Outline Studies in Biology

Enzyme Kinetics

The Steady-State Approach

Second Edition

Paul C. Engel

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Editors' Foreword

The student of biological science in his final years as an undergraduate and his first years as a graduate is expected to gain some familiarity with current research at the frontiers of his discipline. New research work is published in a perplexing diversity of publications and is inevitably concerned with the minutiae of the subject. The sheer number of research journals and papers also causes confusion and difficulties of assimilation. Review articles usually presuppose a background knowledge of the field and are inevitably rather restricted in scope. There is thus a need for short but authoritative introductions to those areas of modern biological research which are either not dealt with in standard introductory textbooks or are not dealt with in sufficient detail to enable the student to go on from them to read scholarly reviews with profit. This series of books is designed to satisfy this need. The authors have been asked to produce a brief outline of their subject assuming that their readers will have read and remembered much of a standard introductory textbook of biology. This outline then sets out to provide by building on this basis, the conceptual framework within which modern research work is progressing and aims to give the reader an indication of the problems, both conceptual and practical, which must be overcome if progress is to be maintained. We hope that students will go on to read the more detailed reviews and articles to which reference is made with a greater insight and understanding of how they fit into the overall scheme of modern research effort and may thus be helped to choose where to make their own contribution to this effort. These books are guidebooks, not textbooks. Modern research pays scant regard for the academic divisions into which biological teaching and introductory textbooks must, to a certain extent, be divided. We have thus concentrated in this series on providing guides to those areas which fall between, or which involve, several different academic disciplines. It is here that the gap between the textbook and the research paper is widest and where the need for guidance is greatest. In so doing we hope to have extended or supplemented but not supplanted main texts, and to have given students assistance in seeing how modern biological research is progressing, while at the same time providing a foundation for self help in the achievement of successful examination results.

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Enzyme Kinetics

The Steady-state Approach

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Contents

Dedication

This book is affectionately dedicated to my father, still active at the laboratory bench at eighty-seven and a continuing source of inspiration.

Acknowledgements

I am deeply indebted to Dr Keith Dalziel FRS and Professor Vincent Massey FRS, who, between them, taught me my trade; to the late Dr William Ferdinand and Professor Walter Bartley, whose thoughtful suggestions greatly improved the manuscript; to Liz, Thomas and Anna for everything else.

Note to the reader

Although rapid-reaction techniques are playing an increasingly important part in the study of enzyme mechanisms, rapid-reaction kinetics has not been included in this book. The topic has been covered admirably elsewhere. It seemed best in the limited space available in the present volume to concentrate on the steady-state approach, which is of interest from the standpoints both of mechanism and metabolism. Whereas rapid-reaction kinetics remains the preserve of the specialist, every biochemist needs a basic understanding of steady-state kinetics.

1 Introduction

1.1 The book

This book makes no claim to be a comprehensive treatise, and it is not intended to compete with books in that category. It is aimed at 'non-believers', that is to say the 90% or so of biochemistry students, and indeed of practising biochemists, who place enzyme kinetics in the same category as Latin and cold showers, character-building perhaps, but otherwise to be forgotten as quickly as possible. Kinetics often appears to bristle with forbidding symbols, and many potential converts are discouraged before they even start. I believe, however, that much of enzyme kinetics can be conveyed and understood qualitatively. Algebra is needed only subsequently to provide rigorous confirmation. Approached in this way the subject is easy to grasp, and the symbols take on real meaning.

I hope also that the book will help to persuade sceptics that enzyme kinetics is of importance to most biochemists and not just to its devotees.

1.2 What are enzymes?

Biochemistry was born in the resolution of the famous conflict between Liebig and Pasteur as to the nature of fermentation. To the chemist the process was evidently chemical. To the biologist it was demonstrably biological. Both, of course, were right, although they could not see that their viewpoints were reconcilable. It was the Buchner brothers who showed that yeast, a living organism, yielded a non-living cell-free extract capable of fermenting sugars. A long line of subsequent investigators showed that this was due to the presence in the extract of many powerful catalysts, each one highly specific for one of the steps in the breakdown of glucose. Similar catalysts, or enzymes, are found in all living things.

