preparative centrifuge.

After each run, it still may be necessary to pipette the two 0.5 ml subfractions and one or more 0.5 ml reference fractions after the second run with the preparative tubes remaining in the buckets. This is recommended if the tubes cannot easily and smoothly be removed with a sterilized haemostat.

H. Special Considerations for all Density Gradient Procedures

Before considering some preliminary results from these subfractionation techniques it is appropriate to make some general comments. First, all procedures involve addition of salt to serum or serum fractions having approximately 5 - 7% content of serum proteins. When such salts are added to these serum solutions and mixed there are usually bubbles formed and there is an increase in solution volume. After the bubbles are removed (by low-speed centrifugation or simply waiting for about 30 minutes) appropriate aliquots must be transferred to the bottom of preparative tubes with great care. Since there is an increase in volume, usually this can be done with a 1, 2 or 3 ml transfer pipette of small tip bore. With the above transfer procedure, there is a calculated loss, which needs to be considered if precise quantification relative to serum lipoprotein concentrations are required. It should be emphasized that contamination on the upper wall of the preparative tube and occurrence of air bubbles in the layered fraction must be scrupulously avoided. If a bubble is discovered in the layered serum solution, it must be removed before overlayering, usually by careful and minimal aspiration with a Pasteur pipette. It is obvious that optically clear cellulose nitrate (or polycarbonate) tubes must be used for these procedure.

Finally, because of the number of fractions involved and the length of fractionation time required, it is not usually practical to completely subfractionate all lipoprotein classes from one single serum sample. If it is

desirable to look at a patient's complete subfractions, such as a type V, it would be advisable to take weekly samples from which one or at most two major classes are subfractionated in succession each week. It is for the above reasons that the examples presented in the next section are from different subjects or patients.

VII. RESULTS AND DISCUSSION OF DENSITY GRADIENT LIPOPROTEIN SUBFRACTIONATIONS

A. $S_f > 400$ Lipoproteins

The examples of subfractionation of the $S_f > 400$ classes are taken from a previous study by Lossow et al. (70). In order to provide sufficient $S_f > 400$ lipoproteins, two subjects were given a 100 g. safflower oil preparation; one was a diabetic with normal blood lipids #735) and the other was a type V hyperlipemic subject (#726). Plasma specimens were obtained 8 hr and 12 hr after the meal for the diabetic and type V patient, respectively. The results of the three stage subfraction were evaluated by electron microscopy, described in detail elsewhere (70). Fig. 29 compares the calculated recovery of sample 735 with the particle size distribution determined by electron microscopy and elemental NCH total mass of each fraction. The measured recoveries, including overlap of fractions, agree well with the calculated recoveries of all fractions except fraction III. Although essentially all the mass of fraction III was recovered with measured diameters larger than that predicted for the lower boundary, approximately 45% of the mass included particles larger than 1175 Å. An almost identical histogram, including the discrepancy in fraction III, was obtained for sample 726. There appears to be no obvious explanation for this discrepancy.

A summary of the elemental NCH and P analysis is given in Table V . The percentage composition of both protein and phospholipid increase with decreasing particle size, in general agreement with other data (68,71). Also given are the corrected serum concentrations of each lipoprotein subfraction, indicating the range of values that might be expected in such subjects following a fat tolerance test.

B. S_f 20 - 400 VLDL Subfractionation

Results from typical subfractionation of a type IV fasting patient are shown in Fig. 30. Lipoproteins greater than S_f 403 were first removed by the procedure described in Section II C, which also partially removes the S_f 20-400 VLDL, as shown below the total VLDL schlieren pattern. As can be seen, the anticipated recovery and overlap agree very well with the schlieren patterns of the three subfractions. Details of these earlier data are given elsewhere (70).

Another more complete example of VLDL subfractionation and analysis is given from a type III and type IV fasting patient. This example contrasts the logarithmic schlieren plots as shown in Fig. 31 with the previous linear plots. As before, lipoprotein recovery in each subfraction is close to that predicted and there is minimal overlap of adjacent fractions. Also, the sum of the three subfractions would yield a pattern very similar to the total VLDL pattern. Since the elemental data on these subfractions are both recent and typical of those obtained for all the fractions reported here, we will report more completely the type of data obtained and the reproducibility of the procedures. Table VI shows the reproducibility of the elemental data, including the

standard error of measurement (for this series) of lipoprotein mass, protein and phospholipid content. The elemental procedure provides an unusual and accurate method for determining lipoprotein mass and wt% protein. Table also shows the corrected serum concentrations of the total VLDL and the subfractions for each subject, indicating almost identical recovery by the two preparative procedures. Although comparison of VLDL in type III and IV patients reveal quantitative differences in the lipid moiety and qualitative differences in the protein moiety (35), our data indicate similar wt% protein and phospholipid content for corresponding subfractions obtained from each hyperlipoproteinemic patient.

Further schlieren analysis of these VLDL subfractions involved an ηF° versus ρ study. Each fraction was analyzed at a density of 1.037 g/ml and 1.063 g/ml NaCl by appropriate solid NaCl addition. Analytic runs were made at 34,000 rpm and results are given in Table VII. These data of S_f° rate, σ and molecular weight, plotted in Figs. 32 and 33 are in general agreement with earlier data on subfractions of similar S_f ranges (68,72,73). For a given S_f rate there is approximately the same molecular weight for both the type III and type IV subfractions. However, at a given S_f rate there appears to be a somewhat higher hydrated density in all type III subfractions, which probably reflects the higher cholesteryl ester and lower glyceride content of all three fractions (35). A more complete analysis including the clinical features of this study are reported elsewhere (35).

C. S_f 0 - 20 Low Density Lipoproteins

LDL subfractionation was performed on plasma from two fasting patients,

clinically characterized as type IV hyperlipoproteinemia. Fig.34 shows the corrected linear schlieren plots of each subfraction from each of the subjects. The observed recoveries agree reasonably well with that anticipated by calculation. The η F° versus ρ studies were done at 52,640 rpm at approximately 1.063 and 1.097 g/ml NaCl (by appropriate solid NaCl addition), yielding S_f° rates, σ's and molecular weights of the major component of each subfraction. A summary of the physical and chemical data are shown in Table VIII. These results show that with increasing S_f° rate, molecular weight increases and both hydrated density and protein content decreases, the latter confirming earlier data (40). Further, the range of molecular weight from 1.9 - 3.3 millions for LDL fractions whose major component varied from S_f° 4 to S_f° 13 agree generally with recent physical data on these lipoproteins (50-52).

