For my first independent research project after my appointment to the Berkeley faculty, I chose to work on the structures of myoinositol-containing phospholipids, a study that led us eventually to the discovery of D-myoinositol 1,4,5-trisphosphate or IP3. Before describing this research, however, I should say how that choice came about. While in graduate school at the University of Wisconsin, I had had the good fortune to study under Karl Paul Link, who was widely renowned for his discovery of dicumarol and the synthesis of related blood anticoagulants such as warfarin, work that was recognized with two Lasker Awards (1). On the side, however, Link remained a carbohydrate chemist at heart, a hobby that had grown out of his studies on plant polysaccharides and uronic acids while a student and then a young faculty member. In fact, Stanford Moore had completed his doctoral dissertation with Link on a method for characterizing aldo-mono-saccharides as benzimidazole derivatives (2).

I arrived at Madison in the fall of 1946, fresh from a stint in the United States Navy, and I found Link’s laboratory bursting at the seams with about 15 ex-GIs, all hard at work trying to make up for lost time. During earlier investigations on the structure-function relationship of coumarin anticoagulants, an attempt to synthesize the glucoside of dicumarol had been frustrated because the acetylated intermediate was degraded in alkali under conditions used for deacetylation (3). Because glycosides are acetals, which are typically acid-labile and alkali-stable, I found the anomaly intriguing and decided to study a variety of synthetic compounds in an effort to understand the structural basis for alkali sensitivity (4). This research formed the core of my doctoral dissertation, and although I failed to recognize it at the time, the chance exposure to carbohydrate chemistry was to have a lasting influence on the direction my career would take.

I continued my indoctrination in sugar chemistry during a postdoctoral year in Edinburgh, Scotland, with E. G. V. Percival in the new Department of Chemistry at Kings Buildings headed by Edwin Hirst. This was a time of economic depression in Britain, which was still suffering the aftermath of the war, and I discovered that I had left a well equipped laboratory in Madison to engage an unexpectedly primitive research environment. Wisely I did not let this change in fortunes discourage me. Instead I undertook a project dealing with the structure of maple sapwood starch and did the best that I could with the available facilities (5). My efforts were well rewarded because, in the process, I became adept at the uses of analytical and preparative filter paper and cellulose column chromatography, skills that were to be extremely valuable in my later research. The greatest challenge to my ingenuity, however, was to construct an electric stirring device from a small board-mounted motor, a couple of wooden pulleys, a piece of string, and a glass rod. The speed of the motor was regulated by adjusting light bulbs that were wired in series with the power cord to draw off electricity, a crude but effective method of control. I have always enjoyed working with my hands, so this mundane project even took on a certain appeal.

Living in a new environment always has its fringe benefits. While in Edinburgh, I developed a special affection for the Scots and a better understanding for the lingering resentment that
reflects a long history of conflict within the British Isles. Thus, I could understand why one of my graduate student colleagues was proud to proclaim, at every opportunity, that he had never been south of the border! It was also during this year that some Scottish separatists sneaked into Westminster Abbey and made off with the Stone of Scone. This symbol of Scottish nationalism, which was taken from Scotland to England by Edward I, had long rested beneath the chair on which British monarchs were crowned. The incident created quite a stir among the local patriots, but after its recovery the stone was returned to the Abbey. (I was recently informed that the Stone of Scone has since been returned to Scotland.)

Although I enjoyed the time, when the year ended I was ready to move on to Berkeley, where I had arranged to study with Hermann O. L. Fischer. Nicknamed “Hermannol,” probably by his friend Claude Hudson as a play on the term “polyol,” Fischer was an expatriate German scientist who had experienced a turbulent career that eventually led him to the University of Toronto. Then, when the new Biochemistry and Virus Laboratory was set up in 1948, Wendell Stanley had recruited him to Berkeley. I was attracted to Fischer in part because of his research on phosphorylated sugars but also because during graduate school I had drawn heavily on the published works of his father, Emil Fischer (6). I guess the idea of being associated with the son of Emil Fischer just seemed “real cool” to me. As it turned out, it also proved beneficial that I happened to go to Berkeley just as the University was entering a period of rapid postwar expansion.