The chemical nature of enzymes remained in dispute for a long time. Willstätter, working with peroxidase, had chosen an enzyme of such high catalytic efficiency that he believed that his active preparations were protein-free. By contrast, Sumner's crystalline urease was of such relatively modest activity that his critics attributed the catalysis to a highly active trace contaminant rather than the purified protein itself. Improvements in fractionation procedures have since allowed the purification of many hundreds of enzymes from diverse sources, leading to the realization that all enzymes are proteins.

Proteins represent both the expression of the genetic blueprint and the means of that expression. Protein synthesis requires enzymes. This apparent paradox raises fascinating questions regarding the origins of life. It is the specificity of proteins, that is to say their capacity for selective

molecular recognition, that gives to life its directionality and order. Enzymes form a rather special category, because they not only recognize substances but also transform them chemically. This enables living things actively to mould and use their environment rather than merely existing as passive accretions of preformed substances. The network of enzyme-catalysed reactions that we call metabolism is subject, moreover, to delicate control minimizing waste, and the properties of key regulatory enzymes form an important part of the control apparatus.

The study of structure has led us first to a knowledge of the 'primary' linear sequence of amino acid residues that make up a protein chain and latterly, through X-ray crystallography, to a clear picture, for some proteins at least, of the three-dimensional arrangement of such linear chains. This, however, is still only a static picture, and leads us, therefore, to the next question.

1.3 What is enzyme kinetics?

Enzyme kinetics is the study of enzymes in action. The extremely high rate of enzyme-catalysed reactions greatly facilitates this study. Consider, for example, the haem-containing proteins, haemoglobin and catalase. Haemoglobin binds oxygen. It may bind and release many oxygen molecules in the course of a minute but they remain oxygen molecules, and at any instant, only one is associated with each haem centre. Catalase, being an enzyme, has a cumulative effect. Again no more than one H_2O_2 molecule will be bound per haem, but while it is bound it may react, and one therefore observes a rapid evolution of oxygen – about a million molecules per minute per enzyme molecule.

The task of enzyme kinetics is the systematic analysis of such processes, involving a study of the dependence of reaction rates on substrate concentration, pH, temperature, ionic strength and other relevant variables.

Kinetic information is obviously of descriptive value. It permits the classification and distinction of individual enzymes. Hexokinase and glucokinase, for instance, both catalyse the phosphorylation of glucose by ATP. No doubt we shall be able before long to specify the difference between these two proteins precisely in terms of their detailed structures, but the metabolic biochemists' longstanding interest in hexokinase and glucokinase stems from a recognition of their *functional* (i.e. kinetic) differences.

To quote another example, enzyme evolution may be simulated by applying selective nutritional pressures [1,2] to bacterial cultures. Mutant enzymes are quickly identified by their distinctive kinetic properties.

The intelligent use of enzymes as tools, for instance for specific metabolite assays in research, clinical diagnosis or food analysis [3], requires sound knowledge of their kinetic behaviour. Purified enzymes are also being applied increasingly in biochemical engineering [4]. Enzyme 'reactors', in which enzymes are covalently attached to solid supports can

be used for syntheses without side reactions. The design of such reactors is evidently a kinetic problem.

It is also sometimes useful to subvert normal enzyme function. This can often be done selectively through the use of 'active-site-directed' irreversible inhibitors [5a]. Work on such inhibitors began in a military context with nerve gases but has since been valuable in the attempt to build a rational pharmacology. It is still probably true that most chemotherapeutic agents have been stumbled upon by chance, but many drugs are in fact enzyme inhibitors. Once their site of action is identified, they may be improved more systematically, as with the anti-depressant monoamine oxidase inhibitors [5b]. Direct studies of the effectiveness of an inhibitor depend, of course, on kinetic measurements (see Chapter 3).

Beyond these purely practical considerations there are the insights that kinetics provides into an enzyme's mechanism of action (to be dealt with in subsequent chapters) and its place in the overall metabolic pattern. In establishing the quantitative significance of a metabolic pathway it is not sufficient to show that the necessary enzymes are present. Each enzyme must also be capable of handling the observed throughput of metabolites. For example Tempest and his colleagues [6] challenged the accepted view that glutamate dehydrogenase is responsible for ammonia assimilation in bacteria by showing that in several species the effective activity of this enzyme is quite inadequate for the task. This led to the discovery of a new pathway and a new enzyme, glutamate synthase, which appears also to be important in plants.

