# W. W. "Mo" Cleland: A Catalytic Life

Debra Dunaway-Mariano,[†] Hazel M. Holden,[‡] and Frank M. Raushel*[,§]

[†]Department of Chemistry and Chemical Biology, University of New Mexico, Albuquerque, New Mexico 87131, United States
[‡]Department of Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706, United States
[§]Department of Chemistry, Texas A&M University, College Station, Texas 77843, United States

ABSTRACT: Professor W. Wallace Cleland, the architect of modern steady-state enzyme kinetics, died on March 6, 2013, from injuries sustained in a fall outside of his home. He will be most remembered for giving the enzyme community Ping-Pong kinetics and the invention of dithiothreitol (DTT). He pioneered the utilization of heavy atom isotope effects for the elucidation of the chemical mechanisms of enzyme-catalyzed reactions. His favorite research journal was *Biochemistry*, in which he published more than 135 papers beginning in 1964 with the disclosure of DTT.
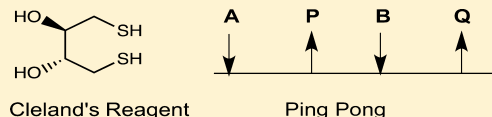
Professor W. Wallace Cleland, the architect of modern steady-state enzyme kinetics, died on March 6, 2013, from injuries sustained in a fall outside of his home in Madison, WI. His untimely departure marked the end to a brilliant academic career that shaped the development of mechanistic enzymology over the past half-century. Cleland's early reputation in biochemistry was built upon a series of three landmark papers published in 1963 that provided a logical and precise terminology for the analysis and interpretation of the kinetics of enzyme-catalyzed reactions involving more than a single substrate and product.[1−3] It is from these papers that the term "Ping-Pong mechanism" was first coined to describe the situation in which one or more products are released into solution before all substrates have been added to the active site. These papers were followed the next year with his invention of dithiothreitol (DTT) as a protective agent for the reduction of disulfide bonds in proteins.[4] This compound has proven to be enormously successful as a replacement for the obnoxious odor of β-mercaptoethanol and is now known and sold commercially as "Cleland's Reagent". At the beginning of his career, Cleland focused on the application of enzyme kinetics to elucidate the reaction mechanisms catalyzed by a series of kinases and dehydrogenases. By the early 1970s, his research focus shifted to concentrate almost exclusively on the development and utilization of isotope effects for the elucidation of enzyme-catalyzed reaction mechanisms.

Cleland spent his entire research and teaching career in the Department of Biochemistry at the University of Wisconsin—Madison (UW), except for two years of military service (1955−1957) at the Medical Nutrition Laboratory in Denver, CO, and two years at the University of Chicago in the laboratory of Eugene Kennedy as a National Science Foundation (NSF) Postdoctoral Fellow. He first arrived in Madison as a graduate student in 1951 after obtaining an undergraduate degree in chemistry from Oberlin College in 1950. As an NSF Predoctoral Research Fellow, Cleland joined the laboratory of Professor Marvin J. Johnson where he conducted his doctoral research on biochemical pathways for citric and oxalic acid formation in *Aspergillus niger*. His two research papers with

Johnson were published in the *Journal of Biological Chemistry*, and he graduated from UW with a doctorate in biochemistry in 1955.[5,6] In the Kennedy laboratory at the University of Chicago (1957−1959), Cleland was supported by an NSF Postdoctoral Fellowship where he discovered galactosyl-sphingosine transferase, an enzyme that converts sphingosine and UDP-galactose to psychosine and UDP.[7] It is likely that Cleland's first encounter with the measurement of enzyme kinetic constants occurred in the Kennedy laboratory.