This was a time of active research on biosynthetic pathways that involved short chain phosphorylated sugars, as exemplified by the studies of Melvin Calvin on photosynthesis, of P. R. Srinivasan and David Sprinson on shikimic acid biosynthesis, and of Bernard Horecker on transaldolase. With Fischer and his colleague, Donald MacDonald, I undertook the syntheses of several such metabolic intermediates, including D-glyceric acid 2-phosphate, D-glyceraldehyde 3-phosphate, dihydroxyacetone phosphate, hydroxypyruvic acid 3-phosphate, and D-erythrose 4-phosphate (7). The novelty of our approach was to prepare stable dimethyl acetal derivatives of the inherently unstable phosphorylated compounds with aldehydo or keto groups. These could be stored indefinitely and then be converted by mild acid hydrolysis of the acetal to the active metabolites as needed. Our success is documented by the fact that today, 50 years later, samples of the preparations have survived in pure crystalline usable form. During these first years in Berkeley, I also became interested in inositol chemistry as a result of studies on the cyclitols in sugar pine heartwood (8). Then, when Elvin Kabat came to Berkeley from Columbia University to spend a sabbatical with Fischer and learn some carbohydrate chemistry we all collaborated on the methylation analysis of galactinol, an α-D-galactoside of myoinositol. This study established that the galactose was linked to the 1,1-position on the inositol ring (9), a fact that I was to put to good use in my later studies.

After my appointment to the faculty in 1955, I was in a position to set up an independent program, and this background led me to undertake a project concerned with the character-
ization of inositol-containing phospholipids. In so doing, I was fortunate to have Finn Wold, Lewis Pizer, and Francis Lane Pizer as my first graduate students. At the time, there was convincing evidence from a number of studies that the lipid known as "phosphoinositide" was a phosphatidylmyoinositol (10), and as expected for such a structure, acid or alkaline hydrolysis of the phosphodiester bond had yielded myoinositol phosphate as one of the degradation products (11–13). Because the chemical hydrolysis of phosphate diesters with neighboring free hydroxyl groups can lead to phosphate migration, however, the position of attachment of the phosphatidic acid unit to the myoinositol ring was uncertain. Important studies at Cambridge University by Brown and Todd (14), showing that the alkaline hydrolysis of the phosphate diester linkage in nucleic acids proceeds via a cyclic phosphate intermediate, suggested to us a strategy to resolve this uncertainty. We subjected pure soybean phosphoinositide to alkaline hydrolysis and isolated the inositol phosphate fraction. It consisted mainly of myoinositol 1-phosphate along with some myoinositol 2-phosphate and other minor products (15). This result indicated that the putative myoinositol cyclic phosphate intermediate had involved positions 1 and 2 on the ring.

Because position 2 of the myoinositol ring lies between two adjacent cis-hydroxyls, called D-1 and L-1, the phosphatidyl group in the lipid could have been attached to position 2 or to one of the adjacent enantiomorphic 1-positions. The choice between these alternatives was suggested by the fact that the myoinositol 1-phosphate we isolated was optically active, $[\alpha]_D -9.8^\circ$ (water, pH 2). This would be expected from the cyclization and reopening of a phosphate diester group originally on the d-1- or l-1-position, because the intermediate cyclic phosphate would be asymmetric if the myoinositol in the starting diester were asymmetrically substituted (Fig. 1). This would not be the result if the original diester involved position 2, which has a plane of symmetry, unless the asymmetry of the glycerol portion were able to exert a directive influence during the reaction.

To complete the characterization, we carried out a definitive synthesis of l-myoinositol 1-phosphate, starting from galactinol (9). For this synthesis, we perbenzylated galactinol, removed the benzylated galactose moiety by acidic methanolysis, and phosphorylated the free l-1-position of the recovered penta-O-benzylmyoinositol. Deblocking of the product by hydrogelenolysis yielded l-myoinositol 1-phosphate (Fig. 2), which showed $[\alpha]_D +9.3^\circ$ (water, pH 2) (16, 17). Because this synthetic l-isomer, which later was found to occur naturally (18), showed a rotation equal to that of the lipid-derived product, but of opposite sign, it must be the enantiomer; and consequently, the 1-phosphatidylmyoinositol (15) must have had had the

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1 For these assignments, the three adjacent cis-hydroxyls of myoinositol are numbered one to three, and the direction of numbering is selected to give substituted positions the lowest possible number. When the ring is represented with these three hydroxyls projecting downward and the direction of numbering is clockwise, the myoinositol configuration is D and if counterclockwise it is L. Note that myoinositol 2-phosphate and 5-phosphate have a plane of symmetry and are meso compounds.
In an important parallel study, Brown et al. (19) degraded horse liver phosphoinositide by the periodate/phenylhydrazine procedure, which avoided the cyclic phosphate intermediate, and they recovered a single myoinositol 1-phosphate with the same optical activity as the isomer we had obtained from the soybean lipid. Together, these studies firmly established that the myoinositol ring was substituted on the D-1-position in phosphatidylinositol from both plants and animals. This was an important result, although it was not surprising because most myoinositol derivatives show chirality.

