W. Wallace Cleland

1930–2013

BIOGRAPHICAL *Memoirs*

A Biographical Memoir by
Perry Allen Frey

NATIONAL ACADEMY OF SCIENCES

WILLIAM WALLACE **CLELAND**

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William Wallace Cleland, professor of biochemistry at the University of Wisconsin-Madison, passed away on March 6, 2013, of injuries sustained in an accident on March 1, 2013. He spent his last days surrounded by his family.

Professor Cleland preferred to be addressed as "Mo." He was born to Elizabeth and Ralph Cleland on January 6, 1930, in Baltimore. The family moved to Bloomington, Indiana, where Mo's father became chair of the Department of Botany and dean of the graduate school at Indiana University.

After graduating summa cum laude with a bachelor of arts degree in chemistry from Oberlin College in 1950, Mo received his graduate education at the University of Wisconsin-Madison, where he studied carbohydrate metabolism in *Aspergillus niger*. He was a National Science Foundation (NSF) predoctoral fellow from 1953 until 1955 and received his master of science and PhD degrees in biochemistry in 1953 and 1955. He then served in the United States Army Medical Corps between 1955 and 1957.

By Perry Allen Frey

Following release from military service, Mo was an NSF postdoctoral fellow from 1957 until 1959 under Eugene P. Kennedy at the University of Chicago, where he researched the enzymatic synthesis of psychosine and cerebrosides. He joined the Department of Biochemistry at the University of Wisconsin-Madison in 1959 as an assistant professor and launched his research career in enzymology, specializing in enzymatic reaction mechanisms. Mo was promoted to associate professor in 1962 and to professor of biochemistry in 1966.

Steady-state kinetics of multi-substrate enzymatic reactions

The development of structure and function in enzymology, beginning with function, proceeded in three main phases: analysis of kinetic mechanisms, analysis of chemical mechanisms, and analysis of molecular structures of enzymes. A complete understanding

of the mechanism of enzyme-action required all three of these aspects to be rigorously determined and integrated into a logical picture.

Mo made influential contributions to enzyme kinetics throughout his career. At the time he began working in mechanistic enzymology, significant information about the biochemical functions of many enzymes was available, sufficient to allow detailed kinetic analysis to proceed. Less was known about chemical mechanisms and very little was known about molecular structures of proteins. Enzymes were known to be globular proteins with definite three-dimensional structures. Amino acid sequences of a few enzymes were known, and protein chemical methods for determining sequences were available. Mo made his most important contributions in the areas of steady-state enzyme kinetics and chemical reaction mechanisms of enzyme-action.

Mo's most widely cited work brought order into the field of multi-substrate, steady-state enzyme kinetics in 1963. Prior to that, steady-state enzyme kinetic mechanisms had been analyzed in various ways by other investigators, who employed varying styles in presenting the results. Consequently, the kinetic mechanisms appeared idiosyncratic, with few unifying principles relating the various studies. Mo analyzed the possible and prevalent kinetic mechanisms in three papers that he published in *Biochimica et Biophysica Acta*. The topics he covered were: nomenclature and rate equations; inhibition nomenclature and theory; and prediction of initial velocity and inhibition patterns by inspection. These papers showed how kinetic patterns and inhibition patterns could be viewed by inspection and lead to conclusions about the appropriate kinetic mechanisms and rate equations.

At the time, steady-state enzymatic rates were conventionally graphed as Lineweaver-Burk double reciprocal plots of rates on the ordinate and substrate concentrations on the abscissa, all measured at a fixed concentration of the enzyme. Most enzymes followed the Michaelis-Menten rate law, and this plot gave a straight line with positive intercept and slope. Multi-substrate enzymes acted on two or more substrates simultaneously and produced two or more products. In these cases, the concentration of one substrate was held constant while that of the other was varied in constructing a double-reciprocal plot. In this way, varying each substrate at several fixed concentrations of the second gave two classes of kinetic patterns.

