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TYPE III HYPERLIPOPROTEINEMIA:

GENETICS, DIAGNOSIS AND VERY LOW DENSITY APOLIPOPROTEINS

by

WILLIAM JOHN PETER GODOLPHIN

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The undersigned certify that they have read, and
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ABSTRACT

Type III hyperlipoproteinemia is a rare genetic disorder of fat metabolism, frequently leading to premature arteriosclerosis in the afflicted. The mode of inheritance is in question, at least partly because of a scarcity of detailed kindred studies. Such studies, in turn, are hindered by the fact that the definitive diagnosis requires equipment and expertise not widely available in clinical laboratories. Most recently, attention has been focussed on the apoproteins of serum lipoproteins and, in some cases, variations in form or concentration of these have given important clues to the pathogenesis of other lipoprotein disorders.

This study has been concerned with a phenotypic analysis of two large and several smaller kindreds; an exploration of alternate (and simpler) methods or diagnosis; and a physicochemical study of very low density apolipoproteins in Type III.

The incidence of Type III in the largest kindred, and in smaller ones, was most compatible with autosomal recessive inheritance. The other large kindred suggested a dominant or polygenic mode. In this family the propositus was the first, and so far only, reported case of Type III in

childhood. This unique early appearance may be due to his inheriting two or more different disorders of lipoprotein metabolism. His father, a diabetic, also exhibited the pathognomonic lipoprotein for Type III, but only when his diabetes was controlled, and in the absence of other clinical symptoms of Type III hyperlipoproteinemia. Evidence presented here suggests that Type III may be more genetically heterogeneous than previously supposed.

Definitive diagnosis of Type III requires preparative ultracentrifugation of fasting plasma to isolate lipoproteins prior to electrophoresis. Unpredictable variations in migration make the electrophoretograms difficult to interpret. It was found that addition of reconstituted normal plasma, from which most lipoproteins were removed, not only resulted in more reproducible migration but also provided a mobility reference marker.

The first new method presented is a single, rapid and simple, analytical ultracentrifugation of plasma after density adjustment. A characteristic bimodal Schlieren peak was observed in all untreated and some treated Type III patients. Another new approach utilizes isoelectrofocussing of plasma in polyacrylamide gels, followed by staining with Sudan Black B. Type III samples produced a unique pattern with a densely staining band at pI 5.44.

An examination of Type III very low density lipoproteins after delipidation revealed an abundance of a 'new' apolipoprotein. By amino acid analysis this protein was shown to be unusually rich in arginine and glutamic acid. It occurs in polymorphic forms by polyacrylamide gel electrophoresis and ion exchange chromatography; and has a molecular weight of 35,000. It is seen in small amounts in normal plasma. The increased amounts of this apolipoprotein plus the suggestion that it has an abnormal form, indicate that it may play an important role in the appearance and/or course of Type III hyperlipoproteinemia.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

Bis	N,N'-methylenebisacrylamide
d	density
EDTA	ethylenediaminetetraacetate
g·min	acceleration due to gravity x minutes
HDL	high density lipoprotein
LDL	low density lipoprotein
LPP	lipid-poor plasma
NaDS	sodium decyl sulphate
PAGE	polyacrylamide gel electrophoresis
PAGE-SDS	PAGE in sodium dodecyl sulphate
SDS	sodium dodecyl sulphate
Sf	Svedberg flotation rate
SF1,SF2,SF3	major fractions eluted by Sephadex G200 gel filtration of apoproteins of very low density lipoproteins.
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane
VHDL	very high density lipoprotein
VLDL	very low density lipoprotein
Vo	void volume
Vt	total bed-volume

LIPOPROTEINS

Suspicion that an elevated serum cholesterol may play a causal role in atherogenesis spurred an early interest in the circulating serum lipoproteins. Development of high resolution techniques in ultracentrifugation and electrophoresis resulted in a classification system during the 1950's which, with some modification, is still in general use. Four major groups are recognized among the normal serum lipoproteins. These can be defined by their hydrated densities and electrophoretic mobilities on a medium such as paper, cellulose acetate or agarose gel.[1] These major classes also have relatively distinct compositional differences in protein, phospholipid, cholesterol (free and esterified) and triglyceride content,[2] and they have specific metabolic roles:

-Chylomicrons, average density (d) <0.94 g/ml, Sf>400*, remain at the origin upon electrophoresis on paper, cellulose acetate and agarose gel.

-Very low density lipoproteins (VLDL), d=0.94-1.006 g/ml, Sf 20-400, prebeta mobility.

-Low density lipoproteins (LDL), d=1.006-1.063 g/ml, Sf 0-20, beta mobility.

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*Sf = flotation rate in Svedbergs (10^{-13} cm/s/dyne/g) in a sodium chloride solution density 1.063 g/ml at 26°.[3]

-High density lipoproteins (HDL), d=1.063-1.21 g/ml, Sf<0, alpha mobility.

In the following work, normal lipoproteins will be referred to by both electrophoretic mobility and ultracentrifugal behaviour, e.g. beta-LDL, prebeta-VLDL, alpha-HDL. Abnormal particles which are found in various disease states will be designated in the same fashion along with the disease, e.g. Type III beta-VLDL, for the abnormal beta-migrating very low density lipoprotein found in Type III hyperlipoproteinemia.

HYPERLIPOPROTEINEMIAS

For some time familial hyperlipidemias have been recognized as predisposing to atherosclerosis but systematic studies were hampered not only by inadequate techniques for lipoprotein analysis but also by lack of uniform terminology for clinical features. A consistent system for the recognition and naming of pathological conditions characterized by elevations in blood lipids did not exist until relatively recent times. A typing system devised by Fredrickson et al. and reviewed in full in their now classic 1967 paper[4] has been widely adopted[5] and popularized,[6] and with some modification[7] will be used here to describe the hyperlipoproteinemias.

Type I-- elevated chylomicrons in fasting plasma (14-16

hours after the last meal of a normal diet); due to a deficiency of post-heparin lipolytic activity;[8] autosomal recessive inheritance; very rare. Fasting chylomicronemia is occasionally seen secondary to other disorders such as hypothyroidism and untreated diabetes mellitus.

Type II-- elevated beta-LDL with accompanying hypercholesterolemia; autosomal dominant inheritance; relatively common in the heterozygous form. There is often a strong family history of premature coronary artery disease associated with this condition. Subclassification into Types IIa and IIb has been made dependent upon the concomitant absence or presence of elevations in prebeta-VLDL. It has been suggested, on the basis of genetic studies, that Type IIb is a completely separate entity.[9] [10] A Type II lipoprotein pattern may also be seen in a number of other conditions: hypothyroidism, liver disease, nephrotic syndrome.

Type III-- characterized by the presence of abnormal VLDL in addition to the usual normal lipoproteins. This abnormal VLDL is beta-migrating upon lipoprotein electrophoresis and its presence often results in a broad fusion of the beta and prebeta bands; hence the synonym 'broad-beta disease'. The disease is rare and the inheritance is undecided. Type III beta-VLDL has been reported to occur transiently in untreated diabetic ketoacidosis and systemic lupus erythematosus.[11] The

disorder   is   aggravated   and   may   be   caused   by
hypothyroidism.[12]

Type IV-- elevated fasting prebeta-VLDL; autosomal
recessive inheritance; very common; has many secondary
causes.

Type V-- combined elevation of prebeta-VLDL and fasting
chylomicrons; autosomal recessive inheritance; very rare;
may be only a nutritional variant of Type IV.[13]

TYPE III - GENETICS

This disorder was recognized by Fredrickson et al. as a
separate and distinct form of hyperlipoproteinemia in their
1967 review.[4] Prior to this most workers did not distinguish
the disease as being a unique entity. Studies by Gofman et
al.[14] in 1954 referring to "xanthoma tuberosum" and by
Borrie[15] in 1957 referring to "idiopathic
hypercholesterolemic xanthomatosis associated with
hypertriglyceridemia" were almost certainly on Type III
patients and some of these have since been restudied.[16]
Generally, though, clear-cut pre-1967 literature cases are
difficult to find. Since establishment of a 'hard'
definition, a number of family studies have been reported
but the mode of inheritance is still unclear. Because of the
technical demands (access to a preparative ultracentrifuge
and a high resolution electrophoresis method) many writers

have used less rigorous definitions of the disorder (clinical symptoms, triglyceride/cholesterol ratios, 'broad-beta' electrophoretic pattern). Hence, it is likely that many of the reported cases of Type III are not that disorder at all. Thus inheritance of Type III and its familial coincidence with other hyperlipoproteinemic types is not well documented.

Nevin and Slack have suggested that the disease is inherited as an incomplete dominant[17][18] but their criteria for establishing the diagnosis was based only on serum lipid levels and xanthomatosis. Matthews concluded that Types III and IV are different phenotypic expressions of the same mutation.[19] However, his criteria for classification were an elevation in beta- and prebeta- lipoproteins on paper electrophoretograms and most of these patients would probably now be called Type IIb or 'mixed type' hyperlipoproteinemics. Fredrickson and Levy have reported 36 kindreds with Type III.[7] In 21 of these Type IV was seen in other members of the family, Type II never, and Type III with vertical transmission in five. They suggest that the likely modes of inheritance are: (1) autosomal dominance with incomplete (Type IV) heterozygous expression, or (2) mixed heterozygosity for more than one mutation with Type IV an expression of one of these, or (3) autosomal recessive with Type IV as the heterozygote phenotype.

A high incidence of Type IV in Type III kindreds is

well documented. However, coincidence of Type II and Type III, (seen in one family in this study), has been rarely reported. Fredrickson and Levy[7] report a complete absence of Type II patterns in the afore-mentioned Type III kindreds. They also claim to have found no cases of Type III in an analysis of more than 200 kindreds of Type II. Lees et al.[20] found only one Type II among more than 100 relatives of Type III probands, a frequency which could be completely fortuitous.

On the other hand, Lasser and Katz[21] reported a well defined Type III with high LDL-cholesterol (a characteristic of Type II). This subject had normal siblings and spouse but four sons were Type II. Hazzard et al.[22] found beta-VLDL associated with high levels of LDL-cholesterol in a patient whose family met their criteria for familial hypercholesterolemia (approximately equivalent to Fredrickson's Type II). In two other hyperlipidemic subjects they found faint beta-VLDL electrophoretic bands in the presence of a predominance of prebeta-VLDL and/or beta-LDL. In this excellent study of survivors of myocardial infarction (which incidentally casts doubt on the genetic validity of the Fredrickson typing system) they suggest that beta-VLDL may not be "a specific marker for a distinct genetic disorder". In this regard it should be noted that Fredrickson and Levy[7] and Lees et al.[20] used paper electrophoresis to search for beta-VLDL, a less sensitive

method than agarose gel electrophoresis used by Hazzard et
al.[22]

netic analyses are always embarrassed when it is
necessary to study a substance or symptom which is a few (or
many) steps removed from the mutant gene product. Such is
the case in Type III. As will be further discussed under
Results and Discussion it seems possible that more than one
defect could give rise to a beta-migrating lipoprotein with
VLDL ultracentrifugal behaviour. In all the subjects of the
study reported here, though, beta-VLDL was a predominant
lipoprotein.*

The only other literature report of Type III occurring
in the same kindred with Type II is that by Strunge and
Trostman.[23] They found Types II, III, and IV among 13 adult
siblings whose father was Type IV. However, serious
discrepancies in their data and a failure to rigorously
define their criteria for diagnosis rule against acceptance
of that report.

Onset of the Type III condition has been associated
with maturity. The mean age of detection is 30-35 years in
males and 45-50 years in females.[24] There have been no

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* A patient diagnosed as Type III at the time of this
writing had clinical symptoms associated with the disease
(planar and tuberous xanthomas), elevated VLDL-cholesterol
and 'broad-beta' electrophoretic pattern; but the major
portion of ultracentrifugally isolated VLDL migrated as a
sharp band between the beta and prebeta regions with only
minor amounts in those regions proper.

indisputable literature reports (other than from this laboratory[25]) of the condition occurring before the end of the second decade.

TYPE III - DETECTION

Type III has been the most difficult of hyperlipoproteinemias to detect because of technical problems associated with laboratory diagnosis. This is unfortunate since the disease is also one of the most satisfying to treat from the clinician's point of view. Affected patients generally respond dramatically to dietary management, alone or with clofibrate (the drug of choice in treatment of this disease[26] [27]). There is evidence of atheromatous regression upon treatment and the prognosis of treated patients is good.[28] It seems possible that there are a good many clinically asymptomatic and undiagnosed people with Type III hyperlipoproteinemia.

There is no report of beta-VLDL appearing in a patient in whom it had previously been shown to be absent. Beta-VLDL has been discovered in asymptomatic relatives during screening of kindreds of symptomatic Type III probands. Beta-VLDL is always present in treated Type III patients whose lipids have normalized. Symptomatic Type III is often associated with and may be precipitated by weight gain.

It appears then, that this disease might be diagnosed

before onset of clinical symptoms and by appropriate management, affected persons could be put at considerably less risk of arteriosclerosis. The major stumbling block to this attractive possibility is the difficulty of laboratory diagnosis.

Definitive diagnosis requires a preparative ultracentrifuge, a lipoprotein electrophoresis system, and a high degree of expertise in the use of this equipment. Routine analysis, then, is both expensive, time consuming and technically demanding. A number of alternative procedures have been proposed but remain either unproven or unreliable.

The simplest of these is the "rule of thumb" devised by Fredrickson and Levy.[29] This requires only serum cholesterol and triglyceride quantitation. In Type III the numerical result of:

[cholesterol(mg/100 ml)] - [triglyceride(mg/100 ml)/5]

is greater than 250. This criterion probably holds true in many but certainly not all cases of Type III. Variations on this theme have been advocated, all of them based on the fact that Type III beta-VLDL is abnormally rich in cholesterol. Hazzard et al.[30] have suggested that a VLDL cholesterol/triglyceride ratio >0.42 (or >0.47[22]) is diagnostic of Type III. Preparative ultracentrifugation to isolate VLDL for lipid analysis is still required, although selective polyanionic precipitation[31] may be possible. A

number of hyperlipoproteinemic subjects without any evidence of beta-VLDL have been positive by this test.[22] Wieninga et al.[32] report the ratio of VLDL-cholesterol/total serum cholesterol in Type III to have a range (0.25-0.50) which does not overlap that seen in other conditions. This method also requires a preparative ultracentrifuge.

Analytical ultracentrifugation of plasma lipoproteins by the method developed at the Donner Laboratory[33] yields a distinctive pattern with Type III plasma. There is an excess of the Sf 12-20 and a depression of the Sf 0-12 class of lipoproteins.[34] The method in its entirety requires rather sophisticated equipment for, and techniques in, preparative and analytical ultracentrifugation, refractometry, and computerization; and the typical Type III pattern generated has been seen in Type IV in the absence of beta-VLDL.[7]

A 'broad-beta' pattern frequently observed on lipoprotein electrophoresis of untreated Type III plasma may be the first hint but is not diagnostic. Other conditions also produce a 'broad-beta' pattern[35] and sometimes it is not seen at all.[36] Simultaneous electrophoresis on polyacrylamide gel and another medium such as paper or agarose gel has been reported to clearly distinguish Type III from other phenotypes.[37] These results, however, conflict with other reports of lipoprotein electrophoresis in polyacrylamide gel,[38] although this may be due to minor methodological differences. Immunologic distinction of Type

III has been made,[39] using an antiserum to apolipoprotein-X
(Lp-X: an abnormal serum lipoprotein found in obstructive
liver disease[40]). The apoprotein of Lp-X is largely albumin
plus the small peptides (molecular weight <10,000,
collectively often called apo-C) found in VLDL. In this
method observation of an immunoprecipitin arc in the beta
region after immunoelectrophoresis is considered diagnostic
for Type III. The antisera, although manufactured
commercially now, is not widely available. Most recently
Wieland and Seidel[41] have reported a method involving the
specific polyanionic precipitation of VLDL after
electrophoresis in agarose gel. Visualization of a
precipitate in the beta region is claimed to be diagnostic
of Type III.

TYPE III - APOLIPOPROTEINS

In the past few years intensive work in a number of
laboratories has elucidated many of the characteristics of
the major apoproteins of normal human plasma lipoproteins.
Table 1 lists some of these characteristics, the name used
in this work and some of the more frequently found synonyms
used in the literature.

The complete amino acid sequences have been published
for apoLp-GlnII, apoLp-Ser and apoLp-Ala.[43] [44] [45] These have
been characterized by a remarkably high incidence of
contiguous basic and acidic residues, a property long

Table 1. Major apoproteins of human plasma lipoproteins.

Name	Synonyms	Terminals N-	-C	MW*	% of protein in VLDL	LDL	HDL*²
apoLp-GlnI	A-I 'apoLP-thr'	Asp	Gln	28000	-**	-	65-75
apoLp-GlnII	A-II apoLP-gln	PCA	Gln	17380	-	-	20-25
apoLp-B	B apoLP-ser apo LDL	Glu	Ser?	25000?	40-45	90+	-
apoLp-Ser	C-I 'apoLP-val'	Thr	Ser	6631	8-10	-	2-4
apoLp-Glu	C-II	Thr	Glu	10000	8-10	-	2-4
apoLp-Ala	C-III	Ser	Ala	8764	30	-	5-10

*Molecular weight.

**Spaces left blank do not necessarily mean that there is none of that apolipoprotein present but most workers have reported less than 1% and this could possibly be due to contamination of lipoprotein preparations by members of other density classes.

thought to be important in protein-lipid binding.[46] ApoLp-GlnI and apoLp-Glu are activators of lecithin:cholesterol acyltransferase[47] and lipoprotein lipase[48] respectively. Relatively little is known about apoLp-B. It tends to aggregate and is very insoluble. Recent reports have suggested that it can be separated into two or three distinct peptides.[49] [50] ApoLp-Ala is found in polymorphic forms with different stoichiometric amounts of sialic acid bound to it.[51] [52] These will be referred to as apoLp-Ala0, apoLp-Ala1, and apoLp-Ala2 for the forms having 0, 1, or 2 moles of sialic acid bound per mole of protein. A number of other minor apolipoproteins have been found but these are not well characterized.[50]

Since Type III plasma contains abnormal VLDL it seemed a possibility that this was due to synthesis of an abnormal apolipoprotein which either had unusual lipid-binding properties or interfered in some other way with lipid metabolism. Chemical, optical and immunochemical studies of apoLp-B isolated from both beta-LDL and total VLDL of Type III plasma yielded no detectable differences from that of normal plasma.[53] Nor were there any remarkable differences in the C apolipoproteins (apoLp-Ser, apoLp-Glu, apoLp-Ala) from Type III total VLDL.[54] Quarfordt et al. have separated and isolated beta-VLDL and prebeta-VLDL from Type III donors by starch block electrophoresis.[55] The prebeta-VLDL was chemically very similar to prebeta-VLDL from normal

subjects. The apoprotein of Type III beta-VLDL on the other hand, was found to consist almost entirely of apoLp-B. Thus if the apoproteins of the total VLDL isolated from Type III plasma were examined one would expect to find the same qualitative composition as in that from normal prebeta-VLDL; with an increase in the proportion of apoLp-B. Previous work in this laboratory on one case of Type III confirmed the increased amounts of apoLp-B.[5] However, it was found that the qualitative composition was not the same in Type III total VLDL and normal or Type IV prebeta-VLDL. There were substantial amounts of protein(s) in Type III total VLDL which were not identical with any of the previously recognized major apolipoproteins of normal alpha-HDL, beta-LDL, or prebeta-VLDL.[5] A second point of conflict arises between the data of Quarfordt et al. and that of another laboratory (Seidel and Greten[39]) in that if Type III beta-VLDL contains only apoLp-B then it should not cross-react (as it was found to do[39]) with antiserum to the apo-C proteins.