D. High Density Lipoproteins

HDL subfractionation was performed on serum from two normal nonfasting adults. The corrected schlieren plots for the two subfractions from each subject are shown in Fig. 35. Although there is considerable overlap between the two fractions, observed recoveries approximately agree with calculated recoveries. The decision to achieve 100% recovery of $F_{1.20}$ 3.0 molecules was somewhat arbitrary and was intended to divide the HDL spectra into roughly equal parts. Actually, fractionation at $F_{1.20}$ 3.5 or 4.0 would probably yield subfractions somewhat closer in identity to the earlier HDL_2 and HDL_3 components. It should be observed that the earlier techniques (11,74) fractionates primarily on the basis of density giving two fractions, HDL_2 ($1.063 < \sigma_2 < 1.125$ g/ml) and HDL_3 ($1.125 < \sigma_3$ 1.20 g/ml). In contrast, our procedure fractionates

essentially on the basis of particle size and therefore the two procedures may be achieving a different type of fractionation within the HDL spectra.

Our η F° versus ρ flotation studies were done at 52,640 in a medium of approximately 1.203 and 1.290 g/ml NaBr. Solid NaBr was added to aliquots of the two subfractions raising densities to the higher values. All the physical and chemical data are summarized in Table IX. These preliminary data show similar hydrated densities but lower molecular weights than earlier data, particularly for the less dense $F_{1.20}$ 3.0 - 9.0 component (74,75). These discrepancies, in part, may be the result of the different fractionation procedures used. However, chemical composition, and in particular protein content (44% - 55%), is in general agreement with the data based on the older HDL_2 - HDL_3 subfractionation. In order rigorously to compare procedures, in the future it will be necessary to determine the density heterogeneity of each subfraction acquired by "cumulative flotation rate" separations.

VIII. ADVANTAGES AND LIMITATIONS OF CUMULATIVE FLOTATION PROCEDURES

It should be emphasized that the earlier preparative techniques continue to have certain advantages in that the use of angle head rotors considerably simplifies methodology and at the same time permits fractionation on a much larger scale. However, there are uncertainties concerning lipoprotein structural stability during prolonged and repeated centrifugations in angle rotors, particularly with HDL (76-78). Many of these angle rotor fractionations of necessity have involved serial centrifugations (75,78) to avoid contamination from albumin and other non-lipoprotein protein. In addition, as a precaution against lipoprotein aggregation, especially for LDL in high salt media, (79) the pH of solutions used for all lipoprotein fractionation should be maintained near

neutrality (above pH 5.5).

On the other hand, our density gradient procedures just described provide lipoprotein fractions in a media of defined small molecule composition with minimal contamination from other plasma lipoproteins and proteins. There is also essentially no contamination from the plasma small molecule components. Upper limits of such contamination can be evaluated, for example, by going through the cumulative flotation isolation procedure for LDL and HDL subfractionation using 1.063 and 1.21 g/ml bottom fractions raised to the appropriate densities. These fractions should have essentially no LDL or HDL lipoproteins present, respectively, but would have all other serum components which could potentially contaminate each lipoprotein subfraction. Table X shows the CHN elemental analysis of the LDL and HDL gradient fractions obtained from the standard isolation procedure (dynamic) and a control gradient allowed to stand at 1 g in a 23 C chamber (static). Considering the error of measurement of sample above matrix, there is no detectable contamination by elemental analysis in the fractions that would contain the lipoprotein subfractions. Where contamination is important, such as in lipoprotein immunological studies (78), such fractionation may help define more accurately the characteristics of the protein moiety within subfractions of all the plasma lipoproteins. In addition, the "cumulative flotation rate" procedure is flexible, allowing the collection of two, three or more subfractions of any desired flotation rate interval within each of the $S_f > 400$, VLDL, LDL and HDL spectra. Where very large fractions from a single plasma are needed, this general technique might be used in a zonal type rotor (80) with reduced resolution.

IX. ELECTROPHORESIS OF LIPOPROTEINS

A. Principles of Lipoprotein Electrophoresis.

The surface electric charge of lipoproteins has become the basis of numerous electrophoretic methods which rate second in importance after ultracentrifugation for lipoprotein analysis. The net charge on a lipoprotein molecule, which primarily determines its electrophoretic mobility, results from the balance of positive and negative charges on terminal and side chain amino acid residues. Additional charge contributions would be expected from adsorbed metal cations and fatty acid anions and to a small extent from those phospholipids which do not exist in the form of zwitterions at electrophoretic pH values (8).

The mobility of lipoproteins in free electrophoresis, i.e., where the lipoproteins are in buffered solution without supporting medium, and in layers or blocks of potato starch granules is identical. However, on paper, cellulose acetate, and various types of gels the migration of lipoproteins according to their net electric charge is modified by two types of interactions between the lipoproteins and the supporting media. Firstly, all of the supporting media other than starch granules possess interstices of a range of sizes which offer resistance to the passage of chylomicra and VLDL. Secondly, chemical interactions between lipoproteins and supporting media probably make at least a small contribution to the electrophoretic pattern and to its variations in different media. Perhaps

the most important such interaction occurs in paper electrophoresis. In barbital buffer, containing EDTA to minimize the effect of metal ion contamination in the paper or reagents, the lipoprotein pattern shows a poorly defined α_1 (HDL) lipoprotein zone, fails to show resolution of the VLDL from LDL, and exhibits considerable trailing of VLDL between the beta region and the origin. The addition of human serum albumin to the buffer solution sharpens the α_1 lipoprotein band, partially resolves pre-beta (VLDL) from beta (LDL), and limits trailing to samples containing large concentrations of VLDL.

Our knowledge at present suggests that the electrophoretic mobilities of HDL, LDL and VLDL are largely determined by the presence of the apoproteins. The apoproteins consist of families of polypeptide subunits including a set A, found in HDL, a set B found in LDL, and a third set in VLDL which contains at least some members of sets A and B plus several additional polypeptides (81-85). The precise pattern obtained in a given electrophoretic medium is then modified by the mechanical and chemical interactions with the media as mentioned above. Figure 36 indicates the relationships in typical normal and abnormal human sera between electrophoretic patterns in agarose gel and ultracentrifugation. Figure 37 illustrates these relationships schematically in the four media principally used in lipoprotein electrophoresis.

B. Electrophoretic Mobility in Various Media.

The correspondence between the electrophoretic zones and the standard ultracentrifugal fractions is summarized in Table XI. These relationships have been determined by electrophoresis of fractions isolated with the preparative ultracentrifuge (86), and by indirect methods.

