Reflections

THE PENTOSE PHOSPHATE PATHWAY

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INTRODUCTION

I received my basic training in enzymology as a graduate student in the laboratory of Professor T.R. Hogness at the University of Chicago, from 1936 to 1939. Hogness had constructed a photoelectric spectrophotometer modeled after the one in Otto Warburg’s laboratory in Berlin-Dahlem. I was assigned a problem on succinic dehydrogenase from beef heart, using the Warburg manometric apparatus, and didn’t get to use the spectrophotometer until Erwin Haas arrived from Warburg’s laboratory in 1939. Haas asked me to join him in the search for an enzyme that would catalyze the reduction of cytochrome c by reduced TPN (now NADP). This reaction was thought to be the missing link in the electron-transport chain from substrate to oxygen and marked the beginning of my interest in what was then thought to function as a direct oxidative pathway for the metabolism of carbohydrate, but is now known as the pentose phosphate pathway.

After I left Chicago, during the Second World War, my experience with the spectrophotometer landed me as job at the National Institute of Health in Frederick S. Brackett’s group in the Division of Industrial Hygiene. Brackett had assembled an automatic recording spectrophotometer in the basement of Building 2 that I was assigned to use to develop a method for the determination of carbon monoxide hemoglobin in the blood of navy pilots returning from combat missions. That and a number of other war-related projects kept me occupied for the next four years.

In 1945, after the end of the war with Japan, I was advised by the Director of the Laboratory, Dr Paul Neal, that I was free to return to research in enzymology. I began studies on the reduction of cytochrome c by the succinic dehydrogenase system, using what was now my own Beckman spectrophotometer. One day, which I consider to be a turning point in my career, Arthur Kornberg, who had been working in Building 4 on the biological role of folic acid, appeared in my laboratory. Arthur had become convinced that enzymes were the key to an understanding of intracellular biochemical processes and suggested that we work together. We began with studies on the effect of cyanide on the succinic dehydrogenase system, since cyanide was known to bind to, and be a general inhibitor of, enzymes containing the heme group. An exception was cytochrome c, which had been reported to be resistant to the action of cyanide. Contrary to these early reports, we found that cyanide did react with cytochrome c and in 1946 published our first paper together, in the Journal of Biological Chemistry, entitled “The Cytochrome c Cyanide Complex”.

MAKING HISTORY IN BUILDING 3

Two years later, in 1948, when Arthur returned from a study leave in the laboratories of Severo Ochoa in New York and Carl Cori in St. Louis, he invited Leon Heppel and me to join him in setting up a new Section on Enzymes in the Laboratory of Physiology, to be housed in Building 3, which was being completely renovated. Leon and
I were about to be transferred, it having been discovered that the Industrial Hygiene Research Laboratory in Building 2 had never been officially part of the NIH, but was in the Bureau of State Services, which was moving to new headquarters in Cincinnati.

In the fall of 1948, while we waited for the of the renovation of laboratories in Building 3 to be completed, we all three worked in Building 2. Arthur and I, both of whose planned research projects would depend heavily on assays using the “pyridine nucleotide” coenzymes DPN and TPN, collaborated in their isolation. In those early years of American biochemistry there were no vendors that supplied these materials. We isolated them from sheep liver, using the unpublished procedure from Warburg’s laboratory that Erwin Haas and I had used in Chicago. In 1948 Arthur and I possessed the world’s supply of TPN, and when Warburg visited our laboratory in 1948, we were able to present the discoverer of TPN with a gift of 25 mg. of that coenzyme.

The new Enzyme Section in the Division of Physiology in Building 3 provided an exciting and stimulating atmosphere. Together with Herbert Tabor from the Laboratory of Pharmacology in Building 4, we organized a daily lunch-hour journal club, during which we reviewed the literature on every facet of enzymology and intermediary metabolism. This was the beginning of a great history for Building 3, continuously occupied by scientists who were to make notable contributions in biomedical science. The first NIH recipients of the Paul Lewis Laboratories Award in Enzyme Chemistry, then one of the most prestigious awards in biological research, were all from Building 3: Arthur Kornberg in 1951, Bernard Horecker in 1952, and Earl Stadtman in 1953. Later, our Section on Enzymes became part of the new Experimental Biology and Medicine Institute, which Henry Sebrell, then the NIH Director, proposed to function as the basic research arm of the NIH. It was later renamed the National Institute of Arthritis and Metabolic Diseases, a change that had no effect on the nature of our research, but resulted in increased funding by Congress. We continued to work in the laboratories in Building 3.