Cleland joined the UW Biochemistry Department in 1959 as an Assistant Professor and was promoted to Associate Professor in 1962, apparently without having published any independent papers up to that time. A photograph of the young Cleland in his office in 1964 is presented in Figure 1 along with two additional photographs from his later years. His initial research interests focused on lipid biochemistry and the substrate specificity of acyl-CoA thioesters in the acylation of glycerophosphate to form phosphatidic acids. His first graduate student was Estela Sanchez Quintanar, the first Mexican woman to earn a doctorate in biochemistry.[8] Cleland's research interests changed significantly after he was asked to teach biochemistry to first-year graduate students. He became dismayed with the presentation of enzyme kinetics and then set out to revolutionize how steady-state kinetics could be used to determine the mechanisms of enzyme-catalyzed reactions. These efforts led to the publication of three papers in *Biochimica et Biophysica Acta* (BBA) in 1963 that have become citation classics.[1−3] Since their publication, these papers have been collectively cited more than 4000 times.

Manuscripts of the three articles were first submitted to *Biochemistry* in 1962, but the Editor-in-Chief at the time, Hans Neurath, rejected them for being too lengthy for the new American Chemical Society (ACS) publication.[9] They were then simultaneously offered on an informal basis to *Journal of Theoretical Biology*, *Journal of Biological Chemistry* (JBC), and

Figure 1. Professor W. W. Cleland at various stages in his career.

BBA. After JBC indicated that they would be receptive to a formal submission, they were submitted to this journal. However, shortly thereafter BBA, having misunderstood the trial basis for the initial submission, accepted the manuscripts pending minor corrections. JBC, after a formal review, wanted substantial revisions. Not wanting to modify the new nomenclature further, he submitted the final corrections to BBA.

The Cleland approach to steady-state kinetics has stood the test of time because the framework of his methodology and nomenclature has allowed an intuitive and visual depiction of what must be happening within an enzyme active site. This is easily seen for the classic Ping-Pong mechanism shown in Scheme 1, where the horizontal line represents the enzyme

Scheme 1. Shorthand Notation for the Ping-Pong Kinetic Mechanism
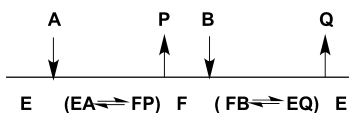
surface, E and F represent stable enzyme forms, EA, FP, FB, and EQ represent transitory enzyme complexes, A and B are the two substrates, and P and Q are the two products of the reaction.

However, the new nomenclature was not so popular in Cleland's own family.[10] Ralph Cleland, Mo's father, then Chairman of the Biology Department at Indiana University, and

a member of the National Academy of Sciences, voiced strong criticism of the "Ping-Pong" terminology, calling it nonprofessional, and thought it should be modified. The young assistant professor was not convinced. The third paper ends with a paragraph that begins, "The value of the methods outlined in this paper is too obvious to need further elaboration here. They have been used routinely in this laboratory for exploring possible mechanisms, and they have proven of special value to students trying to learn something useful about kinetics without getting lost in equations and algebra". The BBA papers of 1963 were published 50 years ago this year and 50 years after the publication of the classic Michaelis–Menten paper in 1913. Cleland was soon transformed from a lipid biochemist to a mechanistic enzymologist. In later years, he was heard to remark, "the UW faculty thought that they were hiring a lipid biochemist, but in fact what they got instead, is a fat biochemist."

Cleland's publications that followed focused on the application of steady-state kinetics and the kinetics of isotope exchange for the elucidation of enzyme mechanisms. Cleland soon realized that the graphical and desktop calculator-based methods then used for data analysis were inadequate for his purposes. In the same year that the three BBA papers appeared, his first computer programs, written in Fortran for use with an IBM 1620 computer, were published in *Nature*.[11] The workhorse in the Cleland laboratory in those early years was the Beckman DU monochomator equipped with a Gilford 200 optical density converter and strip-chart recorder (Figure 2).


Figure 2. Beckman DU spectrophotometer.

