



Britton Chance



Personal perspective

The stopped-flow method and chemical intermediates in enzyme reactions – a personal essay

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Abstract

My perspective does not deal directly with photosynthesis research. However, the methods evolved have played an important role in several areas of biochemistry and biophysics including photosynthesis. Improvements of rapid reaction methods, based upon the pioneering studies of Hamilton Hartridge and F.J.W. Roughton, and Glenn Millikan, required a detailed study of the hydraulics of small-bore tubes and jet mixers, taking into account, that economy of reactants was demanded by the sparcity of enzyme preparations in the 1930s. This Personal perspective recites the steps taken to minimize the volumes expended in rapid flow studies, together with the improvements in electronic technology of rapid and sensitive spectrophotometry of labile intermediates in enzyme reactions. Of the methods studied, continuous, accelerated and stopped flow, the latter was designed for the highest fluid economy and subsequently proved to be of wide use for studying biochemical kinetics. Advances in fast spectrophotometry of dilute solutions led to great improvements in Millikan's dual-wavelength system, both in speed of recording and decreases of background noise level, largely due to electronic stabilization of light sources and sensitive amplifiers. The application of these technologies to studies of hemoglobin, myoglobin and heme enzymes was, more recently, followed by studies of suspensions of organelles, cells, and tissues not only *in vivo* but also at low temperatures with cryoprotectants, leading to the use of flash activation in carbon monoxide inhibited heme enzymes. Laser technology allowed photosynthetic systems to be studied at cryotemperatures, leading to the development of electron tunneling theories for biological reactions (not discussed in this paper). All in all, the many fundamental developments of electronics, optics and hydraulics indicate how technology can respond effectively to the needs of biology in general, and photobiology in particular.

Introduction

Flavoprotein has been one of the more difficult of the respiratory components to detect with visual spectroscopy. Its absorption band in the oxidized state is rather broad, and no band is observed in the reduced state. Thus, flavoprotein is difficult to distinguish from the background of light scattering of the biological structures. In addition, a fundamental difficulty to the visual observations of flavoprotein components has been cited by K.D.

Keilin from a spectroscopic study of heart-muscle preparation. Diffuse absorption bands were observed in extremely dilute preparations and were eventually attributed to flavin or β -carotene in his own eye. These results led him to make the statement that 'observation also serves as a warning against the use of the direct method of spectroscopic examination in the general survey of flavin or carotene distribution in different tissues and in their extracts' [Keilin and Smith (1939)]. One must therefore rely upon sensitive physical

instruments and upon those to whom a cytochrome spectrum is ‘a line drawn on a servo-operated pen’ [Hartree (1963); Mann (1964)]. As some of us remember, Professor Keilin took special interest in the extension of his technique of visual spectroscopy into regions of the spectrum where the eye was either unable to see (DPNH) or unable adequately to discriminate (flavoprotein). (from: Chance 1966)

The concept that an enzyme and its substrate would form a chemical intermediate that resembled the oxygen compound of hemoglobin or myoglobin but was unstable and decomposed to yield products was quite a novel idea in 1936. I probably could not have received a grant to try to measure such a transient intermediate, even if research grants had been available at the time; I doubt that anyone would have sponsored the project. However, Glenn Millikan’s experiences with the combination of oxygen with myoglobin (Millikan 1936) stimulated a preliminary study, which showed that adding H_2O_2 to a crude preparation of peroxidase gave a colored compound that could go on to oxidize a variety of phenols (Chance et al. 1940a). Although, we studied the overall reactions of pyrogallol, leuco-malachite green, and other phenols in detail with a Dubosque colorimeter (see http://www.humboldt.edu/~scimus/HSC.36-53/Descriptions/Color_B&L.htm) and a stopwatch, we initially did not even imagine trying to detect transient enzyme–substrate intermediates, partly because the concept of a steady state for a two-substrate enzymatic reaction was not understood. The rapidity of enzyme reactions also discouraged any attempts to search for intermediates.

The idea that Millikan’s flow apparatus for measuring the formation of oxy-myoglobin could be used to detect short-lived intermediates in enzyme reactions was spurred by Moelwyn-Hughes’ pioneering papers (Moelwyn-Hughes 1933, 1937), which suggested that the rate of reaction of enzyme and substrate would be limited by diffusion at about $10^6 \text{ M}^{-1} \text{ s}^{-1}$. I thought it might be possible to study this process by a flow method if Millikan’s apparatus could be operated at times of milliseconds and if we used concentrations in the micromolar range, rather than the millimolar concentrations he had to use for myoglobin kinetics. My thinking was also stimulated by the Michaelis–Menten theory (Michaelis and Menten 1913) and J.B.S. Haldane’s book (Haldane 1930). These were tantalizing teasers for trying to detect the

enzyme–substrate complex itself, rather than studying the overall reaction by making periodic readings with a colorimeter. But also, there was stimulus from Ernest Rutherford’s observations of the lifetime of ionized gases flowing down a tube and from Hamilton Hartridge and F.J.W. Roughton’s (1923a–c) pioneering studies of the jet mixer. Millikan’s distaste for the stoneware bottles of blood required by Hartridge and Roughton caused him to make an apparatus that was economical enough to study purified myoglobin, and led me to contemplate how to make the apparatus ‘micro.’ The flow apparatus I ultimately constructed was, in fact, as micro as it has ever been made.

