

# Phenotyping

## On Reaching Base Camp (1950–1975)

Donald S. Fredrickson, MD

The search for plasma lipoproteins began at the turn of the century. It was not until 1949 that a meeting of the Faraday Society celebrated the separation of the alpha and beta lipoproteins. At that moment, ultracentrifugists in Berkeley were already busily converting “ $\alpha$ ” to high density lipoprotein and “ $\beta$ ” to low density lipoprotein; the modern era of lipoproteins had begun. Over the succeeding 10 years, a quarrel over whether the level of S<sub>1</sub> 0–20 or cholesterol was the more powerful risk factor ended with an eclipse of the analytical ultracentrifuge and a surge of interest in the biological side of lipoproteins. The postheparin clearing factor became lipoprotein lipase, and free fatty acids were discovered. In 1960, abetalipoproteinemia and Tangier disease suggested that the apolipoproteins must be specific and spurred a hunt for their number and nature. The first amino acid sequences aroused speculation of “amphipathic helices.” By 1970, conversion of hyperlipidemia to five types of hyperlipoproteinemia led to worldwide fascination with electrophoretic patterns, “floating beta,” and “the Friedewald formula” as codes for genetic abnormalities leading to early coronary artery disease. A few years later, the appearance of “familial combined hyperlipidemia” confounded the phenotyping, and the discovery of the low density lipoprotein receptor heralded the coming of true genotypes. This is a Bethesda-based story of the “climb to base camp” preceding the joining of molecular biology with the research on lipoproteins, dyslipoproteinemia, and atherosclerosis. (*Circulation* 1993;87[suppl III]:III-1–III-15)

**KEY WORDS** • lipoproteins • dyslipidemia • hyperlipoproteinemia • Tangier disease • hyperlipidemia • phenotyping • apolipoproteins • free fatty acids • xanthomas • lipoprotein lipase

When the Faraday Society met in Birmingham, England, in 1949, its discussions constituted the first international symposium on lipoproteins. The host was Alastair Frazier, father of one of the prevailing theories on fat absorption and chylomicron formation. Paradoxically, the American scientists Oncley and Gurd<sup>1</sup> were not among the approximately 140 attendees. Their paper describing the reproducible isolation of two distinct classes of lipid-protein complexes from human serum by fractionation techniques using alcohol at low temperatures and low salt concentrations<sup>2</sup> was delivered by Edwin J. Cohn. On the basis of their migration on electrophoresis, these complexes were named the “alpha and beta lipoproteins,” the latter being much larger and considered to have a molecular weight of possibly 1 million.<sup>3</sup> Essentially all of the cholesterol in plasma was accounted for by these two lipoproteins.

There had been many earlier explorations of how nonpolar lipids were able to remain soluble in the extracellular waterways. Those studies were thoroughly reviewed by Theorell<sup>4</sup> in 1930 and included references from as far back as 1902 but omitted the 1901 report of

Nerking,<sup>5</sup> who determined that the removal of all the lipid with ether required proteolysis. Macheboeuf,<sup>6,7</sup> while working at the Pasteur Institute in the 1920s, first reported the reproducible precipitation of a “cenapse,” or lipid-protein complex (most likely an alpha lipoprotein), by treating horse serum with ammonium sulfate. Bennhold,<sup>8</sup> who in 1932 echoed the conviction of earlier colleagues that “die Globuline binden Cholesterin,” was generously credited by Tiselius (the inventor of electrophoresis) as being the first to attempt to isolate lipid-protein complexes using a prototype of this important technique.<sup>9</sup>

Thus, it was through several of the by-products of the blood fractionation scheme developed by Cohn during World War II that the 50-year-long primordial “solvent and salt era” drew to a climactic close. As the curtain then promptly rose on the modern era of lipoproteins, Barr and associates<sup>10,11</sup> at the New York Hospital were adapting Cohn fractionation to a clinical scale. By 1951, they had reported an apparent association between lower levels of alpha lipoproteins and a proclivity toward coronary artery disease. At the time, zonal electrophoresis was also being used for preparative capture of lipoproteins by other workers<sup>12–14</sup> including Esko Nikkilä in Helsinki, who was beginning a distinguished career studying the metabolism of triglyceride-rich lipoproteins that was curtailed by his death in 1990.

The limited interest in plasma lipoproteins in 1950 has grown enormously and has come to exert a great influence on health practices related to cardiovascular disease. This article is a review of some of the events in

From the National Library of Medicine, National Institutes of Health, Bethesda, Md.

Address for correspondence: Donald S. Fredrickson, MD, National Library of Medicine, 1W22, National Institutes of Health, Bethesda, MD, 20892.

The first Donald S. Fredrickson Lecture on Lipoprotein Research was presented at the Ninth International Symposium on Atherosclerosis, October 9, 1991, Chicago.

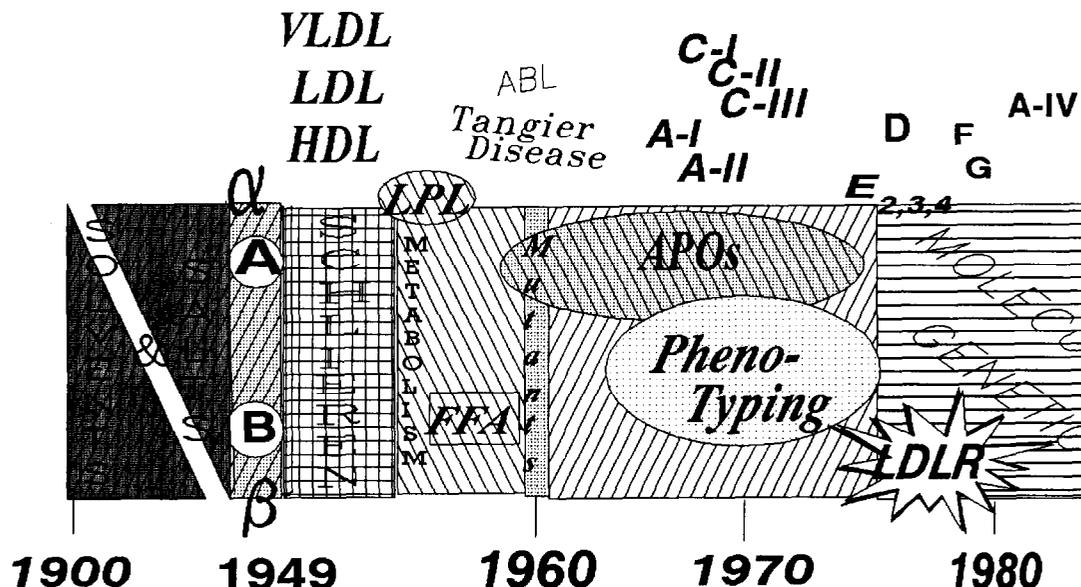


FIGURE 1. With artistic license, depiction of the major episodes in the 90-year history of the search for understanding of the transport of fats and lipids in plasma. VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LPL, lipoprotein lipase; FFA, free fatty acids; ABL, abetalipoproteinemia; APOs, apolipoproteins; LDLR, low density lipoprotein receptors. (See text for explanation of episodes.)

the first 25 years of the modern era and pays particular attention to the first large-scale exercise in phenotyping individuals who had familial abnormalities in plasma concentrations of cholesterol and triglycerides that began in the intramural research program of the National Heart Institute (NHI). This institute, the second spawned in the early pluralization of the National Institutes of Health (NIH, 1948), was given a federal mandate to wage a nationwide war on chronic heart diseases, of which atherosclerosis was a principal component.

Within its first year, the NHI took over responsibility for the Framingham Heart Project, which had started in 1946. Eventually, this long-running observation of citizens in a typical New England community would provide the data that would form the basis of more than a score of randomized interventional trials in the prevention of coronary artery disease. Each of these studies helped to highlight the importance of blood lipids and lipoproteins in the search for ways to reduce the prevalence of premature atherosclerosis.

An episodic view of plasma lipoprotein research is shown in Figure 1, representing some of the major phases of scientific activity from the midcentury to the introduction of molecular genetics around 1975. In this lattermost period, the harvest in understanding of both normal and aberrant mechanisms of fat transport and metabolism has been dramatic, and there is as yet no end in sight.

