Review

Michaelis and Menten and the long road to the discovery of cooperativity

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ABSTRACT

This article sketches the road from the establishment of the principles of enzyme kinetics, at the beginning of the 20th century, to the discovery of regulatory mechanisms and the models to explain them, from the middle of the century onwards. A long gap in time separates the two periods, in which technological advances were made that allowed the discovery of feedback inhibition and cooperativity. In particular, these discoveries and the theory needed to explain them could not have been made without knowledge of the major metabolic pathways and the enzymes and metabolites involved in them.

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1. Introduction

This year marks not only the centenary of the Michaelis–Menten equation, published in Biochemische Zeitschrift [1], but also the half-centenary of the allosteric concept proposed by Monod, Changeux and Jacob in the Journal of Molecular Biology [2] that opened the way for understanding cooperativity and feedback inhibition. As we will see (Fig. 1), a long road separates the two events, as the first reports of feedback effects did not appear until several decades after Michaelis and Menten.

Leonor Michaelis's contribution to enzyme kinetics is enormous, in particular his work with Maud Menten [1], and their article can be considered an inflection point in the curve of the development of the field. In the 19th century researchers had studied chemical reactions that in general proceeded in a single step, so that following the course of reaction presented no great problem. However, when the study of enzyme reactions started, although the idea of the formation of an enzyme–substrate complex already existed [3,4], people continued trying to analyse the kinetics in terms of the progress curve of the reaction, until Michaelis and Menten drew attention to a better strategy. They not only used correct algebra for analysing their data, but also emphasized two important points, the advantages of working in conditions of initial velocity and the necessity of controlling the pH (the pH scale having been introduced a little earlier [5]). If these conditions were satisfied the curve of velocity in function of the concentration of substrate needed to be a hyperbola, defined by two parameters, what we now call the Michaelis constant, \( K_m \), and the maximal velocity \( V \) (or, more appropriately, the limiting velocity) [6]. The reanalysis of Michaelis and Menten’s results in terms of the steady-state interpretation [7] did not alter the fundamental correctness of their approach.

This realization and the establishment of a correct experimental protocol were crucial, because they meant that any deviation from hyperbolic behaviour needed an explanation. The possibility of recognizing deviations allowed enzyme cooperativity to be discovered, and models were developed to explain it. These deviations from hyperbolic behaviour were initially received with surprise and worry, as it was not easy to show that they were not artefacts. Umbarger [8], for example, referred to ‘peculiar kinetic behavior’ and Gerhart and Pardee, although several years later, still talked about ‘complex kinetics’ [9]. By that time, of course, the cooperative binding of oxygen to haemoglobin was well known [10,11] and was a source of inspiration for the development of models that could explain the peculiar kinetic behaviour. It may appear surprising, however, that it took so long, more than 40 years from the time of Michaelis and Menten, to recognize the first cases of deviation from hyperbolic kinetics; in fact it is not so surprising, as discussed below, because to detect a deviation an adequate range of substrate concentrations must be studied and the velocity pre-
ciscely determined. Furthermore, in general only enzymes showing feedback inhibition show cooperativity, so the number of possible examples was not large.

2. Practical problems for obtaining adequate saturation curves

The necessity of working in conditions of initial velocity [1] not only showed how to do kinetic experiments well, but it also imposed a very significant constraint on kinetic studies, because it implied a capacity to detect small amounts of product with sufficient accuracy, or small decreases in substrate concentration, and at that time this was not easy. In practice it required analytical methods sensitive enough to cope with this restriction. Furthermore, as the initial velocity is a measure of the tangent to the progress curve at time zero, which requires a curve with a sufficient number of points, the ideal situation is to use an analytical method whose results could be registered continuously, such as a spectrophotometric test. Nowadays, this is no problem, as any laboratory has such equipment, but that was not the case at the time of Michaelis, nor for a considerable time afterwards. Furthermore, even when people started to have spectrophotometers, many reactions could not be followed directly, as they produced no observable spectrophotometric change. The realization that coupled assays could be used [12–14], where, for example, the oxidation of NAD(P)H or the reduction of NAD(P) could be followed, was an important step in the right direction. Much later, the development of the theory of coupled assays [15–17] allowed a reliable protocol to be established, and this provided a powerful tool for studying enzyme kinetics and contributed strongly to the field.

