After 40 years of cholesterol-watching

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It seems appropriate on the occasion of this 25th Anniversary of the *Journal of Lipid Research* to ask where the lipid field (as we now know it) came from and where it is going. In *this* chapter I intend to tell the story of our laboratory's concentration on the handling of cholesterol by the human body, how we came to be interested, what we think we have learned, and where these findings lead us. If I do so in rather personal terms, I hope to be forgiven.

Early years with lipid-rich translucent plasma

In the very first year of my laboratory life—aiming, I thought then, at a full-time academic career in endocrinology—I discovered that very large amounts of fat can be present in blood plasma that is completely translucent. It never occurred to me that the exploration of that mystery would lead me away from hormones and into the then charmless field of lipids. Using what would surely today be called blunderbuss methods, I was able to show that plasma phospholipids acted as solubilizing agents for the other fats in the blood (1). What we now call Lp_x permits the transport in plasma of vast amounts of free cholesterol and lecithin; these lipids, in combination with a mixture of proteins, transport triglycerides in the plasma in such finely particulate form that they are invisible to the naked eye.

My initiation to laboratory life under the watchful eye of Donald D. Van Slyke was my first hands-on experience with quantitative biochemical methodology, in which, among other things, I learned the difference between accuracy and precision. The editorial work on that experience led to my first encounter with Peyton Rous, then in charge of the *Journal of Experimental Medicine,* whose admiration of the English language and skill in its usage were exemplary.

The gathering together and the studying of patients with fat-rich translucent plasma was also my first experience in clinical research; the product of those three years' work was a very long and detailed description of a "new" form of liver disease that I called Primary Biliary Cirrhosis. My co-authors on that paper in *Medicine* (2) were invaluable in helping with the clinical management of those women desperately ill with PBC: grossly disfiguring xanthomata, massive hyperlipidemia, yet translucent plasma. Henry Kunkel, my laboratory partner, became interested in the peculiar lipoprotein patterns in this disorder; this led him into free electrophoresis with Duncan MacInnes and Lewis Longsworth and later to the invention of zonal electrophoresis with Arne Tiselius. Aside from guiding the clinical aspects of that study, my personal contribution was the performance of scores of fat balance studies and thousands of measurements of the various plasma lipids—free and esterified cholesterol, total lipid phosphorus, and total lipid carbon by Van Slyke manometry, with triglycerides by difference. It was an initiation to scientific medicine that left strong impressions, the most indelible of which was the realization of how rewarding patient-oriented research can be.

Separation—the key to progress

In those years (1946–49) only a few scientists in New York City could call themselves expert in lipids—Warren Sperry, Forrest Kendall, and Erwin Chargaff, all at Columbia, come to mind. It seems to me that my Rockefeller Institute colleagues working on proteins, carbohydrates, and nucleic acids were interested in lipids only in order to get rid of them! The major problem at that time was purification: how to separate lipids from the other compounds commonly associated with them, and from each other. My own introduction to separation methods was expertly guided by Lyman Craig with whom I spent two exciting and warmly friendly years applying his invention of countercurrent distribution to the separation of long-chain fatty acids and then of bile acids. Down the hall were our talented colleagues in separation methodology, Stanford Moore and William Stein, whose papers on column chromatography of amino acids served all of us as models of clarity, thoroughness, and scientific inspiration.

The first real breakthrough for those of us in lipids came from Bengt Borgström in Sweden, who in 1952 re-discovered and re-defined the separating power of silicic acid first described by Trappe 12 years earlier. Soon after, Jules Hirsch joined my laboratory and initiated a rigorously systematic study of silicic acid columns; our report in 1958 (3) took as its model the standard-setting example of Moore and Stein, of course. This

whole process was dramatically miniaturized when, by 1959, we became aware of Stahl's invention of thin-layer chromatography. It is interesting to note that the first issue of the *JLR* (1959) contains several references to silicic acid columns, but none to TLC!

