RECOLLECTIONS

Some early tracer experiments with stable isotopes

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In 1935, I began research as a graduate student in Harold Urey's laboratory. He had just received the Nobel prize for the discovery of deuterium and was busy concentrating other stable isotopes, particularly those of biological interest, namely, ^{13}C , ^{15}N , and ^{18}O . Urey was a man of broad interests ranging from his primary interest in spectroscopy and structure of diatomic and other simple molecules to the isotopic composition of meteors and the application of isotopes to biological problems. For example, when he first succeeded in concentrating ^{18}O , he thought of using H_2^{18}O to determine the body volume of water long before anyone had thought of the isotope dilution method. He chose H_2^{18}O rather than D_2O because he wanted to avoid isotopic exchange known to occur between D_2O and body constituents. His suggestion that I be the subject of the experiment stemmed from the fact that I had the smallest volume of anyone in the lab. Because it was already known that mice imbibing 25% D_2O died, I did not greet his suggestion with enthusiasm in spite of his assurance that theoretically ^{18}O should not perturb physiological systems as deuterium does. For once, I refused to be guided by theory and the experiment was not done.

Urey supplied the isotopes for the animal experiments that were being actively pursued by Schoenheimer and Rittenberg using stable isotopes as tracers of metabolic pathways. Rittenberg had preceded me as a student in Urey's lab and had subsequently joined Schoenheimer in the biochemistry department

of the Columbia College of Physicians and Surgeons. In a brilliant series of papers, they demonstrated the unique ability of labeled metabolites, initially with deuterium as the label, to yield unequivocal answers in intermediary metabolism (Schoenheimer & Rittenberg, 1935). As Schoenheimer stated in one of his lectures, the earlier attempts to trace the fate of compound A in the body by feeding A, followed by finding a new constituent B in the urine, and concluding that A was converted to B, were analogous to putting a penny in a vending machine, out comes a piece of chocolate, and concluding that the penny was converted to chocolate. Perhaps their most unexpected finding was that compounds that had been considered static were indeed turning over continually in the body, leading to a new concept of the dynamic state of body constituents (Rittenberg, 1948–1949).

Fig. 1. Mildred Cohn.

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Mildred Cohn, Professor of Biochemistry and Biophysics, Emerita, at the University of Pennsylvania School of Medicine, was born in New York City and received her undergraduate degree in chemistry from Hunter College. She was awarded the Ph.D. in chemistry from Columbia University under the mentorship of Harold Urey. As a postdoctoral fellow, she worked in biochemistry under Vincent du Vigneaud, briefly at George Washington University and for eight years at Cornell Medical College. From 1946 until 1960, Dr. Cohn was in the biochemistry department at Washington University School of Medicine, initially as a research associate and from 1958 as an associate professor. In 1960, she joined the Johnson Foundation/Department of Biophysics of the University of Pennsylvania, as professor from 1961 and Career Investigator of the American Heart Association from 1963. Dr. Cohn is a member of the National Academy of Sciences, the American Academy of Arts and Sciences, and the American Philosophical Society and a past president of the American Society of Biological Chemistry. In addition to the National Medal of Science, she has received nine honorary degrees.

Fig. 2. Harold Urey.

When Urey succeeded in concentrating ^{15}N , Schoenheimer and Rittenberg came to his laboratory to discuss the quantities they needed for their experiments. Schoenheimer told me with much enthusiasm that they would now be able to label both the carbon chain (with deuterium) and the amino group of amino acids with ^{15}N , thus enabling them to study the metabolic fate of different groups within a molecule (Schoenheimer et al., 1939). Shortly thereafter, I finished my graduate work and was looking for a job, not a very rewarding task in 1937. I had begun to despair when Rittenberg contacted me and asked me whether I would be interested in a postdoctoral position in a biochemistry laboratory. A well-known biochemist, Vincent du Vigneaud, was interested in embarking on tracer studies of amino acid metabolism and had approached Schoenheimer and Rittenberg to enlist their help in finding a postdoctoral fellow who had experience with stable isotopes. I sought Urey's advice on the matter, and with his disappointing efforts in trying to find me a position in physical chemistry and his enthusiasm for tracer work in biology, he advised me to accept. Rather than continue as a postdoctoral fellow in Urey's laboratory, my other option, I decided to switch to biochemistry, and I have never regretted that decision.

