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Review

The origins of enzyme kinetics

Athel Cornish-Bowden

Unité de Bioénergétique et Ingénierie des Protéines, Institut de Microbiologie de la Méditerranée, Centre National de la Recherche Scientifique, Aix-Marseille Université, 31 chemin Joseph-Aiguier, 13402 Marseille Cedex 20, France

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ABSTRACT

The equation commonly called the Michaelis–Menten equation is sometimes attributed to other authors. However, although Victor Henri had derived the equation from the correct mechanism, and Adrian Brown before him had proposed the idea of enzyme saturation, it was Leonor Michaelis and Maud Menten who showed that this mechanism could also be deduced on the basis of an experimental approach that paid proper attention to pH and spontaneous changes in the product after formation in the enzyme-catalysed reaction. By using initial rates of reaction they avoided the complications due to substrate depletion, product accumulation and progressive inactivation of the enzyme that had made attempts to analyse complete time courses very difficult. Their methodology has remained the standard approach to steady-state enzyme kinetics ever since.

1. Introduction

Michaelis and Menten are by far the best known of the scientists who created the subject of enzyme kinetics, but what was their real contribution? Have they simply received the credit for work already published by Brown [1] and Henri [2,3] before their paper of 1913 [4] (Fig. 1), as some authors [5,6] have suggested? Here I shall argue that although earlier authors, especially Henri, made important advances they lacked Michaelis and Menten's insight of realizing that an analysis in terms of initial rates would eliminate the complications that had plagued their predecessors' efforts to interpret time courses.

2. The basic contribution of Michaelis and Menten

In common with numerous researchers of their time Michaelis and Menten studied the inversion of sucrose catalysed by invertase. (The word *inversion* refers to the use of a polarimetric method to follow the reaction, the sign of the optical rotation of "invert sugar", or the mixture of glucose and fructose produced in the reaction, being opposite from that of sucrose.) They expressed the rate v of the reaction in the following way:

$$v = C \cdot \Phi \frac{[S]}{[S] + k} \tag{1}$$

defining Φ as the total molar concentration of invertase, $[S]$ as the sucrose concentration (noting, incidentally, that there is no practical difference between the free and total concentrations of substrate when its concentration is very large compared with that of the enzyme), k as the dissociation constant of the enzyme–substrate complex, and C as a constant of proportionality. At the time they were writing, the convention that capital letters are used for equilibrium constants and lower-case letters for rate constants did not exist, so k here is not a rate constant, whereas K_3 in Eqs. (4) and (5) below is a rate constant.

The equation is nowadays usually written in a form resembling the following:

$$v = \frac{Va}{K_m + a} \tag{2}$$

in which v is the initial rate observed at a total substrate concentration of a, and V, the *limiting rate*, and K_m, the *Michaelis constant*, are constants. As any modern textbook will show, the steady-state mechanism introduced by Briggs and Haldane [7] is now taken as the starting point for interpreting this equation:

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \xrightarrow{k_2} E + P \tag{3}$$

in which E is the enzyme, A is the substrate, P is the product, EA is an enzyme–substrate complex, k_1, k_{-1} and k_2 are rate constants, and the reaction is assumed to be in a steady state in which the rate of production of EA is balanced by the rate of its conversion to

E-mail address: acornish@imm.cnrs.fr

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Die Kinetik der Invertinwirkung.

Von

L. Michaelis und Miß Maud L. Menten.

(Eingegangen am 4. Februar 1913.)

Fig. 1. Title of Michaelis and Menten's paper. Notice the unusual way the English word "Miss" is spelt.

products. With this interpretation $V = k_2 e_0$, where e_0 is the total enzyme concentration, and $K_m = (k_{-1} + k_2)/k_1$. (Briggs and Haldane did not use the symbol K_m, which appeared, however, possibly for the first time, in Haldane's book [8].) This interpretation did not come immediately, and Henri [2,3] and Michaelis and Menten [4] both assumed that K_m was the equilibrium dissociation constant of EA, which would be k_{-1}/k_1 in the symbols used here. (The more usual symbol today for the dissociation constant k_{-1}/k_1 would be K_s rather than K_m.) The question now to be asked is whether Eq. (2) is more appropriately called the Henri–Michaelis–Menten equation or the Michaelis–Menten equation.

