DISCOVERY!
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J Dent Res 82(2):76-81, 2003

Salivary Biochemistry in Buffalo: The Legacy of Michael J. Levine

(AQ)

INTRODUCTION

Occasionally in science, circumstances converge that allow for the gathering of a group of people who together accomplish extraordinary feats. Such were the circumstances between 1975 and 1995 at the University at Buffalo School of Dental Medicine under the guidance of Michael J. Levine (MJL), whose research group helped usher our knowledge of salivary biochemistry from the "pre-technology" era that existed before 1970 into the present post-genomic era.

This article will review MJL's career in Buffalo and will recount his mentorship of an outstanding cadre of productive and creative scientists, many of whom remain actively involved in oral health research and dental education throughout the United States and the world.

FOUNDATIONS

Following completion of his baccalaureate studies at Cornell University in the early 1960s, and after an abbreviated stint in the US Marine Corps (for which he received an honorable discharge), MJL found his way into the laboratory of Dr. Solon (Art) Ellison as a dishwasher (that is certainly "starting at the bottom"!). This evolved into a position as a laboratory technician. Of interest to Dr. Ellison (then the chair of the recently established Department of Oral Biology) was salivary protein research. Before his tenure at Buffalo, Ellison had worked on the faculty of Columbia University with Dr. Irwin Mandel. Together, they published several classic studies that were among the first to identify immunoglobulins in saliva and several unique salivary proteins and glycoproteins. One of their goals was to correlate the concentration of various constituents of saliva with dental caries experience. MJL began by working to isolate the glycoproteins of parotid saliva, together with J.C. Weill (Levine *et al*., 1969). It was upon the foundation begun by Art Ellison that MJL grew and prospered.

KEY WORDS: (AQ)

Received June 4, 2002; Accepted October 2, 2002

Before long, MJL decided to enter a formal program of study. Dr. Ellison recounts the story: "One day he (MJL) asked whether he could be a graduate student, since the lab work he was doing could well be the basis for a dissertation. So he began a program. He then decided that as long as he was going to school, he may as well take a DDS also" (Ellison, 1992).**(AQ)** Thus, MJL entered the newly founded graduate program in Oral Biology at Buffalo, and then the DDS program. He was one of the few combined dual-degree graduates in the United States, completing first the DDS in 1971 and then the PhD in 1972 (see Fig. 1). His PhD thesis focused on both the immunochemistry and the chemical characterization of glycoprotein components of human parotid saliva, particularly what he later named the "proline-rich glycoproteins".

It is now well-recognized that the student who enters such a combined clinical-research training program needs to overcome considerable challenges. This was even more true in the 1960s, since until then few students had ever engaged in such a non-traditional program. As occurs with many students in such a predicament, MJL concentrated his efforts on his research project, to the consternation of many of the clinical faculty. One of his clinical instructors recently recalled MJL as a student who "for the first time made it clear to me that there were alternative career paths for dental students outside of clinical practice."

Enthused by the study of glycoproteins, MJL entered the Periodontal Pathobiology Training Program at Harvard University School of Dental Medicine, following his graduation from Buffalo. He combined this with a post-doctoral fellowship in the laboratory of Dr. Robert Spiro, a distinguished glycoprotein biochemist at the Joslin Diabetes Center. Few dentists in those days had the foresight to pursue post-doctoral training in a medical school setting. This was a pivotal experience for MJL. He learned from Dr. Spiro how to organize and run a productive biochemistry laboratory. He was fond of telling stories of the unique strategies Dr. Spiro used to motivate his post-doctoral fellows. One anecdote involved a fellow who was directed to isolate a specific glycoprotein from bovine kidney basement membranes. The kidneys were obtained from a slaughterhouse outside Boston early in the morning and brought to the laboratory on ice, where they then underwent a lengthy process involving multiple steps of column chromatography to isolate the component of interest from the fresh kidney glomeruli. Complex enzyme assays were performed on each column fraction to identify cell organelles

