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Memories of a Senior Scientist

Sixty years of research: from soil science and the browning of dried apricots to the biochemistry of metabolism

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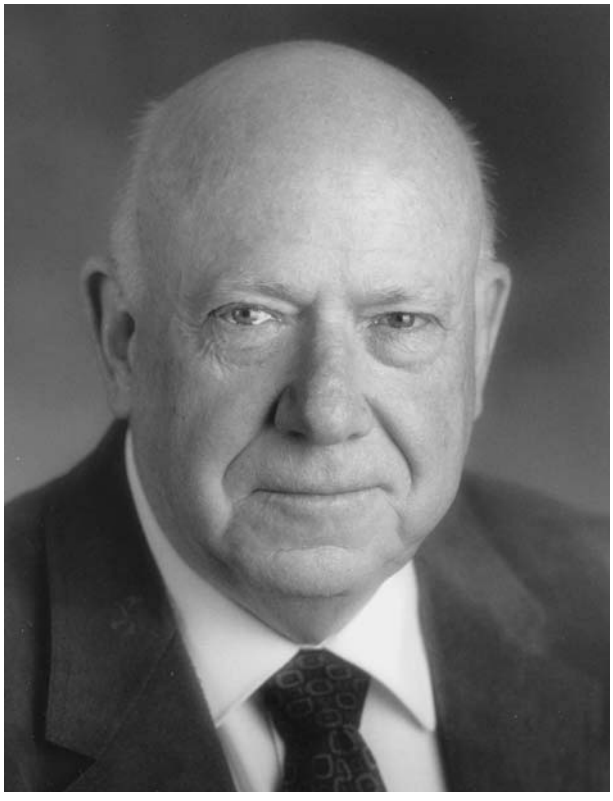

I was born November 15, 1919, in Carrizozo, NM. In 1928, our family moved to Roswell, NM, and in 1930 to San Bernardino, CA, where I attended grammar school (5th and 6th grades), junior high school, high school, and junior college. In 1940, I went to the University of California in Berkeley and obtained a B.S. degree in soil science because I wanted to learn how to analyze soils and determine what kinds of fertilizer should be added to make plants grow better. I learned that you cannot do that. However, this proved to be a rewarding experience because the soil science curriculum included courses in organic, inorganic, and analytical chemistry, physics, bacteriology, human and plant physiology, plant nutrition, soil physics, colloidal chemistry, agronomy, and soil microbiology. The latter course proved to be critical to my scientific development. The course was taught by a highly reputed microbial biochemist, H. A. Barker, for whom I worked as a technician during the last year before I graduated in 1942.

After graduating shortly after the beginning of World War II, I accepted a job as a member of the survey group charged with a survey of the overland route leading from Alaska to Canada, the so-called ALCAN highway. When I returned after nine mnths in Alaska, I visited Dr. Barker, who informed me that the University was encouraging professors to become engaged in war-related projects and that he had agreed to direct a research program on the 'browning of dried apricots' in the Department of Food Technology. This was considered a war project because dried fruit, an important part of the soldier's diet, deteriorated rapidly at the high temperatures in the South Pacific and became inedible after only a few weeks. Barker asked me if I would be interested in working as his assistant on this project. I gladly accepted and for the next five years I worked on the browning of dried apricots. This proved to be a wonderful experience. I learned a lot of analytical chemistry and became familiar with emerging,

forefront technologies. Among other findings, we established that the browning reaction involves conversion of hexose and pentose sugars to furfuraldehyde derivatives that condense with amino groups of proteins to produce intermediates that undergo browning.

Even more important for me was the fact that during the early phases of this research I met Thressa Campbell (Terry), who was also working in the Food Technology Department, and we were married in 1943.

When the war was over, Barker agreed to serve as my mentor for my Ph. D. studies. For my thesis, I carried out studies to elucidate the mechanism of short-chain fatty acid synthesis by *Clostridium kluyveri*, an organism that Barker had isolated from canal mud outside Professor Kluyver's laboratory in Delft, Holland. Previous studies by Barker and his associates had established that *C. kluyveri* catalyzed conversion of acetate and ethanol to a mixture of C_4, C_6, and C_8 fatty acids. I found that soluble enzymes in cell-free extracts of *C. kluyveri* could carry out all of the steps involved in the synthesis and oxidation of these fatty acids. Further studies with these enzyme preparations and subsequent work as a postdoctoral fellow in Fritz Lipmann's laboratory at the Massachusetts General Hospital established that acetyl-Coenzyme A (acetyl-CoA) is formed in the oxidation of ethanol by *C. Kluyveri* and that the synthesis of fatty acids involves the condensation of two equivalents of acetyl-CoA to form acetoacetyl-CoA, which is subsequently converted to butyric acid by a pathway in which β-hydroxybutyryl-CoA, crotonyl-CoA, and butyryl-CoA derivatives are intermediates. This was the first evidence that acyl-CoA derivatives are intermediates in fatty acid synthesis. For reviews, see [1, 2].

