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REACTION KINETICS AND STRUCTURE OF ENZYME
MACROMOLECULES SUBJECTED TO HYDRODYNAMIC
SHEARING FORCES: ENZYME MECHANOCHEMISTRY

A Dissertation Presented

By

MATTHEW TIRRELL

Submitted to the Graduate School of the
University of Massachusetts in partial fulfillment
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August

1977

Polymer Science and Engineering

REACTION KINETICS AND STRUCTURE OF ENZYME
MACROMOLECULES SUBJECTED TO HYDRODYNAMIC
SHEARING FORCES: ENZYME MECHANOCHEMISTRY

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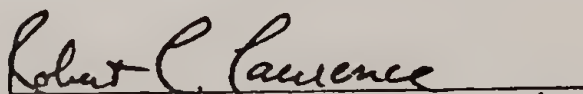
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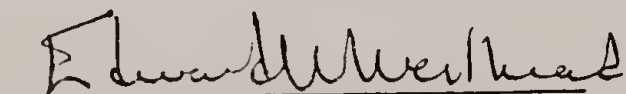
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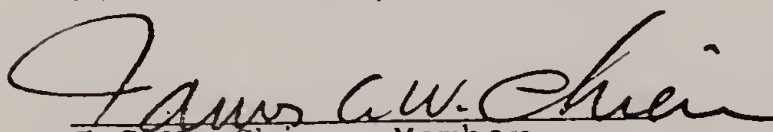
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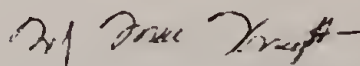
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Für Claudia,
Du hast mein Leben
wunderschön gemacht!

and

for my mother,
who worked much
harder than I did
to make this possible.

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ABSTRACT

Reaction Kinetics and Structure of Enzyme
Macromolecules Subjected to Hydrodynamic
Shearing Forces: Enzyme Mechanochemistry

September 1977

Matthew Tirrell
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Directed by: Professor Stanley Middleman

This dissertation reports on efforts to determine whether hydrodynamic shearing forces, of a magnitude encountered in many practical situations, will affect the structure, and therefore the catalytic activity, of enzyme macromolecules. Some of the situations where this type of effect may be expected to be important are: flow of blood through some extracorporeal treatment device; biochemical reactor systems; mechanochemical systems in vivo such as membranes and muscles; and analytical or rheological measurement conditions involving strong flow fields. With these ideas in mind, the present work deals primarily with the study of the kinetics of the urease catalyzed hydrolysis of urea occurring in deforming reaction media. When effects on the kinetics due to shearing were observed, ancillary analytical techniques were employed in an attempt to learn about the structural changes responsible.

Initially, strong, shear rate dependent, partially irreversible inactivation of urease was found with applied stresses up to 2.5 Pascals. Qualitatively similar results were obtained with urease preparations of three different levels of purity. Protection against this inactivation is provided by millimolar quantities of ethylenediaminetetraacetate (EDTA) and β -mercaptoethanol (β -met). Shearing promotes urease inactivation by parachloromercuribenzoate (PCMB). These three facts form the basis for the proposed mechanism of the irreversible inactivation due to shearing. This mechanism is a shear-promoted, metal ion catalyzed oxidation of an essential sulfhydryl group(s) on urease. Evidence that the metal ion responsible is adventitious ferric ion has been obtained as well. Size exclusion chromatography of urease before and after shearing revealed molecular weight heterogeneity and preferential inactivation of the larger size urease species. Some shear-induced aggregation was noted as well when urease was sheared in relatively concentrated solutions. Circular dichroism studies showed changes in the secondary structure consistent with this after long shear exposure, although no change in the secondary structure was detected after relatively short exposures, even when an activity change was seen.

Reaction media of higher viscosity, including 5 mM EDTA, were investigated by conducting the reactions in glyc-

erin/water mixtures. In this system inactivation was found to be shear stress dependent and reversible up to at least 21.0 Pascals. This effect must be the result of a hydrodynamically induced conformational change in urease. Indications are, in this set of experiments as well as the previous set, that only the aggregated urease species are significantly affected by these stress levels.