Use of the pyridine nucleotides in enzyme assays with the Beckman spectrophotometer required knowledge of the exact extinction coefficients of the 340 nm peaks of the reduced forms. The published values for DPNH showed considerable variation, and there was scant information for TPNH. In the new laboratories in Building 3, Arthur and I designed experiments to determine the true extinction coefficients at 340 nm of both coenzymes, which proved to be identical. That work, published in 1948, made possible quantitative spectrophotometric measurements in reactions involving the pyridine nucleotides and became one of the most frequently cited papers in the biochemical literature.

In the new laboratories in Building 3 Leon Heppel and I also collaborated in the purification of xanthine oxidase from milk, after we found that this enzyme could reduce not only methylene blue, a reaction that I had studied in Chicago, but also cytochrome c. But this reduction occurred only if oxygen was present, a curious observation that was quickly picked up by Fridovich and Handler, who were working at Duke University on the formation of the superoxide anion. The reduction of cytochrome c by superoxide anion became a widely used assay for this species of “active oxygen”. I also returned to
the study of cytochrome c reductase, which Haas and I had isolated from yeast, and accomplished the first isolation of this flavoprotein from mammalian liver. By then, it had become apparent that these cytochrome c reductases did not function in mitochondrial respiration, but rather as components of the cytochrome P-450 system for the metabolism and detoxification of drugs and other xenobiotics.

THE PENTOSE PHOSPHATE PATHWAY

The oxidation of glucose 6-phosphate

When Otto Warburg discovered TPN as the coenzyme required for the oxidation of glucose 6-phosphate to 6-phosphogluconate, the role of the other pyridine nucleotide, DPN, as the coenzyme required for the fermentation of glucose to ethanol in yeast, or the glycolysis of glucose to lactic acid in muscle, had been well established. The finding that the new coenzyme was required for the oxidation of glucose 6-phosphate and also for the further oxidation of the product, 6-phosphogluconate, led Warburg, and also Frank Dickens in England and Fritz Lipmann, then working in Denmark, to propose that there existed an alternate pathway that functioned as a “direct oxidative pathway”.

They had obtained evidence that the products formed in the oxidation of 6-phosphogluconate by TPN were carbon dioxide and an unidentified pentose phosphate. Since carbon dioxide was one of the products, it seemed reasonable to regard this alternate pathway as the one responsible for the oxidation of carbohydrate. Haas and I had already shown that TPN could serve as an electron transport link to the cytochromes and therefore to molecular oxygen.

Twenty years after the pioneering work of Warburg, Dickens and Lipmann, with a new laboratory technician, Pauline (Polly) Smyrniotis, I began studies on the enzymes involved in the oxidation of 6-phosphogluconate and the metabolic intermediates formed in this pathway. We were joined by J.E. Seegmiller, my first postdoctoral student, and he and I worked out a new method for the preparation of glucose 6-phosphate and 6-phosphogluconate, which were not yet commercially available. We purified the enzyme, 6-phosphogluconate dehydrogenase, from brewer’s yeast, the richest source we could find, and, by coupling the reduction of TPN to its re-oxidation by pyruvate in the presence of lactic dehydrogenase showed that the first product of the oxidation of 6-phosphogluconate, in addition to carbon dioxide, was a new pentose ester, ribulose 5-phosphate, which was then converted to ribose 5-phosphate by a pentose phosphate isomerase present in our purified dehydrogenase preparations. The separation of ribulose phosphate from ribose phosphate and the demonstration that their inter-conversion was catalyzed by a pentose-phosphate isomerase was made possible by the recent development at the Oak Ridge National Laboratory of a separation technique for nucleotides called ion-exchange chromatography.

