PREFACE

The increasingly large number of papers on solid-supported enzymes which have recently appeared is a reflection on the many areas of enzymology to which they have been applied. Two particular types of application with which this thesis is concerned are (a) obtaining information on the nature of enzyme-membrane interactions, particularly through a study of model systems; and (b) the use of enzymes attached to tubes for automated analysis of biologically important molecules. In both of these areas, it is important to know how an enzyme's properties are likely to be altered upon immobilization. In case (a) this would help to elucidate the nature of the interaction between membranes and enzymes *in vivo*. In case (b) a study of the kinetics would be of use to ensure improved efficiency in the analytical procedure.

Two theoretical treatments of these systems were developed previously in our laboratory. It remained for their validity to be tested experimentally, and this was the prime object of the work reported in this thesis. Case (b) was of special interest, since the enzyme, L-asparaginase, is currently used in the treatment of certain tumour diseases.

In addition to the above, while many preparations of enzymes embedded in gels have been reported, no thorough study of the reason for the loss of activity of the supported-enzyme preparations has been carried out. Such a study seemed

important for a full understanding of the properties of the immobilized enzyme, and also for the ability to select suitable supports for other solid-supported enzyme preparations.

Some of the work described in this thesis has been submitted for publication, as follows:

(1) "Kinetic Studies on Solid-Supported β-Galactosidase," P.S. Bunting and K.J. Laidler, Biochemistry, 24, 4477 (1972).

(2) "Flow kinetics of L-asparaginase attached to nylon tubing," P.S. Bunting and K.J. Laidler, submitted to Biochemistry.

(3) "Some properties of α-chymotrypsin and β-galactosidase in polyacrylamide gels," P.S. Bunting and K.J. Laidler, submitted to Canadian Journal of Biochemistry.

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ABSTRACT

Considerable interest has arisen in recent years in the field of solid-supported enzymes, for reasons which are discussed in Chapter 1. It is important to establish how the enzyme's ability to catalyze a reaction is affected by immobilization. As is discussed in the thesis, there are four main ways in which the kinetics may be altered, namely through (1) conformational, (2) environmental, (3) partitioning and (4) diffusional effects. This thesis concerns a study of the four effects on the kinetics of enzymes immobilized in membrane- and flow-type systems. The reasons for selecting these two examples are discussed in the first chapter.

Chapter 2 describes the details relating to the experimental aspects of studies with β-galactosidase in polyacrylamide gel slices. This experimental system is used to test a theoretical treatment relating to the kinetics of enzymes in membranes. Details of the results in relation to the theory are presented in Chapter 3. The main findings are: (1) diffusion is important in the kinetics at high enzyme concentrations and at large membrane thicknesses; (2) the dependence of the rate of reaction on the concentration of enzyme in the solid support is the same as that in free solution (i.e. linear) when diffusion is not important in the system; a square root relationship applies, however,

when diffusion is rate-limiting; (3) there is no effect of diffusion on the maximum velocity of the immobilized enzyme, V'_{max}, but the apparent Michaelis constant (K'_m) is increased by an order of magnitude; (4) the effect of partitioning on the distribution of substrate and product is small; (5) the conformational and environmental effects on the immobilized enzyme are to lower both V'_{max} and K'_m by a factor of two in comparison with the values in free solution and (6) quantitative agreement between theory and experiment is found as well as qualitative agreement.

Chapter 2 also contains details of experiments with both β-galactosidase and α-chymotrypsin trapped in polyacrylamide gels. Two different methods of initiating the free-radical polymerization process to form the gels are compared, namely photolysis and radiolysis. These experiments centre on the reasons for the observed decrease in enzymic activity which occurs when the enzymes are immobilized in the gels. It is found that a concentration of acrylamide of 15% (wt/vol) provides optimal activity for the supported enzyme preparations. The drop in activity at higher concentrations of acrylamide is ascribed to an inactivating effect by acrylamide and its polymer on the enzyme. The initial rise in activity at low acrylamide concentrations is the result of protection of the enzyme from the free radicals present during the polymerization process, by acrylamide and its polymer.

Further studies described in Chapter 2 relate to the measurements of diffusion coefficients of small molecules in the polyacrylamide gels, and to the stabilities of the supported enzyme preparations. It is found that the diffusion coefficients are very similar to those found in free solution. The solid-supported enzymes are somewhat less stable to thermal denaturation than the enzyme in free solution.

Studies on the kinetics of the enzyme, L-asparaginase, attached to the inside surface of a nylon tube, are reported in Chapter 4. The results are interpreted in relation to a theoretical treatment, and, again, both qualitative and quantitative agreement between theory and experiment is found. The same general result is obtained as in the membrane system, namely that diffusion has no effect on the value of V'_{max}, but the apparent Michaelis constant is raised by up to three orders of magnitude. The effect of conformational and environmental factors on the kinetic parameters is found to be small, the Michaelis constant being about 50% higher for the immobilized enzyme (free from diffusion effects) compared with the enzyme in free solution.

In Chapter 5 are presented the main conclusions relating to the experimental tests of the theoretical treatments, for the two solid-supported enzyme systems studied. The results are discussed in relation to those reported in the literature, and a number of general principles are suggested as to the manner in which the four factors discussed above may alter the kinetics of the immobilized enzyme.

LIST OF SYMBOLS

α	parameter
A	area
β	parameter in membrane diffusion theory
D	diffusion coefficient
η	utilization factor
E	number of moles of enzyme in a membrane
$[E]_m$	concentration of enzyme within a membrane
F	diffusion factor for enzyme kinetics in membranes
γ	parameter in membrane diffusion theory
k_c'	true catalytic rate constant at high concentration for supported enzyme
$k_c(app)$	observed (apparent) value of k_c
K_m'	true Michaelis constant for supported enzyme
$K_m(app)$	apparent (observed) Michaelis constant
k_L	mass transfer coefficient
L	tube length
ℓ	membrane (slice) thickness
λ	constant in diffusion equation
P	partition coefficient
Pe	product concentration at tube exit
ϕ	dimensionless constants in theory of flow kinetics
ρ	dimensionless constants in theory of flow kinetics
R	tube radius
S	substrate concentration in bulk solution

S'	substrate concentration in the solid support
S_o	initial substrate concentration
τ	time to reach steady-state conditions
V'_m	true maximum velocity for supported enzyme
$V_m(app)$	apparent (observed) maximum velocity
v	rate of reaction
v_F	rate of flow of solution
x	distance through membrane normal to surface

CHAPTER ONE

GENERAL INTRODUCTION

During recent years an increasingly large number of research papers have dealt with the attachment of biologically active molecules to solid supports. Included in these are several reviews on various aspects of the subject (1-8). The interest in these insoluble derivatives stems from the wide variety of different fields in which they may be applied. The types of molecules that have been attached to such insoluble supports include enzymes, antibodies and antigens, and substrates and inhibitors.

This introductory chapter will briefly discuss some of the applications of insoluble biological materials, in order to provide a background to the particular topics which are the main concern of this thesis. Applications are to be found in the areas of isolation and purification of biological compounds, industrial synthesis, detection of biologically important molecules, automated analysis, simulation of biological membranes, and medical therapy, to mention some.

Isolation and Purification

Antigens were among the first biological materials to be attached to solid supports. Supported antigens were used in columns to separate a specific antibody from a sample

containing many antibodies. This could be done because of the specific interaction between antigen and antibody. By the use of this technique of "affinity chromatography", numerous purified samples of both antigens and antibodies have been obtained. Silman and Katchalski have reviewed this earlier work (8).

In a similar way, inhibitors and coenzymes have been attached to solid supports with a view to isolating specific enzymes from a mixture of proteins (such as a crude tissue extract). In this instance, advantage is taken of the specificity of complex formation between enzyme and coenzyme or inhibitor. Numerous examples are discussed in a review by Cuatrecasas (9). Trypsin and chymotrypsin, for instance, have been purified using columns containing solid-supported protein inhibitors specific for these enzymes (10,11). Kasche (12) has been able to separate several forms of chymotrypsin which were produced after denaturation by exposure of the enzyme to gamma radiation, again with the use of a solid-supported inhibitor preparation. DNA polymerase has been purified by attaching a specific DNA to polyacrylamide beads (13). It has even been possible to isolate an active-site peptide from ribonuclease, using a column containing the enzyme on a suitable support (14).

The reverse process has been applied to the purification of inhibitors, by attaching the enzyme to a solid support in a column, as in the cases of the protein inhibitors of trypsin and chymotrypsin (8,10).

Supported enzymes have also been applied to protein sequencing. When a free proteolytic enzyme is used to obtain peptide sequences from the native protein being analyzed, the proteolytic enzyme may remain as a contaminant. If, however, it is used attached to an insoluble support, it is a simple matter to filter off the enzyme after use. Affinity chromatography may also be used afterwards to separate the relevant peptides from unwanted residual protein (15-17).

Detection of Biologically Important Molecules

Many laboratories, hospitals and clinics carry out research which involves the detection of specific biological molecules. In most cases, it is possible to do this using an enzyme which reacts with the molecule in question. Such methods are rapid, simple and specific. Enzymes are, however, expensive. Attaching them to insoluble materials enables them to be filtered off and re-used over prolonged periods of time, thus saving on expense. Enzymes attached to solid supports have been employed in the detection of urea with the use of urease (18,19); glucose with glucose oxidase (20,21); asparagine with asparaginase (22); and amino acids with amino acid oxidases (23), to mention a few.

In many cases, analyses of this nature have been automated, and supported enzymes have been incorporated into automated analysis on several occasions (19,20,22). This is

often conveniently done with the enzyme attached to the inside of a tube. Many automated procedures of analysis involve the use of tubes in a flow system, illustrated schematically in Figure 1. The sample, S, being analysed is allowed to react with the enzyme, E, normally used in free solution. The resultant product is then reacted with reagents R_1 and R_2 to form, for example, a coloured product whose concentration may be determined spectrophotometrically at A. The reactants are all sampled automatically, and pumped through the separate pieces of tubing, allowing them to be mixed in the order required by the particular analytical procedure involved. Waste material is continuously collected in the discard vessel, D. Numerous samples may be analysed sequentially in this way.

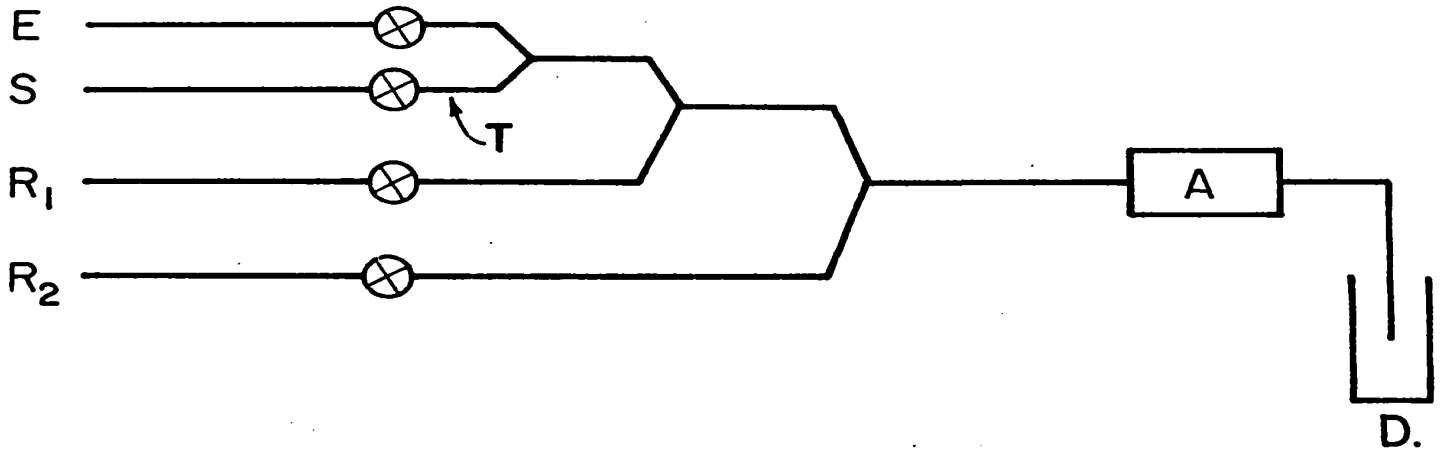
It is a simple matter, however, to replace the enzyme sampler at E by a length of tubing, containing enzyme attached to its interior wall, incorporated into the sample tubing at T. The same enzyme sample may then be used repeatedly, thus reducing expense.

Industrial Applications

Starches are converted into sugars on an industrial scale, with the aid of enzymes as catalysts. The use of solid-supported enzymes is likely to reduce expenditure, as indicated above, as well as improve the overall efficiency of the process. Such experiments have, in fact, proved highly

Figure 1

A schematic diagram of a flow-type automated analysis system, involving the use of tubes (see text).


E
S
T
R1
R2
A
D.

successful (24,25). The process of beer brewing also utilises yeasts and enzymes. If these additives are immobilized on a solid support, they can be filtered off, thus avoiding contamination of the product.

Medical Applications

In many cases, accumulation of certain metabolites in the body (or lack of an enzyme or hormone) may lead to harmful diseases. Enzymes may be used to lower the levels of such metabolites (and enzymes and hormones may be added to compensate for the lack of such). This may be accomplished by direct injection of the enzyme, or hormone, into the body. Several disadvantages are, however, encountered with the enzyme in free solution: (1) The enzyme may not be re-used, and it is very expensive, as high-purity preparations are required; (2) there are often toxic side effects associated with injecting enzymes into the body; (3) the free enzyme may be digested by proteases, and also lose activity by thermal denaturation.

Use of the enzyme (or hormone) attached to a solid support overcomes many of the above problems. The enzyme is not subjected to the action of proteases since it is protected by the support, as demonstrated by experiments with insulin (26). Furthermore, as discussed in Chapter 2, solid-supported enzyme preparations are frequently more stable to thermal denaturation than the enzyme in the free state.

If the immobilized enzyme is used in an extracorporeal shunt, the toxic side effects may be eliminated, since the enzyme is then not in contact with the body for an appreciable length of time. Such shunt systems have been used with microencapsulated enzymes (27). An enzyme-containing tube might also be used in the same way, as is discussed further in Chapter 4.

Biological Applications

Solid-supported enzymes have commonly been used to simulate biological membranes or tissue, which often contain enzymes. It has been demonstrated that, in many cases, detachment of the enzyme from the membrane leads to altered kinetic properties. In some cases, detachment has led to an increase in the apparent activity (28), and in others all activity is lost. The study of enzymes in membranes is one way of investigating the nature of membrane-enzyme interactions, which are clearly of considerable importance. This point will be returned to in Chapter 5.

General Aspects of Supported-Enzyme Kinetics

In view of the wide range of fields to which supported enzymes have been applied, it has become important to know how the mode of action of such enzyme preparations might change upon immobilization. A number of experiments have been carried out with this in mind and, in addition, several

theoretical treatments have been developed which indicate what changes in kinetics may be expected. These aspects will now be discussed.

There are four main reasons why an enzyme may exhibit different kinetic behaviour (such as changes in the Michaelis parameters) when it is bound to a solid support, compared with those parameters in free solution:

(1) The enzyme may adopt a different conformation when attached to the support, from that in free solution. The kinetic behaviour of an enzyme can be considerably altered owing to such conformational changes. One or two investigations have indicated that conformational changes do occur after immobilization of certain enzymes (29,30).

(2) The interaction between enzyme and substrate may take place in a different environment. Enzyme reactions, like ordinary chemical reactions, are known to be affected considerably by factors such as ionic strength, dielectric constants, and the like. It would not be surprising if the kinetics of an enzyme were altered by environmental effects in the solid phase.

(3) The substrate and product of the enzyme reaction will partition themselves between the solid phase and the surrounding bulk solution. Since they may not be equally soluble in the two phases, the substrate within the support may be at a different concentration from that in the bulk solution. For uncharged supports or substrates, the effect is

likely to be small, as has been shown in a number of experiments (for a review, see Laidler and Sundaram (3)). When support and substrate are both charged, however, dramatic changes in the observed Michaelis constant have been recorded. With support and substrate bearing like charges, this constant has been observed to increase. When the charges are unlike, the constant decreases (3). These observations are understandable in terms of the electrostatic interactions which prevail. In the first instance above, a charge-repulsion effect reduces the effective substrate concentration within the solid matrix. In order to get a rate equivalent to that for the enzyme in free solution, much higher substrate concentrations must be used, leading to an increase in the apparent Michaelis constant. The reverse applies in the second instance, with unlike charges on support and substrate, where an attractive electrostatic interaction occurs.

It is a simple matter to measure partition coefficients between solution and support, in the absence of the enzyme, so that the partitioning effect may be taken into account.

(4) Finally, and most central to this thesis, the enzyme reactions may be to some extent diffusion-controlled, since the substrate must penetrate the solid matrix in order to react with the enzyme. Intuitively, one might expect a rise in the apparent Michaelis constant, in the same way as for a repulsive charge interaction discussed above: diffusion will

also tend to reduce the effective substrate concentration of the enzyme in the solid phase. Again (all other things being equal) a higher substrate concentration in the bulk solution is required to overcome this effect and to achieve the same rate as for the enzyme in free solution. This leads to an increase in the apparent Michaelis constant, as illustrated schematically in Figure 2.

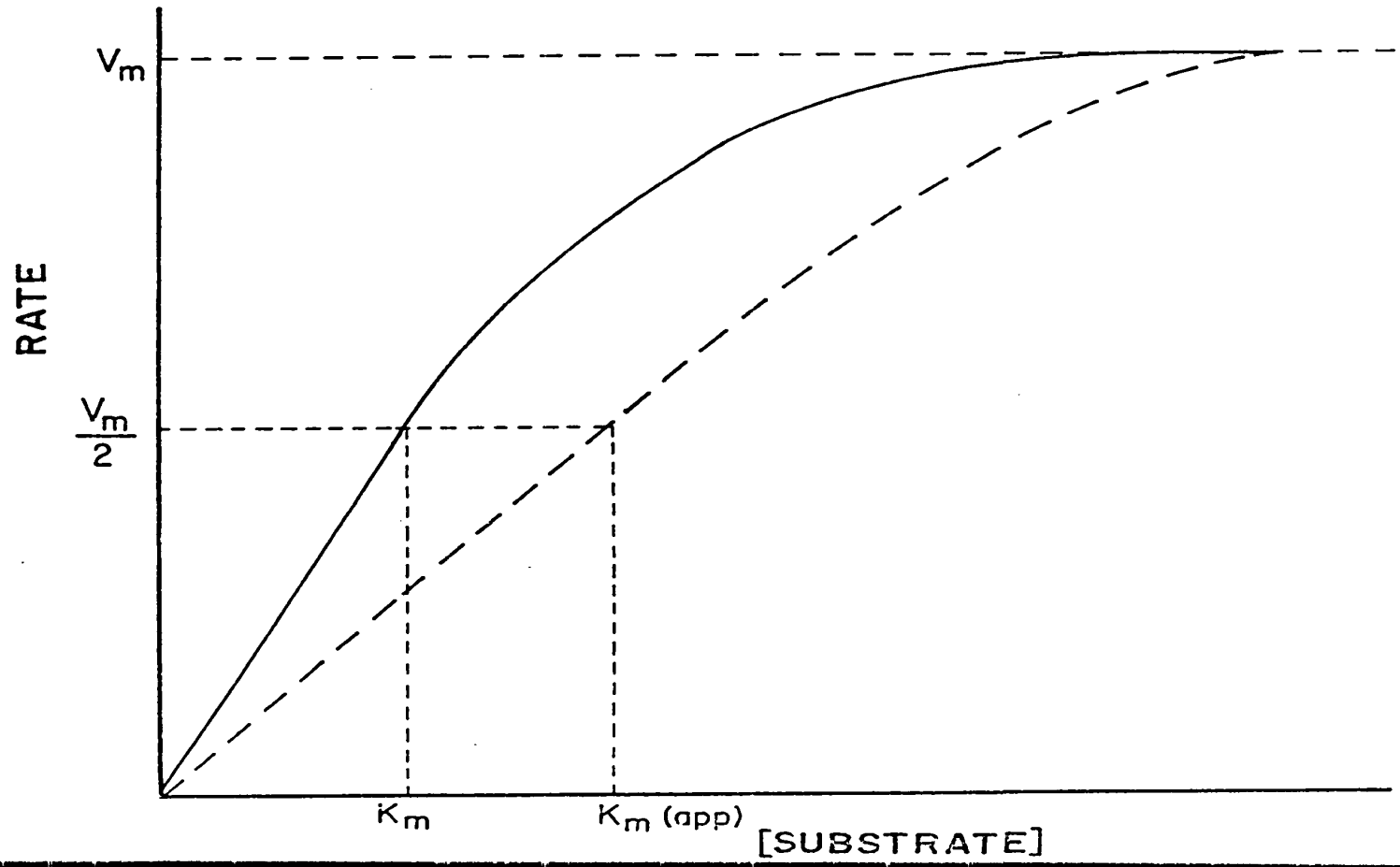
Qualitative Diffusion Effects

A number of qualitative studies have indicated that diffusion effects are important in the kinetics of solid-supported enzymes. Some examples will now be discussed.

Histochemical studies on enzymes in natural membranes and in tissue slices have often used rates of enzyme reactions at high substrate concentrations as a means of determining the enzyme concentration in the membrane. In such studies, it is necessary to ensure that the true activity of the enzyme is being measured, in order to obtain the correct value for the enzyme concentration. In certain cases, however, a diffusion effect on the apparent activity has been indicated, so that an incorrect value for the concentration was obtained (31-34). This effect was demonstrated by the non-linear increase in reaction rate with thickness of the tissue slice, for example, and by the observation of a different activity of the enzyme when different substrates were used. As will be seen below, these observations are not unusual when diffusion control of the enzyme activity is involved.

Figure 2

A plot of rate versus substrate concentration in a schematic illustration of the effect of diffusion on the kinetics of an enzyme obeying the Michaelis-Menten law. The Michaelis constant for the free enzyme is K_m; that for the supported enzyme is $K_m(app)$. Both are defined as that substrate concentration which gives a rate of half the maximal velocity (V_m), which is assumed to be the same for both enzymes.


V_m
$\frac{V_m}{2}$
RATE
K_m
K_m (app)
[SUBSTRATE]

Other indications of diffusion effects in naturally-occurring systems have been reported. The kinetic parameters of adenosine triphosphatase, for instance, when bound to the natural membrane, were observed to be different from those of the enzyme in free solution (35). In particular, the apparent Michaelis constant for the bound enzyme was about four times higher than that for the free enzyme. Such an observation can be accounted for in terms of diffusion effects, although environmental effects cannot be ruled out. Similar changes in the kinetic parameters have been observed for catalase inside erythrocyte membranes compared with the values for the enzyme in free solution (36).

A further complication may arise in such natural membrane systems, however, since the membrane bears a net charge, and a charged substrate will experience partitioning effects such as were discussed above.

Other diffusion effects have been noted in experiments involving simulated enzyme membranes. For instance, carbonic anhydrase attached to silastic membrane was observed to enhance the transport of carbon dioxide across the membrane, owing to facilitated diffusion (37). The rates of two-enzyme systems, with the enzymes catalyzing consecutive reactions, have been found (38) to be greater when both of the enzymes are trapped together within the membrane, than when trapped separately; Mattiasson and Mosbach (39) found a similar effect with a three-enzyme membrane. An environmental

effect could be ruled out, by taking into account the change in rate for the separately-bound enzymes. The rate increase was ascribed to the effective increase in substrate concentration for the final enzyme in the consecutive reaction pathway, since the product of the preceding enzyme could not readily diffuse away, being hindered by the surrounding solid matrix.

In a system involving spherical particles of solid-supported enzyme, Kay and Lilly (40) found that the apparent Michaelis constant was much larger for particles of greater diameter than for the smaller-diameter particles. Since, in such a comparison, the environment is the same for both, diffusion limitation of the rate seemed the most likely explanation for this effect. An additional feature of this work was the shapes of the Lineweaver-Burk plots of reciprocal of rate plotted against reciprocal of substrate concentration. It has been predicted on theoretical grounds (41,42) that in the absence of diffusion such plots should be straight lines, as observed for normal Michaelis-Menten kinetics in free solution; in the presence of diffusion effects, however, the plots should be curved at higher substrate concentrations, meeting at a common point on the vertical (rate) intercept. This was found to be the case by Kay and Lilly (40). Similarly curved Lineweaver-Burk plots have been reported by Gowron et al (43,44) for succinic dehydrogenase in natural membranes, and by Julliard and co-workers (45) for glutamate

dehydrogenase in collagen. Our work, reported in Chapter 3, with β-galactosidase in polyacrylamide gel, produced the same type of result under diffusion-controlled conditions.

In yet another study, Axen and co-workers (46) observed that the apparent Michaelis constant for chymotrypsin bound to sepharose particles was ten times larger than the value for the free enzyme. As was mentioned above, this effect is expected where diffusion control is involved. Upon solubilization of the enzyme, using dextranase to break down the polymer, the Michaelis constant became the same as that for the free enzyme. This result indicated that diffusion was indeed responsible, and that there was no environmental effect on the Michaelis constant.

Other indications of diffusion effects have been reported, in experiments where environmental influences were not involved. Romeo and Barnard (47) found that the activities of several enzymes were masked when they were trapped within a "synthetic cell" membrane. The activities were reduced by about 40% for a cell wall of approximately 150 microns thick. The activities of the free enzymes used for comparison were measured in the presence of the chemicals used to make the synthetic cell.

Schurr and McLaren (48) observed that for the hydrolysis of gelatin by trypsin, the apparent Michaelis constant was six times higher for the gel than for the sol. Here again, the environments of the enzyme in both phases

were similar, so that diffusion control was probably responsible for the change, assuming a negligible partitioning effect.

The above are a few examples of qualitative demonstrations of diffusion effects in supported enzyme systems. In some cases, environmental effects could not be ruled out as being at least partly responsible for the changes observed in the kinetics upon immobilization of the enzyme. This is an important point, which has been discussed in recent reviews (3,49), and will be returned to later. Enzymes in free solution are known to be affected in many ways by their environment, as indicated by studies with mixed solvents on ribonuclease (50), chymotrypsin (51) and trypsin (52). These include changes in the pH-rate profiles, and in the pK's of groups involved in the kinetic reaction. Similar changes in supported enzyme systems have also been reported, as discussed in the above-mentioned reviews.

Partitioning effects have rarely been taken into account in the above experiments; yet in some cases they may be appreciable. Any analysis of diffusion effects on the kinetic parameters of supported enzymes must separate them from environmental and partitioning effects.

Quantitative Diffusion Studies

The need for theoretical treatments of diffusion in solid-supported enzyme systems was clear on the basis of

the above qualitative results; numerous workers responded to this with papers dealing with a variety of different situations, the most important of which are summarized in Table 1. A discussion of each of these is beyond the scope of this thesis; a few comments, however, on those directly related to the type of experimental system used in this work, seem merited. Our experimental work described in Chapter 3 was directed at testing one of these theories, that of Sundaram, Tweedale and Laidler (42). Similar theories have been developed independently by Goldman, Kedem and Katchalski (53) and by Sluyterman and van der Graaf (54). In the last-named two cases, the theory was applied to certain experimental results, with reasonable agreement. On both occasions, however, only three different sets of conditions were used to test the theory; and, more important, the assumption was made that no environmental or partitioning effect contributed to the observed changes in kinetic parameters. The work of Goldman, Kedem and Katchalski (53) utilized three different papain-collodion membranes; that of Sluyterman and van der Graaf (54) involved studies of the activities of three enzymes in the crystalline state. In both cases, the experimental system was shown to be better than 90% free of diffusion effects, subject to the above-mentioned assumptions. In neither case was a thorough study made over a wide range of experimental conditions. This prompted the investigations reported in the next two chapters of this thesis.

TABLE 1

Type of Supported-Enzyme System	Reference
I. Membrane Systems	
a) Diffusion of substrate from one side of a membrane to the other, with enzyme uniformly distributed within the membrane.	Blaedel and Boguslaski (55); Laidler and Sundaram (3); Goldman, Kedem and Katchalski (53); Selegny et al (56).
b) Diffusion of substrate from one and two sides of a membrane layered with adsorbed enzyme.	Goldman, Kedem and Katchalski (53).
c) Diffusion of substrate from both sides of an enzyme membrane, with enzyme uniformly distributed within the membrane.	Sundaram, Tweedale and Laidler (42); Sluyterman and van der Graaf (54); van Duijn et al (34); O'Sullivan (57); Selegny et al (58).
d) Effect of Nernst diffusion layer surrounding an enzyme membrane.	Goldman, Kedem and Katchalski (127).
e) Enzyme membranes carrying out consecutive reactions.	Goldman and Katchalski (59).
II. Particulate Systems	
a) Enzyme attached to outside of particle, or uniformly distributed within particle.	Kasche et al (60); Blum and Jenden (61); Schurr (62); Moo-Young and Kobayashi (41).
b) Effect of Nernst diffusion layer around enzyme immobilized in particles.	Kobayashi and Moo-Young (63).
III. Cylindrical Systems	Blum and Jenden (61).
IV. Flow Systems	
a) Tubes containing enzyme on the interior wall	Kobayashi and Laidler (64).

b) Diffusion effects in continuous flow columns and packed bed reactors.	Lilly and Sharp (65); Lilly, Hornby and Crook (66); Kobayashi and Moo-Young (63,67); Hornby, Lilly and Crook (68).
V. Theories considering <u>inhibition</u>, especially by substrate and product.	Goldman, Kedem and Katchalski (53).
VI. The effect of <u>electrostatic potential</u> on supported enzyme systems.	Goldstein, Levin and Katchalski (69); Shuler, Aris and Tsuchiya (70).

In Chapter 4, kinetic studies on an enzyme attached to the inside wall of a tube, are presented. No previous kinetic study on such a system has been carried out. The importance of this study is apparent from the variety of uses to which the tubes containing enzyme have been put, as discussed above.

CHAPTER TWO

KINETIC STUDIES ON ENZYMES IN POLYACRYLAMIDE GELS

Introduction

In order to test the theory of the effects of diffusion on the kinetics of enzymes supported in membranes, described in Chapter 3, a number of experimental requirements had to be met. The most important of these were: that the enzyme should be uniformly distributed throughout the solid matrix; that membranes of different thicknesses should be readily and reproducibly obtained; that there should be no leakage of enzyme from the support; and that the supported enzyme preparation should be reasonably active and stable.