Viscometric measurements have been combined with a recently proposed theory to show that the urease oligomers are linearly arranged. This may be related to their shear susceptibility.

Preliminary experiments with another enzyme, lactic dehydrogenase, have shown that it is unaffected by stresses up to 2.1 Pascals. It is however irreversibly inactivated about 50% by exposure to 9.4 Pascals. Structural changes, observed in UV and CD spectra, are seen to accompany inactivation. It is thus seen that the comparatively large size of urease is not an essential feature to see shear modification of enzyme kinetics.

A quantitatively good, phenomenological model of shear modification urease catalyzed urea hydrolysis has been developed; unfortunately, the physical significance of some of the parameters remains obscure. Although some conjectures are advanced, a detailed molecular understanding of the shear modification phenomena is a task for the future.

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P R E F A C E

This dissertation is divided into nine chapters.

Chapter I begins by discussing some motivation for the work presented. This is followed by a general discussion of the effects of flow fields on the conformations of macromolecules in fluids, including some pertinent remarks on shear degradation (covalent bond cleavage) of macromolecules. Then, a comprehensive review is made of all work relevant to the effect of mechanical forces on biologically active proteins. Every known example of an imposed mechanical force causing an activity change is included, as well as some work on inactive species structurally similar to enzymes, to obtain some perspective on the effects one might possibly expect.

Since the principal object of study in this work was the enzyme urease, Chapter II is a review of the basics of urease catalysis and structure. Chapter III contains the details of all the experiments done with urease. It is intended to be primarily useful to any future experimentalist pursuing similar lines of research. Conceptually, it is independent of the remainder of the dissertation and may be skipped by anyone more interested in the results themselves than how they were obtained. Sufficient remarks are given with the results on urease, presented in Chapter IV, to make

their origin clear.

Chapter V describes two different kinds of efforts to describe the modifications in kinetics of enzyme catalyzed reactions occurring in deforming media. The first is empirical and the second barely scratches the surface of a still covert molecular understanding.

Chapter VI is a pause to make some general remarks on the interpretation and significance of the results of this dissertation. Chapter VII then digresses a bit to discuss some intrinsic viscosity measurements on urease. These are used, along with a recently proposed theory, to provide new evidence that urease aggregates in a linear fashion. Some preliminary results on shear-kinetic experiments with lactic dehydrogenase are reported in Chapter VIII. Conclusions are enumerated and suggestions for future work made in the final chapter, Chapter IX.

It is hoped that this preface, and the organization of this dissertation which the preface describes, will facilitate an appreciation on the part of the reader for the motivation, structure and principal accomplishments of this work.

C H A P T E R I

INTRODUCTION TO ENZYME MECHANOCHEMISTRY

There are two lines of investigation pursued in this dissertation which unify and motivate the various parts. The first is, what are the nature and magnitude of the effects of hydrodynamic stresses on biologically active proteins, specifically enzymes? The second is, what can be learned about the structure and function of these macromolecules by their physical and chemical responses to these stresses? While more or less classical viscometric structure determination forms a small part of the work reported herein, the stresses applied to the enzyme macromolecules have, for the most part, purposely exceeded the noninfluential (in terms of structural changes) stress levels of viscometry. The objective is to understand the behavior of a biologically active protein molecule when it is subjected to sufficient physical force to alter its activity. A complete realization of this objective would consist of: (1) ascertaining the lower limit of physical force necessary to cause a perceptible activity-modifying effect, and (2) elucidating the mechanism of activity modification, meaning the exact nature of the conformational or chemical changes induced by stress. Included in the latter are any effects of the environment which work in

concert with the applied stress to bring about the activity modification. Among these are effects of surfaces, pH, ions and temperature. As will be shown in subsequent chapters, these have been nearly fulfilled in this work for one enzyme system and some steps have been taken toward study of another enzyme.

Extracorporeal treatment of blood for the purposes of oxygenation or detoxification (Middleman, 1972; Lightfoot, 1974) or for therapy for the multitude of enzyme deficiency diseases (Wacker and Coombs, 1969) constitutes an area of very practical application for this sort of research. Applications to food engineering are possible as well (Charm, 1963; Pradipasena and Rha, 1977). With this in mind, the literature pertinent to this dissertation is now examined, first with a general, and quite lacunary, review of the effect of mechanical forces on the conformations of macromolecules, and second with a thorough review of the previous work relevant to the effects of mechanical forces on the structure and function of biologically active protein macromolecules.