In all fields of science, technological changes have dictated forward movement in research. The start and decline of new paradigms, however, also depend on the people who use the technology to find the answers to particular questions. This is illustrated by the group of scientists in California who succeeded in overthrowing the alpha-beta paradigm of lipoproteins immediately after its belated arrival.

#### The Analytical Ultracentrifuge or Schlieren Period

The word "Schlieren" refers to the optical patterns of substances sedimenting (or floating) in the analytical ultracentrifuge (AnUC) first described by Svedberg and Rinde<sup>15</sup> in Uppsala in 1924. In the mid 1930s, one of his students, A.S. McFarlane,<sup>16-18</sup> improved the optics and carried out lengthy investigations of the ultracentrifugal behavior of serum proteins from many sources, including patients with a variety of diseases. Many of his AnUC plots, especially those from the clinical material, contained a density- and time-sensitive component in the region of the albumin boundary, which McFarlane called the "X protein" to distinguish it from albumin and globulin. Ten years later, K.O. Pederson<sup>19</sup> extended these studies and concluded that this labile component, which appeared soon after acceleration of the cell, was a lipoprotein. He succeeded in partially isolating it by altering the salt concentration of the serum in a preparative ultracentrifuge.

In the late 1940s, John Gofman, a physician and physical chemist, gained a space in the Atomic Energy Commission facilities at the Donner Laboratory, University of California at Berkeley, where he mobilized resources to build an AnUC, and enlisted several graduate students, including Frank Lindgren,<sup>20</sup> in a campaign to master the X protein, which had eluded the Swedish workers (see "Note 1"). Over the next 5-year period, Gofman and coworkers<sup>21-30</sup> drove their preparative and analytical ultracentrifuges day and night to develop a totally new system of plasma lipoprotein analyses and, at the same time, to explore the relations between lipoprotein concentrations and certain aspects of health and disease. Within a short time, they had replaced the alpha and beta nomenclature with new designators based on the densities and flotation characteristics of lipoproteins in the ultracentrifuge. The Gofman group identified multiple subclasses of lighter lipoproteins designated as "S<sub>i</sub>" (Svedberg units of flo-

tation) that soon came to be known as low density lipoproteins (LDL) and very low density lipoproteins (VLDL); they also discovered that the larger of these lipoproteins were converted into those that were smaller and denser. High density lipoproteins (HDL) were also isolated,<sup>24</sup> and the concentrations of HDL<sub>2</sub> and HDL<sub>3</sub> subclasses were measured, which resulted in the observation that the average concentration of HDL<sub>2</sub> was much higher in women.<sup>29</sup>

Enlisting clinical colleagues to provide samples from a large number of patients, Gofman and coworkers undertook an early exploration into the relation between specific classes of lipoproteins and a propensity for premature coronary artery disease. They examined patients with "hyperlipoproteinemia," thereby introducing this term into the literature.<sup>27</sup> A compendium of the findings of the Gofman group was published in the obscure journal *Plasma* in 1954.<sup>30</sup> Copies of this archive became valuable collector's items that novitiates such as myself zealously guarded as if they were breviaries.

Within a year of his first publication—and unfortunately, as events would prove, before their methodology was perfected—Gofman and colleagues<sup>22</sup> suggested that certain lipoproteins in themselves and not their lipid components might be the key cause of atherosclerosis. It was intended as a challenge to an establishment already comfortable with the cholesterol hypothesis that had been promulgated around 40 years earlier by Anitschkow<sup>31</sup> from studies in cholesterol-fed rabbits (see "Note 2"). The National Advisory Heart Council moved quickly to set up a prospective cooperative study of lipoproteins and atherosclerosis to compare cholesterol and lipoprotein measurements as predictors of new (coronary) events.

Five to six bumpy years later, the final judgment was bitter and divided, with the majority choosing the cholesterol concept (see "Note 3").<sup>32</sup> One unfortunate outcome was Gofman's premature departure from the field he had opened so zestfully. It was not unlike the sudden disappearance of a brilliant but brief civilization, and there followed a period when elements of the Donner achievements had to be rediscovered and relearned. Today, there is no quarrel over the measurement of LDL rather than total serum cholesterol as the more powerful indicator of coronary risk. Similarly, the riddle of the relations between the various subgroups of HDL and the metabolism of both cholesterol and triglycerides continues to hold keys to as yet poorly understood but no doubt important mechanisms (see "Note 4").

### Physiology and Metabolism

#### *Bethesda*

The brief Schlieren period, with its intense focus on the AnUC, served as a prism, splitting a single beam to play upon a number of more basic aspects of lipoprotein biology and lipid transport. One of the places housing more diffuse interests was the Bethesda Laboratory of Cellular Physiology and Metabolism, where the NHI intramural research on atherosclerosis was concentrated, beginning around 1950. In characteristic fashion, the then NHI scientific director, James A. Shannon, chose as the leader of this effort a protein chemist, Christian B. Anfinsen, who had a genius for allowing

young scientists to discover themselves (see "Note 5"). I arrived at this laboratory in 1953 as one of eight clinical associates picked by Shannon for the NHI. I had come from the laboratories of Ivan D. Frantz at the Massachusetts General Hospital, where I had learned how to measure cholesterol as the digitonide and to handle the newly available isotopes of carbon and tritium.<sup>33</sup>

We clinical associates all realized that we were the fortunate beneficiaries of converging opportunities. The clinical center had unparalleled resources for combining fundamental laboratory work with clinical investigation. The "in" topics of the 1950s included the structure and synthesis of proteins, and Anfinsen's laboratories were equipped with the latest instrumentation and researchers familiar with their use. The new Moore-Stein amino acid analyzers shared space with the blocks of starch, hoary with urea crystals as protection against denaturation during the isolation of proteins. Enzymatic digestion was followed by high-voltage electrophoresis for identification and further isolation of peptide fragments. The ninhydrin stains on all hands were considered to be the fingerprints of the future.

In such a milieu, those who had matriculated in scientific experiments dominated by lipid solvents also became keenly aware of the potential for studying proteins, including those that mysteriously assisted the solubilization and metabolism of cholesterol and triglycerides. Among the other technologies that had recently arrived, the use of radioactive isotopes offered unlimited opportunities to explore previously hidden territories and to participate in charting metabolic routes accessible for the first time, with the promise of unusual opportunities.

One trend was predictable. The AnUC, with its superior yet very demanding precision, was again regarded as more useful for the study of proteins than lipoproteins. Excursions into the physiological and biochemical realms of lipoproteins required the capture of larger amounts for characterization and analysis. Clinical investigations also benefited from the use of the preparative ultracentrifuge to quantify lipoproteins in different density fractions. After the overnight runs, the plastic tubes were sliced and their contents drawn off for chemical measurements. Adaptation of Lindgren's early methods<sup>24</sup> for preparative ultracentrifugation was one of the first tasks undertaken in Bethesda by Havel, Eder, and Bragdon,<sup>34</sup> and their method remains a staple of lipoprotein laboratories today.

As relatively virgin territory, lipoprotein research was marked by numerous leaps forward. Partly because they involved Bethesda, but also because they were so fundamental in nature, two achievements of this period are briefly described here.

#### *Lipoprotein Lipase*

One of the earlier landmarks was the unraveling of the mystery surrounding the "anti-chylomicron effect"<sup>35</sup> of heparin, which was first observed in the early 1940s.<sup>36</sup> Heparinized blood given intravenously to dogs caused a rapid disappearance of the turbidity in postprandial plasma. Gofman's group<sup>23</sup> had previously shown that this clearing was accompanied by a swift cascade of lighter triglyceride-rich lipoproteins into VLDL of

smaller size and higher density and that, later, there were often lesser rises in the LDL region.

Anfinsen, Boyle, and Brown<sup>37</sup> showed that what they called "the heparin-clearing factor" was a tissue component that had the properties of a protein and was therefore possibly an enzyme; a plasma cofactor also appeared to be required for the clearing action. The problem was then assigned to Edward Korn,<sup>38-40</sup> a postdoctoral student newly arrived in Bethesda. Within 2 years, Korn had isolated the factor released by heparin from tissues and had proved that it was an enzyme; this he termed lipoprotein lipase (LPL). The plasma cofactor remained undiscovered at this time.