Numerous methods have been devised in order to make insoluble enzyme derivatives; these have been discussed in detail in several reviews (3,6,8,49). The following is a brief account of the main methods of attachment, in the light of the above criteria for testing the theory experimentally.

(1) One of the earliest methods tried is insolubilization of the enzyme without the use of a solid-supporting matrix. Some workers used a bifunctional reagent, such as glutaraldehyde, in order to polymerize the enzyme, which ultimately precipitated out of solution. Others modified reactive groups on the enzyme, rendering it insoluble. While the insoluble enzymes prepared by these methods are generally active, their

mechanical properties are not suitable for obtaining membranes of varying thicknesses. This type of approach was therefore not considered for testing the theory.

(2) Another method commonly used is that of physical adsorption to a solid matrix. Supports such as ion exchange resins, cellulose derivatives, clays and even glass have been used in these adsorption methods. Indeed, one of the theories of diffusion relating to immobilized enzymes has been tested using papain adsorbed to collodion (53). For our purposes, however, it seemed a matter of some difficulty to get membranes of accurate and reproducible dimensions, over a wide range of thicknesses. Moreover, physically adsorbed immobilized enzymes often desorb when conditions are changed even moderately, as discussed in the above-mentioned reviews. For these reasons, this method was not used in our experiments.

(3) The method of covalent attachment of enzymes via reactive functional groups on the solid support has met with considerable success in supported enzyme preparative work. The enzyme is irreversibly attached, and different supports provide a variety of environmental and mechanical properties. The procedures used to achieve covalent attachment will not be discussed here, but in general this method has been carried out on supports of a particulate nature (with notable exceptions). In most cases, it has not been shown that uniform distribution of enzyme throughout the support has been achieved. Use of a support with a varying concentration of

enzyme inside would greatly complicate the interpretation of the results. This method was therefore also discarded in the present work.

(4) The fourth method is that of inclusion of the enzyme within the pores of a gel. This method was attractive from two main points of view: (a) Obtaining the gel from a solution ensured uniform distribution of the enzyme; and (b) it seemed a reasonably easy matter to slice up such a gel into disks of reproducible thickness, using a microtome. The main disadvantage is that enzyme may leak out through the pores of the gel, as has been found in many cases. Nonetheless, the gel method was attractive for an additional reason - it was thought possible that the microenvironment of the enzyme in the gel might be the same as that in the solution from which the gel forms, i.e. that the act of gelation would have no effect on the enzyme's properties compared with those properties in the sol. This, if true, would provide an opportunity to study, and perhaps even differentiate between, factors (1) and (2) discussed on page 8.

Having established gel inclusion as the method of preference in these studies, it remained to decide upon the particular type of gel. Starch, gelatin and other thermally-produced gels were discarded, since heating up the solutions to temperatures of 40 and 50 degrees would inactivate the enzyme. (Starch gel has, in fact, been used on one or two occasions (71,72)). A method of chemical polymerization, which could be carried out in the cold, seemed preferable.

γ-radiation has been used in the preparation of many different types of polymers and gels (73). It seemed likely that γ-radiation of a solution of a water-soluble polymer (for example, see Davidson and Sittig (74)) might lead to a general method of preparing supported enzymes bound to hydrophilic solid carriers. This was therefore the method initially adopted. Several polymers were tried in this manner, including polyvinyl alcohol, hydroxyethyl cellulose, methylcellulose and carbowax. No suitable gel was obtained from aqueous solution, except in the case of polyacrylamide from acrylamide monomer. This gel has been employed on numerous other occasions, with the use of a photochemical method of polymerization, for electrophoresis (75, 76) and also for the preparation of solid-supported enzymes (77-80). At the time this work was started, no reports were available in the literature as to the use of γ-radiation in the preparation of immobilized enzymes. It was thought useful to try both photochemical and γ-radiation methods of polymerization, and to compare the activities of the resulting preparations. This work is described below.

Polyacrylamide gels were also favoured because their physical properties have been well characterized for use in protein separation using gel electrophoresis (75). Pore sizes may, for instance, be varied almost at will (81,82). This provides a means of eliminating the major objection to the gel inclusion method, raised above: that of leakage of

enzyme from the gel. Polyacrylamide gels, furthermore, are water compatible, of suitable mechanical rigidity for slicing on a microtome, and, since they are electrostatically neutral, charge-charge interactions, which might interfere with a diffusion study, are minimized.

Preliminary Experiments with Enzymes in Polyacrylamide Gels

A number of preliminary studies were carried out using polyacrylamide gels, prior to the detailed kinetic studies described in Chapter 3 of this thesis. These were done with a view to obtaining a more detailed knowledge of the immobilized enzyme system. Particular attention was paid to the following points:

(1) Two different polymerizing methods, viz. photochemical and γ-radiation methods of initiation of the polymerizing process.

(2) The reasons for the activity losses observed during polymerization.

(3) The activities of the resulting supported enzymes.

(4) The stabilities of the supported enzyme preparations.

(5) Some physical properties of the gels.

(6) The microenvironment of the enzyme within the gel as compared with that in free solution.

In these preliminary experiments, two enzymes were used, namely β-galactosidase and α-chymotrypsin. Most of the work was carried out with the former enzyme, but results with

α-chymotrypsin are included as they confirm and amplify the other results. β-Galactosidase has been immobilized on three other occasions: in cellulose (83,84); in diethylaminoethylcellulose (84); and in polyacrylamide (39). α-Chymotrypsin has been attached to many supports (49), but it has been included in polyacrylamide gel on only one other occasion (78).

Materials

β-Galactosidase from E.Coli, K.12, was a partially-purified preparation obtained from Worthington Biochemical Corporation. Two lots were used, BG OHA for initial experiments, and BG 1EA for all Lineweaver-Burk plots. The substrate for this enzyme, o-nitrophenyl-β-D-galactopyranoside (ONPG) was obtained from Sigma Chemical Co.

α-Chymotrypsin (lot CD1 6JF) was purchased from Worthington Biochemical Corporation. Its substrate, N-acetyl-L-tyrosine ethyl ester (ATEE), was obtained from Mann Research Laboratories.

The cross-linking agents in the gel polymerization, N,N,N',N'-tetramethylethylene diamine (TEMED) and N,N'-methylene bisacrylamide (BIS) were obtained from Eastman Organic Chemicals. Acrylamide was obtained from Eastman, and from Matheson, Coleman and Bell. The ammonium persulphate used in the polymerizations was obtained from Fisher Scientific Co., and disodium hydrogen phosphate from J. T. Baker

Chemical Co. Sodium chloride, used to adjust the ionic strength of the solutions, was obtained from Canadian Laboratory Supplies Ltd.

Methods

(a) In the case of β-galactosidase, all solutions used to assay the enzyme and to prepare the gels were made up in 0.02M Na_2HPO_4 buffer at pH 7.50 ± 0.05, and were adjusted with sodium chloride to an ionic strength of 0.10M. The usual assay procedure for β-galactosidase (86) has been shown by other workers (87) to result in a loss of enzyme activity over a period of several hours. On the other hand, the latter group of workers found that phosphate buffer resulted in constant activity over at least six hours. This result was confirmed in the present work, but only if the buffer was degassed for two hours with nitrogen, which removes oxygen which may inactivate the essential thiol group of the enzyme. Degassing solutions is also important for proper preparation of polyacrylamide gels, since oxygen is a free-radical scavenger (75).

(b) When α-chymotrypsin was the enzyme used, all solutions were made up in distilled water, pH 7.0 ± 0.05, and at an ionic strength of 0.15M, again using sodium chloride.

Enzyme Assays

(a) β-galactosidase: Enzyme weighings were checked by ultraviolet absorption at 280 nm, in buffer at pH 7.50, with an ε_{420}^{1cm} value of 1.44 ± 0.04 litre gm^{-1}; the latter value was determined on four separate occasions. ONPG was used as substrate, and the rate of reaction followed on a Bausch and Lomb Spectronic 600 spectrophotometer, with a Sargent SRLG recorder attached, by the increase in absorbance at 420 nm due to the production of o-nitrophenol. The temperature was kept at 25.0 ± 0.2°C. Rates at pH 7.5 were calculated using ε_{420}^{1cm} = 3,200 M^{-1}. This value was obtained from the experimentally determined pK value of 7.10 for the product of hydrolysis of the substrate at pH 13 for one hour at 100°C (after which there was no further change in absorbance). ε_{420}^{1cm} values of 190 at pH 1.0 and 4,350 at pH 13.0 were obtained. The pK of 7.10 is consistent with that quoted for an ionic strength of zero, 7.22, in the Handbook of Biochemistry, Chemical Rubber Co.; our ionic strength of 0.1 will tend to decrease the pK.

Assays of the solid-supported β-galactosidase preparations were carried out by suspending disks of the enzyme-containing gels in 10 ml of substrate buffer solution. By means of a Watson-Marlow Flow Inducer (Model MHRE 200) the solution was pumped through tygon tubing to a microvolume flow cell (obtained from Hellma Co.) to determine the absorbance. The volume of the solution in the flow cell plus

tubing was less than 10% of the total solution volume. The flow rate was adjusted so as to be faster than the rate of formation of product. The solution was stirred with a small magnetic stirrer, which kept the slices moving around in the vessel. When necessary, to obtain a measurable rate, up to eight slices were suspended in the solution at one time. The rate was always directly proportional to the number of slices.

(b) α-chymotrypsin: This enzyme was assayed with the substrate ATEE, using a pH-stat (51). The enzyme supported in the gel was studied as a slurry of mechanically dispersed particles. All runs with chymotrypsin were carried out in deionized distilled water, at 25°C, pH 7.0 and ionic strength of 0.15M.

Preparation of Gels

For the polyacrylamide gels made by photopolymerization of acrylamide monomer solution (75,76), BIS was used as cross-linking agent, ammonium persulphate as photocatalyst, and TEMED as catalyst and cross-linking agent. Since other work (81,82) had shown that the use of 5% of BIS (with reference to BIS plus acrylamide monomer) produces the smallest pore size in the gel, this percentage was used in our experiments. Stock solutions of 60% w/v acrylamide monomer (including the 5% BIS) were kept for a month at room temperature in the dark. Solutions of TEMED (2% v/v) and

ammonium persulphate (2.8% w/v) in degassed buffer were stable for one week at 4°C in the dark. The following indicates typical concentrations used to make the mixture for polymerization:

Acrylamide (+ 5% BIS)	1.5 ml of 60%
TEMED	0.5 ml of 2%
Persulphate	0.1 ml of 2.8%
Enzyme and buffer	3.9 ml
Total	6.0 ml

The amounts of acrylamide and enzyme were varied somewhat, but those of the other components, and the total volume, were held constant.

The polymerizations were carried out in glass tubes of constant 1.0 cm diameter. The tubes were first dipped into Fotoflow solution (Eastman-Kodak) diluted 1:20 with distilled water; this facilitated subsequent removal of the gels from the tubes (75,76). During polymerization the solutions were cooled by placing the tubes in a beaker of water, covered with a plastic lid. The water was stirred and cooled to 0-4°C in a Stir-Kool apparatus (from Thermoelectronics Unlimited Inc.). Irradiation was done using a fluorescent lamp about two meters away from the tubes; complete gelation took about 45 minutes.

In the experiments involving γ-radiation as the initiator of polymerization, exactly the same technique was used, except that the photocatalyst, ammonium persulphate,

was omitted from the solutions, which were cooled in an ice bath and placed in the sample chamber of a Gammacell 220 cobalt-60 source. Irradiation was continued for various periods of time, at two different dose rates, as will be discussed below.

Polymerization induced by γ-radiation has, to the author's knowledge, been reported (in patents, with scant detail) by only one group of workers (88). This method was attractive, as mentioned above, since it appeared promising as a general method for forming gels from water-soluble polymers. The γ-radiation forms cross-links between the polymer chains, ultimately leading to gel formation. Attempts with materials other than acrylamide failed to produce suitable gels.

Slices were cut from the gels with a Model 880 American Optical Co. microtome (89,90). The gel was first frozen with CO_2 gas which had been cooled by allowing it to escape from a cylinder through a nozzle. The slice thickness was varied by manual adjustment of the blade-to-sample distance. The slices were stirred in buffer for a few minutes to remove adsorbed enzyme; this procedure was repeated between runs to remove substrate and product from them.

The slice thicknesses were measured using a Zeiss optical microscope with a calibrated 12.5x magnification eyepiece and 10x and 40x magnification objectives. The slices were mounted on their sides between coverslips and observed

underwater to prevent drying out and contraction. It was checked that there were no refraction errors. Each slice was examined at three points, and five measurements were made at each point. The slices were found to be uniformly thick.

Partition and Diffusion Coefficients

Partition coefficients for the product, o-nitrophenol, were measured by placing a known volume of gel in a product solution of known concentration. The system was left for two days to attain equilibrium, and the change in concentration of the solute was determined. It was checked that there was no further change in concentration after two days.

Diffusion coefficients were measured by placing a cylinder of gel at the bottom of a tube and placing a solution of the diffusant (such as product) in the tube, in which there was a small stirrer; the whole was then placed in stirred buffer solution from which aliquots were withdrawn at regular intervals and analysed. It was checked that there was no leakage of diffusant, and that a planar diffusion front was evident, by visually following diffusion of a blue dye; the linearity of diffusion with time confirmed this. The diffusion coefficient, D, was then calculated from the formula

$$\text{Diffusion rate} = \frac{AD}{xV} (S_o - S)$$

where A is the cross-sectional area and x the length of the gel, V the volume of buffer solution, and S_o and S the initial

and time-dependent concentrations of diffusant respectively. Care was taken to deal with volume changes when the gels were placed in buffer solution. Stirring of the solutions on both sides of the gel was found to be necessary.

A typical experimental plot for diffusion is shown in Figure 3. The diffusion coefficients obtained from the above equation were compared with those obtained using the "time lag" method (91). This method is illustrated in Figure 3, the lag period arising as a result of setting up a steady state after the pre-steady state section (dotted line). The intercept on the time axis, λ, is given by (91):

$$\lambda = \frac{x^2}{6D}$$

x and D being as previously defined. It is thus a simple matter to determine D. The results obtained using these two methods were in good agreement.

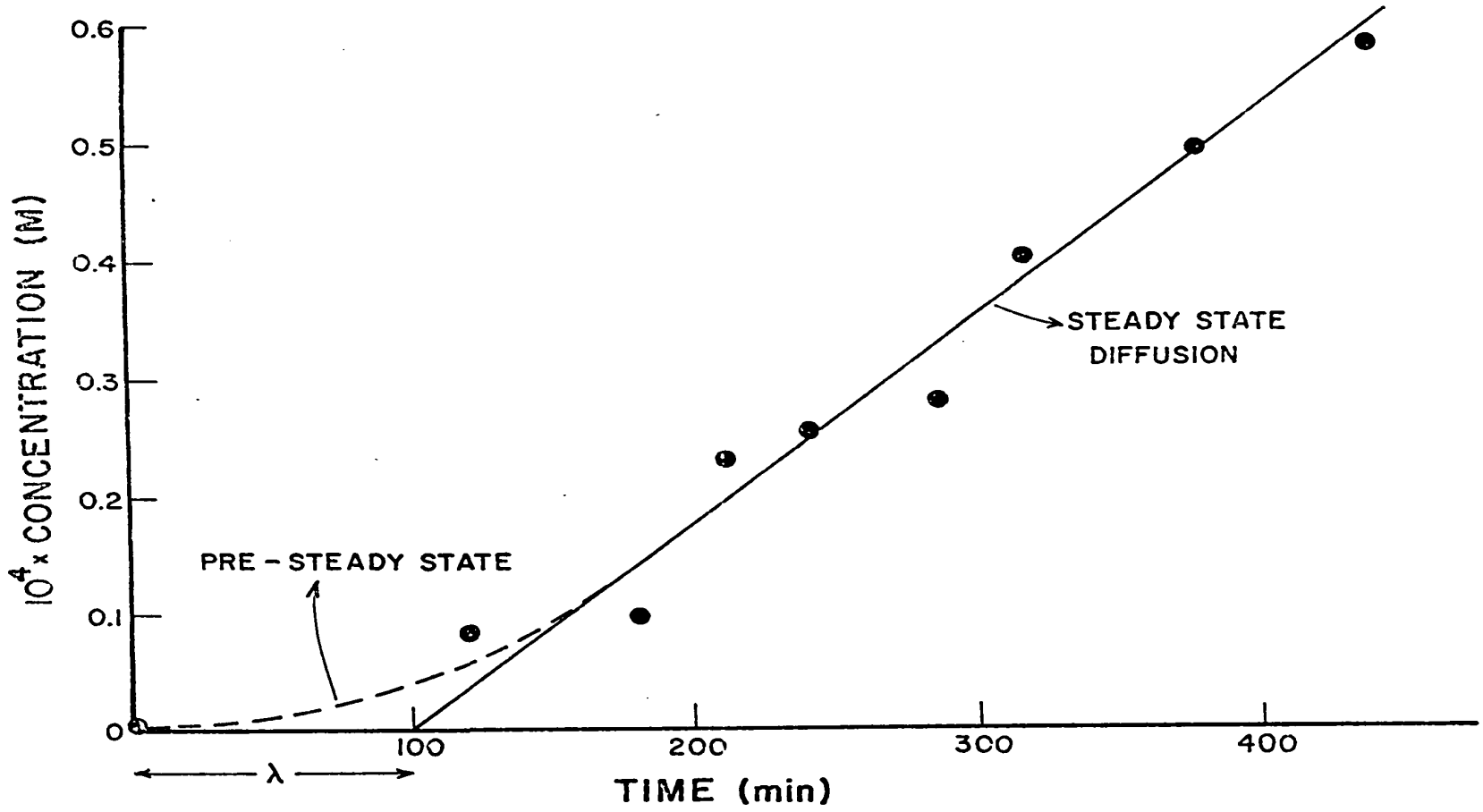
The analysis of rate vs substrate concentration data, to give K_m(app) and k_c(app) values, was carried out by computer using a programme adapted from that given by Cleland (92).

Experimental Results

Enzyme Activity Initial experiments were concerned with obtaining a preparation of β-galactosidase in polyacrylamide gel of suitable activity, with no leakage of enzyme. Leakage could not be checked by use of the normal protein assays such as the Folin-Lowry method (93), since residual acrylamide

Figure 3

A plot of concentration as a function of time in a typical experiment to determine the diffusion coefficient of o-nitrophenol in polyacrylamide gel. The dotted curve indicates the pre-steady state.



monomer interferes with the assay to such an extent that the small increase in colour due to protein leakage could not be reliably determined. Instead, the enzyme activity was assayed as a function of time by removal of aliquots of surrounding buffer over a period of 6-8 hours. There was considerable enzyme leakage for 5% and 7.5% (w/v) acrylamide gels, but negligible leakage for 10% and higher. These results were confirmed by assaying the supported enzyme slices. Similar conclusions have been reached by Degani and Miron (82) for cholinesterase in polyacrylamide gels.

Experiments were carried out to ensure that the free and supported enzymes maintained constant activity over a period of several hours; some results are shown in Figure 4. A check was also made that the activity of the free enzyme was linear as a function of enzyme concentration in acrylamide monomer solution. Results for a 15% acrylamide solution are shown in Figure 5.

Loss of Activity during Polymerization A series of experiments was carried out to determine the effect of acrylamide concentration on the enzyme activity, the object being to minimize the loss in activity during entrapment in the gel. The results for β-galactosidase are summarized in Table 2. The symbol $h\nu$ refers to photopolymerization, γ_H to high-intensity γ-radiation (10 minutes at 2,380 rads per minute) and γ_L to low-intensity γ-radiation (at 720 rads per minute to the same total dose). The right-hand column gives results

Figure 4

Time dependence of the activity of free and supported β-galactosidase. The two curves for the free enzyme correspond to $[\beta GAL] = 1.60 \times 10^{-3}$ mg ml^{-1}; $[ONPG] = 2.74 \times 10^{-4}$ M; pH = 7.5; T = 25.0°C; I = 0.1 M (phosphate buffer). The results with supported enzyme were obtained with 100 μslices in a volume of 10 ml; $[\beta GAL]_{gel} = 0.505$ mg ml^{-1}; [ONPG] = 1.66×10^{-3}M; pH = 7.5; I = 0.10 M; 0.02 M phosphate buffer; T = 25.0°C.

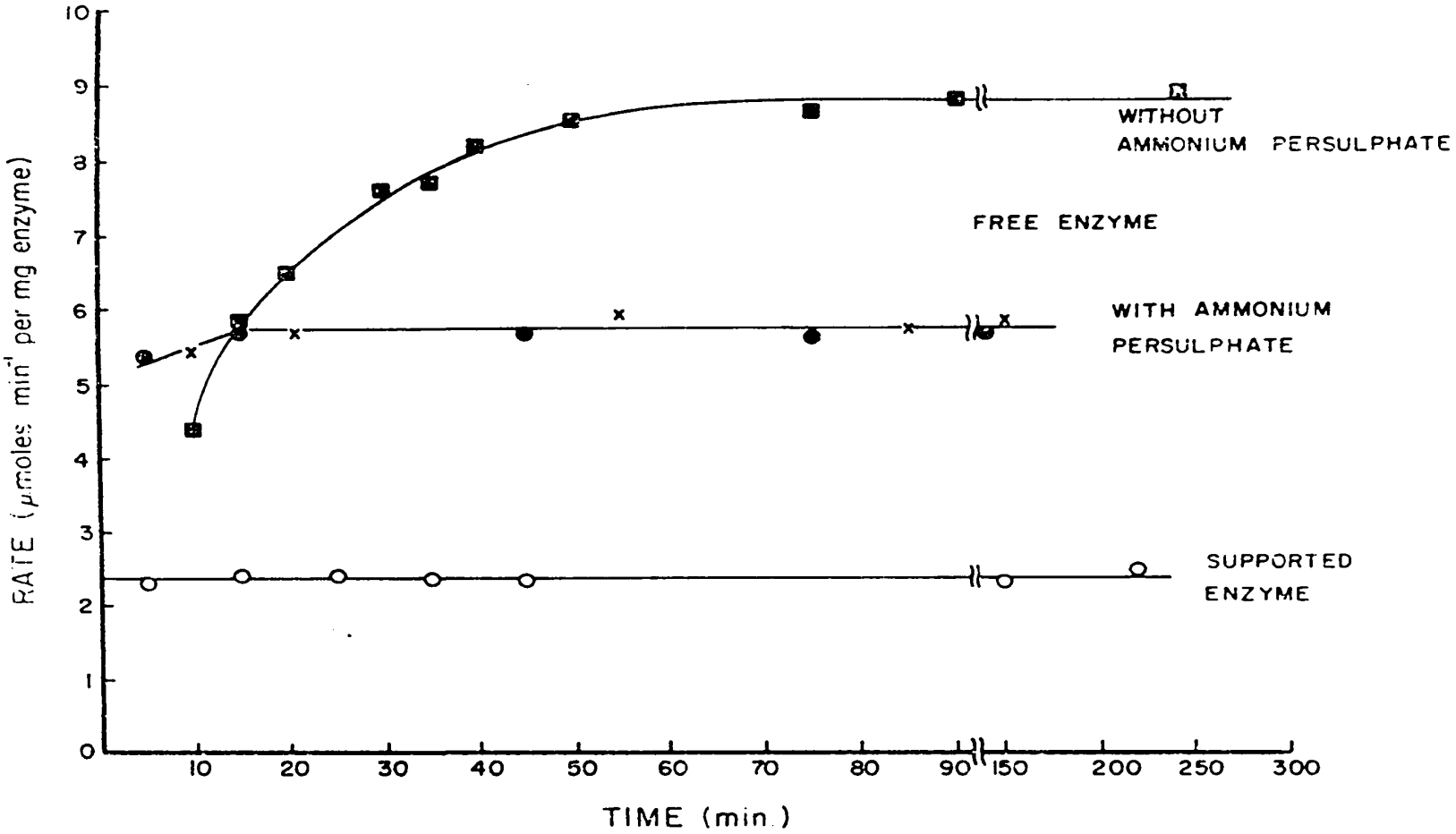


Figure 5

A plot of the rate at high substrate concentration as a function of enzyme concentration for free β-galactosidase in phosphate buffer (■) and in 15% acrylamide monomer (●). [ONPG] = 1.66×10^{-3}M.

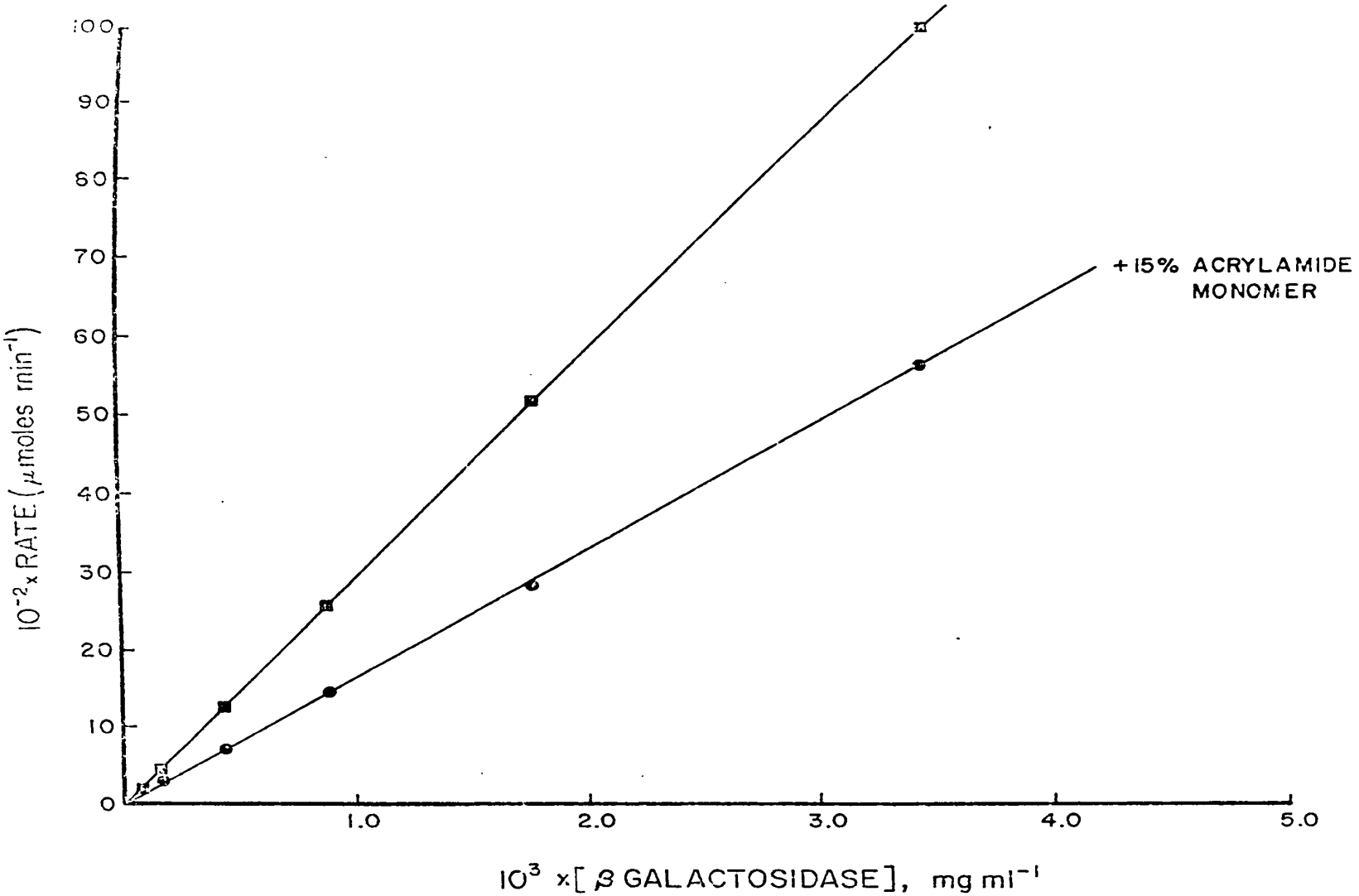


TABLE 2

Enzyme Activities at Various Acrylamide Concentrations

Acrylamide (percent w/v)	Diameter (cm)			Thickness (μ)			Rate (μmoles $l^{-1}min^{-1}$)			Normalized Rate (μmoles min^{-1} per mg enzyme)			Rate (μmoles min^{-1} per mg enzyme)
	hν	γ_H	γ_L	hν	γ_H	γ_L	hν	γ_H	γ_L	hν	γ_H	γ_L	γ_H
30	1.12	1.13	1.13	104	120	125	3.00	1.96	1.93	1.74	1.28	1.21	(a)
25	1.12	1.11	1.12	111	107	115	3.55	2.44	2.19	1.93	1.85	1.52	3.2
20	1.05	1.05	1.08	100	117	113	3.66	2.62	2.42	2.51	2.03	1.84	-
15	1.05	1.06	1.04	108	107	106	3.72	2.89	2.38	2.36	2.40	2.08	4.7
10	1.02	1.03	1.02	102	96	97	3.21	2.15	2.19	2.29	2.11	2.17	5.7
7.5	.95	.99	.99	-	-	-	2.73	1.97	1.90	2.24	2.09	2.00(b)	6.6

(a) Too low to measure

(b) These rates were calculated using same thicknesses as for 10% disks.

Enzyme concentration in gel = 0.33 mg ml for hν; 0.25 mg ml^{-1} for γ_H, γ_L, in irradiated solution of free enzyme.

[ONPG] for assay = 1.66×10^{-4}M for supported enzyme; 1.33×10^{-4}M for free enzyme.

T = 25°C; pH = 7.5; ionic strength = 0.1M.

for the free enzyme, irradiated for 3 minutes at 2,380 rads per minute, in the presence of various concentrations of acrylamide, and refers to 1 mg of free enzyme. The other rates quoted are for experiments with gels of the thicknesses and diameters indicated. The rates are per litre of solution per slice, all normalized to 100 micron thickness and 1.0 cm disk diameter, and to an enzyme concentration of 0.25 mg ml^{-1} of gel.