For synthetic macromolecules it is fairly well established that conformational changes occur when they are subjected to hydrodynamic forces; however, most of the evidence for this is rather indirect, coming from measurements of flow birefringence and viscometry (Janeschitz-Kriegel, 1969; Bird et al., 1977) or inferred from molecular theories (Yamakawa, 1971; Bird et al., 1977). There is some direct evidence from

light scattering that molecular extension does occur (Cottrell et al., 1969; Cottrell et al., 1970; Champion and Davis, 1970). This implies that there is mechanically induced rotation about backbone bonds. Qualitatively, what happens is that the originally spherical statistical shape of the random coil takes on an ellipsoidal shape when subjected to a homogeneous flow field. This shape change is fully reversible on stopping flow. For simple shear flow, deformation occurs in the plane defined by the velocity vector and the direction of the velocity gradient; for simple elongational flow these two directions coincide and molecular deformation occurs in this direction as well. No direct measurements of macromolecular coil deformation in elongation flow have been reported as yet although experimental apparatus for such work is presently under construction (Leal and Pings, 1977). For this reason, the bulk of the remainder of this dissertation will deal with simple shearing flows, except for periodic comments where an elongational flow may be important practically or potentially interesting experimentally. Molecular theories based on models such as the elastic dumbbell (Bird et al., 1977) or the Rouse model (Rouse, 1953) treat the inherent resistance of the molecule to deformation as governed by a Brownian motion-entropic potential. The predictions for $\alpha^2 = \langle r^2 \rangle / \langle r^2 \rangle_{eq}$, where $\langle r^2 \rangle$ is the mean square end-to-end length and the subscript eq refers to equilibrium conditions, as a function of a dimensionless shear