The high density lipoproteins exhibit electrophoretic mobility between albumin and α_1 globulin. When this fraction is relatively abundant, partial resolution into two zones is often seen on both paper and other media. The minor zone (less uptake of fat stains) extends towards the anode from the main zone and at least partially overlaps the albumin band. Some investigators have raised the question whether this minor anodal band of α_1 lipoprotein is really caused by staining of the free fatty acid-albumin complex rather than by a portion of high density lipoprotein. We have recently shown after preparative electrophoresis of human serum in agarose (with G. Nelson and R. Noble, unpublished data) that this zone does contain all of the lipids expected in α_1 lipoprotein and is no more than slightly enriched in free fatty acids. It is still not clear, however, whether the resolution of α_1 lipoprotein on electrophoresis corresponds to the partial ultracentrifugal resolution of HDL_2 and HDL_3, but our evidence does not support such a relationship. M. Nichaman et al. (personal communication) have noted progressive increases in the staining and degree of separation of the minor anodal component in certain sera during storage at 4°C.

The VLDL move in the region of the α_2 globulins in cellulose acetate and various gels. On paper, in barbital buffer containing albumin these lipoproteins move slightly faster than the β lipoproteins -- in the

pre-β region. In addition, Smith has reported (87), and we have confirmed, that isolated lipoproteins of S_f 12-20 (density 1.006-1.019 gm/ml) exhibit an intermediate electrophoretic mobility between those of β and pre-β lipoproteins. However, no practical resolution of this class of lipoproteins by means of paper electrophoresis is possible. In agarose gel the mobility of VLDL in different human subjects is observed to vary from the α_2 into the pre-β regions and occasionally is found to be resolved into two distinct bands (Fig. 38). The latter finding has most often been observed in subjects receiving either certain contraceptive drugs or clofibrate. In polyacrylamide gel, which has a smaller pore size than agarose, the VLDL are trapped just after entering the separating gel from the stacking gel, thus migrating in a "post-β" position.

The LDL move in the β globulin region. In media which are capable of resolving the β_1 and β_2 globulins of serum, the β lipoprotein is found in the β_1 (faster) zone.

Chylomicrons, because of their large size, form a symmetrical band about the origin or point of application on paper. In the various gels they remain in the origin well or in the starting gel. On cellulose acetate, however, it has been found that a part of the chylomicron fraction migrates to the α_2 region, thus producing ambiguity in the distinction between VLDL and chylomicrons.

C. <u>Exceptional Electrophoretic Behavior of Lipoproteins</u>.

Recently, four uncommon exceptions to the usual lipoprotein patterns have been observed. 1) In the Type III hyperlipoproteinemia of Fredrickson

et al. (34) a portion of the lipoproteins of hydrated density less than 1.006 g/ml exhibits β electrophoretic mobility ("floating β lipoprotein"). Recent findings by Fisher suggest that lipoprotein of these characteristics is usually present in patients with elevated pre-β lipoproteins (VLDL) (88). Thus the large amount of "floating β lipoprotein" characteristic of Type III hyperlipoproteinemia may indicate a quantitative, rather than qualitative, difference from Type IV. Quantitative limits may therefore have to be set for accurate differential diagnosis. 2) A new lipoprotein of pre-β mobility and the density of LDL ("sinking pre-β") has been reported by H. Sodhi in a healthy 10-year-old boy (89); and similar lipoproteins were observed in a considerable number of normal individuals in the Framingham study (W.B. Kannell and W. Castelli, personal communication). 3) J. Davignon (personal communication) has observed a "retro-α_1" band rarely in normolipidemic and occasionally in hyperlipidemic individuals, usually in association with liver disease, obesity, or diabetes. Sometimes the band appeared in conjunction with clofibrate therapy. The faintly staining band was located between the β and α_1 zones, usually close behind the main α_1 band. This lipoprotein exhibited a density between 1.063 and 1.21 g/ml, was not precipitated by dextran sulfate, and reacted with α_1 lipoprotein antiserum in immunoelectrophoresis.
4) Patients with obstructive jaundice exhibit hyperlipoproteinemia which results from the presence of a low-density lipoprotein of abnormal composition and properties (90, 91). This lipoprotein migrates with β lipoprotein in agarose, but unexpectedly in a cathodal direction in agar gel (92). One must therefore be aware that exceptional lipoprotein behavior will occasionally appear as the total experience with these electrophoretic methods increases. For certain studies it may be important to carry out preparative ultracentrifugation at densities of 1.006 or 1.063 g/ml in conjunction with electrophoresis.

D. Qualitative Lipoprotein Electrophoresis

Zone electrophoresis in a supporting medium provides the only simple, direct evaluation of the main classes of serum lipoproteins which does not require the use of either an analytical or preparative ultracentrifuge. Qualitative interpretation of lipoprotein patterns has proved to be useful in clinical medicine and human genetics. Recently, a didactic classification of familial hyper- and hypo-lipoproteinemias has been based upon this technique (34). Routine experience with this classification in other laboratories has been reported (93,94). The lipoprotein patterns according to this classification are presented in Fig. 39 for paper and Fig. 40 for agarose gel. Furthermore, numerous secondary hyper- and hypo-lipoproteinemias have been observed in many other diseases (8, 34).

Details and discussion of the paper electrophoresis technique have been presented in a review by Hatch and Lees (8). A recent modification was presented by Moinuddin and Taylor (95). The agarose gel technique has been described in articles by Noble (96), Rapp and Kahlke (97), McGlashan and Pilkington (98), Papadopoulos and Kintzios (99); in a Ph.D. thesis by Irwin (100); and in recent books by Cawley (101) and Houtsmuller (102). An agar gel procedure was published by Iammarino et al. (103). Description of lipoprotein electrophoresis in polyacrylamide gel has been presented by Narayan et al. (104-106), Raymond et al. (107), and Pratt and Dangerfield (108). The techniques used for cellulose acetate electrophoresis have been presented by Colfs and Verheyden (109), Chin and Blankenhorn (110), Farber et al. (111), and Winkelman et al. (112). The technical details of these electrophoretic methods will not be repeated here. Recently, a comparison of several electrophoretic techniques has been presented in abstract form by Maskett et al. In Type III hyperlipoproteinemia (34), correct diagnosis was obtained 50 - 55% of the time when only paper, agarose gel, or polyacrylamide gel was used; whereas with a combination of paper and polyacrylamide gel, correct diagnosis was obtained in over 90% of cases (Maskett et al., 113, and personal communication).