Those graphs in which the lines differing in slope converged at the ordinate were symptomatic of "sequential binding mechanisms," in which the substrates became nonco-

valently enzyme-bound one at a time, either randomly or in compulsory order. The resultant ternary complexes reacted further to produce the products.

Those line patterns displaying identical slopes but different intercepts signaled a mechanism in which one substrate bound to the enzyme, imposed a chemical change on the enzyme, and the first product dissociated. Then the second substrate to bind reacted with the chemically altered enzyme to produce the second product and return the enzyme to its original state. Mo coined the term "ping-pong kinetics" for this pattern, implicating a covalently modified enzymesubstrate intermediate. The ping-pong mechanism became the most chemically revealing kinetic mechanism, in that it neatly divided the chemistry of catalysis into two distinct chemical processes.

Once the basic kinetic mechanism for an enzyme was established, the effects of inhibitors on rates could be analyzed. If an inhibitor was present at a fixed concentration, it would perturb an established double reciprocal plot in a characteristic way; a different line would result, having a different slope and either the same or a different intercept. With several different inhibitor concentrations, several different lines would be found. Inhibitors differed by whether they produced slope effects, intercept effects, or both in kinetic patterns. Competitive inhibitors could bind only in place of one substrate, and they gave slope effects, but not intercept effects, versus that substrate. Noncompetitive inhibitors could bind to either the free enzyme or to the complex of enzyme and the varied substrate, and they gave both slope and intercept effects. Uncompetitive inhibitors could bind only to the complex of the enzyme and the varied substrate, and they gave only intercept effects, resulting in parallel lines of identical slope.

Cleland's rules analyzed the effects of varying concentrations of inhibitors on double reciprocal plots. Inhibitors were classified as competitive, producing only slope effects; uncompetitive, producing only intercept effects; or noncompetitive, producing both slope and intercept effects. These effects were explained by Cleland's rules in terms of the species of enzyme complex to which the inhibitors could bind.

Cleland's reagent: dithiothreitol

Early biochemists purified oxygen-sensitive proteins in the presence of mercaptoethanol to protect cysteinyl sulfhydryl groups against oxidation. Glutathione or thioglycolate also were sometimes used for this purpose. These compounds served as reducing agents with reduction potentials of near –0.2 volts (V).

Mercaptoethanol was used most often because it was inexpensive and very soluble in water. However, the problems with mercaptoethanol were its odor and that relatively high concentrations of it were required, because of the reduction potential and because two molecules were required to reduce a disulfide. Mo reasoned that a dithiol that can form an internal cyclic disulfide, by analogy to dihydrolipoamide, should be superior. He studied the reducing properties of dithiol compounds that would form six-membered cyclic disulfides. Mo found that dithiothreitol (DTT) and dithioerythritol were clearly superior. They were highly watersoluble and displayed even lower reduction potentials than dihydrolipoamide. Mo found the reduction potential of DTT to be –0.332 V, about 0.044 V more negativethan that of dihydrolipoamide. DTT displayed little odor and reduced disulfides quickly and quantitatively. It also proved to be effective at very low concentrations.

It seems ironic that the structure of DTT can be viewed as two molecules of mercaptoethanol joined at carbon-1 by a covalent bond with threo-stereochemistry. DTT is known as Cleland's reagent and can now be found in most biochemical laboratories.

The biologically reactive structure of magnesium-adenosine triphosphate

Before high-resolution x-ray crystal structures of enzymes and enzyme-nucleotide complexes became available, the issue of the structure of the magnesium complex of adenosine triphosphate (ATP) at an enzymatic active site could not be addressed. The complex was routinely written into biochemical equations as MgATP without reference to the actual structure at an active site. Structures of very few proteins were available, and enzymes were known to act stereospecifically on substrates. It was assumed that Mg^{2+} in MgATP was coordinated to the triphosphate group of ATP, but it was not known which of the seventeen possible regio- and stereoisomers of MgATP functioned in enzymatic reactions. The issue extended to the structure of MgADP produced in reactions catalyzed by phosphokinases. Five regio- and stereoisomers of MgADP, with magnesium coordinated to the diphosphate group, were recognized but not identified as enzymatic products. It seemed likely that only a few of the possible structures would be reactive in enzymes, but which one(s) those could be were still a mystery.