In 1950, Terry and I were recruited by C. B. Anfinsen to join the Laboratory of Cellular Physiology and Metabolism of the National Heart Institute at the National Institutes of Health (NIH), in Bethesda, MD, where we have been ever since. In the meantime, I have had the pleasure of carrying out research with more than one hundred highly talented graduate students, postdoctoral fellows, and visiting scientists. Regretfully, due to the space limitation and diversity of the research projects, it is impossible to describe in detail the contributions of each associate. Therefore, this presentation represents a brief summary of the research objectives and major experimental findings of our research during the past 52 years, and includes references to comprehensive reviews in which individual contributions are discussed.

The early studies at NIH focused on the role of CoA in metabolism. These studies led to the demonstrations: (a) that cells contain CoA transferases that can catalyze transfer of the CoA moiety of one acyl-CoA derivative to the carboxyl group of another acid to form the corresponding acyl-CoA derivative; (b) that the CoA-dependent oxidation of some aldehydes can lead to the formation of acyl-CoA derivatives; (c) that cells contain acylthiol transferases that catalyze the transfer of an acyl group from one thiolester to the sulfhydryl group of another compound to form its thiolester derivative; and (d) that extracts of *C. kluyveri* contain an enzyme that catalyzes the acetyl-CoA-dependent acetylation of hydrogen cyanide, which serves as an acetyl donor for the acetylation of amino acid amino groups [3, 4].

In efforts to elucidate basic mechanisms involved in heterocyclic compound metabolism, enrichment culture technology was used to isolate strains of bacteria that could grow on riboflavin as its sole substrate and another strain that could grow on nicotinic acid. Studies with these organisms carried out by various postdoctoral fellows were the basis of a number of research projects in our laboratory for nearly 15 years [5, 6].

In 1960,Terry and I went on sabbatical leave and spent the first six months working in Feodor Lynen's laboratory in Munich and the next 6 months in Paris, where Terry worked in Marianne Manago's laboratory and I worked with Georges Cohen at the Pasteur Institute. In Lynen's laboratory, I initiated a research project to test the hypothesis that vitamin B_{12} and biotin are implicated in the metabolism of propionate by *Propionibacterium shermanii*. I was assisted in this study by two students in Lynen's laboratory, Peter Overath and Herman Eggerer. We established that cell-free extracts of *P. shermanii* catalyzed the biotin-dependent addition of CO_2 to propionyl-CoA to form methylmalonyl-CoA and the vitamin B_{12} coenzyme-dependent intramolecular transfer of the -COSCoA moiety of methylmalonyl-CoA to the methylene carbon atom to form succinyl-CoA. These reactions are implicated in the metabolism of fatty acids with an uneven number of carbon atoms [7, 8].

At the Pasteur Institute, I participated in an ongoing project in Cohen's laboratory to elucidate the mechanism involved in the regulation of aspartokinase in *E. coli*. This enzyme catalyzes the ATP-dependent conversion of aspartate to aspartyl-P, which is an intermediate in the biosynthesis of three different end products – threonine, lysine, and methionine. This study led to the discovery that *E. coli* contains three different aspartokinases [9, 10]. One was specifically and noncompetitively inhibited by L-lysine and its formation was completely repressed by growth in a medium containing excess lysine. One was specifically and competitively inhibited by L-threonine but was not subject to repression by growth in the presence of threonine. The third enzyme was specifically inhibited by homocysteine.

The sabbatical year had a profound influence on my subsequent research at NIH. The studies in Lynen's laboratory prompted investigations on the role of vitamin B_{12} in the anaerobic metabolism of nicotinic acid, ethanolamine deaminase, and ethylene glycol, and also in the conversion of CO_2 to various products in *Clostridium*

thermoaceticum [11]. The study in Cohen's laboratory prompted studies on the regulation of glutamine synthetase (GS) activity in *E. coli*. This enzyme catalyzes the ATP-dependent conversion of NH_3 and glutamate to glutamine, which is the first step in a highly branched metabolic pathway, leading to the formation of adenylic acid, cytidine triphosphate, carbamyl phosphate, glucosamine-6-phosphate, tryptophan, histidine, and some amino acids. The enzyme from *E. coli* was purified to homogeneity and crystallized, and its activity was shown to be only partially inhibited by any one of seven different end products of glutamine metabolism. However, their effects were complementary. Collectively, they could inhibit 90% of the glutamine synthetase activity by a process referred to as 'cumulative feedback inhibition'. Furthermore, it was established that GS is comprised of 12 identical subunits, arranged in two superimposed hexagonal arrays. However, unlike the regulation of other enzymes, it was established that feedback inhibition of *E. coli* GS is regulated by a bicyclic nucleotidyl cascade system. One cycle involves the adenylyl-transferase-dependent covalent attachment and detachment of the AMP moiety of ATP to a unique tyrosyl residue in each subunit of GS and the other cycle involves the uridylyl-transferase-catalyzed covalent attachment and detachment of the UMP moiety of UTP to a tyrosine hydroxyl group in a regulatory protein (M_r = 44,000). The adenylylation and deadenylylation reactions are catalyzed by the same adenylyl-transferase whose adenylylation capacity is specified by binding of the unmodified form of the regulatory protein to the adenylylation site, and its deadenylylation capacity is specified by binding of the uridylylated form of the regulatory protein to the deadenylation site on the enzyme. These site-specific interactions are regulated by the concentrations of the enzyme substrates (ATP, UTP, orthophosphate) and also by allosteric interactions with 2-oxoglutarate and glutamine. Significantly, only the adenylylated subunits of GS are subject to inhibition by end products of glutamine metabolism. Accordingly, the sensitivity to feedback control is determined by the average number of adenylylated subunits per enzyme molecule, which, depending on the cellular environment, can vary from a value of 1 to 12. For reviews of this work, see [12–14].

