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John W. Daly: The Early Years. The NIH Shift and Cyclic-AMP Assays: Early Pharmacological Breakthroughs

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Abstract – Although trained as an organic chemist, John Daly embarked in his early years at NIH on several research projects that involved a significant and sophisticated application of biochemistry and pharmacology. He was able to work with impressive leaders in these fields, including the late Nobel Laureate, Julius Axelrod. In this report, we highlight two aspects of this work—his involvement in the discovery of the NIH shift and the development of a method to quickly assay cyclic AMP biosynthesis. The strong pharmacological component of his research career evolved from these and other early seminal discoveries.

DISCOVERY OF THE NIH SHIFT:

INTRODUCTION

Scientific advances often are most impressive when things appear to be going wrong. Perseverance, keen observations, and intelligence are necessary in order to take advantages of the surprises that research provides. The story of the NIH shift is a good example of this. This is an important chapter in the early research that John Daly conducted. This work involved the input of many NIH scientists and this prompted one of the players, Dr. Bernhard Witkop, Chief of the Laboratory of Chemistry (now the Laboratory of Bioorganic Chemistry), to name the fundamental process that was discovered the NIH Shift.[1]

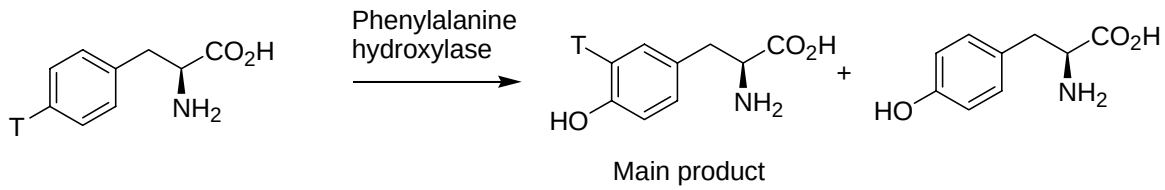
OXIDATIVE ENZYME ASSAYS BASED ON RADIOISOTOPE RELEASE:

John and the late Gordon Guroff, a biochemist in the National Heart Institute (now NHLBI), published an initial observation that turned out to have immense ramifications.[2] Oxidative enzymes catalyze the

incorporation of molecular oxygen in the metabolism of organic compounds and, accordingly, have many important roles. Examples include the β-hydroxylation of dopamine to produce norepinephrine, conversion of proline to hydroxyproline, and the biosynthesis of tyrosine from phenylalanine. In addition, metabolic clearance of endogenous and exogenous substrates often involves initial hydroxylation catalyzed by oxidases. In research involving this important class of enzymes, a strategy had evolved that took advantage of the fact that the transformation involved replacement of a hydrogen atom with oxygen. Thus, if tritium were incorporated into a substrate at the site of oxidative attack, the rate of enzymatic incorporation of oxygen could be followed by the rate of tritium loss. This strategy has been applied, for example, to study the hydroxylation of cholesterol,[3] β-hydroxylation of dopamine,[4] and conversion of proline to hyrdoxyproline.[5]

A PROBLEM WITH AROMATIC SUBSTRATES: THE RETENTION OF TRITIUM

As part of on-going work at NIH on the chemistry and biology of biogenic amines and amino acids, attempts were made to apply tritium release to develop an assay for phenylalanine hydroxylase using 4-tritiophenylalanine as substrate. However, although tyrosine was formed, very little tritiated water was produced.[6] To investigate this problem further, para-hydroxylation of 4-deuterophenylalanine with phenylalanine hydroxylase was examined. The reaction produced tyrosine that retained significant amounts of deuterium. Furthermore, the isotope was shown to be located in the 3-position, as determined by NMR.[2] To quantitate this retention of label, a similar experiment with radiolabeled phenylalanine confirmed that the tritium released from 4-tritio-phenylalanine was far less than the amount of tyrosine formed, and approximately 95% of the radioactivity was retained in the product.[7] Control experiments were carried out to rule out random labeling of the substrate, and iodination of the product tyrosine resulted in loss of tritium, confirming migration of tritium to the position ortho to the newly installed oxygen substituent (scheme 1).[7]



Scheme 1

This phenomenon was studied further with additional enzymes and substrates. Similar to phenyalanine, 4-tritio-amphetamine retained >90% of radiolabel. On the other hand, further hydroxylation of phenolic substrates resulted in essentially complete loss of radiolabel. For example, enzymatic hydroxylation of 3-tritio-tyrosine produced 3,4-dihydroxyphenylalanie (DOPA) with less than 5% of tritium retention.