Loss of Activity at High Acrylamide Concentrations Figure 6 shows that the rates pass through a maximum as the acrylamide concentration is increased. A similar peak has been found by Degani and Miron (82) for cholinesterase, and by Gruesbeck and Rase (25) for glucoamylase, both trapped in polyacrylamide gel, using different approaches. The loss of activity at higher acrylamide concentrations was possibly a denaturing effect similar to that brought about by urea and guanidine hydrochloride. Alternatively, it could also be the result of a competitive inhibition effect of the acrylamide, the substrate being displaced from the enzyme (and the rate therefore being lowered) at higher acrylamide concentrations. A similar reduction in activity was found with experiments using the unirradiated free enzyme, as shown for β-galactosidase in Figure 7. Results for α-chymotrypsin, at two substrate concentrations, are also shown in Figure 7; the presence of substrate at higher concentrations protects the enzyme against the inactivation process. The decrease in rate at higher con-

Figure 6

Effects of acrylamide concentration on the activities of free, photopolymerized and γ-irradiated β-galactosidase. The lower scale refers to rates for the free enzyme, per mg of enzyme; the upper scale refers to rates for the supported enzyme, per liter of solution, per slice. Other conditions: pH = 7.5, I = 0.1 M, T = 25°C.

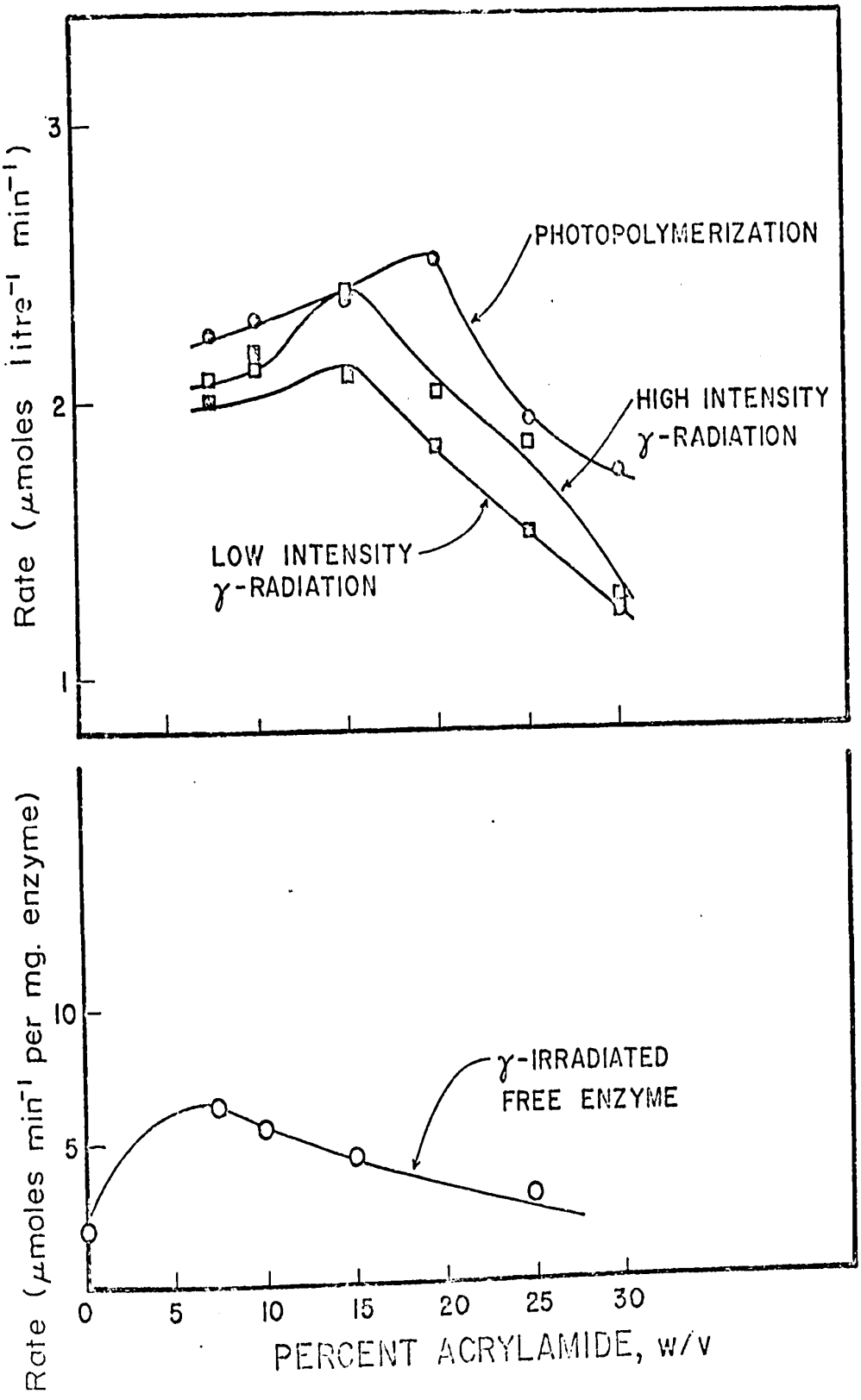
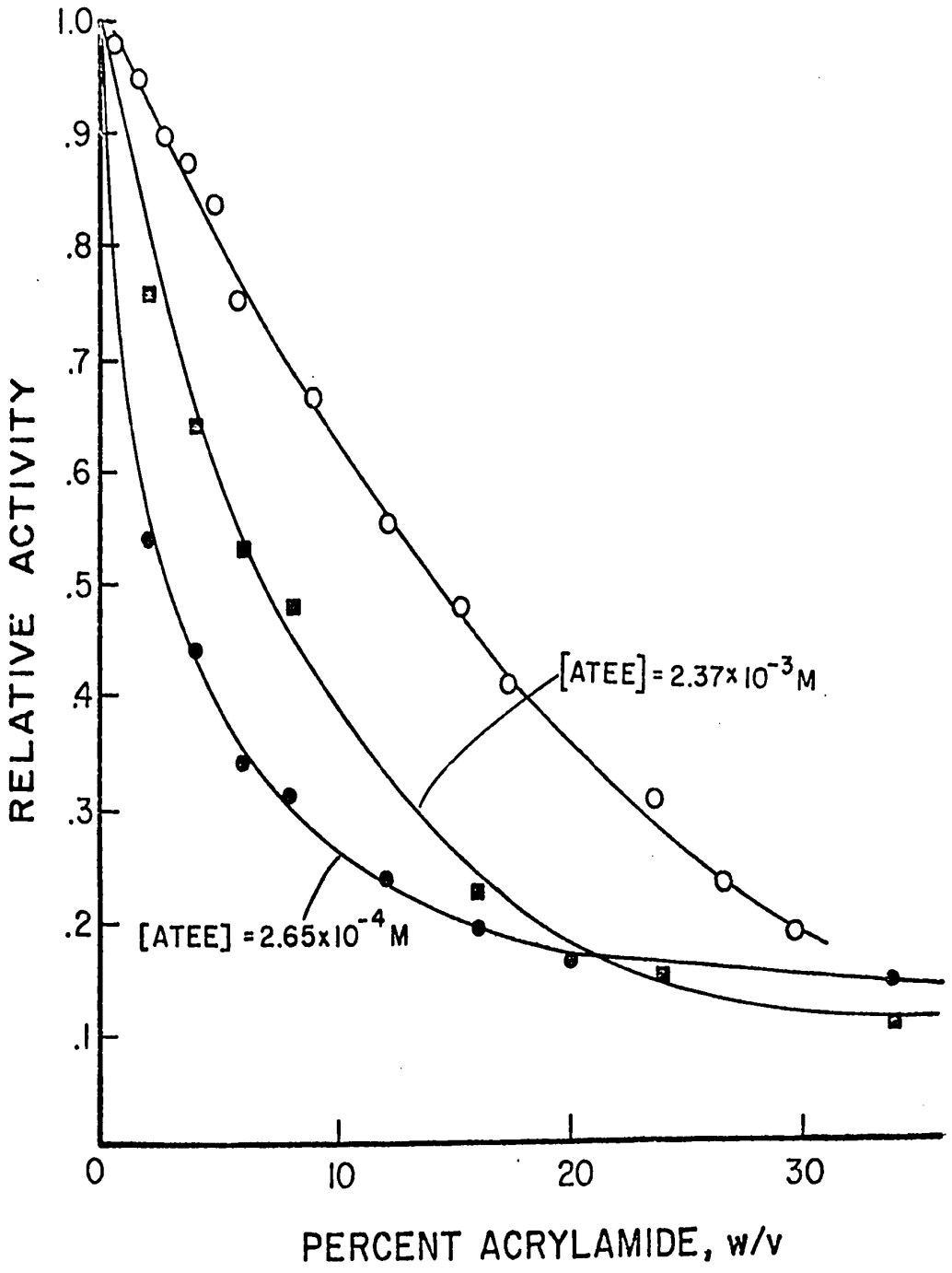


Figure 7

Effect of added acrylamide on the activities of unirradiated β-galactosidase (open circles) and α-chymotrypsin (filled points; two substrate concentrations shown). For β-galactosidase: enzyme concentration = 1.33×10^{-3} mg ml^{-1}; [ONPG] = 4.47×10^{-4} M; T = 25°C; pH = 7.3; ionic strength = 0.02 M. For chymotrypsin: enzyme concentration = 4.3×10^{-7} M; T = 25°C; pH = 7.0; ionic strength = 0.15 M.



centrations of acrylamide is clearly not related to radiation or free radical effects.

In order to try to determine whether the inactivation was due to competitive inhibition, or to denaturation, a series of Lineweaver-Burk plots was made, at different concentrations of acrylamide monomer, using the free enzyme. The results are shown in Figure 8. The Michaelis-Menten parameters are presented in Table 3.

It is to be seen that the kinetics of β-galactosidase in acrylamide solutions is fairly complex. There is little change in V_{max} at different concentrations of acrylamide, but considerable change in the apparent Michaelis constant takes place. This is not, however, a simple competitive inhibition effect, since in the cases of 20% and 30% acrylamide, no intercept on the negative side of the 1/[ONPG] axis is obtained. Moreover, the plots are not linear in the high-substrate region. For the same reasons, simple substrate inhibition cannot explain the results. In fact, the results are very similar to those obtained for diffusion-control of the reaction rates, as will be seen in Chapters 3 and 4.

Diffusion effects for enzymes in polymer solutions have been reported by Laurent (94). According to his view, the enzyme is restricted by the long polymer chains present in solution. Laurent's experimental results show an increase in the apparent Michaelis constant as the polymer concentration is increased. It is quite possible that in the present

Figure 8

Lineweaver-Burk plots for β-galactosidase in solutions of 10%, 20% and 30% acrylamide monomer, and in the absence of free radical initiators.

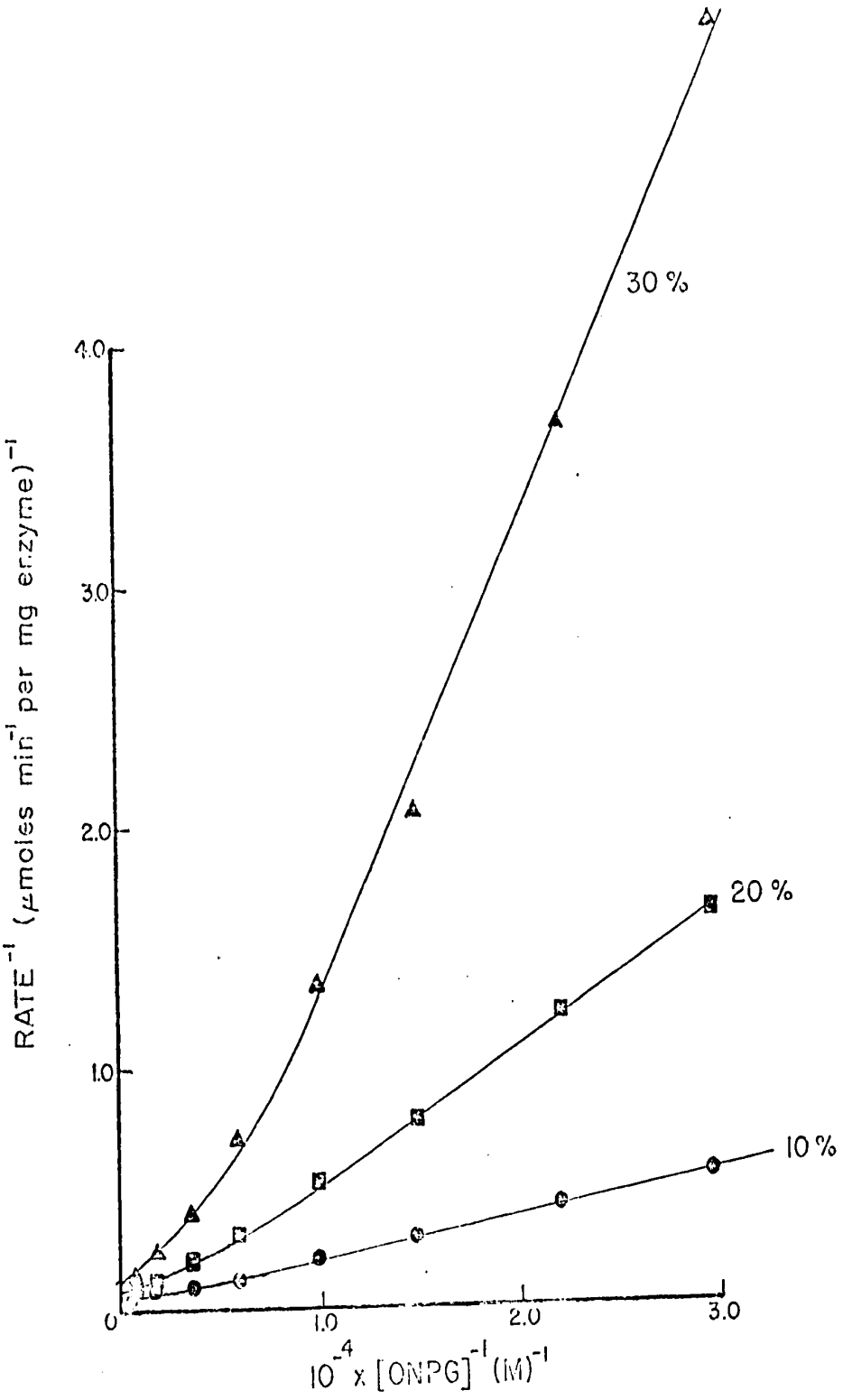


TABLE 3

Michaelis-Menten Parameters for Free

β-galactosidase in Acrylamide Monomer Solutions

Percent Acrylamide (w/v)	$10^4 \times K_m$(app) (M)	V_{max} (μmoles min^{-1} per mg enzyme)
0	1.9	30
10	4.0	27
20	5.6	17
30	25	20

work some of the acrylamide exists in solution as the polymer, since polymerization is photo-catalyzed.

Whatever the actual cause of this rise in K_m(app), it is clear that the fall-off in activity of both the immobilized enzyme and the enzyme in free solution, are related to the acrylamide itself, and not to the γ-radiation or to the free radicals.

Loss of Activity at Low Acrylamide Concentrations Figure 6 shows that with the irradiated systems there is an initial rise in activity at lower acrylamide concentrations; this is not found with the unirradiated systems. These facts suggest that the acrylamide and its polymer provide protection of the enzyme from the free radicals generated by the γ-radiation (mainly of the water molecules) or the photochemical process, and propagated by the polymerization reactions. There appears to be considerable protection; it is only dominated by the inactivation process at about 20% acrylamide in the case of the gels. There appears to be less protection by the monomer than by the polymer, the curve for the free irradiated enzyme starting to fall off at much lower acrylamide concentrations (Figure 6). Similar protection from γ-radiation for trypsin in acrylamide solutions has been reported in a patent by Dobo and Karpathy (88).

Further evidence for this protection is provided by the results shown in Figure 9 for β-galactosidase and in Figure 10 for α-chymotrypsin. These curves show rates plotted

Figure 9

Activity of β-galactosidase as a function of time of γ-irradiation, for unprotected enzyme in buffer solution, and for enzyme protected by 15% acrylamide. Intensity of radiation = 2,380 rads per minute; concentration of enzyme = 1.67×10^{-3} mg ml^{-1}; [ONPG] in assay = 1.33×10^{-3} M; other conditions as in Figure 4.

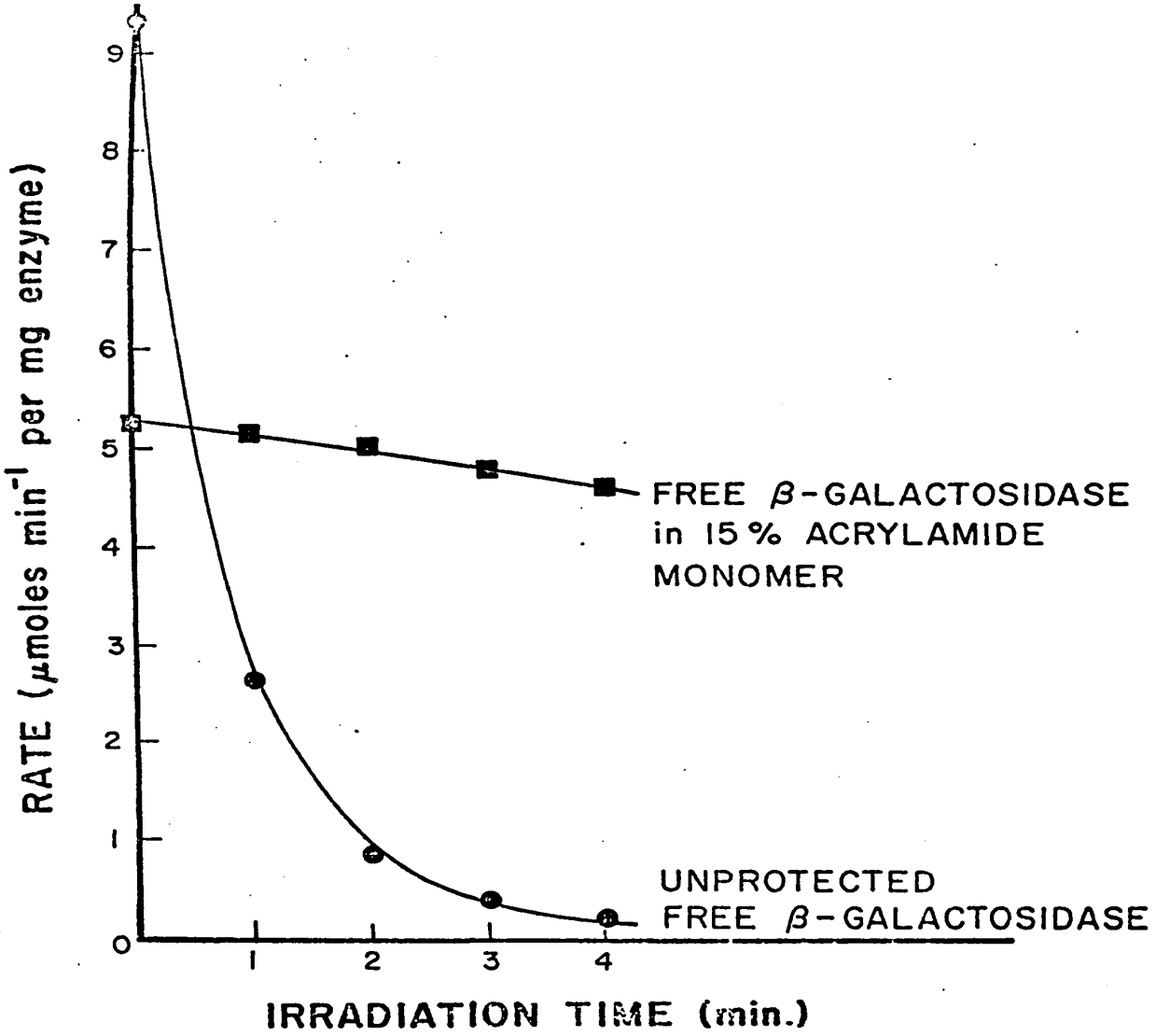
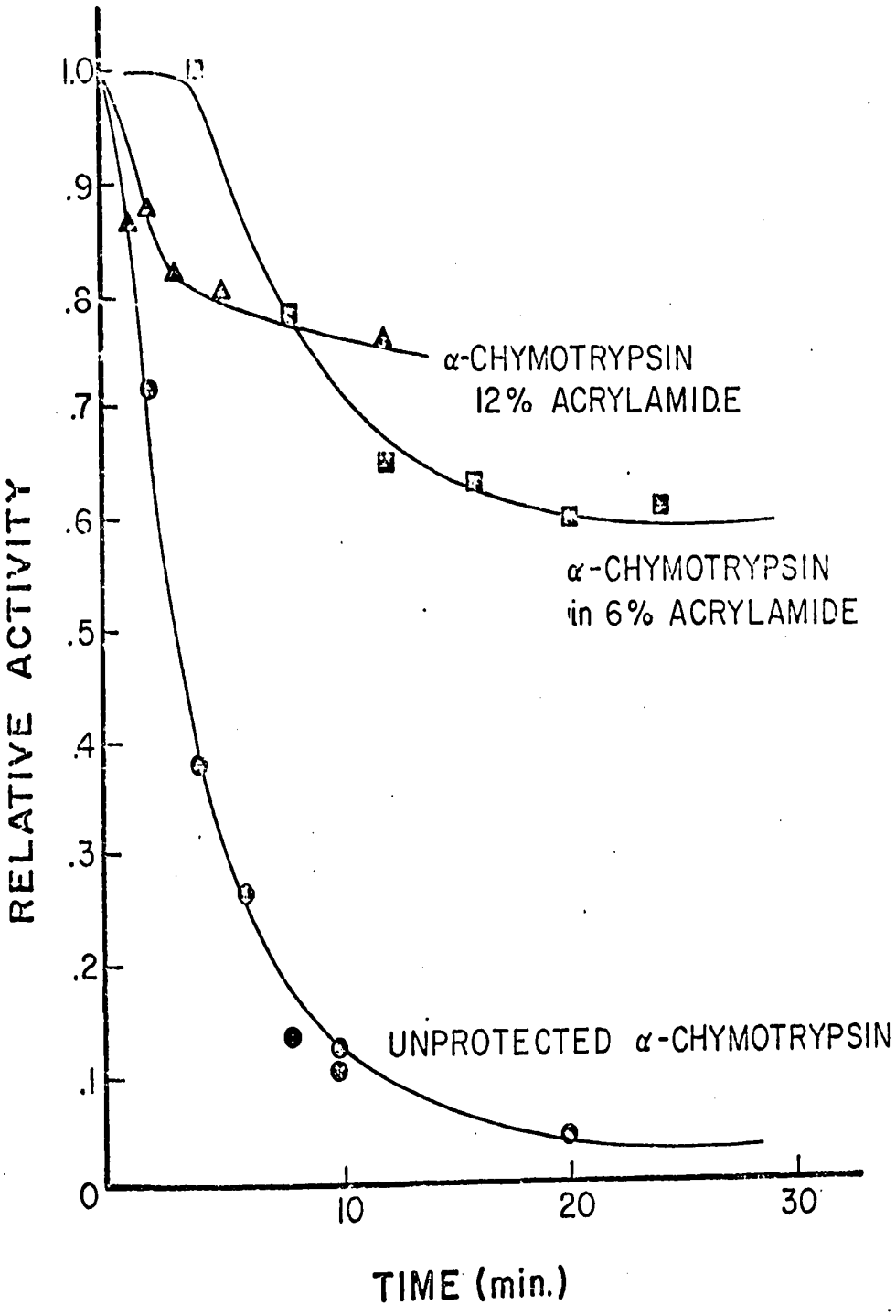


Figure 10

Activity of α-chymotrypsin as a function of time of γ-irradiation, for unprotected enzyme in water, and for enzyme protected by 6% and by 12% acrylamide. Intensity of radiation = 840 rads per minute; concentration of enzyme = 4.3×10^{-7} M (based on a M.W. of 23,800); [ATEE] in assay = 2.0×10^{-3} M; other conditions as in Figure 7.



against time of irradiation. In both cases, the unprotected enzyme rapidly loses activity, whereas in the presence of acrylamide, higher activities for the free enzyme are observed, despite the inactivating effect of the acrylamide.

Activities of the Supported Enzymes Figure 6 demonstrates for β-galactosidase that the supported enzyme prepared by high and low intensity γ-radiation have similar activities; and that these are different from the activity of the chemically polymerized gels. Lineweaver-Burk plots for the photopolymerized and γ-irradiated samples, and for the free enzyme, are shown in Figure 11. The Michaelis-Menten parameters are given in Table 4 and support the above conclusion; these were obtained by computer analysis (92). For α-chymotrypsin, on the other hand, the supported enzyme produced by γ-irradiation was considerably more active than that produced by the photopolymerization, as seen from Figure 12, which shows the rates for the enzyme during the polymerization process.

Some Physical Properties of the Gels Table 5 shows some diffusion and partition coefficients for the product, o-nitrophenol within the gel. It is to be noted that, as expected, the rate of diffusion is, for the gels prepared by photopolymerization, less at 15% acrylamide than at 7.5% acrylamide. This is because the pore size is smaller for gels containing higher concentrations of acrylamide (81). These diffusion constants are of the same order of magnitude as those reported by other workers

Figure 11

Lineweaver-Burk plots for β-galactosidase in 100 micron slices of polyacrylamide gel. For the γ-irradiation the crosses are for low intensity (720 rads per minute), the squares for high (2,380 rads per minute); both samples had a total dose of 23,800 rads. Concentration of β-galactosidase in the support was 0.50 mg ml^{-1}; other conditions as in Figure 4. Filled circles are for the free enzyme.

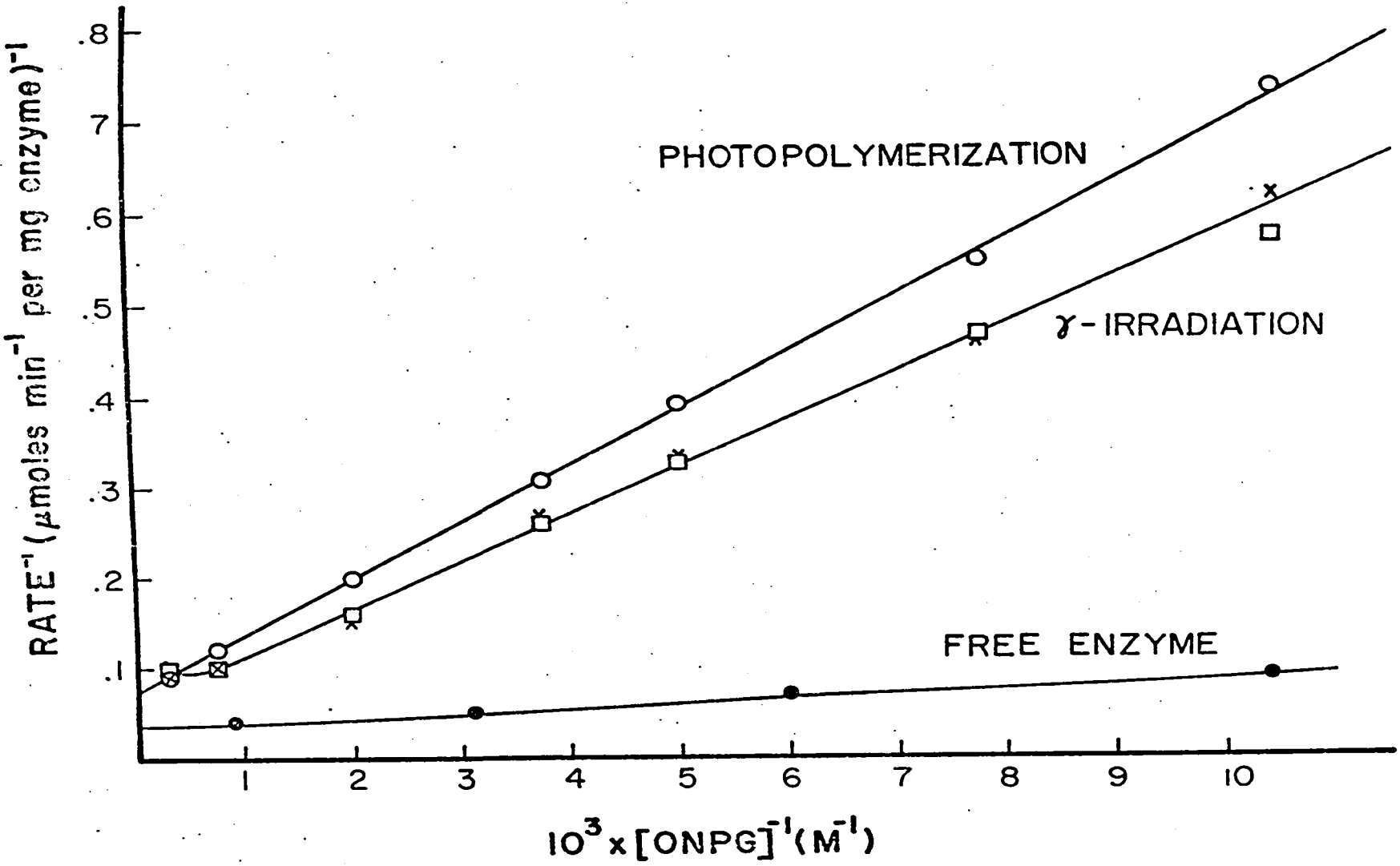


TABLE 4

Michaelis-Menten Parameters for β-Galactosidase under Various Conditions

Conditions	Slice thickness (microns)	Slice diameter (cm)	K_m(app)x10^4 (M)	V_{max}(app) (μmoles min^{-1} per mg enzyme)
Free enzyme	-	-	1.73	30.3
Photopolymerization	120	1.04	8.6	13.8
γ-irradiation (720 rads min^{-1})	107	1.05	8.7	17.1
γ-irradiation (2,380 rads min^{-1}	104	1.06	7.9	15.8

Figure 12

Loss of activity of α-chymotrypsin during the polymerization process, in a 30% w/v solution of acrylamide. γ-irradiation at 830 rads per minute; [ATEE] = 2.0×10^{-3} M; [α-chymotrypsin] in γ-irradiation experiments = 5.0×10^{-7} M; [α-chymotrypsin] in photopolymerization experiments = 4.3×10^{-7} M.