The revelation of LPL in 1955 became the centerpiece of what is still one of the most popular areas of lipoprotein metabolism. At once, numerous reports appeared of measurements of LPL activity in plasma, most of which were nonvalid because none had reckoned on there being more than one enzyme released by heparin.<sup>41-43</sup> The most important of these other lipases was eventually traced to liver cell membranes<sup>44-46</sup> and is now known as hepatic lipase. Measurement of LPL release in plasma soon became critical to the phenotyping activities described below. The method developed in our laboratory by Ronald Krauss in particular<sup>47</sup> to differentiate the various postheparin lipases became the standard until immunoaffinity techniques began to arrive.<sup>48,49</sup>

Another important boost to understanding LPL activity came with the discovery of apoprotein C-II in Bethesda in 1970.<sup>50-52</sup> Within a few months, three independent laboratories recognized it as the missing plasma cofactor considered by Anfinsen and coworkers to be necessary for lipolysis of triglyceride-rich particles by LPL.<sup>53-55</sup> Today, 20 years later, the LPL and C-II genes have been cloned, and extensive study of chylomicronemic patients has revealed approximately a dozen mutational changes in one protein or another that are known to cause defective lipolysis.<sup>56</sup>

#### Free Fatty Acids

Another key discovery in the mid 1950s was that not all of the key mechanisms of fat transport in the blood involved lipoproteins. This knowledge was provided independently by Vincent Dole<sup>57</sup> at the Rockefeller Institute and Robert Gordon<sup>58</sup> of the NHI Bethesda contingent. A tiny fraction of the total mass of fats and lipids in plasma proved to be the most sensitive mechanism for maintaining the body's caloric economy in balance.

The tidal movements of calories, tied mainly to the digestive rhythms, are often far in excess of immediate needs. The storage of excess calories as triglycerides requires less water than glycogen, and the release of ester bonds by hydrolysis demands less energy. When summoned, the fatty acids race through plasma bound to albumin and are available for immediate consumption or are readily returnable to fat depots. Dole called these moieties nonesterified fatty acids, and Gordon preferred unesterified fatty acids. Steinberg of the *Journal of Lipid Research* resolved the dilemma by deciding upon free fatty acids (FFA), all sides agreeing that harmony in nomenclature was worth the chemical inaccuracy of the term.

It was deduced from arterial-venous differences that these small amounts of FFA—for which very sensitive methods of measurement were developed—had a transit time through plasma of seconds rather than minutes. This was confirmed by study of the disappearance of <sup>14</sup>C-labeled fatty acids, and the rapid reappearance of the label in expired carbon dioxide indicated the ready availability of FFA for metabolism.<sup>59</sup> Dietary fatty acids, touring the plasma as triglycerides in chylomicrons, also rapidly reemerged in FFA.<sup>60</sup>

In the late 1950s, the accumulation of new knowledge was so rapid that, accepting an invitation that Anfinsen could not fulfil, Gordon and I wrote for *Physiological Reviews* the first definitive analysis of the emerging subject of the transport of fatty acids.<sup>61</sup> It was yet another sign that this was a renaissance, a time when apprentices carried out the surplus work of their masters.

#### Apolipoproteins and Mutations

Around 1960, I formed a small group called the Section on Molecular Disease. (It was, as far as I am aware, the first appearance of Pauling's felicitous<sup>62</sup> term among the tables of organization of the NIH.) The purpose was to launch a long-range study of genetically determined diseases involving plasma lipoproteins. The theme had become important for several reasons. The departure of Havel for the Cardiovascular Research Institute at the University of California at San Francisco had left me a legacy of patients with abnormal lipoprotein concentrations and a variety of xanthomas, some of whom had been among the first patients studied at the clinical center. Also around this time, I had agreed to join John Stanbury and James Wyngaarden in writing a book with the bold intention to take up from where Garrod<sup>63</sup> had left his descriptions of inherited metabolic disorders in 1908. The book was to be called *The Metabolic Basis of Inherited Diseases (MBID)*, and its chapters were to cover the clinical and genetic manifestations and all of the relevant biochemistry available on such disorders as far as 1960. (In 1988, the sixth edition of this ever-expanding volume appeared under a new team of editors.)

On being immersed in both the care of patients with, and gaining familiarity with the world literature on xanthomatoses and lipidoses, I became acutely aware of the inchoate state of diagnosis and minimal understanding of these disorders. What we had seen in the preceding 7 years in Bethesda convinced us that we were entering a time of sweeping enlightenment in the area of both lipid metabolism and genetics and, thus, opportunity for improvement in both taxonomy and understanding.

#### Abetalipoproteinemia and Tangier Disease

In 1960, as we were embarking on studies of hyperlipoproteinemia, two rare mutations were described that caused hypolipoproteinemia and reinforced our growing faith in the uniqueness of the apolipoproteins. On occasions, the elimination of an effective activity of a particular protein by mutation is the only proof of its function. These discoveries were a stimulus to divert part of our activities to participate further in the study of apolipoproteins.

The first disorder was described by Salt and coworkers<sup>64</sup> in England, who found a young girl whose ability to absorb dietary fats was nearly abolished. On centrifugation, LDL, VLDL, and chylomicrons were missing, and plasma triglycerides were at correspondingly vanishing levels. Fortunately, by this time in the mid 1950s, immunochemical methods were applied that could distinguish the two major antigens of the plasma lipoproteins.<sup>65-67</sup> This patient lacked what came to be called the "B" protein of beta-lipoprotein. This was the first recognized instance of abetalipoproteinemia (ABL).

A scant few months after this report of ABL, we came upon what appeared to be its mirror image.<sup>68,69</sup> A 5-year-old boy diagnosed as having Niemann-Pick disease had been referred to the clinical center. Although I had never previously seen a patient with this disease, having scanned the world's literature in preparation for my definitive chapter on Niemann-Pick disease in the *MBID* allowed me to assert that the diagnosis was incorrect. The patient's plasma cholesterol level was low, whereas the triglycerides appeared to be somewhat higher than normal. There were very low levels of HDL or alpha-lipoproteins in the plasma. The most astonishing abnormality—which had led to the erroneous diagnosis on histology—was the storage of mammoth amounts of cholesteryl esters in reticuloendothelial tissues throughout the body. The patient's tonsils had been removed, but we had the dramatic and rare experience of observing the bright orange tonsils of the boy's only sibling. Among other things, we had discovered the only lipidosis that can be diagnosed by looking into the mouth! We called this condition Tangier disease, after the Chesapeake Bay island home of the patient's family. A carrier status characterized by half-normal levels of HDL in obligate heterozygotes confirmed that this was an autosomal recessive disease.<sup>70</sup>

This finding stimulated a 10-year study of the minute quantities of "A" apoprotein in the plasma of these patients. We now know, however, that rather than being due to a low level of HDL synthesis, these patients can produce normal A-apolipoproteins (apo A), but the turnover rate in plasma is so rapid that the levels remain very low.<sup>71</sup> The reasons for this as well as the exact mechanism(s) of the attendant tissue storage of cholesteryl esters as yet remain obscure, but, someday, someone will reveal the important mechanisms for removal of cholesterol from tissues. The discovery of ABL and Tangier disease served for us believers as a witness that lipids were not cadging rides on any old globulins in plasma but used specially designed carriers for this purpose (see "Note 6").<sup>72</sup> Exciting questions were raised concerning these putative lipid-avid proteins: How many were there, how were they able to perform their tasks, and what had been their evolution?

### *The Hunt for Apolipoproteins*

By the time the two new genetic disorders of apolipoproteins had been found, some effort to characterize these proteins was already under way. Only the hardest experimentalist attempted to begin with the stubbornly insoluble B-apoproteins (apo B), which appeared to constitute all of the LDL protein. Most of the earliest work concentrated on HDL or on the triglyceride-rich particles. Initially, the main centers of this activity were Berkeley, Bethesda, Chicago, and Oklahoma City. De-

termination of terminal amino acids in apoproteins confirmed that the A and B proteins were different<sup>73-75</sup> and suggested that VLDL might have other apoproteins as well.<sup>75</sup> The meager amounts of protein in chylomicrons included the "fingerprints" of A and B and a third unknown print, which Martin Rodbell and I<sup>76</sup> called simply "C."