After spending 2 months in Rittenberg's laboratory learning how to convert organic compounds isolated from rat tissues to water so that their deuterium content could be determined from the density of the water, I joined du Vigneaud's group in the medical school at George Washington University. He was chairman of the biochemistry department, a position he had achieved some five years earlier at the age of 31. The mode of doing research in this laboratory was quite unfamiliar to me. As du Vigneaud had explained when he interviewed me for the position,

I would be a member of a team and he was the chief. It was not until I actually started working there that I realized that it meant that each individual did only a limited part of a research problem, and du Vigneaud coordinated all the parts. Furthermore, if you suggested an experiment within the framework of the problem, he made the decision whether it would be pursued. As a graduate student, I had operated quite independently, and this style of operation required an adjustment. As the only physical chemist in the group, I was also expected to repair a Leeds and Northrup suspension galvanometer, rewire the intercom system so that it could be controlled at the substations, and perform other such tasks beyond my expertise. I had no difficulty in setting up the falling drop method for measuring the density of water to a part per million in order to measure its deuterium concentration because I had done so during my graduate research. The method was first devised by Krogh and Ussing in Copenhagen and required among other things constructing a water bath that held constant to 0.001 °C.

The initial experiment in which I participated made use of two isotopic labels: ^2H and ^{15}N . My share of the team effort included planning the isotopic experiment so that it could yield a quantitatively significant result and performing the isotopic analysis for deuterium. The research was a collaborative effort with Schoenheimer and Rittenberg, and they would analyze for ^{15}N on a mass spectrometer. Although I had used a mass spectrometer for analysis of ^{18}O in my thesis work, mass spectrometers were not available commercially and none was available at George Washington University. It had been established by du Vigneaud that both stereoisomers of an unnatural amino acid, L- and D-phenylaminobutyric acids, were excreted as L-acetyl derivatives. To determine the pathway of the inversion of configuration of the D-isomer, doubly labeled isomers were synthesized with deuterium in the α position and ^{15}N in the amino group. It was found that none of the deuterium appeared in the acetylated product but the ^{15}N was fully retained, thus demonstrating the advantage of using two isotopes rather than one for discriminating among possible pathways (du Vigneaud et al., 1939).

In the spring of that year, 1938, I was present at an encounter of historic dimensions. Professor Urey was attending the annual meeting of the American Physical Society in Washington and I decided to meet him there. As we stood chatting on the lawn of the Bureau of Standards, E.O. Lawrence of Berkeley approached. Urey introduced me to him, describing me as a former graduate student of his who was now applying stable isotopes as tracers in biology. He then continued in a rather uncharacteristic manner to taunt Lawrence by pointing out that they had no radioactive isotopes that were suitable for biological work. "Hydrogen, we have deuterium, you have nothing [tritium had not yet been discovered], carbon, we have ^{13}C , you have ^{11}C with a half-life of 20 minutes, nitrogen, we have ^{15}N , you have nothing and oxygen, we have ^{18}O and you have nothing." I later learned from Martin Kamen that Lawrence returned from that meeting, called his group together, and said "We have to find a radioactive isotope with a sufficiently long half-life to be useful to the biologists." Shortly thereafter, Rubin and Kamen succeeded in producing ^{14}C . When it became available after World War II, it changed the study of intermediary metabolism forever.