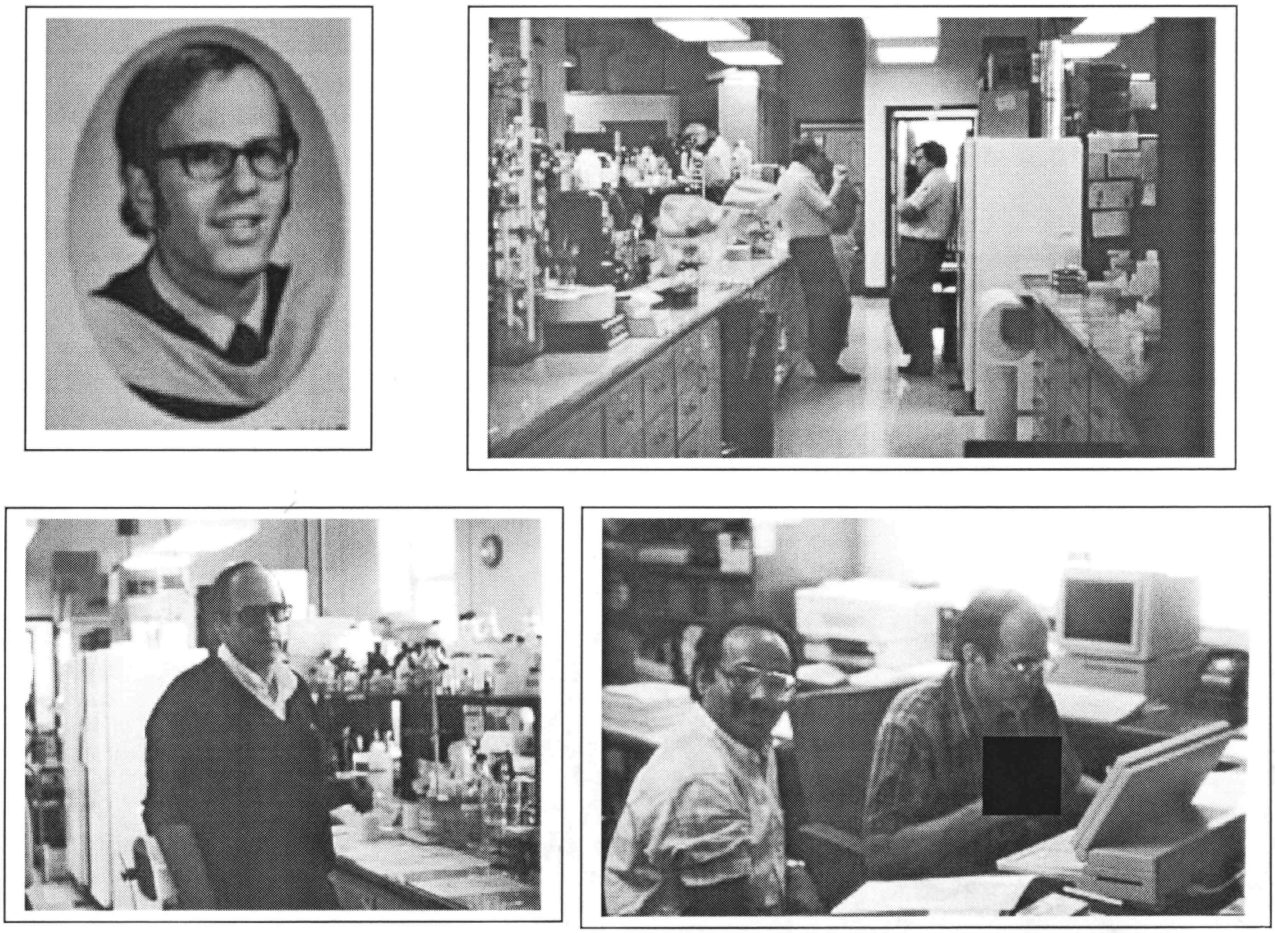

Figure 1. (upper left) MJL graduation portrait from the University at Buffalo Dental School, 1971. (upper right) MJL discussing an "issue" with Bob Cohen, 1987. (lower left) MJL in the lab, *ca.* 1988. (lower right) Writing a manuscript with N. Ramasubbu, 1990. (Guess what is behind the black box!)

