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Author's Choice

Chance and Serendipity in Science: Two Examples from My Own Career

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The usual scientific paper follows a rather narrowly (but not ever rigidly) defined pattern. Both the author and the journal like to see a linear logical presentation of a "story." Seldom does the paper give the reader the "backstory." Where did the idea come from in the first place? How many false leads led down blind alleys? What happened by chance and what by logical planning? Was there an element of serendipity involved? Perhaps as we enter the paperless era and do not have to count words quite so religiously, it may be possible to encourage a more freewheeling scientific paper, but for now, we have to rely on the historians of science and/or those who "tell all" about their own research. "Reflections" seems an appropriate space for the latter. I have chosen two scenarios from my own career in which happy accidents played important roles but, unhappily, received little recognition in my published papers.

Act I: Phytanic Acid Storage in Refsum Syndrome

Scene 1: The Background—In the early 1950s, I was working at the National Heart Institute in Bethesda, Maryland, in the laboratory of Christian B. Anfinsen, under whose guidance I had done my Ph.D. thesis research at Harvard University. The thesis had dealt with protein structure and biosynthesis, but I wanted to get into an area that might use my medical background (M.D. obtained in 1944 from the Wayne State University College of Medicine, Detroit, Michigan) to better advantage. More specifically, I was trying to understand lipid and lipoprotein metabolism in relation to atherogenesis. One of my projects was to explore the feasibility of preventing coronary artery disease by using inhibitors of cholesterol biosynthesis to lower blood cholesterol levels. Donald S. Fredrickson and I had proposed this possible approach and had presented some preliminary findings suggesting that it would work (1, 2). I pulled together the available literature on this and other chemotherapeutic approaches to control of hypercholesterolemia in 1962 (3). It was a bustling field, but nothing very useful had surfaced yet. A new drug, triparanol, had just been approved for lowering blood cholesterol levels, and it was reported to work by inhibiting cholesterol biosynthesis (4). Triparanol (Merrell Dow) had been shown to decrease conversion of labeled acetate to nonsaponifiable lipids *in vitro*. Our laboratory got involved in determining at what step in the synthetic pathway triparanol worked. With DeWitt S. Goodman, Joel Avigan, and other National Institutes of Health (NIH) collaborators, we were able to show that triparanol blocked the very last step in cholesterol biosynthesis, the conversion of desmosterol to cholesterol (5, 6). Unfortunately, triparanol proved to have unacceptable side effects in patients and was withdrawn from the market. All of this is simply to provide background to explain why my antennae were very much tuned into cholesterol biosynthesis at the time I first became aware that there was such a thing as phytanic acid.

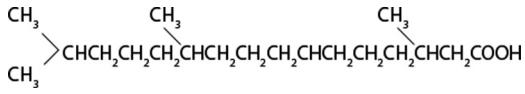

FIGURE 1. **Structure of phytanic acid.**

Scene 2: Refsum Syndrome Is Recognized as a Unique Neurologic Syndrome—In 1946, Sigvald Refsum, a distinguished neurologist in Oslo, described for the first time a syndrome characterized by retinitis pigmentosa, cerebellar ataxia, sensory and motor peripheral neuropathy, and a number of other less constant features (7). The syndrome was very clearly familial. Several siblings were affected in each of the several families under Refsum's care. Most of these came from the west coast of Norway, where consanguineous marriages were fairly common at the time. The villages along the fjords were accessible only by sea and were totally isolated during the winter freeze, a setting likely to increase prevalence of recessive disorders.

This syndrome was one of a very large basketful of similar neurologic syndromes, but Refsum, an astute clinician, was convinced that it was unique. He proved to be right. However, there was no clue yet to the underlying genetic or biochemical basis.

Scene 3: Klenk and Kahlke in Germany Demonstrate That Refsum Syndrome Is a Lipidosis with Accumulation of Phytanic Acid—Donald S. Fredrickson, one of the pioneering editors of *The Metabolic Basis of Inherited Disease*, was a close colleague of mine in the Anfinsen laboratory in Bethesda. One day, I was reading his article on lipidoses, in which he referred to a recent report by E. Klenk and W. Kahlke. They had been sent tissue samples from a patient with Refsum syndrome, a rare neurologic disorder, and found them to be loaded with phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) (8). This highly branched fatty acid accounted for fully 50–70% of all fatty acids present in the liver and kidney! To me, and I am sure to anyone else familiar with the pathway of cholesterol biosynthesis, the structure of phytanic acid, shown in Fig. 1, immediately suggested that the disease might be due to a metabolic error that added a fourth isoprenyl group to farnesol. The resulting 20-carbon compound, geranylgeraniol, could then undergo saturation of its double bonds and oxidation of its hydroxyl group to yield phytanic acid as the end product. If that were the mechanism, then inhibition of the cholesterol biosynthetic pathway could control Refsum syndrome. As mentioned above, Don Fredrickson and I had done studies suggesting the feasibility of inhibiting endogenous cholesterol synthesis to lower blood cholesterol levels. Why not try to do this in these patients, who we assumed were overproducing terpenes derived from the same pathway? At that time, the biosynthesis of geranylgeraniol had been described in plants, but there was only one paper describing it in animals (pig liver) (9). We were not perturbed by that. Refsum syndrome was, after all, the result of a very rare mutation that might be amplifying greatly what was normally only a minor pathway. We decided to push on with it, find some patients, and show that their blood phytanic acid was synthesized, like cholesterol, from acetate via mevalonic acid.