At the time, I was aware of the important work of Jordi Folch at Harvard Medical School, who had isolated a complex phosphoinositide from beef brain (20, 21). This isolation was based on the facts that phospholipids have low solubility in acetone, but they can be extracted from an acetone powder of brain tissue with chloroform, and the inositides can then be precipitated selectively by adding ethanol or methanol. Because strong acid hydrolysis of the material had yielded an “inositol metadiphosphate,” Folch concluded that the original lipid was a polyphosphoinositide. Because it was known, however, that acid treatment could cause phosphate groups to migrate around the inositol ring (15), we decided to reexamine this characterization. A new graduate student from the University of Chile, Carmen Grado, had just joined my group, and I suggested that she should repeat the brain phosphoinositide preparation according to Folch. When Grado subjected this material to strong acid hydrolysis, she observed that the resulting inositol phosphate fraction gave a very diffuse unresolved streak on paper chromatography (22). She then repeated the study, using alkaline degradation of the brain lipid, and found that chromatography of the inositol phosphate fraction gave a well resolved pattern of five components, one mono-, two bis-, and two trisphosphates of myoinositol. This suggested that the Folch brain inositide preparation was a mixture of related substances, and because the myoinositol trisphosphates predominated, we proposed that “the lipid might more accurately be called a triphosphoinositide” (22). At about the same time, Dittmer and Dawson, at Cambridge University, reported the isolation from ox brain of a lipid fraction with the composition expected for a triphosphoinositide (23).

Grado then went on to characterize each inositol polyphosphate in the mixture, using a sequence of periodate oxidation to cleave the inositol ring between free glycol groups, borohydride reduction of the resulting dialdehyde, and dephosphorylation to yield a free polyol. From an inositol bisphosphate, one could expect a tetritol if the phosphate groups were next to each other. If they were in a 1,3-position, a pentitol would result; and if they were in a 1,4-position, the ring would be cleaved in two places to give two molecules of malondialdehyde phosphate, which would be oxidized further by excess periodate to yield inorganic phosphate, formate, and carbon dioxide. From an inositol trisphosphate with the phosphates adjacent to each other, a pentitol would be formed; if in a 1,2,4 arrangement, a hexitol would result; and if in 1,3,5 arrangement, the inositol ring would survive the treatment. Besides indicating the phosphate positions, the identity and optical activity of the resulting polyol would also reveal the chirality of the inositol derivative. Using these methods, Grado characterized the two bisphosphates as D-myoinositol 1,4- and 4,5-bisphosphate and the major trisphosphate fraction as either the D-1,4,5-isomer or the D-1,4,6-isomer, the uncertainty arising because both of these trisphosphates would yield the same D-iditol in the above analytical procedure (22).

2 The convention for assigning configurations to substituted myoinositols was changed during the 1970s, so that what we designated L-myoinositol 1-phosphate in 1959 (16, 17) was later renamed D-myoinositol 1-phosphate. In this article, I have assigned all configurations in agreement with the convention now used.
uncertainty was resolved by Raymond Tomlinson, a graduate student who had made a detailed study of the dephosphorylation of phytic acid (myoinositol hexaphosphate) by wheat bran phytase (24). He observed that alkaline phosphomonoesterase selectively removed phosphate groups flanked by unsubstituted hydroxyls, and he found that the myoinositol trisphosphate isolated by Grado was converted to d-myoinositol 4,5-bisphosphate by this treatment (25). Thus, the complete characterization of d-myoinositol 1,4,5-trisphosphate can be summarized as shown in Fig. 3.

These studies still left open the question of the true nature of the apparently heterogeneous brain phosphoinositide. From the composition of his preparation, Folch (21) had postulated that it could be represented as a meta-diphosphatidylmyoinositol, whereas Hawthorne (26) proposed a cyclic structure of myoinositol meta-diphosphate with monoacylglycerol. I was fortunate at the time to be joined by a postdoctoral co-worker, Hans Brockerhoff, who had studied with Donald Hanahan at the University of Washington. To obtain the water-soluble component(s) of the brain lipid complex with intact phosphodiester linkages, Brockerhoff deacylated the phosphoinositide preparation with hydroxylamine and separated the products on an ion exchange column (27). This yielded three fractions with compositions corresponding to glycerol myoinositol phosphate (20%), glycerol myoinositol diphosphate (22%), and glycerol myoinositol triphosphate (58%) (Fig. 4). Further analysis suggested that these products could be derived from three lipids: 1-phosphatidyl-d-myoinositol, 1-phosphatidyl-d-myoinositol 4-phosphate, and 1-phosphatidyl-d-myoinositol 4,5-bisphosphate. This conclusion was confirmed when Stewart Hendrickson, a postdoctoral fellow who had studied with Herbert Carter at the University of Illinois, developed an ion exchange procedure using a homogeneous chloroform/methanol/water solvent. This solvent dissolved the intact brain phosphoinositide preparation and allowed its separation into three homologs (28), analysis of which agreed with Brockerhoff’s assignments (27). Hendrickson also found that the three lipids were closely related in that each was predominantly acylated by the same mixture of stearic, oleic, and arachidonic acids. Later, Brown and Stewart (29) also characterized purified triphosphoinositide, using the selective degradation procedure Brown and co-workers had exploited so effectively earlier (19).