Early studies on physical chemistry at the University of Pennsylvania, 1937

I began work on a rapid-flow apparatus in 1937 as a graduate student in chemistry at the Johnson Foundation of the University of Pennsylvania. The instrument was constructed in the basement of 436 Montgomery Avenue, Philadelphia, under the supervision of the physical chemist Martin Kilpatrick. Martin knew little about fast chemical kinetics and was working mainly on ‘dilatometry’ (measurements of very slow volume changes during chemical reactions) with Knut Kreiger and John Miller as his much admired and patient graduate students. The flow apparatus included a mechanical syringe drive with thyatron motor control and a tachometer generator to measure the flow velocity. The spectrophotometer was an electronic, low-noise, high-gain version of one that had recently been developed for photoelectric automatic ship steering.

Our first flow apparatus was, for its time, a wonderful instrument (Figure 1; Chance 1940a, b). However,

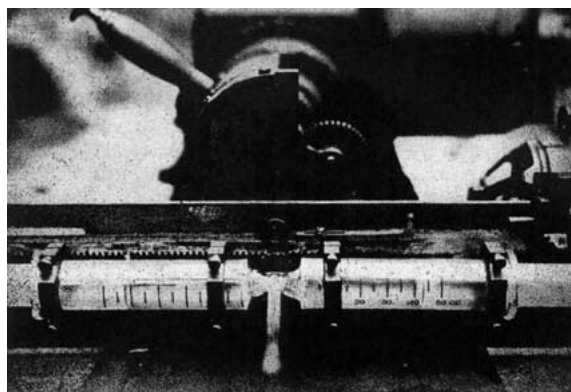


Figure 1. Motor driven syringe unit with horizontal opposed all glass syringe unit and belted generator for flow base. (Reproduced from Chance 1940d.)

the syringes required 150 ml of material and were impractical to use, as they had to be filled through the observation tube. In addition, the measurements of the flow velocity were crude, and we did not have a good understanding of the nature of flow in the capillary or cavitation at the jets. The data display involved mirror galvanometers that were inconvenient and provided only a one-dimensional display of the signal *versus* time. But the results suggested that further work including introduction of a double-beam spectrophotometer might make it possible to measure small changes in light absorption with unprecedented speed (1 ms) and sensitivity (10^{-4} absorbance units).

Off to Cambridge University and to Australia on board MV New Zealand Star, 1938

In March 1938 came a totally unexpected contract with British General Electric Company (British GE) to test my autosteering device on Blue Star Motorship, MV New Zealand Star, a fast, 20000 ton, twin-screw, refrigerator ship plying from London to New Zealand and Australia on the prestigious Blue Star line. The Autosteer first had to be shipped from the USA to Salford, UK, and my wife Jane and I accompanied it in the hope that the trip would provide an opportunity to study at Cambridge University. All my flow equipment remained in Philadelphia because I had no address at Cambridge. I also had no idea what was going on there except that Glenn Millikan had published his outstanding paper on the kinetics of the myoglobin and oxygen reaction with a novel micro flow apparatus and I wanted to study under Hartridge, Roughton and Millikan!

I visited Cambridge shortly after my arrival at GEC with the autosteer equipment. The meeting with Glenn Millikan was felicitous. The examination consisted of treading the cornice of the columns of Nevilles court at Trinity College (no hands), and resulted in my acceptance as a research student. I soon was housed in his very laboratory, cheek to jowl with his huge flow apparatus and cat muscle spectrophotometer, with remains of F.J.W. Roughton and Hamilton Hartridge's enormous 20 l stoneware bottles cautioning me against the use of excessive volumes of reagents. However, Glenn soon departed for a Physiological Congress in Moscow, and Jane and I were off to Australia on the New Zealand Star to test my automatic ship steering gear.

That three-month voyage was one of remarkable intellectual challenge for me, providing time for clear thinking about the problem of rapid reactions and allowing me to explore unknown and unexpected areas of development. In addition to setting up, testing and installing the autosteer on the New Zealand Star, I continued to develop the flow apparatus during the voyage.

The first step chronologically was to design an electronic feedback circuit for regulating the intensity of the measuring light. This was a logical step for me, since the autosteering device also used negative feedback! Using an electronically stabilized lamp instead of a light powered by a storage battery decreased the noise from 1×10^{-3} to 1×10^{-5} . I also adopted a photoelectric amplifier with a differential amplifier, as John P. Hervey had taught me at the Johnson Foundation. Hervey had used such a circuit to amplify D.W. Bronk's 'nerve messages.' E.D. Adrian and coworkers also had used high gain DC amplifiers at Cambridge in the 1930s as I did as a part of the development of automatic ship steering. The physiologists' photokymograph was unsuitable, so to obtain the two-axis data display that I sought, that is, flow velocity against absorbance change, I built a long-persistence, five-inch cathode ray tube oscillograph shielded in a sewer pipe!

I worked out a scheme for measuring the flow velocity by an electronic derivative circuit, a precursor of electronic analogue computing. The idea here was to have movements of the syringe plungers rotate a linear potentiometer that was capacitively coupled to an electronic amplifier. The concept of applying Prandtl-Karman theory flow patterns and cavitation studies was tested in the bathtub of the chief engineer's cabin on the New Zealand Star to and from Australia. This study was essential to analyzing pressure/flow data on small-bore capillaries.

All this culminated in a systems analysis of the many parameters and calculations of criteria for optimal performance to guide my construction of the apparatus when I returned to Cambridge. It was a three-month voyage of design calculations!