Scene 4: Looking for a Patient with Refsum Syndrome and Winding up in Oslo—Excited about the prospect of proving our hypothesis, we immediately began asking neurologists at NIH and around the country if they were seeing any patients with the syndrome. We came up empty, so I wrote to Professor Refsum himself in Oslo and asked if we could interest him in collaborating with us on a project utilizing the patients he continued to follow in his clinic in Oslo. Within a week or so, I was en route to Oslo with a vial of [2-^{14}C]mevalonic acid in my luggage (probably illegal even then, but hopefully the statute of limitations will protect me now, fifty years later). I took a room at the Hotel Bristol in Oslo, and I was warmly welcomed by Refsum and his colleagues, including Lorentz Eldjarn, Oddvar Stokke, and Kenneth Try, biochemists from the Institute of Clinical Biochemistry at the Royal Hospital. One of Refsum's patients, Frau E. T., had already been admitted to the hospital, and within the week, we had administered the [^{14}C]mevalonate. I took blood samples at intervals over the next week and shipped the extracted serum lipids back to my colleague Joel Avigan in Bethesda for analysis. The word came back quickly: there was the expected amount of ^{14}C in the serum cholesterol but absolutely *none* in the serum phytanic acid! My first reaction was that there must be a mistake of some kind, but in each and every sample over the next days, the results were the same: no label in the phytanate but the expected amounts in the cholesterol. The possibility that phytanate might be synthesized by some new pathway independent of mevalonate seemed most unlikely. (Later studies by our group at the Clinical Center in Bethesda would conclusively rule out this possibility by showing that there was no D_2O incorporation either (10).) So, there I was in the Hotel Bristol in Oslo with an attractive hypothesis refuted by some ugly data.

If phytanate was not being biosynthesized endogenously, it must be entering from an exogenous source, almost certainly dietary, and accumulating because of a defect in the metabolic pathway for its degradation