Of course, no discovery appears from nowhere — other, perhaps, than Newton's study of colours [9] — and not only did Brown and Henri contribute, but numerous other developments of the preceding century were also important, including general ideas of chemical kinetics [10], the law of mass action [11], the discovery of a papain–substrate complex [12], and earlier studies of invertase [13,14]. Nonetheless, Michaelis and Menten's paper [4] represented a major turning point in the history of our understanding of enzyme catalysis, and its effects are still relevant 100 years later, because they defined how kinetic experiments need to be done if useful information is to be obtained from them: they were the first to understand the importance of controlling the pH, and the first to recognize that initial rates are easier to interpret than time courses. Their third contribution — taking account of the effects of spontaneous mutarotation on the kinetics observed by polarimetric methods — was important for the study of invertase, but does not have a wider importance beyond the obvious point that if the products of a reaction undergo spontaneous changes that affect the method of assay this needs to be taken into account. Not only did they define how experiments should be done, but they also carried them out rigorously, and obtained results with a precision that can stand comparison with that obtainable today; almost as important, they described what they had done with sufficient clarity and completeness for Johnson and Goody [15] to be able to repeat them and check them nearly a century later. Unfortunately, not all enzyme kinetic experiments are described so clearly today, and that is why the guidelines proposed by the STRENDA Commission of the Beilstein-Institut [16,17] have become necessary.

Two modern translations of Michaelis and Menten's paper are available: one, by Boyde [18], is included in this Special Issue of *FEBS Letters*, and is based on an earlier one by the same author [19]. The other is a downloadable supplement to the recent paper of Johnson and Goody [15]. Boyde [19] also includes translations of some relevant publications of Henri [2,3], Sørensen [20] and others. Various of these (but not Michaelis and Menten's paper) have been translated by Friedmann [21].

3. Advances made by other early authors: the enzyme–substrate complex

3.1. Brown and Henri

At the beginning of the 20th century the nature of enzyme catalysis and kinetics was of widespread interest and was studied by several different authors, most notably Brown [1] and Henri [2,3]. Of these, Brown [1] was probably the first to realize that a mechanism that required passage through an enzyme–substrate complex implied an upper limit on the rate of an enzyme-catalysed reaction, and he can be credited with introducing the first model of enzyme saturation. However, his interpretation was qualitative, unsupported by any algebra. Henri [2,3] criticized it for its completely unrealistic assumption that the enzyme–substrate complex had a fixed lifetime, and derived an equation for the instantaneous rate of a reaction subject to product inhibition:

$$\frac{dx}{dt} = \frac{K_3(a-x)}{1 + m(a-x) + nx} \tag{4}$$

in which a is the total amount of sucrose, x is the amount of product at time t, K_3 is a constant proportional to the amount of enzyme, and m and n are also constants. In his thesis [3], but not in his paper [2], he went on to note that if $x = 0$ when $t = 0$ then this can be simplified to

$$\text{initial rate} = \frac{K_3 a}{1 + ma} \tag{5}$$

which is just the Michaelis–Menten equation in unfamiliar symbols, other than the fact that it expresses the rate at which the amount (not the concentration) of product changes, i.e. the *rate of conversion* [22], whereas today a kinetic equation usually expresses the rate at which the concentration changes. However, apart from noting that this gave a good account of the experimental observations with invertase, Henri took the matter no further: he did not point out that this simpler equation could form the basis of an experimental approach that would allow a far easier analysis than the attempts to use the time course that had long dominated efforts to understand the kinetics of enzyme-catalysed reactions.

Most of the early discussion of the enzyme–substrate complex incorporated two assumptions: that it must necessarily participate as an intermediate in the reaction mechanism; and that it was maintained at equilibrium with the free enzyme and substrate. Although Henri [2,3] thought that its participation as an intermediate was the most likely interpretation, he also considered an alternative possibility, and found that if the complex existed only as a "nuisance complex" in a side reaction the kinetic behaviour would be indistinguishable from that given by assuming it to be an intermediate. That is true so far as the steady state is concerned, but transient-state measurements allow the two possibilities to be distinguished [23,24]. Non-productive complexes can certainly exist, and can complicate the interpretation of data for enzymes that act in nature on large polymers when studied with small synthetic substrates [25], but no examples are known for which Henri's alternative mechanism is the whole explanation of enzyme saturation.