Because of the rapid coordination exchange typical of Mg^{2+}, the regio- and stereoisomers would be interconvertible and at equilibrium in solution. A given enzyme was expected to bind only one of them. Mo addressed the issue of which isomer would bind and react by synthesizing coordination exchange-inert complexes of ATP and ADP. Molecules such

as Cr(III)ATP and Co(III)tetrammine-ATP could be synthesized, separated into isomers, and their structures could be determined by crystallography in collaboration with his colleague, M. Sundaralingham. He could then identify which isomers would preferentially bind to an enzymatic site.

Mo's studies of coordination exchange-inert complexes and their interactions with enzymes contributed to advances in enzyme stereochemistry. However, as the technology of macromolecular x-ray crystallography improved, crystals of enzymes with MgATP bound at the active sites could be obtained and the structures determined by x-ray diffraction.

Enzymatic kinetic isotope effects

The enzyme-substrate binding, product release, and conformational effects intervening among the chemical steps in the action of an enzyme create a fundamental problem in mechanistic analysis. Measurements of heavy-atom kinetic isotope effects (KIEs) are employed by chemists in non-enzymatic reactions to distinguish alternative mechanisms. But binding and conformational effects in enzymes can limit rates, often weakening this method.

In the second half of the twentieth century, most enzymologists employed KIEs in a qualitative way to determine whether a chemical step limited an enzymatic rate. A positive KIE implicated a chemical step in rate limitation and defined that step as part of the chemical mechanism. A negative result did not eliminate the chemical step from the mechanism because a binding process or enzyme conformational change might limit the observed rate. Analysis of KIEs in the Cleland laboratory proceeded quantitatively in several phases and required measurements of equilibrium isotope effects and secondary KIEs, as well as primary KIEs. Equilibrium effects were the effects of the heavy isotopes on equilibrium constants. Secondary KIEs were effects on rates where bonding to the heavy isotope was not cleaved, but the chemical nature of bonding changed, as in conversion of trigonal carbon to tetrahedral carbon.

In early collaborations with Wisconsin colleagues Marion O'Leary and Dexter Northrop, Mo tried to overcome these problems and apply KIEs to analyze chemical mechanisms in enzymatic catalysis. In the process, Mo invented the equilibrium perturbation method for measuring KIEs. This method was brilliantly conceived and enabled KIEs to be measured at chemical equilibrium in a single experiment.

Mo continued with this work and became a master of enzymatic kinetic isotope effects. He neutralized the masking of chemical steps by noncovalent processes through the use of alternative substrates to increase ligand dissociation rates, through the exploitation of pH effects to find conditions where chemical steps limit rates, and through site-directed mutagenesis to make chemical steps rate-limiting, KIEs on k_{cat} (turnover rate constant) could be measured.

Mo chose the internal competition method to determine KIEs on k_{cat}/K_m. In this method, the heavy atom was a trace label at natural abundance. This method gave KIEs on k_{cat}/K_m, the second-order rate constant for reaction of an enzyme with a substrate. This was the only method available for ^{14}C or ^{3}H effects because the radioactive species were always trace labels. Mo did not generally rely on radioactivity measurements. He preferred stable heavy atoms like ^{13}C, ^{15}N, and ^{18}O. Trace labeling with these isotopes often required chemical synthesis and always required chemical degradation of products and isotope ratio mass spectrometry to analyze substrates and products. Mo was never deterred by the required chemistry. Moreover, he obtained exceedingly accurate values of the small KIEs for isotopes such as ^{13}C and ^{15}N.