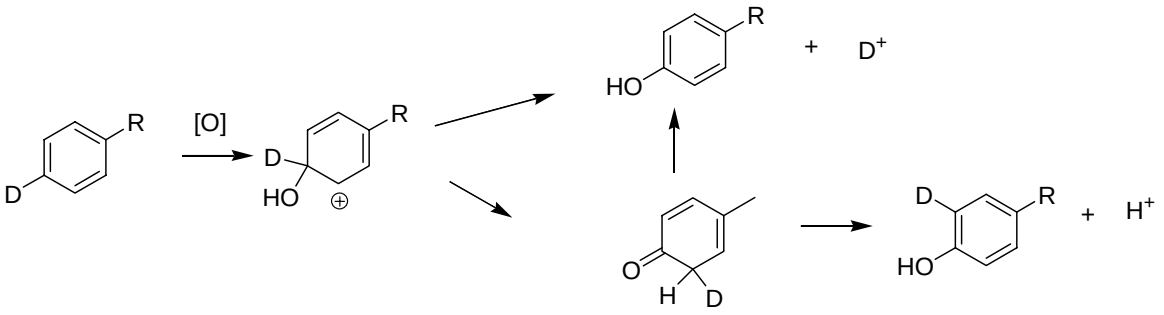
Hydroxylation of 5-tritio-tryptamine led to 5-hydroxy-tryptamine (serotonin) with tritium migration predominantly to the 4-position (85% retention).[1]

Other substituents situated at the site of hydroxylation, for example chlorine, were found to migrate. However, 4-fluorophenylalanine was oxidized to tyrosine with complete loss of fluorine accompanied by no formation of 3-fluorotyrosine.[1]

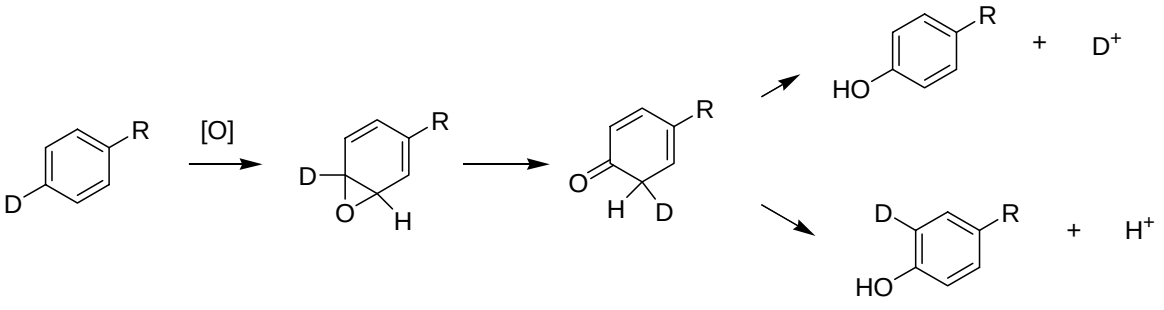
MECHANISTIC PROPOSALS

The migration of radiolabel thwarted the use of tritium label as a strategy to assay enzymes such as phenylalanine hydroxylase. However, this same migration clearly was of substantial importance, since this revealed a new reaction path in aromatic chemistry (and biochemistry). Chemical models of the enzymatic process were developed. In particular, it was demonstrated that peroxytrifluoroacetic acid-mediated hydroxylations of aromatic substrates were accompanied by migrations similar to those observed with microsomal hydroxylations. Other peroxy acids gave similar results.[9]

A cationic mechanism was considered for the NIH shift (Scheme 2). Howeveer, patterns of migratory aptitude seemed to be incompatible with this. Arene oxides had been proposed previously as important metabolic intermediates, for example in the formation of adducts of naphthalene and glutathione. Thus, an alternative arene oxide mechanism was proposed (Scheme 3) and this has received much support in subsequent research.