Paper electrophoresis of lipoproteins has been widely applied in clinical laboratories and in some epidemiological studies. Lipoprotein electrophoresis in other media, with the possible exception of cellulose acetate, has thus far been limited mostly to research laboratories. The advantages of agarose gel over paper, namely greater speed, resolution, and sensitivity, were offset by the need for daily preparation of the gel media and the need for more skillful technique. However, this situation is changing through the introduction of stable, ready-made gels in package form by at least two companies: Analytical Chemists, Inc., Palo Alto, Ca. (product distributed by G. K. Turner Co., Palo Alto), and Bio-Rad Laboratories, Inc.[3], Richmond, Ca. Minor technical and packaging details are still being worked out. However, these products, and others that may follow, should bring the gel technique within the capability of any clinical laboratory that employs paper electrophoresis. Results may be obtained in about one and one-half hr (exclusive of scanning and interpretation), rather than the ten- to 24-hr period required for most of the methods previously available.

E. Semi-Quantitative Lipoprotein Electrophoresis and Comparison with Analytical Ultracentrifugation

Although qualitative interpretation of lipoprotein electrophoretic patterns has become widely accepted and is available in many clinical laboratories, the needs of epidemiologic teams and of clinical investigators using drug or diet therapy are served better by quantitative data presented in digital form.

Paper Electrophoresis. A comparison of quantitative measurements by paper electrophoresis and by analytical ultracentrifugation in 71 normal and abnormal cases was carried out in the authors' laboratories (114). Nonfasting morning serum samples were obtained from 15 healthy employed men aged 38 to 50 years, and 16 healthy employed women aged 36 to 50 years (Livermore G and H series). Fasting serum samples were obtained from 20 male patients aged 29 to 66 years, and 20 female patients aged 27 to 70 years. These patients

exhibited miscellaneous hyperlipoproteinemias and were undergoing a variety of treatments. The three series of samples provided a range of lipoprotein levels in each major fraction that was adequate to test the correlation between the electrophoretic and ultracentrifugal methods.

Paper electrophoresis was performed in barbital buffer at pH 8.6 and ionic strength 0.1, containing 1% (w/v) human albumin, by the method of Lees and Hatch (115). For each sample, duplicate electrophoretic strips were made on different days. The strips were stained for 6 hours at 37° in Oil Red O (saturated solution in 60% ethanol). Details and discussion of this technique are presented elsewhere (8).

Scanning was performed with the Model RB Analytrol densitometer with automatic integration (Beckman Instruments, Inc., Spinco Division).[3] The standard B-5 cam was replaced by a B-2 cam, and a 520 nm filter was used in the front holder with a 500 nm filter in the rear holder. The slit width was 1.5 mm. The calibration set point was determined by interpolation in the table given in the instrument manual, and was used without "color correction." The strip was located in the light path about 1 cm cathodal to the origin mark. The zero point was set and the calibration set point was adjusted with the neutral density filter in the light path. The strip was scanned and the scan was visually examined. If the baseline of the scan was not close to the chart zero line behind the origin, between pre-β and α_1, and in front of α_1 lipoprotein, the zero

point was reset and the scan was repeated. Often the best setting for the baseline was at the lowest point between the pre-β and α_1 lipoprotein zones.

Values obtained from the duplicate strips were averaged for statistical analysis. In a preliminary study on the 15 normal male subjects duplicate scans were made on each strip, duplicate strips were run in the same cell, and duplicate strips were run on different days in order to evaluate the sources of variation in the procedure.

The establishment of criteria for the boundaries of the electrophoretic zones is difficult. Our efforts were aided by data feedback from computer plotted scatter diagrams which correlated the electrophoretic and ultracentrifugal data (116). The trail, and chylomicrons, if present, extended from slightly behind the point of application of the serum to the intersection with the baseline of an extension of the linear portion of the rear of the β peak. The β peak was measured in two ways: by integrated area and by peak height; the latter method was easier and as accurate as the area method in this study. The most important, and the most difficult, demarcation was that of the boundary between β and pre-β zones. The best results were obtained at the midpoint between two lines perpendicular to the baseline, located at the first and second perceptible deviations from the straight line describing the descending limb of the β peak. This rule was inapplicable in those rare cases where the pre-β component was higher than the β component. The leading boundary of the pre-β was marked when the trace

returned close to the baseline. The boundaries for α_1 lipoprotein were usually easy to define.

Under magnification the boundaries were marked along the baseline and their vertical projections located on the integrator trace. Integrator pips were counted and recorded in terms of integration units for each lipoprotein fraction. Peak height (in mm) for β lipoprotein could be converted approximately into integration units by multiplying the values by 0.63.

Correlation and regression relationships between the densitometric scans and the ultracentrifugal analyses were calculated with a CDC-6600 computer and graphs were prepared with a Cal-Comp. Corp. plotter.[3]

Variation between scans of the same strip (mean deviation between duplicates) was much smaller (3 to 9%) than the other sources of variation (13 to 30%), so that duplicate scans were not made in the remainder of the study. Variation between strips run in the same cell was smaller than that between strips run on different days for the trail and pre-β fractions, about equal for the β fraction, and -- unexpectedly -- larger for the α_1 fraction. Thus for reliable results, duplicate strips should be made for each serum sample; but it probably makes little difference whether these are made concurrently or on different days.

Our data relating the amounts of each major electrophoretic and ultracentrifugal fraction in the 71 serum samples are presented in Table XII; the right hand column shows the percentage of the 71 samples in

which the actual ultracentrifugal value was within ± 30 percent of the value predicted by the regression line. We can thus imagine that if we had available only the electrophoretic measurements, we could have predicted the ultracentrifugal values of each major lipoprotein fraction within ± 30 percent from 83 to over 90 percent of the time. Although analytical ultracentrifugation is not entirely error-free, the major part of the deviations from a perfectly linear relationship undoubtedly came from the electrophoretic procedure.

In a more recent series (unpublished), we have found difficulty in measurement of the β zone in sera containing large amounts of very low density (pre-β) lipoproteins. Evidently a significant portion of these lipoproteins lies beneath the β peak, resulting in erroneous increases in both peak height and area of the β zone. For these occasional samples the best estimate of β lipoprotein was obtained by subtracting the height of the chylomicron or trail fraction at the origin from the peak height of β lipoprotein.