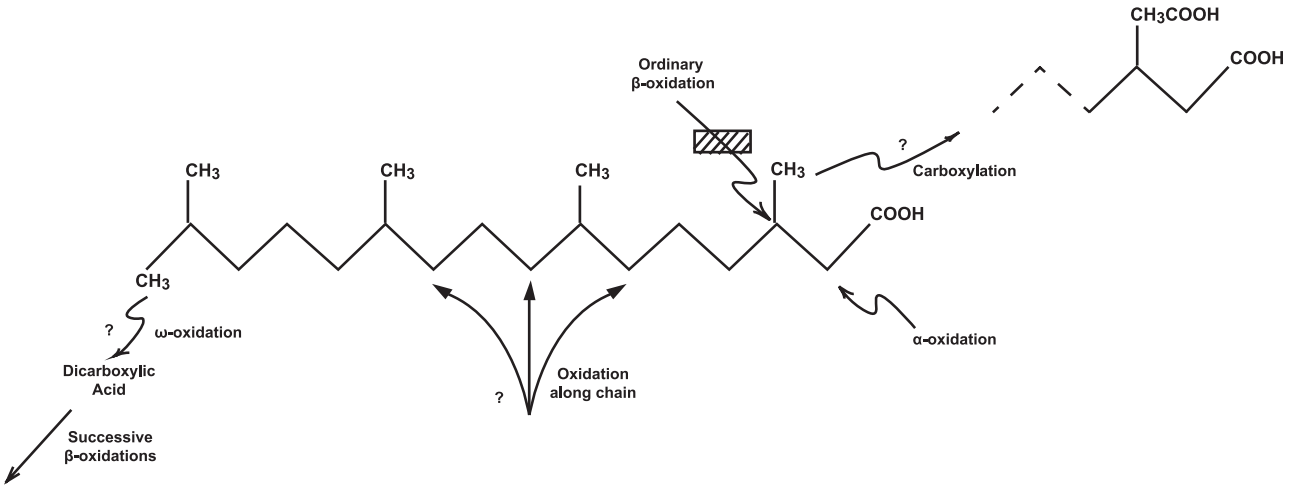

FIGURE 2. **Phytanic acid catabolism.**

and/or excretion. A quick trip to the library confirmed what I suspected, namely that there was absolutely nothing known about the metabolism of phytanic acid and very little about its occurrence in foodstuffs. Did we want to get into an entirely new area of research? This was no longer a problem relating in any way to cholesterol metabolism; inhibitors of cholesterol biosynthesis were not going to help these patients. On the other hand, the pathway for degradation of phytanic acid might not prove too difficult to figure out. If we knew the mechanisms involved in its degradation, we might find ways to accelerate it. Even if we were unsuccessful in that, we might be able to help these patients by removing phytanic acid-containing foods from their diets. After consultation with Avigan, Goodman, and my other colleagues in Bethesda and with the blessings of our scientific director at the National Heart Institute, Robert W. Berliner, we decided to pursue the project. After all, here was one of the first examples of a spinocerebellar disorder with a well defined metabolic basis. Understanding its pathogenesis might shed light on the mechanisms underlying others. As J. B. S. Haldane wrote in his essay on "The Future of Biology," " . . . there is one general law to be noted. The unexpected always happens."

Scene 5: What Is the Normal Pathway for Phytanic Acid Catabolism, and Where in That Pathway Is the Defect in Patients with Refsum Syndrome?—Phytanic acid catabolism had never been explored. The classic β-oxidation pathway would not work because of the methyl substitution at carbon 3 (Fig. 2). Several options suggested themselves.

One option, the one we favored, was that the carboxyl carbon might be removed first by α-oxidation, leaving as the first product 2,6,10,14-tetramethylpentadecanoic acid (pristanic acid). With the first methyl substitution now at carbon 2 instead of carbon 3, β-oxidation could proceed down the full length of the fatty acid. Lorentz Eldjarn and his group at the Royal Hospital in Oslo favored a second option, namely that degradation might proceed from the ω-end after conversion of carbon 16 to a carboxylic acid. They obtained some preliminary evidence for an ω-oxidation pathway, but it proved not to be a major pathway. We had, *by chance*, bet on the right horse and were able to show that pristanic acid was the major product of phytanic acid catabolism (11) and that the following successive β-oxidation steps were as predicted (12, 13). One of the reasons we were able to wrap it up rather quickly was another lucky coincidence.

It so happened that the LKB Instrument Company was displaying their new gas chromatography-mass spectrometry apparatus in Rockville, Maryland, a stone's throw from NIH. The company invited investigators to come and give their magic machine a try. We just happened to have in the freezer a bunch of samples of lipids extracted from the livers of rats fed large amounts of phytol. With our mass spectrometry expert, Henry M. Fales, leading the way, our team went out to Rockville. Within a few hours, we had unequivocally identified the presence of three of the products expected as the result of successive β-oxidations of pristanic acid (Fig. 3) (13).

Sometimes fortune smiles. We immediately bought one of the new LKB machines.

Once the metabolic pathway was established, the location of the defect in Refsum syndrome was easily identified. Cultured skin fibroblasts from patients oxidized [U-^{14}C]phytanic acid to $^{14}CO_2$ at ~1% the rate seen in normal fibroblasts but oxidized [U-^{14}C]pristanic acid at the same rate as normal fibroblasts. So, the block was at the very beginning of the pathway, compatible with the