From the first, there had been the suspicion that apo A might be plural. In 1968, preliminary evidence of this by Cohen and Djordjevich<sup>77</sup> was amply confirmed by Bernard and Virgie Shore,<sup>78</sup> who separated and identified the proteins according to their C-terminal residues as C-Gln and C-Threo. The latter was corrected to C-Gln-II in 1971 by Koster and Alaupovic,<sup>79</sup> who also noted that the N-terminus of the second apo HDL was blocked, providing one basis for the long period of confusion over the number of major proteins present in HDL.

In 1965, Gustafson, Alaupovic, and Furman<sup>80</sup> used nonpolar solvents to extract from human VLDL a third protein that they called "C." Between 1966 and 1969, Virgil Brown in our laboratory set out to purify C in VLDL and isolated three distinct apoproteins, namely, apo VLDL-Val, apo VLDL-Glu, and apo VLDL-Ala (now better known as apo C-I, apo C-II, and apo C-III, respectively<sup>50-52</sup>). As already noted, apo VLDL-Glu was quickly identified as the LPL cofactor (see "Note 7").<sup>81,82</sup>

### *Apolipoprotein Nomenclature*

Those who explore the older literature will be aware that, by 1970, the naming of apolipoproteins was a confused and contentious business. The Shores and our group held to the C-terminal nomenclature. On the other hand, Alaupovic and coworkers<sup>80</sup> introduced the hypothesis that there were several apolipoprotein families, each characterized by the presence of a "single apolipoprotein or its constituent polypeptides."<sup>83,84</sup> By this reasoning, the several apoproteins isolated by Brown from VLDL should all be termed "C" followed by a different Roman numeral, as they are all related in an as yet unelucidated manner. As neither side would yield to the philosophical approach of the others, eventually, journal editors—and common sense—demanded one system. As some of the initial C-terminal determinations had to be corrected,<sup>79,85</sup> and more than one protein in a major lipoprotein class proved to have the same carboxyl-terminal acid, theoretical objections gave way to the practical. We conceded and urged adoption of the ABC system in 1972 (see "Note 8").<sup>86,87</sup>

### **New Phenotypes of Hyperlipoproteinemia**

#### *Confusing Taxonomy*

In the early 1960s, apolipoproteins did not offer a rational approach to the first task assumed by the Section on Molecular Disease: improvement of the phenotyping of hyperlipoproteinemia. Patients with elevated cholesterol or triglyceride levels that baffled their physicians came in increasing numbers to the clinic. From the first, even good generic descriptors were lacking. "Hyperlipemia," for example, had become synonymous with creamy plasma. I therefore chose to use the term "hyperlipidemia" and inserted "familial" as the key modifier in the title of the first *MBID* chapter, but I lacked the courage to jettison the hallowed but useless "essential" or "idiopathic." I elected to relegate

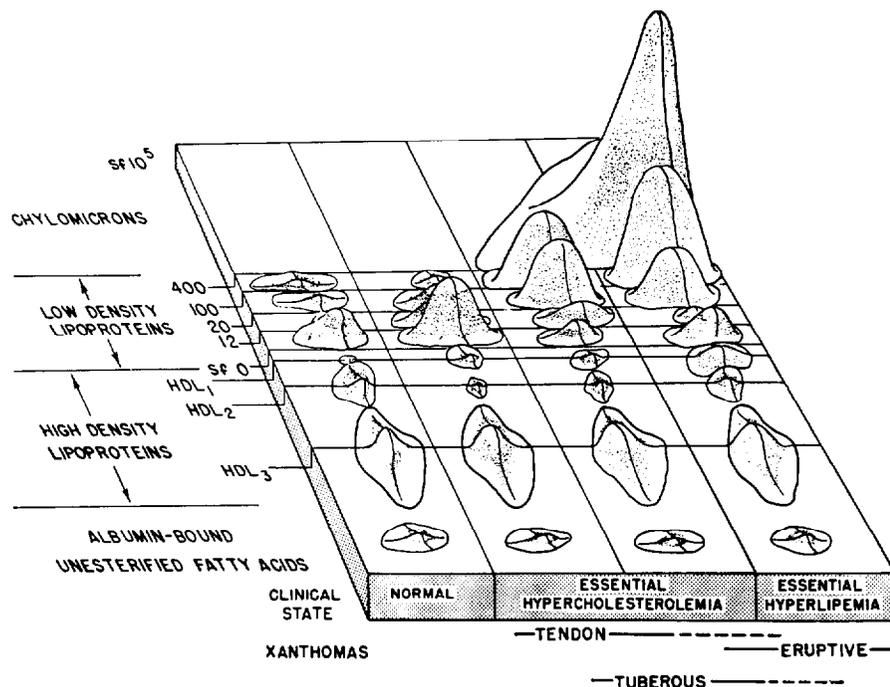


FIGURE 2. Graphic plot of lipoproteins and xanthomas constructed from the data of Gofman et al<sup>30</sup> as it appeared in the first edition of *The Metabolic Basis of Inherited Disease*,<sup>90</sup> 1960. Reproduced with permission.

the xanthomatous lesions to secondary place, especially after noting that, in the 19th century, biliary cirrhosis had mocked nearly all the xanthomas found in hyperlipidemic patients.

In 1958, the doyen of the lipidoses was Siegfried Thannhauser, at that time at Tufts University Medical School (see "Note 9"). Thannhauser had left Germany in the late 1930s, bringing with him a major work by himself and Magendantz on the different groups of xanthomatous diseases published in 1938.<sup>88</sup> The work became the core of Thannhauser's authoritative book published in 1940, its last revised edition appearing in 1958.<sup>89</sup> In this edition, two syndromes of hyperlipidemia were recognized: essential or idiopathic hyperlipemia and essential hypercholesterolemia. Thannhauser was aware from the reports of varying lipid patterns and the different kinds of associated xanthomas in affected patients that greater heterogeneity existed, but a new approach did not appeal to him. In his last edition, he wrote: "Gofman's method is, from the heuristic point of view of the investigation of the multiplicity of the serum protein aggregates, very interesting, but it seems not justified, or at least premature, to attribute the various Svedberg units diagnostic significance in clinical medicine."<sup>89</sup>

Toward the end of the 1950s, I visited Thannhauser to convince him of his error. I had, by then, compiled lipoprotein data from Gofman's *Plasma compendium*<sup>30</sup> into a three-dimensional plot (Figure 2) to use as an illustration in the first edition of *MBID*.<sup>90</sup> This proved to have no power to tempt the ailing professor to recant, however, and I returned to work on my own generation.

From the outset, we had restricted our studies to patients with evidence of familial disorder. I will leave to a footnote the details of methodology that relied upon preparative ultracentrifugation with quantification of the major lipoprotein classes in terms of their cholesterol value (see "Note 10").<sup>91,92</sup> It was soon evident that other simpler methods for classification, particu-

larly in screening family members, would be helpful. In 1961, Ahrens and coworkers<sup>93</sup> at the Rockefeller Institute introduced the terms "fat-induced" and "carbohydrate-induced" hyperglyceridemia and added virtue to the possibilities of rapidly distinguishing between the triglyceride-rich lipoproteins VLDL (carbohydrate-induced or endogenous triglycerides) and chylomicrons (fat-induced or exogenous triglycerides).

#### Paper Electrophoresis

Around this time, a possible new approach for making this distinction in a rapid and simple manner literally came strolling through the door of the laboratory in the form of a new assistant, Robert S. Lees. At the Massachusetts General Hospital, he and Frederick Hatch had altered the conventional system for electrophoresis of plasma proteins and lipoproteins<sup>94</sup> by adding albumin to the usual barbiturate buffer,<sup>95</sup> thereby obtaining as many as four lipoprotein bands from plasma samples after the strips were dried and stained with a fat-soluble dye. The other protein bands remained invisible. When Lees joined us, I insisted that we were interested only in quantitative methods, but I raised no objection to demonstrations of his method.