A deviation from hyperbolic behaviour does not necessarily mean, however, the existence of cooperativity. Enzymes are often unstable, and they can become inactivated in the assay conditions, and as substrates usually act as stabilizing factors, an increase in substrate concentration may produce increases in product formation, above what the Michaelis–Menten equation would predict. In such a case the curve of rate as a function of substrate concentration may appear sigmoidal instead of hyperbolic. The problem of enzyme instability is serious, and it was especially so during the first half of the 20th century when there was not enough knowledge about how to stabilize enzymes. It is not surprising that many of the studies were done with enzymes chosen for their stability, such as extracellular enzymes, usually studied with artificial substrates. As a matter of fact, both Henri [4] and Michaelis and Menten [1] made their pioneering kinetic studies with invertase, an extracellular enzyme secreted by yeast. (Although invertase is little studied in modern biochemistry, it is widely used in the confectionery industry for production of chocolates with liquid centres.)

Extracellular enzymes have also the advantage that they have fewer ‘contaminants’ than intracellular ones and tend to be easier to purify. Obtaining pure enzymes and even partially purified ones was a difficult task, and progress required knowledge of how to stabilize enzymes, and the development of purification techniques and of such appropriate materials as ion-exchange resins and filtration gels. Affinity chromatography, which contributed greatly to the field, only developed in the 1970s.

Something crucial to bear in mind is that at the time of Michaelis and Menten, and for several decades afterwards, metabolic pathways and intermediates had not been well established, and the corresponding enzymes were also not well known: for example, the Krebs cycle was not proposed until 1937 [18], and remained controversial for a considerable time afterwards, with some of the enzymes, such as isocitrate dehydrogenase [19] still needing to be characterized. Many studies were done using crude tissue extracts. Only when knowledge of protein chemistry had advanced sufficiently could the study of intracellular enzymes be accomplished with the use of natural substrates, which were in many cases metabolites, and the phenomena of cooperativity and allostery could be revealed.

An artefactual deviation from hyperbolic behaviour could also be attributed to problems of controlling the concentration of the real substrate. This could happen if, for example, the real substrate was a complex with a metal ion, and the variation of substrate concentration had failed to take this into account. A good example is ATP, for the real substrate is nearly always MgATP.

Furthermore, in order to clearly detect a deviation from the expected hyperbolic behaviour it is necessary to make measurements at several substrate concentrations, both above and below half-saturation. At the lower concentrations the problems of enzyme instability just mentioned and of lacking an adequate detecting method...
can arise, and quite often, experimental studies are done with too few observations at these low concentrations.

For all these reasons it is not surprising that several decades passed after the pioneering work of Michaelis and Menten before the first cases of deviation from the expected behaviour were reported [8,9]. Probably such deviations had been observed before, but experimenters lacked the confidence to report them, and regarded them as artefacts.

3. Feedback inhibition and cooperativity: two faces of the same coin

Among the first enzyme reactions known not to follow the classical hyperbolic behaviour were threonine deaminase [8] and aspartate transcarbamoylase [9], and such enzymes also showed feedback inhibition. In other words cooperativity and feedback inhibition were discovered at the same time.

During the 1950s there was great interest in cybernetics, the study of control systems originated by Wiener [20] and still with some influence today [21]. There were indications that feedback control could exist in living organisms: for example, in Escherichia coli the presence of isoleucine in the culture medium prevented threonine from being metabolized to isoleucine [22]. In 1956 Umbarger shed light on this phenomenon in a classic paper of a single page in Science [8]. He studied the effect of isoleucine on deamination of threonine, the first step in the conversion of threonine to isoleucine, and found that isoleucine was a very strong inhibitor, 100 times stronger than leucine. Surprisingly, in spite of the structural difference with the substrate, the inhibition was competitive. Furthermore, the kinetic results were ‘peculiar’, because to obtain a straight line in the double-reciprocal plot he needed to use the square of the substrate concentration. So, together with finding the existence of deviations from the expected hyperbolic behaviour he found evidence of a negative feedback.