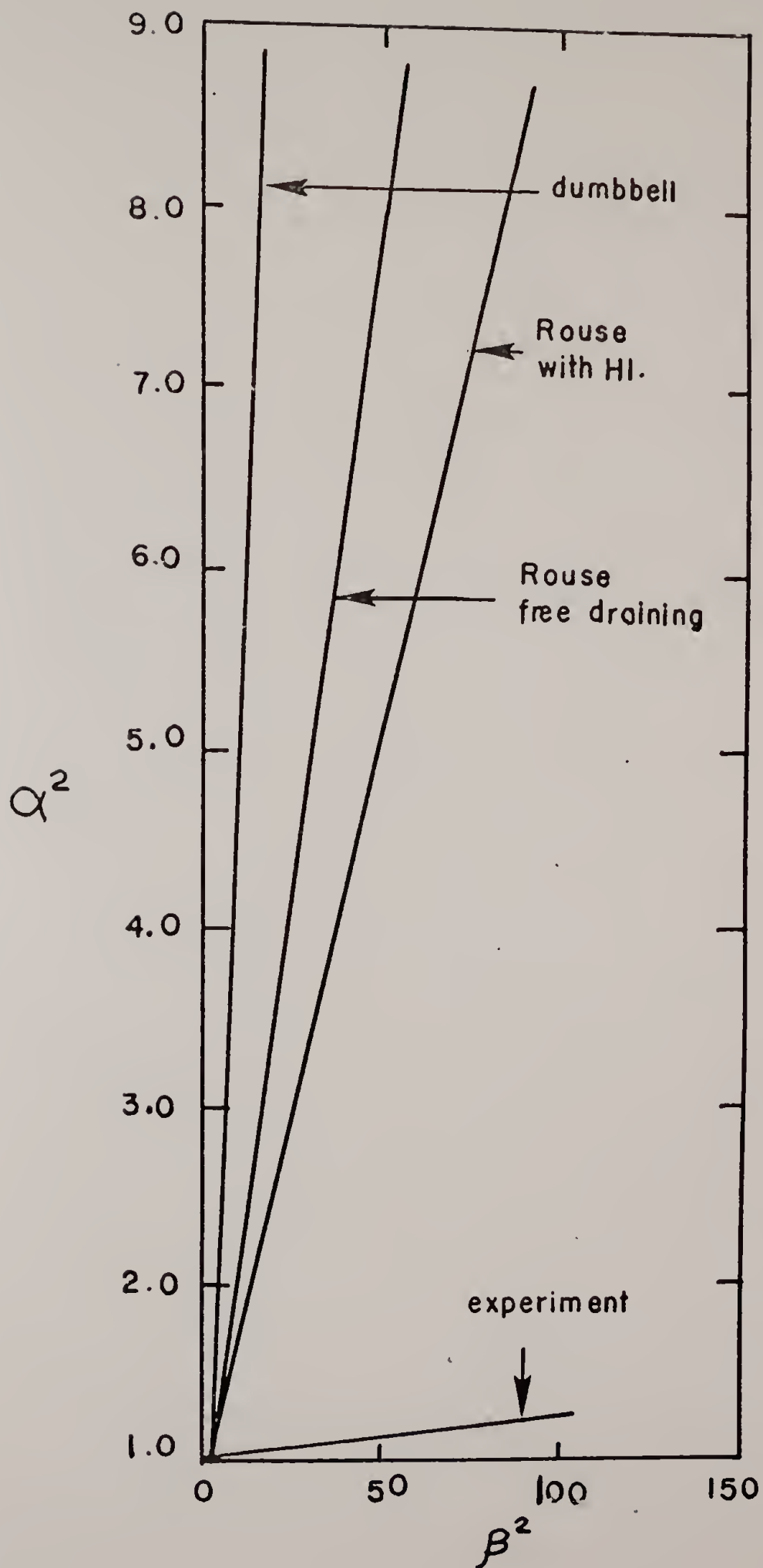
rate $\beta = [\eta]_{eq} \bar{M}_w \dot{\gamma}_0 / RT$, are shown in Figure I-1. All the results of these molecular theories can be expressed in the form $\alpha^2 = 1 + K\beta^2$ where $K = 2/3$ for elastic dumbbells (Bird et al., 1977), $4/15$ for the "free draining" Rouse theory (Peterlin, 1963) and 0.136 for a version of the Rouse theory which treats the domain of the polymer coil as impermeable to solvent flow (Peterlin, 1963). Also shown in Figure I-1 are experimental results of Cottrell et al. (1969) which fit the line $\alpha^2 = 1 + 0.013\beta + 0.000169\beta^2$. Obviously, real polymer coils deform significantly, but much less than predicted by these models. This realization, which had become clear independently of these results, has led to the notions of "internal viscosity" and "inherent rigidity" (Cerf, 1960) in macromolecules. The idea is that there is a force, F_{IV} , opposing conformational change, indicated by the time rate of change of the end-to-end distance, \dot{r} ; this "sluggishness" leads to the defining relation for inherent rigidity, Q :

$$F_{IV} = Q\dot{r} \quad (1)$$

Including this effect (Cerf, 1968) brings the predictions of the molecular theories closer to reality (Cottrell et al., 1969). Values of Q have been estimated for both polystyrene and polyisobutylene at 1.6×10^{-5} g/sec (Cottrell et al., 1969; Peterlin, 1967; Cerf, 1958).

Under extreme conditions of applied hydrodynamic shearing forces, macromolecules may degrade mechanically, re-

Figure I-1. Molecular extension as a function of a dimensionless shear rate for three theoretical models and some experimental data (after Cottrell et al., 1969).



sulting in chain scission and decreasing molecular weight. For macromolecules of sufficient molecular weight and concentration to experience entanglements with other macromolecules in fluid media, degradation is promoted by the mechanical energy stored in the chain links between entanglements. F. Bueche (1960) has presented a theory which treats this phenomenon; the pertinent essential features of his theoretical results are: (1) activation energy for bond cleavage is reduced by the mechanical tension in the bond; (2) degradation is highly molecular weight-sensitive; (3) there is a lower molecular weight below which no mechanical degradation occurs; (4) a reduced parameter, including bulk shear stress, molecular weight and temperature, controls the extent of degradation; and (5) breakage occurs near the middle of the chain molecule.