Rates of product formation were measured with the aid of a homemade light box and an elaborate set of "ship's curves". In 1977, he confidently stated in a review article that "...a new generation of enzymologists, armed with a brain and a Gilford and requiring only modest funds, will ferret out the secrets of how enzymes really catalyze their reactions".[12]

Cleland's most cited paper in *Biochemistry* was published in 1964 on the utilization of dithiothreitol as a protective agent for thiol groups.[4] At this time, his research program was supported by a grant from the National Institutes of Health that focused on determining the substrate specificity of acyl-CoA thioesters for the acylation of glycerophosphate to form phosphatidic acids. Commercial Coenzyme A cost $600 per gram, and 25% of this material was oxidized. At the time, thiols were kept reduced via the addition of low-molecular weight thiols, such as β-mercaptoethanol or cysteine, to solutions through thiol–disulfide exchange reactions. Because the equilibrium constants for these reactions are near unity, a large excess of β-mercaptoethanol is required. Cleland reasoned that if a cyclic disulfide were to form, the equilibrium constant would be

significantly more favorable. The first compound of this type to be tested was 1,4-butanedithiol, but this compound reeked so badly that it was quickly discarded.[13] That experience led to the introduction of hydroxyl groups to the central two carbons for improvement in water solubility and reduced volatility (Scheme 2). DTT worked spectacularly well and remains the reagent of

Scheme 2. Structure of Dithiothreitol in the Reduced and Oxidized Forms
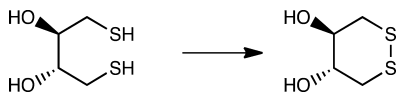
choice for keeping thiols groups in proteins and coenzymes reduced. Calbiochem was the first commercial company to market DTT as "Cleland's Reagent". He apparently never benefited financially from this invention and paid retail price for DTT from the commercial supply houses. The paper has been cited more that 1600 times.

In another highly cited *Biochemistry* article, Cleland introduced the concept of net rate constants as a convenient and easy way to write the expressions of the standard kinetic constants, k_{cat} and k_{cat}/K_m, in terms of the microscopic rate constants.[14] To use this method, the true rate constants for the forward and reverse reactions for a given step are substituted with the equivalent "net rate constant" for an irreversible step that would provide the same flux through that step in the steady state. This is visualized in the example provided in Scheme 3. In

**Scheme 3. Formulation of "Net Rate Constants" for Enzyme-Catalyzed Reactions**
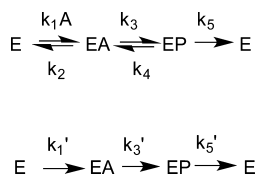
this format, the velocity of the reaction is given by the expression $v/E_t = 1/(1/k_1' + 1/k_3' + 1/k_5')$. For the irreversible steps, the net rate constant is equal to the true rate constant, and thus in this example, $k_5' = k_5$. For reversible steps, the net rate constant is equivalent to the true rate constant for the forward direction multiplied by the fraction of the subsequent enzyme complex that partitions to product. Thus, for the second step, $k_3' = k_3[k_5/(k_4 + k_5)]$. After the expression for k_1' has been written, the entire expression for the steady-state reaction can be derived in a matter of minutes. For many enzymologists, the concept of net rate constants has become the method of choice for the derivation of steady-state rate equations.

The most provocative title for any Cleland paper was most certainly "Enzymology - Dead?" that was published in *Trends in Biochemical Sciences*.[15] In that paper, he laments the fact that some influential segments of the biochemistry community considered enzymology a "dead science" and not worthy of serious attention. At the time, molecular and cell biology were considered by some more fashionable, and the results from these investigations more likely to be publicized in the *New York Times*. He forcefully extolled the virtues of detailed kinetic analyses, the explosion of structural data from advances in X-ray crystallography, and the intricacies of chemical bond formation

that can be gleaned from kinetic isotope effects to proclaim that it was an exciting time to be an enzymologist. Indeed, Cleland's most recent work, focused on elucidating the catalytic mechanism of the multidomain, multifunctional biotin-dependent enzyme pyruvate carboxylase, provides testament to the endurance of the excitement that has been carried forward by the current generation of mechanistic enzymologists.