(*e.g.*, plasma membrane, golgi, etc.). This work took many hours and careful attention. After working through the night for 24 hours, the unfortunate fellow found that one of the columns had become contaminated with bacterial growth. Needless to say, the experiment had to be aborted. On learning this, Dr. Spiro simply said, "Well, if you hustle, you should be able to get to the slaughterhouse in the next couple of hours to pick up some more kidneys to start over again." This expectation, that science "is your mistress", left a lasting impression on MJL and set the tone for how he would run his own laboratory in the future.

"TRUCKIN' BACK TO BUFFALO..."

Following the completion of his training program in Boston, MJL returned to Buffalo in 1975 as an Assistant Professor in the Departments of Oral Biology and Periodontology. He was fortunate to, first, share, and then to inherit, Art Ellison's well-equipped salivary biochemistry laboratory, a great foundation upon which to build his research effort. While it was well-known by this time that saliva contained several previously described proteins—such as amylase, immunoglobulins, lactoferrin, and lysozyme—it was also clear that there were quite a few components that remained uncharacterized (Ellison, 1966).**(AQ)** Thus, he set as his goal to identify and characterize as many of the remaining unknown salivary protein and glycoprotein components as possible. This was a daunting challenge, since, by today's standards, the available methodologies were quite primitive and labor-intensive, and the number of unique components in saliva appeared to be ever-increasing as more sensitive protein-detection methods were developed. Several of these unknown salivary components were particularly enigmatic. One such component was the basic proline-rich glycoprotein (PRG) of parotid saliva (Levine *et al.*, 1969; Li and Levine, 1980). Other major components that were, until then, quite recalcitrant to study were the high-molecular-weight glycoproteins. These components tend to complex with other salivary proteins that, until then, prevented their isolation to homogeneity. Using the knowledge gained from Spiro, and the efforts of many graduate students and post-doctoral fellows, beginning with Mark Herzberg and a bit later Larry Tabak and Akapron Prakobphol, MJL began the formidable task of isolating and chemically characterizing these components. He also benefited from Art Ellison's willingness to share his well-equipped laboratory.

Initial studies found at least two high-molecular-weight components in the mucous secretions, both of which were highly glycosylated and resembled mucins: a higher-molecular-weight mucin, named mucin glycoprotein or MG1 (Levine *et al.*, 1978); and a low-molecular-weight mucin or MG2 (Prakobphol *et al.*, 1982). Around this time, Dr. Molakala S.

Reddy joined the group and added considerable expertise to the characterization of the carbohydrate moieties of these glycoproteins, utilizing gas chromatography-mass spectroscopy (Reddy *et al*., 1985). In approximately three weeks, Dr. Reddy disproved the structure proposed by Larry Tabak in his PhD thesis for the major oligosaccharides derived from monkey extraparotid salivary mucins. Fortunately, appropriate corrections were made prior to the submission of the work for publication! Together, they made substantial progress in characterizing this group of molecules (summarized in Tabak *et al*., 1982; this paper was named a "Citation Classic" by CSI—one of the few oral biology papers to be accorded that distinction). Libuse Bobek eventually accomplished the difficult task of cloning and sequencing of the gene for MG2, now known as MUC7 (Bobek *et al*., 1993).

During the course of this work, a variety of smaller-molecular-weight phosphoprotein components was identified as contaminants of, and subsequently purified from, the mucins. Among these were the "cysteine-containing phosphoproteins", later recognized as cystatins. These were purified and characterized by John Shomers, who worked as a technician for MJL as he also pursued his MS degree on a part-time basis (Shomers *et al*., 1982). The gene for cystatin SN was later cloned and sequenced by Ibtisam Al-Hashimi for her PhD thesis work, while the gene for cystatin S was cloned and sequenced by Libuse Bobek (Al-Hashimi *et al*., 1988; Bobek and Levine, 1992). These proteins have been shown to have cysteine-protease inhibitory activity as well as the ability to influence biomineralization of teeth.

An overriding rationale for the work was, some day, to produce an effective, biologically based artificial saliva for the treatment of xerostomia. The strategy was to simulate the functional characteristics of salivary components by devising synthetic molecules consisting of multiple biologically active or "functional domains". It was imagined that such composite molecules could be "custom-designed" mimics generated from knowledge of the primary sequence and computer-assisted structural predictions of conformation. Promising candidates would then be subjected to *in vitro* and finally *in vivo* testing.