Du Vigneaud's primary interest was the metabolism of sulfur-containing amino acids. At that time it was known that methi-

onine was an essential amino acid for the growth of rats, but it had been reported that homocystine could replace methionine and he wanted to investigate the pathway of the conversion. Therefore, tetradeuterohomocystine was synthesized labeled with deuterium in the α , α' and β , β' positions and incorporated into a pure amino acid diet supplemented with all the pure vitamins known at that time. It is noteworthy that only 10 of the amino acids were available commercially; the others were synthesized or isolated in the laboratory during the summer months—most of the postdoctoral fellows had backgrounds in organic chemistry. The rats fed the deuterated homocystine did not grow. The biochemical community was so distrustful of isotopes at that time that the initial reaction was to blame the deuterium in the homocystine for the failure of the labeled homocystine to support growth. Needless to say, du Vigneaud repeated the experiment with unlabeled homocystine and the rats did not grow. Concurrently, similar growth experiments were being pursued by Rose at the University of Illinois and his rats were growing. Because each laboratory had its own rat colony, they decided to exchange rats. The rats from Washington grew in Urbana and the rats from Urbana did not grow in Washington, whereupon du Vigneaud requested Rose to inform him exactly what the components of his rat diets were. The amino acid composition was identical but the vitamin supplements differed; in du Vigneaud's laboratory pure B vitamins were used, but in Rose's laboratory tikitiki, a rice bran extract, was used as the source of the B vitamins (neither diet contained folic acid; it had not been discovered yet). Du Vigneaud realized that some component(s) of the vitamin supplements must account for the differences in the growth patterns in the two laboratories. Remembering that the rats fed his purified vitamin supplement exhibited fatty infiltration of the liver of the type observed in choline deficiency, he said "Choline, transmethylation from *N*-methyl to *S*-methyl." I was stunned; what an intellectual feat—from tikitiki to transmethylation. Choline was added to the homocystine diets and the rats grew happily. As a footnote, I should add that an occasional rat grew without added choline in the diet. One of the graduate students (Arden Moyer) was assigned the task of staying up all night to monitor the eating habits of the animals, who were housed in metal metabolism cages with a grid as the floor, through which the urine and feces fell. He discovered that the few rats that were growing without choline in the diet were indulging in coprophagy, thereby obtaining the necessary choline, which had been synthesized by microorganisms in their gut. My respect for the intelligence of rats was strongly reinforced.

The transmethylation experiments were pursued further after we moved to Cornell Medical College in the summer of 1938. In preparation for tracer studies of transmethylation, du Vigneaud asked me to prepare CD_3OD . Although I did not generally participate in synthetic work, this was the sort of synthesis a physical chemist was expected to know how to do. Fortunately, I recalled that Professor Zanetti at Columbia had synthesized deuterated methyl alcohol. I contacted him and asked how he had synthesized the compound. When I learned that it required compressing CO and deuterium gas to 10,000 pounds pressure and circulating the mixture over a catalyst at 300 °C, I asked whether I could do the synthesis in his laboratory because we were now conveniently located in Manhattan. He agreed on condition that I determine the physical constants of the product so that he could submit the characterized compound

to Beilstein. After running the reaction for about a month, I had succeeded in producing about 100 g of CD_3OD , which was sufficient for many years of transmethylation experiments. Both choline and methionine were synthesized with deuteromethyl groups and there ensued a long period of tracer studies with them. It was shown that in the rat, the methyl group could be transferred from choline to methionine and that the methyl group of methionine was transferred in the formation of choline, creatine, and anserine. Other experiments involved betaine and sarcosine. The central role of the methyl group of methionine in metabolism was firmly established (du Vigneaud, 1942–1943).

Perhaps the most elegant isotopic tracer experiment that was done in du Vigneaud's laboratory addressed the question of the pathway of the conversion of methionine to cysteine. Because it was known that, of the two sulfur-containing amino acids, methionine was the one essential in the diet of the rat, the question was whether only the sulfur of methionine was used to produce cysteine or whether the carbon chain of methionine was also utilized. The synthesis of the methionine, labeled with ^{13}C in the β - and γ -positions and with ^{34}S , occupied Kilmer, a postdoctoral fellow, for one year. Finally it was fed to a dehaired rat, and after several weeks of feeding of the labeled compound its newly grown hair was clipped and the cystine isolated from it. Eighty percent of the sulfur in the cystine was derived from the dietary methionine and none of the carbon, an unequivocal result (du Vigneaud et al., 1944).