The identification of the sugar in the new pentose ester as ribulose was based on a number of criteria, including comparison with the authentic sugar, prepared by the method of Glatthaar and Reichstein, using a number of chemical and physical criteria, which included X-ray diffraction of the crystalline nitrophenyl hydrazones. In those days discoveries of new sugar phosphate esters were rare events and I felt that it was necessary
to establish its identity beyond a shadow of doubt. The results were first presented at the
American Chemical Society meeting in Boston in 1951, at a symposium honoring Arthur
Kornberg’s Paul Lewis Laboratories Award, and I recall the warm reaction at that
meeting to the work reported from our laboratories in Building 3.

During the following year Jay Seegmiller and I showed that the same products
were formed in the metabolism of 6-phosphogluconate by enzymes from mammalian
tissues.

The further metabolism of the pentose phosphates

An important clue to the further steps in what was later to become known as the
“Pentose Phosphate Pathway” was already in the literature. In 1938 Zaccharias Dische
had demonstrated that red cell lysates catalyzed the conversion of the 5-carbon sugar,
ribose 5-phosphate to hexose-monophosphate, an observation that Seegmiller and I
confirmed in 1952 with rabbit bone marrow extracts. These observations gave rise to the
hypothesis that the oxidative pathway was really a cyclic mechanism for the direct
oxidation of carbohydrate. With each turn of the cycle one molecule of carbon dioxide
would be produced, and the pentose-phosphates formed would be metabolized back to
hexose phosphates to start another cycle. Six turns of the cycle would result in the
complete oxidation of one molecule of glucose.

But the reactions involved in the conversion of the 5-carbon pentose phosphates
to the 6-carbon hexose phosphates were completely unknown. What ensued was a race
involving a number of laboratories, including ours at the NIH and those of Ephraim
Racker at the New York City Research Laboratories, later at Cornell University in Ithaca,
Seymour Cohen at the University of Pennsylvania in Philadelphia, Oliver Lampen at
Washington University in St. Louis, and Frank Dickens in England, to identify the
reactions and metabolic intermediates involved.

It had already been established from the work of Dische and others that one of the
products of pentose phosphate metabolism was the 3-carbon sugar, glyceraldehyde 3-
phosphate, which suggested cleavage of a 5-carbon sugar, probably ribulose 5-phosphate,
between carbon atoms 2 and 3. The 3-carbon fragment, glyceraldehyde 3-phosphate, was
a known intermediate in glycolysis. But what was the fate of the remaining 2-carbon
fragment? Polly Smyrniotis  and I, now joined by my first foreign postdoctoral fellow,
Hans Klenow from Copenhagen, set out to purify the enzyme(s) involved in the cleavage
of pentose-phosphate, using rat liver as the enzyme source and an assay that measured
the appearance of glyceraldehyde 3-phosphate. When we also followed the disappearance
of the five carbon sugar, using Dische’s “orcinol” reaction, we detected the formation of
a new product that also reacted with orcinol, but produced a different color and a visible
absorption spectrum distinctly different from that produced in the reaction with pentoses.
Our clue to the identity of this product came from Melvin Calvin’s laboratory in
Berkeley, where, using radioactive carbon dioxide, they had identified both ribulose
diphosphate and a seven-carbon sugar, sedoheptulose monophosphate, as early
intermediates in the fixation of carbon dioxide in photosynthesis. Authentic
sedoheptulose was available from Nelson Richtmyer’s laboratory in Building 4, and on
Christmas eve, 1951, when everybody else had gone home, I sprayed a paper chromatogram with orcinol and up came the blue spot characteristic of sedoheptulose. I rushed up and down the laboratory hallway clutching the paper chromatogram, but there was nobody there to show it to, so I took it home and hung it under the Christmas tree, singing the little ditty: “It’s sedoheptulose, it’s sedoheptulose, tra la la boom diay, tra la la boom diay”, much to the amusement of my young daughters..

We adopted the name “transketolase”, first suggested by Racker and his co-workers, because it catalyzed the transfer of a two carbon fragment from the keto pentose, ribulose 5-phosphate, to the other 5-carbon sugar, ribose 5-phosphate, to generate the new ketol linkage in the 7-carbon sugar, sedoheptulose 7-phosphate. Thus the unknown 2-carbon fragment never occurred as a free entity. Later, simultaneously with Racker, we showed that the coenzyme carrier for the 2-carbon fragment by transketolase was thiamine pyrophosphate.