The sideshow quickly captured our interest, however, for the fluttering strips, stained with the scarlet dye oil red O, made a fetching display. We performed a few experiments to determine the nature of the bands that often joined the usual alpha (HDL) and beta (LDL) bands. It was shown that the prebeta bands, moving between the beta and the faster alpha bands, were VLDL. Material left at the origin represented chylomicrons.<sup>96</sup> It appeared that here was a ready method for distinguishing two different forms of glyceride transport, an obvious need in the population of abnormal subjects we were investigating.

Soon, we were meeting each morning to "read the wash" on the line where the stained strips were hung to dry. A form of shorthand emerged from the laboratory

banter, numbered types evolving from the order of the most prominent bands on the strips. A great splash of red chylomicrons at the origin and often, little else in the way of other lipoproteins, was called type I. A strongly colored beta band (an excess of LDL) became type II. Type IV was a prominent prebeta band (the classification allowing a peek at the total concentration of plasma triglycerides). Initially, type III was the presence of both prominent beta and prebeta bands, and type V became a useful handle for plasma samples in which fairly high triglyceride concentrations were accompanied by both a prebeta band and a chylomicron smudge at the origin.

#### *Evolution of Types*

These original five types of hyperlipoproteinemia were first publicly displayed to a group of cardiologists in the mid 1960s.<sup>97</sup> In the chapter on hyperlipoproteinemia in the 1966 (second) edition of *MBID*, an important adjustment to this scheme appeared as a last-minute addendum.<sup>98</sup> The genesis of the change was the arrival of Robert I. Levy, who in the course of further examining the nature of prebeta lipoproteins<sup>99</sup> had also checked the completeness of the separations of VLDL from LDL in the "beta-quants," as we called the routine analyses. He found that beta-migrating lipoproteins were occasionally present in the supernatant layer after the overnight centrifugation.

This "floating beta" and the broad beta band it produced on paper electrophoresis proved to be not a random error but the signature of what became type III. Analysis of collections of such patients, who had a unique tendency to collect lipid in the creases of the palms as well as both peripheral and coronary artery disease, led us to reexamine the earlier literature. As we had acknowledged on introducing type III, this phenotype appeared to have a possible correspondence to the abnormal Schlieren patterns described in patients with xanthoma tuberosum in the AnUC studies of Gofman and coworkers.<sup>25,27</sup> In those patients,  $S_f$  0–12 lipoprotein (LDL) concentrations were sometimes depressed, and variable increases in  $S_f$  12–20, 20–100, and 20–400 appeared. They noted that a few of their patterns could not be distinguished from those with essential hyperlipemia. I had included these data in the graph (Figure 2) prepared for my visit to Thannhauser (see "Note 11"),<sup>90,100–104</sup> In 1969, we collaborated with Frank Lindgren<sup>105</sup> to compare all the AnUC patterns in the various types: The type III and xanthoma tuberosum patterns corresponded.

#### *Additional Types*

By the time we thought we were ready to produce a definitive description of the typing system, observations of more patients required that several other adjustments be made. The patients with type II, defined by the excess of beta lipoprotein or LDL, frequently had modest hypertriglyceridemia and distinct prebeta bands. We subdivided type II into IIA and IIB, the latter having a modest increase in triglyceride levels. The bulk of these patients had the familial disorder first described in the latter half of the 19th century<sup>106</sup> and were diagnosed by Wilkinson and coworkers<sup>107</sup> as having essential familial hypercholesterolemia in 1949. The presence or absence of mild hypertriglyceridemia did not

discriminate between the heterozygotes and homozygotes in the data that we had collected from numerous families.<sup>108</sup>

A fifth phenotypic pattern (type V) was added after the observation of numerous patients with severe hypertriglyceridemia who had both prebeta bands and chylomicrons at the origin of the strips. Although a number of the family members with these probands had lesser degrees of hypertriglyceridemia, long observation periods on the wards led us to consider that at least some of the subjects with type IV and others with type V were distinct phenotypes. These subjects responded differently to diet and weight loss, and the type V phenotypes had a higher prevalence of diabetes mellitus, coronary artery disease, attacks of abdominal pain, and pancreatitis.

Moreover, as better tests for measuring postheparin LPL activity were developed, it became readily apparent that the familial hyperchylomicronemia expressed in the type I pattern was biochemically different from the others.<sup>47</sup> In 1960, Havel and Gordon<sup>109</sup> found postheparin lipolytic activity (PHLA) to be very low in the affected members of the first family with essential hyperlipemia seen at the clinical center.<sup>110</sup> By 1965, we had measured PHLA in 15 subjects with typical type I and had confirmed that it was definitely low in 14 of them. Virtually all of the type V subjects had normal PHLA despite their gross chylomicronemia. The management of type I and type V was distinctly different, but both were recognized to be potentially lethal diseases because the bouts of pancreatitis could be life threatening.

From the beginning, it was obvious that the phenotypes being classified by this typing system (Figure 3) were neither homogeneous entities nor true genotypes but were frequently secondary to other conditions. Although these phenotyping studies appeared to distinguish several new familial hyperglyceridemic syndromes, the true usefulness of the approach lay in two other directions. Large numbers of physicians, most of whom had been unaware of lipoproteins, realized that a better way to interpret the disturbing elevations of cholesterol or triglycerides was needed, especially as implications of risk for coronary artery disease began to circulate. The translation of hyperlipidemia to hyperlipoproteinemia attracted adherents who then gained entry to the large body of information being reported from the many laboratories working on lipoproteins. Second, these designations into at least five groups were not abstractions but the first step toward better diagnosis and management of hyperlipidemia, as there were unique clinical features and different responses to both diet and drug regimens in each phenotype and important differences in familial expression and in the complications of the hyperlipidemia.

In 1967, we<sup>111</sup> published a five-part review of all that was then known of lipoproteins and dyslipoproteinemia to assist clinicians in using this rapidly growing body of information (see "Note 12").<sup>112</sup> Many of the basic clinical aspects of the review, as deep as they went at the time, have endured; the information on the lipoproteins per se, however, was out of date by the time of publication.

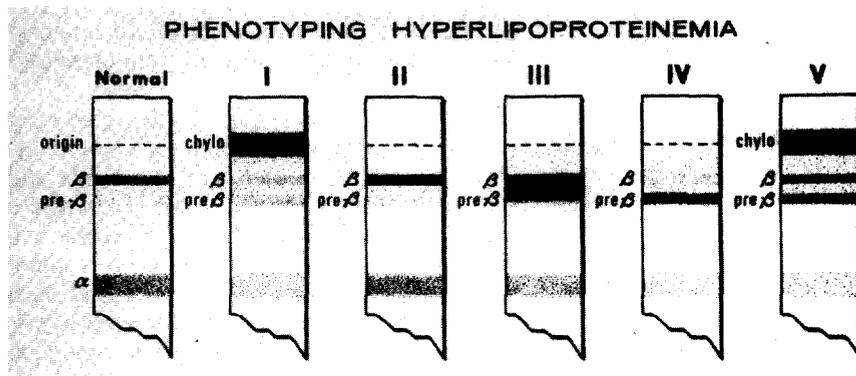


FIGURE 3. The five major phenotypes of hyperlipoproteinemia as defined by electrophoresis, circa 1967.

### Emergence of a New Speciality

The eagerness with which clinicians took to these early exercises in lipidology and their generous referrals of both questions and patients made the development of a new nonspeciality of medicine a stimulating and gratifying experience. Because of the pleomorphism of the abnormalities accompanying hyperlipoproteinemia, many aspects of medicine were involved that appeared at first to be remote from the measurement of lipoproteins. We also occasionally violated the territories of venerable medical specialities that had dealt with the various clinical manifestations of hyperlipidemia. The paramount group was the dermatologists under whose dominion all patients with hyperlipidemia and xanthomas had been observed and managed for nearly a century.

In the 1950s, some rare skin disease specialists began to use the AnUC,<sup>113</sup> although the descriptive aspects remained. In 1968, I gave back-to-back lectures to both the American Academy of Dermatology and the American Dermatological Association without censure of the cavalier treatment of the canons pertaining to xanthomas. Invitations to demonstrate phenotyping before such diverse groups as geneticists, internists, ophthalmologists, orthopedists, otolaryngologists, pathologists, and pediatricians led me to grateful appreciation of the early operational arrangements of the NIH Clinical Center that had allowed us to study patients vertically by age, from infancy to senility, and horizontally across the spectrum of organ manifestations, allowing the broadest perspectives. Ultimately, of course, it was the cardiovascular community that had the greatest need for lipoprotein analyses and interpretations and retained the closest interest in the core of our work. I became a member of the American Society for the Study of Arteriosclerosis at a time of anxiety over its "marriage" to the American Heart Association (AHA). The union has survived, and the major lipoprotein forum in the world today is still the AHA meetings.