It was recognized that an important function of saliva was its lubricity, or ability to lubricate the oral tissues. MJL's group was one of the first to test purified salivary molecules as lubricants. Michael Hatton, while a dental student, and Alfredo Aguirre, then an MS student, constructed an interesting homemade device (based on a previously described device used to evaluate lubricating properties of synovial fluid (Swann *et al*., 1981). It resembled a record player that used a spinning circular glass plate upon which the substance to be tested was placed. An arm having a sensor was then rested on the spinning plate, and the resistance, assumed to be inversely proportional to the inherent lubricity of the material being tested, was measured (Hatton *et al*., 1985; Aguirre *et al*., 1989). It was found that purified glycoproteins such as PRG are lubricative, and that the carbohydrate moieties play a role in this function. It was therefore surprising that later studies found that statherin, a small peptide devoid of carbohydrate thought to contribute to tooth surface mineralization, possesses lubrication properties (Douglas *et al*., 1991). It appeared that statherin's amphipathic nature enables it to function as a boundary lubricant on enamel. These findings contradicted the long-held assumption that lubrication of saliva was dependent on glycosylated salivary components.

SALIVA, PELLICLES, AND DENTAL PLAQUE

Another important function of salivary components is their ability to interact with bacteria. By the mid-1970s, it was recognized that salivary molecules were intimately involved in normal dental plaque formation. Herzberg, Murray Stinson (a member of the microbiology faculty at Buffalo), Tabak, Patricia Murray (as part of her PhD thesis), and Jim Bergey (who later joined MJL as a post-doctoral following training in Stinson's lab) showed that salivary glycoproteins interacted specifically with oral streptococci. A series of elegant biochemical studies demonstrated that the bacteria possessed surface proteins or lectins which specifically bound to salivary glycoprotein carbohydrate moieties such as sialic acid (Levine *et al*., 1978; Murray *et al*., 1982, 1986; Bergey *et al*., 1986). Again, these were among the first demonstrations of lectin-like proteins on oral bacteria. Other studies focused on specific interactions between oral bacteria and amylase (Scannapieco *et al*., 1989, 1993; Tseng *et al*., 1999) and histatins (Raj *et al*., 1990). For his PhD thesis, Aaron Biesbrock extended such studies to medical pathogens such as *Staphylococcus aureus* and *Pseudomonas aeruginosa* and found a salivary mucin-secretory immunoglobulin A (S-IgA) complex to interact with the bacteria (Biesbrock *et al*., 1991).

Another area of interest was the detailed study of salivary pellicles. Ibtisam AI-Hashimi, in her PhD thesis (Al-Hashimi and Levine, 1989), combined (SDS)-PAGE/Western transfer analyses with specific radiolabeling/SDS-PAGE fluorography to identify salivary proteins in early enamel pellicle. Major components identified in pellicles included amylase, cystatins, salivary mucin, and S-IgA. These studies illustrated the selective nature of salivary protein adsorption to the enamel surface. Similar approaches were later used for PhD studies by Steve Bradway to study mucosal pellicle (Bradway *et al*., 1992) and Mira Edgerton for denture-pellicle (Edgerton and Levine, 1992). Indeed, the interesting studies of Bradway demonstrated that the enzyme transglutaminase, originating from oral epithelium, could catalyze the covalent attachment of salivary proteins such as the proline-rich proteins within oral pellicles and microbial surfaces (Bradway, 1992, 1993; Staab, 1999).**(AQ)**

AN ENVIRONMENT FOR LEARNING

From its inception in the mid-1970s with six or seven people, MJL's research group grew exponentially through the 1980s. By the time I arrived as a PhD student in 1985, more than 25 students and post-doctoral fellows were working in the laboratory. It was an exciting time indeed. Spurred on by friendly competition from other salivary biochemistry laboratories, led by Frank Oppenheim at Boston University, Don Hay at Forsyth Dental Center in Boston, and Anders Bennick at the University of Toronto, the laboratory was a beehive of activity. One had the opportunity to learn not only from MJL, but also from many enthusiastic colleagues who, as a group, collectively displayed expertise from classic biochemistry and immunology to molecular and structural biology. The laboratory was also very well-equipped to us "the latest" scientific methods. Through successful grant-writing and the support of Dean William Feagans and Dr. Robert