Two puzzling observations remained to be explained. One was that the configuration of the hydroxyl group on the third carbon atom of the new product, sedoheptulose 7-phosphate was opposite to that on the third carbon atom of the presumed substrate, ribulose 5-phosphate. This lack of stereo-specificity, particularly since we had demonstrated that the reaction was readily reversible, was high improbable for an enzyme-catalyzed reaction. The other unexplained observation was that the cleavage of pentose phosphate by our purified transketolase preparations from rat liver required the presence of aldolase, a crystalline and supposedly pure enzyme from rabbit muscle that catalyzed the condensation of two triose phosphates to form the hexose, fructose 1,6-bisphosphate. The answer to both of these puzzling observations came from the discovery by Paul Stumpf, while on sabbatical in my laboratory, and reported almost simultaneously from the laboratories of Dickens, Racker and Ashwell, of another enzyme, an “epimerase”, that catalyzed the conversion of ribulose 5-phosphate to its “3-epimer”, xylulose 5-phosphate, which had the same stereo-configuratuion at the 3-carbon atom as sedoheptulose phosphate. With this substrate, we confirmed the earlier report by Racker and his coworkers that xylulose phosphate, rather that ribulose phosphate was the true substrate for cleavage by transketolase, and the true donor of the 2-carbon fragment. The requirement for aldolase was explained when we found that crystalline preparations of this enzyme from rabbit muscle contained the epimerase as a contaminant, which could only be removed by many re-crystallizations.

Thus three different pentose phosphates were now shown to be involved in the new pathway: ribulose 5-phosphate, the first product of the oxidation of 6-phosphate gluconate, and xylulose 5-phosphate and ribose 5-phosphate, both formed from ribulose 5-phosphate, one serving as the 2-carbon donor and the other as the acceptor in the reaction catalyzed by transketolase. The addition of any one of these pentose phosphates to crude tissue extracts would result in the formation of an equilibrium mixture of all three.

Completion of the cycle

Still to be discovered, however, was a mechanism that would convert the
products of the transketolase reaction, sedoheptulose phosphate and glyceraldehyde phosphate, to the six-carbon sugars, fructose-6-phosphate and glucose 6-phosphate and complete the cycle. In particular, what was the fate of sedoheptulose phosphate? We found that purified enzyme preparations from liver or yeast would catalyze the formation of hexose monophosphate from sedoheptulose monophosphate, but only if triose phosphate was also present. When I described this finding at one of our luncheon journal club meetings, Horace (“Nook”) Barker, who was visiting from the University of California at Berkeley and working in Kornberg’s lab, suggested that we consider the possibility of another transfer, this time of a 3-carbon fragment, from sedoheptulose phosphate to triose phosphate, to generate fructose 6-phosphate. When we carried out an experiment with carbon-14 labeled triose phosphate, we found that, as predicted, the fructose 6-phosphate formed had radioactivity in the last three carbon atoms, with the first three unlabelled. We named the enzyme “transaldolase” because it catalyzed the transfer of an aldol linkage, rather than the hydrolytic cleavage catalyzed by aldolase.

What remained was to account for the fate of the remaining four carbon atoms of sedoheptulose 7-phosphate. For this work Polly Smyrniotis and I were joined by Paul Marks and Howard Hiatt, two young MDs working as Clinical Associates in the new Clinical Center in Building 10, who had asked to join my group to learn enzymology in their “spare time”, which turned out to be from 5 PM to midnight. We identified the missing fragment as another new sugar ester, the 4-carbon sugar erythrose 4-phosphate, in a number of tests, including its conversion to the 7-carbon sugar sedoheptulose 1,7-diphosphate in a condensation with dihydroxy acetone phosphate, catalyzed by fructrose bis-phosphate aldolase. It was also converted to fructose 6-phosphate in the reaction catalyzed by transketolase.