Development of the tangentially opposed mixer at Cambridge University, 1938

On my arrival back in Cambridge, it became clear that Roughton and Millikan, though pioneers in the flow-apparatus, were not very knowledgeable in electronics. I found this surprising, because E.D. Adrian and later Alan Hodgkin were developing fast amplifiers

for recording action potentials in the basement of the same laboratory and Andrew Huxley was next door! In accordance with time-honored tradition of Cambridge, one just did not speak about one's work outside the lab. It took a foreign body like me to bring fast DC amplifiers into use on the second floor of the staid physiology laboratory, opening up the study of rapid reactions by the 'stop-flow' method. The reversion spectroscopy and the slow galvanometer that Roughton and Millikan had been using were just not suited to the demands of fast recording.

In a complete mathematical analysis of the flow apparatus for continuous-, accelerated-, and stop-flow conditions, I evaluated the signal-to-noise ratio, the response time of the apparatus, the flow velocity and the optical characteristics of the channels, and optimized the parameters for the three methods. The physics of the flow method and the optical properties of the capillary observation tubes were studied in detail. I examined the hydraulics of the flow method to find the conditions for optimal turbulent flow in the observation tube, considering the pressure drops in various types of mixers. This led to an optimal design, which greatly exceeded the performance of the previous designs. Cavitation was studied for the first time and was found to be minimized if the turning radius of the flow was not too great and some back-pressure was applied to the system (Table 1; Chance 1940c; Figure 2; Chance 1940d). Calculations also were made of the time of arrival of the mixed reactants at the point of observation of a stop-flow apparatus as were the effects of optical (longitudinal) slit lengths for a fast reaction at two values of observation using the accelerated flow method. The stop-flow method has subsequently been developed *in extenso* by many laboratories because it requires no knowledge of the flow velocity or its dynamics. However, it assumes that turbulent flow occurs during the filling of the observation chamber, which may not be the case in apparatus built without benefit of similarly detailed calculations.

Table 1. Flow velocity for onset of cavitation in glass mixers and observation tubes. Source: Table III from Chance (1940c, see p. 472)

Syringe	u_0 for onset of cavitation (m s^{-1})	A_0 (mm)	D_j (mm)	K^1
1	6.60	2.27	1.40	0.557
2	8.50	2.27	1.20	0.499
3	7.4	1.23	0.50	0.787

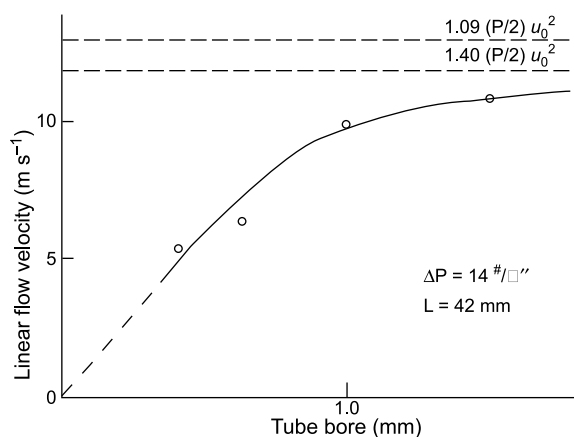


Figure 2. A low resolution diagram of linear flow velocity attainable from observation tubes of different bore for a constant length and pressure. The curve approaches the kinetic energy loss term of the Prandtl-Karman equation for sharp-edged entrances. (Reproduced from Chance 1940c; see p. 471.)

I began constructing the microflow apparatus as soon as I got my hands on some dentist's drills and samples of the novel plastic 'perspex' (PMMA). The design concepts were dominated by a large number of mixing jets that were opposed to each other, plus a right-angle turn at the entrance to the observation chamber. I found that a heated glass capillary could be sealed into the plastic reasonably well, thereby making a connection from the mixer to the observation tube. Small (0.2 mm) holes were drilled in the plastic to make the tangential jet mixer and then were sealed off with heated glass plugs.

Glenn Millikan's 20-ml syringes were cut down to 1-ml syringes that were snug-fitted or threaded into the plastic mixer block. The apparatus delivered a volume of 1 ml from each syringe at a velocity of 20–30 m s^{-1} for a time of 1 s. No motor or hydraulic drive was used and 'finger push' on the syringes was sufficient; an electronic flow velocity meter gave the ordinate of the display.

Most of these novel developments were made in the summer of 1938 while Glenn Millikan was away, and were followed by a large number of tests on inorganic redox reactions. Curiously, Millikan provided no myoglobin to be reacted with oxygen for a one-to-one comparison with his apparatus, even though the study of luciferase O_2 reactions in 1939 showed that deoxygenation and filling under anaerobic conditions were possible. Nor indeed did David Keilin, Thaddeus Mann or Edward (Ted) Hartree offer the brash American a helping hand or some peroxidase preparation. (See Chance et al. 1940a and Chance 1943a for kinetic