3.2. Van Slyke and Cullen

Van Slyke and Cullen [26], who were studying urease at about the same time as Michaelis and Menten's work, did not assume that the enzyme–substrate complex was at equilibrium with the free components; instead they assumed that it would be formed in an irreversible reaction and broken down in a second irreversible reaction to regenerate the free enzyme. They treated the time required for a complete catalytic cycle as the sum of the times required for the two steps, and the steady-state assumption was implicit in their treatment. Processes occurring in series can always be analysed in terms of additive times, but Van Slyke and Cullen's approach has not often been used explicitly in later work. However, it can be very useful, for example, for considering the steps in metabolic processes [27]. Assigning rate constants k_1 and k_2 to Van Slyke and Cullen's two processes allowed the rate equation to be written as follows:

$$v = \frac{Va}{(k_2/k_1) + a} \quad (6)$$

This would be experimentally indistinguishable by steady-state experiments from Eq. (2), though the interpretation of the constant in the denominator is different. In a companion paper Van Slyke and Zacharias [28] extended this analysis to take account of the effects of reaction products and protons on the rate of reaction.

Van Slyke and co-workers did not mention Michaelis and Menten's paper, and were almost certainly unaware of it. Much later in his life Van Slyke made an indirect but important contribution to enzymology when he sponsored publication of the theory of *induced fit* [29]. In 1911, at the beginning of his career, he had worked with Emil Fischer in Berlin, but that did not inhibit him from facilitating the first dent in the long established *lock-and-key* model of enzyme specificity.

3.3. Reassessing Henri's contribution

Segal's classic review [30] provides a more detailed account of the development of understanding of the kinetics of enzyme-catalysed reactions than I have given here, and Mazat [31] makes a somewhat different (though not incompatible) assessment of the relative importance of the contributions of Henri on the one hand and Michaelis and Menten on the other. He points out that although attributing Eq. (2) to Michaelis and Menten alone is too deeply entrenched to be dislodged, it is not too late to give Henri due credit for his considerable contribution, and that Eq. (4) could very properly be called the "Henri equation", the first equation for competitive product inhibition to be proposed, and conceptually important for understanding the effects of products on the invertase-catalysed reaction. In modern symbols the Henri equation would be written as follows:

$$v = \frac{Va}{K_m(1 + p/K_p) + a} \quad (7)$$

in which K_p is the inhibition constant for product, and the other symbols are as in Eq. (2).

4. Michaelis and Menten's experimental approach

4.1. Control of pH

Although the term and definition of pH are rightly associated with Sørensen [20], Michaelis already had a clear grasp of the importance of the concept, and his work on the effect of hydrogen ions on invertase [32] appeared only a short time after publication of Sørensen's analysis. In the previous work on invertase, Henri [2,3] ignored the question altogether, and Brown [1] reported that control of acidity was not necessary. O'Sullivan and Tompson [13] had carried out many experiments (they referred to "hundreds") to determine how much acid needed to be added if reproducible results were to be obtained, but they did not attempt a theoretical analysis and simply made a vague statement that "the acidity was in the most favourable proportion". Presumably Henri and Brown were able to evade the question by using preparations that contained enough natural buffering agents to maintain the pH constant, but it is clear that the reforms introduced by Sørensen [20] and Michaelis [32] are essential for any biochemical experiment today. Michaelis had a long-term interest in hydrogen-ion concentration, and his book [33] became the standard work on the subject. In particular, he introduced the "Michaelis functions" that allow interpretation of bell-shaped pH profiles. As noted already, Van Slyke and Zacharias [28] were also quick to recognize the importance of the hydrogen-ion concentration.

Michaelis's work on hydrogen ions was, in fact, well advanced when Sørensen's paper appeared. He discussed this in his autobiographical notes [34]:

> The method of the hydrogen electrode was developed in order to measure the hydrogen ion concentration. It was shown that the effect of an enzyme such as invertase, trypsin, etc. depends on the concentration of the hydrogen ions, and not on the titration acidity. Just when this work was coming to a conclusion, the paper by Sorensen on the same subject was published. However, being familiar with the method, Michaelis, although deprived of the priority, extended these studies by showing that the dependence of enzyme activity on pH was of the same nature as the dependence of the dissociation of a weak acid on pH. The theory of buffers (under the name of "hydrogen ion regulators") was developed.