Agarose Gel Electrophoresis. A collaborative comparison of both paper and agarose electrophoresis with analytical ultracentrifugation has been published by R. Noble and the authors (116). Recently a collaborative study of a small selected series of normal and abnormal subjects has been carried out by the authors in collaboration with S. Hulley, S. Cook, S. Wilson and M. Nichaman. Both paper electrophoresis and agarose electrophoresis were compared with the analytical ultracentrifuge in 20 selected subjects, including four normal patterns, six of Type II, five of Type IV, and five of Type V with marked elevations of VLDL and chylomicrons. Since the electrophoretic techniques used in the latter study

took advantage of the information gained from those studies previously mentioned and appeared to give significantly better results for agarose electrophoresis, we wish to present the data of this recent study here as an example of the present state-of-the-art of quantitative lipoprotein electrophoresis. An indication of the sensitivity and resolution of these electrophoretic data was their ability to point out a minor deficiency in the standard ultracentrifugal method for measurement of low concentrations of S_f° 20 - 400 lipoproteins (below 150 mg/100 ml). For those samples, an improved analysis of S_f° 20 - 100 lipoproteins was made using the 2' UTS frame as described in Section IV E. (We are indebted to M. Nichaman et al. for making available the agarose electrophoresis data concurrently with their preparation for normal publication.)

The methods of analytical ultracentrifugation employed in the collaborative study were those described in preceding sections of this chapter. Paper electrophoresis was carried out as described by Hatch and Lees (8). Only a single analysis was made on each sample. The agarose electrophoretic technique was that of Noble (96) and was modified in three respects: 1) the strips were one-half the width employed previously and the full width of the pattern was scanned, leading to better reproducibility of duplicate strips; 2) standardization of dye uptake by the electrophoretic strips on different days was improved by making daily dye-uptake controls with aliquots of a frozen, pooled serum and adjusting all values for test sera accordingly; and 3) duplicate analyses were performed and the data averaged. The strips were stained with Oil Red O (prepared freshly every two months) at 40°C as described by Hatch and Lees (8); staining was carried out for 24 hours after preliminary studies showed that this time period provided reproducible and nearly maximal dye uptake by each lipoprotein fraction. The data were analyzed with a computer as described in the article by Noble et al. (116).

The correlation coefficients among lipoprotein separations in agarose gel and paper and the ultracentrifugal analyses are presented in Table XIII. Scatter diagrams are shown in Fig. 41. The results for agarose gel electrophoresis were considerably better than those reported in the previous study, probably owing to the modified strips and more rigorous standardization of dye uptake. The results for paper electrophoresis were substantially the same as obtained previously (Table XII) (114, 116). One useful indication of the accuracy of a method being tested by comparison with an established method is the "standard error of the estimate," Sy.x. This is a measure of the statistical variation of the estimates of the "true" values obtained with the test method, i.e., the variations about the least-squares regression line relating test method values (x-variable) to standard method values (y-variable) (117). A comparison is presented in Table XIV of Sy.x values obtained in the study by Hulley et al. with those of previously published data. The results suggest that, after careful standardization, agarose gel electrophoresis data may be transformable into actual lipoprotein concentration values (mg/100 ml) with an acceptable degree of accuracy.

In order to relate the electrophoretic fractions observed in agarose gel to specific parts of the ultracentrifugal lipoprotein spectrum, correlation coefficients were calculated between the amounts of each electrophoretic fraction and the ultracentrifugally measured lipoproteins at small increments across the low-density (S_f^o 0-400) and high-density ($F_{1.20}^o$ 0-9) flotation spectra (Fig. 42). Highly significant positive correlation was observed between β lipoprotein and lipoproteins of S_f^o 5 to about 12. For pre-β lipoprotein the significant correlation was observed from S_f^o 16 to 400.

For α_1 lipoprotein a significant correlation was observed from $F_{1.20}^o$ 1.5 to 7, which encompasses most of the high-density lipoproteins.

The partial resolution of α_1 lipoprotein into a main band, denoted α_x, and a minor faster anodal component, denoted α_y, was evaluated with the Model 310 Curve Resolver (Instrument Products Div., E.I. DuPont de Nemours Co.)[3] as described previously (116). As shown in Fig. 42, the main α_x component is well correlated with most of the HDL ultracentrifugal spectrum (R = 0.89), without any suggestion of preferential representation of HDL_2 ($F^o_{1.20}$ 3.5-9) or HDL_3 ($F^o_{1.20}$ 0-3.5). However, the minor α_y component is not significantly correlated with any part of the HDL spectrum, despite the fact (mentioned earlier) that this zone contains lipids typical of HDL (R with total HDL = 0.19). In view of the equivocal nature of the α_y zone, the semi-quantitative estimation of the α_1 lipoproteins by agarose gel or paper electrophoresis should perhaps be limited to the main or α_x zone.

Finally, an approximate correspondence has been found between the chylomicron zone at the electrophoretic origin and lipoproteins of $S_f > 400$, determined by ultracentrifugation in a density gradient (Section VI E) (Table XIII). However, the number of sera containing chylomicrons and the range of chylomicron concentrations are still insufficient for a rigorous statistical analysis.

F. Evaluation of Lipoprotein Electrophoresis.

Although a fair estimate of the amounts of β and pre-β lipoproteins can be inferred from chemical measurements of serum cholesterol and triglycerides, such inferences can be seriously in error in certain clinical disorders. The concentration of α_1 or high-density lipoproteins cannot be estimated directly from serum cholesterol and triglyceride levels. Thus a proper understanding of the distribution of the lipoproteins in plasma is best achieved by a direct method of assay. The semi-quantitative correspondence between the relatively simple and inexpensive electrophoretic method and the more reliable, but elaborate, ultracentrifugal method means that plasma lipoprotein patterns can be measured in clinical and epidemiological laboratories. In most individuals with either normal or abnormal plasma lipoprotein levels, the ultracentrifugal value for each major lipoprotein fraction could be estimated within ± 30 percent by paper electrophoresis and within somewhat narrower limits by agarose gel electrophoresis. These electrophoretic methods can be considered to be semi-quantitative. Since the significant clinical disorders and the results of successful therapy generally produce changes of much greater magnitude, this degree of accuracy is tolerable.

There are difficulties in achieving rigorous control over the complete sequence of operations in the semi-quantitative electrophoretic technique. In our experience, the features of the method that require particular attention are: 1) careful addition of serum aliquots to the applicator and

thence to the strips, 2) control of temperature and concentration of the dye solution, and 3) calibration of the scanning densitometer. Bush (118) and Winkelman and Wybenga (119) discuss certain features of quantitative scanning technique.