Hirsch's later work with factice as a solid support showed it to have even greater promise as a separating tool (4), but this innovation never really caught on. (We wanted to see if powdered rubber offered advantages over silicic acid, so we ground up Artgum erasers. Only later did we learn that this product, called factice, in fact was vulcanized soybean oil!) To Hirsch's great credit was his careful demonstration of the sensitivity and precision of differential refractometry for monitoring column eluants; he anticipated the current use of this instrument in high performance liquid chromatography (HPLC) by many years.

The 1950's saw so many innovations in separation methodology that the sleepy old field of lipid research suddenly took off for the moon. Perhaps the most important of all inventions was that of gas–liquid chromatography; it was first described for separations of short-chain fatty acids in 1952 by James and Martin. Later their invention of the gas-density balance made it possible to monitor the GLC outflow continuously; our 1956 home-made apparatus, the first in the USA, was equipped with a density balance built personally by Martin and whipped into reproducible submission by James and Insull; it demanded a charge of only 3 milligrams of mixed long-chain esters! The introduction in 1958 of polar liquid phases by Callen and Orr at Procter and Gamble and by Lipsky and Landowne at Yale made possible the separation of unsaturated methyl esters from their saturated homologs; in 1959 Lovelock's argon ionization detector made the process more sensitive by several orders of magnitude. In our laboratory, Stoffel's painstaking isolation of fish oil fatty acids (5), which are very long-chain and highly unsaturated, showed the immense power of GLC for quantitative analysis of highly complex mixtures. His work (1957–59) was the first to demonstrate the lack of destruction of polyunsaturates at high temperatures, and their obedience to well-defined physical rules, later codified by Woodford in terms of "carbon numbers."

Where GLC was critical to the progress of research in lipids, the contemporaneous development by Gofman and his colleagues of ultracentrifugation for the separation and quantification of lipoproteins was equally as revolutionary.

Dietary fats and plasma cholesterol

The explosion of new methods for dealing with lipids and lipoproteins in the 1950's coincided with the demonstration here (6, 7) that plasma cholesterol concentrations could be predictably affected by the quality of dietary fat. This coincidence of a new physiological finding with the introduction of powerful new separating tools revolutionized our thoughts and actions, and led to a succession of studies hitherto undreamed of, here and elsewhere. The applications of these advances to the field of atherosclerosis research occurred at many levels—epidemiologic, animal models, and basic molecular biology. The numbers of lipid-oriented investigators multiplied exponentially. As funds for research swelled year after year, lipid research hit the Big Time. I feel now—and felt then—that I was terribly fortunate to be in at the beginning of this new field, with important new physiologic findings, new tools, and seemingly unlimited resources at a time when academic medicine was heavily committed to research.

Sterol balance methodology

It was my decision in 1960 to set a new course for our laboratory, namely, to attempt to understand the mechanisms by which plasma cholesterol-lowering followed the ingestion of highly unsaturated fats. To that end, first with Spritz and then with Grundy and Miettinen, we developed a sterol balance methodology (8–10) that allowed us to define with considerable precision the sites of regulation of cholesterol metabolism in man, and the extent to which these regulatory factors vary from one person to another. At the outset we were timid in adopting the techniques of radioisotopic labeling in patients, but the pioneering studies of Gould and Taylor with radiolabeled cholesterol persuaded us to set forth. We have not regretted these decisions, and submit, after 20 years' experience with the combination of isotope kinetics and sterol balance methods, that we can now reliably measure the following regulatory factors in cholesterol metabolism: dietary intake, percent and absolute absorption from the intestine, total body synthesis and its feedback control, conversion to bile acids, pool sizes of exchangable cholesterol, and excretion from any and all sites, best expressed as daily rates per kilogram of body weight.

What have we learned?

A. From the point of view of methodology:

1) Cholesterol is degraded to nonsteroidal compounds (not yet identified) during its transit through the intestine (11); bile acids are not (8).