In a dilute, noninteractive solution, mechanical degradation has also been reported (see for example: Casale et al., 1971; Casale and Porter, 1977). In some cases, degradation in dilute solution can definitely be attributed to capillary entrance effects (Culter et al., 1972) or to turbulence (Virk, 1975) both of which have some elongational flow character from the point of view of the macromolecule. It is not now certain whether a laminar shearing flow is capable of degrading synthetic polymer molecules in the absence of entanglements. It does seem possible to degrade high molecular weight polynucleotides such as DNA under di-

lute laminar conditions (Davison, 1960; Levinthal and Davison, 1961; Harrington and Zimm, 1965; Yew and Davidson, 1968; Bowman and Davidson, 1972; Adam and Zimm, 1976) as well as turbulent ones (Cavalieri, 1957; Cavalieri and Rosenberg, 1958; Doty et al., 1958; Thomas, 1959; Davison, 1959, 1960; Rosenkranz and Bendich, 1960; Mandell and Hershey, 1960; Hershey and Burgi, 1960; Burgi and Hershey, 1961, 1962; Davison et al., 1961; Rubenstein et al., 1961; Kaiser, 1962; Hershey et al., 1963; Hogness and Simmons, 1964; Harrington and Zimm, 1965; Harrington, 1966; Rees et al., 1974; Noll et al., 1975; Lewis et al., 1976). Harrington and Zimm (1965) also found degradation of polystyrene in dilute solution under laminar and turbulent conditions. The shear stresses where degradation begins to occur in laminar flow have been observed to range from about 10.0 to 100.0 Pascals, these observations having been made in solvents of viscosities from 10^{-3} Pa-s to about 10^{-2} Pa-s. If one makes any of a number of reasonable assumptions, the applied mechanical tension necessary to cleave a covalent bond (carbon-carbon for example) in a purely mechanical fashion may be estimated from known bond strengths (Levinthal and Davison, 1961, 1967; Harrington and Zimm, 1965; Norrby, 1975). A value of 10^{-9} Newtons is typically obtained. If the molecular cross-section through which this force acts is taken to be in the range 10^2 to 10^3 nm, this leads to an estimate of the necessary shear stress to produce rupture of 10^3 to 10^5 Pascals.

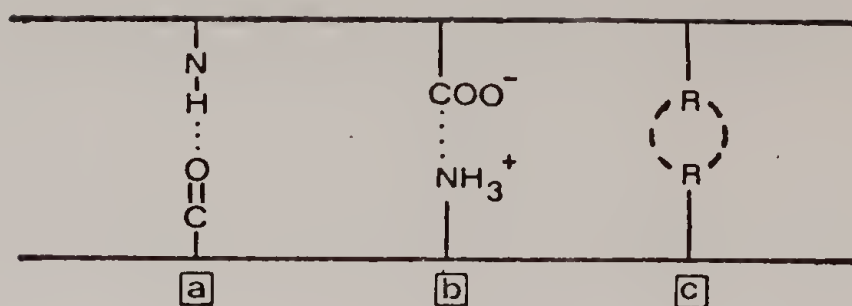
We see that there is a discrepancy of 1 to 4 orders of magnitude between the estimated and observed critical stresses. This raises a question, still without a general answer and very germane to this dissertation: in the cleavage reaction, do the hydrodynamic forces provide most of the energy for the chemical reaction or do they function to decrease somewhat the activation energy of a thermal reaction or of a chemical reaction, such as hydrolysis or solvolysis, so that shear breakage is properly viewed as a mechanically assisted thermal or chemical reaction? Put another way, more appropriate to our subsequent discussion of the effect of shear on biologically active macromolecules, are the hydrodynamic forces directly causative of some modification reaction or rather do they simply precondition the macromolecule to subsequent modifying events? This dissertation presents evidence indicating that the latter may be true for some protein molecules at moderate stress levels.

However, to put this work in proper perspective, the existent literature on the effect of shear on proteins will be reviewed. We begin with some essential features of protein structure.