In the following work it was proposed to:

(1) examine the genetics of available Type III kindreds, especially that of the first reported case of the disorder appearing in a child.[25]

(2) explore new approaches to making a laboratory diagnosis of Type III hyperlipoproteinemia by methods which

did not require preparative ultracentrifugation.

(3) /re-examine the question of the apolipoproteins in Type III VLDL especially to see if the 'new' proteins[56] were consistently present in other cases of Type III and if they could be isolated and characterized.

METHODS AND MATERIALS

SAMPLES

Plasma samples for chemical determinations and for examination of the composition or character of lipoproteins were prepared from blood drawn by venipuncture, after a 12-14 h fast, into Vacutainer tubes containing tripotassium EDTA (Becton, Dickinson & Co., Clarkson, Ont).

Samples for the preparation of apolipoproteins were drawn, after fasting, into Fenwal RT-204CRC Transfer Packs (Baxter Laboratories, Malton, Ont), containing acid-citrate-dextrose.

Plasma was stored at 4° and preserved by adding buffered 1.5% Thimerosal at a rate of 4 ul/ml.[57] Plasma from distant points was packed on ice in insulated containers and usually shipped by air express. Chemical determinations and isolation of density fractions were done within a day or two of drawing the sample. Longer storage than this resulted in noticeable deterioration of electrophoretic patterns of whole plasma although isolated fractions were much more stable.

ANALYTICAL ULTRACENTRIFUGATION :Plasma lipoproteins

Plasma samples were centrifuged in a Beckman Model E analytical ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif) using the following procedure.* To 1.0 ml of EDTA plasma was added 0.41 g KBr to yield a solution of density 1.27 g/ml. This was centrifuged at $20°$ and 44,000 rpm with the Schlieren pattern recorded every 2 min for 12 to 16 min after attainment of full speed. Flotation constants, uncorrected for concentration, were calculated from the resulting time/distance curves.

PREPARATIVE ULTRACENTRIFUGATION :Plasma lipoproteins

Lipoproteins were isolated from plasma by minor modification of the method of Hatch and Lees.[57] Ultracentrifugation was done in a Beckman L2-65B ultracentrifuge using various rotors, (60Ti, 65, or 30.2), depending on the volume of sample. In every case the speed and time of running was adjusted so that isolation and washing of lipoproteins was under the following conditions:

Chylomicrons -- 7.8×10^4 g•min at native density, room temperature.

VLDL -- 1.7×10^8 g•min at d=1.006 g/ml, $17°$.

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* K. A. Evelyn, Strong Laboratory, Department of Medicine, University of British Columbia, personal communication.

LDL -- 1.4×10^8 g•min at d=1.063 g/ml, 17°.

HDL -- 2.7×10^8 g•min at d=1.20 g/ml, 7°.

Densities were determined by pycnometry at room temperature and adjusted with solid NaBr. Chylomicrons were often removed by placing the plasma at 4° overnight and aspirating the creamy layer that formed at the top of the sample. After chylomicrons were removed the VLDL fraction was isolated by putting the plasma in a centrifuge tube, carefully overlaying this with salt solution density = 1.006 g/ml and centrifuging for the appropriate time. The floating layer (VLDL) was removed by tube-slicing. The central clear solution in the tube was discarded and the infranatant (containing LDL, HDL and plasma proteins) was adjusted to density = 1.063 g/ml and centrifuged to isolate LDL. The corresponding procedure was followed to isolate HDL at density = 1.20 g/ml. Isolated fractions were usually mixed with two volumes of salt solution at the appropriate density and washed twice by centrifuging.

Washed lipoproteins to be used for apolipoprotein preparation were dialyzed against 0.01% disodium EDTA, pH 8 at 4°.

Lipid-poor plasma (LPP) used in agarose electrophoresis was prepared by adjusting the density of pooled normal plasma to 1.21 g/ml with NaBr, centrifuging as if to isolate HDL and slicing off and discarding the floating

lipoproteins. The infranatant (LPP) was dialyzed against normal saline, filtered and stored at -20°. Lipoprotein electrophoresis of LPP yielded only a lightly staining band in the alpha region, probably due to minor amounts of HDL or very high density lipoproteins (VHDL).[58][59]

LIPID ANALYSIS :Plasma lipoproteins

A semi-automated method for the simultaneous determination of triglycerides[60] and cholesterol[61] in an isopropanol extract was used. This was performed at the University Hospital using the same standards and control sera as for the routine clinical analysis.

AGAROSE GEL ELECTROPHORESIS :Plasma lipoproteins

Electrophoresis of plasma lipoproteins was done by the procedure previously reported from this laboratory.[62] Agarose (0.5%, w/v) in 0.05M barbital buffer, pH 8.6 was poured onto motion-picture leader film and allowed to set. Slots were cut into the gel and filled with 10 ul of sample. Electrophoresis was performed 6 V/cm for 45 min. Staining with Sudan Black B (C.I. No. 26150) and destaining was carefully standardized and controlled. Previous studies have shown that there is an excellent correlation between the dye uptake and the amount of lipoprotein present in each fraction.[54][63] In order to obtain reproducible migration of ultracentrifuge-isolated lipoprotein fractions it was

necessary to replace other serum proteins to the amount removed during isolation. This was done by adding lipid-poor plasma to the sample.

DELIPIDATION :Lipoproteins

Lipid-free apolipoprotein (<1% phospholipid, no detectable triglyceride or cholesterol) could be obtained by lipid extraction into ethanol-diethyl ether (3:1, v/v).[64] Salt free samples of isolated lipoproteins were lyophilized (Automatic Freeze-Dryer Model 10-010, The Virtis Company, Gardiner, NY) in 50 ml round bottom centrifuge tubes. Ethanol-ether (40 ml) was added, the lipoprotein cake dispersed by shaking and the stoppered tube mounted on a rotator. Extraction was continued at 4°. After 8 h extraction the mixture was centrifuged (2000 rpm, 15 min), the supernatant decanted and the sedimented protein resuspended in ethanol-ether for a further 8 h extraction. This procedure was repeated until the protein was completely white -- usually three to five times. The protein was then washed three times in ether and dried under a stream of nitrogen. The resulting apolipoprotein was soluble in 0.2M Tris HCl, pH 8.2, 0.1M sodium decyl sulphate to a concentration of 30 mg/ml.

A report by Scanu and Edelstein[65] suggested that substantial protein losses could occur during ethanol-ether extraction. However, treatment of the supernatants in the

above procedure according to their recommendations yielded no protein. Apparently such losses depend upon the presence of water in the extracting solvents (Scanu and Edelstein used lipoprotein preparations directly without lyophilization).

COLUMN CHROMATOGRAPHY :Apolipoproteins

Tris (tris[hydroxymethyl]aminomethane) buffer was prepared using Trizma and TrizmaHCl (Sigma Chemical Co., St. Louis, Mo) mixed in ratios according to the manufacturer's Bulletin No. 106B. A stock solution of unit molarity and pH 8.2 was prepared containing 70.8 g TrizmaHCl and 66.8 g Trizma/l. This was diluted appropriately to yield buffers of the required concentration. Buffers were put through a 0.8 um filter (Millipore Filter Corporation, Bedford, Mass) and stored at 4°. Urea (ACS certified grade, Fisher Scientific) was prepared as 8M solution, filtered and stored at 4°. Immediately prior to use it was run through a column of Rexyn I30C (Fisher) to produce a solution having conductivity <1 umho/cm. Sodium decyl sulphate (NaDS) purchased from Schwarz/Mann, Orangeburg, NY, was used as a solubilizing detergent in preference to sodium dodecyl sulphate (SDS) since NaDS can be more completely removed by dialysis.[66] Water for solutions and dialyses was distilled and deionized. Apolipoproteins dissolved in Tris-NaDS buffers were dialyzed in 18/32 dialysis tubing (Union

Carbide). Proteins in urea solutions were dialyzed in Spectrapor Membrane Tubing 3, MW cut-off 3500 (Spectrum Medical Industries Inc., Los Angeles, Calif). This low molecular weight cut-off dialysis tubing was used since in urea solutions significant losses of protein (especially apoLp-Ser) occurred using the 18/32 tubing. Dialysis of pooled fractions from column chromatography was at 4° against water, usually continued until the protein precipitated. These fractions were then lyophilized and stored dry at -20° or in buffer solution at 4°.

Absorbance was read in a Beckman DU spectrophotometer equipped with a Gilford optical density converter. Protein concentrations were approximated by the formula

$$1.55 \ A280 - 0.74 \ A260 = mg/ml.[67]$$

This agreed with dry weight within ±10%.

Gel filtration chromatography. Sephadex G200 (Pharmacia (Canada) Ltd., Montreal, Que) was swollen, degassed, packed, equilibrated and eluted according to the manufacturer's instructions[68] with 0.2M TrisHCl, pH 8.2, 0.002M NaDS, 0.003M sodium azide in a 2.5 x 100 cm glass column (Pharmacia K25/100 with flow adapters) at room temperature. Elution was by pump-driven upward flow at 20 ml/h. Fractions were collected by drop counting (50 drops = 2.2 ml/tube). Protein samples (5-100 mg) were applied dissolved in 1-4 ml 0.2M TrisHCl, pH 8.2, 0.1M NaDS. Void volumes (Vo) and

effective bed volumes (Vt) were determined with Blue Dextran 2000 (Pharmacia) and KI, respectively.

Ion-exchange chromatography. DEAE-cellulose (Whatman DE52 microgranular, preswollen) was equilibrated according to the manufacturer's instructions[69] in starting buffer 0.005M TrisHCl, pH 8.2, 8M urea and packed under pressure into a column 0.9 x 26 cm (Metalloglass Inc., Boston, Mass) at 4°. A thin slurry of DEAE-cellulose was prepared in 0.1 M Tris and titrated to pH 4 with HCl. This was degassed under vacuum and titrated back to pH 8.2 with solid Tris. The slurry was allowed to settle in a graduated cylinder for one hour and the supernatant (including fines) was aspirated. The cellulose cake was resuspended in 0.005 M TrisHCl, pH 8.2, allowed to settle, and decanted (repeated three times). This cake was stored at 4° for up to a week. Prior to a chromatographic experiment sufficient DEAE-cellulose cake was resuspended in 100 ml of the starting buffer at 4°, allowed to settle, and decanted (repeated twice) before packing. Starting buffer was pumped through the column until the conductivity and pH of the in-going and out-coming buffers were equal. Protein samples (5-70 mg dissolved in and dialyzed against starting buffer) were pumped onto the column followed by 10 ml starting buffer. Eluting buffer was pumped at 20 ml/h from a closed 255 ml mixing flask (initially filled with starting buffer) connected to an open reservoir of limiting buffer. The limiting buffer volumes

and sequence were usually:

    0.1M TrisHCl, pH 8.2, 8M urea for 400 ml,

    0.2M TrisHCl, pH 8.2, 8M urea for 200 ml,

    0.4M TrisHCl, pH 8.2, 8M urea for 100 ml,

and finally NaCl added at a rate of 1 mole/l to the mixing flask.

Fractions were collected by drop counting (75 drops = 4.1 ml/tube) and conductivities read with a conductivity bridge (YSI Model 31, Yellow Springs Instruments, Yellow Springs, Ohio).

POLYACRYLAMIDE GEL ELECTROPHORESIS : Apolipoproteins

Disc gel electrophoresis (PAGE) was performed in a tap water cooled Buchler apparatus (Buchler Instruments Div., Nuclear Chicago Corp., Fort Lee, NJ). Gels were cast in glass tubes 5 x 75 mm which had been treated with a 0.5% solution of Photo-Flo 200 (Eastman Kodak Co., Rochester, NY) and dried. The running gel was 47 mm and the stacking gel 15 mm long. A buffer system similar to that of Reisfeld and Small[70] was used. Gel preparation and composition was:

Running gel (lower), pH 8.75 (measured pH of the buffer

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*$T=(a+b)/m \cdot 100$ [%], $C=b/(a+b) \cdot 100$ [%]; where a= acrylamide (g), b= Bis (g), m= volume of solution (ml).[71]

solution), T=7.7%, C=2.4%*.

3 ml catalyst solution containing 3.6 g ammonium persulphate/l in water, freshly prepared.

1.5 ml buffer containing 453.76 g Tris, 600 ml 1N HCl, 6 ml TEMED (N, N',N'-tetramethylethylenediamine, Eastman #8178)/l in water, kept as a stock solution.

10.5 ml acrylamide solution containing 107.2 g acrylamide (Eastman #X5521), 2.72 g Bis (N,N'-methylenebisacrylamide; Eastman #8383)/l in deionized 8M urea, freshly prepared.

This mixture was degassed under high vacuum, poured into the tubes and overlayed with a few millimetres of water. Polymerization was at room temperature for 30 min.

Stacking gel (upper), pH 6.55, T=2.2%, C=9.1%.

2 ml catalyst solution containing 25 mg riboflavin, 0.6 g ammonium persulphate/l in water.

0.5 ml buffer containing 111.5 g Tris, 640 ml 1M phosphoric acid, 5 ml TEMED/l in water.

7.5 ml acrylamide solution containing 28.6 g acrylamide, 2.73 g Bis/l in deionized 8M urea.

After degassing, pouring and overlaying with water this gel was photopolymerized for 1 h.

Upper (cathode) buffer -- 0.05M Tris, 0.06M glycine, pH

8.9, containing 0.3 mg bromophenol blue/l.

Lower (anode) buffer -- 0.06M TrisHCl, pH 8.1.

Samples (usually 100 ul of buffer solution containing 5-200 ug of protein) were made 20% in sucrose, and if not already in urea solution. they were made 8M in urea with solid urea (Ultra-Pure #04000-9200, Schwarz/Mann). Samples were layered on top of the stacking gel under cooled upper buffer and electrophoresed at 2.5 mA/gel until the bromophenol blue tracking dye just reached the bottom of the gel, 1.5-2 h. After removal from the tube each gel was placed in 5 ml fixing solution (5% trichloroacetic acid, 5% sulphosalicylic acid) and 0.25 ml of a 1% solution of Coomassie Brilliant Blue (C.I. No. 42660, #B-0630, Sigma) in ethanol was added. Gels were stained overnight then destained by gentle rocking in several changes of the fixing solution. They could be stored indefinitely, in the dark in 7.5% acetic acid.

Gels were arranged in grooves cut into a slab of white translucent plastic supported over a fluorescent lamp and photographed on Polaroid Black & White Land Pack Film Type 107 through a #56 Klett filter.

POLYACRYLAMIDE GEL ELECTROPHORESIS IN SODIUM DODECYL SULPHATE :Apolipoproteins

The same system as for PAGE was used with the following variations.

A 62 mm long running gel was used without a stacking gel. The acrylamide solution was made up with 0.3% SDS instead of urea solution. Gels were made with T=10% and T=15% by increasing the amount of acrylamide and Bis proportionately. SDS was added to the upper buffer to a concentration of 0.2%. Samples were prepared by mixing 10 ul of protein solution (made up in 1% SDS at a concentration of 1 mg/ml) with 50 ul of upper buffer, 20 mg sucrose and 5 ul 2-mercaptoethanol. Electrophoresis was continued until the bromophenol blue marker was about 5 mm from the bottom of the gel. After extruding the gels from their tubes the marker band was stabbed with a pin dipped in india ink. The staining and destaining solutions of Weber and Osborn[72] were used but destaining was done by extensive washing rather then electrophoretically. Relative mobilities were calculated by dividing the measured distance that the bromophenol blue band migrated by that which the protein migrated.

ANALYTICAL GEL ISOELECTROFOCUSSING :Plasma lipoproteins and Apolipoproteins

The procedure used for isoelectrofocussing in polyacrylamide gels was essentially the same for both native lipoproteins and apolipoproteins; only the staining procedure differed. A gel electrofocussing apparatus M137 with plastic gel tubes 10 x 0.3 cm (MRA Corp., Boston Mass) was used. Ampholine was a 40% w/v solution, pH 3-10 (LKB - Produkter AB, Bromma, Sweden). All solutions except catalyst have been stored at 4° for up to 6 months. The resulting gel contains 2% carrier ampholytes with T=3%, C=2.6%. Solutions were mixed in the following order:

1.06 ml acrylamide solution containing 300 g acrylamide, 8 g Bis/l. With some batches of Acrylamide this solution may require up to one month's storage at 4° in order to yield gels which polymerize to a consistency convenient for handling.

1.00 ml TEMED solution containing 2.3 ml TEMED/l.

4.00 ml catalyst solution containing 1.4 g ammonium persulphate/l, freshly prepared.

0.54 ml Ampholine.

1.08 ml glycerol.

3.22 ml water.

This mixture was quickly degassed and poured into the gel tubes covered at the lower end with Parafilm. After 4 h polymerization at room temperature the tubes were covered with Parafilm and stored at 4°. Best results were obtained if gels were made at least 24 h before using. Just before an experiment the Parafilm was removed and the lower end of the tube covered with fine mesh gauze held in place with a rubber band. Gel tubes were placed in the apparatus maintained at 3°-6° by a circulating water bath. The lower (anode) solution was 0.01M phosphoric acid and the upper (cathode) 0.02M sodium hydroxide. Gels were prefocussed for 30 min at 0.5 mA/gel. After prefocussing an equal volume of sample and a solution of 0.5 g sucrose, 0.2 ml Ampholine/ml were mixed. 6 ul of this was layered on top of the gel under 15 ul of a solution of 0.2 g sucrose, 0.05 ml Ampholine/ml.

In the case of native lipoproteins the sample was either EDTA plasma or ultracentrifugally-isolated lipoproteins.

Apolipoprotein samples were made up in buffer and about 5-15 ug of protein applied to the gel.

Isoelectrofocussing was at 0.5 mA/gel until the voltage reached 400 V (about 1 h) at which time the power supply was switched to constant voltage and the run continued for 18 h. After partial removal from the tubes the lower 5 mm of the gel was dipped in india ink to permanently mark the anodal

end. Excess ink was washed off with water and gels extruded with a pipette bulb into fixing or staining solution.

For native lipoproteins the gels were put directly into a staining solution of Sudan Black B.[73] This staining solution was prepared by dissolving 250 mg Sudan Black B in 10 ml acetone and adding 7.5 ml acetic acid, 40 ml water. After stirring for 30 min this was centrifuged to remove undissolved dye and used within 12 h (unstable). Gels were stained overnight at room temperature and destained by three 15 min washes in 10 ml of acetone-acetic acid-water (20:15:65, v/v/v). Photographic records were kept as for PAGE but using a #62 Klett filter.

For apolipoproteins the gels were extruded into 20% trichloroacetic acid and washed for 5 h with frequent changes to remove the carrier ampholytes, which are stained by Coomassie Blue. Gels were then stained in the same stain as used for PAGE-SDS and destained by washing in 20% trichloracetic acid.

Isoelectric points (pI) of the focussed bands were determined by transversely slicing the gel into 3 mm segments, eluting these for 1 h with 0.5 ml water, determining the pH and finally staining the segments.

PROTEIN HYDROLYSIS :Apolipoproteins*

Proteins were hydrolyzed in 1-2 ml 6N HCl containing 1 drop of 2-mercaptoethanol or in p-toluenesulphonic acid by the method of Liu and Chang.[74] Hydrolysis was at 110°, usually for 22 h, in sealed evacuated tubes with known amounts of norleucine present as internal standard. After hydrolysis in HCl any precipitate was centrifuged out and the hydrolysate transferred to and dried on a rotary flash evaporator. The residue after drying was dissolved in starting buffer for the amino acid analyzer. After hydrolysis in p-toluenesulphonic acid, the pH of the hydrolysate was adjusted to that of the starting buffer with 2N lithium hydroxide and the sample applied directly to the column.