During the 1960s when we were conducting the above studies, very little was known about the cellular function(s) of the inositol phospholipids. Mabel and Lowell Hokin at the University of Wisconsin had investigated the possible role of these lipids in cellular secretion (30), and they, along with others, had studied the incorporation of $^{32}$P$_1$ into the brain lipids (31, 32). These studies had yielded only limited information owing, in part, to uncertainty about the actual structure of the brain inositide. From the insight we had gained by our structural work, it appeared to us likely that the three components would be interconvertible in cells by an enzyme-catalyzed process of cyclic phosphorylation-dephosphorylation. When Brockerhoff investigated the incorporation of $[^{32}P]phosphate$, $[^3H]$myoinositol, and $[^{14}C]$glycerol into the individual inositides in brain tissue slices, the results proved to be consistent with such a pathway (33, 34). Thus, the monoester phosphate groups turned over rapidly, whereas the glycerol, myoinositol, and phosphodiester groups were much more stable. Moreover, turnover of the monoester phosphate groups was not random, because partial enzymic dephosphorylation of 1-phosphatidylmyoinositol 4,5-bisphosphate to the next lower homolog occurred by the selective removal of the 5-phosphate group, indicative of a specific 5-phosphomonoesterase in brain tissue (35).

I became eligible for a sabbatical leave in 1961, and because our work on the brain polyphosphoinositides was going well, I asked Edgar Lederer if I could spend a year with him to study the glycosphingolipidosides of mycobacteria. He welcomed me in his very gracious
manner, and he even arranged the rental of a spacious apartment in Paris on rue Pierre Curie (later renamed rue Pierre et Marie Curie). Thus, I had only a short stroll to catch the Ligne de Sceaux at Luxembourg Station for the daily ride to his CNRS laboratory at Gif-sur-Yvette. Myoinositol, as a lipid constituent, was first reported by R. J. Anderson to occur in the phospholipids of mycobacteria (36), and Lederer subsequently described a dimannosyl phosphoinositide from the same source (37). While at Gif, I collaborated with his colleague, Erna Vilkas, on experiments to establish the linkages of both the phosphatidyl and the mannosyl groups to the myoinositol ring (38). In later investigations by Yuan Chuan Lee, a postdoctoral student from the University of Iowa, we determined the structures of the family of mannosyl phosphoinositides in Mycobacterium smegmatis (39, 40). Like the other phosphoinositides, the phosphatidyl group was found attached to the D-1-position of myoinositol, whereas a single mannose was linked to position 2 and one to four mannoses were linked to the D-6-position (Fig. 5). This phospholipid was later found to serve as an anchor for the lipoarabinomannan in mycobacteria (41), and it is interesting that the glycolipophosphoinositide protein anchor has the analogous structure in which a carbohydrate chain is also attached to position 6 of myoinositol (42).

In a report to the International Congress of Biochemistry on the “Structure of Myoinositol Phospholipids” (43), I summarized the results of our studies and observed that: “In attempting to assess the role of phospholipids in cellular metabolism, one can place primary emphasis on the lipid end of the molecule and its modification according to the type of fatty acid there esterified. Or, one can direct attention to the hydrophilic end. In the case of the inositol phospholipids, we find, in the great structural variability of the inositol part, evidence that herein may lie the prime functional center of these molecules.” I have never considered myself a clairvoyant, and as it turned out, both the polar and nonpolar ends of the inositides have important regulatory functions. Today, we can look back and see that our earlier studies were significant mainly in helping to prepare the groundwork for the explosive developments concerning the cellular functions of the phosphoinositides that followed upon the important discoveries described in the review by Berridge and Irvine (44).

In these Reflections, I have limited myself to that early period of the 1960s in which I was directly involved, and I have referred only peripherally to the many subsequent important developments. I can’t avoid reference, however, to the role that has been discovered for 1-phosphatidylmyoinositol 3-phosphate and its derivatives (45), substances we never encountered in our investigations. I should also admit that I am a little disappointed that I never encouraged my co-workers to pursue a detailed study of the enzymes involved in the metabolism of the polyphosphoinositides. My only excuse is that we were drawn in other directions by our discovery of the mycobacterial polymethylpolysaccharides (46, 47), which were found later to act as regulators of fatty acid synthesis in this microorganism (48), and to investigations on the genetic control of yeast mannoprotein structure (49). Both of these developments are traceable to the sabbatical leave I spent at Gif in 1961, a testament to the unpredictable influence such an experience can have. Despite the minor doubt expressed above, I must say that I enjoyed a wonderful ride with the phosphoinositides, and it was all great fun! I am especially grateful to the many co-workers who shared this journey with me.

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