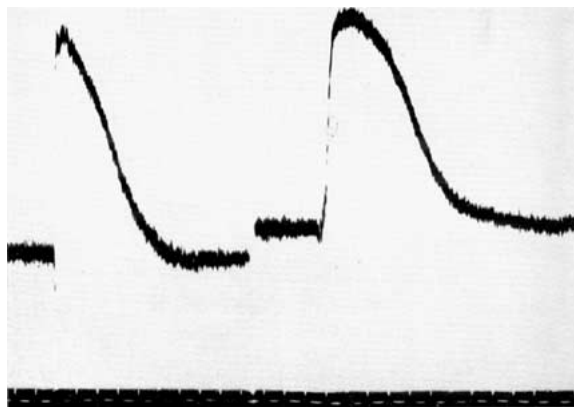


Figure 3. Stopped flow kinetics: mirror oscillograph recording of the production of malachite green (left) and the corresponding kinetics of the enzyme–substrate compound (right). Time markers at bottom (in white, not easily seen), 200 ms. Peroxidase = 1×10^{-6} mol of hematin Fe per liter in one syringe, $\text{H}_2\text{O}_2 = 4 \times 10^{-6}$ mol per liter, leucomalachite green = 15×10^{-6} mol per liter, pH = 4.0 in the other syringe. (Reproduced from Chance 1943b; see p. 558.)

studies on enzyme (peroxidase)–substrate complex.) The situation was strikingly different after the war, when it was noted that I had developed the bombing computer that kept the nearby Alconbury bombers going, as well as the precision timer used in anti-aircraft radar.

The name ‘stop-flow’ actually was not used in this early work. We first used the term in 1940 in a study of bioluminescence flashes (Chance et al. 1940b) [See Figure 3 for an example of kinetic studies on malachite green (Chance 1943b).] Interestingly, although my applications focused mainly on heme proteins, the second major application of the stop-flow technique was a study of bioluminescence by Richard (Dick) DeSa and J.W. (Woody) Hastings (1968).

Clouds of war: return to Penn, 1939–1940

Jane and I returned to Pennsylvania in 1940 to show off our offspring proudly to our parents. When the onset of World War II prevented our return to idyllic Cambridge, D.W. Bronk most generously accepted me back at the Johnson Foundation; I started over again to build version 3 of a rapid-flow instrument!

The light-control circuits in the new design included the reliable 2A3 vacuum tubes operating at 110 Volt DC, with negative feedback from plate to grid. The feedback photometer was based upon Johnson and Lewellyn’s (1934, 1935) formula for signal-to-noise ratio, and required an input signal of more

than 50 mV for shot-noise limitation. A 921 photo-tube with a $100\text{M}\Omega$ resistor was connected to the grid of a low-microphonic pentode in what was essentially a cathode-follower circuit. The response time was <0.01 ms. This complicated electronic system involved over half a dozen vacuum tubes in a DC circuit with highly regulated voltages, and gave as output push–pull deflection to a home-made cathode ray tube display. The plastic flow apparatus was transformed into glass through the skills of James (Jimmy) Graham, who made the mixer and flow tube by using acid to dissolve 0.5-mm copper wires embedded in the glass.

I carried out a detailed analysis of the signal-to-noise ratio of a spectrophotometer with a regulated light intensity, and developed equations for determining the flow velocity by the electronic derivative scheme and displaying it on a home-made 5-inch cathode ray tube. Using a flow velocity of 25 m s^{-1} , I obtained a time resolution of 0.3 ms with a reagent extenditure of only 0.5 ml. Kinetic constants were measured for reactions with half times of 0.4 ms. This allowed me to measure a second-order rate constant of $8.6 \times 10^5\text{ m}^{-1}\text{ s}^{-1}$, approaching the limit set by Moelwyn-Hughes (1933, 1937). The fastest reaction studied was that of ceric sulfate and hydrogen peroxide. This provided a suitable model for the on-coming studies of peroxidases and hydrogen peroxide, which react with a rate constant of $10^7\text{ M}^{-1}\text{ s}^{-1}$ [see my 1940 PhD thesis (Chance 1940a); it was published in *The Journal of the Franklin Institute* (Chance 1940b–d)].

These developments were summarized in a paper that was published (belatedly, because of war work) in the *Review of Scientific Instruments* in 1942 (Chance 1942). The paper described a three-color colorimeter for measuring the enzyme–substrate compound differentially in the region of maximum extinction coefficient (the Soret band). The overall reaction was measured simultaneously in the red region of the spectrum by the oxidation of leuco-malachite green to a colored product, malachite green. The performance was illustrated by a measurement of micromolar concentrations of peroxidase (5×10^{-10} mol enzyme in 0.5 ml solution) with a rise time of 10 ms and a stop-flow recording over 20 s, which afforded a complete curve for both the overall reaction and the enzyme–substrate intermediate.

The rate constant for the ‘on’ reaction of peroxidase with H_2O_2 was found to be $9 \times 10^6\text{ M}^{-1}\text{ s}^{-1}$ over a range of time from 10 to 200 ms. The reaction exhibited strictly second-order (bi-molecular)

behavior, suggesting that the dominant intermediate in this time range was the red 'complex I' of peroxidase and peroxide. The lifetime of the enzyme-substrate compound was strictly proportional to the peroxide concentration. Perhaps most importantly, it was possible to superpose the measured curves for the intermediate and product concentrations on differential analyzer solutions of the non-linear differential equations for enzyme action. This result unquestionably identified the role of the intermediate compound in enzyme action.

The mechanical differential equation solver, 1939

The flow apparatus had revealed the kinetics of formation and decomposition of an enzyme-substrate compound and it was necessary to prove that the traces followed a Michaelis-Menten mechanism. While steady-state solutions were possible for the peak of the formation of the enzyme-substrate compound, it was not satisfying to me to fit the data at only one point in time. But try as I could, I found no ready solution for the non-linear differential equations. Without an analytic solution of the complete kinetic curves for the formation of the intermediate, disappearance of substrate and appearance of product, I had no way to portray theoretically what could be determined experimentally. Thus, I turned to a mechanical differential equation analyzer.