Stained protein bands isolated by PAGE were hydrolyzed in HCl by the same procedure. Gels were first washed extensively in 7.5% acetic acid to remove all glycine (from the PAGE upper buffer) and then slices of gel containing the desired protein band were hydrolyzed.[75] The p-toluenesulphonic acid method could not be used for PAGE gels since the acrylamide dissolved completely and produced a solution too viscous to apply to the amino acid analyzer.

AMINO ACID ANALYSIS :Protein hydrolyzates

A single column Technicon Amino Acid Autoanalyzer with Chromobeads B (Technicon Instruments Corp., Chauncey, NY) was used with the lithium citrate buffer system of Perry et al.[76] A more sensitive and stable colour reagent was made up of 18 g ninhydrin, 23 ml propionic acid, 250 ml 2M sodium propionate and 500 ml ethylene glycol monomethyl ether/l; and 0.004M hydrazine sulphate was used as a reducing reagent.[77] Amino acid separation was improved (especially of valine and cystine) by changing the buffer system of Perry et al. as follows: Chamber 4 - 37.5 ml pH 2.80 buffer + 37.5 ml pH 3.80 buffer; Chamber 6 - 37.5 ml pH 3.80 buffer + 37.5 ml pH 6.10 buffer.

TERMINAL ANALYSIS :Apolipoproteins

Amino terminal determination using the dansyl reaction was performed according to Gray[78] in 8M urea-sodium bicarbonate and/or SDS-N-ethylmorpholine solution. Thin layer chromatography was on polyamide sheets using the solvent system of Hartley.[79]

Carboxy terminal analysis using carboxypeptidase A (COA DFP 9KA, Worthington Biochemical Corp., Freehold, NJ) was done by the method of Ambler[80] using an enzyme substrate ratio of 1:20 at 37° in 0.2M N-ethylmorpholine acetate, pH 8.5 with incubations up to 5 h.

ANTISERA AND IMMUNODIFFUSION :Apolipoproteins

Apolipoproteins isolated by DEAE-cellulose chromatography were dissolved in 0.2M TrisHCl, pH 8.2, 0.002M NaDS to a concentration of 1 mg/ml. This solution (0.6 ml) was emulsified with an equal volume of Freund's complete adjuvant (Difco Labs., Detroit, Mich) and injected subcutaneously at multiple sites on the backs and necks of 4-5 pound male New Zealand White rabbits. Ten days later the rabbits were boosted with an intravenous injection of 0.4 ml of the same antigen solution containing no adjuvant. Ten days after boosting the rabbits were bled. Antisera were concentrated five times in a Minicon Macrosolute Concentrator (Amicon, Lexington, Mass).

Double immunodiffusion by the Ouchterlony technique[1] was performed on glass microscope slides. These slides were evenly coated with 1.5 ml of hot 1.5% agarose in 0.05M barbital buffer, pH 8.6. Holes (3 mm diameter) were punched in the cooled agarose and filled with 5 ul of antigen solution or antiserum. Diffusion was allowed to continue overnight or longer in a humidified chamber. Slides were then placed in normal saline (0.85% NaCl) overnight to leach out unprecipitated protein and then in methanol for 1 h to remove salts. After drying, slides were immersed in 0.6% Amido Schwartz (C.I. No. 20470) in methanol-acetic acid-water (45:10:45, v/v/v) for 5 min and destained in several changes of the stain solvent. Dried and stained slides were

preserved by spraying with an acrylic resin (Labcote-. Nutritional Biochemicals, Cleveland, Ohio).

SUBJECTS

Plasma samples were treated as described under Preparative Ultracentrifugation and Agarose Gel Electrophoresis and as summarized in Figure 1.

A summary of the plasma lipoprotein electrophoresis patterns, sexes, ages, serum cholesterol and triglyceride analyses and absence or presence of xanthomas (where known) at the time of presentation is shown in Figure 2.

J.O'D., male, age 23 has been extensively reported in the literature by Dr. W.R. Hazzard, Harborview Medical Center, Seattle, Washington.[30][82]

J.Kl., male, age 41 was diagnosed by and is a patient of Dr. J.A. Little, St. Michael's Hospital, Toronto, Ontario.

E.Li., male, age 53 was diagnosed on a referral sample after lipoprotein electrophoresis at Calgary Medical Laboratories, Calgary, Alberta, showed a 'broad-beta' pattern. He had small tendinous xanthomas and was overweight. Lipoprotein electrophoresis revealed a distinct and separate prebeta band. This medical doctor was self-treated and showed a much improved lipid pattern six months
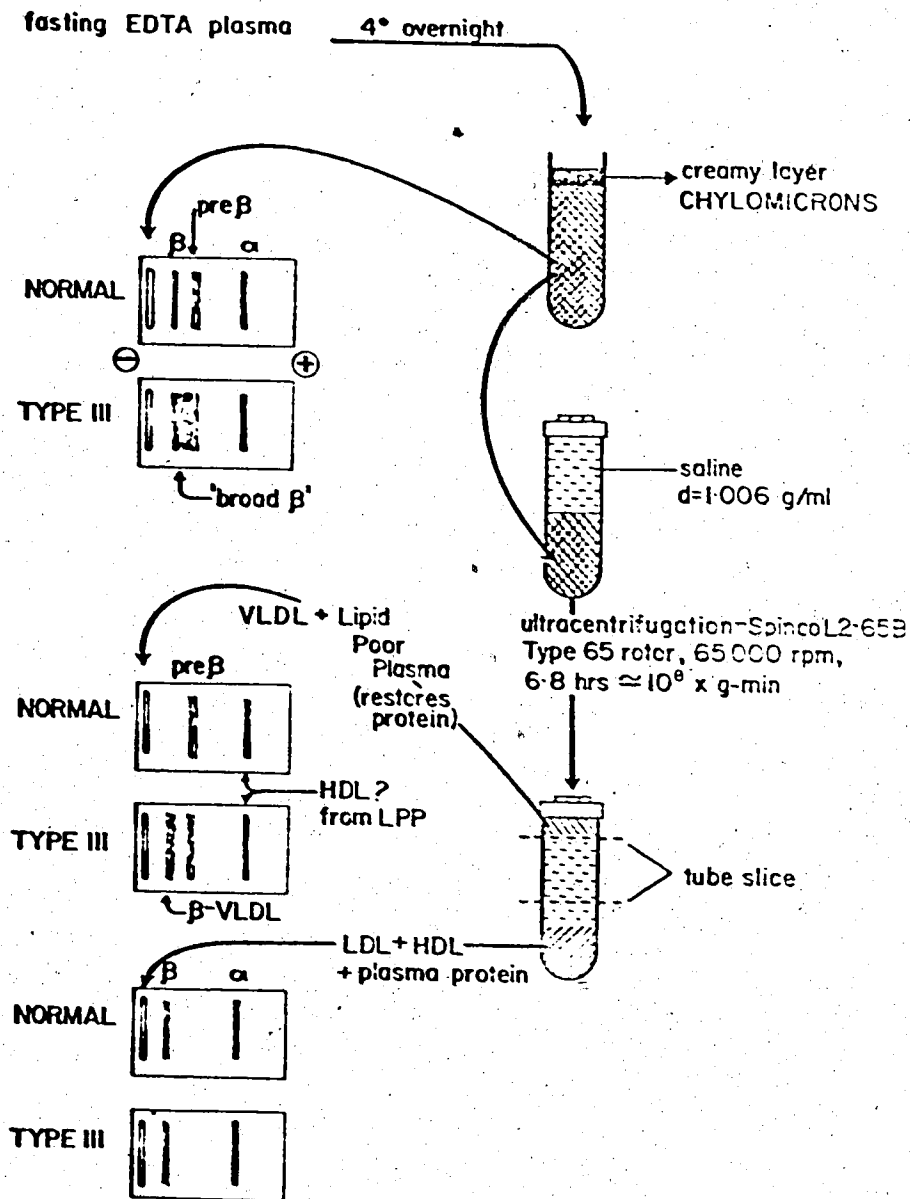
Figure 1. Procedure for the identification of Type III hyperlipoproteinemia by preparative ultracentrifugation and electrophoresis of plasma lipoproteins.
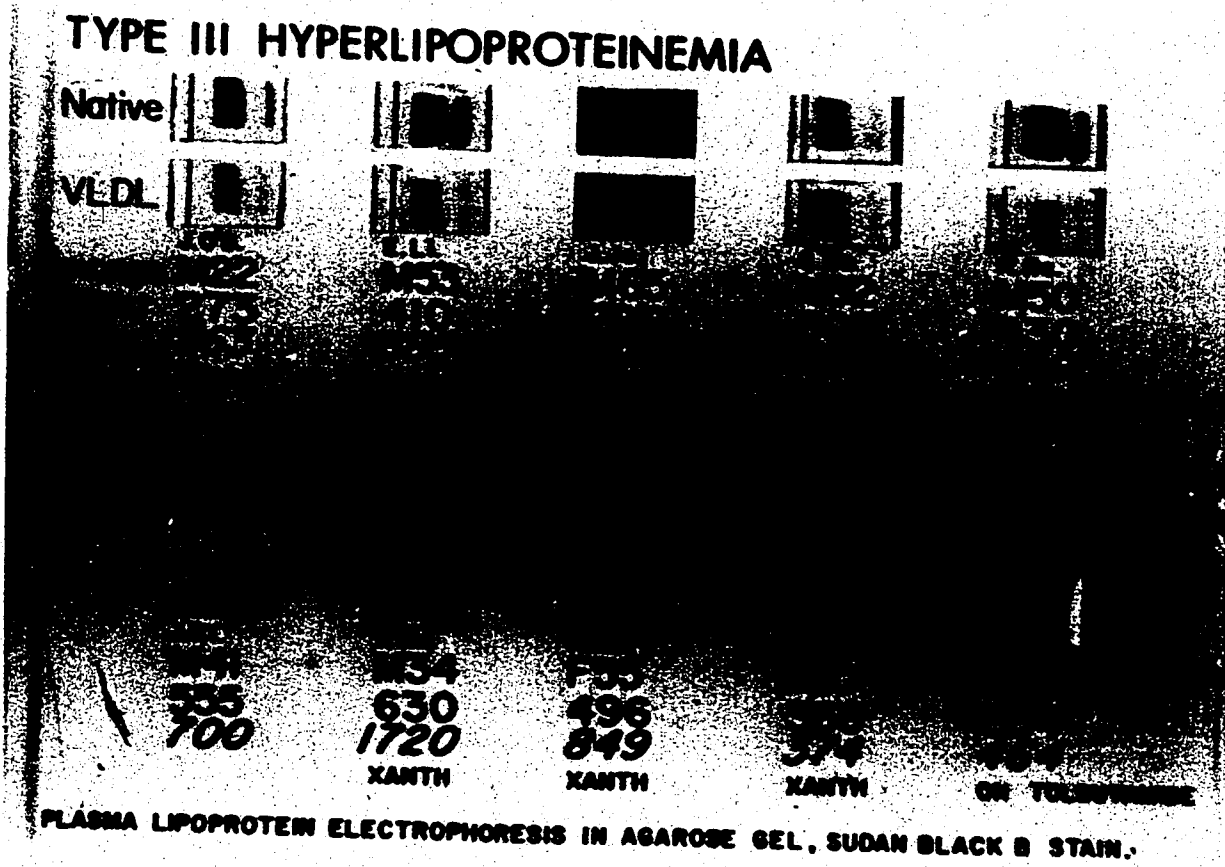
Figure 2. Plasma lipoprotein electrophoresis in agarose gel, stained with Sudan Black B. The upper electrophoretogram of each pair is EDTA plasma, the lower is ultracentrifuge-isolated VLDL diluted with lipid-poor plasma. Under the electrophoretogram is the patient identification, sex and age (M= male, F= female), serum cholesterol [mg/100 ml], serum triglycerides [mg/100 ml], and the presence of xanthomas (xanth) at presentation, or medication at time of sampling.

after diagnosis. He has been lost to follow-up.

T.An., male, age 34 was diagnosed on a referral sample after lipoprotein electrophoresis at the Royal Alexandra Hospital, Edmonton, showed a 'broad-beta' pattern. He had tuberoeruptive xanthomas on his elbows, the palms of his hands and under his toes. He had first noticed these about four years previously. He suffered some shortness of breath and was overweight. Fasting plasma (20 h after the last fat meal) contained substantial amounts of chylomicrons. Plasma lipoprotein electrophoresis produced a separate and distinct prebeta band. He has two young sons whose plasma lipids are normal and a younger brother who suffered a "heart attack" at age 28. T.An. had been a professional football player until his late twenties and his first symptoms (xanthomas) began to appear only after he ceased regular exercise and began to gain weight. He was treated with clofibrate and given a diet for weight reduction and maintenance designed for Type III.[83] He has been lost to follow-up and his response to treatment is not known.

J.Jo., male, age 65 was diagnosed on a referral sample after lipoprotein electrophoresis at Vancouver General Hospital, Vancouver, British Columbia, produced a 'broad-beta' pattern. He has large tuberous xanthomas on the extensor surfaces of his elbows and knees. He is hypertensive, suffers intermittent claudication with a lack of pulse in the small arteries of both legs, and has a

severe diminution of his renal reserve. His family history will be discussed under Results and Discussion - Family Studies.

H.Ri., female, age 55, sister of J.Jo. was diagnosed upon screening of that kindred. She had been treated for possible angina about 15 years ago. She is overweight and had noticed a few small xanthomas on her elbows about nine months prior to diagnosis. Plasma lipoprotein electrophoresis yielded a dense fusion of the beta and prebeta regions.

S.De., male, age 52 has previously been studied in this laboratory[5] and was originally discovered through routine lipid analysis at the University Hospital. He suffered a myocardial infarction at age 44. He has two or three small xanthomas on his right elbow which have only appeared since diagnosis. He is not overweight. His fasting plasma has always had a fairly thick layer of chylomicrons after refrigeration overnight and lipoprotein electrophoresis produces a 'broad-beta' band with no clear cut band in the prebeta region. There is often a pronounced streaking from the beta region back to the origin. He has two unaffected normal sisters and a normal son. There is no family history of diabetes or heart disease although his mother may have had a stroke at age 79. His condition has been refractory to treatment with thyroxine, clofibrate, cholestyramine and controlled diet.

J.Ra., male, age 10 has previously been reported in brief.[25] He presented with obesity, lipemia retinalis, tuberoeruptive xanthomas on elbows, knees and in the creases of his buttocks. He was the only one of these Type III patients to also have the nearly-diagnostic[8][*] planar xanthomas in the creases of his palms and between his toes. Plasma lipoprotein electrophoresis produced a 'broad-beta' band and a fairly distinct prebeta band. A glucose tolerance test was normal. His family history will be discussed under Results and Discussion -- Family Studies. His response to dietary treatment alone (loss of excess weight and maintenance), has been remarkable. Tuberous xanthomas rapidly disappeared and after eight months there were only traces of the planar xanthomas remaining. His plasma lipids remain at relatively normal levels although they are liable to sudden increases, especially in the summer when he sometimes indulges his fondness for icecream. This is in keeping with the frequently-noticed extreme carbohydrate inducibility of the disease.[7]

R.Ra., male, age 50, father of J.Ra., was diagnosed upon screening of that kindred. He is of normal weight and has no xanthomas. He has diabetes mellitus which is controlled by diet and tolbutamide. A remarkable phenomenon occurs in R.Ra. when his diabetes is allowed to go out of control. His plasma lipoproteins then take on a fairly typical Type IV pattern and there is no evidence of beta-

VLDL. When tolbutamide is administered and his diabetes controlled, beta-VLDL returns (Figure 2). Although beta-VLDL has been reported as a transient phenomenon in uncontrolled diabetic ketoacidosis[11] its occurrence in R.Ra. seems to be quite the opposite.

RESULTS AND DISCUSSION

FAMILY STUDIES

Extensive family studies were done on the kindreds of
J.Jo. and J.Ra. In the accompanying pedigrees the lipid
values given for hyperlipoproteinemic subjects are those
observed before treatment was begun. Members for whom no
lipid values are given were not examined, but according to
interviews with others of the family, they were well.

The upper limits of normal for serum cholesterol [250
mg/100 ml] and triglycerides [150 mg/100 ml] shown in the
pedigrees may be questioned. These are the tentative normal
limits given by the University Hospital Clinical Laboratory
and are used here only as rough guidelines.

Serum lipid levels are strongly age- and sex-dependent
and serum triglycerides tend to have a log-normal
distribution. For comparison, the upper normal limit (value
exceeded by 5% of the population) according to various
published studies[85 86] for cholesterol ranges from 240
mg/100 ml (male age 20-29, ref. 86) to 330 mg/100 ml (male
or female age 50-59, ref. 7). The upper limit for
triglycerides ranges from 140 mg/100 ml (male or female age
20-29, ref. 7) to 310 mg/100 ml (male age 40-49, ref. 86).
Thus the upper limit values of 250 mg/100 ml for cholesterol
and 150 mg/100 ml for triglyceride are about the lowest of
estimates provided by large population studies.

Compounding the problem of deciding cut-off limits for 'normality' are: (1) significant variations between different analytical methods commonly used; (2) day to day variations in serum lipid levels of the individual and a marked effect of diet which is not completely negated by a 12-14 h fast; (3) lack of adequate analytical control material, especially as regards triglycerides; (4) a coefficient of variation of the analytical methods of 5-10%.

For these reasons the Fredrickson typing system has not been strictly adhered to and members of these pedigrees have only been 'Typed' if their lipid or lipoprotein levels were strikingly abnormal. All plasma samples were checked for beta-VLDL by ultracentrifugation and agarose electrophoresis.

J.Jo. kindred. (Fig. 3) The father (I-1) of J.Jo. was essentially well all his life. The mother (I-2) had had hypertension for many years. Attempts at relieving her high blood pressure resulted in syncopal attacks. A sister (II-3) died at age 49 of a heart attack after a long history of angina and hypertension. The brother (II-2) is well. There is only one certain Type IV in the family (III-7) but the lipid levels of a few others are upper normal or slightly elevated (II-2, III-6,8,11,12).