Our semi-quantitative electrophoretic data have been reported in terms of integration units, or mm of peak height in the case of β lipoprotein. Within the limits of attained accuracy these data could be related to the mg per 100 ml values from ultracentrifugation -- the latter method involves specific refractive increment values that were standardized gravimetrically. Laboratories wishing to establish semi-quantitative lipoprotein electrophoresis have these options for standardization: 1) local assay of series of healthy males and females covering a range of ages, with establishment of local norms in terms of integration units, 2) performance of a small series of direct comparisons with a reliable ultracentrifugal laboratory over a broad range of lipoprotein concentrations and subsequent conversion to the gravimetric units, or 3) if facilities for preparative ultracentrifugation and precision refractometry are available, standardization of the clinically important pre-β and β fractions with S_f 20-400 and S_f 0-20, respectively, may be accomplished (23, and Sect. III).

Of course, all of the usual admonitions about defining "normal" lipoprotein levels apply to these data. Our values were obtained from a healthy middle-aged population in which the males, especially, can be assumed to have a great deal of occult atherosclerosis. This limitation must be recognized by everyone who generates, or interprets clinically, semi-quantitative data from lipoprotein electrophoresis.

X. CONCLUSIONS

We have considered in some detail the present state of the art of two widely-used and important tools for lipoprotein research: ultracentrifugal fractionation and analysis, and lipoprotein characterization and semi-quantification by the latest electrophoretic techniques. Throughout our presentation we have emphasized and illustrated the usefulness and frequent necessity of employing computer techniques in many aspects of instrumentation and data analysis. Scientists and students looking for a historical and broad introductory treatment of the subject of plasma lipoproteins are referred to a Scientific American article by W. J. Lossow (120).

Currently there is an active ferment in the fields involving the study of serum lipoproteins and their metabolism. Many of these studies are concerned with such fundamental questions as the structure and physical-chemical characterization of lipid-protein complexes. Other questions relate to the detailed characterization of the protein moieties, their substructure and conformation within each class of the serum lipoproteins. All such studies should increasingly contribute toward our understanding of how specific elevations and abnormalities of these important and complex molecules relate to states of health and disease.

We have presented results which show that the relatively simple and inexpensive electrophoretic methods correlate rather well with analytical ultracentrifugation. This means that semi-quantitative measurement of the major lipoprotein fractions can be carried out in clinical and epidemiological laboratories, where there is great demand for these analyses for studies of human genetic disorders, cardiovascular risk factors, secondary metabolic disorders,

and evaluation of the results of drug and diet therapy.

In most scientific research, the results of carefully planned experiments ultimately depend on the validity, reproducibility and reliability of the methods employed to acquire and analyze the data. Therefore, it is our hope that by emphasizing the unique capabilities, as well as the limitations, of ultracentrifugation and electrophoresis, we will assist the more biologically and metabolically oriented scientists working in lipoprotein research.

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The authors thank the American Oil Chemists' Society for permission to reproduce or reproduce with minor revisions Figs. 7, 14-19 (from reference 50), Fig. 8 (reference 56), Fig. 9 (reference 29), Figs. 20, 23 and 24 (reference 57), Fig. 9 (reference 29), and Tables I-IV (from reference 50). Similarly we thank: The Review of Scientific Instruments (reproduction of Figs. 4-6 from reference 27), The Journal of Clinical Investigation (data for Figs. 10 and 11 from reference 32), The Journal of Lipid Research (Figs. 25, 26, 29 and 30 from reference 70, data for Table V from reference 70, and Fig. 1 from reference 23), and Biophysica et Biochimica Acta (reproduction of Fig. 31, and data for Table VI from reference 35).

FOOTNOTES

1. Unless otherwise indicated, all densities are given at 20°C.

2. If very high serum concentrations of chylomicra are present, a similar centrifugation of 6 ml whole serum at 1.92×10^6 g min (23 C) quantitatively removes $S_f > 1000$ into the top 1/2 ml. The 5.5 ml subnatant may be easily used for preparing fractions for analytic ultracentrifugation (Section IIB), with appropriate corrections for partial removal of S_f 0 - 400 (Section IV E).

3. Reference to a company or product name does not imply approval or recommendation of the product by the University of California or the U. S. Atomic Energy Commission to the exclusion of others that may be suitable.

4. S_f refers to uncorrected flotation rate, expressed in svedbergs (10^{-13}cm/sec/dyne/g), in a NaCl medium of1.063 g/ml. F rate refers to uncorrected flotation rate at any given density. S_f° refers to flotation rate fully corrected for effects of concentration dependence and to standard conditions of 1.745 M NaCl at 26 C (ρ = 1.0630 g/ml). Similarly, $F_{1.20}^\circ$ refers to HDL flotation rate fully corrected for concentration dependence and to standard conditions of 0.195 M NaCl - 2.774 M NaBr at 26 C (ρ_{26} = 1.2000 g/ml).

5. As summarized in reference 9 by S. Margolis, defatted LDL and VLDL contain as much as 5 - 9% carbohydrate of which 1.5 - 3.5% is N containing glucosamine and sialic acid; and the remainder consists of neutral sugars. De-lipidized HDL contains approximately 3% carbohydrate of roughly similar composition (9).

FOOTNOTES

5. continued

The protein content reported here is uncorrected for the presence of this carbohydrate, although lipoprotein carbohydrate CHN is included in our analysis. Assuming 5% carbohydrate (2½% galactose and 2½% glucosamine) in all delipidized lipoprotein fractions, we have calculated approximate errors for lipoprotein mass and protein content. For $S_f > 400$, VLDL, LDL and HDL, lipoprotein mass is underestimated by 0.07%, 0.21%, 0.5% and 1.3%, respectively; similarly, protein content is overestimated by 0.04%, 0.14%, 0.4% and 0.9%, respectively.