The Johnson Foundation was by no means barren of stimuli for using a differential analyzer to study enzyme kinetics. In fact, Alan Burton had built such a fluid-flow model of consecutive reactions and had applied it to a series of consecutive reactions. Although this system was unrelated to enzyme action, it readily demonstrated the steady state and it provided a vivid model of a kinetic system with dynamic inputs and outputs. The intellectual stimulus for a quantitative treatment of enzyme kinetics thus was already present, and communications with Dr J.G. Brainerd, subsequently Dean of the Moore School, elicited great enthusiasm for the project. Using appropriate kinetic constants and concentrations, we were able to plot out the complete solutions to the non-linear differential equations for substrate disappearance, product formation, and the concentration of the enzyme-substrate compound. Simulations of the reaction were run under six conditions that overlapped the experimental conditions, and the computer solutions agreed well with

the experimental measurements (for catalase and cyanide reaction, see Chance 1943c). Although complete profiles of the uncertainties in the parameter values were not made, changing the parameters by $\pm 10\%$ resulted in altered kinetic profiles that clearly justified the choice of parameters for fitting the experimental results. This was the first study of enzyme kinetics by a machine computer.

The mechanical equation solver, approximately 75 feet long, consisted of geared multipliers and ball-and-disc integrators that set up each parameter and turned out mechanical solutions of the differential equations for exhaustion of substrate and formation and decomposition of the enzyme-substrate compounds (Figure 4 shows a photo of this 'monster'). The solutions simulated the experimental data over the whole time range when the rate constant of the 'on' reaction of peroxidase with H_2O_2 was taken to be $1.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, showing that this was essentially an irreversible reaction in contra-distinction to the assumptions of the Michaelis-Menten theory. The turnover of the enzyme was found to be proportional to the hydrogen donor concentration, and for the reaction with leuco-malachite green in acetate buffer had a rate constant of $3 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$. The Michaelis constant for peroxide was found to be a few micromolar in the presence of $\sim 10 \mu\text{M}$ leuco-malachite green. The activity of peroxidase could thus be described well by a second-order reaction of the enzyme with H_2O_2 , followed by a second-order irreversible reaction for the reaction of the enzyme-substrate intermediate with the hydrogen donor. Extension of the Michaelis-Menten theory to a second-order reaction



Figure 4. Mechanical computer, used by the author, with J.G. Brainerd, in 1939, to solve the non-linear differential equations representing enzyme-substrate kinetics at the Moore School of Engineering at the University of Pennsylvania.

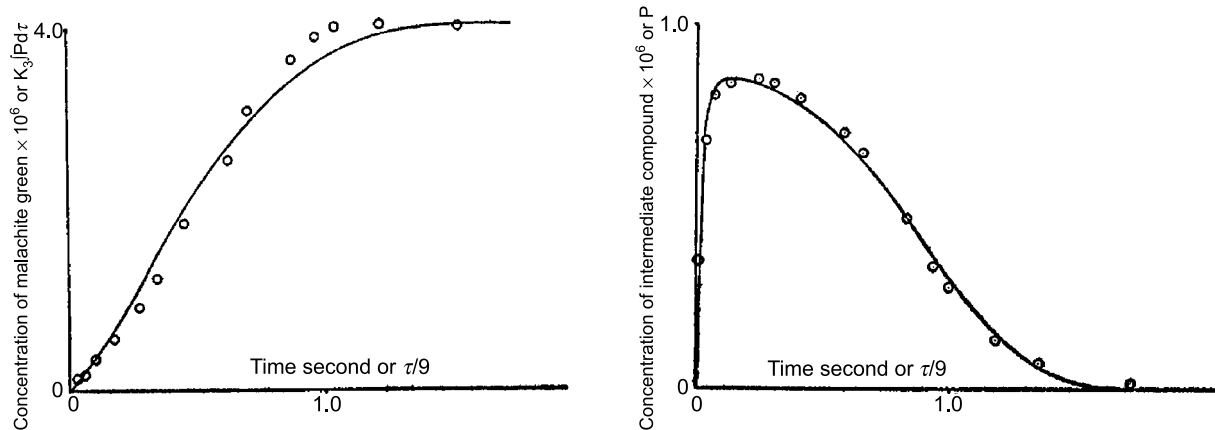


Figure 5. Comparison of experimental enzyme-substrate and 'over-all' kinetics (circles) with a mathematical solution of the Michaelis theory for experimentally determined reaction velocity constants and concentrations (solid lines). *Left*: concentration of malachite green; *right*: concentration of an intermediate compound. (Reproduced from Chance 1943a, see p. 569.)

of the enzyme-substrate compound led to a completely different interpretation of the theory, which could be tested rigorously for the first time. Furthermore, the Michaelis-Menten theory as represented by Thaddeus Briggs and J.B.S. Haldane was solved, not only for the steady state, but also for the transient conditions preceding establishment of the steady state (see Figure 5; Chance 1943a).