2) The outflow rate of intestinal contents (usually referred to as variations in fecal flow) varies greatly from person to person, and from day to day in any one person. However, these degradations and variations can be adequately corrected through oral administration of markers (chromic oxide for fecal flow and sitosterol for cholesterol degradation) (11, 12). These corrections must be made in all balance studies, whether in animals

or in humans and regardless of the type of diet ingested, first to test whether these effects occur in the subject under study and then to make the appropriate corrections when they do.

3) The major route of sterol excretion in man is via feces, with very small amounts (less than 5% of the total daily output) excreted in the urine (as steroid hormones) or via the skin (13). Skin surface lipids are rich in cholesterol that is almost entirely synthesized in the skin; only trivial amounts of skin surface cholesterol are in equilibrium with plasma cholesterol (14).

B. From the point of view of physiology:

4) Balance studies and isotope kinetics are applied appropriately only in the metabolic steady state (10), that is, when there is no net change in total body cholesterol pools during the study period. Almost acceptable is the *steadily* unsteady state, that is, when net changes in pool sizes occur slowly but steadily, as in human growth, or during chronic administration of a drug, or with long-term imposition of a dietery change. Accordingly, we have not put much quantitative reliance on sterol balance studies and isotope kinetics in patients with unstable thyroid disease or diabetes mellitus, or in patients undergoing rapid weight loss. Such data may, however, be useful qualitatively.

5) The substitution of polyunsaturated for saturated fat in the diet (at eucaloric intakes) in patients who are normolipidemic or simply hypercholesterolemic causes no significant change in cholesterol synthesis rates or in excretion of steroids from the body (15). In some (but not all) patients with hyperglyceridemia, there is an increased excretion that is greater in amount than that needed to explain their reductions in plasma cholesterol levels (16). The available data are consistent with the proposal that diets rich in polyunsaturates usually cause the transfer of cholesterol out of plasma, probably into the main bulk tissues (muscle, connective tissue, and adipose tissue). Since the amount thus displaced would not significantly increase the cholesterol concentrations in those tissues, it has not been possible to verify this hypothesis. The speed with which plasma cholesterol levels can be made to rise or fall makes it highly unlikely that the "movable" cholesterol goes in and out of the artery wall; this untestable supposition is made credible by the clinical trials of high P/S diets, none of which has shown an *increased* incidence of coronary heart disease.

6) The mechanistic effects of a few lipid-lowering drugs have been defined in terms other than changes in plasma levels. Clofibrate (17) causes no change in absorption or synthesis, but does cause a slow steady efflux of cholesterol from body stores; a rise in cholesterol content of bile leads occasionally to gallstone formation. Neomycin (in low dosage) (18) and sitosterol (in pharmacologic dosage) (19) interfere with cholesterol absorption, which in turn leads to an increase in cholesterol synthesis; nevertheless, a reduction of stored cholesterol probably follows as xanthomata resolve. Cholestyramine (20) interferes with the re-absorption of bile acids with resultant increase in cholesterol synthesis; net efflux of stored cholesterol may occur, but this has not been adequately tested. Ileal bypass surgery (20) causes the same changes as cholestyramine, but to a greater degree.

7) Portacaval anastomosis in patients with familial hypercholesteremia (21) has been shown to cause a marked reduction in plasma cholesterol levels (but not into the "normal" range), with marked reduction in cholesterol synthesis rates and net efflux of stored cholesterol. These advantages have not been of sufficient magnitude to prevent the progression of cardiovascular disease.

Recognition of heterogeneity

I firmly believe that one of our most gratifying contributions is the demonstration in quantitative terms of the heterogeneity that exists from person to person in the regulation of cholesterol metabolism. I will illustrate what I mean in three figures.

Fig. 1 shows how reproducible the effects of a corn oil formula regimen were in any one individual when repeatedly challenged with the same regimen (7); in contrast, **Fig. 2** shows how different the responses were to the same regimen in different individuals. Conclusion: individuals respond to a given dietary fat regimen in the same way month after month; but, given the same regimen, the responses of different individuals vary markedly.