### Phenotyping in Crescendo

The popularization of phenotyping as a means toward better understanding and management of the patient abnormalities grew even further when the AHA, the NHI, and other groups became activists in enlisting physicians into organized campaigns to reduce the risk of premature coronary artery disease. After 1960, numerous dietary trials—some on a large scale—were begun; however, after a decision by the NIH in 1969 that a vast

controlled clinical trial of dietary changes was impractical, drug trials became inevitable (see "Note 13").<sup>114-118</sup>

### Lipid Research Clinics

In 1970, a panel on hyperlipidemia and premature atherosclerosis was convened by the then director of the National Heart and Lung Institute (NHLI), Dr. Theodore Cooper (see "Note 14"). The panel arrived at several conclusions,<sup>119</sup> including unanimous agreement that translation of plasma lipid concentrations into lipoproteins was desirable and that it was possible to agree upon a standard classification of lipoprotein patterns for diagnosis and management of hyperlipidemia. Another decision was that a national network of lipid research clinics should be established for the development of population standards and final methodologies over the next 5 years. A further conclusion was that one of the purposes of the clinics would be to carry out a randomized prospective trial to determine the effect of the treatment of hyperlipidemia on the incidence of coronary artery disease. Dr. Robert Levy became the head of the new lipid research clinic program.

A few weeks after the Bethesda meeting, the World Health Organization issued a report recommending the phenotyping system as a worldwide standard (see "Note 15").<sup>120</sup>

### Continued Furbishes

As we entered the 1970s, we continued in our attempts to make the phenotyping system more practical for physicians.<sup>121,122</sup> No practitioner had access to a preparative ultracentrifuge, and few were able to obtain satisfactory electrophoretic patterns in type III, although local or regional laboratories all over the world began to enjoy a "land-office" business in lipoprotein patterns. The diagnoses on printouts sent to hospitals and physicians from these laboratories assumed an undeserved authority.

For this reason, various algorithms and other devices were sought to encourage thinking about lipoproteins while using readily available information and a minimum of extra laboratory analyses. An example was the popularization of the practice of examining the patient's plasma after allowing it to sit overnight at 2°C. The finding of a creamy layer floating over the clear infranatant layer was an infallible hallmark of full-blown type I. In type V, the cream was always overlying a milky layer of variable turbidity.

### Diagnosis of Type III

Type III, the rarest form of hyperlipoproteinemia, received much attention whenever it was recognized. It



FIGURE 4. Three examples of the diets developed for each phenotype of hyperlipoproteinemia and the accompanying handbook published by the National Heart Institute<sup>131</sup> and sent to physicians on request. An estimated 7 million copies were distributed.

was quickly found to carry a high risk of both coronary artery and peripheral vascular disease. Once recognized and treated, its extraordinary xanthomatous skin deposits melted away within a few weeks. A precise diagnosis of type III was available only to specialized laboratories such as ours, and our search for other rules of thumb to assist the clinicians sometimes approached the baroque and were focused on using plasma lipid analyses alone for screening purposes. A ratio of cholesterol to triglyceride approaching 1 was, and still remains, a valid clue to the presence of type III. In 1975, after a review of about 100 type III patients out of a total of nearly 1,000 patients in our registry,<sup>123</sup> we concluded that the ratio of VLDL cholesterol to plasma triglycerides clearly distinguished the abnormal VLDL of type III.<sup>124</sup> This was consistent with a previous report by Hazzard and colleagues,<sup>125</sup> which used the slightly more cumbersome measurement of VLDL lipids only.

Fortunately, at about this time, the mystery of this rare and always intriguing phenotype began to clear due to interventions involving apoproteins. During 1971–1972, again with particular thanks to the sleuthing of the Shores,<sup>126</sup> a new “arginine-rich peptide” was detected in VLDL apolipoproteins.<sup>127</sup> Two years later, Havel and Kane<sup>128</sup> reported a predominance of arginine-rich protein in type III plasma (see “Note 16”). Gerd Utermann<sup>129</sup> then isolated what he called apoprotein E from the abnormal VLDL. Utermann’s discovery included the separation of E by isoelectric focusing into three major components: E<sub>2</sub>, E<sub>3</sub>, and E<sub>4</sub>. The discovery of the inheritance of these phenotypes, the appearance of the E<sub>2</sub>/E<sub>2</sub> homozygote in type III, and the single point mutations in the E proteins that alter the catabolism of lipoproteins bearing the mutant E make a fascinating story but unfortunately are beyond the scope of this essay.

#### *The Friedewald Formula*

Of all of these physicians’ do-it-yourself lipoprotein guides, the most durable was published in 1972. Judging

from the composition of average VLDL particles provided by numerous laboratories and from observations made in hundreds of patients with hypertriglyceridemia, we sought a way to estimate LDL concentrations without an ultracentrifuge. Levy and I popularized a crude rule (obvious to the cognoscenti) stating that, from the concentrations of total plasma cholesterol (C) and triglycerides (TG) and the concentration of HDL cholesterol (HDL-C), the amount of LDL cholesterol (LDL-C) can be estimated as  $LDL-C = C - [HDL-C + TG/5]$ , provided that the patient does not have type III. This was hardly higher mathematics but, to establish the validity of this rule, we asked William Friedewald, a biometrics expert in the NHLI, to check this formula independently. Even after the designation of type III will have faded from memory, the Friedewald formula<sup>130</sup> may be remembered forever as the most durable relic of the phenotyping period.

#### *Our Best Sellers*

One of the most useful features of the phenotyping system was that it allowed guidance to more specific treatments for each type. The fundamental first step in therapy was to adjust the diet, including a focus on the patient’s body weight. Physicians, including ourselves, are inept at changing diets, but we were fortunate at the clinical center to have the ample and invaluable help of nutritionists, including, in particular, Ernestine Bou, Mamie Bonnell, Nancy Ernst, and Edith Jones.<sup>131</sup> Their standard diets were often sought by physicians who made their requests in large quantities. Through the courtesy of the U.S. Government Printing Office, we had five different diet booklets (Figure 4) in five differently colored covers designed to respond to the calls from the clinics. When the distribution was finally suspended in the late seventies, more than 7 million (royalty-free) copies are believed to have been requested and distributed.

### Evolution

At a meeting on lipids in Greece in the late 1960s, Hugh Sinclair, a don of Magdelene College, was presiding in his inimitable way at an informal session of lipidologists. While gazing at the Temple of Poseidon at Sunion, he intoned, "Regard these magnificent ruins still standing after 2,000 years, and you, Fredrickson, your classification will be lucky to survive another five!" He was partly right; the end was predictable although it endured for more than 20 years. Its limitations included having ignored HDL, having no claim to genotype, and relying on a nonstandardized test for the diagnosis of type III; we stood naked in the path of the march of molecular genetics toward a modern basis for genotyping.

While an old-timer in the laboratory can make use of the shorthand for an abnormal pattern, the departure of the typing system from the curricula of medical schools here and abroad has spelt relief for many students.

The long-desired specificity of mechanisms as a replacement for this durable expedient arrived gloriously with the revelation of the LDL receptor (LDLR, see Figure 1) in 1974.<sup>132,133</sup> Several years earlier, the appearance of familial combined hyperlipidemia and its mockery of phenotypic variation within families<sup>134-136</sup> further weakened the foundations of a structure that even I had ceased to support, although my disillusionment had been of another kind.