Umbarger’s observation on threonine deaminase was very important, as it is one of the first cases of deviation from the expected hyperbolic kinetic behaviour to be reported. However, as this could have been an artefact, for the reasons mentioned earlier, he added: ‘Further experiments are in progress in an effort to decide whether this peculiar kinetic behavior is apparent or real.’ These results were confirmed later [23], but they continued to be worried. In relation to their Fig. 5 the authors said: ‘It is to be noted that the abscissa is 1/S^2 rather than the usual 1/S since this reaction appears to be bimolecular with respect to both substrate and inhibitor. Attempts to alter the conditions of assaying enzyme activity so as to obtain the usual monomolecular kinetic behavior have been unsuccessful.’ [23].

Umbarger’s observations [8,23] were later confirmed by Jean-Pierre Changeux during his thesis work [24] using a derepressed mutant which produced more than ten times more enzyme than the wild type. Like Umbarger, he found that the kinetics of L-threonine deamination, both in the absence and presence of L-isoleucine, were ‘somewhat complex’ and ‘does not follow simple Michaelis–Menten kinetics’. Furthermore, he found that the inhibitory effect of isoleucine was competitive with respect to threonine, even with purified enzyme (Umbarger’s experiments were done with crude extracts). The complexity of the kinetics induced him to postulate that distinct binding groups would exist on the surface of the enzyme, and that consequently it would be possible to desensitize the enzyme, that is to have a threonine deaminase that was still active but insensitive to isoleucine. p-Chloromercuribenzoate proved to be very effective for achieving this, indicating that partially different groups are involved in the binding of threonine and isoleucine in spite of the competitive character of the inhibition. So here are the roots that would lead to the concept of the allosteric site [2]. Another concept that also emerges from the work of Changeux and his observation of the deviation of Michaelis–Menten kinetics is the idea of a threshold, very important in metabolic regulation. Thus he said ‘it is worth noting that as a result of the “bimolecular” kinetics of inhibition the effect of metabolite becomes significant above a threshold value. In other words, the intracellular metabolic pool should become effective in the feedback system only when the concentration rises above a critical level’.

Another early case of feedback inhibition was the control of pyrimidine biosynthesis in E. coli by cytosine derivatives: these inhibit formation in vivo of the pyrimidine intermediate ureidosuccinic acid [25], now known as carbamoyl aspartate. Experiments in vitro with crude extracts showed that cytidine, and especially cytidine-5-phosphate, acted as competitive inhibitors with respect to aspartate for the formation of ureidosuccinic acid, the first reaction unique to pyrimidine biosynthesis [25]. Thus a nucleotide end product was able to compete with a structurally very different substrate, an aminoacid. As these experiments, like Umbarger’s, were done in crude extracts one could argue that the inhibition by cytosine derivatives could be indirect: for example, these derivatives could have been transformed in the extract to the real inhibitor. Afterwards aspartate transcarbamoylase, the enzyme responsible for forming carbamoyl aspartate, was purified and the experiments repeated with a highly purified enzyme [9]. Gerhart and Pardee confirmed that CTP inhibits competitively with respect to aspartate [9]. Furthermore, CTP appeared to bind to a second site different from the active site, which they called the feedback site, as the enzyme could be desensitized without losing catalytic activity [9]. This led them to postulate that ‘the bound end product may be an allosteric inhibitor by deforming the enzyme so that the latter has a low affinity for the substrate’. This beautiful classic paper, which had great influence on my own research (see below), gives a very good illustration of what Monod, Changeux and Jacob would call an allosteric site [2]; it also describes an activator, ATP. However, something that I find very puzzling is that although the deviation from hyperbolic behaviour leaps out at the modern reader’s eye, with significant and very noticeable cooperativity with respect to aspartate in Figs. 2,3 and 5 of that paper, they downplayed this observation: in relation to their Fig. 2 they only said ‘Despite the complex kinetics…’, adding in a footnote that ‘Further kinetic studies and possible explanations for their anomalous appearance may be found in the thesis of J.C. Gerhart, University of California, 1962’. In relation to the desensitized enzyme (their Fig. 3) they just said that ‘the dependence of velocity on aspartate concentration followed a curve unlike the unusual sigmoidal dependence of the native enzyme’. This lack of emphasis on the cooperativity may perhaps be because they did not have any explanation for it, whereas they did have a plausible mechanism for explaining the feedback inhibition. As late as 1962, therefore, the observation of sigmoidal dependence was seen with some suspicion, as it went against the ideas established in the article of Michaelis and Menten, despite the fact that the cooperativity of oxygen binding to haemoglobin had been known since 1910 [10,11]. It is a great pity that they did not pay more attention to the sigmoidicity because their article also illustrates very clearly the idea that effectors modify the degree of cooperativity with respect to the substrate, inhibitors by increasing it and activators by decreasing it: CTP (inhibitor) increased cooperativity with respect to aspartate (their Fig. 2) whereas ATP (activator) decreased it (their Fig. 5).