4.2. Characterization of inhibitors

As noted already in Section 3.3, the Henri equation (Eqs. (4) and (7)) takes account of product inhibition, and other predecessors of Michaelis and Menten were also aware that the products of a reaction could have an inhibitory effect on its rate. However, it was Michaelis and his collaborators Rona [35] and Pechstein [36] who first made a systematic study of inhibitors. They classified them as *competitive* if they increased the apparent value of K_m with no effect on V, as they observed for the effect of fructose on invertase, and *non-competitive* if they decreased the apparent value of V with no effect on K_m. On that basis they reported that glucose was a non-competitive inhibitor of invertase, but in reality its effect is more complicated than that. Moreover, their classification is unfortunate for a different reason, as we now realize (probably first emphasized by Cleland [37]) that the rational classification is in terms of effects on the apparent values of V/K_m (competitive) and of V (*uncompetitive*): thus the opposite extreme from competitive inhibition is uncompetitive inhibition, and Michaelis's non-competitive inhibition is just a particular case of *mixed* inhibition, in which there are effects on both parameters.

4.3. Initial rates

Although Henri [3] derived the equation for the initial rate of an enzyme-catalysed reaction, he did not use it as the basis for a method of analysis. Instead, Michaelis and Menten [4] recognized that extrapolating the rate to zero time would overcome several problems:

1. Complications due to the progress of the reaction vanish: inhibition by accumulated products, loss of activity of the enzyme, and, in the case of the polarimetric methods used for studying invertase, spontaneous mutarotation of the products.
2. The reverse reaction can be ignored, because it cannot occur until some products have had time to appear.
3. An initial-rate equation is much simpler to derive and use than an equation for the full time course of a reaction.
4. There is no drift in the pH or other conditions at zero time.

Most of these points are still valid today, though the justification for ignoring the reverse reaction is often given incorrectly, not only by students in answers to examination questions, but sometimes even in textbooks [38] and recommendations of an international union [39]: the reverse reaction cannot occur if no products are present; there is no assumption about the magnitudes of the reverse rate constants; $k_{-2}p = 0$ if $p = 0$, regardless of the magnitude of k_{-2}. Even mutarotation is still important if it is regarded as a

specific instance of a general problem, that the products of a reaction may undergo spontaneous reactions that affect the validity of the assay. Today mutarotation, and indeed the whole inversion process, can be much more conveniently followed by nuclear magnetic resonance spectroscopy [40], which allows all of the species in the reaction to be separately monitored, but, polarimetry was an advanced technique for its day, and offers an early example of the use of a physical method for following a chemical reaction.

It is perhaps less true that time courses are too complicated to use. It has long been known how to integrate the Michaelis–Menten equation itself, but methods for integrating steady-state equations for other cases were introduced in an *ad hoc* way, each mechanism being treated as a special case [41], and Boeker's general method [42,43] appeared surprisingly recently. In modern practice the use of integrated equations for deducing kinetic parameters from time courses remains unusual. On the one hand even quite small effects of products can lead to large errors in the parameters [44], so the analysis is less straightforward than the initial-rate method introduced by Michaelis and Menten [4]. In addition, although it is easy to integrate Eq. (2), the resulting equation expresses the time t in terms of the concentration of product p and the initial concentration of substrate a_0:

$$t = \frac{1}{V}\left[p + K_m \ln\left(\frac{a_0}{a_0 - p}\right)\right] \tag{8}$$

whereas one would usually prefer to express p in terms of t. Goudar et al. [45] have shown how this can be achieved.

4.4. The foundation of steady-state kinetics

The importance of Michaelis and Menten's experimental approach lies in the fact that it was a general procedure, readily applicable to other cases, and easily extensible to take advantage of improvements in techniques and knowledge: if it were no more a method for studying invertase it would be forgotten by now. As already mentioned, Michaelis himself played a large part in developing methods for studying pH dependence [32,33], as well as methods for characterizing enzyme inhibitors [35,36].

The apparent conflict over the interpretation of the constant in the denominator of the rate equation (K_m in Eq. (2), $1/m$ in Eq. (5), k_2/k_1 in Eq. (6)), was resolved by recognizing that it was more realistic to regard it as function of three or more rate constants [7], so it defines the concentration of the enzyme–substrate complex in a *steady state*, not necessarily at equilibrium. This reinterpretation required no fundamental change in Michaelis and Menten's methodology.

The basic theory of steady-state enzyme kinetics could then be regarded as complete, and provided a firm foundation for the later development of methods for studying reactions with multiple substrates, reversibility and specificity. Although Haldane mentioned multiple substrates in his book [8], they were first studied in depth in the 1950s [46–48], but this work had comparatively little impact until it was brought into wide use by a set of landmark papers by Cleland [37,49,50]. These appeared just at the midpoint between Michaelis and Menten and the present, and thus have their 50th anniversary this year. Sad to report, Mo Cleland had a fatal accident only a few days after he had agreed to contribute to this Special Issue of *FEBS Letters*.