Of interest but uncertain significance is a comparison of the lipid levels of the children of J.Jo. (II-1) and

TYPE III HYPERLIPOPROTEINEMIA – J.Jo., VGH '72

I

II

290
300

243
176

496
849°

III

146
65

135
74

182
52

175
118

195
155

236
550

227
206

220
138

142
175

200
180

IV

153
57

195
40

120
40

136
30

142
43

158
57

177
107

187
78

Age, years

250-serum Cholesterol, mg/100 ml, normal = 75-250
150-serum Triglyceride, mg/100 ml, normal=50-150

■ TYPE III

□ TYPE IV

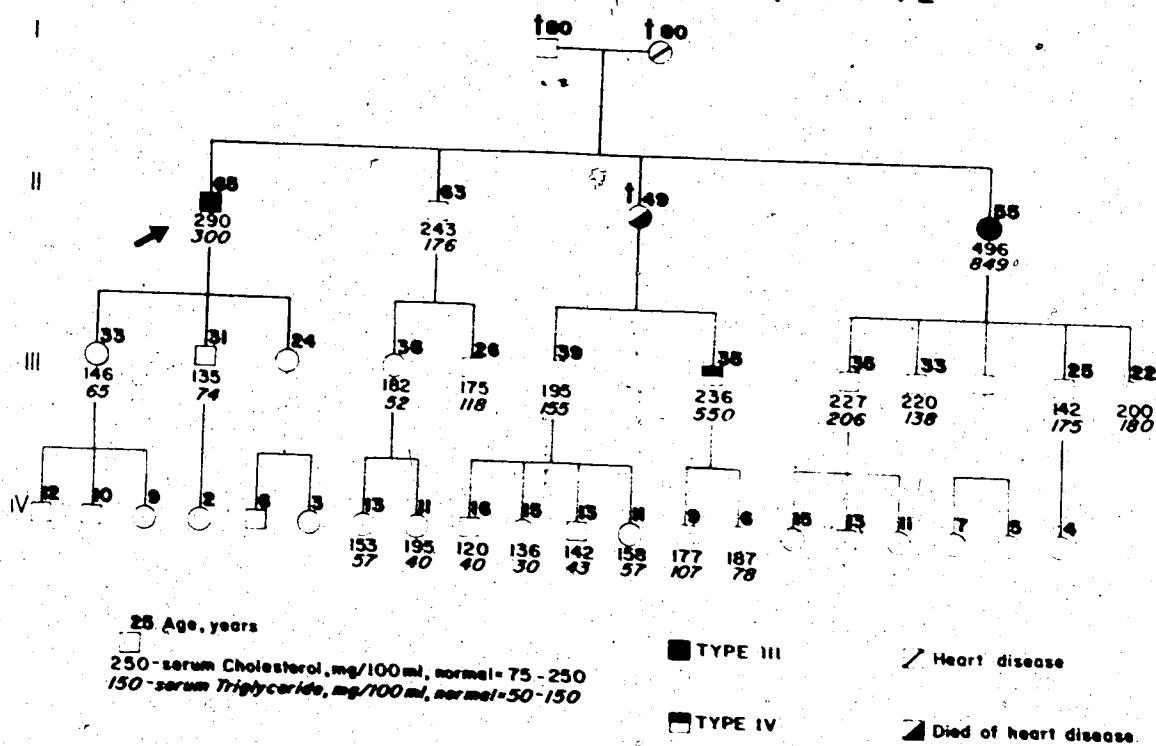/ Heart disease

◪ Died of heart disease

Figure 3. Pedigree of the J.Jo. kindred. The arrow indicates the propositus. Deceased members are marked with a cross.

those of H.Ri. (II-4); and of the children of III-6 and those of his Type IV brother (III-7). This apparent similarity of lipid values among siblings may, of course, be due to familial factors other than genetics, such as eating habits or conscientious adherence to instructions requesting a 12-14 h fast before blood was drawn.

The pedigree of J.Jo. is compatible with any of the forms of inheritance suggested by Fredrickson and Levy[7] for the Type III disorder. However, there appears to be a lower incidence of Type IV than they report in their larger Type III kindreds.

J.Ra. kindred, (Fig. 4) The paternal grandparents (I-1,2) were relatively well all their lives and did not die of heart disease. Maternal grandparents (I-3,4) on the other hand both suffer(ed) heart trouble. Unfortunately little is known of the lipid status of the three uncles (II-1,2,3) who died of heart disease. One of the paternal aunts (II-5) has hypertension, the other (II-4) is well. The proband's father (R.Ra., II-6) is diabetic and displays the Type III lipoprotein disorder when his diabetes is controlled (Fig. 2). The youngest maternal uncle (not shown on the pedigree in Fig. 4) died of "rheumatic heart disease" at age 11 y. The genetic defect for hypercholesterolemia (Type II hyperlipoproteinemia) is present in the maternal side of the family. The dominant mode of inheritance of Type II is illustrated by a comparison of a maternal uncle (II-12) and

TYPE III HYPERLIPOPROTEINEMIA – J.Ra., UAH '71

I

II

III

IV

236
83

305
215

35-
464

252
163

328-
162

254
163

364
147

207
235

316
98

275
167

36

193
93

10

358
374

22

287
82

25 Age, years

250 = serum Cholesterol, mg/100ml, normal = 75-250
150 = serum Triglyceride, mg/100ml, normal = 50-150

■ TYPE III

▨ Heart disease

▣ TYPE III

◪ Died of heart disease
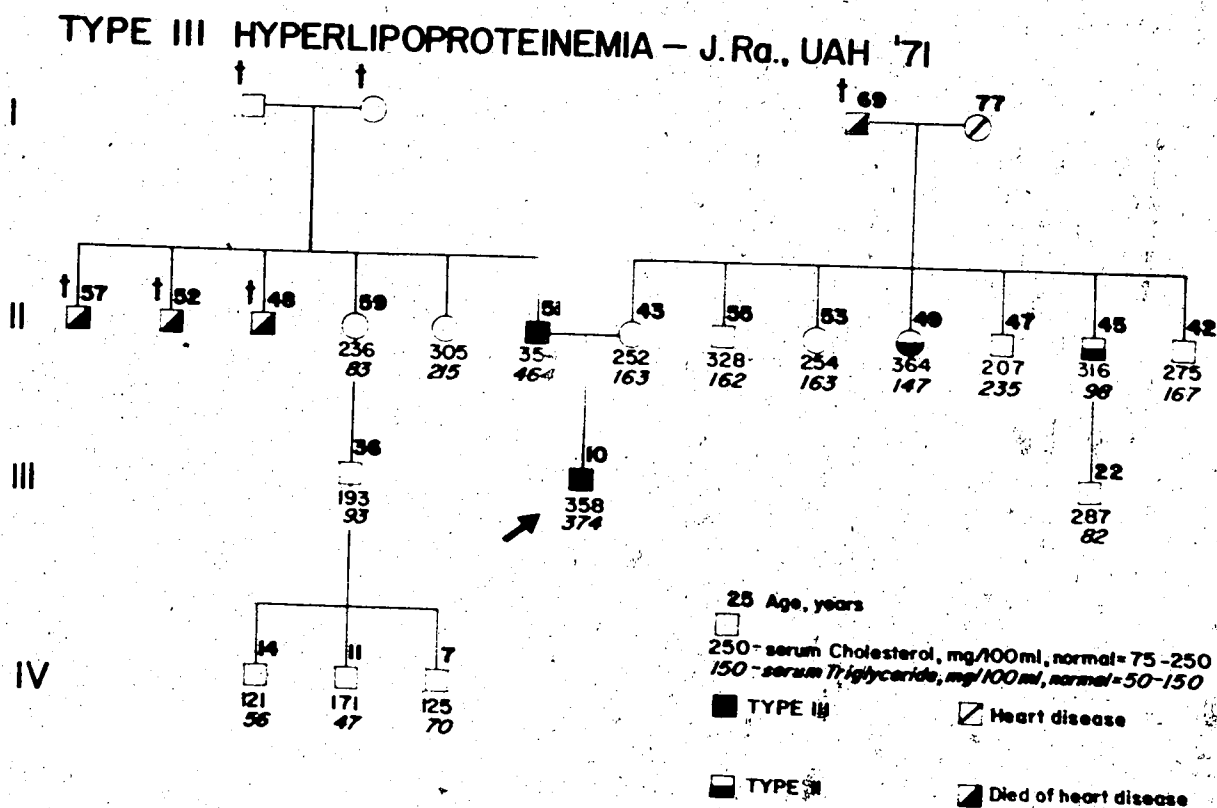
14

11

7

121
56

171
47

125
70

Figure 4. Pedigree of the J.Ra. kindred. The arrow indicates
the propositus. Deceased members are marked with a cross.

his son (III-3) both of whom have elevated cholesterol, low normal triglycerides and an elevated beta-LDL on electrophoresis. As well as two definite cases of Type II (II-10 and II-12) nearly all the aunts and uncles have either elevated or upper normal serum lipids.

It could be hypothesized that the unique early appearance of the Type III disorder in J.Ra. indicates that he inherited a lipoprotein abnormality from each of his parents: Type III from his father, and Type II from his mother. The J.Ra. pedigree is compatible with a dominant mode of inheritance of the Type III mutation. If the three paternal uncles who died of heart disease at ages 57, 52, and 48 also had the Type III abnormality the mutation seems to have taken a particularly virulent form in this family.

DIAGNOSTIC METHODS

Lipoprotein electrophoresis

A major problem in laboratory diagnosis of Type III by electrophoretic demonstration of beta-VLDL is the variability of migration of lipoprotein components. In the course of performing lipoprotein electrophoreses in the routine clinical laboratory it has been observed that freshly drawn specimens often produce slightly different patterns from samples that have been stored for a day or two at 4°. Samples which have come by post may have been exposed to extremes of heat or cold and sometimes produce very peculiar artifacts. It was found that the isolated fractions of VLDL often migrated at different rates than did the VLDL in the native plasma. Hence, comparison of migration rates in isolated fractions was frequently a complex affair, often requiring a number of electrophoreses or even isolations from separate plasma samples before coming to a reasonable conclusion regarding presence or absence of an abnormal component.

Compounding the problem is the presence, in suspect specimens of very high lipoprotein concentrations which produce such large, dense bands that separation of species may be unclear. Samples from some suspect Type III subjects have contained what are apparently very large VLDL particles

which are small enough to enter the gel but are subject to so much sieving that they produce heavy streaking between bands and even back to the origin, further confusing interpretation.

Dilution of samples with saline alleviated the problems of high concentration and streaking but not the variable and unpredictable migration of isolated fractions. It was found that dilution with lipid-poor plasma (to restore plasma proteins) usually resulted in a migration behaviour of isolated fractions which was very similar to that of the native plasma sample. Addition of LPP had the added advantage that the small amount of alpha-migrating material in LPP could be used as a reference marker, and relative migration rates determined from it. If the LPP-diluted isolated VLDL appeared to be migrating prebeta when compared to the electrophoretogram of the whole plasma, this could be quickly confirmed or contradicted by comparing the migrations of the alpha material in the two strips.

By using LPP as a diluent it was found that from nearly all Type III specimens, isolated VLDL produced two separate and distinct bands, one beta- and the other prebeta-migrating (Fig. 2).

Analytical ultracentrifugation

It was previously noted that the analytical ultracentrifugation procedure used at the Donner Laboratory[33] gives a fairly reliable diagnosis of Type III, but because of its complexity it is impractical for most clinical laboratories. It was found, however, when whole plasma was raised to a density of 1.27 g/ml with KBr (a density at which all lipoproteins float fairly rapidly in the ultracentrifuge) a distinctive bimodal peak (Fig. 5) was observed in the case of both treated and untreated Type III samples. Unfortunately no simple quantitative limits could be established for the flotation rates of the peaks. Flotation rates for these peaks and those observed in samples from normal, Type II and Type IV samples are given in Table 2. These rates are uncorrected and it is conceivable that with careful density and concentration corrections some sort of quantitative criteria could be derived which would be diagnostic of Type III. However, this would again have put the method out of reach of most clinical laboratories. In any case, the bimodal peak has so far been seen only in otherwise proven cases of Type III. This pattern was obtained with samples from all untreated Type III subjects (n=5). Treated Type III cases gave variable results (5 positive, 3 negative), and all samples from normal subjects and those with other types of hyperlipoproteinemia (n=10) were negative. The method is

Table 2. Analytical ultracentrifugation of 1 ml EDTA plasma + 0.41 g KBr (final density = 1.27 g/ml) at 20°, 44,000 rpm. -S values are negative sedimentation-coefficients uncorrected for concentration effects. Major peaks are starred (*).

Sample	-S, 20°, d=1.27 g/ml			
Type III		54*	31*	23	4*
Type III		47*	33*	23	4*
Type III	76*	50*	27		4*
Type III		51*	33*		4*
Type III		44*	34*		3*
Type III		48*	33*		3*
Type II		52	35*		4*
Type IV	120*	47	28*		3*
Normal			36*	21	5*
Normal			28*		4*
Normal		52*	36		5*
Normal	93		29*	21	4*

simple, fast and available to anyone with access to an analytical ultracentrifuge.

Isoelectrofocussing

Isoelectrofocussing is a relatively new technique which is rapidly becoming widely used both as a preparative and analytical tool for separating proteins on the basis of differences in their isoelectric points. Preparative isoelectrofocussing is most commonly done in sucrose density gradients and analytical focussing in polyacrylamide gel.

It was thought that preparative focussing[87] might be an approach to isolating fairly large quantities of Type III beta-VLDL and to this end it was tried on isolated lipoprotein fractions. This approach was explored both in sucrose-water and sucrose-ethylene glycol-water solutions[88] using various concentrations, column loading procedures and focussing times.[89] All proved unsatisfactory. The chief problem was that during the final stages of focussing precipitation and subsequent flocculation occurred when any useful amounts of lipoprotein were applied to the column. Since, however, the behaviour of Type III VLDL during preparative focussing appeared to be somewhat different than that of normal or Type IV VLDL, the method was pursued on an analytical scale in polyacrylamide gels. In a gel medium precipitation was not a problem since once focussed, even if precipitation occurred, the lipoproteins remained in sharply

defined bands.

Kostner et al.[90] have reported a method for isoelectrofocusing of lipoproteins in polyacrylamide gel. Their results, however, are not comparable to those reported here since they used a prestaining method in which their stain and gels contained 33% ethylene glycol, and their gels were 5% acrylamide.

A great heterogeneity of position and density was observed in the stained lipoprotein bands of isoelectrofocussed EDTA plasma from normal and abnormal subjects (Fig. 6). On the other hand, Type III specimens produced a single, dense band in the lower region of the gel, (Fig. 7). This band has a measured pI 5.44 (range 5.38-5.48). A similar pattern was produced by specimens from six Type III patients diagnosed in this laboratory and one known Type III sample sent from Toronto by ordinary mail (from Dr. J.A. le).

With the co-operation of Dr. W. R. Hazzard (North West Lipid Research Clinic, Seattle) a blind study was done to assess the method. Plasma specimens from 20 patients were mailed from Se (at ambient temperature, arriving two to three days aft sting) in several batches, identified only by sample numbers. All of the patients were previously known to, and had been 'typed' in, that laboratory. Part of each sample was retained by Dr. Hazzard and subjected to a
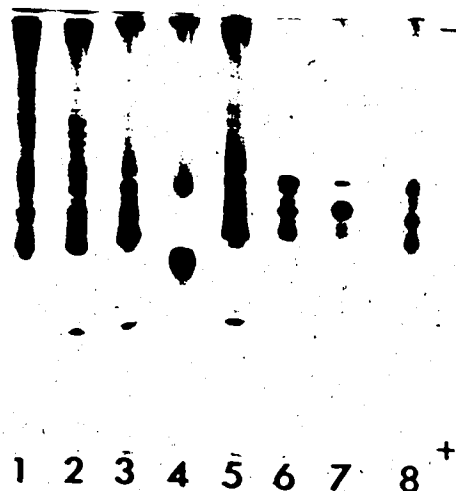
1 2 3 4 5 6 7 8

Figure 6. Analytical isoelectrofocussing (pH 3-10 Ampholine) in polyacrylamide gels, of plasma lipoproteins. 3 ul of EDTA plasma (or isolated fraction) was applied to each gel. The focussed lipoproteins were stained with Sudan Black B. (1)Type IV; (2)Type IV; (3)Type III; (4)Type III VLDL; (5)Type III + Type III VLDL; (6)Type IIa; (7)Normal; (8)Type IIb. The pH range of the gels was from about pH 4 (anode) to pH 8 (cathode), with the prominent band of the Type III specimen at pH 5.44. The gels were run simultaneously

Figure 7. Analytical isoelectrofocussing in polyacrylamide gels, of EDTA plasma from various Type III subjects. Conditions are the same as in Fig. 6.

routine analytical procedure (ultracentrifugal fractionation, cholesterol and triglyceride analysis, lipoprotein electrophoresis on agarose and polyacrylamide gel).

All samples were isoelectrofocussed in this laboratory and the interpretation sent to Dr. Hazzard. He had classified the subjects as Types IIa(n=1), IIb(n=5), III(n=7), IV(n=5) and normal(n=2). Six gave a pattern like those in Fig. 7 and were reported (correctly) as Type III by the isoelectrofocussing method. A seventh Type III was missed -- the sample produced a sharp dense band in the same region as other Type III specimens plus another fairly prominent band in the upper part of the gel. This patient was being treated with Atromid(2g/d), had a normal serum cholesterol (163 mg/100ml), moderately elevated serum triglycerides (226 mg/ml), and agarose lipoprotein electrophoresis showing much more prebeta- than beta-migrating material (the sample did, however, give a Type III pattern by the analytical ultracentrifuge method described above).

Although a great variety of patterns was seen with the isoelectrofocussing method, a remarkable similarity often occurred with samples from first degree relatives. This was noticed among members of the J.Jo. kindred where siblings were sometimes seen to be similar to one another but different from their parents; or one parent and some, but

not all, siblings would be nearly the same. In the blind study, two Type IV first-degree relatives of a Type III patient gave patterns which were reminiscent of Type III i.e., had a well defined band at the same position that of Type III. However, this was not as dense as that of Type III, and other prominent bands were seen in the upper part of the gel. More extensive studies are required but there is a strong suggestion here that analytical gel isoelectrofocussing is sensitive to genetic variations not seen by any other available lipoprotein analytical method.

This method appears to be a fairly accurate way of detecting Type III. It has the advantages of not requiring preparative ultracentrifugation or very expensive apparatus. The amount of sample used is small and does not need special handling (samples gave a reproducible pattern, with little deterioration, over a period of two week's storage at 4° and several days at room temperature). Disadvantages are that gels are fragile and not easily stored after staining. Focussed, stained bands diffused and changed colour fairly rapidly although gels could be kept about two days (for comparison with other runs) in just enough water to keep them wet. Permanent records must be in the form of photographs or possibly, with an appropriate apparatus, as densitometric scans.

APOLIPOPROTEINS OF TYPE III VLDL

It was recognized at the beginning of this work that the most valid approach to determining the presence or absence of an apoprotein variation in Type III would be to separately examine VLDL fractions _after_ they had been separated into beta- and prebeta-migrating species. Attempts to do this by starch and Pevikon block electrophoresis failed to provide larc enough quantities for detailed analyses (VLDL is only about 10% protein by _dry_ weight). Fractionation by preparative isoelectrofocussing was also unsuccessful (see Results and Discussion - Diagnostic Methods).

In a number of Type III samples examined here, the major portion of VLDL was the beta-migrating species (Fig. 2 and Table 3), so efforts to separate the two species were abandoned and apoprotein preparations were made from the ultracentrifuge-isolated total-VLDL. Of course, the final apolipoprotein preparations contained a mixture of proteins derived from both the beta- and prebeta-VLDL but the contribution of prebeta-VLDL was relatively small.

Other laboratories have reported a reasonably consistent and characteristic pattern when normal prebeta-VLDL apoproteins are run on PAGE. Greater heterogeneity has been pointed out in some reports[42 52 91] but all seem agreed that the bands shown in Fig. 8a are the major ones. One of

Table 3. Proportion of VLDL, from Type III subjects, which is beta-migrating. Calculated from densitometric scans of agarose electrophoretograms of ultracentrifuge-isolated VLDL shown in Fig. 2.