Scheme 2



Scheme 3

IMPLICATIONS OF THE NIH SHIFT IN THE METABOLISM OF POLYCYCLIC AROMATIC HARDORCARBONS

Discovery of the NIH shift had many ramifications. This was particularly important in the study of the chemistry and biochemistry of arene oxides that are formed during the oxidative metabolism of aromatic compounds, most notably polycyclic aromatic hydrocarbons (PAH). For example, the mechanistic details of the NIH shift provided a basis for explaining why certain PAHs are highly carcinogenic and others are less so.[10] The impressive research of Don Jerina and his Section on Oxidation Mechanisms in LBC, NIDDK, research that evolved from studies on the NIH shift, dramatically attests to the importance of this seminal discovery by John Daly, Gordon Guroff, Bernhard Witkop, Sidney Udenfriend, and others at NIH.

CYLIC AMP-LABELLING AND SUBSEQUENT RESEARCH.

INTRODUCTION

The discovery of Rall and Sutherland in the late 50s of a soluble heat stable factor responsible for the "formation of liver phosphorylase", i.e. 3'-5' cyclic AMP (cAMP), represents the birth of the field of cellular signaling and mechanism of hormone action. The earliest method for the quantification of cAMP was described by Rall and Sutherland in 1958.[11] This elegant, albeit cumbersome, procedure involves the incubation of fractions of liver homogenate containing liver phosphorylase with ATP, $MgSO_4$, caffeine in a buffer containing glucose 1-phosphate. The products measured upon the addition of cAMP are inorganic phosphate (released from glucose 1-phosphate) and glycogen, measured with iodine. The reported titration range for this assay was 0.08 to 0.2 micromolar.

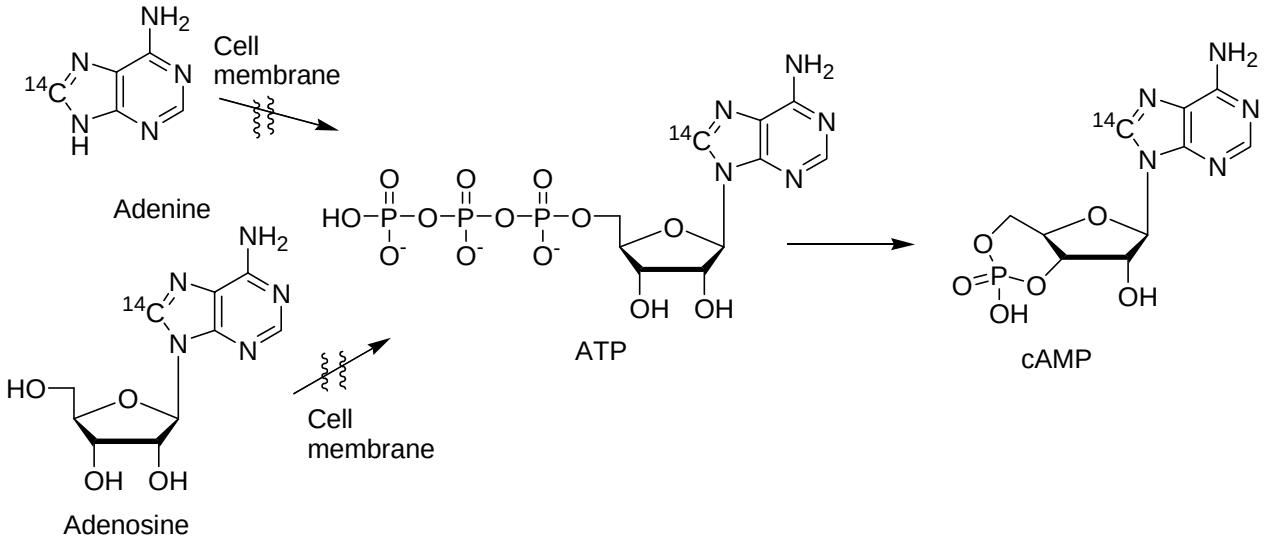
In the late 1960s and early 1970s methods were developed for the direct measurement of cAMP, including radioimmunoassay[12] and methods based on displacement of protein-bound cAMP.[13] Assays based on cAMP binding proteins involve the use of anti cAMP Abs (RIAs) or the protein kinase A regulatory subunit as a binding protein. RIAs are the most sensitive assays available with limits of detection in the femtomole range.[12]