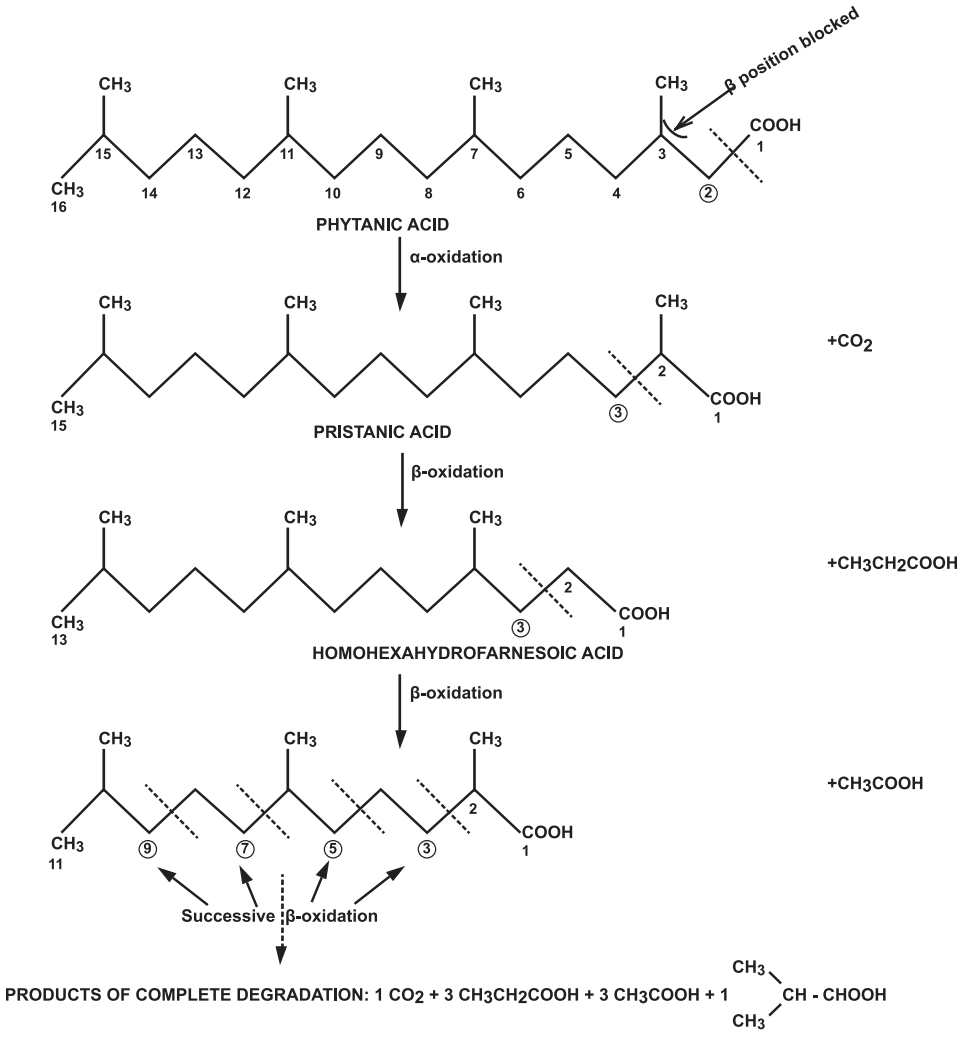

FIGURE 3. ***β*-Oxidations of pristanic acid.**

observation that lower molecular weight degradation products of phytanic acid were not found in the tissues of patients with the disease. Joel Avigan (Fig. 4) was a major contributor to these studies.

Scene 6: What Are the Dietary Sources of Phytanic Acid, Does Reducing Dietary Intake Reduce Body Stores of Phytanic Acid, and Does That Affect the Clinical Course of the Disease?—The library offered little information about food sources of phytanic acid. It was first discovered as a component of beef fat and other ruminant fats. Cheese, butter, cream, and milk were known to be major sources. Also phytol, a component of the chlorophyll molecule, had to be considered as a potential indirect source. Phytol could, in principle, be converted to phytanic acid by simply oxidizing the terminal hydroxyl group to a carboxylic acid and reducing the 2,3-double bond. Indeed, in 1965, we were able to show that both rats and humans can readily convert free phytol to phytanic acid (14, 15). That was quickly confirmed by Klenk and Kremer (16) and Stoffel and Kahlke (17). They, too, were considering phytol to be the most likely source of the phytanic acid in Refsum syndrome patients. And indeed, *unconjugated*

FIGURE 4. **Author (*right*) and Joel Avigan at a conference (circa 1968) where they presented their results on phytanic acid storage disease.**

phytol was a good precursor. However, later studies in our laboratory at NIH showed that only a very small percentage of intact chlorophyll is digested and absorbed; the *covalently linked* phytol simply exits with the feces. In ruminants, however, the rumen bacteria presumably break down the chlorophyll and release *unconjugated* phytol. None of this was known in 1965, so we concocted a diet containing no green vegetables, no ruminant meats, and no dairy products! A nutritionist's nightmare.

Two of Refsum's patients were started on this weird diet, one at the Royal Hospital in Oslo and one as an outpatient, in January 1965 (18). Serum phytanic acid, which is barely detectable in normal serum, was present at 30–40 mg/dl initially. Over the first several months, the plasma phytanic acid level did not budge, and there were long faces all round in Oslo and Bethesda. Finally, after about six months, the levels began to fall, and by one year, they were down to 20–35% of the starting values. The hypothesis was still alive. Presumably, the initial lag represented the slow mobilization of phytanate out of tissue stores. Case 1, a 25-year-old man, showed a striking increase in ulnar nerve velocity (from 8 meters/s in January 1965 to 14 meters/s in October 1965 and 19 meters/s in January 1966). The clinical impression of the neurologists was that his ataxia had improved somewhat and that peripheral nerve function might have improved. Case 2, a 45-year-old woman, showed no clear signs of clinical improvement.

To test more critically whether or not mobilization of phytanic acid stores really improved clinical manifestations, we recruited two patients from Ireland, J. S. and K. S., a brother and sister who had advanced disease (19). They had high serum phytanate levels and were willing to come and stay in the NIH Clinical Center for at least a year on the phytanic acid-"free" diet. They were admitted in June 1966, and after a base-line period, during which their neurologic status was carefully evaluated, they began the diet. Over the next year, plasma phytanic acid levels dropped by >50%, and there was objective evidence of clinical improvement. There was an increase in ulnar nerve conduction velocity and muscle strength; a return of some previously unobtainable reflexes; an improvement in pain, light touch, and position sense; and an improvement in objective tests of coordination (19). Unfortunately, but not surprisingly, there was no improvement in vision or hearing.