Cleland's laboratory published work beginning in the late 1960s that led to what would become a long-term fascination with phosphoryl transfer. His group employed isotope exchange, in combination with initial velocity patterns, to distinguish between sequential and Ping-Pong mechanisms, and the inhibition patterns of substrates, products, and dead-end inhibitors to define binding order and identify dead-end complexes. Dead-end inhibitors were particularly effective as kinetic probes, yet at the time, none were available for targeting ATP binding sites in enzymes or for Cleland's intended use, mimicking the substrate-active MgATP complex in kinase-catalyzed reactions. The challenge for inhibitor design was to block phosphoryl transfer from ATP. Two independent solutions were ultimately developed. In the laboratory of Ralph Yount, the oxygen atom that bridges the γ- and β-phosphoryl groups was replaced with a nitrogen atom to produce adenylyl imidodiphosphate (AMP-PNP).[16] In the Cleland laboratory, ATP was reacted with $Cr^{3+}(H_2O)_6$ to produce the exchange inert $Cr(H_2O)_nATP$ complex.[17] $Cr(H_2O)_nATP$ was first used in the Cleland laboratory as a kinetic probe, and then the positional and stereochemical (Δ or Λ screw-sense) coordination isomers were separated from one another and used to define the structure of the kinase-active MgATP substrate.[18] The structures of the Δ and Λ screw-sense isomers of β,γ-CrATP are presented in Scheme 4.

Scheme 4. Structures of the Λ and Δ Isomers of β,γ-CrATP



(Λ)-β,γ-CrATP (Δ)-β,γ-CrATP
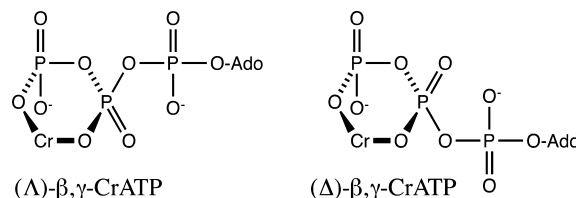
Around 1972, Cleland began to focus his research efforts on improving our understanding of how heavy isotope effects on enzyme-catalyzed reactions could be used to unveil even greater details of transition-state structures within the active sites of enzymes. Pigeon liver malic enzyme was the first protein to be interrogated using isotope effects in his laboratory. It was in these very early days that Michael Schimerlik, a graduate student, and Cleland invented the equilibrium perturbation methods for measuring very small isotope effects on enzyme-catalyzed reactions.[19] In this methodology, the substrates and products of an enzyme-catalyzed reaction are mixed together in a cuvette at concentrations that approximate those that would satisfy the thermodynamic equilibrium constants for the overall reaction. However, one of the substrates contains a heavier isotope at the bond to be cleaved, while the corresponding product contains the lighter isotope. After the enzyme is added, the equilibrium is perturbed because there is net flux from the side of the reaction with the lighter isotope to the side containing the heavier isotope. This perturbation continues until the isotopic driving force is balanced by the net change in

the relative concentrations of substrates and products. The net flux then reverses course, and the reaction returns to the original concentration of substrates and products. Prior to the addition of enzyme, the system is at chemical equilibrium but not isotopic equilibrium. After the addition of enzyme, the system gradually attains both chemical and isotopic equilibrium. This methodology can be used with nearly any enzyme-catalyzed reaction that can be followed optically. The sensitivity is such that isotope effects using ^{13}C and ^{15}N that rival those that can be determined using an isotope ratio mass spectrometer can be measured. The groups of Dexter Northrop (Pharmacy) and Marion O'Leary (Chemistry) at UW also began intense efforts using heavy atom isotope effects to interrogate enzyme-catalyzed reactions.