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TABLE I RESULTS, S_f 0-12 MAJOR COMPONENT, NORMAL SUBJECTS, NONFASTING (LIVERMORE G AND H)

Age (mean ± S.D.)	Males (16) 44 ± 3 years		Females (16) 43 ± 4 years		Difference
1. S_f°	6.20	± 0.96	7.05	± 0.83	$p < .01$
2. σ (g/ml)	1.0304	± 0.0035	1.0284	± 0.0031	N.S.
3. Mol wt (millions)	2.12	± 0.20	2.36	± 0.16	$p < .01$
4. S_f° 20-400, mg/100 ml (VLDL)	118	± 105	36	± 50	$p < .01$
Correlations					
1. S_f° vs σ	-0.95*		-0.95*		
2. S_f° vs mol wt	0.87*		0.76*		
3. σ vs mol wt	-0.69*		-0.52†		
4. S_f° vs VLDL	-0.72*		-0.76*		

* $p < 0.01$, † $p < 0.05$

TABLE II LIPID COMPOSITION AND LIPID σ, S_f 0-20 LIPOPROTEINS, NORMAL MALES AND FEMALES[a]

Component[b]	PL	CE	TG	FC	σ(lipid)[c] g/ml
Males (n = 16)	24.4 ± 2.3	55.1 ± 1.5	9.4 ± 2.4	11.0 ± 0.3	0.9803 ± 0.0016
Females (n = 16)	26.7 ± 1.8	53.4 ± 1.5	9.3 ± 2.3	10.6 ± 0.3	0.9798 ± 0.0015
Difference	$p < 0.01$	$p < 0.01$	N.S.	---	N.S.

[a] Mean and standard deviation, values are wt % of total lipid; free cholesterol is assumed to be 0.198 x cholesteryl ester.

[b] PL, CE, TG and FC are abbreviations for phospholipid, cholesteryl ester, triglyceride and free cholesterol, respectively.

[c] Calculated assuming additivity of densities for PL, CE, TG and FC; individual values used are 0.97, 0.99, 0.92 and 1.067 g/ml, respectively.

TABLE III CORRELATIONS OF S_f° RATE, σ AND MOLECULAR WEIGHT, WITH S_f 0-20 LIPID COMPOSITION, NORMALS[a]

Parameter	PL	CE	TG	σ(lipid)
S_f° rate	0.79* (0.27)	-0.02(0.07)	-0.61† (-0.30)	0.47(0.26)
σ(lipoprotein)g/ml	-0.70* (-0.29)	-0.04(0.03)	0.58† (0.23)	-0.47(-0.17)
Mol wt	0.70* (0.23)	-0.11(0.21)	-0.46 (-0.36)	0.34(0.36)

[a] Normal females and males (in parenthesis). $^*p < 0.01$, $^\dagger p < 0.05$.

TABLE IV RESULTS, S_f 0-12 MAJOR COMPONENT, KAISER CLINICAL REFERRALS, FASTING

Age (mean ± S.D.)	Males (n = 16) 49 ± 11 years	Females (n = 19) 51 ± 10 years	Difference
1. S_f°	5.85 ± 1.41	6.74 ± 0.97	$p < 0.05$
2. σ (g/ml)	1.0327 ± 0.0053	1.0293 ± 0.0037	$p < 0.05$
3. Mol wt (millions)	2.16 ± 0.28	2.29 ± 0.23	N.S.
4. S_f° 20-400, mg/100 ml (VLDL)	289 ± 285	108 ± 108	$p < 0.01$
Correlations			
1. S_f° vs σ	-0.96*	-0.90*	
2. S_f° vs mol wt	0.89*	0.72*	
3. σ vs mol wt	-0.73*	-0.35	
4. S_f° vs VLDL	-0.62†	-0.74*	

* $p < 0.01$, † $p < 0.05$

TABLE V ELEMENTAL ANALYSIS OF $S_f > 400$ LIPOPROTEIN SUBFRACTIONS

Fraction	Case	Calculated Recovery	µg(N+C+H)/0.1 ml*	wt% PL	wt% Protein*	$S_f > 400$ Subfractions† Mass % of Total $S_f > 400$	mg/100 ml Serum
I	735	S_f 2719- 10^5	390	2.8	1.17	43.3	75
	726	S_f 2694- 10^5	941	3.0	1.02	29.1	182
II	735	S_f 908-3176	210	5.5	1.81	23.5	41
	726	S_f 902-3146	1192	2.1	1.98	36.9	230
III	735	S_f 324-1068	296	9.4	2.90	33.1	57
	726	S_f 324-1068	1100	7.7	2.81	34.0	213

† $S_f > 400$ lipoprotein mean mass = 1.1287 (Σ N+C+H), fractions are concentrated $5.84c_o$ over serum.

* Duplicate analysis.

TABLE VI REPRODUCIBILITY, ELEMENTAL NCH AND P ANALYSIS OF VLDL

	μg NCH/.1 gm	N/NCH	% PL	% Protein	μg LP/.1 ml	Mean Value at 1 c., mg/100 ml
Case 853 (Type III) Fasting						
S_f 100-400	1,632	0.01221	12.6	5.46	1,855	464
	1,633	0.01244	12.4	5.60	1,856	
S_f 60-100	1,072	0.01799	18.2	7.84	1,217	305
	1,073	0.01814	19.2	7.92	1,219	
S_f 20- 60	1,000	0.02529	22.7	11.94	1,132	282
	996	0.02495	22.2	11.75	1,127	Sum 1,051
S_f 20-400*	1,227	0.01817	17.0	8.24	1,389	1,044
	1,231	0.01766	20.2	7.95	1,393	
Case 870 (Type IV) Fasting						
S_f 100-400	637	0.01417	16.4	6.21	724	181
	636	0.01437	15.6	6.32	723	
S_f 60-100	1,006	0.01913	17.8	8.85	1,143	285
	1,000	0.01917	18.6	8.87	1,136	
S_f 20- 60	1,178	0.02597	21.1	12.45	1,333	331
	1,161	0.02606	21.7	12.50	1,314	Sum 797
S_f 20-400*	928	0.02114	18.8	10.10	1,050	794
	942	0.02128	18.0	10.10	1,066	
		S.E.M.	(0.92%)	(0.10%)	6.6(0.54%)	

* Recovery from a single stage VLDL density gradient run, 201 x 10^6 g min at 23C.

TABLE VII VLDL SUBFRACTIONATION, PHYSICAL DATA, TYPE III (853) AND TYPE IV (870) FASTING PATIENTS

Case	S_f° Range	σ(ρ Inter.) g/ml	S_f° Rate (Component Measured)	Mol Wt. (Millions)
853	(I) S_f 100-400	0.9550	113.4	25.7
853 rerun		0.9551	115.2	26.4
870		0.9502	103.6	20.9
870 reread		0.9514	103.7	21.3
853	(II) S_f 60-100	0.9666	67.5	14.2
870		0.9588	63.0	11.3
853	(III) S_f 20- 60	0.9753	56.5 (fast)	12.6
853		1.0013	24.3 (slow)	6.20
870		0.9630	49.9	7.75

TABLE VIII LDL SUBFRACTIONATION, TYPE IV FASTING SUBJECTS

Case	S_f Range	$\sigma(\rho$ Inter.) g/ml	Peak S_f° Rate (svedbergs)	Mol. Wt. (Millions)	Protein* Wt. %	PL* Wt.%
	(I)					
876	10.4-20.0	1.0079	13.5	3.04	16.9	24.8
877		1.0110	13.5	3.34	17.9	22.2
	(II)					
876	5.7-12.0	1.0272	6.89	2.17	22.6	23.3
877		1.0281	6.87	2.25	23.8	22.6
	(III)					
876	3.5- 6.5	1.0348	4.96	1.91	25.5	22.3
877		1.0393	4.13	1.88	26.6	21.2

* Duplicate elemental NCH and P analysis.