As an example of the tailoring of enzymes to a metabolic role one may consider the aldolases of muscle and liver [7] which both catalyse reactions (1a) and (1b).

$$\text{fructose 1,6-diphosphate} \rightleftharpoons \text{glyceraldehyde-3-phosphate} + \text{dihydroxyacetone phosphate} \qquad (1a)$$

$$\text{fructose 1-phosphate} \rightleftharpoons \text{glyceraldehyde} + \text{dihydroxyacetone phosphate} \qquad (1b)$$

Muscle aldolase is primarily concerned with the catabolism of glucose via the glycolytic pathway, which requires the splitting of fructose 1,6-diphosphate (reaction (1a) in the forward direction). Liver, however, has to make glucose, and also deals with dietary fructose. This it does by making fructose 1-phosphate, splitting it (reaction (1b)), phosphorylating the resultant glyceraldehyde, and recombining the two triose phosphates to give fructose 1,6-diphosphate (reaction (1a) in reverse). A kinetic comparison of liver and muscle aldolases shows that the liver form is much better adapted for the back reaction, and that in the forward direction it is much better than the muscle enzyme at catalysing reaction (1b).

It must also be obvious that the overall regulation and integration of metabolism can only be understood through a study of the control characteristics of the separate components. Since enzymes are the working parts of the metabolic machine, their properties must define the perform-

ance of that machine. We have accumulated a large body of information about the kinetics of individual enzymes, but are only gradually feeling our way towards adequate descriptions of integrated systems involving many enzymes.

1.4 To purify or not to purify?

It is difficult to be certain that a given chemical conversion is due to the action of a single enzyme unless the enzyme is pure. This is especially true where a reaction forms part of an extended metabolic sequence. It was originally thought, for instance, that the beta-oxidation of fatty acids might involve conversion of the saturated acid to a beta-hydroxy acid in a single step. Only by separating the various enzymes was it ultimately established that the conversion involves a dehydrogenation and a hydration catalysed by different enzymes, and that these enzymes work not on the free fatty acids but on their thioesters.

The substrates and products of an enzyme under study are usually also substrates for other enzymes, and this may seriously hinder measurements of activity in crude cell extracts. The enzymologist aims, therefore, at a 'pure' preparation as judged by various criteria – ultracentrifugation, electrophoresis, chromatography, constant specific activity, immunological assay, absence of known contaminant activities etc.

This approach is always open to the criticism that it is 'non-physiological'; the enzyme may have been irreversibly altered during purification, which sometimes involves harsh treatment with heat, organic solvents, acid or other drastic agents. *In vitro* conditions, moreover, provide, at best, a poor approximation to the *in vivo* environment. Membrane-bound enzymes such as succinate dehydrogenase present especial difficulty: the problems of solubilization and purification are such that different laboratories frequently obtain *in vitro* preparations with different properties.

This is an inherent and inescapable dilemma in biochemistry, akin to Heisenberg's Uncertainty Principle. By the very act of investigating, we disturb and perhaps distort that which we wish to investigate. We can be absolutely certain however, that if we do not investigate, we shall find out nothing. One always hopes, therefore, that the results of careful *in vitro* work may lead to a first approximation of events *in vivo*, thereby indicating the relevant definitive experiments to be done on intact systems. The striking progress in biochemistry over the past 40 years is a vindication of that hope.

It is worth adding that apparent discrepancies between *in vitro* and *in vivo* work often reflect the imperfection of our grasp of metabolism rather than the disruptive nature of our purification methods. Mammalian glutamate dehydrogenase provides an example. The purified enzyme works almost equally well with NAD(H) or NADP(H), an unusual property among nicotinamide nucleotide-linked enzymes. It was claimed, nevertheless, for some years that in the mitochondrion this enzyme uses only NADPH and not NADH. This startling discrepancy turns out to be

due to differences in the availability of the two coenzymes in the mitochondrion under the conditions of experimentation, rather than to alteration of the properties of the purified enzyme [8].