Thus, in the three years available to carry out my thesis work for the PhD degree in Physical Chemistry at Penn, I had studied the hydraulics of mixing and fast flows, designed electronic equipment that exceeded the state of the art in lamp stabilization (one part in 10^5) and allowed electro-optical recording of an absorbance change of one part in 10^5 (near the theoretical limit) in less than 1 ms, and validated the ability to measure second-order reaction rate constants up to $10^5 \text{ M}^{-1} \text{ s}^{-1}$ using inorganic substances. For the Cambridge PhD thesis, I put forward the construction of the apparatus for ready filling of syringes, rapid and very sensitive optical recording, together with a spectrophotometer that measured at three wavelengths and provided kinetics of the enzyme-substrate compound and the overall reaction. These studies permitted me to correlate the kinetics of the formation and disappearance of the enzyme-substrate compound of horseradish peroxidase and peroxide in the presence of leucomalachite green with the conversion of leucomalachite green to malachite green. Thus, for the first time it had been possible, not only to demonstrate the existence of the enzyme-substrate compound, but to validate its func-

tion in an oxido-reduction reaction involving a colored product.

The above data were so significant that they demanded simulation of the differential equations for the formation and disappearance of the enzyme-substrate compound, which required use of computer technology to model the system properly and obtain transient solutions for the non-linear differential equations representing the enzyme action. The combination of these technologies conclusively demonstrated that well-defined enzyme-substrate compounds with clear-cut optical characteristics were formed transiently in the enzyme reaction. I received the Paul Lewis award in enzyme chemistry for this work and in 1940 was appointed acting Director of the Johnson Foundation during Dr Bronk's 1-year sojourn to Cornell University (Ithaca, New York). I also was involved in the construction of a cyclotron for the Penn Medical School. In June 1941, I was drafted by the secret radar lab at the Massachusetts Institute of Technology (MIT), ending my enzyme research until 1947.

The war years (1941-1946)

While working up to 80h a week at the MIT radar laboratory, I somehow found time to write papers on the flow measurements for the Review of Scientific Instruments (Chance 1942) and the *Journal of Biological Chemistry* (JBC; Chance 1943c), and to publish an abstract on the reaction of catalase and cyanide. To my good fortune, F.J.W. Roughton, a 'refugee'

from Cambridge, UK, worked nearby at the Fatigue Laboratory. We spent every Tuesday night after dinner discussing the JBC paper, for which he made many helpful suggestions.

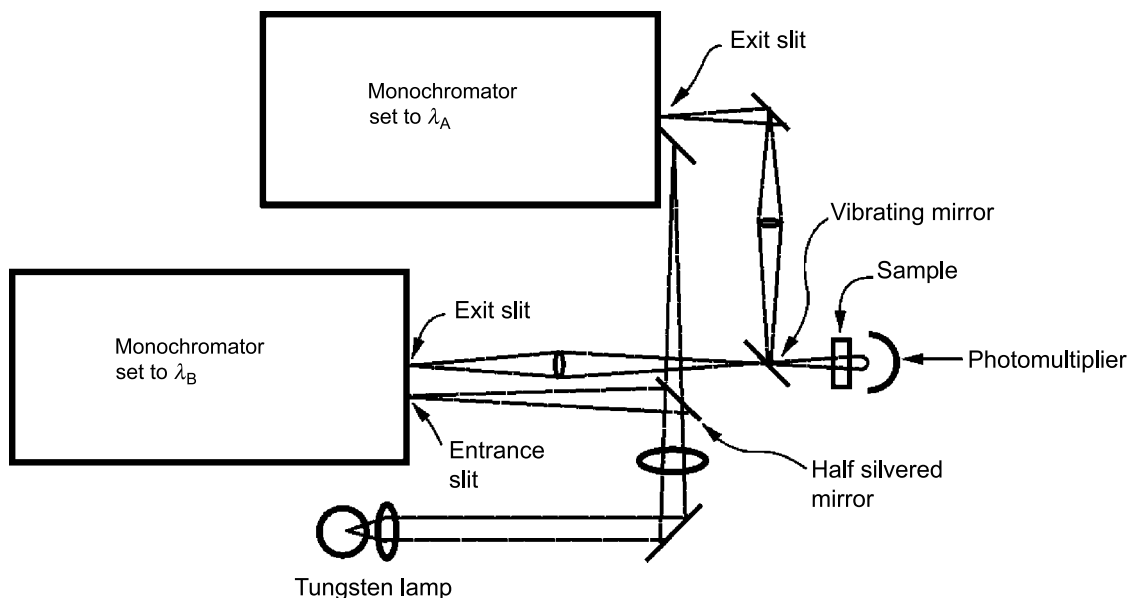
The Radar Laboratory exploited and amplified much of what I had been doing on electronic circuit development. I soon found myself in charge of a very large group called Precision Circuit Development, which focused on extremely precise measurements of time over very long ranges. Our aim was to measure, to better than 0.1% accuracy, the time between transmission of a radar pulse and the return of the reflected pulse, not only for reflections from stationary objects, but of course for moving ships and airplanes. Collaborators on the project included Albert Grass (Grass Instruments) and a number of others, particularly Ted MacNichol, Ivan Greenwood and Richard Close. I had a heavy commitment to the late Ivan Getting's automatic-tracking aircraft radar system, which served so well at Anglo and Normandy beach heads, to say nothing of the buzz bomb terror in the last year of the war. Here the development of a highly linear delay multi-vibrator was essential and was installed in nearly all radars and Loran (Long Range Navigation) as well (see <http://www.radarpages.co.uk/mob/navaids/loran/loran1.htm>). But my main interest was in the development of an airborne bombing computer; my interest was piqued not only by the urgency of the problem, but also by Richard Close's previous experience with computers. Together, we developed an analogue computer for solving the airborne bomb-dropping problem using the novel 3-cm radar APQ-13. The computer allowed the operator to distinguish strategic or tactical targets and to avoid bombing the civilian population, as had occurred frequently when the optical Norden bomb sight was used through the heavy cloud cover of middle Europe.