Once the first cases of deviation from the hyperbolic behaviour were confirmed (sigmoidal dependence of velocity as a function of substrate concentration), others were reported, and the 1960s and 1970s produced several examples. Enzyme cooperativity was thus discovered simultaneously with feedback inhibition, and the driving force was the desire to understand feedback mechanisms, which appeared to have a clear physiological importance. It was therefore not by chance that both properties were discovered at
the same time, as they are two faces of the same coin, and both required molecular explanations. It is quite probable that what evolution selected was feedback inhibition of enzymes involved in pathways controlled by demand, with substrate cooperativity being a side effect, as suggested by Hofmeyr and Cornish-Bowden [26]. In fact, substrate sigmoidicity is often small, but it is increased and can even be induced by an allosteric inhibitor [27], whereas feedback inhibition (i.e. allosteric inhibition) does tend to be cooperative, probably to allow the possibility of a threshold in the response to demand.

4. Models to explain cooperativity and allostery

Models were then derived to explain the observed sigmoidicity and the effects of inhibitors that were structurally unlike the substrates, i.e. allosteric. As new ideas do not come out of the blue, but arise in a definite context, it is not surprising that the first models were based on binding: that is, the cooperative binding of substrates, inhibitors or activators. The importance of haemoglobin in the development of these ideas is great: not for nothing is it called an ‘honorary enzyme’. At the time when models were developed little was known about the quaternary structure of enzymes with cooperativity. One of the few X-ray structures available was that of haemoglobin [28]. Structures of allosteric enzymes such as aspartate carbamoyltransferase came much later [29].

Two principal models appeared in the mid-1960s to explain cooperativity and allostery: the allosteric model, also called the symmetry model or the concerted model, proposed by Monod et al. [30], and the sequential model proposed by Koshland, Némethy and Filmer a year later [31]. Both attach a functional importance to multiple conformations, an idea that originated with Koshland’s induced fit hypothesis [32].

A central feature of the allosteric model is conformational regulation. Thus, an equilibrium exists between two conformational states, usually known as the R (‘relaxed’) and T (‘tense’) states, in the absence of any ligand. Different ligands bind preferentially to one of the two states, and thus perturb the equilibrium between them. Furthermore, all of the subunits in an oligomeric protein can change between the R and T states in a concerted manner, so symmetry is maintained.

In contrast, the sequential model did not require conformational symmetry, as the conformational change of each subunit occurs only when ligand is bound. Thus in this case it is the ligand that induces the conformational change. In the absence of ligand there is a different conformation.

As both models explained the observed kinetic cooperativity on the basis of cooperativity of substrate binding, this implied that the enzyme should be oligomeric, or there must be more than one active site per enzyme molecule. However, as there is usually only one active site per monomer, with rare exceptions such as vertebrate hexokinase B or II [33,34], this meant that monomeric enzymes should not be cooperative: they could show allostery, but not cooperativity.

This limitation encouraged some people in the 1960s, such as Rabin [35], to seek models that could explain kinetic cooperativity without needing cooperative binding: this could be based, for example, on enzyme isomerization during the course of the reaction [35]. This was initially a sort of intellectual challenge as no monomeric enzymes showing deviations from hyperbolic behaviour had been described experimentally, at least, none with natural substrates.

5. Glucokinase: a monomeric enzyme with cooperativity, but no feedback inhibition

In 1975 this view changed after Hermann Niemeyer and colleagues in Chile described sigmoidal kinetics for ‘glucokinase’ (i.e. hexokinase D or hexokinase IV) [36], the enzyme responsible for glucose phosphorylation in hepatocytes, whose activity level in liver depends on diet and hormonal regulation [37]. This glucose uptake is an essential physiological process, crucial for glucose homeostasis. It has acquired greatly increased interest and importance with the rise in recent decades of diabetes type 2 as a major problem of human health [38,39] (the name ‘glucokinase’ is almost universal in the literature, so I shall use it here, but it gives a misleading impression of the specificity [37,40]).