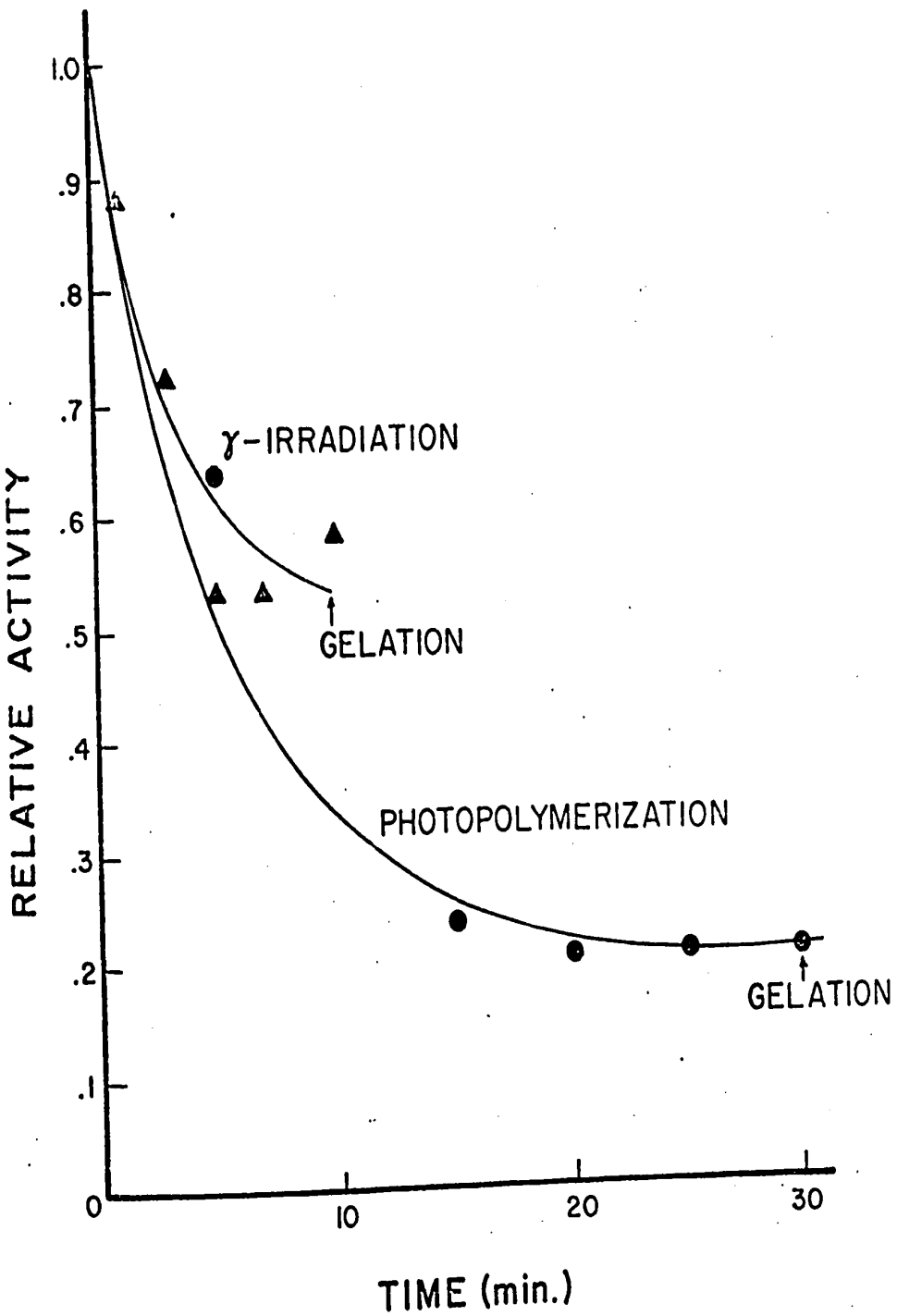


TABLE 5

Diffusion and Partition Coefficients for the Product, o-nitrophenol within the Gel

Conditions	% Acrylamide	Partition Coefficient	Diffusion Constant $x10^6$ ($cm^2 s^{-1}$)
Photopolymerization	7.5	1.4	5.6 ± 0.5
Photopolymerization	15.0	0.89 ± 0.01	3.7 ± 0.3
γ-irradiation	7.5	-	4.7 ± 0.7
γ-irradiation	15.0	-	4.3

in the field of immobilized enzymes (95). The partition coefficients are not very different from unity.

Stability of the Supported Enzymes Studies were made of the rates of loss of activity of the free and supported enzymes, while incubated at 0 and 41°C. Results for β-galactosidase are shown in Figure 13. It is to be seen that at both temperatures the supported enzyme preparations are significantly less stable than the free enzyme. The photopolymerized sample appears to be slightly more stable than that prepared by γ-irradiation.

There is little information in the literature about the stabilities of solid-supported enzyme preparations. Goldman, Goldstein and Katchalski (49) have reviewed some of the available data, but in many cases comparisons with the stability of the free enzyme have not been presented.

Several stability studies have been concerned with enzymes in polyacrylamide gels. Cholinesterase and urease (96), steroid hydroxylase (97), glucose oxidase and lactate dehydrogenase (79), and orsellic acid decarboxylase (98) were all reported stable to storage for several months, when supported in polyacrylamide gels. No comparison with the free enzyme was presented in these instances, however. Phosphoglycerate mutase (77) is equally stable when supported and when free. Enolase (98) and glucoamylase (25) are more stable when supported in polyacrylamide than when in free solution, including at higher temperatures.

Figure 13

Loss of activity with time of free and supported enzymes. In the γ-irradiated systems the intensity was 2,380 rads per minute for 10 minutes. Enzyme concentration in the gels = 0.50 mg ml^{-1}; free enzyme concentration = 2.0×10^{-2} mg ml^{-1}; [ONPG] = 1.33×10^{-3} M.

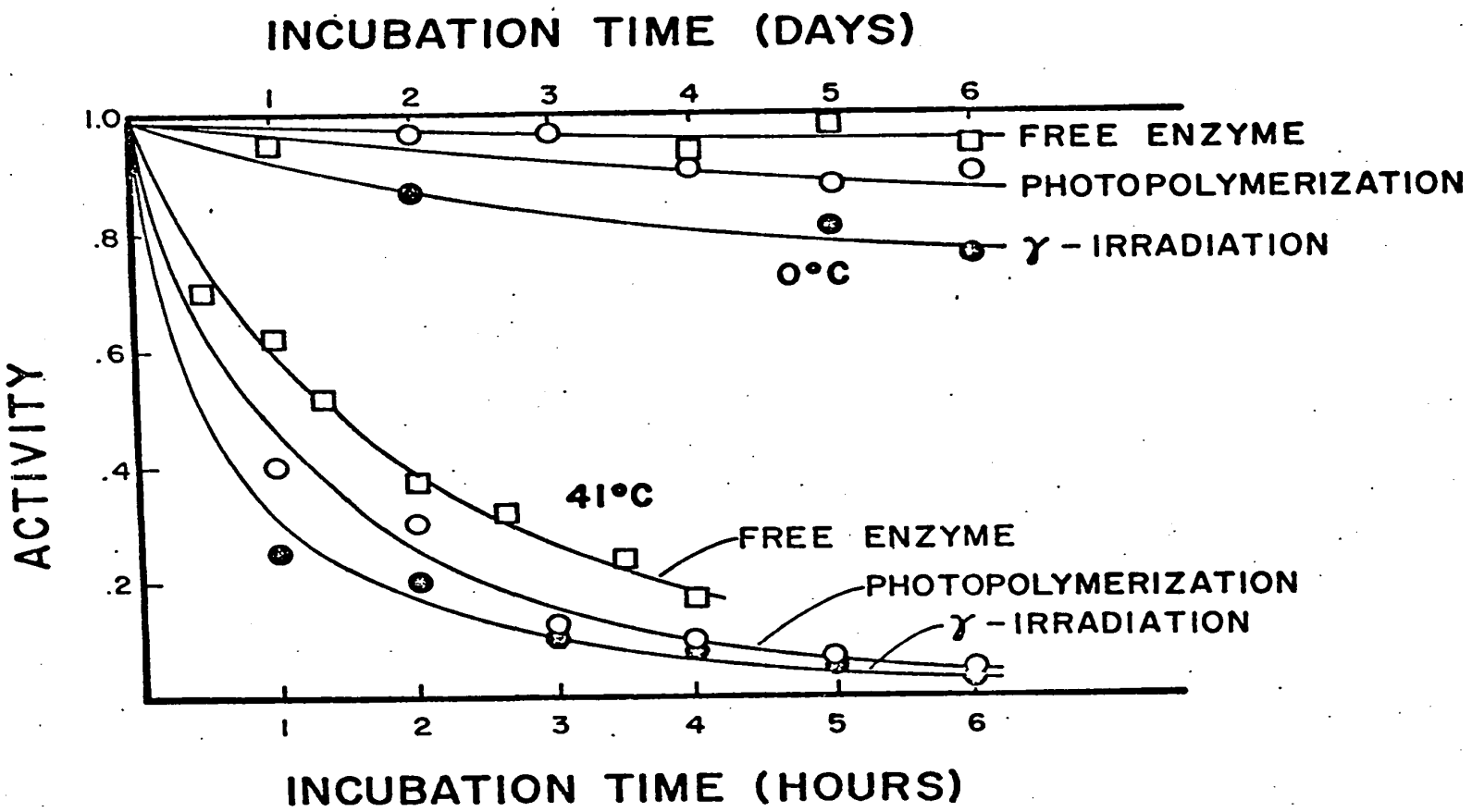


Table 6 is a list of experiments concerning the stability of supported enzyme preparations, which include a comparison with the enzyme in free solution, for various kinds of support. A list of this nature is not available in the literature, and is presented here as a guide to further stability studies. It is to be seen that the stability of a given preparation is not easily related either to the enzyme or to the type of support used. No single support or enzyme yields uniformly stable derivatives, and the stability of a given preparation cannot be predicted in advance.

Effect of Microenvironment Figure 6 shows that the activity of β-galactosidase in free solution is not the same as that in the gel; even the shape of the activity curve is different. This indicates that the microenvironments of the two enzyme preparations are probably different, so that factors (1) and (2) (page 8) probably could not be separated as had been hoped. This is supported by experiments in which gel slices and free enzyme were both assayed in (a) buffer solution and (b) 15% acrylamide monomer solution. With both free and supported β-galactosidase, about 46% of the activity was lost under condition (b) compared with (a), suggesting that the microenvironment of gel and sol are indeed different.

TABLE 6

Stabilities of Supported Enzyme Preparations

Enzymes which are more stable when supported

Type of Support	Enzyme	Temp.	Reference
Nylon micro-capsules	Asparaginase	0, 37	27, 100
	Urease	0, 37	27, 101
	Catalase	0, 37	27, 102
Nylon tubing	Asparaginase	4, 25	22
	Urease	4, 75	19
	Urate oxidase	R.T.	103
	Several dehydrogenases	4	104
Carboxymethyl-cellulose	Ficin	2, 66	69
	Chymotrypsin	Several	105
Cellulose	Lactate dehydrogenase	35 to 65	106
Cellulose derivative	Chymotrypsin	R.T.	108
	Aminoacylase	80	107
	Trypsin	55	109
Cellophane	Glucose oxidase	37	110
Agarose	Elastase	37	111
Sephadex	Luciferase	135	112
	Glycerol dehydratase	110	113
Starch derivative	Papain	40	114
	Subtilopeptidase A	37	110,115
	Trypsin	37	115
	Glyceraldehyde-3-phosphate dehydrogenase	R.T.	116
Silastic	Acetylcholinesterase	60	117
Ethylene-maleic anhydride copolymer	Trypsin	R.T.	118

Table 6 continued

Maleic anhydride-bis-(vinyloxy)butane	Trypsin	55	109
None - polytyrosyl enzyme derivative	Trypsin	25	119
Glass	Papain	88	121
Polyacrylamide	Trypsin	100	120
	Enolase	R.T.	99
	Glucoamylase	R.T.	25
Enzymes which are less stable when supported			
Carboxymethyl-cellulose	Ribonuclease	R.T.	108
	Papain	R.T.	122
	Glucose oxidase	5, 23	123
	Peroxidase	R.T.	124
	Trypsin	R.T.	125
Collodion	Alkaline phosphatase	60, 80	126
	Glucose-6-phosphate dehydrogenase	27	127
	Papain	30 to 80	95
Ethylene-maleic anhydride copolymer	Carboxypeptidases A and B, and Kallikrein	R.T.	128
Leucine-p-aminophenyl-alanine copolymer	Papain	20 to 90	122
Ni/NiO-aminopropyl-ethoxysilane	Glucose oxidase	R.T.	129
Calcium phosphate	Leucine aminopeptidase	55	130
Various	Papain	R.T.	131
Silane derivative	Trypsin and chymo-trypsin	R.T.	121

Table 6 concluded

Enzymes equally stable when supported

Carboxymethyl-cellulose	Acetylcholinesterase	R.T.	132
	Bromelain	60, 70	133
Agarose	Cholinesterase	25,37,60	134
Polyacrylamide	Phosphoglycerate mutase	0 to 60	78
	Aldolase	R.T.	135
Collodion micro-capsules	Catalase	R.T.	102
Various	Cholinesterase	various	133

CHAPTER THREE

KINETIC STUDIES ON β-GALACTOSIDASE IN POLYACRYLAMIDE MEMBRANES

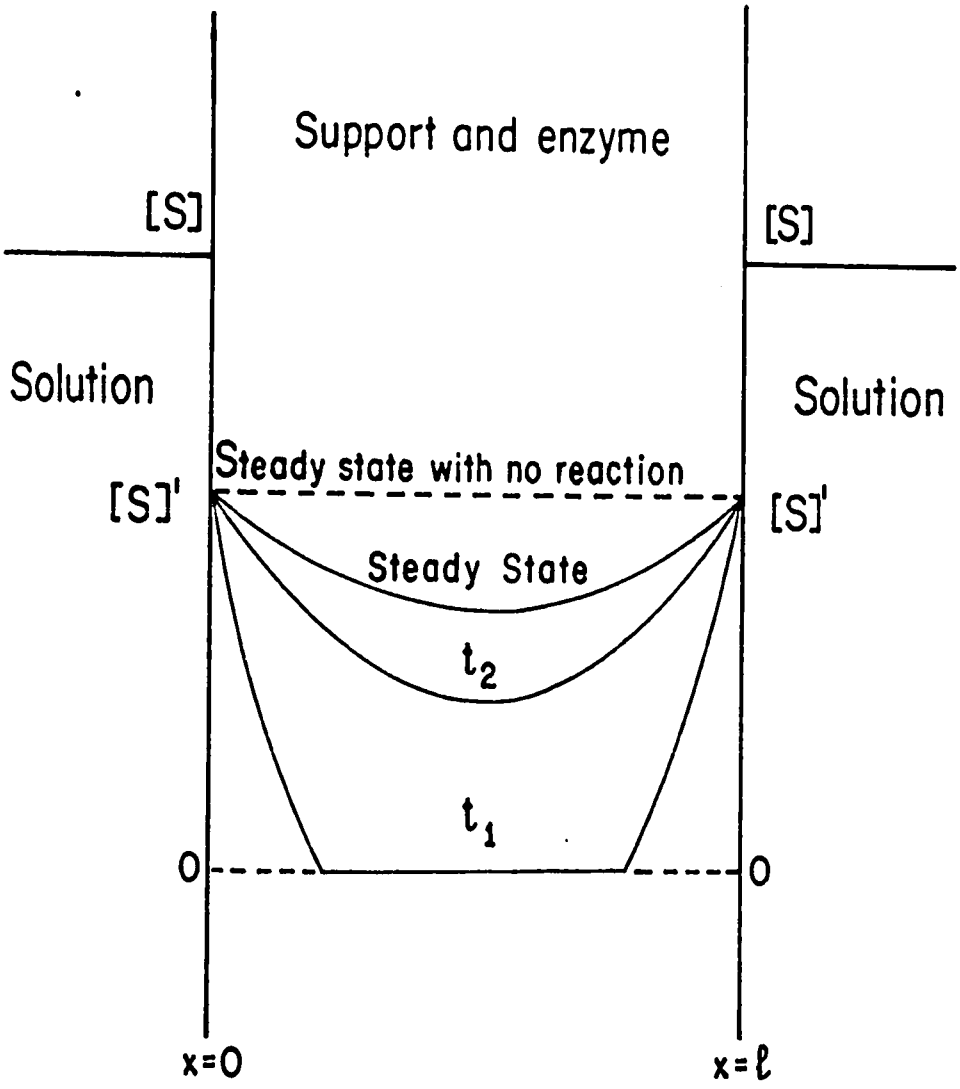
In this chapter, the experimental results relating to the kinetics of β-galactosidase in a membrane system will be presented. Since the experiments were designed as a test of the theoretical treatments mentioned previously, the main points of the theory will first be outlined. The treatment follows that of Sundaram, Tweedale and Laidler (42), although similar approaches have been used by Goldman, Kedem and Katchalski (53) and by Sluyterman and van der Graaf (54). Additional aspects have been explored in a review by Laidler and Sundaram (3).

Theory of Diffusion in Solid-Supported Enzyme Membranes

Figure 14 shows the manner in which the concentration of substrate is expected to vary in an enzyme-containing membrane. The substrate concentration in the bulk solution is S, and that in the solid support is S'. P is the partition coefficient relating the two ($P = S'/S$). After a short period of time, t_1, the substrate concentration will fall sharply to zero at each interface, owing to slow diffusion through the solid matrix, and also to utilization of substrate by the enzyme. After a longer period of time, the curve corresponds to that of t_2 in Figure 14. Ultimately, a steady state con-

Figure 14

The variation in substrate concentration in a membrane of thickness, ℓ, at different times in the approach to a steady state.



centration distribution is obtained. This is a horizontal line if no reaction takes place, or for a very inactive membrane (or for a very thin one). It is this substrate distribution curve which gives rise to the diffusion effects discussed below.

It is important to know how long it will take for such a system to reach the steady state under different conditions. The non-steady state equation for the variation in substrate concentration, s, with time, t, is given (136) by:

$$S=S'\left|1-\frac{2}{\pi}\sum_{n=1}^{\infty}\frac{1-\cos n\pi}{n}.\sin\left(\frac{n\pi x}{\ell}\right)\exp\left(-\frac{Dn^2\pi^2 t}{\ell^2}\right)\right| \qquad (1)$$

where x (cm) is the distance along the membrane (from x = 0 to x = ℓ), ℓ is the membrane thickness (cm), and D is the diffusion coefficient ($cm^2 sec^{-1}$) for the substrate. Taking the leading term of n = 1 as being predominant near the steady state, the time (τ) required to come within 10% of the steady state substrate concentration reduces to:

$$\frac{4}{\pi}\exp\left(\frac{D\pi^2\tau}{\ell^2}\right)=\frac{9}{10} \qquad (2)$$

or

$$\tau = 0.257\,\frac{\ell^2}{D} \qquad (3)$$

As will be seen below, for membranes thinner than 100 microns, the steady state is probably established within seconds. For much thicker membranes (e.g. 1000 microns), the steady state may take ten to fifteen minutes to be established.

The equation for the rate of an enzyme reaction

within the membrane is:

$$v' = \frac{k'_c . E_m . S'}{K'_m + S'} \qquad (4)$$

S' being the concentration of substrate within the support, and E_m the concentration of enzyme within the membrane, in moles per litre. The experimentally measured rate, with respect to the bulk solution, is given by:

$$v = \frac{k_c(app)\ E.S}{K_m(app) + S} \qquad (5)$$

to a good approximation, as indicated by computer calculations (42). Here, E is the number of moles of enzyme in a membrane of cross-sectional area A (cm^2) and thickness ℓ (cm). (As will be seen below, this equation is not strictly correct, since true Michaelis-Menten kinetics probably do not apply).

The theoretical treatment (42) attempts to relate these two equations.

In the case of a membrane of high activity (i.e. high enzyme concentration, or a fast-acting enzyme), there will be a significant variation of the substrate concentration within the membrane. In the steady state, the enzyme rate may be equated with the rate of diffusion (given by Fick's second law):

$$\frac{k'_c . E_m . s}{K'_m + s} = D\left(\frac{\partial^2 s}{\partial x^2}\right)_t \qquad (6)$$

where s has been defined by equation 1. Equation 6 cannot be solved analytically, except under two limiting conditions, (a) and (b) below.

(a) High Substrate Concentrations ($s >> K_m'$)

The equation to be solved is:

$$\frac{\partial^2 s}{\partial x^2} = \beta$$

where $$\beta = \frac{k_c' E_m}{D} \text{ moles cm}^{-5} \quad (7)$$

Application of appropriate boundary conditions leads to:

$$s = S' - \frac{1}{2}\beta x(\ell - x) \quad (8)$$

The rate for a membrane of cross-sectional area, A cm^2 is thus

$$v' = \beta AD\ell \text{ moles sec}^{-1} \quad (9)$$

whence the rate per litre of support is

$$v' = k_c' E_m \text{ moles litre}^{-1} \text{ sec}^{-1} \quad (10)$$

The above equations lead to three qualitative features of the kinetics under this limiting condition:

(1) The rate per litre of support is proportional to the enzyme concentration within the membrane (eq. 10).

(2) The rate per membrane slice is directly proportional to the slice thickness (from eq. 9).

(3) No diffusion effect is predicted for V_{max} ($=k_c'E$). The two equations (4 and 5) are related as follows:

$$k_c(app) = k_c'$$

(b) Low Substrate Concentrations ($s << K'_m$)

The equation to be solved is now:

$$\frac{\partial^2 s}{\partial x^2} = \alpha^2 s \tag{11}$$

where

$$\alpha = \left(\frac{k'_c E_m}{K'_m D}\right)^{1/2} \text{ cm}^{-1} \quad \text{from eq. 6} \tag{12}$$

Applying the appropriate boundary conditions, we have:

$$s = S' \cdot \frac{\sinh\alpha x + \sinh\alpha(\ell - x)}{\sinh\alpha\ell} \tag{13}$$

whence the rate per unit volume of support is:

$$v' = \frac{k'_c E_m}{K'_m} \cdot S' \cdot \frac{2}{\alpha\ell} \cdot \frac{\cosh\alpha\ell - 1}{\sinh\alpha\ell} \tag{14}$$

A similar expression has been derived by Goldman, Kedem and Katchalski (53). If the substitution is made that $\gamma = \frac{\alpha}{2}$, the above expression reduces to

$$v' = \frac{k'_c E_m}{K'_m} \cdot S' \cdot F \tag{15}$$

where

$$F = \frac{\tanh\gamma\ell}{\gamma\ell} \tag{16}$$

and

$$\gamma = \left(\frac{k'_c E_m}{4DK'_m}\right)^{1/2} \tag{17}$$

Since

$$S' = PS,$$

$$v' = \frac{k'_c E_m}{K'_m/PF} \cdot S' \qquad (18)$$

Rates at low substrate concentration, therefore, are affected by diffusion to an extent which is dependent upon the value of F. Figure 15 is a plot of F versus $\gamma\ell$. It is to be seen that F is close to unity (i.e. no diffusion effect is involved) at $\gamma\ell$ values less than about 0.1. This is the equivalent of a low-activity membrane. If experimental conditions are adjusted to bring this about, the true Michaelis constant, K'_m, may be determined, uncomplicated by diffusion, if P is known. It is to be further noted that for higher values of $\gamma\ell$ (greater than about 2) F is given by $1/\gamma\ell$ since $\tanh\gamma\ell \to 1$. Under this condition, the rate of reaction is proportional to $E_m^{1/2}$ since (from equations 16, 17 and 18) the rate per unit volume of <u>support</u> is:

$$v' \propto \frac{E_m}{\gamma\ell} \qquad (19)$$

$$\propto E_m^{1/2}$$

Furthermore, there is no dependence upon thickness of the rate per unit volume of <u>solution</u> at constant values of E_m. This arises, since E and E_m are related to each other as follows:

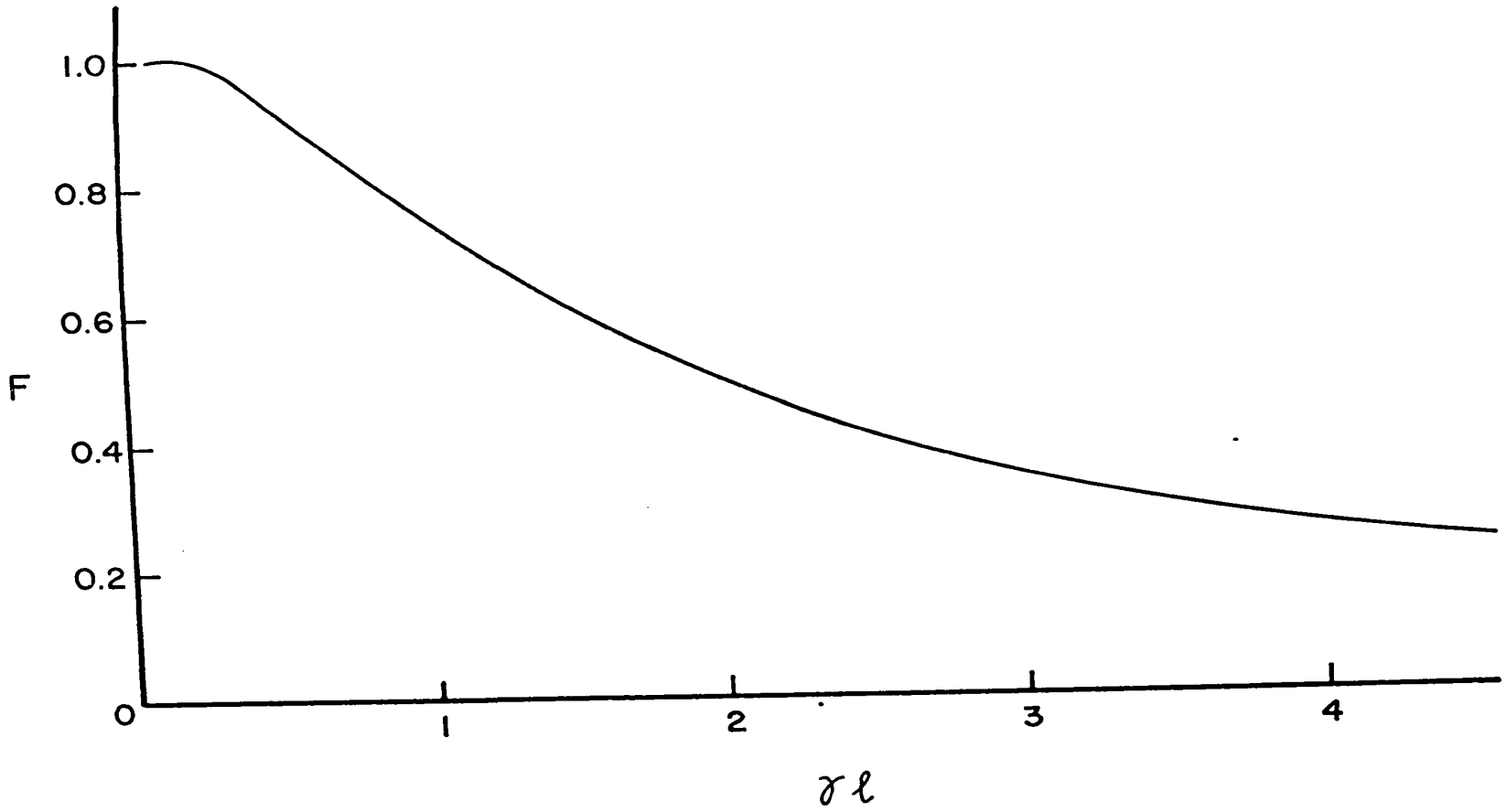
$$E = 10^{-3} E_m . A\ell \quad \text{moles}$$

and since the rate per slice is given by:

$$v' = \frac{k'_c E_m A\ell/10^3}{K'_m/PF} \cdot S'$$

Figure 15

A plot of F against $\gamma\ell$.



i.e.

$$v' \propto \frac{E_m A \ell}{\gamma \ell} \quad \text{at high values of } \gamma\ell. \tag{20}$$

A pair of parameters similar to α and ℓ has been obtained for the kinetics of enzyme reactions inside spherical particles (60), leading to a similar dependence of the rate upon E_m and $E_m^{1/2}$.

In summary, the following qualitative features emerge for the kinetics under the limiting condition of low substrate concentrations:

(1) The rate may be to some extent diffusion-controlled, the diffusion affecting the value of $K_m(app)$.

(2) The rate of reaction per unit volume of support is proportional to $\sqrt{E_m}$, in contrast with case (a).

(3) The rate of reaction is independent of thickness at constant values of E_m, again in contrast with case (a).

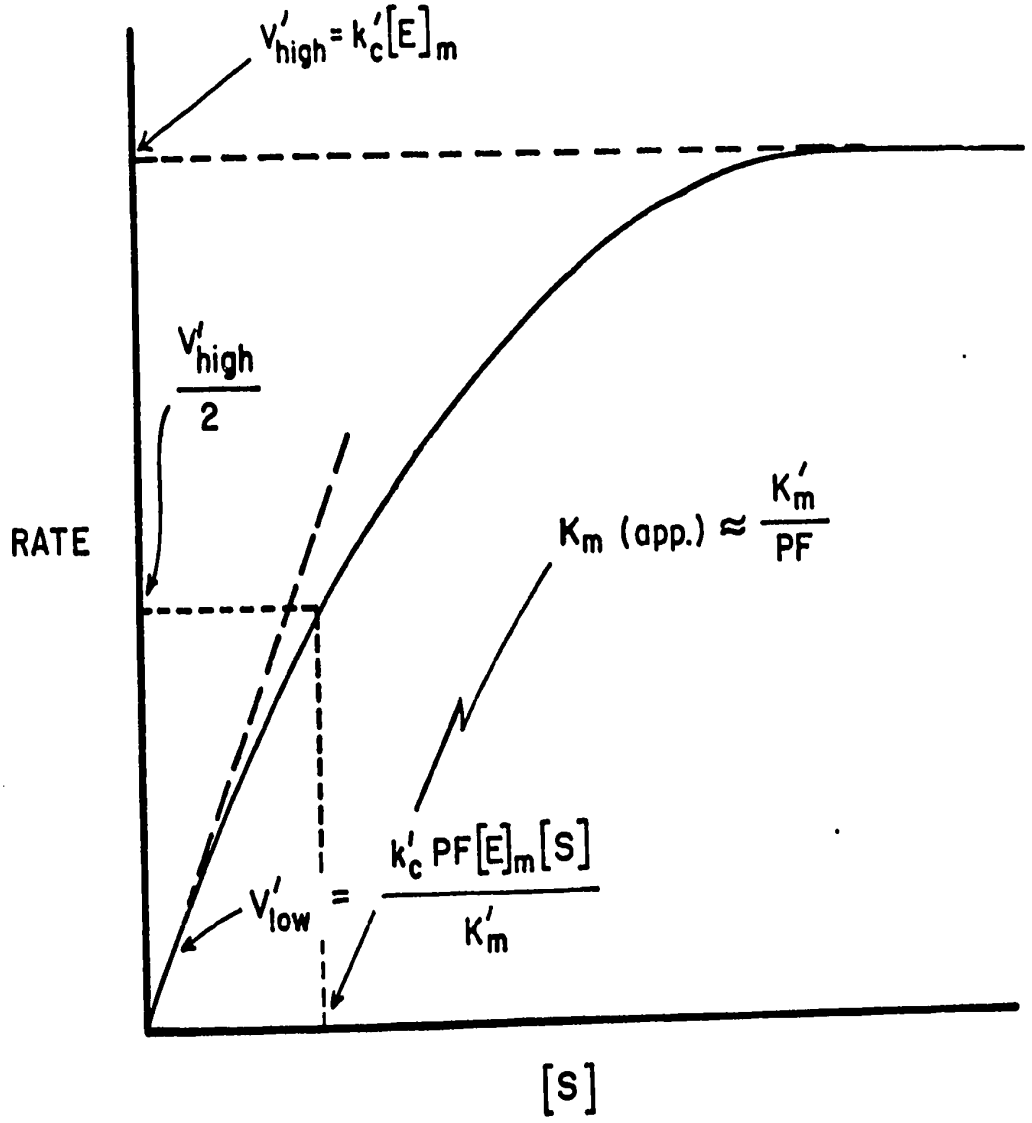
Figure 16 summarizes the results under both sets of limiting conditions. Equations 4 and 5 are related as follows:

$$k_c(app) = k_c' \tag{21}$$

$$K_m(app) = \frac{K_m'}{PF} \tag{22}$$

Figure 16

A schematic plot of the rate of a reaction catalyzed by a solid-supported enzyme against the substrate concentration in the external solution. The effective parameters for the enzyme within the support are k_c' and K_m', and $[E]_m$ is its concentration in the support. P is the partition coefficient and F the function plotted in Figure 15 (cf. eq. 16).