Fig. 3 illustrates the heterogeneity of human responses to changes in cholesterol intake (22); an increased intake and absorption of dietary cholesterol was shown to be either *a*) *well compensated* through increased biliary excretion of cholesterol, or through reduced cholesterol synthesis, or *b*) *poorly compensated,* with net retention of absorbed cholesterol and hence increased cholesterol stores. These various responses were not reflected in or predicted by changes in plasma cholesterol levels; Fig. 3 shows that these plasma increments and decrements were small compared to the other changes we measured.

Such studies in small numbers of inpatients have been extended recently by performance of 76 studies in 51 hypercholesterolemic male outpatients (who were otherwise well). They were fed high and low P/S diets, and on each dietary regimen the effects of high and low cholesterol intakes were measured in terms of absorption, feedback control of synthesis, and plasma levels. As

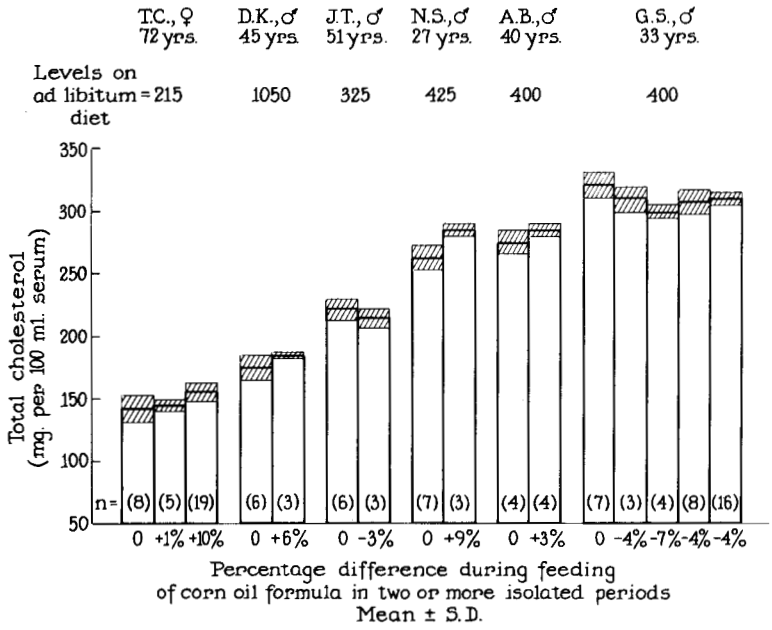

Fig. 1. Reproducibility of plasma cholesterol levels in six patients re-tested on corn oil formula (40% of calories as fat), with other regimens in intervening periods. Bars show mean levels during steady state, hatched area one SD, n = number of data measured weekly during each steady state. Percentage differences along base-line were calculated with reference to mean levels of first feeding periods. (Reprinted with permission of *The Lancet*, ref. 7, Fig. 2.)

expected, plasma levels were always higher in each patient on the low P/S regimen, but in only 20% of trials were the plasma levels increased on the higher cholesterol intake. Seventy percent of the trials showed decreased cholesterol absorption and/or reduced cholesterol synthesis on the higher cholesterol intake, i.e., there was evidence of good feedback control in most of these high-risk hypercholesterolemic patients. None of