The elucidation of the pentose phosphate pathway had now been accomplished. It consisted of two branches, an oxidative branch in which the hexose, glucose 6-phosphate was converted to pentose phosphate and carbon dioxide, with the reduction of two molecules of TPN, and a non-oxidative branch, in which three molecules of pentose phosphate (15 carbon atoms) were reconverted to two and one-half molecules of hexose phosphate (15 carbon atoms) in a series of fully reversible reactions. Our contributions included the discovery of three new sugar phosphate esters, ribulose 5-phosphate, sedoheptulose 7-phosphate, and erythrose 4-phosphate, and three new enzymes, transketolase, transaldolase and pentose-phosphate 3-epimerase. We shared with Racker the discovery of transketolase and confirmed his finding that xylulose 5-phosphate, rather than ribulose 5-phosphate, was the 2-carbon donor in the reaction catalyzed by that enzyme. We also shared with McLean and Dickens, working in England, the discovery that fructose 6-phosphate was also a substrate for transketolase. If the pathway operated as originally envisioned, six turns of the “cycle” would result in the oxidation of one molecule of six-carbon sugar to six molecules of carbon dioxide.

Functions of the Pentose Phosphate Pathway

The function(s) of the new pathway, however, turned out to be quite different from the pathway for the direct oxidation of carbohydrate that we had expected. It provides two mechanisms for the production of ribose 5-phosphate. One is the “oxidative branch” of the
pathway, which also generates two equivalents of TPNH (NADPH). Ribose 5-phosphate can also be formed directly from hexose and triose phosphates, by the non-oxidative rearrangements catalyzed by transketolase and transaldolase. Where large quantities of NADPH are required, as in the synthesis of fatty acids or sterols, the excess pentose phosphates produced would be recycled back to hexose monophosphates.

To assist medical students in memorizing the reactions, someone composed the following song:

THE PENTOSE PHOSPHATE SHUNT
(Tune: “MacNamara’s Band”)

If you’re converting carbohydrate into triglyceride,
If you need pentose moieties to make nucleotide,
You’ll find that Embden-Meyerhof is not the game to play
And you’ll do your biosynthesis the pentose phosphate way.

Chorus: With transaldolase, transketolase, G6PDH too,
Six times six gives five times six plus six of CO₂
Carbons passing to and fro, the back becomes the front,
Did you ever see a pathway like the pentose phosphate shunt?

First G6P is oxidized, NADP reduced
To give gluconolactone, (as might have been deduced).
The lactone is then hydrolyzed to make the gluconate
And decarboxylated to its metabolic fate.

There ends the oxidative phase, now multiply by three,
An intermediate balance sheet by way of summary,
Six NADPH are formed, three CO₂ set free,
Three ribulose 5-phosphate formed from three of G6P.

One isomerization from ketose to aldose
Turns ribulose 5-phosphate to the phosphate of ribose”
The other two epimerized, inverted at C3,
Two xylulose 5-phosphates formed (hence called Xu5P).

Two carbons from Xu5P transferred from the ketose to aldose
(Transketolase needs TPP as everybody knows),
Thus three plus seven made to meet transaldolase attack,
Three C’s from sedoheptulose the GAP gets back,

Glyceraldehyde 3-phosphate thus becoming F6P
Leaves erythrose 4-phosphate looking for some company,
But Xu5P number two has two top C’s to spare,
Transketolase negotiates their transfer as a pair.
So we’ve made another F6P, a triose phosphate too, 
To see what we have now achieved let’s multiply by two, 
Four F6P’s, two GAP’s, by glycolytic tricks, 
Give five glucose 6-phosphates, when we started cut with six!

(Author unknown)

The first discovery relevant to the new Pentose Phosphate Pathway, namely the formation of ribulose and ribose phosphates as products of the oxidation of 6-phosphogluconate, was announced in the spring of 1952, at the annual meeting of the American Chemical Society in Chicago. The outline of the complete pentose phosphate cycle, including the reactions catalyzed by the new enzymes transketolase and transaldolase, was published in 1955 in a review written in collaboration with I.C(Gunny) Gunsalus and W.A.(Woody) Wood for Bacteriological Reviews entitled “Pathways of Carbohydrate Metabolism in Microorganisms”. The existence of the cycle in mammalian liver and in plant leaves was confirmed in experiments with carbon labeled ribose 5-phosphate in two papers published with Martin Gibbs of the Brookhaven National Laboratory, describing work carried out there during the summer of 1953.