The application of heavy atom isotope effects on enzyme-catalyzed reaction remained Cleland's primary research interest until the end of his life and led indirectly to the identification of "low-barrier hydrogen bonds" in enzyme catalysis. In 1987, Cleland proposed that cysteine residues in the active sites of enolase and adenosine deaminase were responsible for certain proton transfer reactions that occurred within these enzymes during the conversion of substrates to products. In the case of enolase, it was shown that 2-deuterated tartronate semialdehyde phosphate had a dissociation constant double that of the unlabeled inhibitor.[20] With adenosine deaminase, it was demonstrated that there was an inverse solvent isotope effect of 0.77 during the deamination of the substrate.[21] In both examples, it was postulated that the sulfhydryl group of an active site cysteine was required for these proton transfers because this is the only amino acid for which the fractionation factor (tendency for deuterium to enrich in a site relative to its content in water) is significantly less than unity. Much to Cleland's surprise, no cysteine residues were observed in the active site when the X-ray crystal structures of these two enzymes were determined later. These results forced a rethinking of the catalytic mechanism and the origin of the low fractionation factors observed for these two enzymes. A solution was found within the prior work of Kreevoy, who had studied hydrogen bonding situations in which the hydrogen is shared between two groups of nearly equal pK_a values. As the distance between the two heteroatoms is compressed to less than 2.55 Å, the hydrogen can move freely between these atoms and the fractionation factor becomes very low. Because these low-barrier hydrogen bonds are significantly stronger than "ordinary" hydrogen bonds, Cleland and Kreevoy have postulated that the formation of such hydrogen bonds during enzyme-catalyzed reactions makes an important contribution to the rate enhancements that are observed.[22]

Cleland's long-standing interest in phosphoryl transfer reactions led to the development of a clever approach for assessing the transition-state structures of these reactions via heavy atom isotope effects using remote labels and an isotope ratio mass spectrometer.[23] For phosphodiesterase-catalyzed reactions, Hengge and Cleland synthesized substrates with *p*-nitrophenol leaving groups that were differentially labeled with ^{15}N and [18]O as illustrated in Scheme 5.[24] One of the compounds was synthesized with [18]O at the nonbridging position and ^{15}N in the nitro substituent. The other compound was synthesized with [16]O at the nonbridging position and ^{15}N-depleted nitrogen within the nitro substituent. The two compounds were mixed in the ^{15}N/^{14}N natural abundance ratio and allowed to react with the enzyme until the reaction had proceeded to ∼50% completion. The *p*-nitrophenol was

Scheme 5. Remote Labeled Substrates for Measuring Secondary [18]O Isotope Effects in Phosphodiesterases
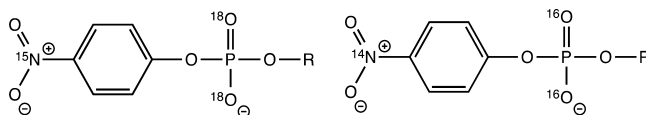
isolated and the nitro substituent quantitatively converted to N_2, in which the ^{15}N/^{14}N ratio could be accurately determined using the isotope ratio mass spectrometer. The change in the ^{15}N/^{14}N ratio provided an indirect measure of the change in the ^{18}O/^{16}O ratio and thus a determination of the secondary [18]O isotope effect for phosphoryl transfer. A similar approach was used by Caldwell et al., to measure the primary and secondary [18]O isotope effects for enzymatic and nonenzymatic phosphotriester hydrolysis.[25]

Isotope effects were also used by Cleland to devise a unique approach to the determination of whether an enzyme-catalyzed reaction was concerted or stepwise.[26] In the malic enzyme-catalyzed reaction, malate is oxidatively decarboxylated to pyruvate and CO_2 using $NADP^+$ as the oxidant. For this reaction, the mechanistic question was whether oxaloacetate is an obligatory intermediate as illustrated in Scheme 6. This issue