Subject	beta-VLDL [% of total VLDL]
J. O' D.	25
J. Kl.	40
E. Li.	86
T. An.	90
J. Jo.	60
H. Pi.	84
S. De.	71
J. Pa.	89
R. Pa. (off tolbutamide)	C
R. Pa. (on tolbutamide)	67

the problems encountered in studying the 'new' proteins found in this laboratory in Type III apo-VLDL[s] was that they migrated on PAGE in the same area of the gel as apoLp-Ser. This difficulty was overcome by lowering the gel concentration and changing the buffer system slightly so that apoLp-Ser did not enter the running gel in the time required to complete the electrophoresis, (Fig. 8b). The results of PAGE runs of apo-VLDL from various Type III samples are shown in Fig. 9. The identities of the bands were confirmed by slicing out the stained protein and determining amino acid composition. The group of bands, which are a major component in Type III apo-VLDL (the 'new' proteins) proved to have an amino acid compositon most similar to the arginine-rich protein (to be called apoLp-'Arg rich' hereafter) found by Shore and Shore[92] in DEAE-cellulose fractionated apo-VLDL from "hyperlipemic serum" (Fig. 9, Table 4)

When the apoproteins from Type IV or normal total VLDL (all of which was prebeta-VLDL) were fractionated on Sephadex G200 an elution profile similar to that reported by Brown et al.[95] was seen (Fig. 10) According to the convention of those workers major peaks will be called SF1 (at the void volume, Vo, containing mostly apoLp-B) and SF3 (the second major peak, containing the apo-C proteins). They also named the very small peak and plateau region between these large peaks SF2. A number of preparations from various

stacking gel

apoLp-B
apoLp-Ser

group of major
apoproteins in
Type III VLDL

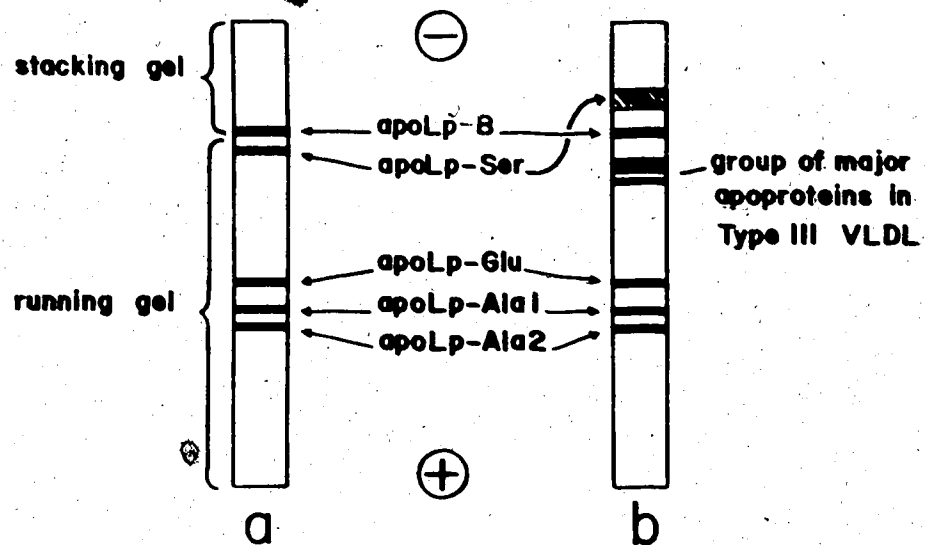apoLp-Glu
apoLp-Ala1
apoLp-Ala2

running gel

a   b

Figure 8. Behaviour of apoproteins of VLDL upon
polyacrylamide disc gel electrophoresis in urea. (a) As most
authors report them. (b) As they appear using the system
reported here. Note that apoLp-B, Glu, Ala1, and Ala2 have
the same behaviour but that in this work apoLp-Ser does not
enter the running gel.

Figure 9. Polyacrylamide gel electrophoresis in 6M urea of
the apoproteins of Type III VLDL.

Table 4. Amino acid composition of stained protein from PAGE of Type III apo-VLDL in moles/1000 moles of amino acids. Column headings (B-1, B-2, etc.), refer to numbered bands in Fig. 9. Column headings naming the protein refer to the composition as reported in or recalculated from data given in the respective references. (tr) trace, (-) not done.

Amino acid	B-1	apoLp-Ser**	B-2	apoLp-B9 3	B-3	B-4	B-5	apoLp-Arg rich9*
Asp	90	88	105	106	61	59	50	48
Thr	43	53	60	64	39	15	38	38
Ser	127	123	72	82	52	58	50	54
Glu	161	158	128	124	214	250	230	233
Pro	15	18	33	38	42	31	28	27
Gly	35	18	50	48	76	77	73	58
Ala	51	53	70	61	87	100	101	108
Val	33	35	56	61	70	79	71	68
Cys	0	0	tr	7	0	0	0	0
Met	14	18	16	17	19	17	18	24
Ile	48	53	58	61	22	19	12	13
Leu	100	105	120	118	124	119	123	109
Tyr	3	0	31	30	17	17	14	14
Phe	49	53	50	53	20	18	14	14
Trp	-	18	-	6	-	-	-	28
Lys	150	158	84	67	36	38	38	48
His	0	0	25	25	19	11	9	13
Arg	58	53	41	32	103	93	130	106

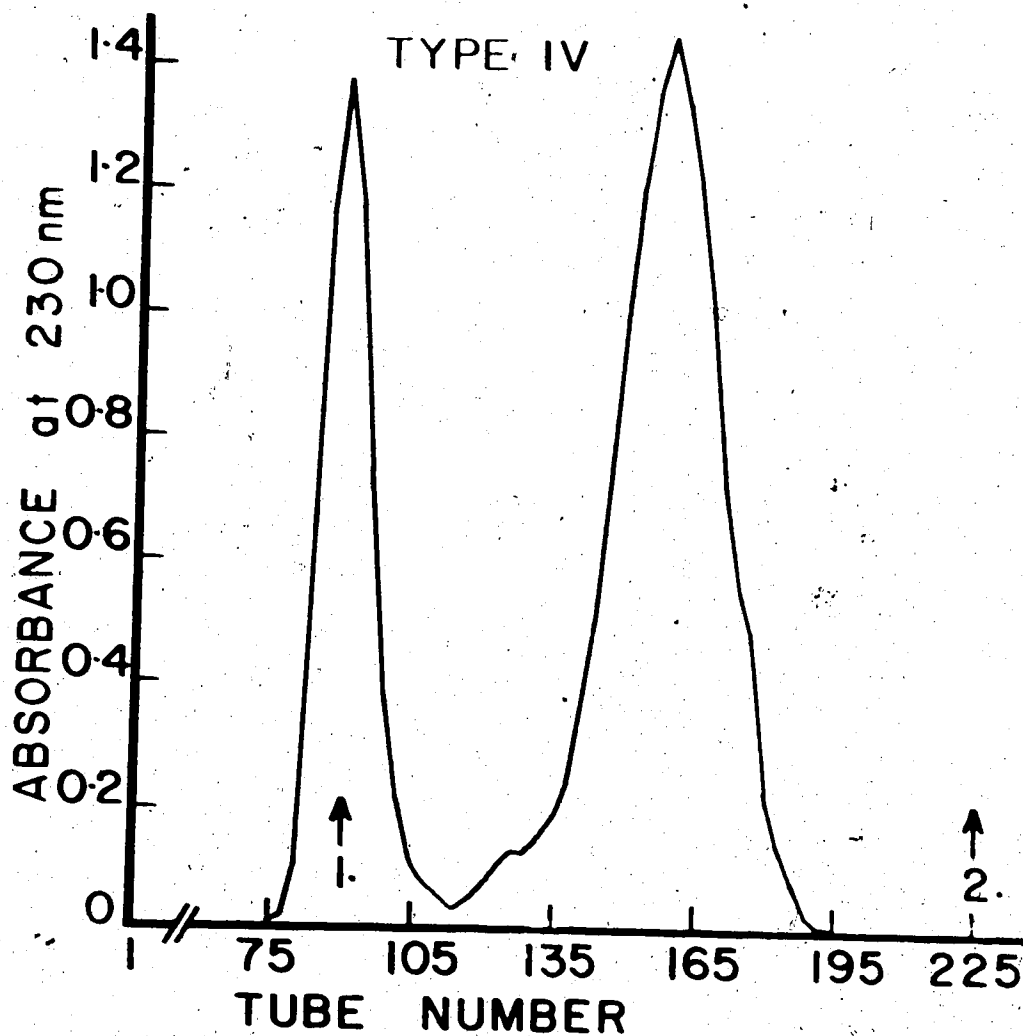|	B-6	apoLp-Glu51	B-7	B-8	apoLp-Ala45
Asp	70	67	96	91	89
Thr	103	109	63	69	63
Ser	123	122	135	127	139
Glu	174	188	132	136	127
Pro	46	50	33	28	25
Gly	27	30	47	53	38
Ala	89	93	138	130	127
Val	57	52	81	77	76
Cys	0	0	0	0	0
Met	30	25	27	23	25
Ile	9	11	0	0	0
Leu	106	107	73	67	63
Tyr	51	60	24	26	25
Phe	33	27	60	48	51
Trp	-	0	-	-	38
Lys	72	53	79	80	76
His	0	0	15	18	13
Arg	12	9	29	29	25

Figure 10. Gel filtration chromatography of Type IV VLDL apoproteins on Sephadex G200 in 0.2M TrisHCl, pH 8.2, 0.002M NaDS. 1.=Vo= elution volume of Blue Dextran 2000. 2.=Vt= elution volume of KI.

Type IV and normal samples yielded patterns which were remarkably similar to Fig. 10, with a protein ratio SF1/SF3 in the range 0.5-0.7. When, on the other hand, the apoproteins cf Type III total VLDL (which were mostly from beta-VLDL) were run, quite a different profile resulted (Fig. 11). Two striking differences were noted:

(1) the SF1/SF3 ratio in Type III samples ranged from 0.8-1.7, reflecting the increased amount of apoLp-B in beta-VLDL.[55]

(2) the presence of a 'shoulder' on the SF1 peak, the relative size of which appeared to correlate with the amount of beta-VLDL in the sample, (compare J.O'D. and T.An. in Figs. 2 and 11 and Table 3).

The runs shown in Fig. 11 were done on gel columns which had been packed at different times with varying bed volumes, hence the varying tube numbers (abscissa), however, calculated Kd* values for the peaks were very similar. When the 'shoulder' was pooled, dialyzed, lyophilized, redissolved and rerun on the column it came off as a separate peak with the same elution volume. When run on a column which had been calibrated with standards of known molecular weights the 'shoulder' gave an apparent molecular

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*Kd= (Ve-Vo)/(Vt-Vo), where Ve= elution volume of substance in question; Vo= elution volume of a completely excluded molecule; Vt= elution volume of a completely included molecule. Kd is an exponential function of the molecular radius of the eluted substance.[96]
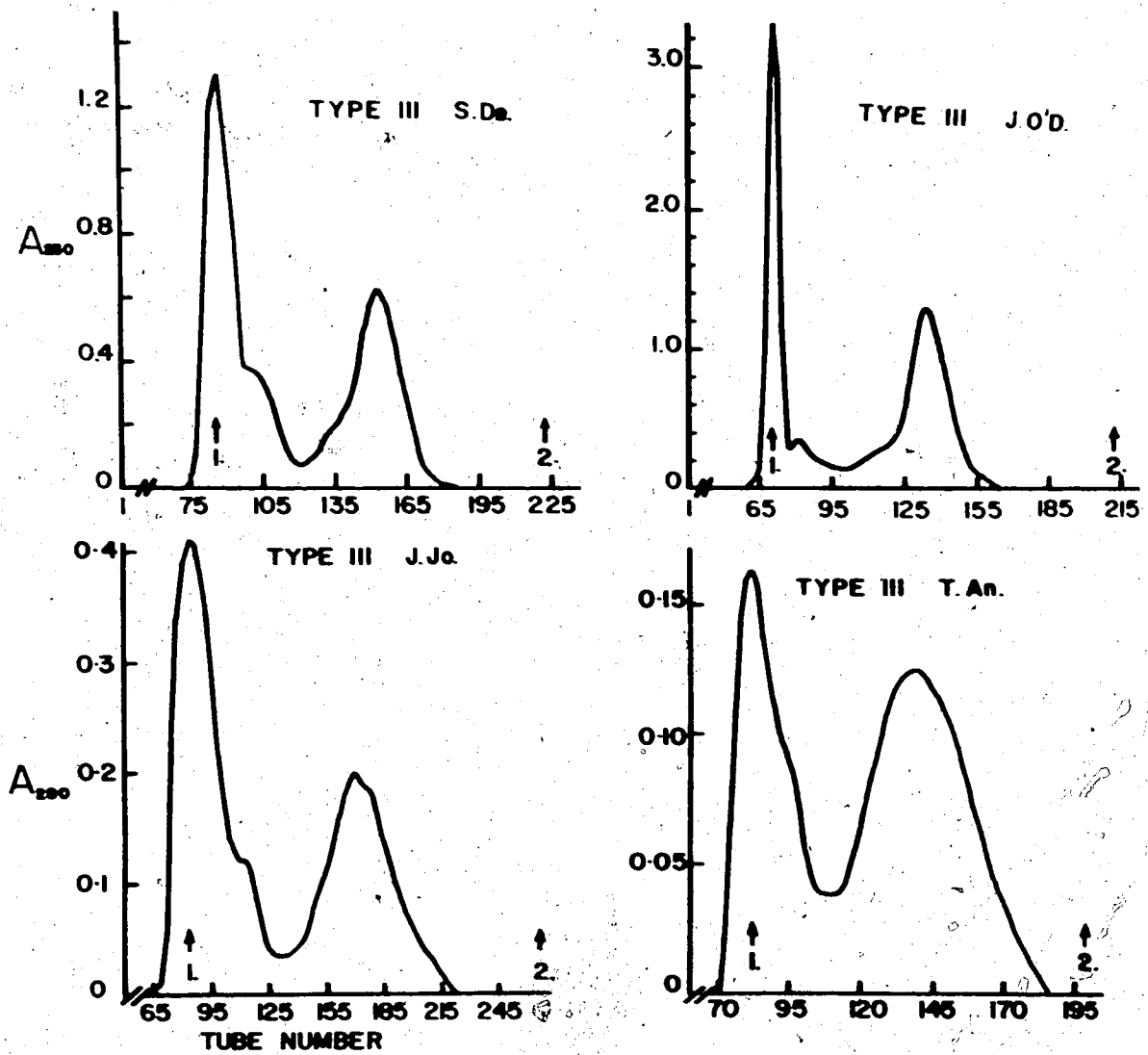
Figure 11. Gel filtration chromatography of Type III VLDL apoproteins. See Fig. 10 for conditions.
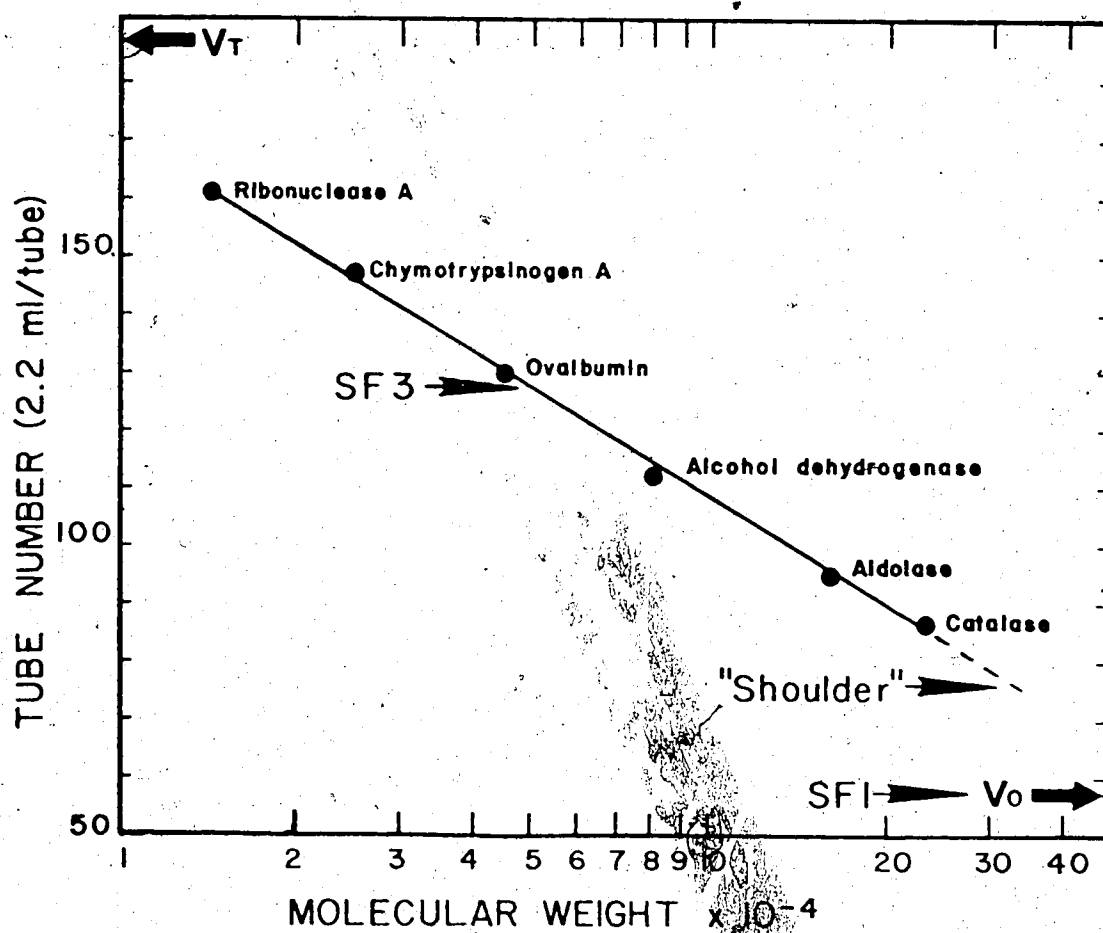
Figure 12. Elution behaviour of Type III VLDL apoproteins and molecular weight standards from Sephadex G200 in 0.2M TrisHCl, pH 8.2, 0.002M NaDS. SF1, SF3 and 'Shoulder' refer to the elution profile in Fig.13. Vt and Vo are the elution volumes of KI and Blue Dextran 2000 respectively.

weight of approximately 300,000 (Fig. 12). The SF1 peak (apoLp-B) always eluted at the void volume indicating aggregation (extensive molecular weight determinations by other methods in a number of laboratories have yielded estimates between 250,000 and 25,000[7]). The SF3 peak elution volume corresponded to an apparent molecular weight 5,000-50 . This again probably reflects aggregation since the known molecular weights (Table 1) of the apoproteins in SF3 (apoLp-Ser, apoLp-Glu, apoLp-Ala) are all ≤10,000. The NIH group have reported the SF3 peak at a greater elution volume[6, 98] (equivalent to a molecular weight 25,000-30,000) whereas previous more detailed work in this laboratory[5] gave results very similar to those reported here. The reason for this difference is not known. In any case the apparent large molecular weight of the 'shoulder' is probably due to aggregation, a phenomenon seen with all the other VLDL apolipoproteins in dilute NaDS or SDS buffers at slightly alkaline pH.

Protein recoveries as measured by absorbance at 280 nm were always about 100%.