It was recognized early on that the brain contains the highest enzymatic capability among all tissues to generate cAMP.[14] Indeed, in brain membranes, formation of cAMP from ATP was readily detected using a radioactive precursor.[15] In brain slices, however, ATP could not be used as a precursor since it does not penetrate cell membranes, and available methods to measure endogenous cAMP formation were not very sensitive in detecting hormone or neurotransmitter induced activation.

THE METHOD

Hirotoshi Shimizu joined John Daly's group at the Laboratory of Chemistry of the National Institute of

Arthritis and Metabolic Diseases, NIH in the late 1960s. Dr. Shimizu addressed the problem of brain slices measurement of cAMP using a simple and elegant procedure based on the knowledge of the metabolism of the precursors involved. The "Shimizu method" can be summarized as follows (Scheme 4):



Scheme 4

Radiolabeled ATP failed to enrich the substrate pool for adenylate cyclase due to its lack of cell permeability. Thus, ^{14}C labeled adenine was utilized. This precursor is cell permeable and was reported to be actively transported and quantitatively converted to adenosine nucleotides.[16] Intracellular conversion of adenine to AMP occurs via phosphoribosylation catalyzed by adenine phosphoribosyltransferase and using PRPP[17] as a substrate. Further phosphorylation would proceed via adenylate kinase (to generate ADP) and through the energy metabolism to ATP. As was discussed in the early Shimizu papers, the very robust cAMP responses observed with different stimuli using this method suggested that "after incubation with adenine ^{14}C, brain slices contain pool(s) of adenine ^{14}C nucleotides which are excellent precursors of cAMP-^{14}C".[18] The nature of these nucleotide "pools" for cAMP generation was further studied by Shimizu[19] although it has not been completely clarified yet.

It was somehow surprising that ^{14}C adenosine was equally effective as a precursor for the intracellular adenine nucleotide pool when added to the brain slices.[20] Later, Shimizu demonstrated that brain adenosine kinase is responsible for the phosphorylation of adenosine to AMP and thus drives the uptake of external adenosine into the tissue.[21]

PHARMACOLOGICAL FINDINGS

The Shimizu method promptly became a useful tool to demonstrate neurotransmitter induced generation of cAMP in brain slices. In the process of investigating depolarizing stimuli (using high K^+, and sodium channel activators), several novel observations were made. Selected examples are presented below.

Depolarization induced the highest magnitude of any response on cAMP conversion in brain slices. At the time of these discoveries, John Daly was involved in the elucidation of the mechanism of action of batrachotoxin, a sodium channel alkaloid activator that he had isolated from neotropical frogs. It is not surprising that batrachotoxin and other sodium channel agents were tested in the Shimizu protocol. The responses observed with these agents were dependent on the presence of calcium, suggesting that the release of a neurotransmitter was involved. Although the release of acetylcholine was initially suspected to mediate the responses, it was soon found by Daly's group and others that adenosine release played a major role in the depolarizing effects triggering cAMP formation. In one of the earliest papers,[18] the release of radiolabeled adenosine was detected after depolarization of brain slices, and a dose dependent stimulation of adenosine of cAMP conversion was reported shortly after.[22] Sattin and Rall[23] initially proposed the presence of an adenosine receptor that regulated adenylate cyclase in the brain, and Daly's work substantiated the idea.[24] From this point on adenosine receptors became a major interest in Daly's laboratory. The field was new at the time and findings were published at a high pace. Other reviews in this issue will cover Daly's contributions to the adenosine receptor research field.