1.5 Methods of measuring enzyme activity

The next problem is that of how best to measure the activity of the enzyme to be studied. One may wish ultimately to study the activity of the purified enzyme over a wide range of conditions, but in order to purify it in the first place one needs a single set of conditions for convenient, sensitive, specific and reproducible monitoring of activity. A suitable buffer must be chosen, preferably one in which the enzyme is reasonably stable. One must decide in which direction to measure the reaction, if it is reversible. It may be necessary, especially in the early stages of purification, to include controls to check for interfering enzyme activities. The most important element in the design of an assay, however, is the selection of a suitable physical or chemical technique for following the appearance or disappearance of one of the reactants. An assay that allows continuous recording is preferable to one that requires repeated sampling of the reaction mixture. For this reason, assays that depend on optical properties (light absorption, fluorescence or rotation) are the most widely used. If the normal reactants have no convenient optical properties, it may be possible to substitute an artificial chromophoric substrate or one which yields a chromophoric product. This device is often used in the study of hydrolases. For instance, in the case of trypsin, an endopeptidase, neither the natural substrates nor the natural products lend themselves to simple, reliable measurements, but the enzyme also works well with low molecular weight 'model' substrates such as the nitrophenol ester of N-α-carbobenzoxylysine, which gives a large optical density change upon hydrolysis.

An alternative is to devise a 'coupled assay' in which one or more auxiliary enzymes are present in order to form an ultimate product that does have useful optical properties. Thus glutamate-oxaloacetate transaminase, which catalyses reaction (2), is conveniently assayed by including in the reaction mixture malate dehydrogenase, which catalyses reaction (3), and the missing reactant, NADH. As fast as the oxaloacetate is produced by reaction (2), it is removed by reaction (3), and one can therefore monitor the rate by following the decrease in the strong absorption or fluorescence of NADH. It is important in such an assay to ensure that the coupling reaction never becomes rate limiting [9].

$$\begin{matrix}\text{L-aspartate} & & \text{oxaloacetate} \\ +\text{2-oxoglutarate} & \rightleftharpoons & +\text{L-glutamate}\end{matrix} \qquad (2)$$

$$\begin{matrix}\text{oxaloacetate} & & \text{L-malate} \\ +\text{NADH} + \text{H}^+ & \rightleftharpoons & +\text{NAD}^+\end{matrix} \qquad (3)$$

Whatever the method of assay, the time course of reaction will be curved; as the concentrations of substrates decrease, and products

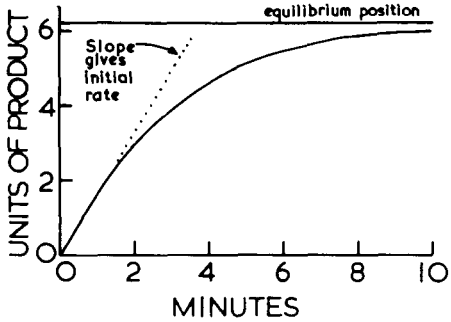

Fig. 1.1 Typical reaction time-course

accumulate, the net forward rate decreases, approaching zero at infinite time (Fig. 1.1). The rate of reaction at any given time is the slope of the tangent to this curve. Most assays are based on an estimate of the 'initial rate', the slope of the tangent at time zero. Tangents to an experimentally obtained curve, often overlaid with instrumental 'noise', are not easy to draw accurately, however, and the first few seconds of reaction are usually lost because of the time taken for mixing. Conditions are therefore chosen to minimize the curvature. This may often be achieved simply by decreasing the concentration of enzyme and increasing the sensitivity setting on the detecting instrument (Fig. 1.2). Occasionally the curvature is due to enzyme instability rather than substrate depletion, in which case the stratagem just mentioned is likely to make matters worse. One must then either add a suitable stabilizing agent or change the buffer completely.

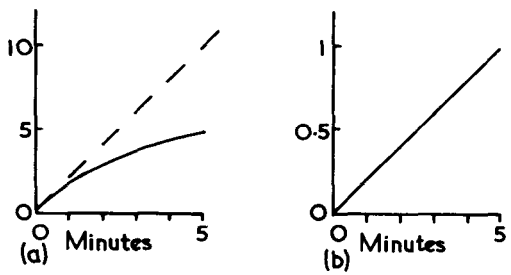

Fig. 1.2 Influence of instrumental sensitivity and enzyme concentration on the determination of initial rates. The trace in (a) is obviously too curved for accurate estimation of the initial rate. In (b) the instrumental sensitivity has been increased 10-fold so that the early linear part of the time course occupies the full vertical expanse, and only 1/10 as much enzyme is added, so that the linear portion is also stretched out along the time axis to give optimal display with a slope of about 45°.