By 1970, I had lectured on phenotyping literally around the world. On such a journey visiting tiny hamlets in New Zealand, great cities in Australia, clinics in India, and many other places between those and America, I had found some things to cheer. Certainly, one was the nearly ubiquitous photographs on the laboratory walls of the six tubes of plasma, one for each type. Decidedly unpleasant, however, was the booming commercial market everywhere for electrophoretic strips stained with oil red O, and they were badly interpreted. Two years later, even institutes in the Soviet Union had fallen under the spell, and yet the conventional laboratory was unable to confirm a diagnosis of type III. In 1975, I dispatched an editorial to *Circulation* entitled "It's time to be practical":

"Ten years ago, we recommended in this journal the use of lipoprotein patterns for identification of different groups of familial hyperlipidemia . . . Technical shortcomings (still) exist . . . Commercial laboratories cannot provide reliable or quantitative lipoprotein patterns, and preparative ultracentrifugation is still necessary to make a certain diagnosis of type III hyperlipoproteinemia . . . Upon finding a subject with hyperlipidemia, the physician is likely to wonder if he should now obtain a lipoprotein electrophoretic analysis. At present, my answer, directed to the generalist, is negative . . ."<sup>137</sup>

By the time the editorial appeared, I was occupying the chair of the director of the NIH. Those exhilarating days among the lipoproteins were over. As I reflect upon the grand and competitive struggle that it has been, I realize that it had been a salient advance, working upward over newly opened routes to establish a base camp. From there would begin the scaling of the high peaks that slowly became visible as we climbed. Our successors in these steeper ascents needed the magic of molecular biology, a technology beyond our

imagining in the early 1950s. The result has been an astounding trail of achievements already left by our successors—the LDL receptor, the determination of the structure of apoprotein B and the regulation of the synthesis of its B<sub>100</sub> and B<sub>48</sub> components, the emerging view of the relation between HDL and triglyceride-rich remnants as subtended by the cholesterol transfer protein, and the chromosomal localization of the apoprotein genes and abnormalities under genetic control—these are only some of the enormously gratifying scientific advances since 1975.

The extension of scientific knowledge and epidemiological data concerning lipoproteins into health practices is, of course, an even more significant contribution to humanity. The paths yet to be traversed remain an impressive challenge. I owe a great debt to those who climbed with me ("Note 17"); I don't think any of us would have missed it for the world.

### Notes

#### Note 1

To John Gofman, Pederson's lengthy report of his struggles with the X protein was an irresistible challenge. His successful conquest of this problem was also attributable to the talents of E.N. Pickel for building his first instrument and to the support of J.H. Lawrence, head of the Donner Laboratory, who found a way to pay for another centrifuge and donated space in his laboratory. Lindgren, a physical chemist and Gofman's very able "right hand," has described<sup>20</sup> how the way to resolve Pederson's difficulties with the pile-up distortions at the albumin boundary came to him one night when he had fallen asleep on the AnUC while working in the Donner Laboratory. In 1990, the laboratory met once again, and Gofman left a valuable transcript describing old memories from the early 1950s. I am grateful to Ron Krauss for an opportunity to peruse these memoirs.

#### Note 2

I had the opportunity to meet academician N.N. Anitschkow in 1963 as a member of one of the first cardiovascular groups to visit the USSR in the post-Stalin period. We walked up the street of life from the obstetrics pavilion to the morgue at the medical school in Leningrad. There, dressed in a white surgical gown and cap, Anitschkow handed out rabbit aortas stained with oil red O. When asked about his constant cigarette smoking, he said "Cholesterin ist Alles."

#### Note 3

Some called it the "uncooperative cooperative study." Four laboratories—the Donner, the Cleveland Clinic, the University of Pittsburgh, and Harvard School of Public Health—examined more than 15,000 men aged 40–59 years and studied 4,914 of them in a 4-year prospective study. The cast of characters involved was stellar. The new events were judged by no less a panel than Paul D. White, Samuel Levine, and Howard Sprague.

The challenge had been given and accepted prematurely. The Gofman team was forced to change the rules as they gained more knowledge. Starting with the S<sub>f</sub> 10–20 class, they then insisted that 12–20 was the

proper one; later, they insisted on inclusion of the 12–100 band. Corrections for the effect of concentrations on flotation rates were also eventually demanded, and, finally, only the Donner group was measuring  $S_f$  100–400 lipoproteins.

The divided conclusion pitted the opinion of laboratories in the East against those in the West, although there appeared to be agreement that cholesterol appeared to be the most effective in separating out the new events, and  $S_f$  12–20 was the least so. With Gofman in a minority position, his introduction of an “atherogenic index” triggered further polemics.

#### Note 4

After 1955, the Donner Laboratory continued to produce important studies of lipoproteins and is still doing so today. Lindgren has recently retired, but Alexander Nichols and Trudi Forte, who made some of the first electronmicroscopic studies of lipoproteins, are still there, and Ronald Krauss, one of our valued coworkers in Bethesda, is now in charge. During the 1960s and 1970s, Bernard and Virgie Shore, at the Livermore Laboratory, collaborated with the Donner group in many key advances in our knowledge of apolipoproteins.

#### Note 5

Dr. Shannon became the director of the NIH in 1955 and served until 1968, the period of the most rapid growth of the extramural programs of the NIH. Anfinsen was awarded a Nobel Prize for Chemistry in 1972 for studies showing how the primary structure of a protein also dictated its conformation. Before we clinical associates arrived, Anfinsen’s recruits had included Raymond Brown, Edwin Boyle, Joseph Bragdon, Daniel Steinberg, Martha Vaughan, James Baxter, Howard Goodman, and Majorie Horning. Soon, Howard Eder joined us from New York and Edward Korn arrived with a fresh degree in biochemistry, later followed by Martin Rodbell, who shared the lab with Korn.

#### Note 6

In 1965, I was invited to Mosbach by Professor Ernst Klenk to address the annual gathering of the Gesellschaft für Physiologische Chemie on the subject of lipoproteins.<sup>72</sup> I took along the figures showing lipoproteins that today appear absurdly simple and used ABL and Tangier disease again to emphasize the special functional nature of these “no doubt” specific proteins, called simply A and B. After I had finished, Professor Klenk’s docent, Willi Stoffel—now Klenk’s successor—said to me, “You did all right, but they don’t believe a thing you said!” At a later meeting in Cologne in 1970, I was gratified when Klenk introduced me with the admission that I had “made him believe in lipoproteins.”

#### Note 7

Eventually, we were to reap the reward for having devoted so much laboratory space to equipment and to other preparations for sequencing an apoprotein that might finally be caught; this was considered one of the higher prizes of the competition in those days. The first two sequences (apo A-II and C-III) became lab trophies in 1972–1974.<sup>81,82</sup>

#### Note 8

In preparing this history, I noted from a Harvey Lecture<sup>86</sup> and another presentation<sup>87</sup> in 1972 that I was using both terms for each apoprotein while not sparing the reasons for our reluctance to adopt a system that was somewhat metaphysical. When I telephoned Petar Alaupovic recently to jog my memory of the precise moment of surrender, he immediately and graciously gave me the answer. Apparently, I had sent a coworker to deliver a paper at a meeting on serum lipoproteins in Austria in October 1973. Alaupovic has even supplied me with a copy of the abstract containing the concession. Noting that (Peter Herbert’s) “presentation was very well delivered and accepted by all participants, but especially my colleagues and myself,” Alaupovic gives more details of the great feast afterward and of Herbert’s toast, in which the latter purportedly said, “You know, Fredrickson sent me into this ‘enemy’ camp to deliver his message that I had not even had time to digest properly on the flight . . . I was really scared at first, but now I find you to be quite all right, at least after a few glasses of wine and beer.” (P. Alaupovic, 1991; personal communication). I suppose that we may denote this brief footnote in the history of lipoproteins as the Concordance of Graz.

#### Note 9

My first meeting with Thannhauser was in 1949 at the instigation of my prospective mother-in-law, who was visiting from the Hague. I shall never forget when, over the dinner table at his home, the professor was asked by Mevrouw Eekhof-Bos what it meant that I intended to pursue a career in biomedical research. “It means, Madame, that your daughter must learn a gainful occupation!” replied Thannhauser.

#### Note 10

All our diagnostic samples were obtained after an overnight fast, with the patients retaining a more or less stable weight. Samples from NIH staff volunteers and several younger cohorts were used to set 90% cutoff points above and below the means in 10-year age increments as normal standards. All values for VLDL, LDL, and HDL were expressed in terms of the content of cholesterol.