Results

Qualitative Aspects of the Theory

All experimental details were the same as those described in Chapter 2. Polymerization was brought about photochemically only.

The influence of slice thickness on the rate of reaction, at constant enzyme concentration within the gel, is shown in Figure 17, for three different enzyme concentrations. Initially, the curves are a linear function of slice thickness, as expected under limiting condition (a). This is followed by a levelling-off when the reaction becomes diffusion-controlled, and limiting condition (b) is attained, when the rate is expected to be independent of thickness, as discussed above. (It is to be noted that the higher the enzyme concentration, the smaller is the thickness at which diffusion becomes rate-limiting). A simple calculation from the results shows that the changeover from condition (a) to (b) in each case occurs when the apparent K_m is from five to ten times larger than the substrate concentration.

Figures 18 and 19 give the variation of the rate with the concentration of enzyme within the polyacrylamide gel. In Figure 18, the rate is plotted against the first power of the enzyme concentration; it is to be seen that there is a linear dependence for the 100-micron slices, but not for slicesof 200 microns and above. A square-root

Figure 17

Influence of slice thickness on the rate (per liter of solution), at three enzyme concentrations. [ONPG] = 1.66×10^{-3} M; other conditions as before.

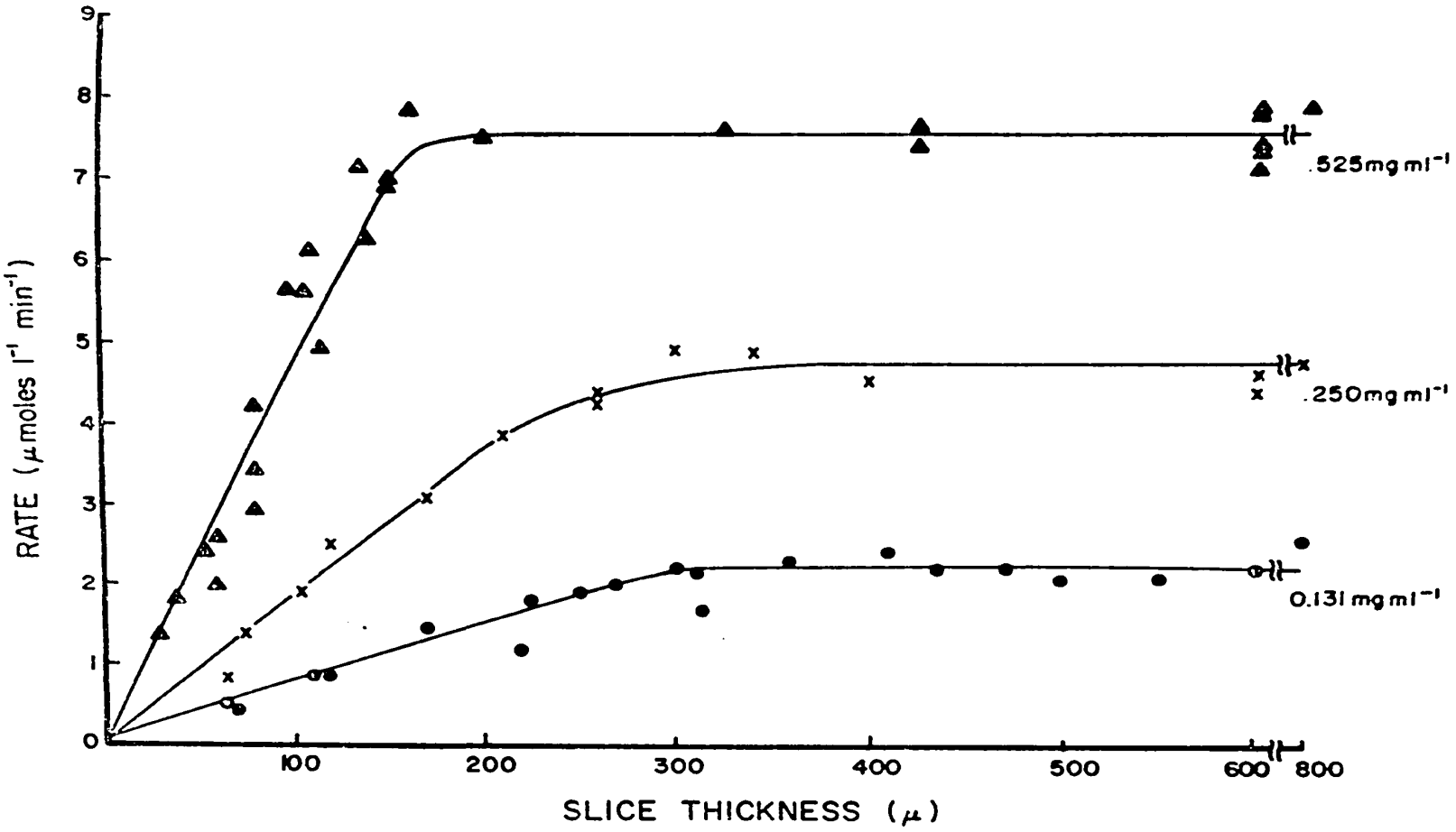


Figure 18

Plots of rate (per liter of solution) against enzyme concentration within the support, for four slice thicknesses.

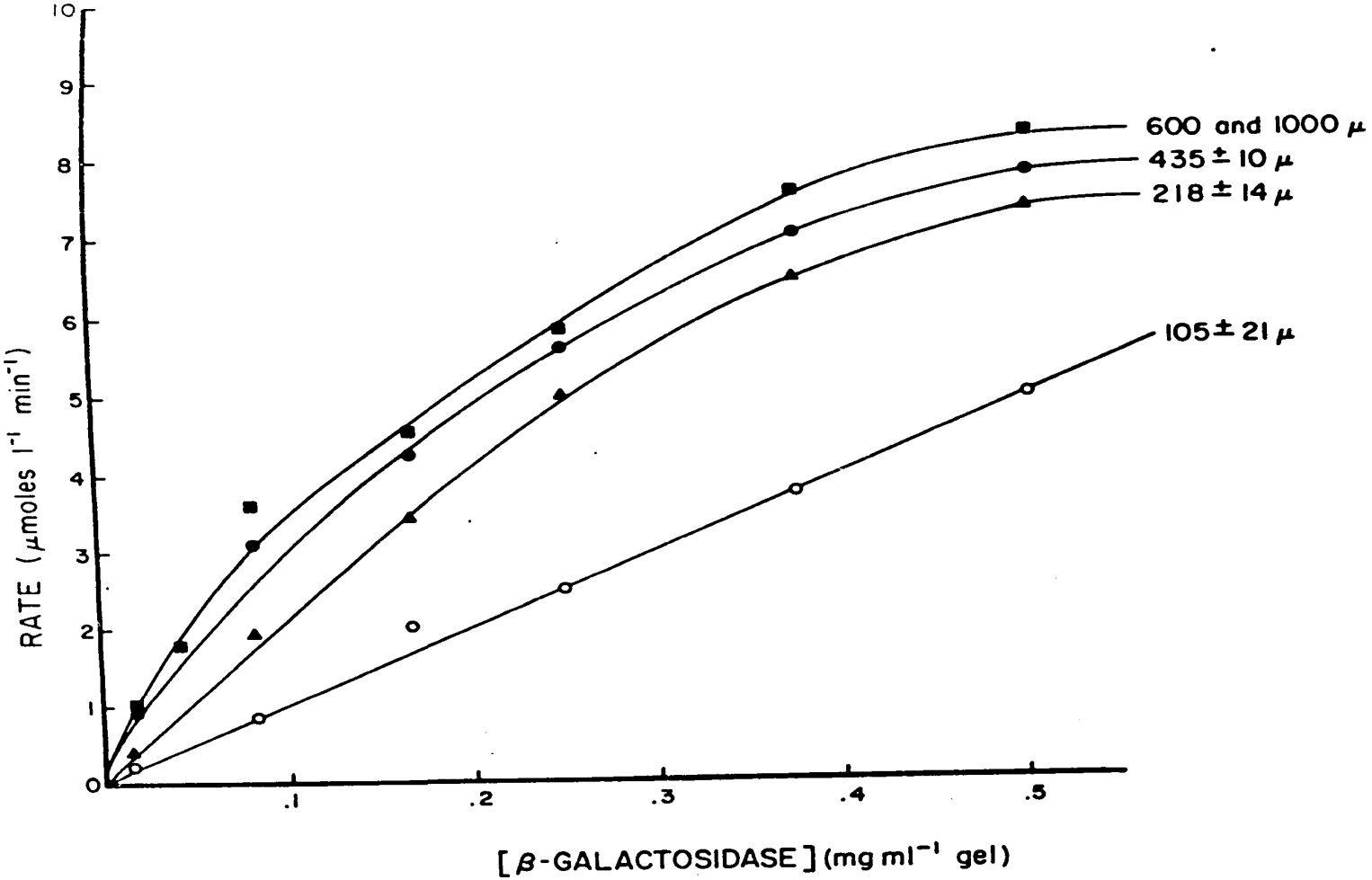
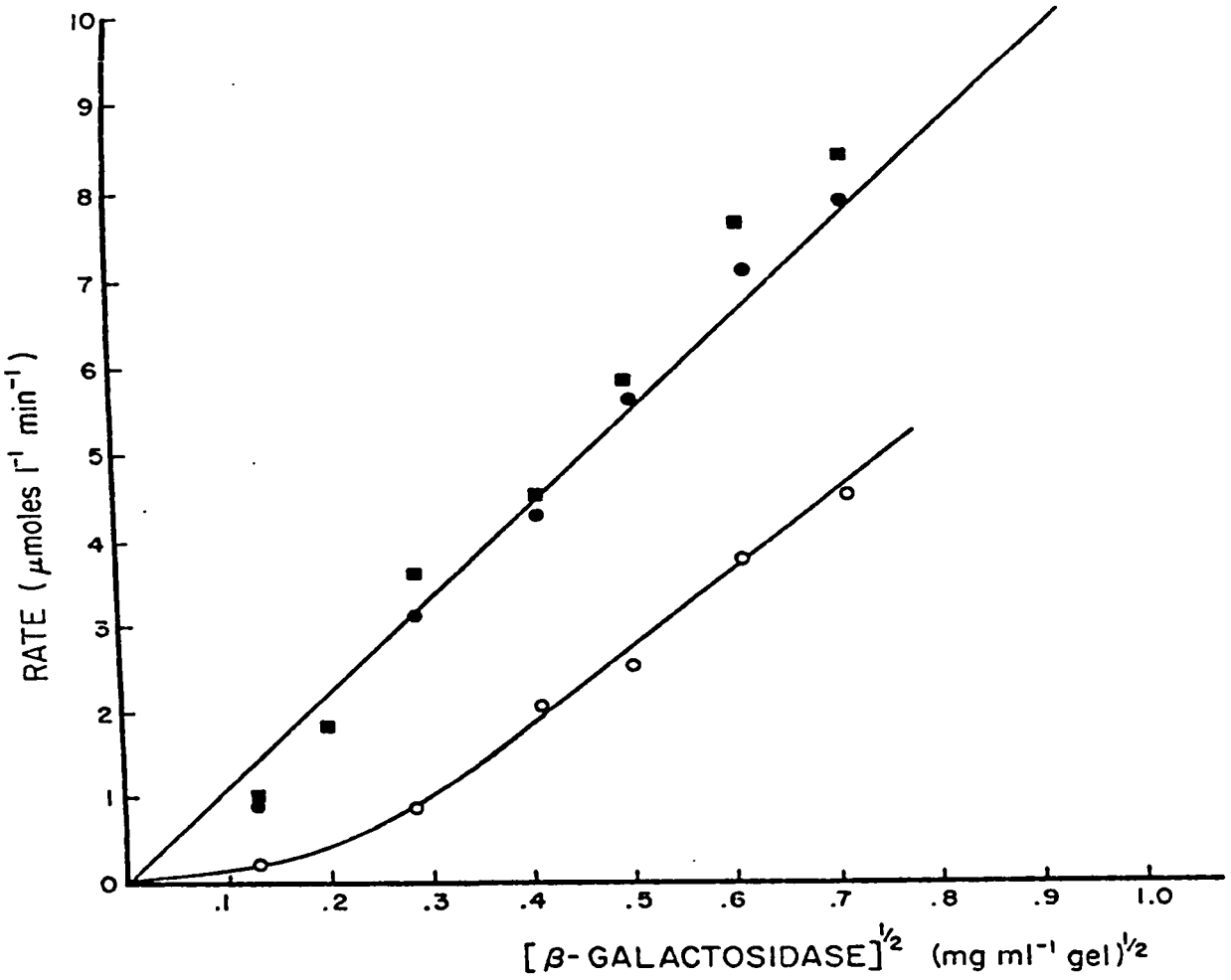


Figure 19

Plots of rate against the square root of the enzyme concentration. The open circles refer to 100μ slices; the filled circles to 400μ slices; and the filled squares to 600μ and 1000μ slices.



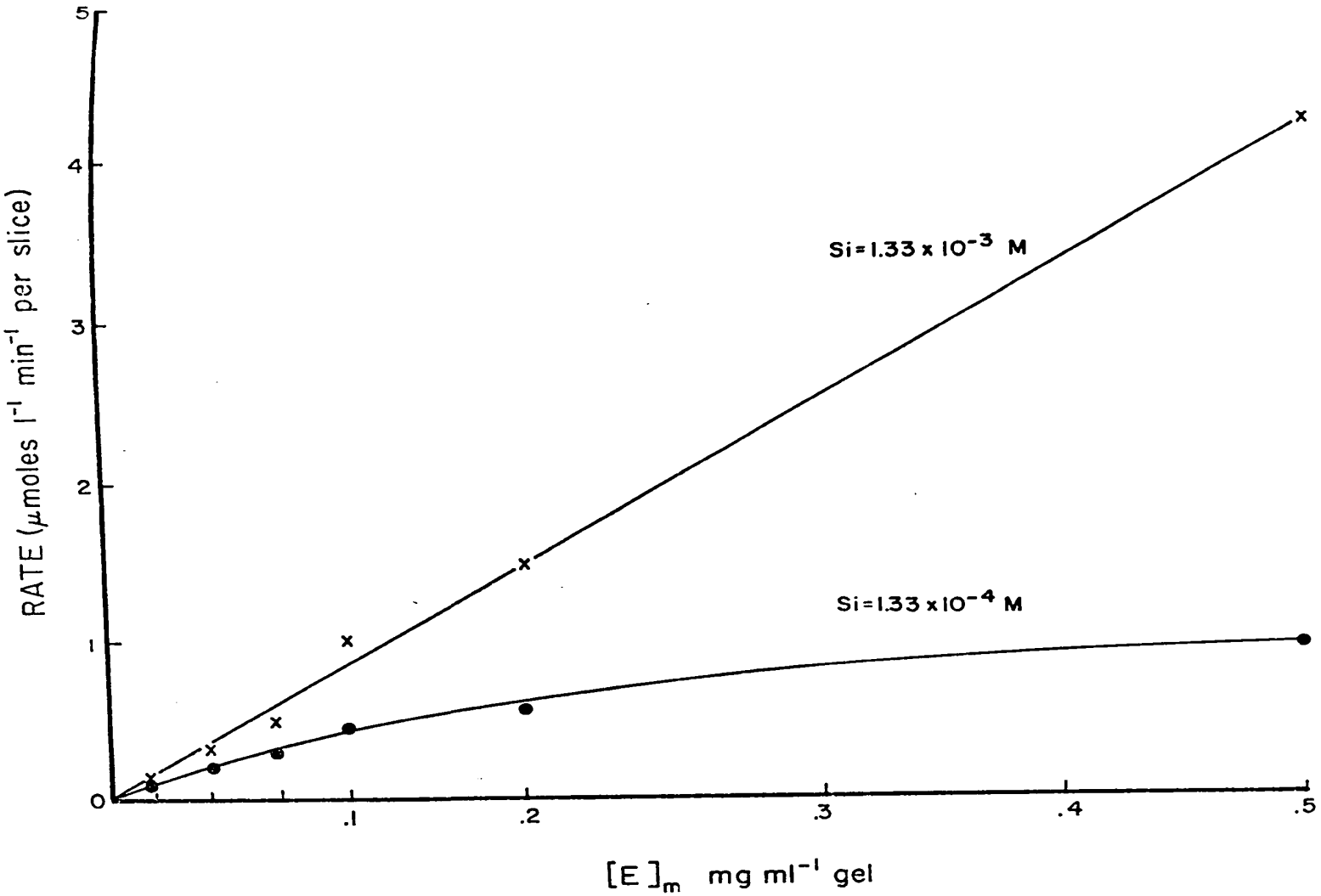
dependence applies to the thicker slices, as shown in Figure 19. This is predicted on the basis of the theory discussed above: the 100-micron slices conform to limiting condition (a), while the thicker slices correspond to condition (b). At the highest concentration of enzyme, the substrate concentration used is considerably greater than the apparent Michaelis constant for the 100-micron slices; but for the others it is of the same order of magnitude, so that the rate is diffusion-controlled.

Figure 20 is a comparison between rates at high and low substrate concentrations for slices 100 microns thick, the two concentrations differing by a factor of ten. It is to be seen that the rate is directly proportional to the enzyme concentration for the high substrate case, as expected for 100-micron slices. At low substrate concentration, however, the square-root relationship is once again obeyed. While the apparent Michaelis constant remains unchanged, the substrate concentration has become so low that condition (b) now applies.

A check was made to confirm that for the free enzyme, in the presence of acrylamide monomer, the rates were directly proportional to the concentration of enzyme. This is displayed in Figure 5. The results above are therefore probably not subject to environmental or other factors, but to diffusion effects.

Figure 20

A plot of rate against concentration of β-galactosidase in 100μ slices of gel, at high (x) and low (o) substrate concentrations.



Lineweaver-Burk plots of 1/v against 1/[ONPG] are shown for various slices in Figures 21 and 22. Such plots were also made for the free enzyme, yielding the following values for the kinetic parameters:

$$K_m = 1.73 \times 10^{-4} \quad (\pm 4\%)$$

$$k_c = 273 \text{ sec}^{-1} \quad (\pm 2\%)$$

The value for k_c was obtained on the assumption of a pure enzyme preparation, and a molecular weight of 540,000 (137). The parameters differ somewhat from those quoted in the literature (86), but it is to be noted that assay conditions (e.g. ionic strengths) are different and that Mg^{2+}, which activates the enzyme, was not used in the present experiments.

The kinetic parameters obtained in the solid-supported enzyme experiments are shown in Table 7. With regard to these values, it should be mentioned that the results with the 1000 micron slices probably do not correspond to attainment of the steady state. The theory (42) predicts that roughly 12 minutes are required for the establishment of the steady state; a linear rate was observed in all experiments for only a few minutes, so that rates for these slices probably correspond to pre-steady state conditions. For the 400-micron slices, only about two minutes are needed to establish the steady state, so that the results are fairly reliable as representing steady-state conditions. For the 100-micron slices, a steady state is established

Figure 21

Lineweaver-Burk plots for 120,420 and 1030μ slices; each point is an average of 6-7 determinations, for which the average error was ∿12%; other conditions as before.

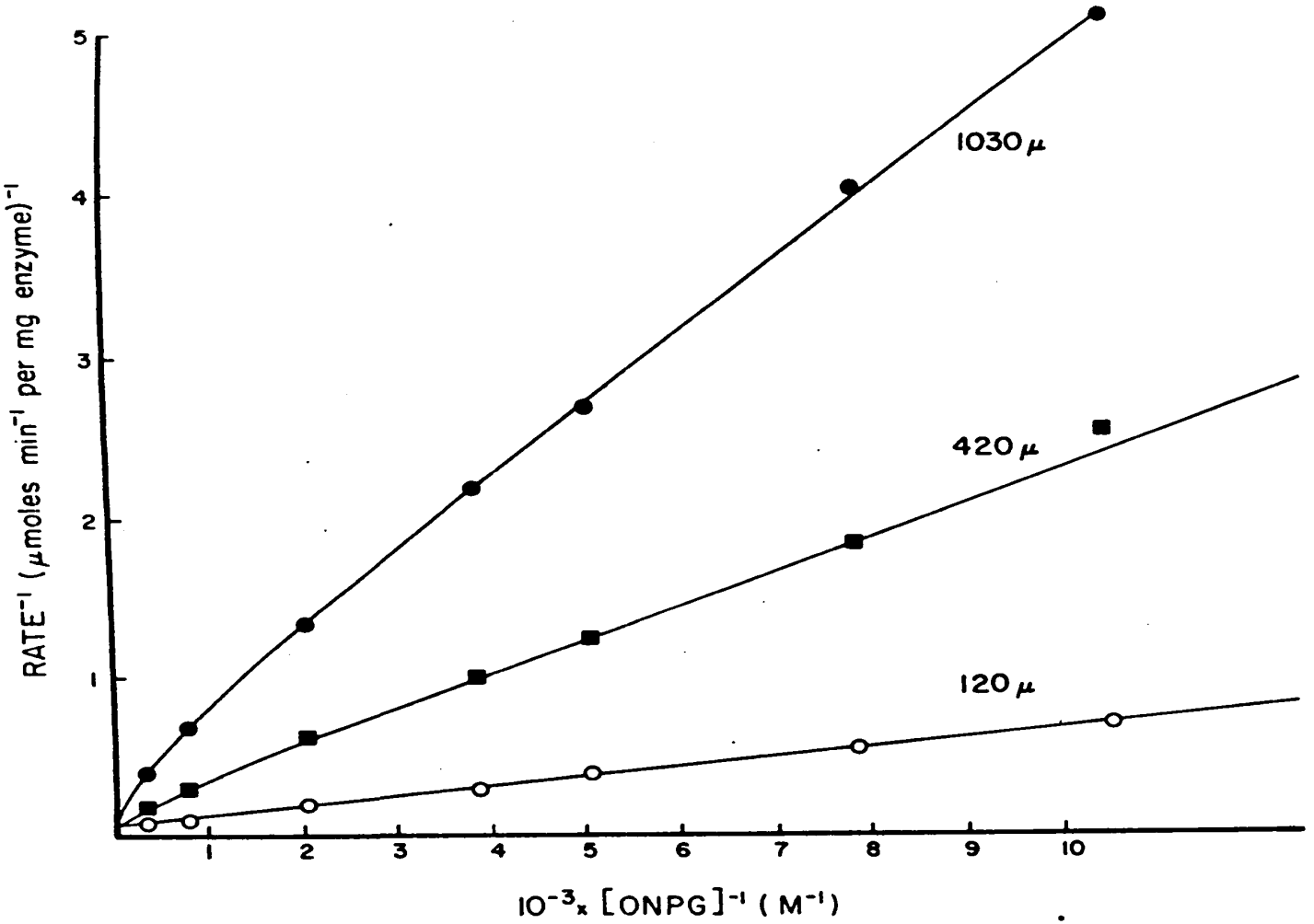


Figure 22

Lineweaver-Burk plots for the supported enzyme, for 110μ slices and at three enzyme concentrations. Each point is an average of three determinations; conditions as previously.

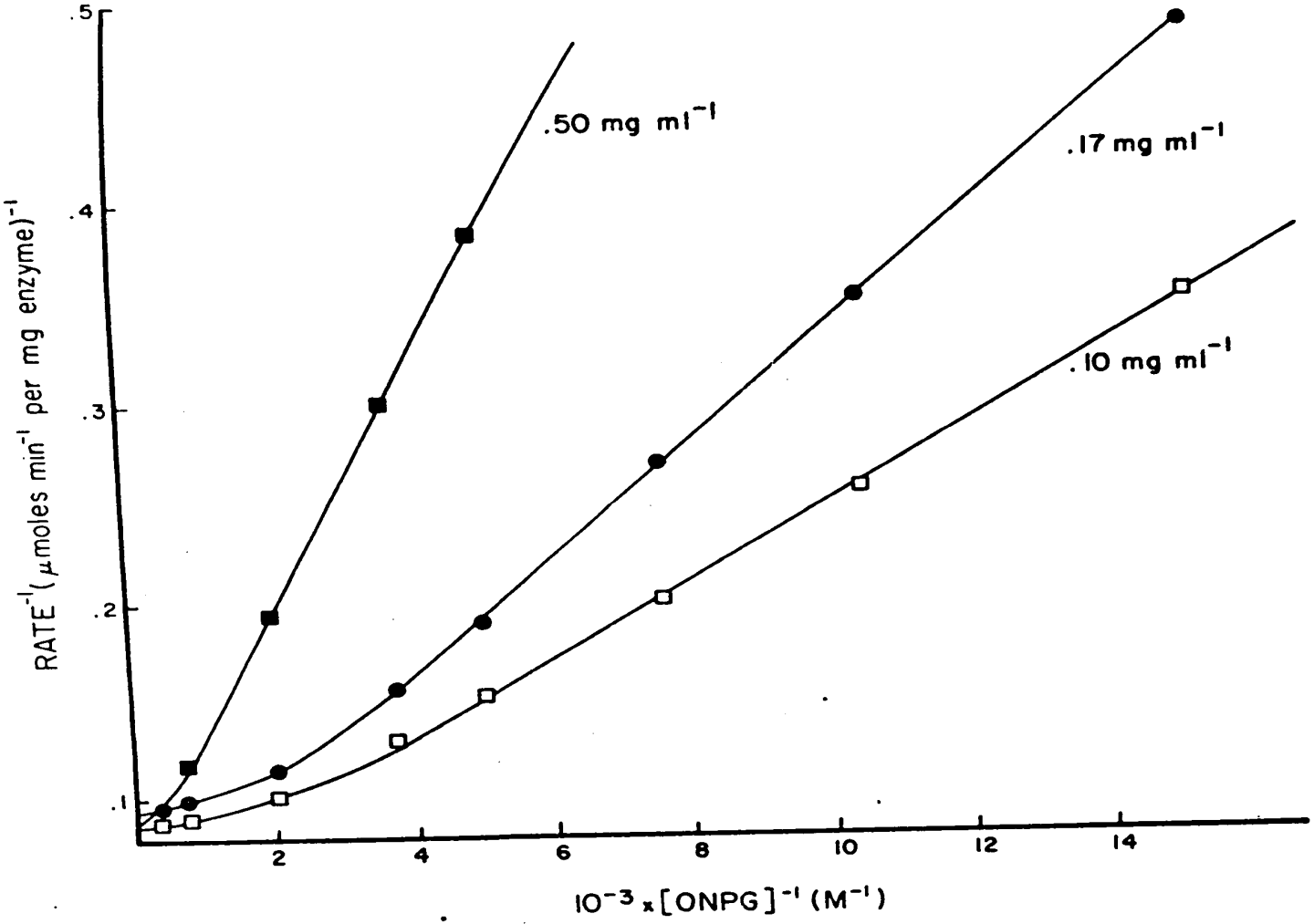


TABLE 7

Values of V_{max}(app) and K_m(app) for a range of enzyme concentrations in the gel

Slice thickness (microns)	Slice diameter (cm)	$[E]_m \times 10^7$ (moles liter^{-1} of gel	V_{max}(app) (μmoles min^{-1} per mg enzyme)	K_m(app)$\times 10^4$ (M)
1030±20	1.04±.01	9.26	2.5±6%	10.0 ±8%
420±10	1.03±.01	9.26	5.6±8%	12.4 ±10%
120±7	1.04±.01	9.26	14.1±12%	8.65±14%
110±10	1.01±.01	3.09	16.9±6%	4.6 ±10%
110±11	1.03±.004	1.85	17.9±4%	3.6 ±6%
105±9	1.02±.02	1.31	10.8±6%	2.1 ±13%
110±8	1.03±.005	0.77	9.9±3%	1.2 ±8%
110±8	1.04±.005	0.31	7.9±8%	1.0 ±10%

V_{max}(app) values are per liter of solution.

The enzyme concentration was calculated assuming pure enzyme and a molecular weight of 540,000 (137).

within a few seconds. It is possible that the curvatures in the Lineweaver-Burk plots (Figure 21) for the thicker slices (400 and 1000 microns) are related to this lack of establishment of the steady state. The theoretical treatment of Kobayashi and Moo-Young (41) predicts that these plots for supported enzymes should, when there is diffusion control, be somewhat convex to the 1/[S] axis; this is observed for all of the 100 micron slices in our experiments (Figure 22).

As a result of the curvatures at high substrate concentrations, the V_{max}(app) values vary for the different slices; this is shown in Table 7. Extrapolation of these plots yields a constant value of 100 ± 20 sec^{-1} for k_c', as expected from the theory. This procedure is, however, unreliable; a more accurate k_c' value of 81.5 sec^{-1} (±5%) was obtained from the slope of a graph such as that for the 100-micron slice of Figure 20.