To allow comparison between experiments, crude rates must be converted to 'specific' rates. For an impure preparation this involves dividing by the protein concentration or some other relevant indicator of the amount of material added; for a pure enzyme, if the molecular weight is known, the molar concentration of enzyme may be used instead. The conversion in either case implies the important assumption that the rate is strictly proportional to enzyme concentration. This has to be tested. If the assumption turns out to be unjustified, this may mean that the enzyme preparation contains an inhibitor or activator, or possibly that it dissociates into subunits upon dilution. Dixon and Webb ([39] p. 49–55) discuss a number of cases of apparent non-proportionality.

In the past, enzyme activity has often been expressed in arbitrary and mysterious units. The Enzyme Commission, seeking standardization, introduced the international 'Unit' of enzyme activity, that amount of enzyme which converts *one μmole of substrate per minute* under standard conditions of assay. This is now widely used. Specific activities are expressed on this basis in Units per mg. protein. More recently [10] the Commission has suggested that the old 'Unit' be replaced by the 'katal', the amount of enzyme that converts *one mole of substrate per second* (1 Unit = 16.67 nkat). Specific activities should now be given in katals per kilogram.

Finally it must be emphasized that kinetic properties of different enzyme preparations can only be validly compared if they are determined under identical conditions – unless, of course, the variation with conditions is the object of the comparison. It is sometimes claimed that two preparations contain kinetically distinct 'isoenzymes' (different proteins catalysing the same reaction), when in fact they have been studied at different temperatures, pH values etc. Such comparisons are meaningless.

For the rest of this book we shall be concerned with the variation of enzyme-catalysed reaction rates, not as a matter of technical detail, but as a subject in its own right. We shall also assume from now on that the enzymes under discussion have been satisfactorily purified and are free of interfering activities.

2 One-substrate kinetics

2.1 Saturation kinetics

If we consider a hypothetical reaction S ⟶ P obeying first-order kinetics, the rate is given by:–

$$-\frac{d[S]}{dt} = \frac{d[P]}{dt} = k[S]$$

i.e. the chance of a molecule of P being formed at any given instant is directly proportional to the concentration of S. Similarly, for a second-order reaction S + T ⟶ P + Q, the rate would be proportional to the concentration of each of the reactants S and T:–

$$-\frac{d[S]}{dt} = -\frac{d[T]}{dt} = k[S][T].$$

In either case a plot of *initial* rate against varied concentration of S would be a straight line, of slope k in the first case and $k[T]$ in the second (Fig. 2.1). (Chemical kineticists tend, in fact, to use the full time course of reaction rather than the initial rate.)

If these reactions were enzyme-catalysed, the dependence of the initial rate on substrate concentration would be different. The rate would probably be proportional to [S] for low values of [S], but with higher values of [S] the rate would asymptotically approach a maximum. This is called saturation. The plot of initial rate against [S] is hyperbolic (Fig. 2.2).

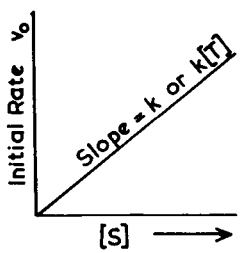

Fig. 2.1 Dependence of initial rate on reactant concentration for a simple first- or second-order chemical reaction.

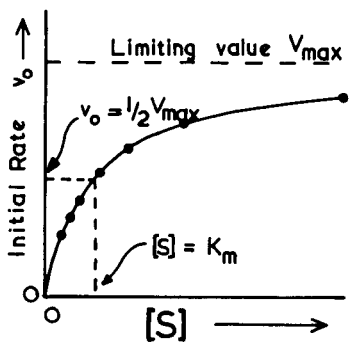

Fig. 2.2 Dependence of initial rate on substrate concentration for a typical enzyme-catalysed reaction.