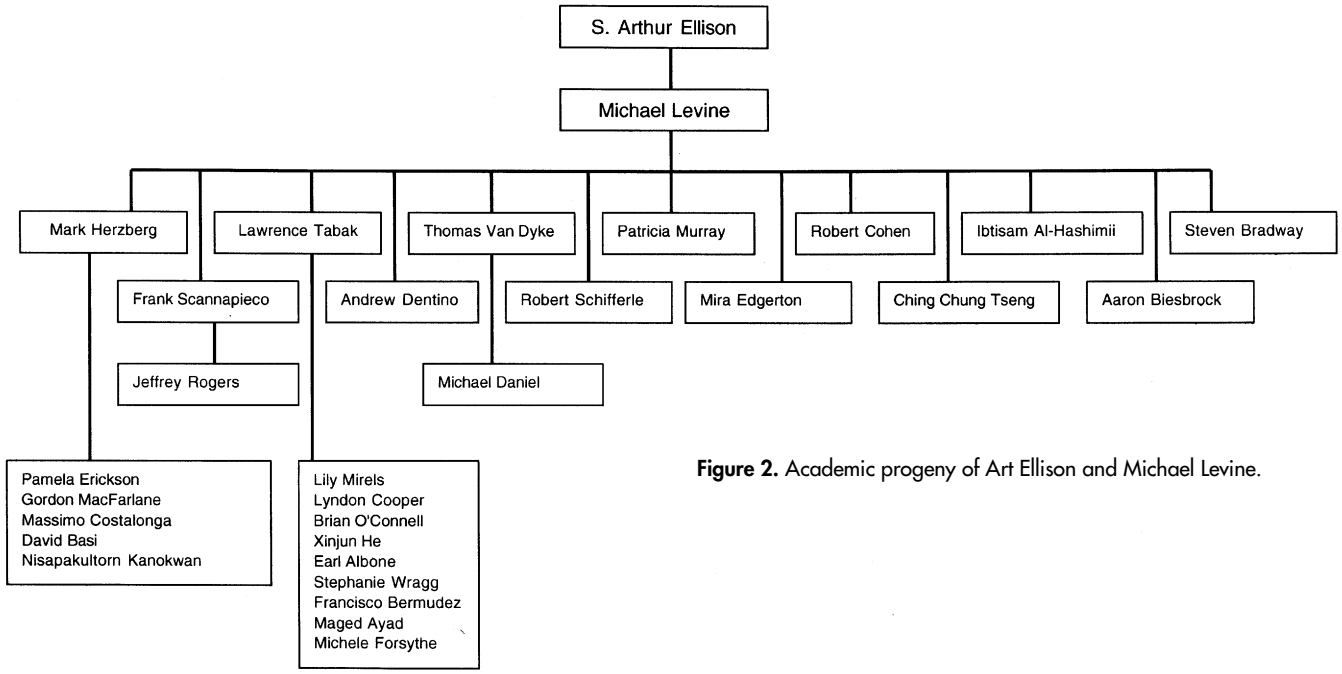

Figure 2. Academic progeny of Art Ellison and Michael Levine.

Genco, Chair of Oral Biology, MJL was able to muster considerable resources to equip the laboratory well. Indeed, one could theoretically select a molecule to be studied, isolate it, determine its amino acid composition and sequence, clone and sequence its gene, express the recombinant protein, study its function, and determine its structural characteristics using a variety of sophisticated spectroscopic methods, all in the same room! While this may seem rather routine today, it was a remarkable range of activities for a single laboratory in the 1980s, especially one in a dental school. MJL believed that it was essential that, if possible, all of the work be done within the confines of the laboratory. This unique facility enabled MJL and colleagues to pursue sophisticated structural biological studies, among the first to be performed for salivary proteins. Researchers such as Ron Loomis, Krishna Bhandary, Antony Raj, and Narayanan Ramasubbu brought structural biology expertise to the laboratory that allowed for the NMR and crystallographic analysis of mucins, amylase, histatins, and cystatins (Loomis *et al.*, 1987; Raj *et al.*, 1990; Bobek *et al.*, 1994; Ramasubbu *et al.*, 1996). For his PhD thesis, Bob Cohen outfitted the laboratory to develop monoclonal antibodies against mucins—again, state-of-the-art technology for the time. He was able to localize the distinct species of mucin to different regions of salivary glands (Cohen *et al.*, 1990, 1991). MJL also embraced the digital age: Every person in the laboratory had access to or owned a personal computer. Soon the laboratory was networked and was one of the first in the University to have Internet access. All of these technological advances allowed the research group to acquire and utilize cutting-edge techniques to advance the science at hand.