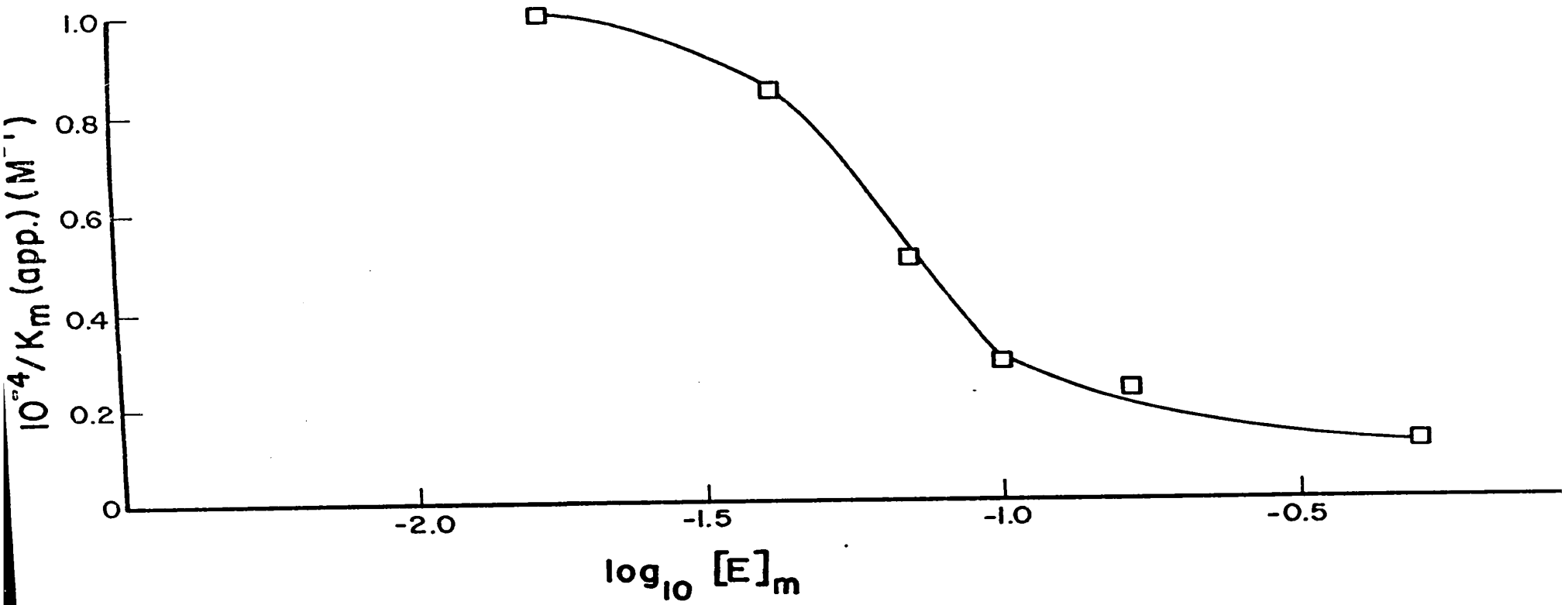
Since slices thinner than 100 microns could not be handled for repeated kinetic analysis, enzymic activity was reduced by lowering the enzyme concentration within the gel, in order to approach the diffusion-free limit. Figure 23 shows a plot of $1/K_m$(app) against $\log_{10}[E]_m$; this plot is discussed below.

Values of k_c(app) and K_m(app)

The combined effect of factors (1) and (2) on the kinetic parameters is readily seen from the results. The value of k_c is reduced in the supported enzyme from 273 sec^{-1}

Figure 23

A plot of $1/K_m(app)$ against $\log_{10}[E]_m$.



for the free enzyme to 81.5 sec^{-1}. The value of K_m is reduced from 1.73×10^{-4} M to 0.90×10^{-4} M. These values for the supported enzyme are referred to as k_c' and K_m'.

The K_m' value (cf. eq. 22) is of considerable importance in a comparison of theory with experiment, and corresponds to the case where F is unity, i.e. where diffusion control is not evident. The shape of the curve in Figure 23 suggests that the results at the lowest enzyme concentration are very close to the diffusion-free limit, since the curve levels off. This particular plot was made because eq. 22 shows that $F \propto 1/K_m(app)$ and eq. 17 shows that $\gamma\ell \propto E_m^{1/2}$; hence a plot of $1/K_m(app)$ against $\log_{10}E_m$ should show the same falling off as does a plot of F against $\gamma\ell$ (Figure 15). A comparison of Figures 15 and 23 shows that this is the case.

On the assumption that little diffusion control is involved at the lowest enzyme concentrations, a value of K_m' can be estimated from eq. 22 as

$$K_m' = PFK_m(app) = 0.90 \times 10^{-4} \text{ M}$$

with F = 1 and P = 0.89 (Table 5). With this value of K_m', and the $K_m(app)$ values at higher enzyme concentrations, it was possible to calculate the F-values over the range of enzyme concentrations and slice thicknesses. The values obtained in this way are shown in the next-to-last column of Table 8. In Figure 24 these values are shown plotted as solid circles. The agreement is very satisfactory. There is still some slight diffusion control at the lowest $\gamma\ell$ value,

Figure 24

The solid line is a plot of the function F (see eq. 16) against $\gamma\ell$. The points are experimental (see text).

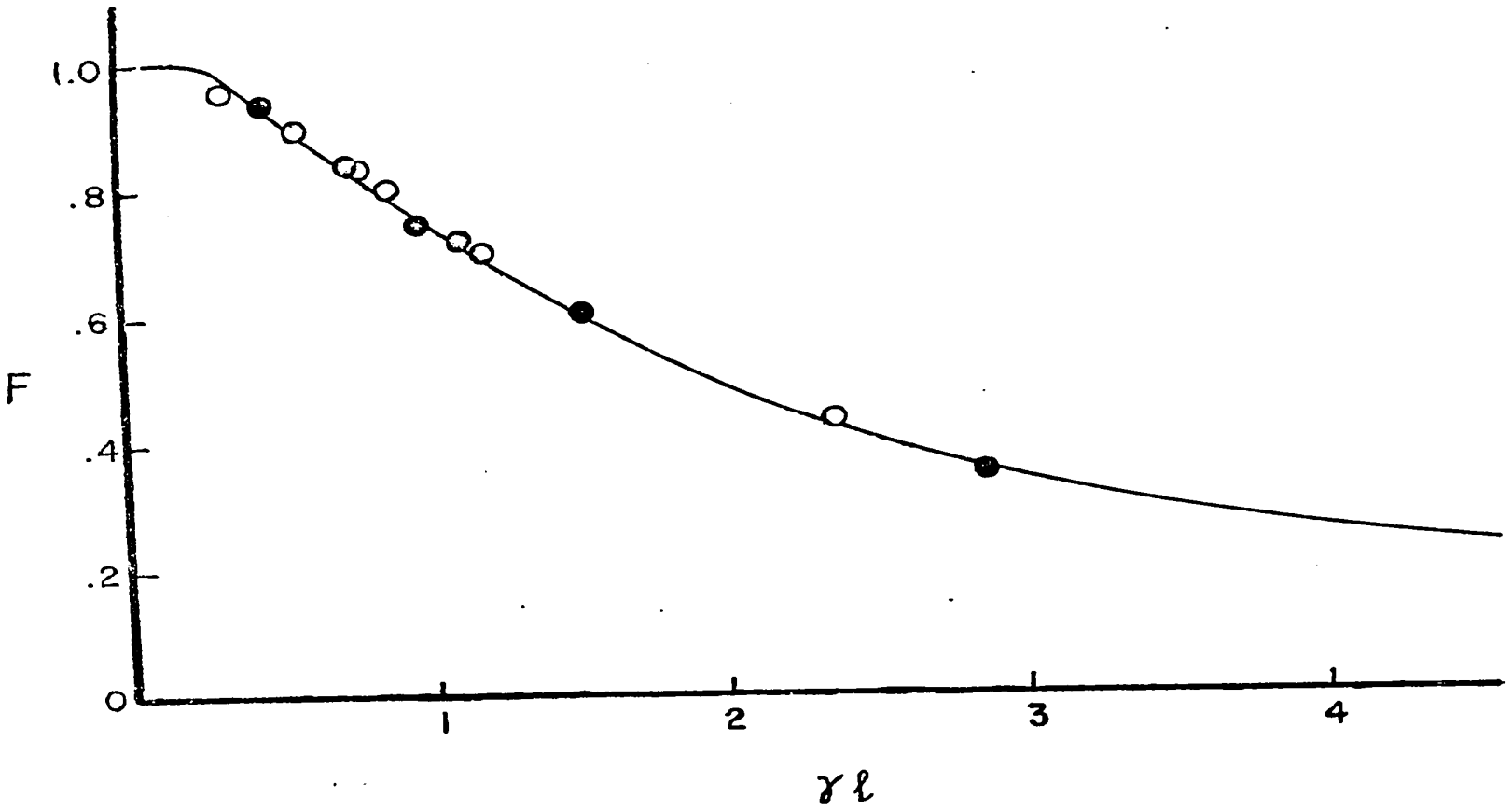


TABLE 8

Calculated F values for a range of enzyme concentrations in the gel

Slice thickness (microns)	Slice diameter (cm)	$[E]_m \times 10^7$ (moles liter^{-1} of gel)	F	
			$K'_m = 0.90 \times 10^{-4}$M	$K'_m = 1.46 \times 10^{-4}$M
1030	1.04	9.26	0.041	0.05
420	1.03	9.26	0.10	0.13
120	1.04	9.26	0.35	0.45
110	1.01	3.09	0.60	0.70
110	1.03	1.85	0.77	0.84
105	1.02	1.31	0.72	0.80
110	1.03	0.77	0.84	0.90
110	1.04	0.31	0.93	0.96

the highest value of F being 0.93. This may be an experimental error; it may also be that a sufficiently inactive membrane was not produced.

A further factor to be considered is the effect of the unstirred Nernst diffusion layer, bordering the membrane. This effect has been investigated by Goldman, Kedem and Katchalski (126) and also by Kobayashi and Moo-Young (62). Even when the F-value of our theory is close to unity, the Nernst diffusion effect can lead to increased values in apparent K_m. This effect may be the reason for failure to obtain completely diffusion-free kinetics. It is unlikely to be too important, however, since no effect of stirrer speed was observed on the rates of the immobilized enzyme. If a Nernst diffusion effect is operative, an increase in stirrer speed should increase the rate, since the diffusion barrier will be lessened.

The open circles in Figure 24 correspond to F values calculated on the basis of an alternative K_m' value, use being made of Method I described in the paper of Kobayashi and Laidler (151). This method led to a value of 1.46×10^{-4} for K_m'. The F values calculated on this basis are shown in the last column of Table 8. The open circles in Figure 24 again lie very satisfactorily on the theoretical curve.

All in all, the main results obtained in the present experimental study are in very satisfactory agreement with the theory of Sundaram, Tweedale and Laidler (42). This agreement is best exhibited by Figure 24.

CHAPTER FOUR

KINETICS OF ASPARAGINASE ATTACHED TO NYLON TUBING

Introduction

In Chapter 1 the use of enzymes attached to the interior wall of tubes was discussed in relation to automated analysis of biochemical metabolites, and to clinical treatment of certain diseases. The enzyme, L-asparaginase (EC 3.5.1.1), which was used in the work described below, was selected because it is currently used in the treatment of certain tumour diseases (138). An immobilized derivative would facilitate such treatment considerably, as was previously discussed.

Asparaginase lowers the concentration of L-asparagine present in the patient with the tumour, by hydrolyzing it to the harmless products, aspartic acid and ammonia. The hydrolysis leads to the regression of the tumour (138), since asparagine is an essential growth nutrient for the abnormal cells.

The practical importance of this enzyme in the treatment of tumours has led to a number of immobilized preparations. The enzyme has been attached to carboxymethyl dextran (139), and has been trapped in polyacrylamide gel (140) as well as within nylon microcapsules (141). Shortly after the present work commenced, a paper by Allison and coworkers (142) appeared describing the attachment of

asparaginase to the inside of a nylon tube, in much the same way as we have done. Allison and co-workers briefly studied the kinetics of the preparation, and observed that the apparent Michaelis constant was increased by a thousandfold compared with that of the enzyme in free solution. This effect was ascribed mainly to diffusion, and also to a charge repulsion effect, between support and substrate, as was discussed in Chapter 1. The effects of pH and temperature on the enzyme were also investigated. In the present study, therefore, attention was focussed on the kinetic aspects, and in particular, on the role of diffusion in the kinetics. The diffusion study was carried out in order to test a theoretical treatment which has recently been proposed by Kobayashi and Laidler (64) and which describes the effect of diffusion on the Michaelis parameters of an enzyme attached to the inner walls of a tube.

Experimental Section

Materials. The enzyme, L-asparaginase (E.C.3.5.1.1) lot 91C-6910, was obtained from Sigma Chemical Company. The substrate, L-asparagine, lot 100645, was obtained from Calbiochem. Boric acid, used as a buffering agent, was Analar grade, obtained from British Drug Houses, Ltd. Standard solutions used for ammonia determination were made using ammonium sulfate obtained from Anachemia. Sodium chloride for adjusting ionic strength was obtained from Canadian Laboratory Supplies Ltd. The Type 6 nylon tubing to which the asparaginase was attached

was a gift from John Tullis, Tullibody, Alloa, Scotland. The aspartic acid used in the inhibition studies was lot 101C-0720 from Sigma Chemical Co. Trichloracetic acid, used in the ammonia assay, was Analar grade, lot 33975, obtained from British Drug Houses. Nessler's reagent (Koch and McKeekin type) was obtained from Harleco. All chemicals used in the procedure for attaching the enzyme to the tube were reagent grade. All solutions were made up with doubly-distilled water, which had been degassed for several hours with a brisk stream of nitrogen.

Ammonia Assay. The extent of the action of the enzyme on L-asparagine was determined by measuring, by means of Nessler's reagent, the amount of ammonia released, solutions of ammonium sulfate being used as standards. To these standards was added 0.25 ml of the Nessler's reagent, followed by rapid mixing with a Deluxe Mixer (Scientific Products). The absorbance at 430 nm was read after 30 minutes on a Bausch and Lomb Spectronic 600 spectrophotometer, with attached Sargent SRLG recorder. A pair of microvolume cells obtained from Hellma Co., was used to read the absorbance (0.1 ml volume, 1 mm diameter and 1 cm pathlength). Sample solutions were analyzed for ammonia in the same way. In all assays 0.1 ml of trichloracetic acid was added in order to keep the ammonia as NH_4^+ and to inactivate free enzyme.

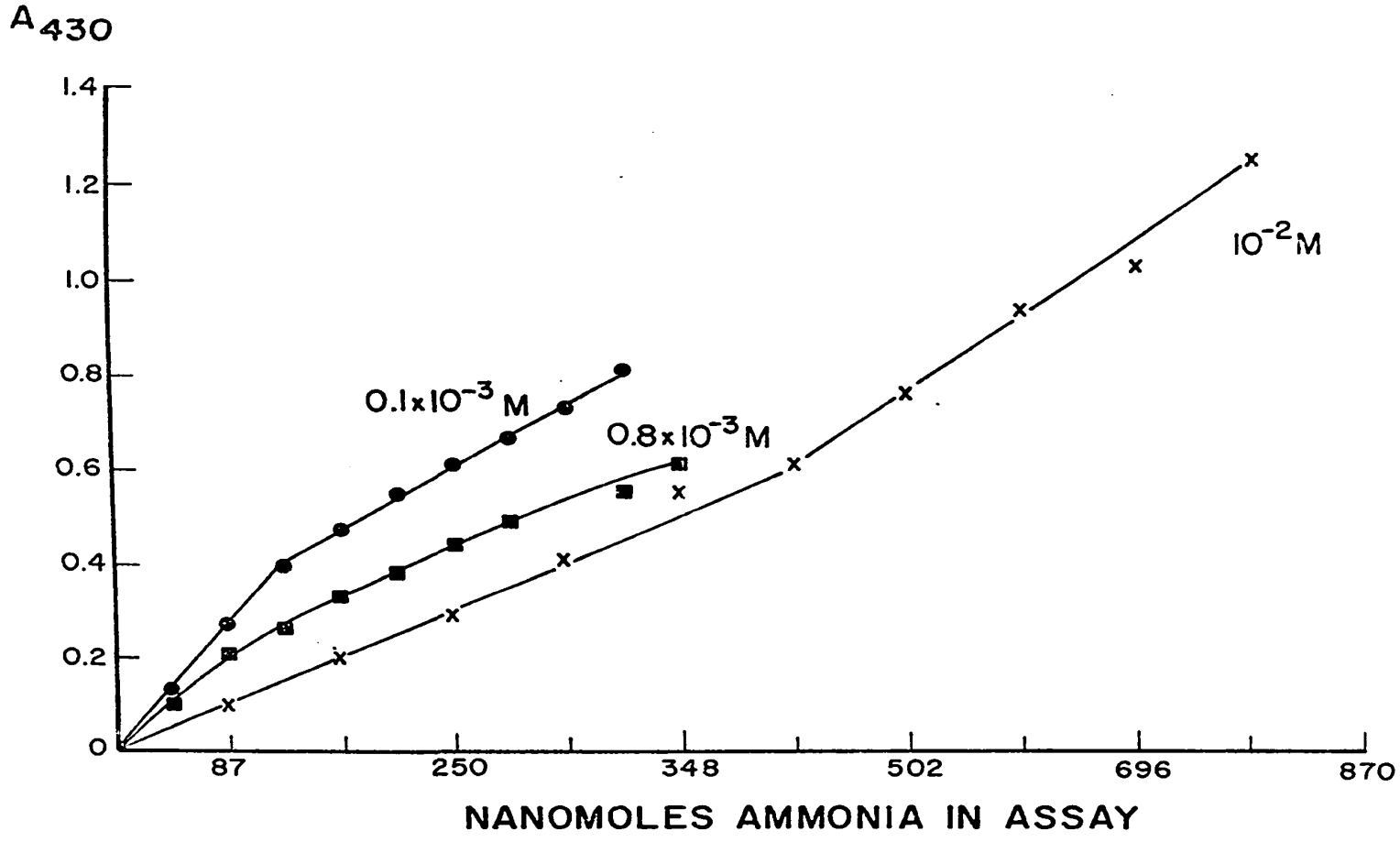
Substrate interfered with the absorbance, and it was therefore necessary to run a separate series of standards

for each substrate concentration. Since absorbance was not always linear with ammonia concentration, it was necessary to run standards covering a range of ammonia concentrations. Typical standard curves are shown in Figure 25. When small amounts of ammonia were being measured, standards and samples were run in quadruplicate; for higher concentrations, duplicates were sufficient.

Attachment Procedure. The procedure used to attach asparaginase chemically to the inner surface of nylon tubing was essentially that of Sundaram and Hornby, (19). The tubing was used as a tightly wound coil. A two-meter portion was partially hydrolyzed on the inside by passing 4.5 M HCl for 15 minutes at room temperature at a flow rate of 5 ml/minute; this procedure releases amino and carboxyl groups, the former subsequently reacting with the glutaraldehyde. Hydrolysis was stopped by perfusing with distilled water for 15 minutes. (At this stage the tubing becomes quite opaque, indicating successful hydrolysis; lower HCl concentrations do not always hydrolyze properly). The remaining steps were carried out with the tube and solutions in crushed ice. Perfusion with 0.2 M $NaHCO_3$ at pH 9 was carried out for 2 minutes at the same flow rate; there was then perfusion of a 5% (v/v) solution of glutaraldehyde in the same buffer for 15 minutes at a flow rate of 2 ml/minute. The tubing was then rinsed with 0.05 M boric acid buffer, pH 8.55, for 5 minutes, and then perfused with 800 units of asparaginase in 10 ml of the same buffer for 6

Figure 25

A plot of absorbance against ammonia concentration in a typical Nessler assay, at three different asparagine concentrations, as indicated on the lines.



hours. (Sundaram and Hornby, (19), took only 1.5 hours over this step). Uncoupled enzyme was removed by washing with 0.2 M $NaHCO_3$, with 0.1 M NaCl and with water for 10 minutes each at 5 ml/minute. When not in use the tube was stored at 0-4°C.

Kinetic Procedure. All substrate and inhibitor solutions were made up in 0.05 M boric acid buffer, at pH 8.55 ± 0.05. Solutions were stored at 0-4°C when not in use, and all kinetic runs were carried out at 37.0 ± 0.1°C.

For work with the free enzyme, a stock solution of L-asparaginase of 0.5 mg/ml was made up in 0.85% (w/v) NaCl in distilled water. A stock solution of L-asparagine in buffer, at 10^{-2} M, was also prepared. 0.25 ml of the stock enzyme solution was caused to react with asparagine at an appropriate dilution, in a reaction volume of 20 ml. Aliquots of 0.1 ml were removed at intervals, and analyzed for ammonia as described above.

For initial experiments with bound enzyme, a 2-meter length of the coiled nylon tubing, containing attached enzyme, was employed; the internal diameter was 1 mm. Subsequent experiments were done with 0.8-meter lengths. Tygon tubing was attached to each end of the coiled tube, one end being connected to a pump. A Watson-Marlow Model MHRE 200 pump was used for high flow rates, and an LKB Varioperpex Peristaltic Pump for lower flow rates. The range of flow rates was 12 cm/sec (6 ml/min) to 0.28 cm/sec (0.13 ml/min).

The other end of the tube dipped into the substrate solution; the concentration range of asparagine was 0.25×10^{-3} M to 10×10^{-3} M. The concentration of product is constant at a given flow rate (since there is continuous flow, with no mixing of substrate and product). Flow rates were determined for each run.

The average experimental error for the product concentration was around 20%. This is due partly to variations in flow rate, but more to the inadequacy of the Nessler assay procedure.

Theoretical Considerations

A detailed treatment of the flow kinetics of an enzyme attached to the inner surface of a tube, with full derivations, has been presented by Kobayashi and Laidler (64). Only the essential features of the theory will be outlined here, with an account of three procedures for applying the theory to the experimental results.

The model considered is as follows (cf. Figure 26a). A tube of length L (cm) and uniform internal radius R (cm) has enzyme uniformly immobilized along its inner surface. Substrate solution of inlet concentration S_o (moles/liter or moles/cc) is pumped through the tube at a constant flow rate of v_F (cm/sec). The product concentration, at the exit of the tube, is P_e (moles/liter or moles/cc).

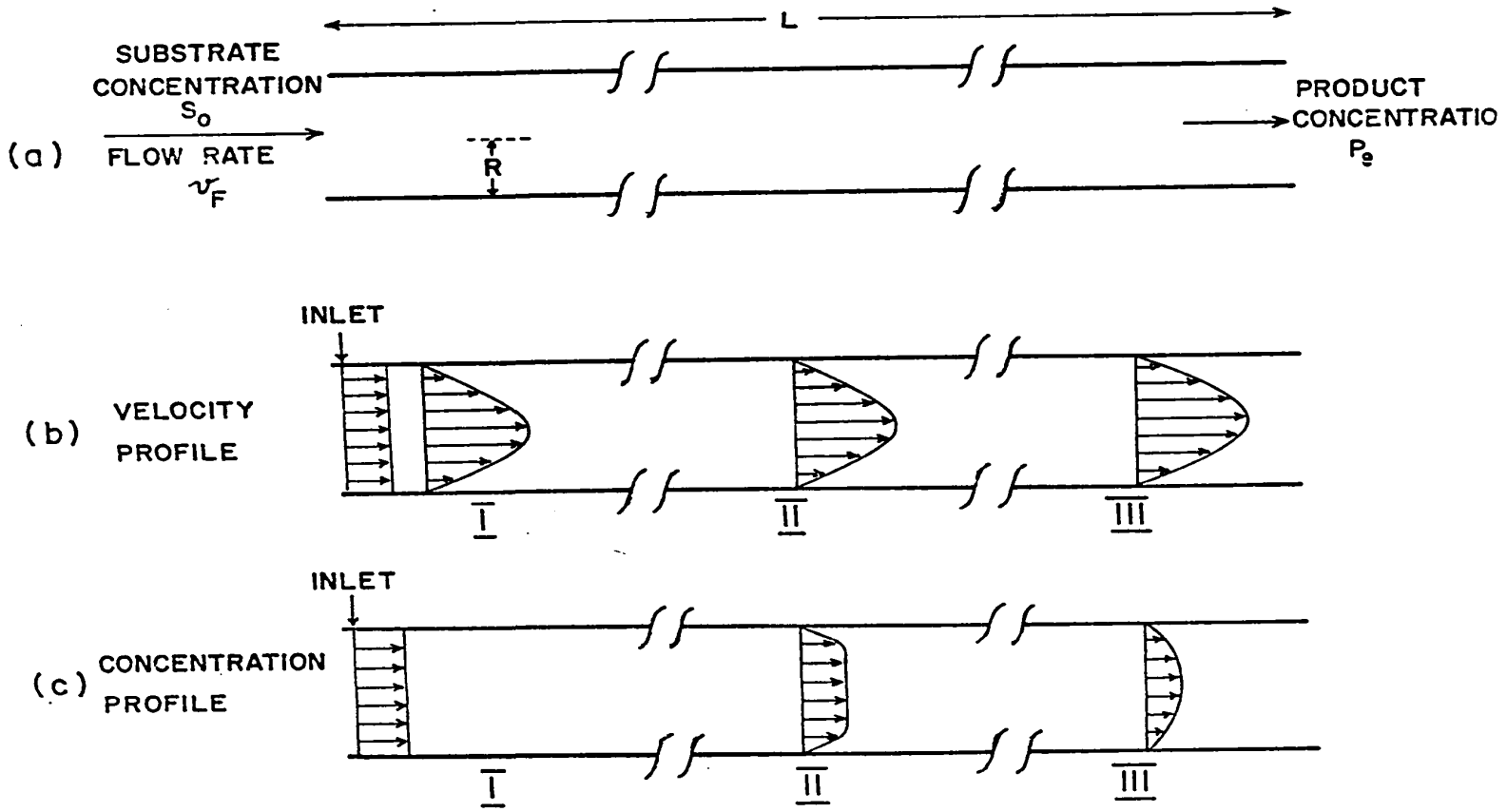
Laminar flow is assumed, and the velocity and

Figure 26

(a) Schematic diagram of a tube showing length, etc.

(b) The velocity profiles in various parts of the tube.

(c) The concentration profiles in various parts of the tube.



concentration profiles are as shown in Figure 26b and 26c. Three sections of the tube are to be distinguished; I, the hydrodynamic inlet section; II, the intermediate section and III, the fully-developed section. At the inlet, the flow velocity is uniform across the tube, but it rapidly adjusts itself to the pattern shown (with zero velocity at the walls) and remains in that configuration throughout the length of the tube. The concentration profiles are as shown in Figure 26c.

A diffusion boundary layer is established at the walls of the tube. Its width depends on the magnitude of the mass transfer co-efficient, which is a measure of the rate at which substrate diffuses in a direction perpendicular to the line of substrate flow, towards the enzyme at the walls. The theory leads to the following expression for this coefficient:

$$k_L = \frac{3}{2} \cdot \frac{\sqrt[3]{12}}{\Gamma(1/3)} \left(\frac{D^2 v_F}{RL} \right)^{1/3} \qquad (23)$$

where $\Gamma(1/3)$, the Gamma function of 1/3, is 2.67 and $D(cm^2/sec)$ is the diffusion coefficient of the substrate. It is to be seen that k_L is higher, i.e. the diffusion boundary layer is thinner, at higher flow rates. Diffusion effects are therefore predominant at very low flow rates, and of less importance at high flow rates when the diffusion layer is very thin. The dependence of k_L on L arises as a result of the changing concentration patterns along the tube.

The theory was developed for simple Michaelis-Menten kinetics as well as for substrate and product inhibition; only the simple case is considered here, since it applies to L-asparaginase. According to Sundaram, Tweedale and Laidler (42), the basic kinetic law for an immobilized enzyme system is

$$v = \frac{k'_c E.S_o}{K_m(app)+S_o} \qquad (24)$$

where E is the enzyme concentration, k'_c is a catalytic constant modified by conformational and environmental effects (but not influenced by diffusion) and $K_m(app)$ is an apparent Michaelis constant; it is influenced by conformational, environmental and diffusional effects. Two limiting cases are of importance:

Case (a). If S_o is high ($S_o >> K_m(app)$) the rate is independent of S_o and has its limiting high value; the rate depends on k'_c and no diffusional effects are found.

Case (b). At low substrate concentrations, such that $S_o << K_m(app)$ the rate depends on S_o and on $k'_c/K_m(app)$, and therefore involves a diffusional effect. The apparent Michaelis constant $K_m(app)$ in fact will increase as diffusional control of the reaction becomes more important, and the rate at low S_o therefore diminishes.

In the case of flow through a tube, the theory predicts that $K_m(app)$ will increase with increasing diffusional control according to the equation

$$K_m(app) = K'_m + \frac{V'_m}{2k_L} \qquad (25)$$

Here K'_m is the diffusion-free value of the Michaelis constant (moles/cc) and V'_m (moles cm^{-2} sec^{-1}) is the maximal velocity for the immobilized enzyme.

The theory further leads to the result that, for Case (b) and full diffusion control, the concentration of product formed at the exit, P_e, is given by

$$P_e = 2.56 \left(\frac{DL}{R^2 v_F}\right)^{2/3} \cdot S_o \qquad (26)$$

Also, the rate v (moles/sec) is given by

$$v = 8.06 \, (v_F D^2 R^2 L^2)^{2/3} \cdot S_o \qquad (27)$$

It is to be noted that expressions 26 and 27 do not involve the concentration or activity of the enzyme; this is the case if the rates are entirely diffusion controlled. It is assumed that there is enough enzyme at the surface to convert such substrate as comes in contact with it.

The theoretical conclusions outlined above lead to the following three procedures for relating the experimental results to the theory:

Method A. Equations 26 and 27 permit the product concentrations, and rates, to be calculated and compared with experiment. Also, product concentrations may be plotted against flow rate, as double-logarithmic plots. If there is full diffusion control eq. 26 shows that the slope should be -0.67.

If there is no diffusion control P_e would be inversely proportional to flow rate, so that the slope of the plot would be -1.0. The slope of an actual experimental plot therefore leads to a conclusion as to the extent of diffusion control.