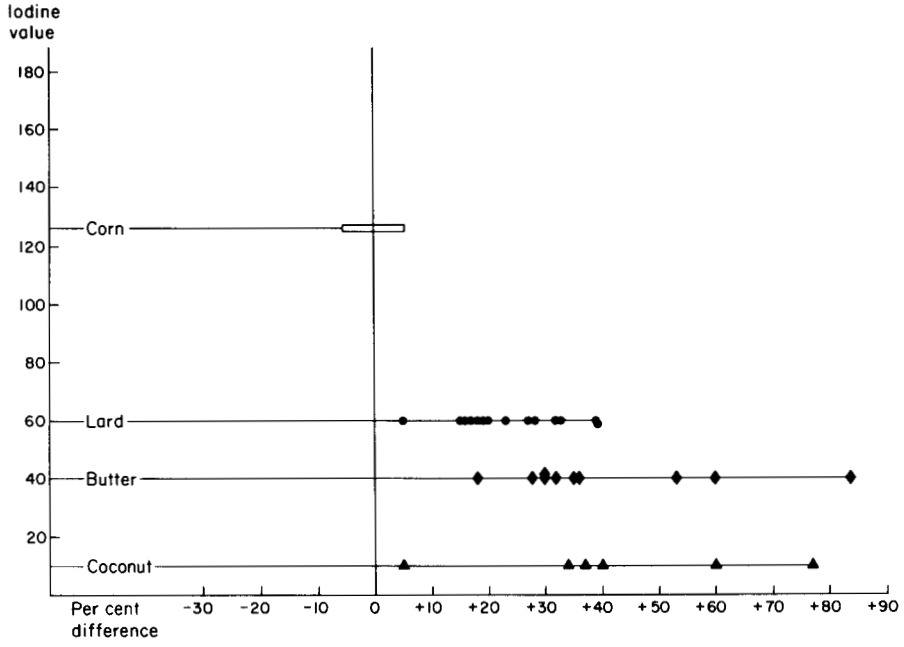

Fig. 2. Heterogeneity of responses in 28 patients' plasma cholesterol levels to formula feeding of three different dietary fats (at 40% of total calories) in eucaloric state. Vertical axis = iodine values of fed fats. Open bar at I.V. 126 = reproducibility of corn oil base-line (see Fig. 1). Horizontal axis = percentage differences in plasma cholesterol levels from base-line established in each patient during ingestion of 40% corn oil formula.

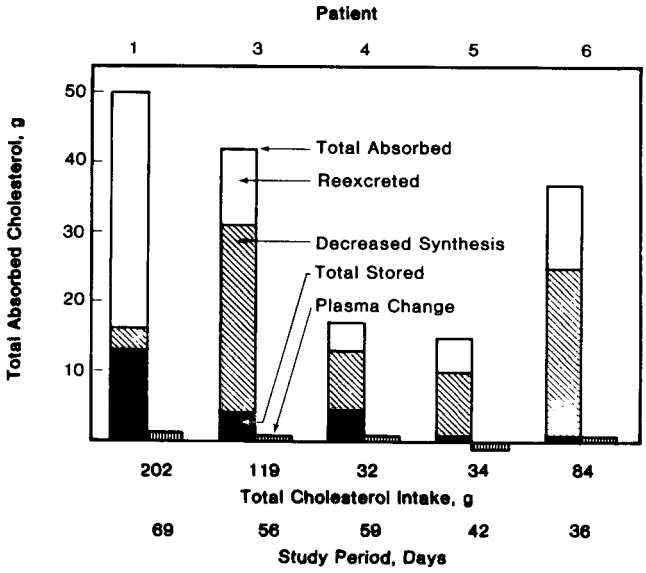

Fig. 3. Heterogeneity of compensatory responses in five patients fed added cholesterol (presented in terms of absorption, re-excretion, decreased synthesis, total retention (storage), and change in plasma content). Note the excellent compensation in patients 5 and 6: the amount absorbed was precisely balanced by decreased synthesis and increased re-excretion. Contrast these findings to those in the other patients, who showed significant retention of absorbed cholesterol. Changes in total plasma content were always small and unrelated to the other parameters. (Data plotted from Table 5, ref. 22; the figure was reprinted with permission of *Archives of Internal Medicine*.)

these responses, nor their magnitude, could have been predicted from their plasma lipid/lipoprotein levels.

Why do I think these conclusions are important? Why are we *splitting*, when one of the driving impulses in medicine and in science is *lumping*? In clinical medicine the student learns to associate a set of signs and symptoms with a history of antecedent events in order to arrive at the most likely diagnosis. In contrast, the experienced practitioner, having learned early to make diagnoses in this way, comes to concentrate on the specifics that make that illness in that patient a little, or sometimes a lot different from the same disorder in other patients. Lumping is characteristic of early training; splitting comes with experience.