These tracer experiments were carried out during World War II. Although du Vigneaud was involved with a war project, as were many of the postdocs, du Vigneaud also continued basic research with the women and the draft-exempt men to maintain continuity until the war would come to an end. He decided that we should have a mass spectrometer so that we would not be limited to deuterium as a tracer, and I was given the task of constructing one because none was available commercially. Harry Thode, with whom I had overlapped in Urey's laboratory, had recently built a mass spectrometer at McMaster University in Canada. During World War II, he was the only scientist in the British Commonwealth who had the expertise to do so. He very generously supplied a replica of the main components, consisting of the ion gun, the collectors, and the glass tube. In addition, I had to get a magnet, supply the electronics, and assemble the parts. The difficulty in obtaining materials was considerable during the war, but I learned that a mixture of chutzpah and ingenuity were necessary to accomplish my goal. For example, to fabricate the metal shield in the 60° glass envelope, I decided to use flexible Greenfield cable made of Nichrome V—the difficulty was to find a company that would make it for me. After contacting several local companies and getting replies such as "Lady, don't you know there's a war on?" in desperation I tried the nation's biggest company in the industry, Anaconda Copper. They not only agreed to fabricate the cable but refused payment from a nonprofit institution. At that time there were no power supplies available with the necessary constancy, and I used a large array of storage batteries as a source of constant current. A miniature spot welder needed to weld the platinum leads to the Nichrome plates in the ion gun was assembled from a high-current step-down transformer connected to small electrodes mounted on a wooden test tube holder. To avoid purchasing an astronomically expensive timing device for the welder, the desired timing was accomplished with fuses of different ratings, which reliably expired in reproducible times, thus costing

five cents per weld. The instrument eventually was completed but it performed somewhat erratically.

After the end of World War II, when ^{14}C and ^{32}P and the instrumentation to measure radioactivity became commercially available, the stable isotopes lost their preeminence as metabolic tracers. In limited areas, where tracers for oxygen and nitrogen were desired, ^{18}O and ^{15}N found a role. The replacement of deuterium by tritium as a tracer was delayed until the introduction of scintillation counting, although deuterium continued to be used for specialized purposes. The era of the study of intermediary metabolism with the stable isotopes as probes was largely superseded because the radioactive isotopes had the advantages of relatively high sensitivity and comparative ease of measurement. In human metabolic studies, stable isotopes continued to be used to avoid exposure to radioactivity.

When I arrived at Washington University in 1946, Carl Cori had just assumed the chairmanship of the biochemistry department, and he gave me the opportunity to work independently, applying isotopes to the study of enzyme mechanisms. One of the problems that I suggested was to determine which bond of glucose-1-phosphate was cleaved in the hydrolytic reactions catalyzed by either acid or alkaline phosphatase and in the phosphorylase reactions by using the ^{18}O . It was not known at that time whether these reactions involved glucosyl or phosphoryl transfer. Although these experiments were simple in concept, the conduct of the experiments was far from simple. A mass spectrometer was needed for analysis of ^{18}O , a procedure for preparing ^{18}O -phosphate was required, and a method for converting the oxygen in inorganic phosphate to a gaseous form such as CO_2 had to be devised for mass spectrometric analysis of the oxygen. The second mass spectrometer was easier to build and a more successful instrument than the first one because Professor Nier of the University of Minnesota not only generously made available the excellent design that he had developed during the war but also had the magnet and the tube built in his shop. Also, Dr. Cori put an excellent electronics engineer (Mr. Loeffel) at my disposal. Materials were difficult to obtain for some time after the war and again I had to appeal to Anaconda Copper Co. for the necessary copper tubing. Finally, after approximately 2 years, all the methodology was in place and I was able to demonstrate that the phosphatases cleaved the P-O bond of glucose-1-phosphate but phosphorylase cleaved the C-O bond (Cohn, 1949).

I planned to extend this approach to many other enzymatic reactions of organic phosphates and I and other investigators subsequently did (Cohn, 1964); stable isotopes have served and continue to serve as excellent probes of enzyme mechanisms (Rose, 1995).