My most valued achievement was a sophisticated bombing computer that involved what was called regenerative tracking. This device was given first place among a number of heavy competitors from Bell Labs (Charles Townes and Dean Wooldridge and others). I also participated in the design of radar data transmission in Project Cadillac, which developed an airborne early-warning system that was a precursor of the present AWACS system, for which I used my precision delay multi-vibrator to develop the technique of pulse code modulation. Finally, and perhaps more important, I tutored Presper Eckert and Kite Sharpless in the design of circuits for ENIAC (Electronic

Numerical Integrator and Computer), which usually is considered to be the first general-purpose electronic computer. For all this, I was made a Fellow of IEEE (the Institute of Electrical and Electronic Engineers) and received the Morelock Award and President Truman's Certificate of Merit, and a Guggenheim Fellowship!

The Guggenheim fellowship and post-war biophysics and biochemistry, 1946–1948

Fortunately, the Guggenheim Foundation under the aegis of Warren Weaver recognized that many scientists had been displaced voluntarily from their academic research by the war, and in some cases, under patriotic motivation had left their chosen field and joined the war effort for which they felt best qualified. Weaver offered rehabilitation fellowships to those who had made such sacrifices, and in 1947 I was one of the lucky ones. I decided to extend my leave from Penn and accept a two-year Guggenheim Fellowship in Stockholm and join Hugo Theorell. I had time and facilities at the Rad Lab to construct a portable wavelength scanning stop flow, which proved essential to time resolve the green and red enzyme-substrate compounds. This began a most fruitful collaboration and enduring friendship with Theorell. Our studies of highly purified peroxidases and catalases revealed that the reaction with peroxide occurred in two steps, with a green intermediate containing pentavalent iron preceding formation of the red quadrivalent compound studied previously. Since the steady-state concentration of the green compound was very low, the fits to the kinetics of the red compound with the mechanical differential analyzer were still valid. However, the kinetics stimulated the later development of an electronic analogue computer at Penn under the aegis of Joseph Higgins (see below) (Chance et al. 1952). A completely unexpected consequence of my work with Hugo Theorell was spurred by his interest in alcohol dehydrogenase where we, together with the group in Stockholm, discovered the role of enzyme-DPN (diphosphopyridine nucleotide, later called NAD, nicotinamide adenine dinucleotide) compound. We made a complete study of the kinetics of this compound in alcohol-aldehyde interconversion, and developed what came to be called euphoniouly the TC (Theorell-Chance) mechanism.



BC-X 92

Figure 6. A diagram of the original time-sharing system for the rapid measurement of optical density changes occurring at two different wavelengths. (Reproduced from Chance 1951; see p. 634.)

Returning to Penn from the Guggenheim, I became Professor of Biophysics and Physical Biochemistry and Director of the Johnson Foundation in 1949.

My visit to Stockholm included a side trip to David Keilin's laboratory in Cambridge. I greatly admired Keilin's work, and our interactions led me to study peroxidases and catalases under the continuous generation of H_2O_2 by the glucose oxidase 'notatin' system. These studies (Chance 1949a-c) provided the basis for the glucose-ometers (glucosometers) that now are sold with considerable profit by drug companies. The visit to Keilin's laboratory also stimulated me to take up the study of the cytochrome chain and to follow the work of E.C. Slater on the Keilin-Hartree heart muscle preparation. This was to become the chief topic of my work from the early 1950s through the 1970s, and led to another important technological development, the dual-wavelength spectrophotometer.

The concept of time-shared dual- and now multi-wavelength spectroscopy of mitochondrial suspensions and photosynthetic reaction centers arose with some difficulty. Using steady illumination as with the Millikan colorimeter was cumbersome, but a Beckman salesman said he had a 'couple of monochromators' (only) for sale 'cheap.' So Vic Legallais coupled a common light source to both monochromators (using

a single source was essential), and I mounted a very light mirror on top of a vibrating reed that resonated at 60 Hz. The synchronous, transformer-coupled detector gave the essential 'common mode' rejection and the 3-section Wallman filter gave microvolt sensitivity (square root Hz)! We never really changed the design concept of the dual-wavelength time-shared spectrophotometer after that, although we did vastly improve the illuminator by using Bausch and Lomb grating monochromators, and many of the spectrophotometers were made and still are in use (Julie DeSa Lorenz, personal communication).

Dual wavelength spectrophotometers (Figure 6) (Chance 1951) gave the first NADH, flavoprotein and cytochrome difference spectra of phosphorylating mitochondria (Figure 7) (Chance and Williams 1955). (For a discussion and application of dual-wavelength spectroscopy in the role of cytochromes in photosynthesis, see Derek Bendall, this issue.) These spectrophotometers have been used widely in other laboratories, and have been sold commercially by several companies including AMINCO, Hitachi, and now Olis, but neither I nor the University received any financial return for the invention. I did not patent the idea because Newton Richards, the Vice President for Medical Affairs at Penn (who also happened to be