In contrast to synthetic macromolecules, whose conformations are governed by entropic-Brownian motion considerations, the conformations of protein macromolecules are dictated by specific energetic interactions. This makes a general theory of mechanical deformation of proteins very

difficult because of the differences among proteins of these energetic interactions. Some attempt toward this has been made by some Russian workers for globular protein enzyme molecules (Khurgin et al., 1967) although they actually only present a conceptual rather than quantitative discussion. Hydrogen bonding, van der Waals forces, electrostatic forces, hydrophobic forces and intramolecular covalent crosslinks each play important roles in the construction of a protein molecule. The net result of these forces is the natural or "native" conformation of the protein. Some of these interactions are illustrated and their relative energetic contribution to the stability of the protein structure compared in Figure I-2. In the native state, each polypeptide chain of a genetically distinct protein does not form a family of random coils; rather, it exhibits a unique and stable three-dimensional structure (Haschemeyer and Haschemeyer, 1973). This structure is present, in almost identical form, in every molecule of that protein. For convenience in our discussion of protein structure, it is helpful to differentiate certain levels of structure. Primary structure refers to the sequence of amino acids (residues) in the polypeptide chains which make up the protein. Both secondary and tertiary structure refer to elements composing the three-dimensional conformation. There are decided shortcomings in the arbitrariness of the distinction to be made, but operationally, the distinction is made as follows. Secondary structure re-

Figure I-2. Contributions to protein structural stability.



UNFOLDED CHAINS \rightleftharpoons NATIVE STRUCTURE

	<u>ΔG (kcal)</u>
CONFORMATIONAL ENTROPY	+ 340
CONFORMATIONAL ENTHALPY	- 100
HYDROPHOBIC BONDS [c]	- 130
HYDROGEN BONDS [a]	- 10
IONIC BONDS [b]	- 10
TOTAL	+ 90

fers to structural elements that involve interactions between amino acid residues fairly close to one another in the sequence. Because of the great diversity in types of near neighbor interactions in proteins, this definition has proved to be less useful for native proteins than it is for synthetic polypeptides. In a number of special cases (the triple helical structure of collagen), the exact meaning of secondary structure depends on the context in which it is used. More generally, secondary structure in proteins represents arrangements of the polypeptide chain that form more or less regular hydrogen bonded structures, in particular, α -helices and the parallel (or antiparallel) arrangement of chain segments known as pleated sheet or β -structures. Often, an arbitrary division of the secondary structure into these categories is made by quoting a "percent α -helix" and a "percent β -structure" for some protein, meaning the percent of the residues in the polypeptide chain participating in each of these types of structures. Whatever fraction is left over is usually termed "random chain," implying some degree of conformational freedom, although it is certainly not "as random" as chain segments in a synthetic polymer molecule. As noted above, there are also a small number of other less important secondary structures. Tertiary structure refers to the three-dimensional structure of the polypeptide chain that results from interactions between amino acid residues relatively far apart in the sequence. It may, more generally, be

regarded as that arrangement of the chain in three dimensions that is not regular (i.e., not composed of repeating structural elements). Intramolecular disulfide bonding is often a crucial element of a protein's tertiary structure. In proteins containing more than one polypeptide chain, the term is used in reference to the structure within each chain, independently. For proteins containing multiple identical or nonidentical subunits, or which tend to oligomerize, the total three-dimensional structure is described by the term quaternary structure, which refers specifically to that structure resulting from the interactions between distinct polypeptide chains. The electrostatic and, especially, hydrophobic forces are important here. Some individual proteins do exhibit a small number of multiple molecular forms, for example, different quaternary structures, as in the case of a distribution of oligomers or different combinations of nonidentical subunits. These multiple molecular forms are termed isozymes. These definitions will be used throughout this dissertation, although, to avoid confusion, the terms conformation, shape, or three-dimensional structure will often be used to include all types of structure above the level of primary structure.