Epilogue—So, we started out with a logical but erroneous hypothesis, expecting to find a previously undescribed variation in the cholesterol (isoprenoid) biosynthetic pathway and a genetic defect that enhanced it. Instead, we wound up working out the details of a previously undescribed pathway for the degradation of a minor dietary fatty acid. Did I or do I regret this diversion from my major focus at the time: cholesterol metabolism and atherosclerosis? Not at all. The several years spent working out the mechanism involved in this disease were full of excitement. We showed that the clinical manifestations were directly related to stored phytanate by demonstrating clear-cut improvement in objective tests of neurologic function when dietary sources of phytanic acid were sharply reduced and the patients' tissue stores of phytanate fell. I particularly treasure the letter I received from our Irish patient J. S. about seven years after he and his sister left the Clinical Center. A few excerpts will make it clear why.

> Dear Professor Steinberg,
>
> This is your patient, Jim . . .
>
> I sure have a lot to be thankful for, and a very great lot of thanks goes to Professor Steinberg. What a change from not been able to tie my boots and fasten buttons, or hardly type, now I can work again with very small screws, like electric plugs, I can put on the wires . . . Professor Steinberg, I've some very big news for you . . . Are you sitting down? ha! I got married early in January . . . Bridie is expecting our baby this month.

Today, neurologists are on the alert for the rare syndrome described by Refsum and initiate diets free of phytanic acid as soon as the diagnosis is made. For an update on phytanic acid storage disease, the role of peroxisomes, and the molecular details of phytanic acid oxidation, I suggest some excellent recent review articles (20, 21).

One last comment: none of this might have happened had I not been working at NIH in Bethesda. One of the incentives for establishing the Clinical Center was to make possible long-term studies like this study of unusual patients with rare disorders. Where else would we have been able to tie up two hospital beds for over a year to prove that a low-phytanic-acid diet not only reduced plasma levels but also led to objectively demonstrable clinical improvement?

Act II. Oxidatively Damaged LDL and Its Role in the Pathogenesis of Atherosclerosis

Scene 1: The Background—Atherosclerosis, the arterial disease responsible for most heart attacks, begins with the appearance of the so-called fatty streak. This lesion is characterized by an accumulation of cholesterol-loaded macrophages just beneath the monolayer of endothelial

cells lining the artery wall. These macrophages are called foam cells because of the frothy appearance of their fat droplets under the microscope. They are derived from circulating monocytes that have penetrated into the subendothelial space and taken up residence there, and most of the cholesterol they contain has been shown to come from circulating LDL. In 1979, Joseph L. Goldstein, Michael S. Brown, and co-workers made the surprising observation that monocytes/macrophages incubated even with very high concentrations of native LDL took it up very slowly. Moreover, they progressively decreased expression of their LDL receptors as their cellular cholesterol level started to increase, thus preventing any further cholesterol accumulation. They simply could not be converted to foam cells, at least under these *in vitro* conditions (22, 23). Now, here was a paradox. Monocytes/macrophages were generally acknowledged to be the precursors of the cholesterol-loaded foam cell, and the bulk of the cholesterol in them was known to arise ultimately from plasma LDL. To resolve the paradox, Brown and Goldstein proposed that plasma LDL must first undergo some kind of structural transformation into a form more readily recognized and taken up by the macrophage. They set out to identify such a modified form and in the process discovered that chemically acetylated LDL was recognized and taken up by the monocyte/macrophage much more readily than native LDL, rapidly enough to generate foam cells. The binding was specific, and they attributed it to a "scavenger receptor," which was later cloned and fully characterized (SRA1) by Tatsuhiko Kodama in the Krieger laboratory (24). However, acetyl-LDL has never been reported in blood or tissues. Despite intensive efforts, the nature of the postulated biological modification that converted native LDL to a form that could generate foam cells remained unknown (25).