Experimentalists have paid careful attention to phenomena observed in laboratory animals: particular organs, cell homogenates, or subcellular particles; major insights have been gained. For convenience and economy, small animals are commonly used, rats, mice, guinea pigs, hamsters, etc. Not only have pure-bred strains been developed, but hundreds of strains with specific genetic defects. By this selective process variations in metabolic responsiveness are minimized: small standard deviations around the mean are indicative of homogeneity. But larger animals, dogs, cats, sheep, nonhuman primates, etc., are rarely pure-bred, and the investigator is faced with impressive inhomogeneities. Consider the recent classic study of plaque regression by Clarkson and his colleagues in monkeys: because of large variations in responses of these individual animals (not only to the atherogenic stimulus but also to the regression regimens), very large numbers of animals were required in order to achieve the statistical power to distinguish clearly whether regression was demonstrable—a 10-year effort with 250 autopsied animals! This experience calls to mind the cost problems of proof-getting in human experimentation (where autopsies are not a part of the protocol!). The recent LRC-CPPT experiment required 3800 high-risk males aged 35–59 years, treated with cholestyramine and minimal dietary changes for up to 10 years, in order to demonstrate ($P = 0.04$, one-tailed test) that there was a difference in the rate of new events of coronary disease. The difficulties of this praiseworthy experiment, and its high cost in time, effort, and dollars, were due in some part to the fact that the study was not carried out in a homogeneous strain of pure-bred human beings. There were marked variations in the responses of these men to *1*) the diet, *2*) the drug, and *3*) the clinic situation (with all that that implies for compliance). Now, if the trial had been carried out in men known to be minimally responsive to the diet prescribed, but maximally responsive to the drug used (even if only in terms of plasma cholesterol-lowering) and maximally compliant with the protocol, far fewer men would have been required, perhaps fewer years of study. But even *that* design would not have taken note of the existence in the test groups of the heterogeneity that I have illustrated in Fig. 3—that is, drug-response differences in terms of cholesterol absorption, rates of cholesterol synthesis, and rates of efflux of stored cholesterol. If a test population could have been assembled in which these critical regulatory factors had been used in patient-selection, considerable reductions in sample size could have been accomplished.

Sharper selection, better trials

Is such a selection process achievable today? Almost. I have noted above that we can now measure intake (23), absorption (24, 25), and feedback control of cholesterol synthesis (26–28) in outpatients. Work in progress here at this time, using ^{18}O-labeled sterols, appears to promise that we will soon be able to measure these and other key factors without need for use of radioisotopic labels. If we are successful in this, it will be practical to screen patients (men, women, and children) for their suitability as test subjects in trials of drugs or diet. In addition, another hard endpoint will soon be available (quantitative coronary/femoral arteriography) that will allow still further economies in requirements for numbers of test subjects.

It has often been said that diet trials are unaffordable and thus beyond any agency's capacity. Is it conceivable to carry out a convincing diet trial in today's world? Certainly. Given the expected advances in endpoint criteria and given the appropriate recognition of patient heterogeneity, the number of patients required to establish whether changes in diet can reduce the rate of atherogenesis will be very much smaller than we thought at one time. I chaired a National Heart Institute panel that recommended in 1969 a number of alternative designs for diet trials to follow up the Diet-Heart Feasibility Trial; all were terribly expensive because they demanded very large numbers of patients (29). However, at that time we failed to recognize the existence and critical importance of heterogeneity. Our designs were wrong: we intended to apply the same dietary regimen to all subjects, provided they were male and had elevated plasma cholesterol levels. The Heart Institute's Task Force (1971) was correct in setting aside our recommendations, saying the costs were too high. They were right but for the wrong reasons. The question whether to undertake a diet trial of the Lipid Hypothesis in today's world, given current understanding and updated methods, should be freshly evaluated; a trial in diet-responsive individuals could be small enough, I submit, to be both affordable and conclusive.