Haldane's analysis of reversibility [8] had come earlier: it later opened the door to analysis of the relationships between the thermodynamics and kinetics of enzyme catalysis [51–53]; see also the article by Noor et al. [54] in this issue. Just two important other components remained to be added to the basic theory to allow such development. The first was a convenient method for deriving non-trivial rate equations, which was provided by King and Altman's graphical method [55]; this is readily converted to algorithmic for computer implementation [56] and usable with modern packages such as *Mathematica*™ [57]. The second was the introduction of statistically satisfactory methods of data analysis, which were supplied by Wilkinson [58] and Johansen and Lumry [59] and brought into wide use with Cleland's computer programs [60].

A topic that took a surprisingly long time to be well understood was enzyme specificity, which was often discussed in vague terms related to the kinetic parameters measured for pure substrates *in vitro*, until Fersht [61] pointed out that the only useful physiological meaning would be one that defined the capacity of an enzyme to discriminate between substrates that are simultaneously available. In terms of the parameters of Eq. (2), this means that specificity is determined by V/K_m, not by either V or K_m alone.

5. The impact of Michaelis and Menten's paper

Not surprisingly, Michaelis and Menten's paper has been very heavily cited (Fig. 2), though perhaps less heavily read — a citation error in the well known paper of Lineweaver and Burk [62], which gave the first page as 1333 rather than 333, has been reproduced in at least 27 later publications between 1938 and 2007. After a rapid growth in citation frequency after 1945, the level remained relatively stable until the huge increase that has occurred in the 21st century, with around 30% of all the citations occurring since 1999. This parallels the rise of systems biology, metabolic modelling and kinetic studies with single molecules, and, astonishingly, the year that has seen the greatest number of citations until today is 2011, and every complete year after 2005 has shown a higher level than the peak of 1953.

Examination of the papers that cited Michaelis and Menten [4] in two representative years 10 years apart, 2000 and 2010, indicates that although there was indeed a large increase in the number of papers that can be classified as systems biology, from one in 2000 to 19 in 2010, the increase is not nearly large enough to account for the whole of the increase in citations, from 21 in 2000 to 74 in 2010. Methods for the study of single molecules were largely non-existent in 2000 but had become a major area of research by 2010, as exemplified by a study of the packaging motor of the bacteriophage ϕ29 [63]. Drug development was certainly not non-existent as a topic in 2000, but apparently none of the papers concerned with it cited Michaelis and Menten [4], whereas several did in 2010. One may hope that this change reflects an increased awareness of the importance of understanding the kinetics of enzyme-catalysed reactions for creating new drugs. Over the same period there were also increases in the numbers of papers concerned with enzyme engineering and with the use of ideas from enzyme kinetics to described observations in ecology. Taken together, these five areas of research seem to account for much of the increase in interest in Michaelis and Menten's paper in the 21st century (illustrated graphically in Fig. 3), together, perhaps, with a revival of interest in enzymes over the whole of biochemistry, as it has become more and more evident that molecular biology alone cannot solve all current problems.

Other papers by Michaelis related to enzyme kinetics, especially those already mentioned [32,35,36] have also been highly cited (Fig. 2b). In the first part of the 20th century these were as heavily cited as that of Michaelis and Menten [4], and despite a decline after 1955 they continue to be cited from time to time today. Michaelis's output in the years leading up to the First World War was enormous, high even by today's standards: 1913 was, in fact, the least productive of the five years from 1910 to 1914, which saw 94 publications, including his book on hydrogen ion concentration [33], and four other books. His later work in Japan [64] and in the USA included numerous major contributions.