Electrophoreses by PAGE of Type III total-VLDL apoproteins eluted from Sephadex G200 indicated that apoLp-'Arg rich' was eluted in two fractions (Figs. 13 and 14). It appeared first of all in the 'shoulder' and then again on the leading edge of SF3. There was also an electrophoretic difference between these forms of apoLp-'Arg rich' in that
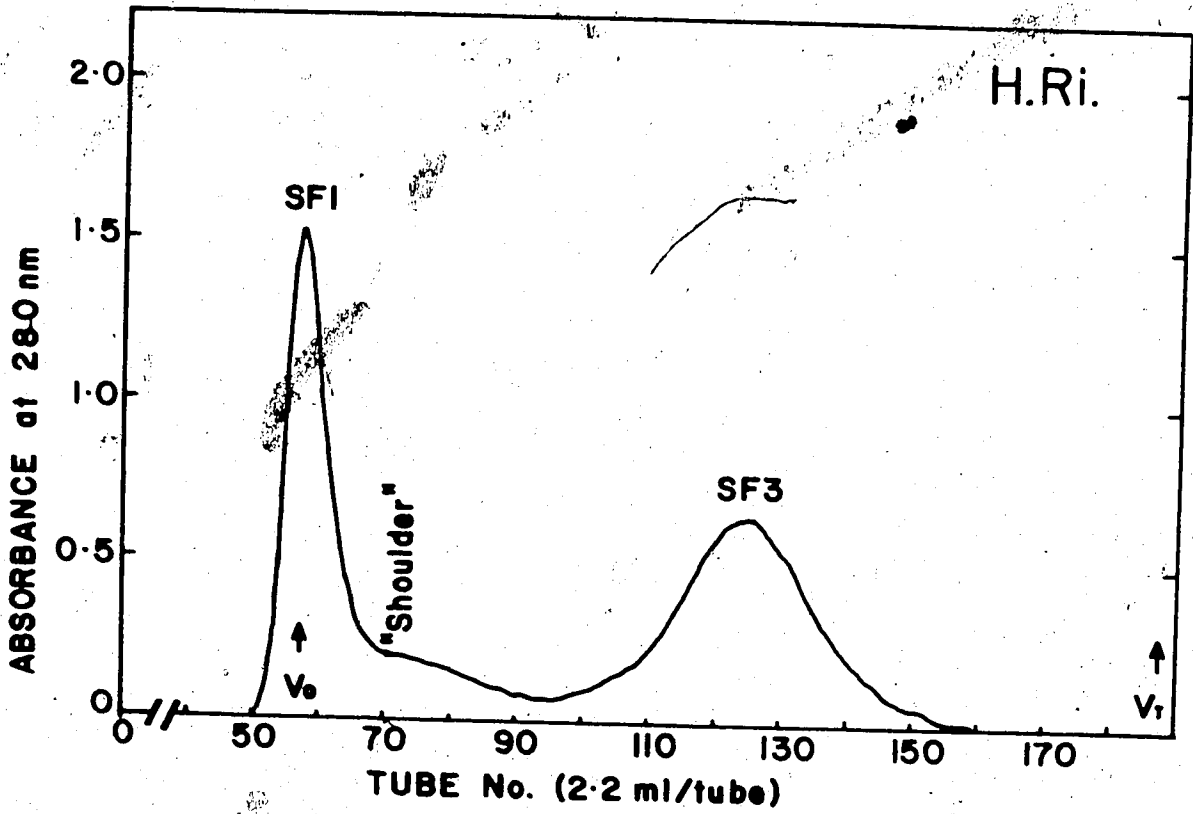
Figure 13. Gel filtration chromatography of Type III VLDL apoproteins. See Fig. 10 for conditions.
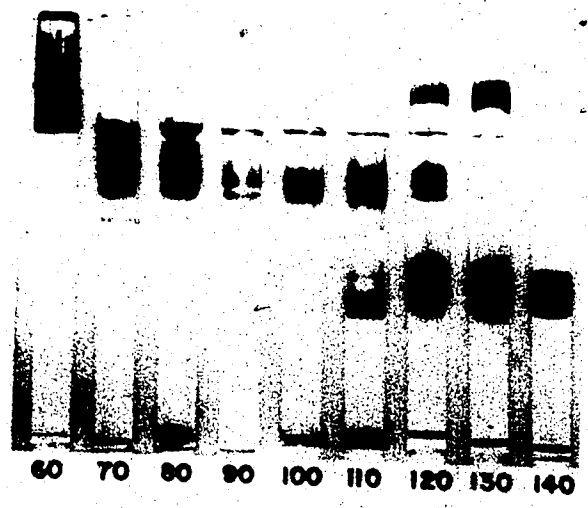

Figure 14. Polyacrylamide disc gel electrophoresis of equivolume samples from fractions eluted during Sephadex chromatography of Type III VLDL apoproteins. Numbers refer to tube numbers in Fig. 13.

the SF3 form migrated slightly faster than the major bands of the 'shoulder' form.

Prebeta-VLDL apoproteins from some Type IV samples when run by PAGE also gave fairly prominent bands in the apoLp-'Arg rich' region; but when these same samples were subjected to gel filtration all the apoLp-'Arg rich' came off in the second position (leading edge of SF3).

It seems then that apoLp-'Arg rich' is not unique to Type III VLDL but:

(1) there are increased amounts of it present in this disorder compared to normal or other types of hyperlipoproteinemic VLDL,

(2) much of this increase is accounted for by the species which have a smaller partition coefficient (i.e. larger apparent molecular weight) when isolated under the conditions outlined here.

The increased amounts of apoLp-'Arg rich' in Type III VLDL has recently been confirmed by Havel and Kane.[99] They used a technique developed in their laboratory[100] for delipidating and solubilizing the apolipoproteins directly by applying whole lipoprotein fractions in tetramethyl urea to polyacrylamide gels, and determining apoprotein concentrations by densitometry of the stained bands after electrophoresis. They found the mean ratio of apoLp-'Arg

rich'/apoLp-B in prebeta-VLDL from normolipidemic, Type IV and Type III patients was 0.25. In Type III beta-VLDL this ratio was 0.4.

The presence of apoLp-'Arg rich' in apo-VLDL from hyperlipoproteinemic subjects has also been recognized recently by Herbert et al.[101] They found that when apo-VLDL was fractionated on Sephadex G200 in Tris-NaDS buffer, a small peak eluted on the leading edge of SF3 in the region previously[95] named SF2 by that laboratory.

Further purification of the apoprotein fractions from gel filtration experiments was achieved by DEAE-cellulose chromatography in 8M urea.

An example of the fractionation of SF3 is shown in Fig. 15. The general profile is similar to that reported by others.[94] [95] In this work it was found that the relative sizes of the major peaks varied considerably from patient to patient. The fractions were identified by their migration on PAGE and/or amino acid composition. Fig. 16 shows the PAGE results from 100 ul of the peak tubes in each of the numbered fractions of Fig. 15. Fraction 1 was apoLp-Ser. Fractions 2, 3 and 4 were not investigated further. Bands with PAGE migration similar to that of 3 and 4 were not seen upon electrophoresis of the unfractionated SF3 apoproteins and it is suspected that these fractions represent either very minor components or degradation products of the major
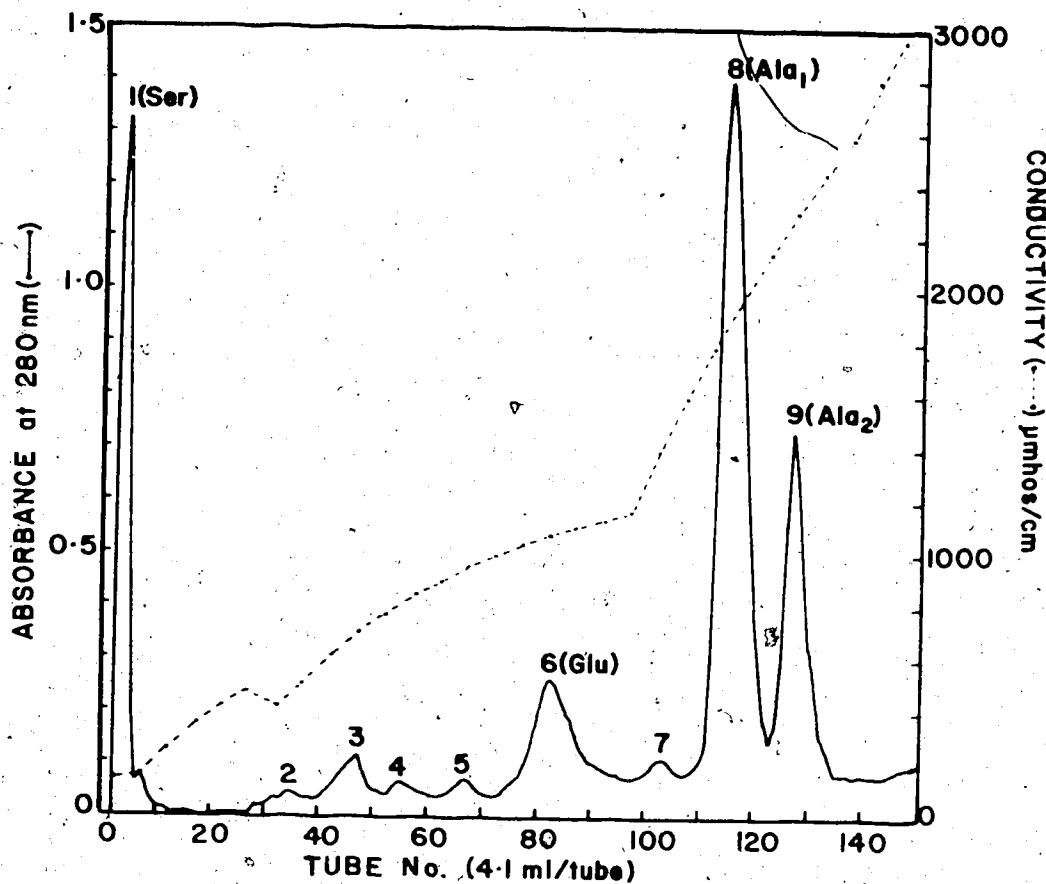
Figure 15. DEAE-cellulose chromatography in 8M urea at 4° of Type III VLDL apoproteins eluted in Sephadex G200 fraction 3 (SF3). Elution was with a TrisHCl, pH 8.2 buffer concentration gradient from 0.005M Tris to 0.4M Tris, 1M NaCl. Ser= apoLp-Ser, Glu= apoLp-Glu, Ala1= apoLp-Ala1, Ala2= apoLp-Ala2.
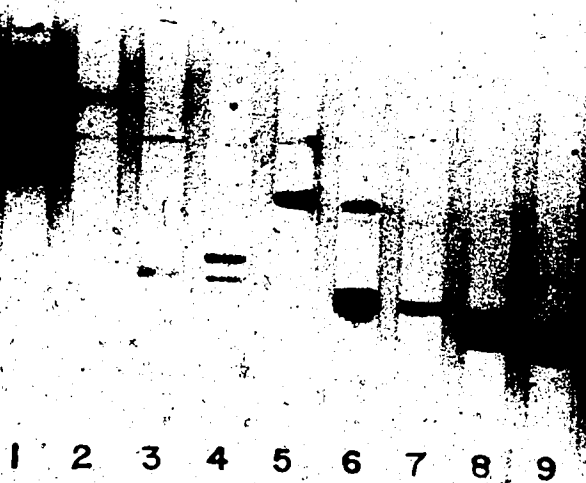

Figure 16. Polyacrylamide disc gel electrophoresis of equivolume samples from the corresponding numbered fractions in Fig. 15.

apoproteins. Fraction 3 had a much lower absorbance at 230
nm than at 280 nm and so is unlikely to be protein. By amino
acid analyses fractions 5, 6, 8 and 9 were identical to
apoLp-'Arg rich', Glu, Ala1 and Ala2 respectively. Fraction
7 was not characterized but is probably identical to the C-X
fraction of Herbert et al.[101] which they found consistently
but in varying amounts and with an amino acid composition
varying between those of apoLp-Glu and apoLp-Ala. Recoveries
from DEAE-cellulose chromatography of SF3 preparations were
55-75% as measured by absorbance at 280 nm.

After further purification of the 'shoulder' (Fig.13)
by recycling through Sephadex G200, this fraction
(containing about 30% apoLp-B) was chromatographed on DEAE-
cellulose. Recoveries of this fraction were so low (20-30%)
that in order to obtain adequate amounts of purified protein
for subsequent experiments it was necessary to pool the
'shoulder' fractions from a number of Type III patients. The
elution profile is shown in Fig. 17. Equal volumes from each
of the lettered fractions were run on PAGE (Fig. 18).
Fraction A had an amino acid composition similar to but with
some significant differences from that of apoLp-B. This is
of interest because it had been previously found that under
the same conditions almost no protein was eluted when apoLp-
B isolated from LDL was applied to the column. Fraction B
had a much lower absorbance at 230 nm than 280 nm and is
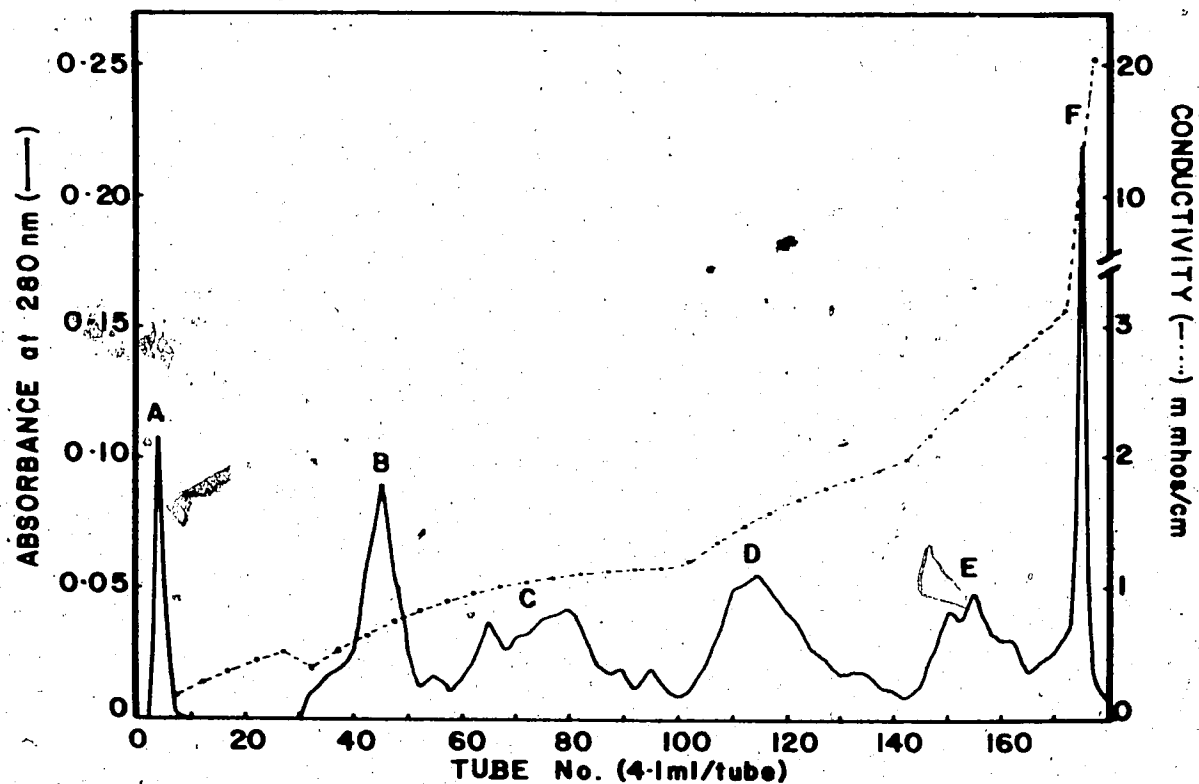probably comparable to fraction 3 from DEAE-cellulose

Figure 17. DEAE-cellulose chromatography in 8M urea at 4° of Type III VLDL apoproteins eluted from Sephadex G200 as a 'shoulder' on the first fraction (SF1). Elution buffers were identical to those used in Fig. 15.
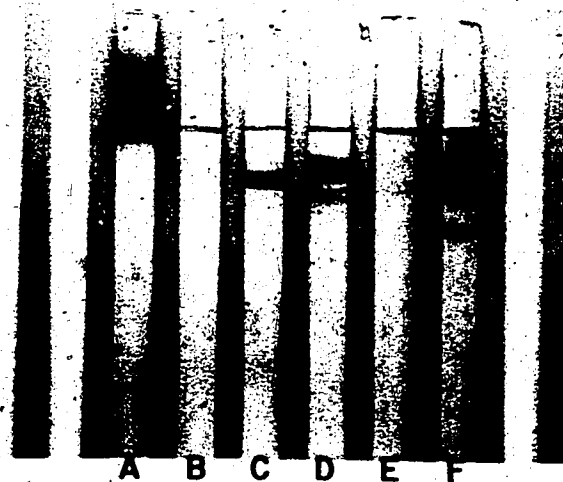

Figure 18. Polyacrylamide disc gel electrophoresis of equivolume samples from the corresponding lettered fractions in Fig. 17.

chromatography of SP3 (Fig.15). This material was not retained by the dialysis tubing (molecular weight cut-off 3500). Fraction E was similar to B and probably not protein. Fraction F, eluted by 1M NaCl, was not further investigated. Fractions C and D were identified as apoLp-'Arg rich' by amino acid analysis. On PAGE fraction C produced one major band with a very faint minor band above and below it. Fraction D was more heterogeneous with one major component and four or five minor ones.

When a preparation of the 'shoulder' from one Type III donor was run alone on DEAE-cellulose an elution profile very similar to Fig. 17 resulted except that the sharp peak on the leading edge of fraction C was more pronounced. When varying amounts up to 75 ug of protein from this single sharp peak were electrophoresed on PAGE only a single band with no minor components was observed. The protein from this sharp peak was used for antiserum preparation.

The elution position of D is of interest in that it is in the same region as apoLp-Ala1 from SP3 (Fig. 15). This may account for anomalous amino acid compositions reported for apoLp-Ala by Shore and Shore,[9] who chromatographed apo-VLDL directly on DEAE-cellulose without prior fractionation by gel filtration.

Polyacrylamide gel electrophoresis in SDS (PAGE-SDS) of both fraction C and D yielded a molecular weight about

35,000 (Fig. 19). When fractions C and D were run on PAGF-SDS in the presence of a reducing agent (2-mercaptoethanol) they each migrated as a single band. When the reducing agent was omitted, fraction C had the same behaviour but fraction D produced a number of larger molecular weight species (Fig.20). The apparent faster migration of C with no reducing agent is not significant since migration of molecular weight standards under the same conditions was increased proportionately.