Mo defined the KIE for a multi-step enzymatic mechanism in terms of the equation

$$^{X}(V/K) = (^{X}k + cf + cr^{X}K_{eq})/(1 + cf + cr),$$

where V/K is k_{cat}/K_m; X is an identifier of the heavy isotope, D for deuterium, 13 for ^{13}C, 15 for ^{15}N, etc; K_{eq} is the overall equilibrium constant; and cf and cr are forward and reverse commitment factors. The commitment factors were defined as the ratio of the rate constant for the bond-breaking step to the net rate constant for release of the first product from the enzyme.

The Cleland laboratory proceeded to develop methods for defining enzymatic transition states, especially of pyridine nucleotide (NAD/NADH)-dependent dehydrogenases. Mo and his associates developed multiple KIE methods to refine transition states. As a general approach to a pyridine nucleotide-dependent dehydrogenase, the primary KIE for deuterium transfer from a substrate to NAD would be measured. In such a reaction, the hydride-accepting site, trigonal pyridine-C4 in NAD, is initially bonded to hydrogen, and upon accepting a hydride from the substrate, pyridine-C4 becomes tetrahedral. When pyridine-C4 is bonded to deuterium ([4-^{2}H]NAD), the change to tetrahedral carbon leads to a secondary deuterium KIE. Another secondary KIE might be the effect of deuterium placement in a non-transferring position in the substrate. The ^{13}C-KIE

would be measured with the heavy isotope at the hydride-donating position of the substrate. Another KIE would be a deuterium KIE measured in D_2O, where deuteride ion (D^+) is transferred in place of a proton (H^+).

In studies of multiple isotope effects, the Cleland lab was able to define transition-state structures for a number of pyridine nucleotide-dependent dehydrogenases. In the multiple isotope approach, they synthesized substrates with deuterium in place of the transferable hydrogen or ^{13}C in place of ^{12}C at a site remote from dehydrogenation but engaged in the overall chemical transformation. For example, dehydrogenation at a substrate site often potentiates decarboxylation at another site, either beta to the dehydrogenation site or linked through double bond conjugation to the dehydrogenation site. After measuring the deuterium-KIE and the ^{13}C-KIE, they synthesized the doubly labeled substrate and repeated the measurements. In this way they could determine the degree of coupling between hydrogen transfer and decarboxylation.

Employing these techniques, the Cleland group could distinguish step-wise mechanisms from concerted mechanisms. The malic enzyme, which catalyzes oxidative decarboxylation of malate, proceeds by a stepwise mechanism, with dehydrogenation preceding decarboxylation. In contrast, based on research in the Cleland group, the reaction of prephenate dehydrogenase clearly functions by concerted hydride transfer and decarboxylation.

In an outgrowth of the multiple isotope method, the Cleland group first described coupled hydrogen motion and the earliest case of hydrogen tunneling in glucose-6-phosphate dehydrogenase. These experiments measured primary and secondary deuterium KIEs on hydride transfer. The Cleland group beautifully characterized the changes in transition states for the actions of formate dehydrogenase resulting from the use of pyridine nucleotide analogs thio-NAD and aceylpyridine-NAD in place of NAD.

Strong hydrogen bonding in enzymatic catalysis

In the course of measuring equilibrium isotope effects, the Cleland group uncovered examples of low deuterium fractionation factors. The deuterium fractionation factor describes the ratio of deuterium to hydrogen at equilibrium and at a defined site in a molecule, where both deuterium and hydrogen can compete. The deuterium fractionation factor is essentially the equilibrium ratio of deuterium to hydrogen at a site with equal access to both isotopes. Low deuterium fractionation factors can arise in a number of ways. One example is that of thiol (SH) groups often found in proteins in the form of

cysteine residues. Molecules with—SH substituents exposed to 1/1 mixtures of H_2O and D_2O undergo proton exchange with the medium but incorporate little deuterium into the thiol group. Thus, they display low deuterium fractionation factors.