The first kinetic studies on glucokinase did not report this sigmoidal behaviour because of the difficulties referred to above: previous studies from various groups had too few observations, and so although the possibility of deviations from hyperbolic behaviour was in the air, it was far from being clear [41,42]. When I joined Niemeyer’s group and started to work with this enzyme, my night-mare was that the apparent positive cooperativity that I was observing could be an artefact, especially because the degree of cooperativity was rather small, with a Hill coefficient of about 1.5, and the enzyme rather unstable. So the paper of 1975 [36] described several experiments that were intended to convince ourselves that the apparent cooperativity was real, as the enzyme appeared to be a monomer (the form of enzyme with a higher molecular mass mentioned in that paper may have been a complex of glucokinase with the regulatory protein, discovered several years afterwards [43]). The fact that the liver glucokinase obtained from different mammalian species, and also from reptiles and amphibians, had a similar degree of cooperativity, despite significant variations in the half-saturation values, tended to support the idea that the cooperativity was not an artefact, and could have a physiological value. It was a relief, therefore, that a year later a group in England described a similar degree of cooperativity [44], this time with the pure enzyme, and showed that it was a monomer [45]. Of course, the crucial question from the point of view of a plausible cooperative mechanism was the quaternary structure in the assay conditions: it also proved to be monomeric [46].

Glucokinase was thus the first monomeric enzyme with sigmoidal kinetics. Influenced by the work of Gerhart and Pardee [9] I tried to ‘desensitize’ it, thinking that it could have an allosteric site capable of binding glucose. All the efforts were in vain, as there is no such site: as long as the enzyme retained activity it continued to show sigmoidicity. In the absence of interacting sites positive cooperativity requires a kinetic mechanism in which binding of substrates is not at equilibrium in the steady state; it can only be a kinetic property that cannot occur at thermodynamic equilibrium. Two models were postulated at the time for explaining the cooperativity: the mnemonical model [47,48] and the slow-transition model [49,50], as we have reviewed [51]. Briefly, in both models the enzyme exists in two distinct forms, E and E’ that are interconverted relatively slowly, with the more stable form E’ predominating in the absence of glucose. In the mnemonical model the less stable form E is the one released at the end of the catalytic cycle. The slow-transition model is somewhat more complicated, as both conformations can accomplish a catalytic cycle, but with different kinetic parameters. In both models, as glucose binds in two different steps, the full rate equation contains terms in the squared concentration of glucose, thereby allowing deviations from Michaelis–Menten kinetics.

Besides being monomeric and showing cooperativity, glucokinase is unusual in that it does not show feedback inhibition. As it is an enzyme controlled by supply [52,53] and not by demand, evolution appears not to have selected a feedback mechanism, whose function in demand-driven pathways is to displace the control from the first enzyme to the demand for the end-product [54], and is crucial for maintaining homeostasis of intermediates. This lack of feedback appears to have dramatic consequences for human health, because drugs that activate it, which were seen as potential medicines for diabetes, as they are effective for decreasing
glycaemia, at least temporarily, produce undesirable effects in the long term [55], probably because there is no feedback that controls the system.

6. Forty years in the wilderness

The large gap in time visible in Fig. 1 is at first sight surprising: why did it take so long for regulatory mechanisms to be discovered? This gap, however, corresponds to the period in which metabolic pathways were being elucidated, intermediates being identified, methods for purifying and stabilizing proteins being developed, as well as methods for following the progress curves of the reactions with high sensitivity. This period of about 40 years after Michaelis and Menten – 40 years in the wilderness – illustrates very well that progress in science has two requirements, not only a guiding vision, but also an adequate technology.

As Woese [56] commented in relation to the biology of the 21st century, not only a guiding vision, but also an adequate technology – illustrates very well that progress in science has two requirements, not only a guiding vision there is no pathway, there is no way to advance the pathway of progress is blocked, and without an adequate guiding vision there is no pathway, there is no way to advance ahead’.

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