Key problems for the future

In science we are accustomed to the unfolding and then the eclipse of new discoveries. In the 1940's we were excited by advances in the enzymology and stereochemistry of cholesterol biosynthesis; then we became fascinated by new understandings of cholesterol and lipoprotein metabolism; now we are marveling at the recent discoveries in the molecular biology and genetics of lipoproteins. It is a truism that each generation of research workers stands on the shoulders of its predecessors; accordingly, the future of cholesterol/lipoprotein research looks bright. For me, the most important challenge to students of human cholesterol metabolism is to find an explanation in genetic terms of the heterogeneity that seems to exist throughout the animal world, and accordingly to pattern our efforts at disease prevention in man to the *specific* metabolic defects that individual patients display.

But I have another dream, a less practical and more basic wish than that just described—one that could well unite the sterol people and lipoprotein people, now working along lines too separate—which addresses the following dilemma: does the biosynthesis of apolipoprotein in the liver precede that of the cholesterol it is destined to carry, or follow it, or proceed simultaneously? In the vernacular, which is the chicken, which the egg? The two biosynthetic apparatuses are separately located in the liver cell, separately controlled, separately fed-back; so which takes precedence, which is the leader? do they occur simultaneously, even though their catabolisms proceed at different rates? does the synthesis of one guide that of the other? I know of no evidence on this fundamental question. Nevertheless, I consider it basic to our understanding of the assembly of VLDL; the answer might help us to know whether to attend more to the feed-back control of the synthesis of the one rather than of the other.

Patient-oriented research

Early in this essay I commented on the rewards of patient-oriented research. What I have described as advances in our understanding of the physiology of cholesterol metabolism could only have come about through the study of patients, not just their lipids/lipoproteins, or their fibroblasts, or their receptors. Reductionists and integrationists approach their goals differently, but they must eventually work hand-in-hand. Both approaches are rewarding, but in different ways; one tells us what *can* happen, the other what *does* happen. They are not just complementary; neither is sufficient without the other.

TABLE 1. Colleagues working together in this laboratory (1952–85)

Ahrens	Stein	Theodor	Schreibman	Kibata
Insull	McAuley	Lees	Nikkari	Back
Blankenhorn	Spritz	Salen	Nakamura	McNamara
Tsaltas	Knittle	Kappas	Liu	Schaefer
Hirsch	Goldrick	Pertsemlidis	Samuel	Lummis
Borgström	Hofmann	Mishkel	Curran	Proia
Blomstrand	Woodford	Klein	Oppenheimer	Most
Peterson	Grundy	Anderson	Sedaghat	McVie
James	Miettinen	Crouse	Boucher	Davidson
Stoffel	Davignon	Smith	Hyman	Young
Lindstedt	Paulson	Kirchman	Park	Parker
Zilversmit	Simmonds	Leibetseder	Palmer	Kolb
Farquhar	Jones	Panveliwalla	Saudek	Brown
Oette	Quintão	Nestel	Barth	Hudgins

Collegiality

I cannot end this retro-prospective without noting that my association with my colleagues has been just as important to me as the work we have accomplished. The Rockefeller Institute (now University) has a long tradition as a training center for biologists and takes great pride in its "graduates." My own "graduates" are listed in **Table 1.** All have had an experience in our group that has left its mark on them and on me. The benefit each has carried away has primarily come from the institution itself, truly an unique place; but their relationshps with each other also have been immeasurably fruitful and memorable. I myself have grown up with each of them and been enriched by that association.

My point in closing on this note is simply this: we are what we have become through the many examples experienced in our younger years. With luck, skill, and hard work we become what we admire; we leave behind us a trail of co-workers whose originality redounds to our credit; *their* credit is *our* credit. I am intensely proud of the people whose names are listed in Table 1. I firmly believe that their prowess and their contributions have been shaped to some degree by my own faith in the beauty and power of our sharpest tool, the English language, and in the desire to communicate with each other that can only occur face to face in an atmosphere of mutual trust, friendliness, and sharing.

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