The most attractive explanation for the behaviour of fraction D would seem to be that during isolation (or perhaps naturally) the molecule has had sulphydryl groups partially oxidized (resulting in an altered affinity for DEAE-cellulose) and that during PAGE-SDS experiments in the absence of reducing agent there is formation of disulphide linkages, resulting in increased molecular weight species. However, amino acid determination after performic acid oxidation[102] yielded no cysteic acid. This may not be definitive here since the amounts of protein available were such that amino acid analyses were done on samples which approached the limits of detection of the analyzer (<2 nmoles). Earlier amino acid determinations where as much as 20 nmoles of protein was applied to the column after hydrolysis without performic acid oxidation showed no trace of cystine. Shore and Shore[92] [94] have also reported the absence of cysteic acid after performic acid oxidation and
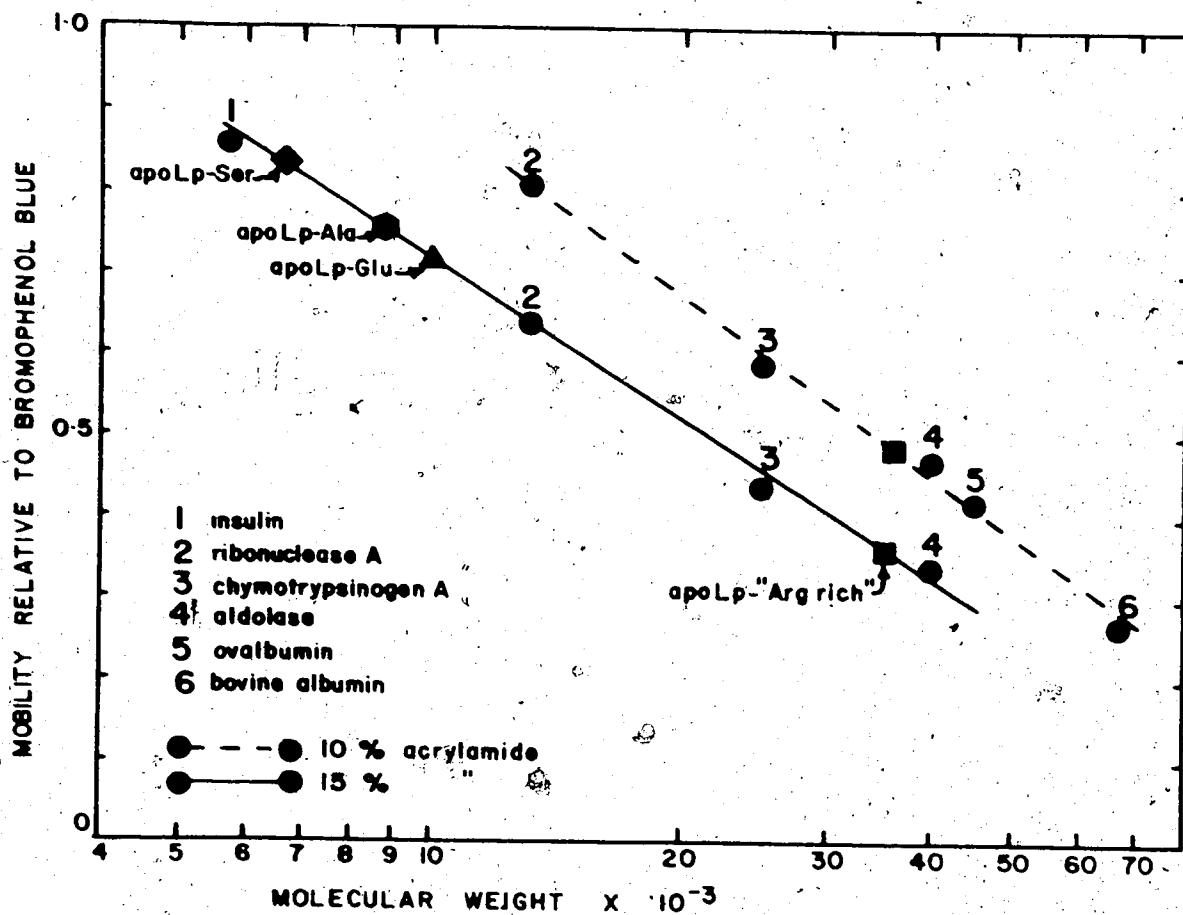
Figure 19. Polyacr[illegible] disc gel electrophoresis in sodium dodecyl sulphate o[illegible]II VLDL apoproteins and molecular weight standards.
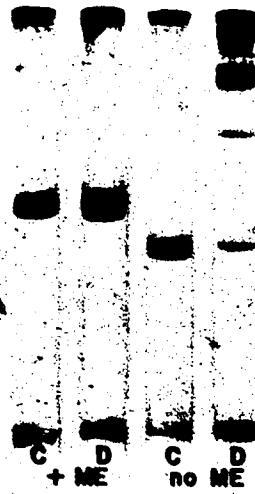
Figure 20. Polyacrylamide disc gel electrophoresis in sodium dodecyl sulphate of apoLp-'Arg rich' fractions C and D in Fig. 17. ME= 2-mercaptoethanol, 5 ul added with sample.

subsequent hydrolysis of apoLp-'Arg rich'.

Numerous attempts to determine an amino terminal using samples from both fractions C and D were unsuccessful although ortho-dansyl-tyrosine and epsilon-dansyl-lysine were produced in readily detectable amounts. By the same method samples of apoLp-Ala and apoLp-Glu yielded their appropriate N-terminals.

Carboxypeptidase A digests of fractions C and D yielded no carboxy terminal although preliminary experiments using apoLp-Ala as a substrate yielded large amounts of alanine and valine (the C-terminal sequence of apoLp-Ala is -Val-Ala-Ala-COOH[5]). Shelburne and Quarfordt have recently reported[103] the isolation of an apoprotein of VLDL by Sepharose 6B chromatography in 6M guanidine HCl. They found it was homogeneous by DEAE-cellulose chromatography, had a molecular weight 33,000 by PAGE-SDS, was insoluble in aqueous buffers, had a high arginine content and N-terminal glycine by dansylation, C-terminal alanine by carboxypeptidase digestion. It is difficult to assess their work as it was published in abstract form only, but it seems likely that the apolipoprotein they report is apoLp-'Arg rich'. If it is, there are several interesting differences between their report, the findings of Shore and Shore,[92] [94] and those reported here.

The studies reported here and those of Shore and Shore

indicated a considerable heterogeneity in apoLp-'Arg rich' both on DEAE-cellulose chromatography and PAGE in urea solution. Shore and Shore[94] report excellent solubility in aqueous buffers and here apoLp-'Arg rich' was found to be easily soluble in N-ethylmorpholine acetate and TrisHCl buffers without detergent. Shore and Shore[92 94] have not reported any results of N- or C- terminal determination. It is possible that the inability to find an N-terminal here was due to alpha-amino carbamylation. Shelburne and Quarfordt worked in 6M guanidine HCl rather than urea solutions. However, the greatest possible care was taken in these studies to avoid carbamylation by using freshly deionized urea, working at 4° and promptly removing urea by dialysis after fractionation. Since only enough purified protein was available to do one set of experiments with carboxypeptidase A it was not possible to pursue the C-terminal with incubations at varying pH or with other carboxypeptidases.

The absorption spectra of fractions C and D were very similar with maxima at 280 nm and minima at 254 nm. The calculated extinction coefficient for a 1% solution at 280 nm was 10.3.

Analytical isoelectrofocussing of all apoLp-'Arg rich' fractions yielded nearly identical patterns with a single major fraction and some very minor heterogeneity.

Antibodies raised against the purest form of apoLp-'Arg rich' isolated gave a reaction of identity with all other preparations of this protein (Fig. 21).
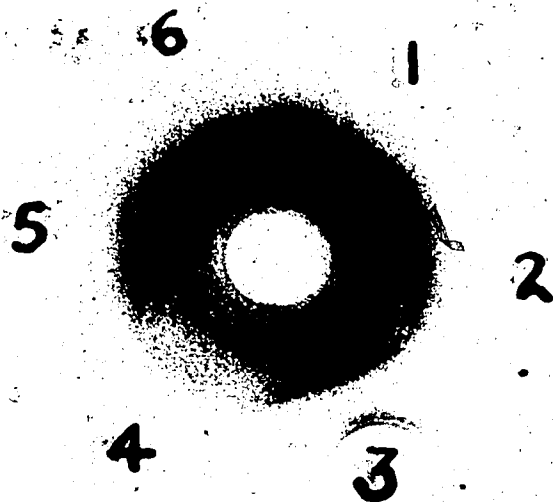
Figure 21. Ouchterlony immunodiffusion of rabbit antibodies
to apoLp-'Arg rich'. Centre well contains antiserum.
(1)fraction 5, Fig. 15; (2)fraction D, Fig. 17;
(3)fraction C, Fig. 17; (4)original antigen used to raise
the antiserum; (5)same as well (2). (6)equivalent to
fraction C, Fig. 17, but isolated from a single patient;
The innermost precipitin band is nonspecific and was also
produced when this antiserum was run against other
apolipoproteins. The faintness of the outer band from well 4
(original antigen) is due to there being an inadequate
amount of antigen available.

GENERAL DISCUSSION

Any attempt to explain the occurrence or fate of beta-VLDL in Type III must consider what is known of normal metabolism of human plasma lipoproteins. Although major advances in our knowledge of such metabolism have been made in recent years the details are still speculative. Metabolic studies are hampered by the fact that lipoproteins, as they are seen after isolation, are the result of complex enzymatic and spontaneous reactions and lengthy separation procedures; thus they are likely to differ significantly from the original secretory or metabolic product.

Chylomicrons and prebeta-VLDL are together responsible for the majority of the body's triglyceride transport. Although originating in different organs in response to different stimuli their secretion mechanisms are similar.[104] Chylomicrons are assembled in the intestinal mucosa absorptive cell in response to dietary fat. ApoLp-B, the 'carrier' protein, is newly manufactured in the same cell along with cholesterol esters which act as a core for the 'nascent' chylomicron. The apoA and apoC proteins may be donated by HDL or VHDL which can enter the extracellular space by leakage from the plasma. Some cholesterol may also be acquired in this fashion.[105] In any case the lymph chylomicrons contain a little of each of the well-recognized apolipoproteins shown in Table 1.[106] ApoC proteins have also been shown to transfer from HDL to chylomicrons in _in vitro_

studies and during alimentary lipemia in normal subjects.[107] After (or while?) the chylomicrons are catabolized in the plasma compartment (by the concerted action of lecithin:cholesterol acyltransferase and lipoprotein lipase[108] [109]) the apoA and apoC peptides are transferred back to HDL probably to be reutilized.[107] The result is a chylomicron 'remnant' relatively rich in cholesterol ester and apoLp-B, which is scavenged by the liver,[110] the cholesterol perhaps going into a pool destined for recirculation in other lipoproteins.[111]

Prebeta-VLDL are secreted by the liver in response to endogenous lipid, also using newly made apoLp-B and probably, to some extent at least, recycled apoA and apoC proteins.[104] The circulating VLDL are catabolized in a fashion similar to chylomicrons with a resulting shift of apoC proteins to HDL.[112] During catabolism VLDL loses most of its triglyceride and apoproteins other than apoLp-B, and becomes relatively rich in cholesterol ester.[113] [114] At the same time it passes through an 'intermediate' density range (1.006-1.019 g/ml, Sf 12-20) and becomes beta-LDL (1.019-1.063 g/ml, Sf 0-12). LDL is scavenged by the liver and the cholesterol may enter a catabolic pool for biliary excretion.[111] Prebeta-VLDL and beta-LDL then bear a precursor-product relationship.[115]

Beta migrating material with a triglyceride/cholesterol ratio = 1 has been reported after starch block

electrophoresis of a VLDL fraction (Sf 20-30) isolated from subjects with endogenous lipemia (Type IV).[116] Fisher has described an Sf 20 particle, with beta-migration, among the lipoproteins of Type IV subjects.[117] Tracer studies with [14C]free fatty acid have indicated a precursor - product relationship of prebeta-VLDL and beta-VLDL in Type III.[118] These results have led some workers to suggest that Type III beta-VLDL is identical to the normal 'intermediate' VLDL catabolic product, and that the Type III disorder is the result of a "deficiency in the second step of VLDL remnant formation i.e. conversion of an intermediate density lipoprotein fraction to LDL".[119] The discovery of greatly increased amounts of apoLp-'Arg rich' in VLDL from Type III patients requires some modification of this idea. Type III beta-VLDL has been found in all density ranges of VLDL, not just the lower Sf fractions. Indeed, one of the best apoLp-'Arg rich' sources in this study was S.De. who by analytical ultracentrifugation proved to have a large proportion of his VLDL in the upper Sf range. Although apoLp-'Arg rich' is found in normal and hyperlipoproteinemic plasma other than Type III, in none of the density ranges from these is it present in the striking amounts found in Type III.*

What is the origin and function of apoLp-'Arg rich'? An

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*R. J. Havel, University of California School of Medicine, personal communication.

enrichment of ▓▓▓▓ 'remnants' through loss of other apoproteins see▓ likely since this would require not only a loss of the apoC proteins but also of apoLp-B, and as emphasized previously there is an _increased_ amount of apoLp-B in Type III VLDL. There appears to be good evidence for a precursor - product relationship between prebeta- and beta-VLDL in Type III (in J.Ra. 'this was especially striking in that during treatment and at times when his strict diet was relaxed the prebeta levels were the most labile, with beta-VLDL levels following). If excess apoLp-'Arg rich' is acquired from some site in the circulation at a critical metabolic step (uptake of cholesterol ester by the liver for example, or removal of other lipid components during triglyceride hydrolysis) then the possibility exists of a 'mutant' form of the protein in Type III. Thus a defect in the conversion of prebeta-VLDL to beta-LDL may give rise to an intermediate particle with unusual avidity for apoLp-'Arg rich'; or a defective form of this protein may be responsible for such a block.

Although fasting chylomicronemia was said not to be a feature of Type III in the original definition[*] it has been described in several cases since and was certainly present in a number of the patients reported here. The finding that these chylomicrons were cholesterol rich[20] and contain excess apoLp-'Arg rich'[99] suggests either that these substances were acquired by exchange during prolonged

circulatory contact with beta-VLDL or that VLDL and chylomicrons share a common catabolic step involving apoLp-'Arg rich'. (A) common triglyceride removal pathway has been reported.

At least one step in plasma lipoprotein degradation is mediated by tissue lipoprotein lipase. Following intravenous administration of heparin an increased lipolytic activity (PHLA) is observed in normal plasma. Decreased PHLA and subsequent hypertriglyceridemia is seen during insulin withdrawal in diabetics.[121] Although low PHLA levels are not a common characterisitic in Type III it has been suggested that this could account for the transient appearance of beta-VLDL during diabetic ketoacidosis.[1] This, of course, cannot explain the disappearance of beta-VLDL in R.Ra. during tolbutamide withdrawal. It may be that at least one insulin mediated step is required to produce a particle capable of being metabolized further e.g. to beta-VLDL. Alternatively, there may be more than one possible lipoprotein catabolic product with beta electrophoretic mobility and density <1.006 g/ml. Beta migration could be a function of either a predominance of apoLp-B or of apoLp-'Arg rich', and a particle might be triglyceride rich (the main determinant of density class) for a number of reasons. Thus patients diagnosed as Type III could have different basic diseases producing different 'kinds' of beta-VLDL.

CONCLUSIONS

The appearance of Type III in the J.Jo. kindred is most compatible with, but not proof of, a recessive mode of inheritance of a single gene defect. The pattern in the J.Fa. kindred fits either a dominant or polygenic inheritance. A possible explanation for the early appearance of Type III in J.Ra. is that he also inherited Type II. If catabolism of VLDL in Type III is:

prebeta-VLDL --1--> beta-VLDL --2--> beta-LDL --3--> ?

then a defect at step 2 (Type III) could feasibly be exacerbated by a simultaneous defect at step 3 (Type II). The unusual response to tolbutamide in R.a. could also be explained in terms of this scheme if during insulin withdrawal the rate-limiting reaction is step 1 (mediated by lipoprotein lipase) but during insulin sufficiency step 2 is rate-limiting.

The Type III subjects reported here present a number of anomalies:

the relative good health, lack of xanthomas and normal weight of S.De. in the face of consistently very elevated plasma lipids as well as his complete lack of response to treatment.

the appearance and disappearance of beta-VLDL in unison with good or poor diabetic control in R.Ra., in the absence of other symptoms characteristic of Type III, e.g.

xanthomas.

the peculiar 'fast' beta-VLDL remarked upon in the most recently discovered case of Type III (footnote, Introduction - Type III Genetics). The above findings are in contrast to the classic appearance of the disorder in T.An., J.Jo., H.Fi., E.Li. and J.Ra. (except, of course, for his young age). In all of these there was xanthomatosis precipitated or aggravated by weight gain, good or even excellent response to treatment and a persistence of true beta-VLDL. This would seem to indicate either that there is more than one 'type' of Type III i.e., multiple causes for beta-VLDL, or that a basic common defect can be enormously modified by other, as yet undefined, physiological factors.

The analytical ultracentrifuge diagnostic procedure presented here is unlikely to be as reliable as the much more exhaustive method devised at the Donner Laboratory.[33] However, in cases where Type III is suspected on clinical grounds or in conjunction with a 'broad-beta' electrophoretic pattern then a Type III pattern on this simple and rapid technique probably allows a presumptive diagnosis. Negative results, however, would not permit ruling out the disorder.

Analytical isoelectrofocussing of plasma lipoproteins proved to be a fairly reliable way of diagnosing Type III. It has at least two major advantages over preparative ultracentrifugation - electrophoresis: (1) it requires very

little sample and no special handling of specimens; (2) the apparatus and skills are more available to clinical laboratories. A remarkable similarity was observed in the focussed lipoprotein patterns of first degree relatives and although still under development, the method offers promise as a research tool for examining genetic variations of lipoproteins.

The new apolipoprotein, apoLp-'Arg rich', described and partially characterized here, has previously been unrecognized or ignored by most workers. It is present in small amounts in normal VLDL but in Type III its concentration is greatly increased. Is the excess apoLp-'Arg rich' merely acquired through non-specific exchange during prolonged circulation of VLDL, or does it actually play a causative role in the development of Type III i.e., does the production of an abnormal form of apoLp-'Arg rich' (as suggested by this work) result in a lipoprotein which is metabolized with difficulty?

The answers must await further developments in the complete characterization of this apolipoprotein and probably in more specific techniques for labelling and tracing the metabolism of apolipoproteins in general. However, the discovery of apoLp-'Arg rich' opens many new lines of attack for the study of lipoprotein metabolism in health and disease and in the search for a basic cause of Type III hyperlipoproteinemia.