While salivary biochemistry was the major focus of MJL's laboratory, other students in the department were encouraged to utilize its resources, both intellectual and material. So, for example, Tom van Dyke, and later Ernie DeNardin and Drew Dentino, pursued biochemical studies of neutrophil chemotaxis as related to localized juvenile periodontitis (Van Dyke *et al.*, 1983; DeNardin *et al.*, 1990; Dentino *et al.*, 1991). For their PhD theses, Joe Zambon purified and characterized the polysaccharide capsule from *Actinobacillus actinomycetemcomitans* (Zambon *et al.*, 1984), and Bob Schifferle the capsule from *Porphyromonas gingivalis* (Schifferle *et al.*, 1989, 1993a,b). Ken Miyasaki used high-performance liquid chromatography to purify myeloperoxidase from human neutrophils and study its role in host defense against periodontopathogens (Miyasaki *et al.*, 1987). Many other students, post-doctoral fellows, and faculty took advantage of the unique resources provided by MJI's laboratory.

LEVINE'S LEGACY

Notwithstanding MJL's achievements in and providing the materials and technology for research, his most outstanding contribution to oral health science was his mentorship of PhD students (13) and post-doctoral fellows (24). Most of these individuals are presently active in academic or research positions throughout the world. Indeed, many of his students have gone on to train their own cadre of students (see Fig. 2). Through these progeny, it is obvious that MJL's influence will be felt by the oral health research community for some time to come.

EPILOGUE

MJL's effectiveness as a mentor and researcher was the result of extremely hard work and intelligence. He often "burned the candle at both ends" and expected—no, insisted on—the same of all who worked with him. In the early days, if you did not reach the lab before Mike arrived, he would barely acknowledge your existence for the rest of the day. The problem was that he usually arrived by 5:30 AM! He was extremely blunt and honest, and often used his quick (and usually acerbic) wit. You always knew where you stood with MJL. If he liked you, he teased you mercilessly. He assigned everyone a nickname (for example, most people knew Larry Tabak as "Bear" and M.S. Reddy as "Bubba"?but few other

nicknames can be printed on these pages!), and most were the butt of his often caustic (and usually very funny) comments. He was extremely finicky about cleanliness and suffered "sponge neurosis", traveling the laboratory cleaning the benches at least once a day. Woe unto the "slob" who left a mess from the previous day's work! Yet he set a tone that encouraged hard work and achievement. He demanded only the very best from his "mentees". His science was strict: He expected that every observation would be verified by more than one methodology. He was especially attentive and helpful with grant- and manuscript-writing. As a result (or in spite) of this attention to detail, there was a tremendous sense of camaraderie and cooperation in his laboratory. Colleagues were always very accommodating and willing to help in any way possible. There were routine laboratory meetings that often went on for hours. It was a tough crowd. If one survived an MJL laboratory meeting, one could present with confidence to colleagues "in the real world".

Is it possible that such a research environment could evolve again? The financial limitations of state governments and universities, the vagaries of grant-funding, and the unfortunate lack of interest on the part of contemporary dental student graduates in basic research suggest that the probability of such a group coming together in the future is remote indeed.

MJL's hard work and extensive accomplishments in research and dental education did not come without a price. After 25 years on the faculty at Buffalo, he retired from full-time research activity in 2000. He continues to spend two days a week teaching periodontics to junior and senior dental students at the University at Buffalo (punctuated by frequent trips to Hawaii). While he was never very active in teaching undergraduate students when he was engaged in full-time research, he has embraced clinical teaching and is now a favorite of the dental students. Some things never change: He continues to tease, cajole, entertain, and inspire his students to do more work than they ever wanted to do!

ACKNOWLEDGMENTS

The author thanks Mark Herzberg and Larry Tabak for reading the manuscript and providing important details of the early history of the MJL research group.

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