Scene 2: A Chance Convergence of Interests and a Little Game of Musical Chairs—Intrigued by the findings of Goldstein and Brown, we at the University of California San Diego in La Jolla, probably along with investigators in many other laboratories, were trying various modifications of LDL in search of the postulated but elusive modified form favored by the macrophage. One of the things we tried was subjecting LDL to partial proteolysis (26), but that did not work, nor did a number of other enzymatic treatments. Also, the nature of the macrophage receptor for acetyl-LDL and its properties remained unclear. I felt I needed a better understanding of macrophage biology and wrote to Zanvil Cohn, an outstanding biologist and a world expert on the immune system, asking if I could spend a mini-sabbatical with him at The Rockefeller University. Meanwhile, at almost exactly the same time, a young investigator at the Royal Hospital in Oslo, Tore Henriksen, was studying the behavior of endothelial cells under various culture conditions. He observed that vascular endothelial cells growing in a medium containing LDL deteriorated over a 24-h incubation, lifted off the dish, and began to die (27). Independently, and again at about the same time, similar findings were reported by James R. Hessler, Abel L. Robertson, Jr., and Guy M. Chisolm III at the Cleveland Clinic (28). Henriksen wrote me to tell me about his findings and to ask if he could come to La Jolla to learn more about lipoproteins. The third party in this game of musical chairs was Eileen M. Mahoney, who had just completed her Ph.D. thesis under Zanvil Cohn's direction. She wanted to come to La Jolla as a postdoctoral fellow. So, Henriksen came to La Jolla from Oslo, Mahoney came to La Jolla from New York, and Steinberg went to New York from La Jolla (Fig. 5).

FIGURE 5. **Author (*center*) with Eileen Mahoney and Tore Henriksen, the group that published the first paper on endothelial cell oxidation of LDL in 1981.**

Scene 3: Overnight Incubation with Vascular Endothelial Cells Drastically Alters LDL Structure and Properties—Henriksen came to La Jolla hoping to figure out how LDL damages endothelial cells. He duplicated his Oslo experiment for us, showing that 24 h of exposure to LDL was enough to cause a large fraction of the cells to lift off the dish; by 48 h, many were necrotic. We set up a microscope in the warm room to do time-lapse photography, and the film was awesome. Within a few hours, the cells began to show blebbing, and as you watched those blebs, they occasionally seemed to be rushing up at you from the cells' surfaces. If LDL was omitted from the medium or if just 5% fetal calf serum was added along with

the LDL, the cells looked just fine. We urged Henriksen to first explore what the endothelial cells might be doing to the LDL during these dramatic incubations. Well, he and Mahoney quickly showed that the LDL at the end of the incubation was radically altered (29–31). It was much denser, had an increased negative charge, and had lost a large fraction of its phospholipid. This modified LDL, which we unimaginatively named "endothelial cell-modified LDL," filled the bill for the postulated modified LDL recognized by the macrophage scavenger receptor. It was taken up rapidly by the macrophage and caused cholesterol to accumulate, and unlike the LDL receptor, it was not down-regulated as the macrophage cholesterol content increased. However, we were still in the dark as to the mechanism(s) involved in this endothelial cell-induced modification.

Scene 4: Two Happy Accidents Put the Finger on Oxidative Damage as the Basis for Generation of Endothelial Cell-modified LDL and Point Us to an Effective Antioxidant for in Vivo Studies—As mentioned above, Chisolm and his colleagues had independently reported on the cytotoxicity of LDL previously incubated with endothelial cells (28). They went on to show that the cytotoxicity depended on oxidative damage to the LDL (32, 33). Now, we had considered oxidative damage as a possible mechanism for the altered affinity of endothelial cell-modified LDL for the macrophage scavenger receptor. In fact, Henriksen had tried to prevent the endothelial cell damage by incubation under anaerobic conditions. Those efforts failed probably, in retrospect, because traces of oxygen remained in the flasks even though they had been flushed with nitrogen. In any case, we were looking for alternative mechanisms when a happy accident put us on the right track.

One of our postdoctoral fellows came to us one day puzzled because his experiment had failed: the LDL he had harvested after overnight incubation with endothelial cells was not taken up rapidly by macrophages. Another fellow, Urs P. Steinbrecher, working with Joseph L. Witztum, pointed out that the negative experiment had been done using a different medium (DMEM) than that used in all our previous studies with endothelial cells (Ham's F-10 medium). Comparison of the compositions showed that F-10 contained more copper compared with DMEM, and LDL oxidation was known to be very efficiently catalyzed by copper. We were quickly able to show that the addition of antioxidants (*e.g.* vitamin E or probucol) to the medium completely blocked the endothelial cell modification of LDL (34). The next logical step was to test whether an antioxidant could prevent or slow atherogenesis in an experimental animal model. But which antioxidant compound should we use? There were no guidelines. Effectiveness *in vitro* certainly need not predict effectiveness *in vivo*. There were too many choices.

Chance entered yet again. One of our postdoctoral fellows, Marek Naruszewicz from Poland, was studying the mechanism of action of a new drug, probucol, which had been approved by the Food and Drug Administration for clinical use in lowering plasma cholesterol levels. Initial clinical results with the drug had been encouraging, especially a report that treatment of patients with familial hypercholesterolemia caused objectively demonstrable regression of tendon xanthomas (35). Naruszewicz was using cholesterol-fed LDL receptor-deficient rabbits as his model, so he could provide lots of LDL for other investigators in the laboratory. One day, one of the young investigators in the laboratory, perhaps Sampath Parthasarathy, carried out a study on endothelial cell oxidation of LDL but got no changes at all! It turned out that the LDL he had used was from one of Naruszewicz's probucol-fed rabbits. We were able to show quickly that probucol was indeed a very effective blocker of LDL oxidation (36). We immediately decided to go with probucol, a compound already known to be safe and effective in clinical use.