REFERENCES

1. Skipski VP: Lipid composition of lipoproteins in normal and diseased states, Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism. Edited by GJ Nelson. New York, Wiley-Interscience, 1972, pp 471-583

2. Havel RJ, Eder HA, Bragdon JH: The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J Clin Invest 34:1345-1353, 1955

3. Gofman JW, Lindgren F, Elliott H, Mantz W, Hewitt J, Strisower B, Herring V: The role of lipids and lipoproteins in atherosclerosis. Science 111:166-171, 1950

4. Fredrickson DS, Levy RI, Lees RS: Fat transport in lipoproteins - an integrated approach to mechanisms and disorders. N Engl J Med 276:34-44, 94-103, 148-156, 215-225, 273-281, 1967

5. Beaumont J, Carlson LA, Cooper GR, Pejfar Z, Fredrickson DS, Strasser T: Classification of hyperlipidaemias and hyperlipoproteinaemias. Bull WHO 43:891-908, 1970

6. Berg RH: Watch out for triglyceride, there's more than cholesterol behind heart attacks. Look 35(3):51-54, 1971

7. Fredrickson DS, Levy RI: Familial hyperlipoproteinemia, The Metabolic Basis of Inherited Disease. Edited by JB Stanbury, JB Wyngaarden, DS Fredrickson. New York, McGraw-Hill Incorporated, 1972, pp 545-614

8. Schreibman PH, Arons DL, Saudek CD, Arky RA: Abnormal lipoprotein lipase in familial exogenous hypertriglyceridemia. J Clin Invest 52:2075-2082, 1973

9. Brown HB, Lewis LA, Page IH: Mixed hyperlipemia, a sixth type of hyperlipoproteinemia. Atherosclerosis 17:181-196, 1973

10. Rose HG, Kranz P, Weinstock M, Juliano J, Haft JI: Inheritance of combined hyperlipoproteinemia: evidence for a new lipoprotein phenotype. Am J Med 54:148-160, 1973

11. Stern MP, Kolterman OG, McDevitt H, Reaven GM: Acquired type 3 hyperlipoproteinemia. Arch Intern Med 130:817-821, 1972

12. Hazzard WR, Bierman EL: Aggravation of broad-beta disease (type 3 hyperlipoproteinemia) by hypothyroidism. Arch Intern Med 130:822-828, 1972

13. Brunzell JD, Hazzard WR, Porte D, Bierman EL: Evidence for a common saturable triglyceride removal mechanism for chylomicrons and VLDL. J Clin Invest 52:1578-1585, 1973

14. Gofman JW, deLalla O, Glazier F, Freeman NK, Lindgren FT, Nichols AV, Strisower EH, Tamplin AR: The serum lipoprotein transport system in health, metabolic disorders, atherosclerosis and coronary artery disease. Plasma 2:413-484, 1954

15. Borrie P: Essential hyperlipaemia and idiopathic hypercholesterolaemic xanthomatosis. Br Med J 2:911-915, 1957

16. Borrie P: Type III hyperlipoproteinemia. Br Med J 2:665-667, 1969

17. Slack J, Nevin NC: Hyperlipidaemic xanthomatosis. I. Increased risk of death from ischaemi heart disease in first degree relatives of 53 patients with essential hyperlipidaemia and xanthomatosis. J Med Genet 5:4-8, 1968

18. Nevin NC, Slack J: Hyperlipidaemic xanthomatosis. II. Mode of inheritance in 55 families with essential hyperlipidaemia and xanthomatosis. J Med Genet 5:9-28, 1968

19. Matthews RJ: Type III and IV familial hyperlipoproteinemia: evidence that these two syndromes are different phenotypic expressions of the same mutant gene(s). Am J Med 44:188-199, 1968

20. Lees RS, Wilson DE, Schonfeld G, Fleet S: The familial dyslipoproteinemias. Prog Med Genet 9:237-290, 1973

21. Lasser NL, Katz S: The occurrence of type II and type III hyperlipoproteinemia in a single kindred. Clin Res 20:549Abs, 1972

22. Hazzard WR, Goldstein JL, Schrott HG, Motulsky AG, Bierman EL: Hyperlipidemia in coronary heart disease. III. Evaluation of lipoprotein phenotypes of 156 genetically defined survivors of myocardial infarction. J Clin Invest 52:1569-1577, 1973

23. Strunge P, Trostmann AF: The lipoprotein pattern in a Danish family. Acta Med Scand 189:73-78, 1971

24. Eisenberg S: Type III hyperlipoproteinemia. Clin Endocrinol Metab 2:111-125, 1973

25. Godolphin WJ, Conradi G, Campbell DJ: Type III hyperlipoproteinaemia in a child. Lancet 1:209-210, 1972

26. Levy RI, Langer T: Hypolipidemic drugs and hyperlipoproteinemia. Ann NY Acad Sci 179:475-480, 1971

27. Strisower EH, Adamson G, Strisower B: Treatment of hyperlipidemic states. Med Clin North Am 54:1599-1613, 1970

28. Zelis R, Mason DT, Braunwald E, Levy RI: Effects of hyperlipoproteinemias and their treatment on the peripheral circulation. J Clin Invest 49:1007-1015, 1970

29. Fredrickson DS: Mutants, hyperlipoproteinaemia, and coronary artery disease. Br Med J 2:187-192, 1971

30. Hazzard WR, Porte D, Bierman EL: Abnormal lipid composition of very low density lipoproteins in diagnosis of broad-beta disease (type III hyperlipoproteinemia). Metabolism 21:1009-1019, 1972

31. Burstein M, Scholnick HR, Morfin R: Rapid method for the isolation of lipoproteins from human serum by precipitation with polyanions. J Lipid Res 11:583-595, 1970

32. Wybenga DR, Ibbott FA, Winkelman JW: The indirect confirmation of hyperlipoproteinemia phenotypes II, III, IV. Clin Chim Acta 40:121-127, 1972

33. Ewing AM, Freeman NK, Lindgren FT: The analysis of human serum lipoprotein distributions. Adv Lipid Res 3:25-61, 1965

34. Fredrickson DS, Levy RI, Lindgren FT: A comparison of heritable abnormal lipoprotein patterns as defined by two different techniques. J Clin Invest 47:2446-2457, 1968

35. Wood PDS, Stern MP, Silvers A, Reaven GM, von der Groeben J: Prevalence of plasma lipoprotein abnormalities in a free living population of the Central Valley, California. Circulation 45:114-126, 1972

36. Aubry F, Lapierre Y, Noel C, Davignon J: Ultracentrifugal demonstration of floating beta

lipoproteins in type III hyperlipoproteinemia. Ann Intern Med 75:231-237, 1971

37. Masket BH, Lev ... Fredrickson DS: The use of polyacrylamide gel ... is in differentiating type III hyperlipoproteinemia. J Lab Clin Med 81:794-802, 1973

38. Naito HK, Wada M, Ehnhart LA, Lewis LA: Polyacrylamide-gel disc-electrophoresis as a screening procedure for serum lipoprotein phenotyping. Clin Chem 19:228-234, 1973

39. Seidel D, Greten H: Hyperlipoproteinamie - typ III eine moglichkeit zur immunologischen diagnostik. Clin Chim Acta 30:31-36, 1970

40. Seidel D, Alaupovic P: An abnormal low-density lipoprotein in obstructive jaundice. Ger Med Month 15:665-670, 671-675, 1970

41. Wieland H, Seidel D: Improved techniques for assessment of serum lipoprotein patterns. II. Rapid method for diagnosis of type III hyperlipoproteinemia without ultracentrifugation. Clin Chem 19:1139-1141, 1973

42. Fredrickson DS, Lux SE, Herbert PN: The apolipo-proteins. Adv Exp Med Biol 26:25-56, 1971

43. Brewer HB Jr, Lux SE, Ronan R, John KM: Amino acid sequence of human apo Lp-Gln-II (apo A-II), an apolipoprotein isolated from the high-density lipoprotein complex. Proc Natl Acad Sci USA 69:1304-1308, 1972

44. Shulman R, Herbert P, Wehrly K, Chesebro B, Levy RI, Fredrickson DS: The complete amino acid sequence of apo Lp-ser: an apolipoprotein obtained from human very low density lipoprotein. Circulation 46:Suppl II:246, 1972

45. Brewer HB Jr, Shulman R, Herbert P, Ronan R, Wehrly K: The complete amino acid sequence of an apolipoprotein obtained from human very low density lipoprotein (VLDL). Adv Exp Med Biol 26:280, 1971

46. Salem L: The role of long-range forces in the cohesion of lipoproteins. Can J Biochem Physiol 40:1287-1298, 1962

47. Fielding CJ, Shore VG, Fielding PE: A protein cofactor of lecithin:cholesterol acyltransferase. Biochem Biophys Res Commun 46:1493-1498, 1972

48. LaRosa JC, Levy RI, Herbert P, Lux SE, Fredrickson DS: A specific apoprotein activator for lipoprotein lipase. Biochem Biophys Res Commun 41:57-62, 1970

49. Kane JP, Richards EG, Havel RJ: Subunit heterogeneity in human serum beta lipoprotein. Proc Natl Acad Sci USA 66:1075-1082, 1970

50. Shore VG, Shore B: The apolipoproteins: their structure and functional roles in human-serum lipoproteins, Blood Lipids and Lipoproteins: Quantitation, Compostion, and Metabolism. Edited by GJ Nelson. New York, Wiley-Interscience, 1972, pp 789-824

51. Brown WV, Levy RI, Fredrickson DS: Further characterization of apolipoproteins from the human plasma very low density lipoproteins. J Biol Chem 245:6588-6594, 1970

52. Albers JJ, Scanu AM: Isoelectric fractionation and characterization of polypeptides from human serum very low density lipoproteins. Biochim Biophys Acta 236:29-37, 1971

53. Gotto AM, Brown V, Levy RI, Birnbaumer ME, Fredrickson DS: Evidence for the identity of the major apoprotein in low density and very low density lipoproteins in normal subjects and patients with familial hyperlipoproteinemia. J Clin Invest 51:1486-1494, 1972

54. Baynton RD: A biochemical and genetic study of type III and type IV hyperlipoproteinemia. Ph.D. thesis, University of Alberta, 1972

55. Quarfordt S, Levy RI, Fredrickson DS: On the lipoprotein abnormality in type III hyperlipoproteinemia. J Clin Invest 50:754-761, 1971

56. Godolphin W, Campbell DJ: Heterogeneity of type III very low density apolipoproteins. Clin Res 19:760Abs, 1971

57. Hatch FT, Lees RS: Practical methods for plasma lipoprotein analysis. Adv Lipid Res 6:1-68, 1968

58. Phillips GB, Wille LE: The phospholipid composition of human serum lipoprotein fractions separated by electrophoresis on agarose gel. Demonstration of a fraction with high lysolecithin content. Clin Chim Acta 49:153-160, 1973

59. Alaupovic P, Sanbar SS, Furman RH, Sullivan ML, Walraven SL: Studies of the composition and structure

of serum lipoproteins. Isolation and characterization of very high density lipoproteins of human serum. Biochemistry 5:4044-4053, 1966

60. Kessler G, Lederer H: Fluorometric measurement of triglycerides, Automation in Analytical Chemistry, Technicon Symposia. Edited by L Skeggs. New York, Mediad, 1965, pp 341-344

61. Black WD, Jarrett JK, Levine JB: Use of a single color reagent to improve the automated determination of serum total cholesterol, Automation in Analytical Chemistry, Technicon Symposia. Edited by L Skeggs. New York, Mediad, 1965, pp 345-347

62. Irwin WC, Campbell DJ: Agarose gel electrophoresis of lipoproteins, Standard Methods of Clinical Chemistry. Vol.7. Edited by GR Cooper. New York, Academic Press, 1972, pp 111-126

63. Hulley SB, Cook SG, Wilson WS, Nichaman MZ, Hatch PT, Lindgren FT: Quantitation of serum lipoproteins by electrophoresis on agarose gel: standardization in lipoprotein concentration units (mg/100 ml) by comparison with analytical ultracentrifugation. J Lipid Res 12:420-433, 1971

64. Brown WV, Levy RI, Fredrickson DS: Studies of the proteins in human plasma very low density lipoproteins. J Biol Chem 244:5687-5694, 1969

65. Scanu AM, Edelstein C: Solubility in aqueous solutions of ethanol of the small molecular weight peptides of the serum very low density and high density lipoproteins: relevance to the recovery problem during delipidation of serum lipoproteins. Anal Biochem 44:576-588, 1971

66. Gotto AM, Levy RI, Fredrickson DS: Preparation and properties of an apoprotein derivative of human serum beta-lipoprotein. Lipids 3:463-470, 1968

67. Operating and Service Manual, Models 4010/4011 Electrophoresis System. Oak Ridge, Tenn, Ortec Incorporated, 1972, p 13

68. Sephadex Gel Filtration in Theory and Practice. Pharmacia Fine Chemicals AB, Upsalla, Sweden

69. Whatman Advanced Ion-exchange Celluloses Laboratory Manual. Edited by CM Thompson. Springfield Mill, Maidstone, Kent, England, W & R Balston (Modified Cellulose) Limited

70. Reisfeld RA, Small PA: Electrophoretic heterogeneity of polypeptide chains of specific antibodies. Science 152:1253-1255, 1966

71. Maurer HR: Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis. New York, Walter de Gruyter, 1971, p 1

72. Weber K, Osborn M: The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem 244:4406-4412, 1969

73. Maurer HR: Disc Electrophoresis and Related Techniques of Polyacrylamide Gel Electrophoresis. New York, Walter de Gruyter, 1971, p 76

74. Liu T-Y, Chang YH: Hydrolysis of proteins with p-toluenesulfonic acid. J Biol Chem 246:2842-2848, 1971

75. Houston LL: Amino acid analyis of stained bands from polyacrylamide gels. Anal Biochem 44:81-88, 1971

76. Perry TL, Stedman D, Hansen S: A versatile lithium buffer elution system for single column automatic amino acid chromatography. J Chromatogr 38:460-466, 1968

77. Eveleigh JW, Winter GD: Amino acid composition determination, Protein Sequence Determination. Edited by SB Needleman. New York, Springer-Verlag, 1970, pp 91-123

78. Gray WR: End group analysis using dansyl chloride. Methods Enzymol 25:121-138, 1972

79. Hartley BS: Strategy and tactics in protein chemistry. Biochem J 119:805-822, 1970

80. Ambler RP: Enzymatic hydrolysis with carboxypeptidases. Methods Enzymol 25:143-154, 1972

81. Clausen J: Immunochemical techniques for the identification and estimation of macromolecules, Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 1. Edited by TS Work, E Work. New York, John Wiley & Sons, Incorporated, 1969, pp 397-556

82. Hazzard WR, Lindgren FT, Bierman EL: Very low density lipoprotein subfractions in a subject with broad-beta disease (type III hyperlipoproteinemia) and a subject with endogenous lipemia (type IV). Chemical composition and electrophoretic mobility. Biochim Biophys Acta 202:517-525, 1970

83. Dietary Management of Hyperlipoproteinemia Type III. Bethesda, Maryland, National Heart and Lung Institute, 1970

84. LaRosa JC: Hyperlipoproteinemia 1. Diagnosis and clinical significance. Postgrad Med 51:62-70, 1972

85. Goldstein JL, Hazzard WR, Schrott HG, Bierman EL, Motulsky AG: Hyperlipidemia in coronary heart disease. I. Lipid levels in 500 survivors of myocardial infarction. J Clin Invest 52:1533-1543, 1973

86. Harlan WR, Shaw WA: Interpretation of hyperlipidemias. CRC Crit Rev Clin Lab Sci 3:451-480, 1972

87. Haglund H: Isoelectric focusing in pH gradients - a technique for fractionation and characterization of ampholytes. Methods Biochem Anal 19:1-104, 1971

88. Pearlstein E, Aladjem F: Subpopulations of human serum very low density lipoproteins. Biochemistry 11:2553-2558, 1972

89. Freedman MH, Painter RH: Isolation and characterization of electrophoretically homogeneous rabbit antihapten antibody populations. 1. Separation and properties of homogeneous anti-p-azophenyltrimethyl ammonium antibodies. J Biol Chem 246:4340-4349, 1971

90. Kostner VG, Albert W, Holasek A: Analytische isoelektrische fokussierung der humanserum-lipoproteine. Hoppe-Seyler's Z Physiol Chem 350:1347-1352, 1969

91. Shore B, Shore V: Isolation and characterization of polypeptides of human serum lipoproteins. Biochemistry 8:4510-4516, 1969

92. Shore B, Shore V: Apoproteins and substructure of human serum lipoproteins, Atherosclerosis: Proceedings of the Second International Symposium. Edited by FJ Jones. New York, Springer-Verlag, 1970, pp 144-150

93. Margolis S, Langdon RG: Studies on human serum beta-lipoprotein. I. Amino acid composition. II. Chemical modifications. III. Enzymatic modifications. J Biol Chem 241:469-493, 1966

94. Shore VG, Shore B: Heterogeneity of human plasma very low density lipoproteins. Separation of species differing in protein components. Biochemistry 12:502-507, 1973

95. Brown WV, Levy RI, Fredrickson DS: Further separation of the apoproteins of the human plasma very low density lipoproteins. Biochim Biophys Acta 200:573-575, 1970

96. Fischer L: An introduction to gel chromatography, Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 1. Edited by TS Work, E Work. New York, John Wiley & Sons, Incorporated, 1969, pp 151-396.

97. Smith R, Dawson JR, Tanford C: The size and number of polypeptide chains in human serum low density lipoprotein. J Biol Chem 247:3376-3381, 1972

98. Eisenberg S, Bilheimer D, Lindgren F, Levy RI: On the apoprotein composition of human plasma very low density lipoprotein fractions. Biochim Biophys Acta 260:329-333, 1972

99. Havel RJ, Kane JP: Primary dysbetalipoproteinemia: predominance of a specific apoprotein species in triglyceride-rich lipoproteins. Proc Natl Acad Sci USA 70:2015-2019, 1973

100. Kane JP: A rapid electrophoretic technique for identification of subunit species of apoproteins in serum lipoproteins. Anal Biochem 53:350-364, 1973

101. Herbert PN, Shulman RS, Levy RI, Fredrickson DS: Fractionation of the C-apoproteins from human plasma very low density lipoproteins. Artifactual polymorphism from carbamylation in urea-containing solutions. J Biol Chem 248:4941-4946, 1973

102. Moore S: On the determination of cystine as cysteic acid. J Biol Chem 238:235-237, 1963

103. Shelburne FA, Quarfordt SH: A new apoprotein of human plasma very low density lipoprotein. Fed Proc 32:547Abs, 1973

104. Hamilton RL: Synthesis and secretion of plasma lipoproteins. Adv Exp Med Biol 26:7-24, 1971

105. Reichl D, Simons LA, Myant NB, Pflug JJ, Mills GL: The lipids and lipoproteins of human peripheral lymph, with observations on the transport of cholesterol from plasma and tissue into lymph. Clin Sci Mol Med 45:313-329, 1973

106. Kostner G, Holasek A: Characterization and quantitation of the apolipoproteins from human chyle chylomicrons. Biochemistry 11:1217-1223, 1972

107. Havel RJ, Kane JP, Kashyap ML: Interchange of apolipoproteins between chylomicrons and high density lipoproteins during alimentary lipemia in man. J Clin Invest 52:32-38, 1973

108. Margolis S, Capuzzi D: Serum lipoprotein synthesis and metabolism, Blood Lipids and Lipoproteins: Quantitation, Composition, and Metabolism. Edited by GJ Nelson. New York, Wiley-Interscience, 1972, pp 825-880

109. Glomset JA, Norum KR: The metabolic role of lecithin:cholesterol acyltransferase: perspectives from pathology. Adv Lipid Res 11:1-65, 1973

110. Redgrave TG: Formation of cholesteryl ester-rich particulate lipid during metabolism of chylomicrons. J Clin Invest 49:465-471, 1970

111. Sodhi HS, Suchodkar BJ: Correlating metabolism of plasma and tissue cholesterol with that of plasma lipoproteins. Lancet 1:513-519, 1973

112. LaRosa JC, Levy RI, Brown WV, Fredrickson DS: Changes in high-density lipoprotein protein composition after heparin-induced lipolysis. Am J Physiol 220:785-791, 1971

113. Bilheimer DW, Eisenberg S, Levy RI: The metabolism of very low density lipoprotein proteins. I. Preliminary in vitro and in vivo observations. Biochim Biphys Acta 260:212-221, 1972

114. Eisenberg S, Bilheimer DW, Levy RI: The metabolism of very low density lipoprotein proteins. II. Studies on the transfer of apoproteins between plasma lipoproteins. Biochim Biophys Acta 280:94-104, 1972

115. Langer T, Bilheimer D, Levy RI: Plasma low density lipoprotein (LDL): a remnant of very low density lipoprotein (VLDL) catabolism? Circulation 17:Suppl 3:III-7, 1970

116. Hazzard WR, Porte D Jr, Bierman EL: Heterogeneity of very low density lipoproteins in man: evidence for a functional role of a beta migrating fraction in triglyceride transport and its relation to broad-beta disease (type III hyperlipoproteinemia). J Clin Invest 49(6):40a, 1970

117. Fisher WR: The characterization and occurrence of an Sf 20 serum lipoprotein. J Biol Chem 245:877-884, 1970

118. Quarfordt SH, Levy RI, Fredrickson DS: The kinetic

properties of very low density lipoprotein triglycerides in type III hyperlipoproteinemia. Biochim Biophys Acta 296:57 .76, 1973

119. Levy RI, Bilhei DW .senberg S: The structure and metabolism .of ch/l microns and very low density lipoproteins (VL), Plasma Lipoproteins, Biochemical Society Symposiu 'o 33. Edited by RMS Smellie. New York, Academic Press, 1971, pp 3-17

120. Hazzard WR, Porte D Jr, Bierman EL: Abnormal lipid composition of chylomicrons in broad-beta disease (type III hyperlipoproteinemia). J Clin Invest 49:1853-1858, 1970

121. Bagdade JD, Porte D, Bierman EL: Acute Insulin withdrawal and the regulation of plasma triglyceride removal in diabetic subjects. Diabetes 17:127-132, 1968

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