We also arbitrarily eliminated all patients with fasting triglyceride levels >200 mg/dL, as there was little normal data available at the time. When, 15 years later, the Lipid Research Clinics compiled their normal standards for thousands of patients, only our triglyceride levels deviated significantly from the means of the new standards.

Occasional technical improvements have allowed a reduction in the complexity and tedium of the original analytical systems. One of the most useful was the discovery by Bernfield<sup>91</sup> and Burstein,<sup>92</sup> the latter at the Centre Transfusion in Paris, and with whom I visited for firsthand observation of his work. It appeared that polyelectrolytes could be used to precipitate LDL and most of the triglyceride-rich lipoproteins, reducing the routine analyses to one overnight ultracentrifugation of plasma at its own density. The tubes were sliced, and the cholesterol in the supernatant was VLDL. LDL was precipitated from the

infranatant fraction, leaving the HDL cholesterol to be determined. LDL was calculated as the difference between plasma C-(VLDL-C+HDL-C).

#### Note 11

There were 23 patients with tuberous xanthomas included in the Donner data, and two were siblings, proving the familial occurrence of the syndrome.<sup>25</sup> The Gofman reports, as did our own, applied reproducible chemical analyses to descriptions of skin lesions that were subjective, historically old in origin, changing over time, and of various etiology.

The vitiligoidea tuberosa of the mid 19th century<sup>100</sup> was due to obstructive biliary disease. At the end of the century, Pollitzer,<sup>101,102</sup> a renowned New York City dermatologist, categorically distinguished xanthoma tuberosum from xanthoma multiplex, and, then, in 1912, published several papers on xanthoma tuberosum multiplex.

We found that tuberous xanthoma lesions over the skin of the elbows and other surfaces were not unique to type III but occurred in patients with familial hypercholesterolemia. Far more characteristic of type III are the yellow-orange deposits of lipid in the creases of the palms and other limbs, which we called planar xanthomas.

Later, following the advice of the Dutch dermatologist Polano, we used the term "xanthoma striatum palmaris."<sup>103</sup> Using a classification scheme that was different from and not compatible with ours, they also found these lesions in several phenotypes.<sup>104</sup> By whatever name, however, these lesions melted away quickly when the patients were treated by diet or administration of clofibrate.

#### Note 12

For several years, Joseph Garland of the *New England Journal of Medicine* had been requesting that we write a review of our work. The rapid development of the field and the priority given to the *MBID* foreclosed a response for many months, but something had to be done. In 1966, I went as usual to the Atlantic City Federation of American Societies for Experimental Biology meetings although this time, I remained in a motel room, filling sheets of foolscap paper and making frequent calls to Levy and Lees for information to fill in the gaps. Garland's acceptance note of the product, with a line stating "Your ms has passed from loving hand to loving hand" was the nicest comment I had ever received from an editor.

The *New England Journal of Medicine* had, at that time, an agreement with Little, Brown & Co. that offered authors an opportunity to reproduce the longer reviews as a monograph, provided that no reprints were issued. I requested that Robert Berliner allow us to order 10,000 reprints instead. He agreed, and 95% were gone within a year. In Garland's "citation classics" for the years 1961-1975, I was flabbergasted to note that, no doubt as a result of this publication, I had emerged as the "most cited physiologist" during that period.<sup>112</sup>

#### Note 13

The Framingham Study added LDL and HDL measurements to its annual analyses in the late 1960s. The key question at this time was whether the diet of the population should be swayed toward more unsaturated fats. In 1969, the first such attempt to compare polyun-

saturated with ordinary fat in a diet was reported in a Veterans Administration domiciliary population.<sup>114</sup>

Before this report, the council of the NHI had created an executive committee on diet-heart disease, which recommended that a trial of diet in free-living populations should be undertaken in America.<sup>115</sup> I was particularly involved because during 1966-1968, I had assumed the directorship of the NHI while still running my laboratory, with the able help of Robert Levy. By this time, Irvine Page was undertaking feasibility studies in 2,000 subjects as preparation for a massive trial of perhaps 100,000 volunteers. Doubts as to the cost-benefit of such an undertaking arose, and I convened a diet-heart review panel under the chairmanship of Edward H. Ahrens Jr. to reconsider the feasibility of the full trial. The panel concluded that such a trial was economically unwarranted.<sup>116</sup> This, in turn, was considered by an NHLI Task Force on Arteriosclerosis, which decided in 1971 that such field trials should also involve other agents than diet alone to lower cholesterol.<sup>117</sup> This opened the way to large-scale drug trials of prevention of coronary artery disease. During this time, the famed NHLI biometrician, Jerome Cornfield, a man without an advanced degree, was the most influential person on the scene and, for the institute director, an invaluable tutor of the art of clinical trials.<sup>118</sup>

#### Note 14

I left the directorship of the NHI to resume full-time work at the laboratory in 1969, although I was then also director of intramural research of the institute, succeeding Robert Berliner. When Theodore Cooper asked me to convene and chair the panel on hyperlipidemia to explore the question of setting up the lipid clinics, I took pains to invite critics as well as aficionados of the phenotyping system. Among the former, I counted my valued colleague Richard Havel. In addition to Havel, the decision makers included Edwin Bierman, David Blankenhorn, William Castelli, William Connor, Gerald Cooper, Seymour Dayton, Howard Eder, Ivan Frantz, Tony Gotto, DeWitt Goodman, William Friedewald, Frederick Hatch, Peter Kuo, Robert S. Lees, Robert I. Levy, Robert P. Noble, Isadore Rosenfeld, and Daniel Steinberg. A questionnaire was issued to the members in advance and written to afford opportunity for sharp criticisms. We had no desire to include the NHLI Molecular Disease Branch, but we were proud that it was the basic model for the new network.

#### Note 15

Dr. Zdanek Fajfar, a Czechoslovakian cardiologist, as the head of the cardiovascular desk, convened a meeting consisting of J.L. Beaumont, a professor of medicine from Paris, Lars Carlson, head of the King Gustave Institute, Gerald Cooper of the Centers for Disease Control (CDC), T.R. Strasser of the World Health Organization, and myself. It was the humid season in Geneva, which added to the heated discussions on the validity and usefulness of the typing system. Beaumont, then a dean of a medical school, was of the persuasion that much of hyperlipoproteinemia had an immunological basis. Carlson, the proper Swedish skeptic, was not enthusiastic about lipoproteins. Cooper, who was the head of the standards group at the CDC, was helpful in smoothing tempers as we slowly achieved some accord.

A year or two after our affirmative report was issued, I visited Carlson's laboratories in Stockholm and was pleased to be invited to the conference room where, surrounded by piles of lipoprotein strips, everyone was engaged in "reading the wash."

#### Note 16

Havel and Kane used the term dysbetalipoproteinemia for this syndrome rather than type III. Today, the general usage is to reserve the former term for the apoprotein abnormality in the far more numerous members of the families of the very few probands that express the full type III phenotype with hyperlipidemia and its frequent accompaniments. The reason for the expression of the abnormal phenotype in apparently genotypically identical family members is a mystery not yet penetrated.

#### Note 17

I am grateful for the collegiality and contributions of the following who worked in molecular disease: Paul H. Altrocchi, Gerd Assmann, Thomas P. Bersot, David W. Bilheimer, Mariel Birnbaumer, Conrad Blum, Jan L. Breslow, H. Bryan Brewer Jr. (now chief of the laboratory), Nancy Briggs, W. Virgil Brown, James Carter, L.L. Davis, Steven J. Demosky, Schlomo Eisenberg, Charles J. Glueck, Antonio M. Gotto, Heiner Greten, Robert J. Heinen, Lloyd O. Henderson, Peter Herbert, Anne Houser, Kathryn John, Antonio Jover, Ronald M. Krauss, Peter O. Kwiterovich Jr., Terry Langer, John D. LaRosa, Robert S. Lees, Robert I. Levy, Samuel E. Lux, Robert Mahley, Betty H. Masket, Duncan L. McCollister, Michael B. Mock, Joel Morganroth, Arne Norman, Katsuto Ono, Nancy Priddy, Stephen H. Quarfordt, Allen Rider, Basil Rifkind, Rosemary Ronan, T. Shiratori, Richard Shulman, Howard R. Sloan, Neil J. Stone, Katherine Wehrly, Lee Witters, and Oscar Young.

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