In early studies, observations by the Cleland group of low deuterium fractionation factors in enzymes signaled the possibility of the participation of acysteinyl residue in catalysis. When in later research the crystal structures of such enzymes failed to implicate a cysteine residue, an alternative explanation for the low deuterium fractionation factors was needed. In collaboration with Maurice M. Kreevoy at the University of Minnesota, Mo advanced the hypothesis in 1994 that short-strong or low-barrier hydrogen bonds (LBHB) could participate in enzymatic catalysis. LBHBs were known by physical chemists and physical organic chemists to display low deuterium fractionation in small molecules. Another known physicochemical property of LBHBs was a characteristic down field ^{1}H-NMR chemical shift. Mo's hypothesis, coupled with the NMR properties of the LBHB, led directly to the assignment by Perry Frey and his associates of an LBHB in the action of chymotrypsin. The LBHB-proton linking His^{57} and Asp^{102} in chymotrypsin had been observed twenty-two years before in the laboratory of Robert G. Shulman using ^{1}H-NMR, but it was not assigned as an LBHB. Subsequent research in the Frey and Cleland laboratories consolidated the assignment of the LBHB in the central, tetrahedral intermediate in the action of chymotrypsin.

Recognitions and honors

Mo was widely honored for his contributions to science. At the University of Wisconsin-Madison, Mo served as the M. J. Johnson Professor of Biochemistry and as the Steenbock Professor of Chemical Sciences, and he was honored with the Hilldale Award in the Physical Sciences in 2001. At the national level, Mo was elected a fellow of the American Academy of Arts and Sciences in 1977 and to the National Academy of Sciences in 1985. He was a Fulbright Scholar in 1987. Mo received the Merck Award from the American Society for Biochemistry and Molecular Biology in 1990, the Alfred Bader Award in Bioinorganic or Bioorganic Chemistry from the American Chemical Society in 1993, the Repligen Award in the Chemistry of Biological Processes from the American Chemical Society in 1995, and the Moore and Stein Award from the Protein Society in 1999.

Activities and interests

Mo lived a full life that included sailing, musical arts, and stamp collecting. He had a passion for sailing, and in his younger days he enjoyed sailing and ice boating on Lake Mendota in Madison and around Wisconsin. He owned and sailed E boats, which were named the *Dorothy O* and later, *Mo's Miss*. He served as the commodore of the Mendota Yacht Club in 1966. Mo also enjoyed ice boating and crewed on a Class A ice boat on Lake Winnebego. Asked about it, Mo once replied, "I do remember sailing on the *Mary B* at Oshkosh in a real blow. That was the race where we made one downwind leg (2 miles) in one minute flat. Quite a thrill!"

Mo appreciated classical music and annually supported the Madison Symphony Orchestra. The symphony honored him for fifty consecutive years of subscriptions to symphony performances. He was an opera lover and knew all of the operas in the standard repertoire. He supported Madison Opera and other opera companies in Chicago; Sarasota, Florida; Logan, Utah; and Seattle.

Mo was a world-class philatelist. He held many leadership positions in the United States Stamp Society, including its presidency in 1992. He published more than three hundred articles in the *Canal Zone Philatelist* and the *United States Specialist*, and he received the Hopkinson Memorial Literature Award in 1986, 2002, and 2006. He also received the Smithsonian Institution's Philatelic Achievement Award in 2008; and in 2009, the Stamp Society inducted Mo into the United States Stamp Society Hall of Fame.

Mo was a devoted parent, with his former wife Joan Cleland, to Elsa Cleland and Erica Shepard; and he was a grandparent to Max, Finn, and Griffin. He was exceptionally generous to colleagues worldwide who consulted him on enzyme kinetics, responding quickly to requests for assistance from students, young professionals, and senior researchers. Mo's friends regarded him as a kind and generous adviser and as a preeminent enzymologist.

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