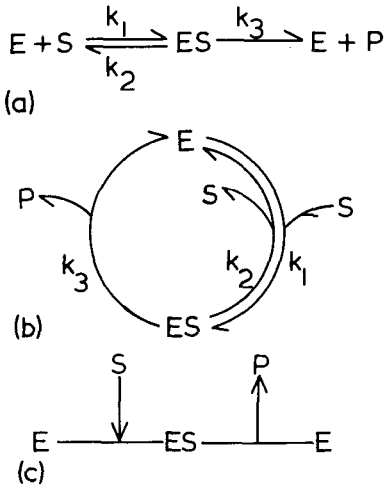

Fig. 2.3 Three different schematic representations of a one-substrate mechanism.

The major credit for realizing the the significance of this pattern goes to A. J. Brown who proposed in 1902 that invertase forms a stoicheiometric complex with its substrate [11]. It is only this complex, he suggested, that breaks down to the products, simultaneously regenerating free enzyme (Fig. 2.3). The nearest analogy to this pattern in classical chemistry is that of surface catalysis.

Brown's kinetic inference of the existence of a one-to-one enzyme-substrate complex was made long before the chemical nature of enzymes was known, 40 years before the spectrophotometric detection of such complexes, and 60 years before their 'visualization' through X-ray crystallography. In the intervening period this one concept has provided the foundation for our understanding of biochemical specificity. This despite the fact that, like all inference from kinetics, it was tentative, consistent with the facts rather than logically inescapable.

The reaction scheme in Fig. 2.3a may also be depicted as in Fig. 2.3b to emphasize the cyclic nature of catalysis, or in the notation developed by Cleland (Fig. 2.3c), which highlights the enzyme-containing complexes rather than the chemical reactants.

Now in this mechanism the initial rate of formation of P is proportional to the concentration of ES, the enzyme-substrate complex. At low concentrations of S the small amount of ES formed is proportional to [S], and so the overall rate is also proportional to [S]. As [S] is raised, however, the balance between E and ES shifts until virtually all the enzyme is present as ES, i.e. the enzyme is 'saturated' with S. In this state the enzyme is working flat out. As fast as ES breaks down to release E and P, or E and S, more S jumps on to re-form the enzyme-substrate complex. Since the enzyme is present at a fixed concentration, e, the rate cannot exceed $k_3 e$ (Fig. 2.2).

The whole of enzyme kinetics is little more than an extension of the simple idea introduced above, that any substance that can be bound to an enzyme distributes the enzyme between two forms or states, free and complexed. The number of different states increases if one introduces inhibitors, or more than one substrate, but the basic idea remains unaltered.

2.2 The Michaelis-Menten equation

A simple mathematical treatment of the one-substrate reaction scheme was given by Michaelis and Menten [12]. It rests on the following assumptions:–

(i) the concentration of enzyme is very small compared to [S], so that formation of ES does not significantly deplete [S]. This condition is met in most catalytic experiments. If, for example, the concentration of substrate is 10^{-3} M and that of the enzyme is 10^{-9} M, then clearly, even if all the enzyme is present as ES, it makes an imperceptible difference to [S].

(ii) the concentration of P is effectively zero. This is the 'initial-rate' assumption, and implies not only that P is absent at the outset, but also that the amount of P formed in the time required for a rate measurement is too small to give rise to a significant reverse reaction;

(iii) although the product-releasing step is fast, it is nevertheless so much slower than the alternative reaction in which S is released from ES, that E and ES may be considered to be effectively at equilibrium. This third, somewhat arbitrary assumption distinguishes the Michaelis-Menten treatment from that later adopted by Briggs and Haldane.

Setting up the equilibrium expression we have:–

$$k_1 [E] [S] = k_2 [ES]$$

$$\therefore [E] = [ES] \frac{k_2}{k_1 [S]} \tag{2.1}$$

But we also have another equation relating the two unknown variables, [E] and [ES]. It is the so-called 'enzyme conservation equation', which states in essence that we know how much enzyme was put in, although we may not know precisely where it has gone. Thus:

$$[E] + [ES] = e \tag{2.2}$$

where e is the total enzyme concentration. We can now substitute for [E] from equation (2.1):

$$\therefore e = [ES] \frac{k_2}{k_1 [S]} + [ES] = [ES] \left(1 + \frac{k_2}{k_1 [S]}\right). \tag{2.3}$$

Now the overall rate, v, is equal to $k_3 [ES]$, since the reverse rate may be ignored.