THE PATH OF CARBON IN PHOTOSYNTHESIS

When, in 1952, Calvin’s group at the University of California at Berkeley reported evidence for ribulose 1,5-diphosphate as the CO2 acceptor for the formation of 3-phosphoglyceric acid, the first CO2-fixation product in photosynthesis, we were excited by the possibility that the pentose phosphate pathway might serve as the mechanism for regenerating this key intermediate from hexose monophosphates. Art Weissbach, a newly arrived postdoc from Columbia, and Polly Smyrniotis carried out the first experiments to identify the enzymatic mechanisms involved. They were able to show that with crude extracts from spinach leaves ribose 5-phosphate was a unique substrate for the formation of phosphoglyceric acid and they purified a kinase from spinach leaves that they used to prepare the barium salt of ribulose 1,5 bisphosphate (RUDP).

In the fall of 1954 we moved from Building 3 to new laboratories on the 9th floor of the NIH Clinical Center, where, joined by Jerry Hurwitz, we isolated the enzyme phosphoribulokinase, responsible for that reaction, as well as the enzyme ribulose bisphosphate carboxylase, which catalyzed the formation of 2 moles of phosphoglyceric acid from ribulose bisphosphate and CO2. Working in laboratories across the hall from each other, Art, Jerry and I divided responsibilities. Jerry was charged with the purification of phosphoribulokinase, I took on the task of preparing pure ribulose 1,5-bisphosphate, and Art went after the most important enzyme, the ribulose bisphosphate carboxylase. The last effort deserves a special comment. Although the enzyme was purified only 10-fold from the crude spinach leaf extracts, by all the criteria that we could apply it appeared to be a pure protein, which meant that it constituted about 10% of the soluble protein in the spinach leaf. Later work in other laboratories around the world confirmed this finding and this enzyme, now known as “Rubisco” for ribulose-
bisphosphate-carboxylase/oxygenase) is now considered to be the most abundant protein on earth.

Our work was published in three back-to-back papers in the February 1956 issue of the JBC, entitled: “Spinach Phosphoribulokinase”, “The Enzymatic Synthesis and Properties of Ribulose 1,5-Diphosphate” and “The Enzymatic Formation of Phosphoglyceric from Ribulose Diphosphate and Carbon Dioxide”. With this work, and our earlier demonstration of the reversible reactions for the interconversion of pentose and hexose phosphates, with sedoheptulose phosphate as a prominent intermediate, all of the enzymes for the reactions of the Calvin Cycle were identified.

CONCLUSION

The Pentose Phosphate Pathway in animals, as discussed earlier, fulfils two important cell requirements: 1) for ribose 5-phosphate for the synthesis of nucleotides and nucleic acids and 2) for reducing power in the form of NADPH. In photosynthesis, it functions to regenerate the primary CO2 acceptor, ribulose bisphosphate, from the hexose phosphates produced. Chloroplasts utilize radiant energy to produce ATP, required for the production of ribulose 1,5-bisphosphate from ribulose 5-phosphate and also for the reduction of 3-phosphoglyceric acid to glyceraldehyde 3-phosphate. The reducing agent for the latter reaction, NADPH, is also generated by the action of light in the chloroplasts. In both animals and plants NADP, rather than NAD, appears to function as the coenzyme for reductive synthesis.

COMMENT

Because these were personal “reflections” they have mainly described work from my laboratory. Calvin and his coworkers provided the first clues leading to the leading to the development of the photosynthetic cycle and also the conclusive evidence for its function as the path of carbon in intact photosynthesizing cells. These pioneering experiments, as well as important contributions from many other laboratories, are cited in a review that I published in 1957 with Wolf Vishniac and Severo Ochoa in Advances in Enzymology. (See reference 13).

REFERENCES


13. Vishniac, W., Horecker, B.L. and Ochoa, Enzymatic aspects of photosynthesis (1957) in *“Advances in Enzymology”* **XIX**, 1-77
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