Synergistic responses: There was a substantial synergism in the cAMP conversion response when depolarization was combined with a receptor agonist.[18] Thus, depolarization conditions (either high K^+ or sodium channel activation) combined with either adrenergic, histaminergic or serotonergic stimulation resulted in much higher than additive responses. It was initially shown that addition of adenosine could mimic the effect of depolarizing agents in synergizing with neurotransmitters. The synergisms could not be accounted for, however, on the adenosine release alone. For example, norepinephrine and serotonin induced a synergistic stimulation of cAMP conversion when given combined, but only a negligible response when given individually.[22] The norepinephrine participation in the synergistic responses became a focus of Daly's work. It was demonstrated that in the brain, alpha adrenergic activation synergizes with beta adrenergic, H_2 histaminergic and A_2 adenosine receptor mediated responses.[25] Such alpha responses were also shown to be dependent on calcium[26] and prostaglandin E_2.[27] The alpha adrenergic stimulation of phospholipase C, with the concomittant generation of intracellular calcium and activation of protein kinase C were identified as mediators of the synergism of cAMP responses.[28] Moreover, sodium channel activators, in addition to the mediating the release of adenosine, also induced phospholipase C activation that contributed to the synergistic responses.[29]

FINAL REFLECTIONS

The generation of the adenine labeling method for cAMP determination represents a scientific milestone

in the field of molecular pharmacology. It is also a landmark in John Daly's impressive scientific legacy.

- It is the initial point of his laboratory's very productive pharmacological component.
- It is the driver for his future activity in the adenosine receptor field, where he became a world leader.
- It provided early examples of electrical activity, through ion channel activation, inducing biochemical changes.
- It generated one of the first examples of the now known as "cross-talk" signaling in cells.

Finally, the good fortune to have worked in John Daly's group invites oneself to reflection. What was so special about John? He was unassuming without equal, he transmitted wisdom without demands, he was unyieldingly passionate. He was a wonderful mentor. He was generous, attentive to new ideas and willing to discuss any new scientific digression.

John Daly's scientific legacy is remarkable by the wide range of areas covered. His persona has left a mark in a significant number of scientists the world over. March 5, 2008 has left a vacuum. We miss you John.

ACKNOWLEDGEMENTS

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Dr. Kenneth L. Kirk received his BA degree in chemistry from DePauw University, Greencastle, Indiana, in 1959 and his Ph.D. in organic chemistry from the University of Wisconsin, Madison, in 1963. He carried out one year of postdoctoral work at the Technische Hocshule, Brauschweig, Germany, with Professor Gerhard Quinkert and a second year at Cornell University with Professor Jerrold Meinwald. He then joined Dr. Louis Cohen's research group at NIH in 1965 as a Staff Fellow in what is now the Laboratory of Bioorganic Chemistry (LBC) of the National Institute of Diabetes, and Digestive and Kidney Diseases (NIDDK). In 1967 he was granted tenure as a Research Chemist, and became a Section Chief in 1985, Deputy Laboratory Chief in 1991, and Chief, Laboratory of Bioorganic Chemistry, NIDDK, in October 1997. He retired in 2008, is now Scientist Emeritus and remains involved in chemistry. His research interests include synthesis and biological evaluation of fluorinated analogues of several classes of biologically important molecules including imidazoles, catecholamines, indoles, and others.

Dr. Fabian Gusovsky received his diploma in pharmacy and biochemistry from the University of Buenos Aires, Argentina in 1980 and his Ph D in Pharmacology from Rush University in Chicago, IL in 1984. He joined the Laboratory of Bioorganic Chemistry, NIDDK at the NIH in 1984 as a visiting post doctoral fellow in John Daly's laboratory. He remained associated to John in the LBC as an independent investigator until 1992. That year he left the NIH to join Eisai Research Institute (ERI), in Andover, MA a subsidiary of Eisai Co, Ltd (Japan) as a Senior Scientist. He is currently Therapeutic Area Head, Immunology and Atopic Diseases at ERI. Since working with John Daly his interests have included the study of the pharmacology of natural products with focus on actions on ion channels and second messenger systems. At ERI he has followed this interest in the context of drug discovery in the area of autoimmune and other immune related diseases.