Using LDL receptor-deficient rabbits as the model, Carew *et al.* (37) showed that probucol dramatically slowed atherogenesis. Lesions in the aortas of the treated group were reduced by >60%. Because probucol treatment did lower LDL, albeit not markedly in these receptor-deficient animals, we added a small amount of extra cholesterol to the feed of the probucol-treated group so that the plasma LDL levels were the same in the two groups. Kita *et al.* (38) in Kyoto independently carried out a similar study with similar results, although they did not try to equalize the plasma LDL levels in the two groups. Needless to say, these results caused quite a stir in the atherosclerosis field and encouraged a large number of laboratories to begin exploring various aspects of the oxidative modification hypothesis.

Scene 5: Oxidized LDL Is Strongly Implicated in the Pathogenesis of Atherosclerosis in Experimental Animals—The hypothesis that oxidative modification of LDL might be an important process in atherogenesis proved to be strongly heuristic (39–41). In the first decade after it was put forward, in 1981, PubMed listed about seventy papers under "oxidized LDL and atherogenesis." And in the next decade, that number grew to more than 1000. During the past ten years, an additional 2000 papers have

been published and listed under "oxidized LDL and atherogenesis." This is not the place to try to review this vast literature, but a few key points may be in order as context for the following scene. A number of detailed reviews are available (42–45). In brief, what has been shown is (*a*) that oxidized LDL is present *in vivo*, in humans and in experimental animals, both in plasma and in atherosclerotic lesions; (*b*) that plasma levels of oxidized LDL are higher in atherosclerotic animals and in patients with extensive atherosclerotic disease; (*c*) that incubation with cells overexpressing 12/15-lipoxygenase can convert LDL to a form like that found after incubation with endothelial cells; (*d*) that knocking out the gene for 12/15-lipoxygenase reduces the extent of lesions in apolipoprotein E-deficient mice; (*e*) that several different antioxidants, antioxidants with widely differing structures and with different mechanisms of action, can slow progression of experimental atherosclerosis; and (*f*) that atherosclerosis in several different animal models has been shown to respond to antioxidants, including LDL receptor-deficient rabbits, cholesterol-fed rabbits, LDL receptor-deficient mice, apolipoprotein E-deficient mice, and cholesterol-fed monkeys and hamsters.

By 1991, the evidence supporting the oxidative modification hypothesis was so strong that the National Heart, Lung, and Blood Institute convened a workshop to discuss the advisability of planning clinical trials (46). The expert committee recommended that clinical trials were clearly warranted by the available basic and experimental findings. Because vitamin E and β-carotene were known to be safe, it was recommended that the first trials should be done with those agents. Disappointingly, with almost no exceptions, these clinical trials failed to show benefit. However, as discussed elsewhere (47, 48), the oxidative modification hypothesis is not refuted by the clinical trials to date. Briefly put, the antioxidants used may not have been the most effective in the human disease, the doses may have been inappropriate, or the patients may have been too old and the disease too far advanced to respond to antioxidant therapy. Hopefully, further research at the basic level will provide a framework for revisiting the hypothesis as it may relate to the human disease.

Scene 6: Chance Calls Attention to Immunogenicity of Oxidized LDL and to the Potential Role of Adaptive Immune Systems in Atherogenesis—An important factor contributing to the widespread acceptance of the oxidative modification hypothesis was the seminal work of Joseph L. Witztum (Fig. 6) on the role of the immune system in the pathogenesis of atherosclerosis. How did he get into that? Yes, by chance. He was doing kinetic studies in humans to quantify the percentage of plasma LDL removed via the LDL receptor pathway *versus* the percentage removed by nonspecific pathways. He did this by comparing the rate of removal of native LDL with the rate of removal of heavily glucosylated LDL. The latter is not recognized by the LDL receptor, so its disappearance rate is a measure of the contribution of the nonspecific pathway(s). In most subjects, as expected, the native LDL disappeared more rapidly, accounting for ~75% of total LDL removal (49). However, paradoxically, the glucosylated LDL actually disappeared at a much faster rate compared with the native LDL in three cases. All three of these individuals had diabetes mellitus, and Witztum inferred that they must have endogenous autoantibodies against glucosylated LDL. He confirmed this (50) and went on to show that even the most minimal structural modification of LDL (*e.g.* methylation) was enough to confer immunogenicity (50, 51).