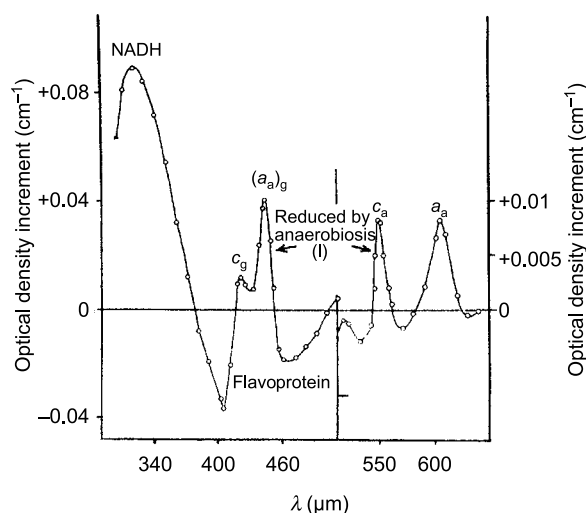


Figure 7. Partial separation of the spectra of components of respiratory chain of rat liver mitochondria by various chemical treatments. The difference spectra were recorded by the dual wavelength spectrophotometer which plots the difference between the absorption of the reference sample of mitochondria (2 mg of protein per ml) and the experimental one. In the trace, the difference is caused by anaerobiosis, which gives the oxidized minus reduced difference spectrum of mitochondria. The absorption peaks reading from right to left are caused by the reduction of cytochromes a_a , c_a , $(a_a)_g$, and c_g , and DPN (diphosphopyridine nucleotide, now called NAD, nicotinamide adenine dinucleotide). The trough at $465 \mu\text{m}$ is caused by the reduction of oxidized flavoprotein. (Figure No. BC-X 156a92 from the Archives of Britton Chance.) (Adapted from Chance and Williams 1955; see p. 395.)

on the Board of Directors at Merck), told me 'don't bother about patents; if it is any good, Merck will manufacture it.' Universities have changed since then!

Further developments of stop-flow

Contributions to stop-flow instrumentation also came from Sheffield, where Quentin Gibson put together a stop-flow of his own particular design (Gibson et al. 1955). Quentin's apparatus incorporated the jet mixer that Millikan and I had developed, and a stopping jamb that gave quick stops for studying hemoglobin. Dick DeSa, Woody Hastings and Quentin Gibson tested this instrument for measurements of bioluminescence (DeSa and Hastings 1968), the same system as I had used three decades earlier (Chance et al. 1940b). Further developments of the stop-flow by Gibson and his coworkers focused on flash photolysis of carboxymyoglobin and cytochrome-*c* oxidase. But their main interest was to build a computer-controlled flow machine. DeSa's company (Olis) replaced the oscilloscope and camera that I had used by digitiz-

ation of the photo-detector output, and added computer analysis software and a computer display of the data. In modern instruments, the dual-wavelength optical system is replaced by a scanning monochromator (Figure 8). For an earlier account of optical spectroscopy, see Chance (1994). Stop-flow measurements continue to be useful in a variety of kinetic studies, sparked particularly by the current interest in protein folding.

Computer simulations

The mechanical differential analyzer study of peroxidase reactions was followed by the development by Joe Higgins of an electronic analogue computer that was dedicated specifically to enzyme kinetics. Joe and I used this computer to simulate multisite control of electron transfer in the respiratory chain as a multi-enzyme system (Chance et al. 1958). This led to the 'cross-over theorem' for identifying sites of energy coupling in the respiratory chain and showed that the three coupling sites are associated with NADH dehydrogenase, cytochrome-*b* and cytochrome-*c*, *c*₁ oxidase. Computer simulation centers subsequently were established all over the world, and now nearly any PC or laptop can be programmed for enzyme kinetics or other biochemical problems. Nevertheless, it is interesting to note that, in a recent paper from Les Dutton's laboratory (see Moser et al. 1992), the water model of consecutive reactions that Alan Burton developed in the 1930s has been useful to demonstrate principles of multiple enzyme systems in the respiratory chain.

Concluding remarks

In summary there was a continuous development of electro-optical technologies from the photoelectric autosteering device through to the fast flow apparatuses and the dual wavelength spectroscopy to ruby laser-activated photolysis. The parallel track of computer development started in the 1930s and greatly accelerated in the war years with bombing and navigational computers, followed by contributions to ENIAC design and construction of real time electronic analogue computers to multi-enzyme computer programs for state of the art digital computers. Finally, Hartridge's 'carburetors' found their way to Roughton's Stoneware Bottle continuous-flow apparatus and to Millikan's portable continuous flow, both



Figure 8. A photograph of the current version of a computerized dual-wavelength spectrophotometer (DW-2 Conversion). This photograph was provided by Julie DeSa Lorenz and John David Sturgill of Oils Company.



Figure 9. Left: the author (top left) at the Olympic Gold Medal ceremony in 1952 with sailing crew members (M. Schoettle, E. White and S. White Jr) of the COMPLEX II 5-5-meter sail boat. Right: the author tests an early heterodyne frequency domain oximeter for brain studies with diffusing near-infrared light.

with mirror galvanometer readout. The fast electronic read-out of modern electronics makes the stopped flow the current and perhaps future method of choice for the study of rapid chemical and biochemical reactions.

At the suggestion of Govindjee,¹ I have included two photographs of myself at two interesting events in my life, one at the time of receiving the Gold Medal for the Yacht Race in 1952 at Helsinki, Finland (Figure 9, left) and the other when my head was being probed by 'cognoscope' (Figure 9, right).

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Note by the editor

¹I recommend that the readers read A.G. Yodh and B.J. Tromberg (2000) Celebrating Britton Chance. *Journal of Biomedical Optics* 5: 115–118 to feel the impact of Brit's several contributions – Govindjee.

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