TABLE IX HDL SUBFRACTIONATION, NORMAL SUBJECTS, NON-FASTING

Case	$F_{1.20}$ Range	$\sigma(\rho$ Inter.) g/ml	Peak $F^{\circ}_{1.20}$ Rate (svedbergs)	Mol Wt.	Protein Wt. %	PL Wt.%
879	(2.7-9.0)	1.0988	4.30	265,000	43.6	33.1
881		1.0950	4.14	236,000	43.4	31.4
879	(0.8-3.0)	1.1437	1.73	170,000	53.9	27.0
881		1.1346	1.84	148,000	55.0	25.7

TABLE X EVALUATION OF POTENTIAL CONTAMINATION IN LIPOPROTEIN SUBFRACTIONS

Fraction	Depth (ml)	LDL, 12 ml Using 1.063 g/ml Bottom Fraction µg/0.1 gm		HDL, 7 ml Using 1.21 g/ml Bottom Fraction µg/0.1 gm	
		Dynamic	Static	Dynamic	Static
I	0.0-0.5	1.3	1.2	-1.0	-1.8
II	0.5-1.0	-0.1	-0.9	1.4	-0.4
III	1.0-1.5	-0.8	-0.5	1.8	0.4
IV	1.5-2.0	-1.4	-1.4	0.7	0.7
	2.0-2.5	-0.5	-1.8	0.5	0.2
	2.5-3.0	0.3	-1.2	2.2	2.2
	3.0-4.0	-2.1	-2.8*	19.6	5.9*
	4.0-5.0	-1.1	-2.2*	1083	160*
	5.0-6.0	0.2	-0.7*	1585	1165*
	6.0-7.0	6.5	2.6*	232	1822*
	7.0-8.0	13.9	4.6*		
	8.0-9.0	36.1	81.2*		
	9.0-10.0	67.5	896*		
	10.0-11.0	130	2823*		
	11.0-12.0	6034	3461*		

* Single determinations, all others duplicate analyses. Matrix above catalyst (CAT) 7.8 µg/0.1 gm, CAT above baseline 3.9 µg/0.1 gm. S.E.M. of sample minus matrix, ± 1.0 µg/0.1 gm (calculated as methyl stearate, the standard).

TABLE XI

CORRESPONDENCE OF ELECTROPHORESIS ON PAPER AND AGAROSE GEL AND ULTRACENTRIFUGATION

	Electrophoretic Zone	Ultracentrifugal Fraction		
Origin	Chylomicrons	S_f^o	> 400	(Density Gradient)
↓	Trail	S_f^o	20-400	(When Markedly Elevated)
	β	S_f^o	0- 20	
	Pre-β	S_f^o	20-400	
	α_1	$F_{1.20}^o$	0- 9	
Anode				

TABLE XII

SEMI-QUANTITATIVE PAPER ELECTROPHORESIS OF SERUM LIPOPROTEINS[a]

3 Populations -- 71 Subjects

Electrophoresis	Ultracentrifuge	Correlation Coefficient	Cases Within ± 30% of Regression Line
(X)	(Y)	(R)	(%)
β	S_f^o 0- 20	0.76	87
Pre-β	S_f^o 20-400	0.97	90[b]
β + Pre-β	S_f^o 0-400	0.90	96
α_1	$F_{1.20}^o$ 0- 9	0.75	83

[a] This table originally appeared in an article by F. T. Hatch et al., Clin. Biochem. 3, 115-123 (1970), and is reprinted with permission of the copyright owner.

[b] At very low levels, scatter is greater than ± 30%, but not of practical significance.

TABLE XIII

CORRELATIONS BETWEEN LIPOPROTEIN ELECTROPHORESIS AND ANALYTICAL ULTRACENTRIFUGATION[a]

Methods	β or S_f 0-20[b]	Pre-β or S_f 20-400	Chylo. or S_f >400	α_1 or HDL
Agarose vs Ultracentrifuge	0.978	0.996	0.854	0.862
Paper vs Ultracentrifuge	0.726	0.956	0.888[c]	0.693
Agarose vs Paper	0.758	0.971	0.942[c]	0.780

[a] Data for agarose gel are the mean of duplicate determinations; for paper electrophoresis and ultracentrifugation single determinations were made. All correlations are $p < 0.01$.

[b] The comparisons are based upon 17 cases, except for β lipoprotein. Comparisons involving β are based upon 15 cases because agarose gel data in four Type V cases had to be excluded, owing to obvious errors believed to result from partial trailing of Pre-β beneath the β peak.

[c] In paper electrophoresis the chylomicron fraction includes material trailing between the origin and β zone.

TABLE XIV

MEANS AND STANDARD ERRORS OF THE ESTIMATE (Sy.x)
OF ULTRACENTRIFUGAL VALUES FROM ELECTROPHORETIC DATA

Elect. Fraction	Study: / Medium: / No. Cases:	Hulley *et al*. / Agarose / 20 / mg/100 ml	Noble *et al*. / Agarose / 28 / mg/100 ml	Hulley *et al*. / Paper / 20 / mg/100 ml	Hatch *et al*. / Paper / 71 / mg/100 ml
β [a]	$\bar{y}$ [b]	385	535	385	455
	Sy.x [c]	32	73	111	110
Pre-β	$\bar{y}$	520	125	520	165
	Sy.x	104	50	258	75
α_1	$\bar{y}$	276	344	276	337
	Sy.x	38	70	48	78

[a] Comparisons involving β lipoprotein in the two series of Hulley *et al*. are based upon 16 cases. Data in four Type V cases had to be excluded, owing to obvious errors believed to result from partial trailing of pre-β beneath the β peak.

[b] Mean concentration in mg/100 ml of the corresponding ultracentrifugal fraction.

[c] Standard error of the estimate derived by least-squares regression analysis of the relationship between electrophoresis (x-variable) and ultracentrifugation (y-variable).