Scheme 6. Stepwise Mechanism for Malic Enzyme
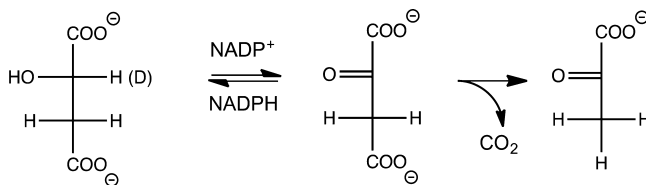
was resolved by measurement of the ^{13}C isotope effect for the decarboxylation at C4 using malate labeled with hydrogen and then deuterium at C2. Equations were derived to demonstrate that if the C−H and C−C bond cleavage reaction occurred within the same step (concerted) of the reaction then deuteration of malate would increase the size of the ^{13}C isotope effect for decarboxylation. If C−H bond cleavage occurred in a step prior to C−C bond cleavage, then the ^{13}C isotope effect for decarboxylation would be expected to decrease with deuteration of the malate because the decarboxylation step would be less rate-limiting with a substrate that was deuterated at C2. With malic enzyme, Hermes et al. demonstrated that the ^{13}C isotope effect decreased from 1.031 to 1.025 upon deuteration of malate, thus demonstrating that oxidation at C2 preceded decarboxylation.

The application of isotope effects to unveil mechanistic issues in enzyme-catalyzed reactions remained the main focus of the Cleland laboratory until the very end. The laboratory continued to collaborate with a significant number of research groups for more than two decades. These included Paul Fitzpatrick (D-amino acid oxidase and tryptophan 2-monooxygenase), George Reed (enolase), Donald Hilvert (prephenate dehydratase), Paul Atwood, John Wallace, and Ivan Rayment (pyruvate carboxylase), Nigel Richards (oxalate decarboxylase), Hazel Holden (perosamine *N*-acetyltransferase and glucose-1-phosphate cytidylyltransferase), Richard Wolfenden (cytidine deaminase), Perry Frey (chymotrypsin and dTDP-glucose 4,6-dehydratase), John Denu (nicotinamidase), Alvin Hengge (phosphoryl transfer), and Paul Cook (6-phosphogluconate dehydrogen-

ase). In addition to these collaborators, Cleland had a fascination with Australia and a long-term friendship with Professor John Morrison of the Australian National University.

Biochemistry was Cleland's favorite journal for publication of his research efforts. With approximately 136 papers published in this journal, he currently ranks fifth as the most prolific author. He was elected to the American Academy of Arts and Sciences in 1977 and to the National Academy of Science in 1985. His received the Merck Award from ASBMB, the Alfred Bader Award and Repligen Award from the ACS, and the Stein and Moore Award from the Protein Society. His love of enzymes, keen intellect, and enthusiastic support of science will be missed.

The scientific legacy left behind from Cleland's passing is, indeed, enormous, and most biochemists are well aware of his outstanding research achievements. There is more to Cleland than science, however. He was born January 6, 1930, in Baltimore. He was named after the Scottish hero William Wallace. His father was then a Professor at Goucher College, and his mother was an organic chemist. According to his brother Charles Cleland, he was called Wallace or Wal as a young boy. Wal then morphed into Wal-Mo because of a family friend, and later the Wal was dropped leaving Mo, a nickname that endured for the next seven decades. He was an accomplished sailor at the Mendota Yacht Club in Madison, loved classical operas, and was an acclaimed philatelist. He received the Smithsonian Institution Philatelic Achievement Award in 2008 and was inducted into the United States Stamp Society Hall of Fame in 2009. In the stamp world, he was known as Wallace.

During his later years, Mo became very active in his neighborhood community and joined the Northside Planning Council. One member of the council commented, "Mo was always there". As Ellen Barnard of the Northside News so aptly stated, "Mo was deeply generous when it came to the people and the causes that he loved and volunteered his time as generously as his money. He would tell you a joke that would catch you by surprise, and his head was full of exotic bits and pieces of information that he shared to 'delight and amuse' people. He was opinionated, soft-hearted and a person one would be proud to call 'friend'".

■ AUTHOR INFORMATION

Corresponding Author
*Department of Chemistry, P.O. Box 30012, Texas A&M University, College Station, TX 77843. E-mail: raushel@tamu.edu. Telephone: (979) 845-3373.

Notes

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