The conformations of protein molecules which function as enzymes, or other proteins whose biological function involves small molecule binding, have as part of their structure a "binding site," or in the specific case of enzymes,

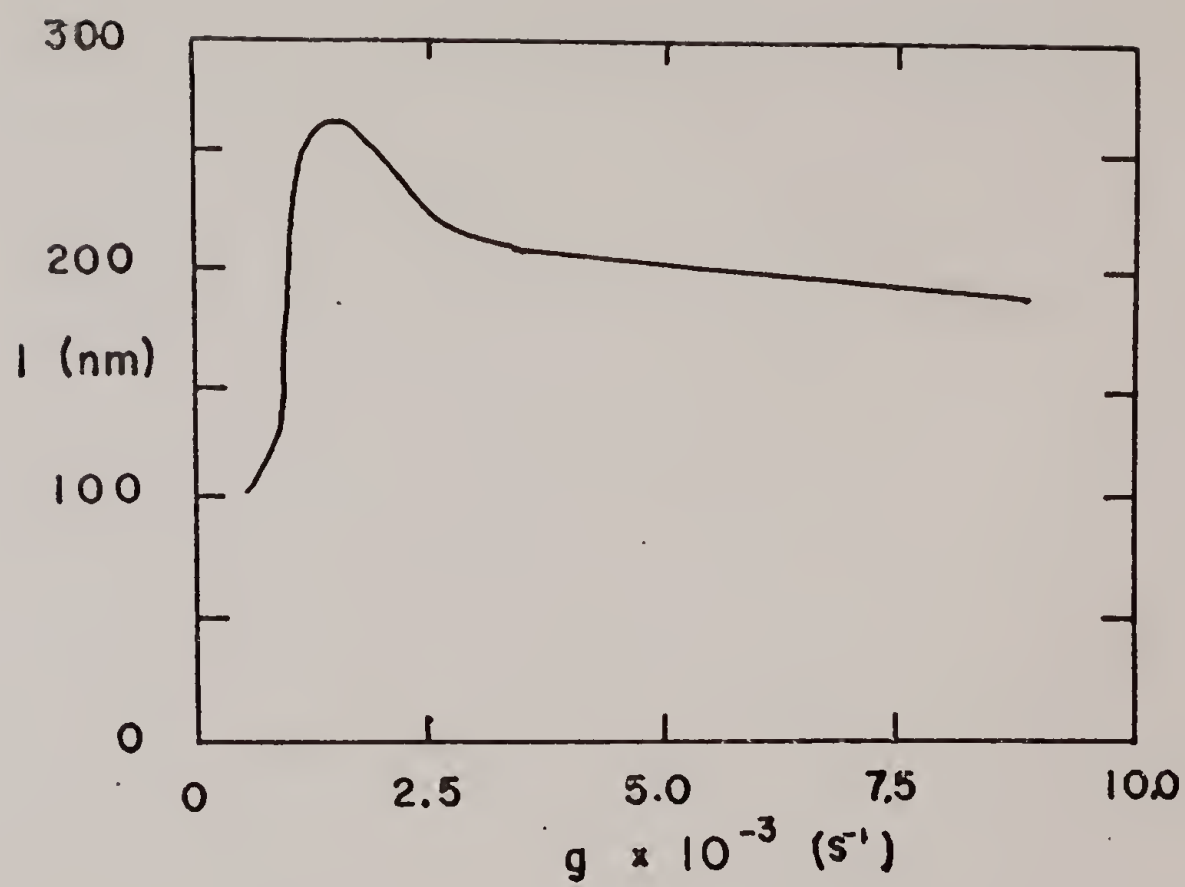
an "active site." This site has elements of the protein structure arranged so that groups responsible for binding and catalysis are appropriately positioned. This is not to imply however that the protein molecule is totally rigid; on the contrary, conformational adaptability on small molecule binding (Citri, 1973) and other aspects of the dynamics of protein conformations are now recognized (Steitz, 1968; Haschemeyer and Haschemeyer, 1973).

The first significant research program attacking the question of shear-induced conformational changes in proteins was that of Joly and Barbu (Joly, 1948, 1949; Joly and Barbu, 1949; Barbu and Joly, 1950). Their initial work examined aqueous solutions of horse serum albumin (HSA) and tobacco mosaic virus (TMV), at concentrations from as low as 0.025% for TMV to as high as 2.5% for HSA, under velocity gradients (g) from 27 s^{-1} to 9900 s^{-1} , in a circular Couette device fitted to measure flow birefringence. Measurement of the extinction angle, χ , for a fixed g , can be used to determine the rotational diffusion constant, D (Scheraga et al., 1951), which can in turn be used to calculate the effective length of the species, ℓ . Their initial findings were that increasing g in the range 0 to 2000 s^{-1} produced an increase in ℓ . This is due to a flow-induced aggregation process. It can, in fact, be described by the collision coagulation theory of Smoluchowski (1918) modified (Joly, 1958, 1964) to account for various interaction strengths between the particles. For

ellipsoidal molecules of small aspect ratio such as HSA, aggregation takes place in an end-to-end fashion where as Joly (1948, 1949) claims that the rodlike TMV molecules aggregate laterally.