Method B. Lineweaver-Burk plots of $1/v$ against $1/S_o$ also give an indication of the extent of diffusion control. Under Case (a), discussed above, there is no diffusion control; at high S_o values, therefore, straight-line Lineweaver-Burk plots are expected, and the Michaelis parameters are determined in the usual way. At low S_o, however, (Case (b)), the Lineweaver-Burk plots are predicted by eq. 27 to be straight lines passing through the origin. One can therefore first determine V'_m from results at high S_o and at high flow rates, when the behaviour will be diffusion-free. From this value, $K_m(app)$ values can be determined as those substrate concentrations at which the rate is half V'_m (these $K_m(app)$ values will not necessarily be diffusion free). The $K_m(app)$ values so obtained can then be plotted against $v_F^{-1/3}$. Combinations of eq. 23 and 25 leads to

$$K_m(app) = K'_m + \frac{2}{3} \cdot \frac{\Gamma(1/3)}{\sqrt[3]{12}} \cdot \frac{V'_m}{2} \left(\frac{RL}{D^2 v_F} \right)^{1/3} \qquad (28)$$

so that such a plot will yield K'_m as the intercept; this is the diffusion-free value. The slope yields the diffusion-free V'_m.

Method C. A special procedure for determining the extent of diffusion control is provided by the treatment of Kobayashi and Laidler (64), who defined two dimensionless variables:

$$\phi = \frac{P_e}{S_o}\left(\frac{v_F R^2}{DL}\right)^{2/3} \tag{29}$$

$$\rho = \frac{K_m(app)}{S_o} \tag{30}$$

Figure 27 shows the relationship between ϕ and ρ for simple Michaelis-Menten kinetics, with or without diffusion. This diagram shows three regions:

Region 1: essentially diffusion-free rates (better than 95% free from diffusion)

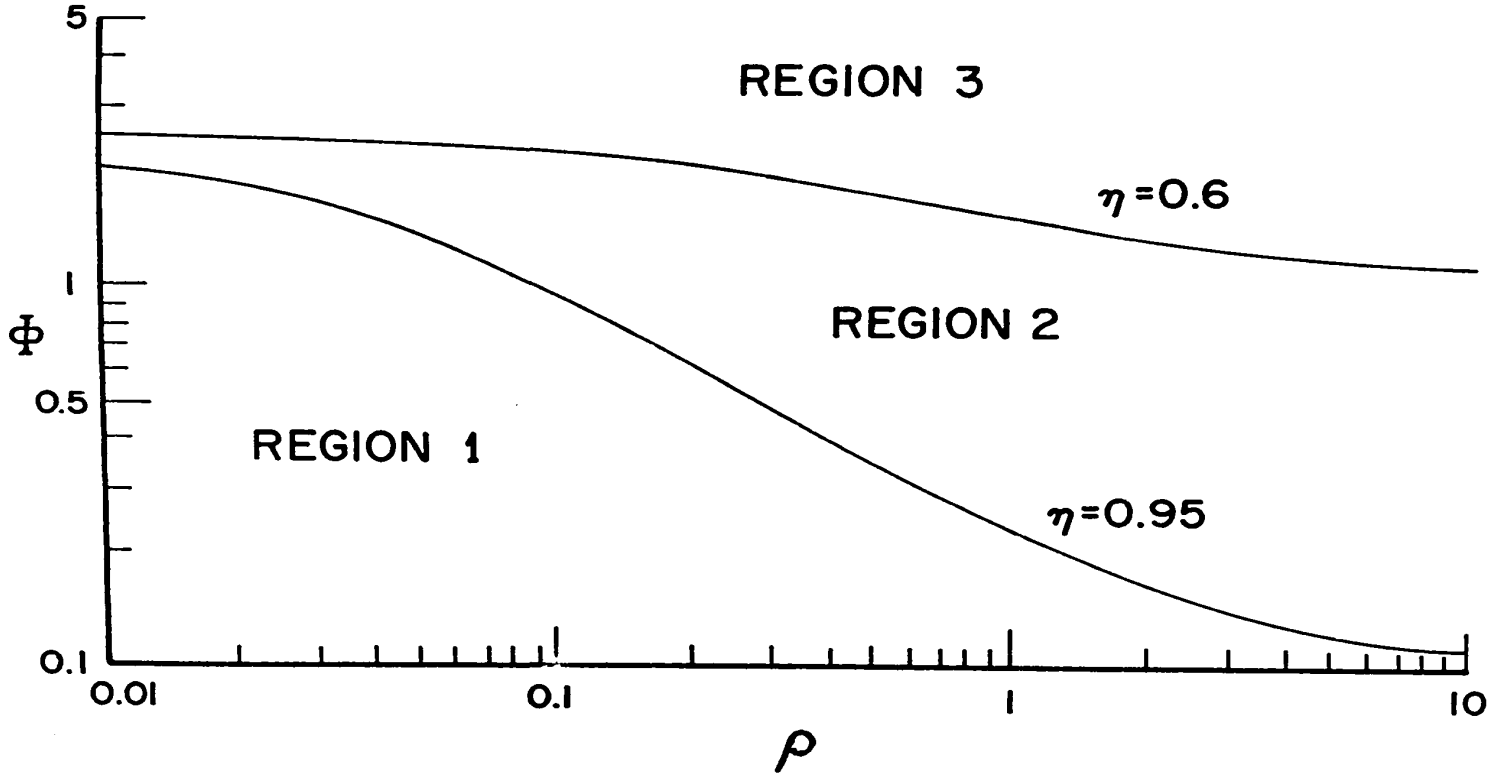
Region 3: dominated by diffusion control (60% or more diffusion controlled)

Region 2: intermediate between 1 and 3.

All of the quantities in 29 and 30 are experimentally obtainable, so that a plot of the data on the diagram in Figure 27 indicates the region. This diagram may be used once the $K_m(app)$ values have been determined; these involve the use of a value for V_m'. Values of V_m' may be checked on Figure 27 to determine whether they are diffusion free. Even so, some error is liable to arise in the determinations of $K_m(app)$, as will be seen below.

Figure 27

Relationship between the parameter ϕ and ρ, which are defined by eq. 7 and 8. The lines separate the diagram into three regions: 1, in which the rates are >95% diffusion free; 3, in which they are >60% diffusion controlled; 2, the intermediate region.


5
1
0.5
0.1
Φ
REGION 3
REGION 2
REGION 1
η=0.6
η=0.95
0.01
0.1
1
10
ρ

Results and Discussion

It was established at the outset that a steady state was set up and maintained throughout each kinetic run, at all concentrations of substrate and at all flow rates. This was done by determining the amounts of product produced in various time intervals, in runs lasting for a total of one hour. The product concentration was always found to be constant with time.

The theory of Kobayashi and Laidler (64) dealt with inhibition by substrate and product, as well as the simple Michaelis-Menten case; the outline above was confined to the simple case. The free enzyme is not subject to product or substrate inhibition (143), and experiments were carried out to verify that this also was the case with the immobilized enzyme. Runs carried out at two extreme substrate concentrations (3.3×10^{-3}M and 0.33×10^{-3}M) with a concentration of 1.0×10^{-3}M of the two products of reaction, ammonia and aspartic acid, showed no inhibition.

The results will now be presented and discussed in terms of the three methods, A, B and C, for applying them to the theory.

Method A. The raw data are presented in Table 9 as concentration of product formed at the tube exit (millimoles per liter) for the two-metre tube. The results for the 0.8-metre tube are tabulated in Table 10. In both tables, the values in brackets have been calculated according to equation 26,

TABLE 9

Concentrations of Ammonia (mM) Produced at the Exit of 2-meter Tube at Various Substrate Concentrations and Flow Rates (Values in Brackets are Calculated Values, using eq. 26)

$[ASN] \times 10^3$	Flow Rate, v_F ($cm\ sec^{-1}$)					
(M)	12	6.0	3.4	2.0	1.0	0.73
.25	0.040 (0.055)	0.082 (0.093)	0.18 (0.13)	0.19 (0.19)	0.23 (0.29)	0.24 (0.38)
.33	0.064 (0.074	0.13 (0.12)	0.26 (0.17)	0.29 (0.25)	0.31 (0.38)	0.34 (0.49)
.45	0.84 (0.10)	0.18 (0.16)	0.31 (0.24)	0.38 (0.35)	0.42 (0.55)	0.41 (0.68)
.67	0.13 (0.15)	0.27 (0.25)	0.43 (0.35)	0.53 (0.50)	0.55 (0.78)	0.57 (0.98)
1.1	0.21 (0.25)	0.43 (0.40)	0.81 (0.58)	1.1 (0.84)	1.2 (1.3)	1.3 (1.6)
3.3	0.21 (0.74)	0.43 (1.2)	1.2 (1.7)	2.1 (2.5)	3.1 (3.8)	3.1 (4.9)
10.0	0.21 (2.3)	0.43 (3.8)	1.3 (5.3)	2.1 (7.8)	3.1 (12)	5.2 (15)

TABLE 10

Concentrations of Ammonia (mM) Produced at the Exit of a 0.8-metre Tube at Various Substrate Concentrations and Flow Rates (Values in Brackets Calculated using eq. 26)

10^3 x [ASN]	Flow Rate, v_F (cm sec^{-1})			
(M)	6.5	1.0	0.72	0.28
0.25	.027 (.048)	0.12 (0.16)	0.14 (0.20)	0.18 (0.38)
0.33	.035 (.060)	0.16 (0.21)	0.17 (0.28)	0.24 (0.50)
0.45	.040 (.083)	0.17 (0.28)	0.20 (0.38)	0.32 (0.70)
0.67	.047 (.120)	0.21 (0.43)	0.25 (0.54)	0.40 (1.0)
1.1	.071 (0.20)	0.30 (0.70)	0.43 (0.89)	0.69 (1.7)
2.0	0.11 (0.37)	0.32 (1.30)	0.43 (1.6)	0.88 (3.1)
3.0	.073 (0.61)	0.29 (2.1)	0.37 (2.8)	0.87 (5.0)
10	.085 (1.90)	0.33 (6.3)	0.40 (8.0)	0.88 (15)

the value for the diffusion constant being taken as 4×10^{-6} cm^2 sec^{-1}. A value for D of this nature was obtained in the work previously described in Chapter 3, and similar values have been obtained by other workers (126).

It is to be seen from Tables 9 and 10 that agreement between theory and experiment is quite good. Not only are the correct trends predicted by the theory, but the actual values agree within a factor of two. At high flow rates and at high concentrations of substrate, agreement is less satisfactory; this is to be expected, however, since diffusion control is absent under these conditions, and equation 26 is not valid.

The results are shown plotted on a log-log scale in Figure 28 for the 2-metre tube and in Figure 29a for the 0.8-metre tube. The figures show that at low substrate concentration, the slope of the plots is -0.67, as expected for diffusion control; at high substrate concentration, however, the slopes are close to -1.0, indicating no diffusion control. (Some of the lines for the 2-metre length of tubing become horizontal at low flow rates; this is because complete hydrolysis of the substrate has taken place).

Tables 11 and 12 show the results as reaction velocity (moles per second) for the two lengths of tubing. The values in brackets were calculated according to equation 27. The agreement follows the same patterns as the product values discussed above.

Figure 28

Double-logarithmic plots of product concentrations against flow rates, for the 2-meter tube and for various substrate concentrations, as follows: ●, 10 mM; O, 3.3 mM; Δ, 0.67 mM; x, 0.33 mM; ■, 0.25 mM.

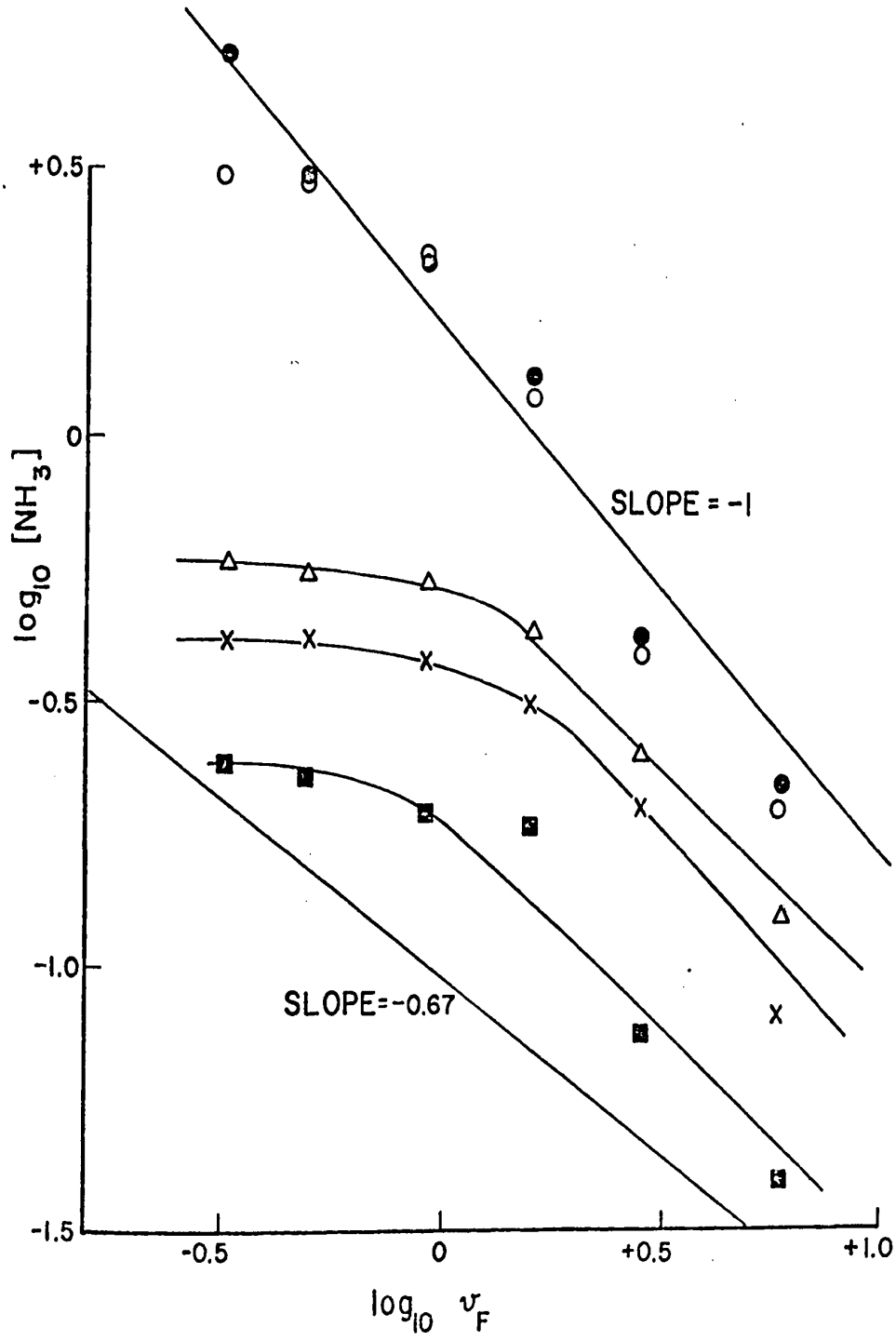

+0.5
0
-0.5
-1.0
-1.5
$\log_{10}$ [NH_3]
SLOPE = -1
SLOPE=-0.67
-0.5
0
+0.5
+1.0
$\log_{10}$ ν_F

Figure 29

Double-logarithmic plots of product concentrations against flow rates: (a) for the 0.8-meter tube at low ionic strength and (b) for the 0.8-meter tube at high ionic strength. The different substrate concentrations are: ● 10 mM; □ 3.3 mM; x 0.33 mM and O 0.25 mM.

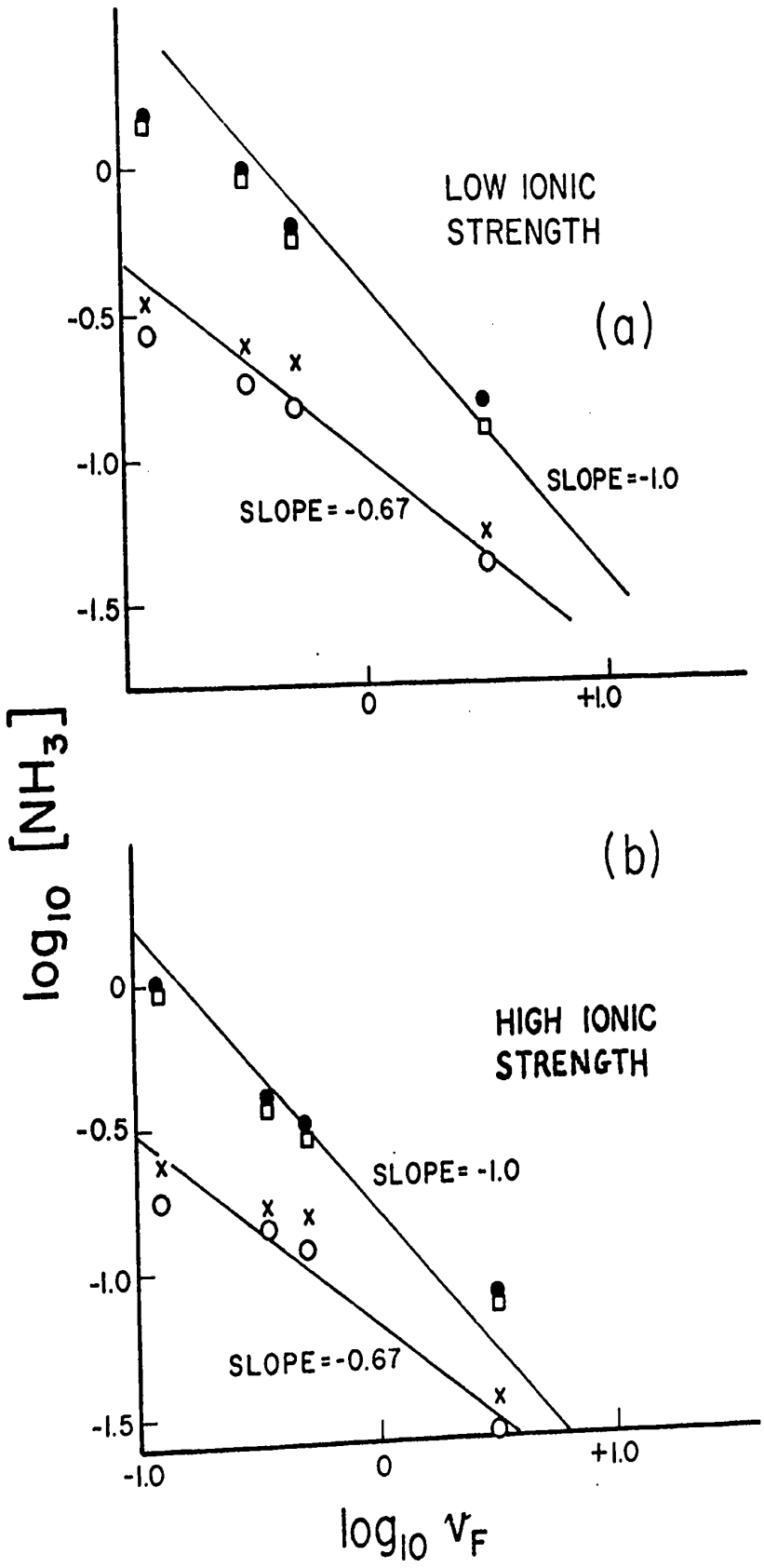

LOW IONIC
STRENGTH
(a)
SLOPE=-1.0
SLOPE=-0.67
0
-0.5
-1.0
-1.5
0
+1.0
(b)
HIGH IONIC
STRENGTH
SLOPE=-1.0
SLOPE=-0.67
0
-0.5
-1.0
-1.5
-1.0
0
+1.0
log10 [NH3]
log10 νF

TABLE 11

Rates of Reaction (10^9 x moles sec^{-1}) for the 2-metre Tube, at Various Concentrations of Substrate and at Different Flow Rates

(Values in Brackets are Calculated According to eq. 27)

10^3 x [ASN]	Flow Rate, v_F (cm sec^{-1})					
(M)	12	6.0	3.4	2.0	1.0	0.73
0.25	3.9 (5.5)	4.0 (4.3)	4.8 (13.5)	3.0 (3.0)	1.9 (2.5)	1.4 (2.3)
0.33	6.3 (7.3)	6.4 (5.8)	7.0 (5.3)	4.6 (4.0)	2.5 (3.3)	1.9 (2.8)
0.45	8.3 (10)	8.5 (7.8)	8.2 (6.3)	6.0 (5.5)	3.4 (4.3)	2.3 (4.0)
0.67	13 (15)	13 (12)	11 (9.4)	8.3 (7.8)	4.5 (6.3)	3.2 (5.6)
1.1	21 (24)	21 (19)	22 (15)	17 (13)	9.8 (10)	7.4 (9.2)
3.3	20 (73)	21 (58)	32 (48)	33 (40)	25 (36)	17 (43)
10	21 (215)	21 (170)	35 (140)	33 (118)	25 (95)	30 (75)

TABLE 12

Rates of Reaction (10^9 x moles sec^{-1}) for a 0.8-metre Tube, at Various Substrate Concentrations and Flow Rates

(Values in Brackets Calculated According to eq. 27)

10^3 x [ASN]	Flow Rate, v_F (cm sec^{-1})			
(M)	6.5	1.0	0.72	0.28
0.25	2.2 (2.4)	1.3 (1.3)	1.1 (1.1)	0.58 (.83)
0.33	2.8 (3.3)	1.8 (1.7)	1.4 (1.5)	0.77 (1.1)
0.45	4.0 (4.3)	2.2 (2.3)	1.8 (2.1)	0.93 (1.5)
0.67	4.8 (6.4)	3.1 (3.4)	2.5 (3.0)	1.4 (2.2)
1.1	7.0 (10)	4.4 (5.6)	3.9 (5.0)	1.9 (3.6)
3.3	6.5 (33)	5.0 (17)	4.5 (15)	3.2 (11)
10	7.8 (95)	5.0 (53)	4.5 (45)	3.2 (33)

Method B. Lineweaver-Burk plots are shown in Figure 30 for the 2-metre tube, and Figure 31 for the 0.8-metre tube. The Michaelis parameters obtained from these plots are presented in Table 13, which also contains the results for the high ionic strength experiments which will be discussed below. In all cases, the rates are lowest for low flow rates and low substrate concentrations, when the theory indicates diffusion-control to be most likely. At high substrate concentrations, the curves approach a common value of V'_m, although there is some spread since at the lower flow rates even these rates are partly diffusion-controlled. The V'_m value at the highest flow rate is, however, free from diffusion effects, as will be seen below. The value obtained from the graph is 22×10^{-9} moles/sec for the 2-metre tube, and 18×10^{-9} moles/sec for the 0.8-metre tube. These values are subject to a certain amount of error owing to the extrapolation procedure adopted in their determination, as is apparent from Figures 30 and 31.

Using these values of V'_m, it was possible to determine K_m(app) at different flow rates. The values were found to increase as the flow rate decreased, as predicted by equation 25. A similar effect of diffusion on K_m(app) was found in the experiments described in Chapter 3 with membrane slices containing enzyme.

The K_m(app) values are plotted in Figure 32 as a function of $v_F^{-1/3}$. The intercept on the vertical axis yields a value of K'_m of around 20×10^{-6} moles per litre. This result

Figure 30

Lineweaver-Burk plots for the 2-meter tube, at various flow rates (cm/sec) which are indicated on the lines.

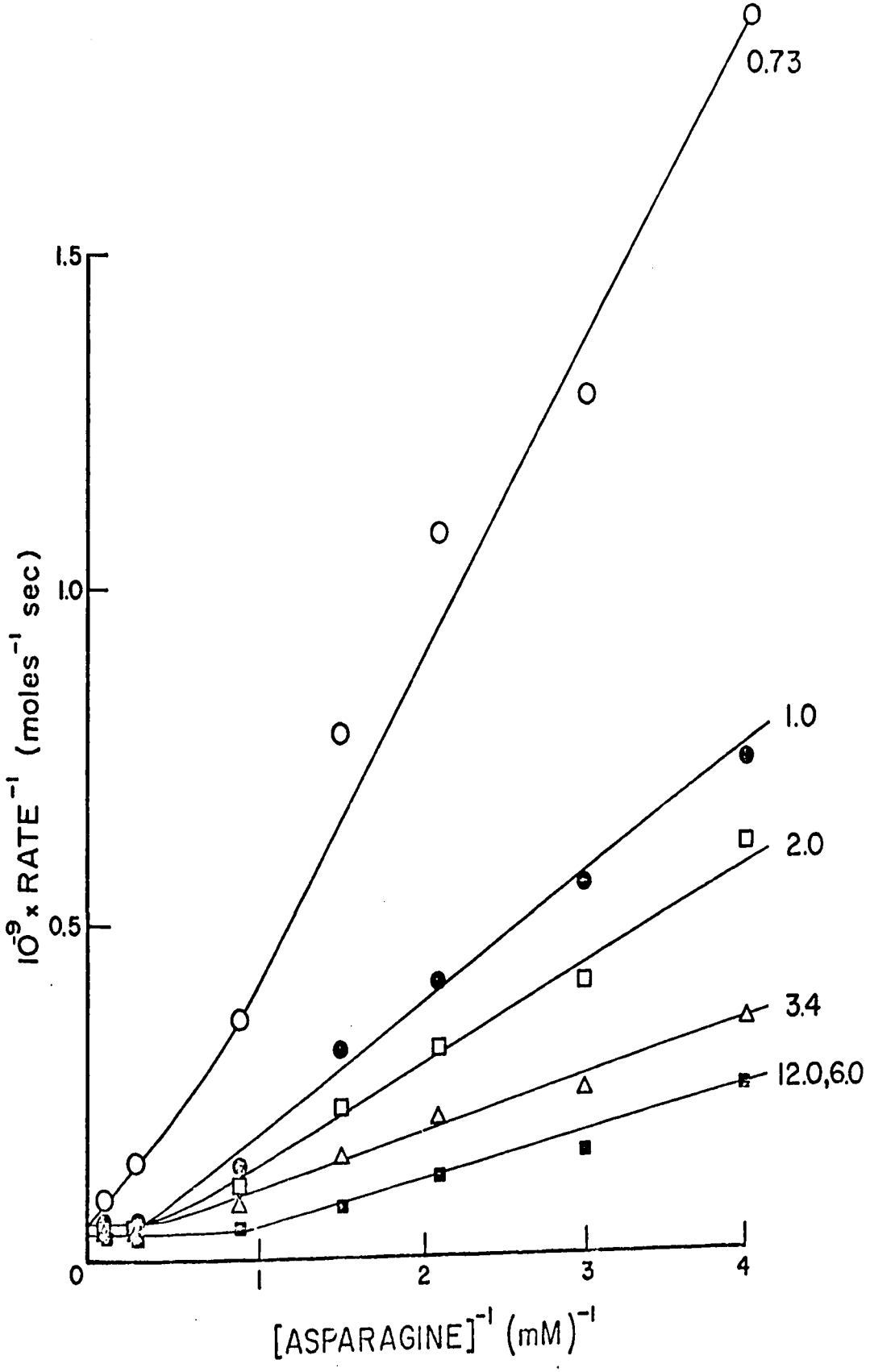

0.73
1.0
2.0
3.4
12.0,6.0
1.5
1.0
0.5
0
1
2
3
4
10^{-9} x RATE^{-1} (moles^{-1} sec)
[ASPARAGINE]$^{-1}$ (mM)$^{-1}$

Figure 31

Lineweaver-Burk plots for the 0.8-meter tube, at various flow rates (cm sec^{-1}), which are indicated on the lines.