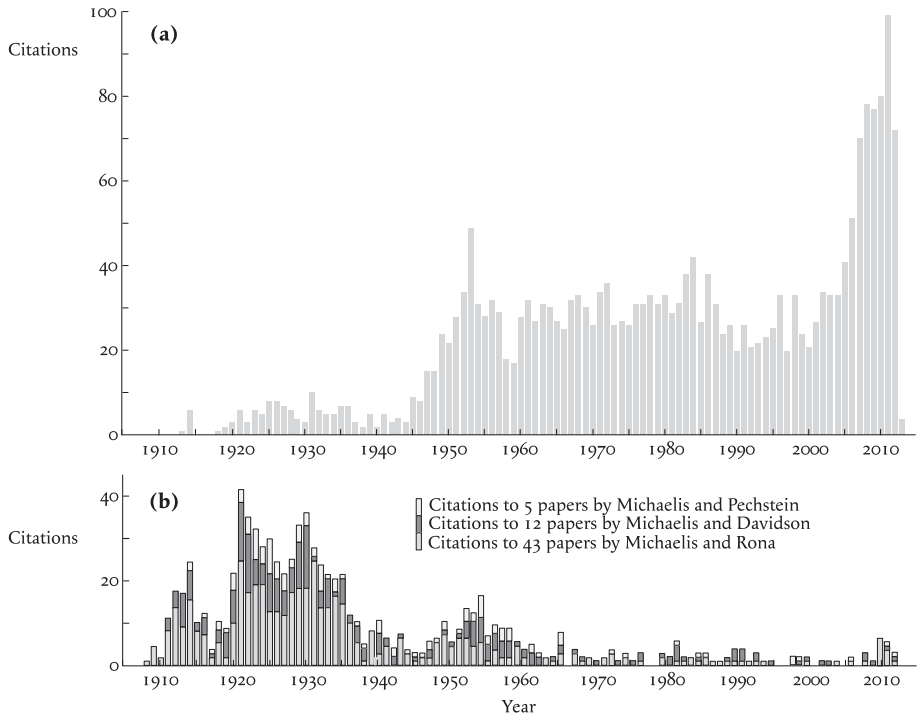

Fig. 2. Citations to Michaelis's papers. (a) Citations to Michaelis and Menten's paper of 1913 [4]; (b) citations to Michaelis's papers with Rona, Davidsohn and Pechstein. The figure was drawn from data in *Web of Science*, checked in February 2013, so the data for 2013 are very far from complete, and those for 2012 are probably incomplete also.

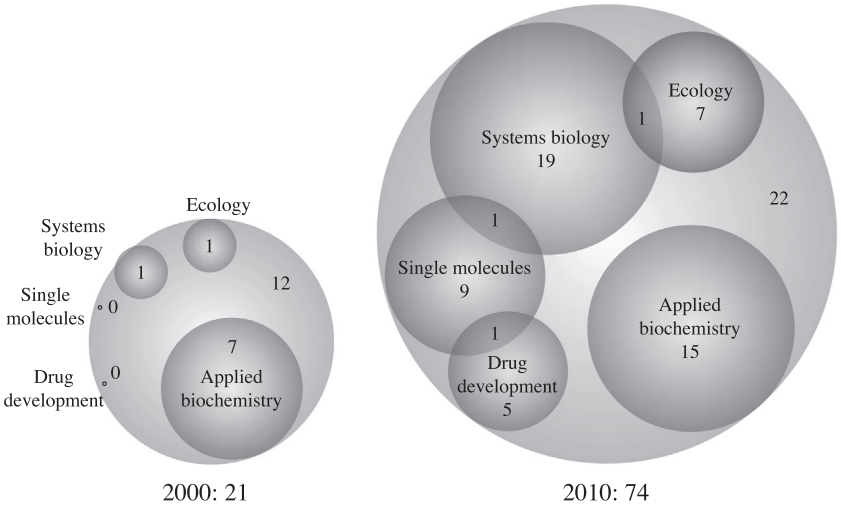

Fig. 3. Growth in citations to Michaelis and Menten [4] in the first decade of the 21st century. The great development of several subjects — systems biology and single-molecule studies, together with applications of enzyme kinetics to applied biochemistry, ecology and drug development — accounts for much but not all of the growth.

The place of Michaelis in the history of enzyme kinetics is thus assured, but what of Menten? She had a long and distinguished career at the University of Pittsburgh, but the work that she published after her brief period in Berlin is not well known by enzymologists, as it took her away from kinetics, and especially into pathology. Her obituary by Stock and Carpenter [65] stated that her reputation rested on three pieces of work in addition to the paper with Michaelis: the discovery of the hyperglycaemic effects of *Salmonella* toxins [66], an azo-dye coupling method to detect the presence of alkaline phosphatase in the kidney [67], and, in the same year, a method based on sedimentation and electrophoresis to show that the differences between adult and foetal haemoglobins in humans was due to multiple molecular forms ([68]). This last work, potentially important, was overshadowed by a later but far better known application of a similar approach to show the molecular nature of sickle cell anaemia [69], and unfortunately it appears not to have survived modern scrutiny of the methods used (M. Brunori, personal communication).

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