FIGURE 6. **Author (*right*) and Joseph L. Witztum, his longtime collaborator on oxidative modification of LDL in atherosclerosis.**

It was against this background that Witztum launched a systematic study of the autoantibodies against various epitopes on oxidized LDL (52, 53). He showed that there was a positive correlation between the levels of antibodies against LDL and the severity of atherosclerosis both in animals and in the clinic (52, 54, 55). To explore this relationship more definitively, his group immunized LDL receptor-deficient rabbits with malondialdehyde-conjugated LDL (a model for oxidized LDL), which raised the plasma titers of antibodies. It was expected that this would increase lesion severity. However, paradoxically, boosting the titers of antibodies decreased rather than increased lesion severity (56). The mechanism of this protection is not clear but could reflect a slowing of oxidized LDL uptake into macrophages induced by the antibodies. The involvement of the immune system is obviously complex, but there is little doubt about its involvement.

Scene 7: Potential Role of the Innate Immune System Is Revealed by Chance—LDL has a molecular weight of >2,000,000 and contains protein, phospholipids, cholesterol, cholesterol esters, triglycerides, and some other minor component lipids. During oxidation, all of these components will be modified to varying extents, and individual LDL molecules may be affected differently. All of the components, lipid and protein, undergo some degree of oxidative modification. Each of them could represent a separate class of neoantigen (oxidation-generated epitope) that could give rise to corresponding structurally different antibodies. Witztum and co-workers (52, 53) decided to explore this potential heterogeneity of the antibodies against oxidized LDL by cloning splenic cells from cholesterol-fed, apoE-deficient mice; fully one-third of all the hybridomas isolated were producing IgM reactive with oxidized LDL, indicating the intensity of the immune response. Shaw *et al.* (57) then began sequencing the monoclonal antibodies so derived. They were amazed to find that seven of them had precisely the same sequences in the variable regions of the light and heavy chains responsible for antigen binding. Moreover, these sequences were identical to those of a germ-line antibody (T15) described some years previously as a natural antibody against the phosphocholine covalently bound to the cell-wall polysaccharide of *Streptococcus pneumoniae*!

Then everything fell into place. Our earlier studies had shown that one of the oxidation-specific epitopes recognized by the macrophage scavenger receptor(s) and also by monoclonal antibodies against oxidized LDL was oxidized phosphatidylcholine (58–60). Oxidative damage to cell membranes, whether induced artificially (58) or by apoptosis or necrosis (61), can generate similarly oxidized phosphatidylcholine moieties recognized both by scavenger receptors and by antibodies to oxidized LDL. The T15 antibody (E06 in our nomenclature) is an innate antibody preserved in evolution to protect against damage by microorganisms, such as *S. pneumoniae*, and against damage by apoptotic or necrotic cells by virtue of its recognition of phosphocholine-containing antigens. Interestingly, the innate C-reactive protein (CRP) also binds to these same phosphocholine antigens (oxidized phospholipids and phosphocholine on the cell coating of microorganisms) (62).

Epilogue—Chance and serendipity deserve co-authorship at several points along the zigzag pathway we have followed. 1) In 1979, there was a chance convergence of interests and observations involving Oslo, New York, and La Jolla that brought Tore Henriksen and Eileen Mahoney to our La Jolla laboratory and started us on our way to appreciating the potential role of oxidatively modified LDL in atherogenesis. 2) A mistaken use of the wrong incubation medium by a postdoctoral fellow forced us to re-evaluate the role of oxidative processes in converting LDL to a form recognized by the scavenger receptor. 3) While trying to understand the mechanism of action of probucol as a cholesterol-lowering agent, we accidentally discovered that it was also a highly potent antioxidant. We then used this drug to show for the first time that an antioxidant could markedly slow the progression of experimental atherosclerosis. 4) We first became aware of the way in which even very minor changes in the LDL molecule can render it immunogenic as a result of studies with a very different focus. We were using LDL conjugated to glucose to estimate the fraction of LDL disappearing via nonspecific pathways. By chance, we had included a few diabetic subjects, and in these patients, instead of disappearing more slowly than the native LDL, the glucosylated LDL actually disappeared much more rapidly than the native LDL. We interpreted this to mean that these subjects had circulating autologous antibodies that reacted with glucosylated LDL. Further studies established that even the most minimal chemical modification of LDL was enough to make it an effective antigen. These findings led directly into an extended series of studies on the immunogenicity of oxidatively modified LDL. 5) Antibodies against oxidized LDL were cloned from the spleens of hypercholesterolemic apoE-deficient mice and sequenced. Many of these clones were making an antibody identical to a well known germ-line or natural antibody, T-15. This unexpected finding established a role for the innate immune system in atherosclerosis and led us to consolidate and clarify our understanding of the nature of the epitopes in oxidized LDL recognized by macrophage scavenger receptors, their chemical nature, and their relationship to epitopes on apoptotic or necrotic cells and on certain microorganisms.

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⌘ *Author's Choice*—Final version full access.
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