One of my colleagues, Graham Webster, challenged me. He pointed out that the reactions I was studying were already fairly well understood, and why didn't I tackle a reaction pathway that was completely unknown, namely the formation of ATP from inorganic phosphate in oxidative phosphorylation. Thus challenged, I approached Ed Hunter in the pharmacology department, who was studying oxidative phosphorylation, and we used my ^{18}O -phosphate with his respiring mitochondria and I dis-

covered that a rapid exchange of the oxygen of phosphate with water was catalyzed in this system, unlike any of the other enzyme systems that I had investigated. At the time, I was leaving for a year (1950–1951) at Harvard Medical School and I told Cori that I planned to pursue the ^{18}O reactions in oxidative phosphorylation. His response was "Good. If you find out anything of interest, you will make David green with envy." During that year in Boston, I learned how to make viable mitochondria, and Cori was much amused by my pride in this accomplishment. The next couple of years were spent showing that this exchange reaction did not occur during substrate level phosphorylation (Cohn, 1951) and was characteristic of oxidative phosphorylation coupled to electron transport (Cohn, 1953). After a decade of experiments that did not reveal the mechanism of ATP formation, I gave up. Fortunately, Paul Boyer, who had early entered the field, did not give up. With ingenious ^{18}O experiments and dedication, he did uncover the mechanism of ATP formation from inorganic phosphate (Boyer, 1993), which has been confirmed by X-ray crystallography (Abrahams et al., 1994).

In recent years, stable isotopes have come into their own again in the context of nuclear magnetic resonance studies, particularly in structural studies of macromolecules. In these applications, it is not the mass of the nuclei that is the relevant parameter, but the magnetic properties of the nuclei, including ^2H , ^{13}C , and ^{15}N . Just as the stable isotopes opened up new concepts in the study of intermediary metabolism and enzyme mechanism, they have now made a comeback and are opening new vistas in the determination of macromolecular structure in solution.

References

- Abrahams JP, Leslie AGW, Lutter R, Walker JE. 1994. Structure at 2.8 Å resolution of $\text{F}_1\text{-ATPase}$ from bovine heart mitochondria. *Nature* 370:621–628.
- Boyer PD. 1993. The binding change mechanism for ATP synthase. *Biochim Biophys Acta* 1140:215–250.
- Cohn M. 1949. Mechanisms of cleavage of glucose-1-phosphate. *J Biol Chem* 180:771–781.
- Cohn M. 1951. A study of oxidative phosphorylation with inorganic phosphate labeled with oxygen 18. In: McElroy WD, Glass B, eds. *Phosphorus metabolism, vol 1*. Baltimore: The Johns Hopkins Press. pp 374–376.
- Cohn M. 1953. A study of oxidative phosphorylation with O^{18} -labeled inorganic phosphate. *J Biol Chem* 201:735–750.
- Cohn M. 1964. Applications of O^{18} to biochemical studies. In: Craig H, Miller SL, Wasserburg GJ, eds. *Isotopic and cosmic chemistry*. Amsterdam: North-Holland Publishing Co. pp 45–59.
- du Vigneaud V. 1942–1943. The significance of labile methyl groups in the diet and their relation to transmethylation. *Harvey Lect* 38:39–62.
- du Vigneaud V, Cohn M, Brown GB, Irish CJ, Schoenheimer R, Rittenberg D. 1939. A study of the inversion of D-phenylaminobutyric acid and the acetylation of L-phenylaminobutyric acid by means of the isotopes of nitrogen and hydrogen. *J Biol Chem* 131:273–296.
- du Vigneaud V, Kilmer GW, Rachele JR, Cohn M. 1944. On the mechanism of conversion in vivo of methionine to cystine. *J Biol Chem* 155:645–651.
- Rittenberg D. 1948–1949. Dynamic aspects of the metabolism of amino acids. *Harvey Lect* 44:200–219.
- Rose IA. 1995. Isotopic strategies for the study of enzymes. *Protein Sci* 4:1430–1433.
- Schoenheimer R, Ratner S, Rittenberg D. 1939. The metabolic activity of body proteins investigated with L(–) leucine containing two isotopes. *J Biol Chem* 130:703–732.
- Schoenheimer R, Rittenberg D. 1935. Deuterium as an indicator of intermediary metabolism. *J Biol Chem* 111:175–181.