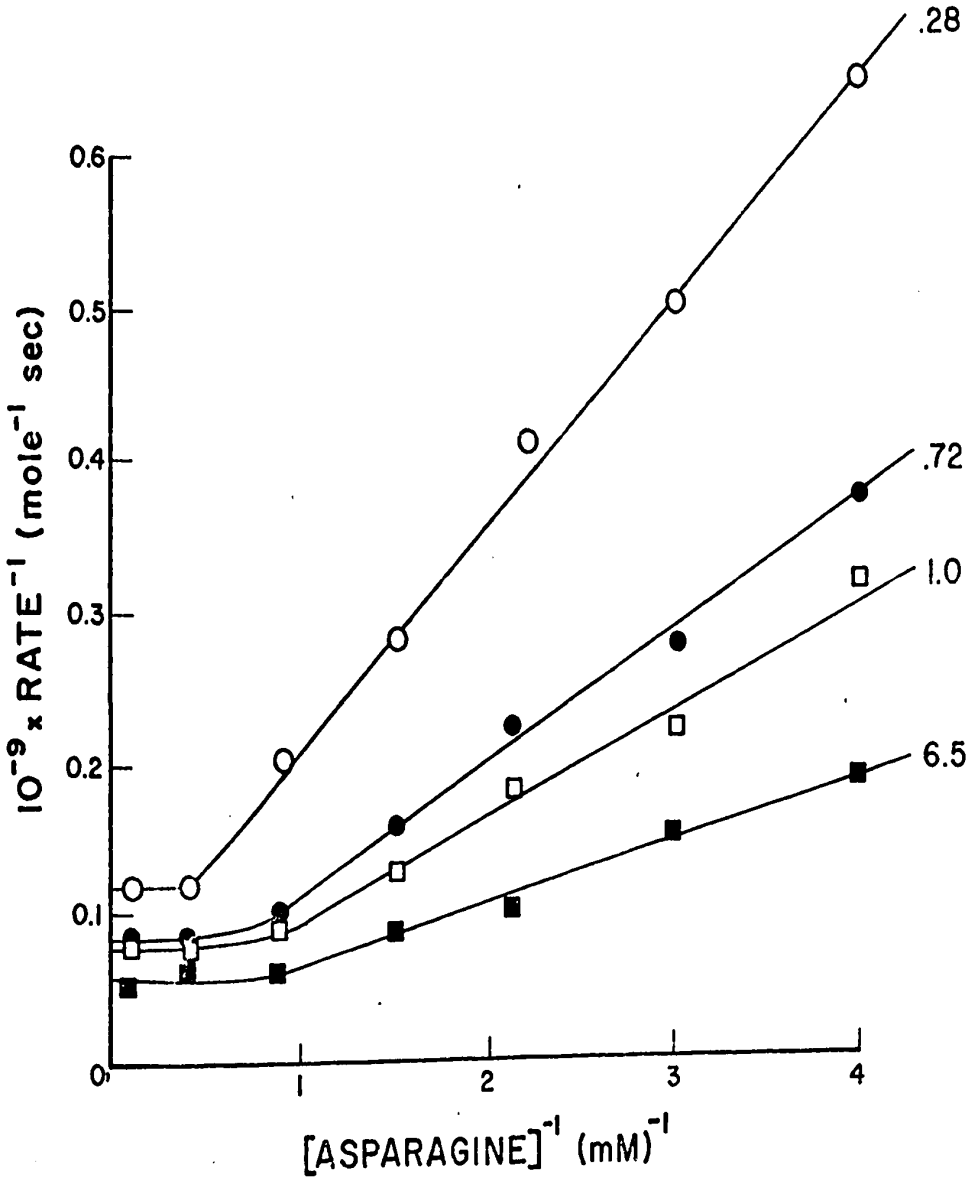

.28
.72
1.0
6.5
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
10^{-9} x RATE^{-1} (mole^{-1} sec)
[ASPARAGINE]$^{-1}$ (mM)$^{-1}$

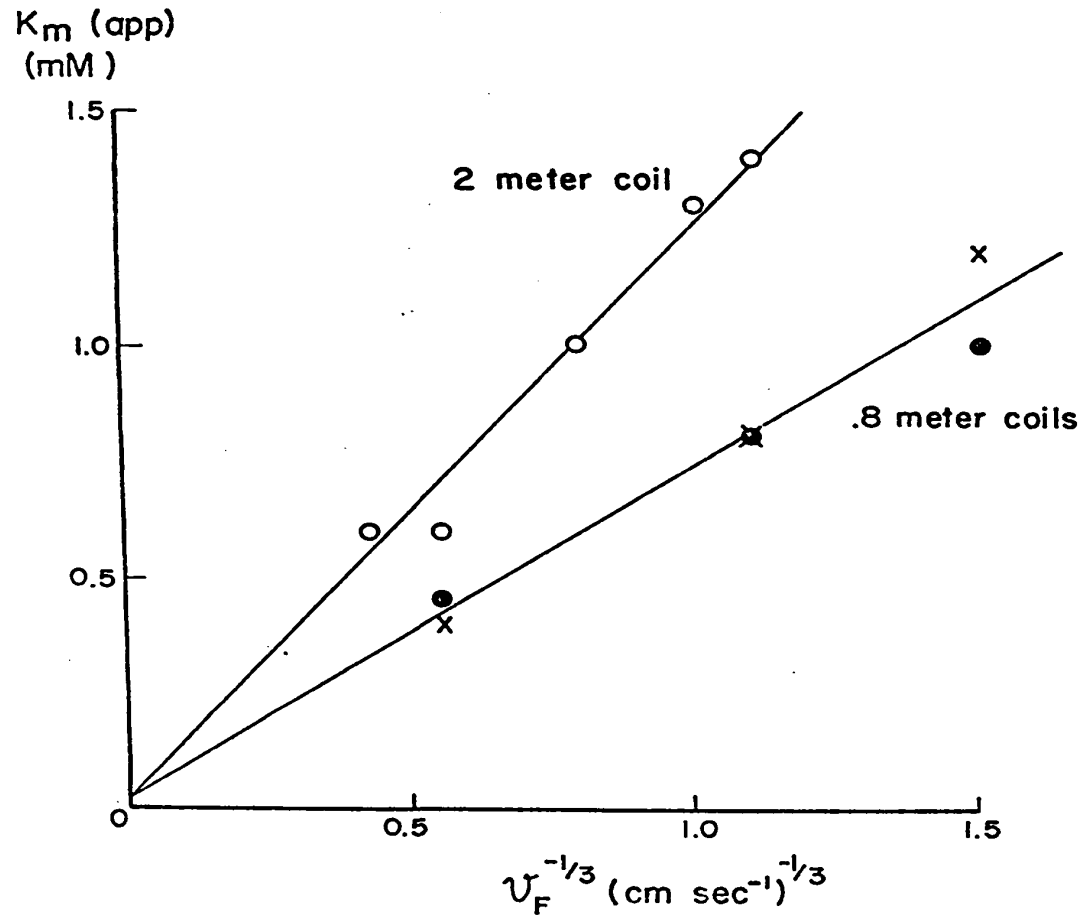
TABLE 13

Michaelis Parameters for Asparaginase Attached to Nylon Tubing, at Different Flow Rates

Flow Rate ($cm\ sec^{-1}$)	$10^3 \times K_m(app)$ (M)			$10^9 \times V_{max}(app)$ (moles sec^{-1})		
	2m	0.8m (low μ)	0.8m (high μ)	2m	0.8 m (low μ)	0.8 m (high μ)
12	0.6	-	-	24	-	-
6.0	0.6	0.45	0.35	24	7.4	10
3.4	1.0	-	-	21	-	-
2.0	1.3	0.8	0.8	21	5.3	6.4
1.0	1.4	0.95	1.2	19	4.8	5.9
0.73	6	-	-	16	-	-
0.28	-	5	5	-	3.5	5.0

Figure 32

Plots of the different Michaelis constants, K_m(app), against the flow rate v_F to the power of -1/3, for the 2-meter and 0.8-meter tubes.


K_m (app)
(mM)
1.5
1.0
0.5
0
2 meter coil
.8 meter coils
0.5
1.0
1.5
$v_F^{-1/3}$ (cm sec^{-1})$^{-1/3}$

is subject to an error of a factor of two or so, since the extrapolation procedure is not very accurate, and the V'_m values used to obtain the apparent K_m results were also subject to error. Nonetheless, it is significant that both tubes give approximately the same intercept. The K'_m may be compared with the Michaelis constant for asparaginase in free solution, determined by an isotopic method, of 13×10^{-6} moles per liter (144). (Unfortunately, the Nessler method of determining ammonia concentration is not sensitive enough to allow such a determination in our case.) It would appear that diffusion is the major factor responsible for the change in apparent Michaelis constant upon immobilizing the enzyme, and that its true K'_m when attached to the tube is not much different from the value for the enzyme in free solution. Other experiments, described below, support this conclusion.

The value of V'_m obtained from the slope of the graph in Figure 32, is 0.38×10^{-9} moles cm^{-2} sec^{-1} (24×10^{-9} moles sec^{-1}) for the two-metre tube. The corresponding values for the 0.8-metre tube are 0.29 moles cm^{-2} sec^{-1} and 7.4×10^{-9} moles sec^{-1}. The values for the two-metre tube agree very well with those in Table 11. Agreement for the 0.8-metre tube (Table 12) is less satisfactory, and indicates the possible errors in the extrapolation procedures.

Method C. Tables 14 and 15 show some of the results in terms of ϕ and ρ. The regions that the values fall into (cf. Figure 27) are indicated in brackets. It is to be seen that

TABLE 14

Values of the Parameters ϕ and ρ, for the 2-metre Tube, at Different Substrate Concentrations and Flow Rates. The Numbers in Brackets Indicate the Regions (Figure 2)

[ASN] x 10^3 (M)	Flow Rate, v_F (cm sec^{-1})							
	12.0		6.0		2.0		1.0	
	ϕ	ρ	ϕ	ρ	ϕ	ρ	ϕ	ρ
0.25	4.8	2.4	5.8	2.4	3.1	5.2	4.3	5.6
	(3)		(3)		(3)		(3)	
0.67	5.4	0.9	7.1	0.9	2.7	1.9	4.2	2
	(3)		(3)		(3)		(3)	
10.0	0.57	0.06	0.76	0.06	0.59	0.13	1.2	0.14
	(1)		(1)		(1)		(1)	

TABLE 15

Values of the Parameters ϕ and ρ, for the 0.8-metre Tube, at Different Substrate Concentrations and Flow Rates. The Numbersin Brackets Indicate the Regions (Figure 2)

[ASN] x 10^3 (M)	Flow Rate, v_F (cm sec^{-1})							
	6.5		1.0		0.72		0.28	
	ϕ	ρ	ϕ	ρ	ϕ	ρ	ϕ	ρ
0.25	6.6	1.8	6.2	3.2	5.9	4.0	4.6	4.0
	(3)		(3)		(3)		(3)	
0.67	4.8	0.67	5.8	1.2	5.3	1.5	3.9	1.5
	(3)		(3)		(3)		(3)	
10.0	0.56	0.05	0.63	0.08	0.64	0.01	0.63	0.10
	(1)		(1)		(1)		(1)	

most of the results lie in Region III, which corresponds to diffusion-controlled rates. The only results lying in Region I are those at high substrate concentration, and at the highest flow rates, as expected for diffusion-free rates. Tables 14 and 15 confirm the discussion above, that the values selected for determination of V_m' were indeed diffusion-free.

The Effect of High Ionic Strength

In general, the results agree well with the theoretical predictions. It was also of interest, however, to determine whether any other factors might be involved in causing the large increase in apparent Michaelis constant. Allison and co-workers (142) suggested that the increase might, in part, be the result of an electrostatic repulsion effect. This was quite feasible, since the substrate is likely to be negatively charged at pH 8.55, and the same applies to the residual carboxyl groups on the wall of the tube. This could lead to an increase in apparent Michaelis constant, as was discussed in Chapter 1.

A series of experiments was therefore conducted with the 0.8-metre length of tubing, at a higher ionic strength (the 0.05M boric acid buffer was made 1.0M in NaCl, to give a total ionic strength of 1.25M). High ionic strength should lower the apparent K_m value (i.e. increase the rates), by reducing the electrostatic repulsion effect. The results are shown as product concentration in Table 16, along with

TABLE 16

Concentrations of Ammonia (mM) Produced at the Exit of a 0.8-metre Tube at Various Substrate Concentrations and Flow Rates, at High Ionic Strength (Values in Brackets Calculated using eq. 26)

10^3 x [ASN]	Flow Rate, v_F (cm sec^{-1})			
(M)	6.5	1.0	.72	.28
.25	.044 (.048)	0.15 (0.16)	0.19 (0.20)	0.27 (0.38)
.33	.055 (.060)	0.22 (0.21)	0.25 (0.28)	0.35 (0.50)
.45	.081 (.083)	0.27 (0.28)	0.31 (0.38)	0.43 (0.70)
.67	.096 (0.12)	0.38 (0.43)	0.44 (0.54)	0.63 (1.0)
1.1	0.14 (0.20)	0.54 (0.70)	0.69 (0.89)	0.88 (1.7)
3.3	0.13 (0.61)	0.62 (2.1)	0.80 (2.8)	1.5 (5.0)
10	0.16 (1.90)	0.62 (6.3)	0.80 (8.0)	1.5 (15)

the calculated values (in brackets) from equation 26. They are presented in Figure 29b as a log-log plot. Table 17 displays the results as velocities (moles per second), together with the rates calculated according to equation 27 in brackets. Figure 33 shows the results as Lineweaver-Burk plots.

Comparison of these results with those for the 0.8-metre length of tubing at lower ionic strength shows that the rates were all lowered by a factor of two. This indicates that no significant charge repulsion effect applies. Control experiments with the free enzyme also produced a slight lowering of the rate at higher ionic strength, by about 7%. It thus appears that the immobilized enzyme does in fact have a very similar K_m' value to that of the enzyme in free solution; and that the dramatic increase in K_m(app) upon immobilization is almost entirely the result of diffusion effects. The slight difference between the two Michaelis constants for the bound and free enzymes is probably due to environment and conformational differences, which, as discussed in Chapter 5, generally lead to relatively small changes in this constant.

In summary, the predictions made by the theory on the kinetics of enzymes bound to the wall of a tube, agree well with the results obtained. The effect of diffusion on this system is very similar to the effect on membrane systems, i.e. the Michaelis constant increases, while the maximum velocity remains unchanged. The significance of this main result is discussed in relation to the results of other workers in the next chapter.

TABLE 17

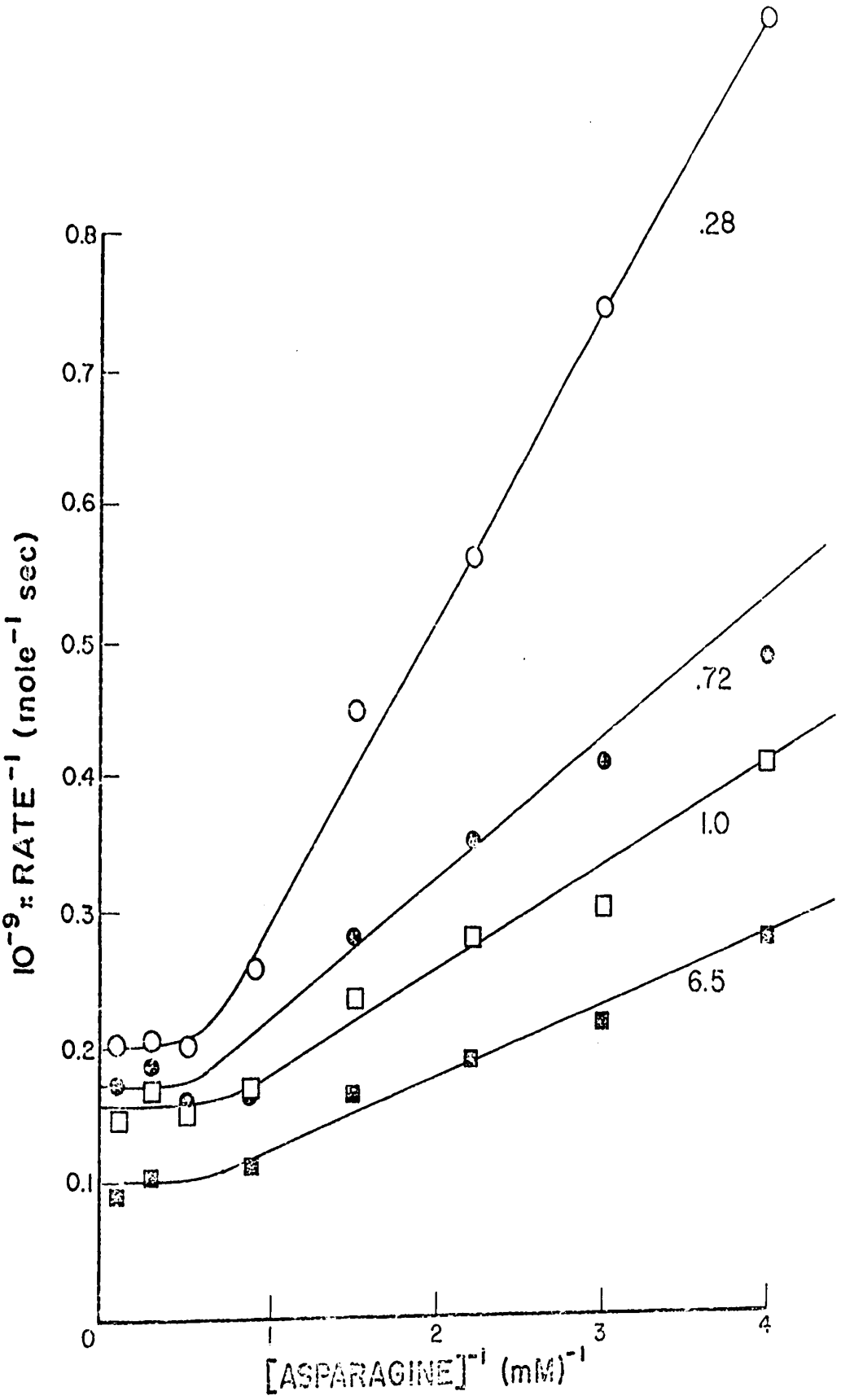
Rates of Reaction (10^9 x moles sec^{-1}) for a 0.8-metre Tube at High Ionic Strength, and at Various Substrate Concentrations and Flow Rates

(Values in Brackets Calculated According to eq. 27)

10^3 x [ASN]	Flow Rate, v_F (cm sec^{-1})			
(M)	6.5	1.0	0.72	0.28
0.25	1.3 (2.4)	0.98 (1.3)	0.79 (1.1)	0.39 (0.83)
0.33	1.7 (3.3)	1.3 (1.7)	0.96 (1.5)	0.52 (1.1)
0.45	1.9 (4.3)	1.4 (2.3)	1.1 (2.1)	0.69 (1.5)
0.67	2.3 (6.4)	1.7 (3.4)	1.4 (3.0)	0.87 (2.2)
1.1	3.4 (10)	2.4 (5.6)	2.4 (5.0)	1.5 (3.6)
2.0	5.3 (19)	2.6 (11)	2.4 (9.2)	1.9 (6.7)
3.3	3.5 (33)	2.4 (17)	2.1 (15)	1.9 (11)
10	4.1 (95)	2.7 (53)	2.3 (45)	1.9 (33)

Figure 33

Lineweaver-Burk plots for the 0.8-meter tube, at various flow rates (cm sec^{-1}) which are indicated on the lines. The data are for high ionic strength (1.25 M).


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
1
2
3
4
.28
.72
1.0
6.5
$10^{-9} \times RATE^{-1}$ (mole^{-1} sec)
[ASPARAGINE]$^{-1}$ (mM)$^{-1}$

CHAPTER FIVE

GENERAL DISCUSSION

This final chapter is concerned with general conclusions and discussion related to the experiments presented in Chapters 3 and 4. The discussion centres around the changes in K_m(app) which are observed when enzymes are immobilized on solid supports. These changes will be analyzed in terms of the four factors mentioned in Chapter 1, which are likely to alter the kinetics of immobilized enzymes. For simplicity, factors (1) and (2) are combined into a simple Factor I in this chapter, since conformational and environmental changes are difficult to separate. Factor II is the partitioning effect, and attention is focussed on the electrostatic aspect. Factor III is the effect of diffusion.

A number of experiments by other workers have been carried out in which the apparent Michaelis constant for the enzyme on a solid support has been compared with the constant for the enzyme in free solution. A list of some of these is presented in Table 18. The examples are grouped into four different categories, as will be discussed below. Numbers 1 to 4 represent instances where partitioning is known to be involved; numbers 5 to 15 involve diffusion effects; and numbers 16 to 23 involve Factor I. Numbers 24 to 34 represent instances where the different factors cannot be identified

TABLE 18

Apparent Michaelis Constants for Solid-Supported Enzyme Preparations
(the electrostatic charge is indicated in brackets)

Enzyme	Substrate	Support	$K_{m(app)}$ (mM)	Reference
Partitioning Effects				
(1) Ficin	BAEE(+)	carboxymethyl cellulose (-)	2.0	68
		none	20	
(2) Trypsin	BAA(+)	maleic acid/ethylene copolymer (-)	0.2	69
		none	6.8	
(3) Bromelain	BAEE(+)	carboxymethyl cellulose (-)	49	146
		none	110	
(4) ATP creatine phosphotransferase	ATP(-)	carboxymethyl cellulose (-)	7.0	68
		none	0.65	
Diffusion Effects				
(5) Chymotrypsin	ATEE	sepharose	30	46
		none	3.3	

Table 18 cont'd

(6) Chymotrypsin	ATEE	DEAE-cellulose (large particles)	K'_m	40
		none	$<K'_m$	
(7) Invertase	sucrose	polyamine ion exchange resin	170	67
		none	160	
(8) β-Galactosidase	ONPG	polyacrylamide (thick slices)	1.24	This work
		none	.174	
(9) Asparaginase	asparagine	nylon	.015	This work
		none	.013	
(10) Alkaline Phosphatase	PNPP	collodion	1.80	126
		none	.034	
(11) Asparaginase	asparagine	nylon	3.0	140
		none	.013	
(12) Trypsin	gelatin	gelatin	3100	49
		none	600	
(13) Alkaline phosphatase	SPP	polyacrylamide (thick film)	4.0	34
		none	1.0	

Table 18 cont'd

(14) Succinic dehydrogenase	chlor-succinate	natural membrane	inferred from shape of L-B plots	43,44
		none		
(15) Potato apyrase	ATP	various	400-1000	132
		none	90	
Environment Effects				
(16) Invertase	sucrose	polyamine ion exchange resin	115	67
		none	160	
(17) β-Galactosidase	ONPG	polyacrylamide	0.09	This work
		none	0.174	
(18) Asparaginase	asparagine	nylon	0.020	This work
		none	0.130	
(19) Chymotrypsin	ATEE	sepharose (solubilized)	3.0	46
		none	3.3	
(20) Chymotrypsin	ATEE	DEAE cellulose (small particles)	K'_m	40
		none	K'_m	
(21) Urease	urea	nylon	3.5	19
		none	3.5	

Table 18 cont'd

(22) Xanthine oxidase	xanthine	intact cells	0.018	145
		none	0.020	
(23) β-Fructo furanosidase	sucrose	polystyrene: tube beads	45 40	144
		none	20	
(24) ATP creatine phosphotransferase	ATP(-)	p-aminobenzyl-cellulose (+)	0.80	68
		none	0.65	
(25) Chymotrypsin	ATEE	carboxymethyl cellulose	0.56	68
		none	0.27	
(26) Papain	BGEE	starch derivative	34	
		none	18	
(27) Subtilopetidase A	ATEE	starch derivative	17	
		none	5.4	
(28) Catalase	not cited	natural membrane	K'_m	16
		none	K'_m	
(29) Potato apyrase	ATP(-)	cellulose	0.25	147
		none	0.10	

Table 18 concluded

(30) Glucose oxidase	glucose	cellophane	13.0	148
		none	13.0	
(31) Adenosine triphosphatase	ATP(-)	natural membrane (-)	0.40	35
		none	0.10	
(32) Glucose oxidase	glucose	nylon	36	20
		none	13	
(33) Trypsin	BAA(+)	maleic acid/ethylene copolymer (-)	7.0	69
		none	6.8	
(34) Papain	BAEE(+)	p-aminophenylalanine/ leucine copolymer (+)	K'_m	143
		none	K'_m	

ABBREVIATIONS

ATEE	N-acetyl-L-tyrosine ethyl ester
ATP	Adenosine triphosphate
BAA	N-benzoyl-L-arginine amide
BAEE	N-benzoyl-L-arginine ethyl ester
BGEE	N-benzoyl-glycine ethyl ester
ONPG	o-nitrophenyl-β-D-galactopyranoside
PNPP	p-nitrophenylphosphate
SPP	disodium phenylphosphate

with any certainty. As will be seen, analysis of the examples in Table 18 leads to a number of interesting generalizations and predictions relating to the extent to which the three factors may be expected to influence the kinetics of an immobilized enzyme.

Partitioning

The effect of partitioning on the apparent Michaelis constant is perhaps most easily identified, and for this reason instances conforming to this pattern of behaviour are listed first in Table 18. Absence of a partitioning effect of an electrostatic nature may be claimed if either the support or the substrate is neutral. Furthermore, if such an effect is suspected, it may be reduced or eliminated by carrying out the kinetic study at high ionic strength, as discussed in Chapter 4. Examples (see reference (3)) are known where no change in K_m(app) is observed for an enzyme in a charged support, acting on a neutral substrate. On the other hand, if the same enzyme is attached to the same charged support, and allowed to react with a charged substrate, a significant change in that constant takes place (3).

It should be borne in mind that effects due to non-electrostatic partitioning may also occur. Since few workers have measured such partition coefficients, this effect will not be discussed.

Examples 1 to 3 in Table 18 illustrate the

significant decreases in K_m(app) which result when an attractive electrostatic effect is involved. (The electrostatic charges are given in brackets). Example 4 is a case of electrostatic repulsion between support and substrate, with ensuing increase in the K_m(app).

Diffusion

The effect of diffusion on the kinetics of supported enzymes is to increase the K_m(app) value, as discussed in Chapters 1, 3 and 4. Numbers 5 to 15 of Table 18 represent instances where diffusion control has been shown to be at least partly responsible for the rise in K_m(app) compared with the value for the enzyme in free solution. In numbers 5 and 6, environmental changes were shown to be small, as discussed in Chapter 1, and electrostatic effects were not involved. The rise in K_m(app) in examples 7-10was shown to be the result of diffusion by comparison of the results with the relevant theoretical treatment, other effects being accounted for. In example 10, diffusion was certainly partly responsible for the rise in K_m(app), but it is possible that environmental factors were involved. Example 11 involves diffusion, by analogy with number 9. The environments in the gel and the sol were the same for example 12 (trypsin acting on gelatin), so that diffusion is clearly implicated. For example 13, the partition coefficient is near unity, by analogy with our results with polyacrylamide reported in Chapter 2. While

environmental factors may be involved as well, diffusion is partly responsible for the increase in K_m(app), as shown by comparison with the theory presented in the same paper (34). In case 14, the Lineweaver-Burk plots were curved in a manner similar to that discussed in Chapters 1, 3 and 4; this indicates diffusion control. Again, environmental factors may also be involved. Example 15 is included, even though diffusion was not actually demonstrated, since the increase in K_m(app) is too large to be explained by environment alone (see below), and electrostatic effects were not involved.

Environment

The effect of environment (Factor I) on the apparent Michaelis constant is the most difficult to determine, since partitioning and diffusion must first be accounted for. In many instances, when no change in K_m(app) is observed, it is assumed that no environmental effect is present. An example of this type is invertase acting on sucrose (number 7 of Table 18); but diffusion was shown to be involved. The same system, when free from diffusion effects (example 16), had a decreased value for K_m(app). The presence of diffusion (number 7) caused the value to be very similar to that for the enzyme in free solution. It is clear that, in this case, the two Michaelis constants were equal by coincidence; no conclusion as to environmental effects in number 7 can be drawn.

For examples 16 to 18, diffusion effects were taken into account with the aid of theoretical treatments, so that an environmental effect could be demonstrated. The changes in K_m(app) due to environment are fairly small. For examples 19 and 20, diffusion was ruled out, as discussed in Chapter 1. Again, the effect of environment is small. In the case of example 21, Kobayashi and Laidler (64) showed that the rates all fell into Region I of Figure 27, so that diffusion effects were not involved. Since there is no partitioning in the tube experiments, Factor I was the only effect remaining; but its influence on K_m(app) was negligible. For xanthine oxidase (number 22), the activation energies for the free and bound enzyme were found to be the same. Since a diffusion-controlled activation energy would be expected to be much smaller than the observed values, Factor I alone was involved, there being no electrostatic effect. Again, the effect is very small. For β-fructofuranosidase (number 23) the K_m(app) values for two types of nylon-supported enzyme were very similar. It is unlikely that exactly the same extent of diffusion control applies to two different systems (particlesand a tube) and the environments for both supported enzymes are the same, as are their K_m-values. In this case, there is an increase in K_m(app) by a factor of two, compared with the free enzyme, with Factor I being the most likely cause.

Undecided

In examples 24 to 34, the various factors could not be separated with any degree of certainty as being responsible for the observed changes in K_m(app). Numbers 24 to 29 have an increase in this constant for the bound enzyme compared with the free enzyme. This could be the result of diffusion and/or environment. In case 30, diffusion and environmental effects could cancel each other out, leading to no observed change in K_m(app). There is again an increase in this constant for numbers 31 and 32. In the former case, either diffusion or charge repulsion are likely factors, as well as Factor I. In the latter instance, diffusion and environment are again possible factors. Case 33 is another instance of almost no change taking place in K_m(app) upon immobilization; this could be the result of electrostatic attraction and diffusion effects cancelling out, since support and substrate are oppositely charged. Finally, example 34 involves electrostatic repulsion between support and substrate, which should produce an increase in the apparent Michaelis constant. The fact that no increase was observed suggests that Factor I is involved, lowering the K_m(app) to some extent, and cancelling out the electrostatic partitioning effect.

General Principles

The examples in Table 18 lead to a number of general principles in terms of the qualitative effects of Factors I, II and III on the apparent Michaelis constant of the bound enzyme compared with that of the free enzyme. When diffusion effects are known to be present, the largest changes in K_m(app) take place, increases of up to a thousand-fold being observed. When electrostatic partitioning effects are involved, large changes of an order of magnitude in the apparent Michaelis constant are not uncommon. It is reasonable to suggest that the converses are also true, i.e. that a large change in K_m(app) is probably due to diffusion and/or electrostatic effects. These may be separated by experiments at high ionic strength as previously discussed.

Smaller changes in the apparent Michaelis constant are more difficult to assign, however, since they may result from Factor I, or cancellation of two or more of the effects discussed.

Generalizations of this nature are, of course, only rough guidelines. A reliable interpretation of any change in K_m(app) can only be obtained from a detailed study utilizing the appropriate quantitative theoretical treatment to interpret the results of the particular system under study.

The experimental work described in Chapters 3 and 4 of this thesis has substantially verified the theoretical treatments of the kinetics of enzymes embedded in membranes

and attached to tubes. Other theories are available for enzymes immobilized in particulate and other systems (cf. Table 1). Utilizing such theories, the effect of diffusion on an experimental value for K_m(app) may be taken into account. If partitioning effects are evaluated experimentally, the effect of Factor I on the enzyme behaviour may readily be determined.

In future experiments, therefore, it will be possible for even semi-quantitative results to be interpreted in a reasonably reliable manner in terms of the various factors likely to influence the kinetics. Use of a reliable theory becomes important for studying enzymes attached to natural membranes, for instance, where parameters such as enzyme concentration, membrane thickness and the like cannot be varied at will, as is the case in a model system.

CLAIMS TO ORIGINAL RESEARCH

1. A method has been developed for preparing immobilized derivatives of enzymes uniformly distributed throughout a solid matrix, in such a way that accurate and reproducible membrane slices may be obtained. This method involves the formation of a gel (polyacrylamide) from a solution (acrylamide monomer) containing the enzyme, by means of chemical polymerization. The gel is subsequently sliced with a microtome.
2. Two methods of initiating the polymerization of acrylamide have been studied, namely photolysis and radiolysis. The latter method of preparing solid-supported enzymes has not been thoroughly studied previously.
3. The manner in which the supported enzymes lose their activity after being immobilized, and the stabilities of the preparations, have been examined.
4. The diffusion and partition coefficients for o-nitrophenol in polyacrylamide gels have been measured under various conditions.
5. β-Galactosidase trapped in polyacrylamide gel has been utilized in order to test a theoretical treatment of the way in which the Michaelis-Menten parameters of a solid-supported enzyme are affected by the surrounding solid matrix. The theory has been tested for the first time over a wide range of membrane thicknesses, and of enzyme and substrate concentrations.

6. The enzyme L-asparaginase, has been attached to the inside walls of a nylon tube. This tube has been used in a flow system in order to test a theoretical treatment of the effect of diffusion on the Michaelis-Menten parameters of the enzyme.
7. The results obtained in the above experiments have been discussed in relation to results of other workers in the field, and certain general conclusions have been drawn as to the effects of immobilization of an enzyme on its kinetic properties, in comparison to the properties of the enzyme in free solution.

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