In the course of our studies on the regulation of glutamine metabolism, I became aware of the fact that the cellular levels of various enzymes are strongly influenced by dietary factors. Under conditions of nitrogen or carbon starvation, the levels of some enzymes increase, some decrease, and others remain the same. In an effort to elucidate the mechanisms involved in the selective degradation of particular enzymes, we measured the activities of 15 enzymes in *E. coli* that had been grown under nitrogen or carbon starvation conditions. We found that under both conditions, five of the enzymes decreased in activity, five increased in activity, and five remained the same; however, the enzyme changes observed under nitrogen starvation were different from those observed under carbon starvation. Because glutamine synthetase was one of the enzymes that decreased under nitrogen starvation, we followed the loss of this enzyme in cell-free extracts of the nitrogen-starved cells. This led to the discovery that the loss of GS activity was dependent upon the presence of molecular oxygen, an electron donor (NADH or NADPH) and a transition metal ion (Cu^{2+} or $Fe^{3\%}$), i.e. by a classical mixed-function oxidation system. Subsequent studies confirmed that the oxidative modification of enzymes by any one of several different mixed-function oxidation system marks them for proteolytic degradation [15, 16]. Many of the enzymes we found to be readily oxidized by mixed-function oxidation systems had been shown by other workers to accumulate as catalytically inactive, or less active, more heat-labile forms during normal animal aging. Therefore, we proposed that oxidation is involved in the accumulation of altered forms of enzymes during aging [17–19]. In the meantime, we demonstrated that the level of oxidized protein increases under conditions of oxidative stress, during aging, and in the development of many diseases, and that the accumulation of oxidized protein in tissues is due in part to the loss in ability to degrade the oxidized proteins [20].

Our studies and those in other laboratories showed that surface-exposed methionine residues of proteins are particularly sensitive to oxidation to methionine sulfoxide by many different reactive oxygen species. But unlike most other types of oxidation, the oxidation of methionine can be reversed by the action of reductases that catalyze the thioredoxin-dependent reduction of methionine sulfoxide back to methionine. This led to the proposition that cyclic oxidation and reduction of methionine residues of proteins may be involved in the scavenging of reactive oxygen species and thereby protect cells from oxidative damage [21]. In the meantime, this thesis is supported by our further studies and those in other laboratories [22].

In conclusion, during the past 60 years of basic research, I have been very fortunate to have had an opportunity to carry out research in a number of basic disciplines. Although most of this time was spent at the National Institutes of Health in Bethesda, MD, from 1954 to 1961 I taught a night school graduate course in Biochemistry sponsored by the U.S. Department of Agriculture and, in the intervening years, as a Visiting Professor, I participated in the teaching of graduate courses at a number of universities, including a course in Biochemistry at Georgetown University (1956–1958), a course on Regulation of Metabolism at the University of Pisa, Italy (1966), a series of lectures on the Mechanism of Enzymic Regulation of Metabolism at the University of Camerino, Italy (1972), a series of lectures in a graduate course in

Biochemistry at Johns Hopkins University as part of the joint program sponsored by the Johns Hopkins University and the Foundation for Advanced Education in the Sciences, a course on Cellular Regulation in the Department of Chemistry at the University of Illinois, Urbana, IL (1967), in the Department of Chemistry at Pennsylvania State University, University Park, PA (1969), and in the Department of Biochemistry (Battelle Research Center) at the University of Washington, Seattle, WA (1973). In addition, I participated as a Visiting Professor in teaching graduate courses at West Virginia University, Morgantown, VA (1975), in the Department of Biochemistry and Molecular Biology at the University of Florida, Gainesville, FL (1977), and in the Department of Biochemistry and Nutrition at the University of North Carolina, Chapel Hill, NC (1980). These associations have played an important role in my scientific career since interactions with students at these institutions have led to recruitments of a number of talented postdoctoral fellows.

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