For shear rates in excess of about 2000 s^{-1} , the shearing force breaks up the aggregates. Figure I-3 illustrates this behavior for HSA. Subsequently, Joly and Barbu demonstrated qualitatively similar behavior for a variety of other proteins (Joly, 1952a, b, c; Barbu and Joly, 1953; Joly, 1956, 1958). The picture they present of what is happening at a molecular level is that shearing increases the collision frequency of the molecules in solution. If they have "sticky" surfaces, either naturally or through a shear induced conformational change (this point has not been clarified), low velocity gradients will promote aggregation. Aggregation decreases χ and hence increases the apparent length, since the flow-produced multiplet will align more readily with the flow direction. At some critical shear rate, the hydrodynamic forces are sufficiently strong to cause rupture of the aggregates. This technique has been suggested as a means of studying the magnitude of physical forces involved in protein quaternary structures, aggregates or isozymes (Reithel, 1963). The quantitative behavior varies, depending on the particular protein, concentration, temperature, pH and ion content of the medium. More elongated molecules will aggregate more extensively, at lower

Figure I-3. Apparent molecular length as a function of velocity gradient for a 2.5% HSA solution (after Joly, 1958).



shear rates and concentrations, than molecules of smaller aspect ratio. For fully native protein preparations, these processes are usually irreversible but do not lead to precipitation. This is one of the indications that this process is flow induced aggregation rather than unfolding of the protein globule. However, for HSA which has been heated for 10 min at 70°C prior to shearing, undoubtedly producing some degree of denaturation, there are small but significant, reversible shear induced changes in the apparent length (Joly, 1956). This is an indication that shearing can unfold an at least partially "loosened" protein coil.

Some interesting insight into the kind of conformational change that can occur when proteins are sheared in solutions concentrated enough so that intermolecular interactions are important is provided by the research of Go and coworkers (Go et al., 1968a, b, c; Nakamura et al., 1968; Kondo et al., 1969a, b). They prepared aqueous buffered solutions of polypeptides: poly-L-glutamic acid, poly-L-tyrosine and several copoly(L-glutamic acid-L-alanine)s of 0.1 to 0.2%. Each of these polypeptides undergoes a rather sharp helix-coil transition upon increasing pH; pH 5.5 to 6.0 for poly-L-glu, pH 10.0 to 11.0 for poly-L-tyr and pH 3.8 to 4.5 for the copolypeptides. Each was subjected to mechanical forces in solution both by shaking and in circular Couette flow in a viscometer. What was found for the homopolypeptides was that for pH below the helix-coil transition,

shear rates from 500 to 8000 s^{-1} (or unquantified mechanical shaking) produced denaturation, meaning molecular coagulation and precipitation. Close to the transition (within about 1 pH unit) this denaturation was reversible upon removing the shearing force. No attempt was made to determine a lower critical shear rate for the onset of this phenomenon. Above the transition pH, no permanent effect of shear rate on structure or solubility was seen, the macromolecules behaving as random coils. Under conditions where mechanical denaturation occurred it was found, by optical rotatory dispersion (ORD), infrared spectroscopy and x-ray diffraction, that the process of mechanical denaturation is one in which the α -helical conformation changes to an intermolecular β -structure. This means that there is a shear-induced rearrangement of the hydrogen bonds. These same observations were also made with the copolypeptides, with some additional interesting features. Copoly(L-glu-L-ala) (19:1) followed the above pattern; however, copolymers of lower glu:ala ratios denatured (coagulated) but did not make a complete $\alpha \longrightarrow \beta$ transformation. A (9:1) copolymer maintained some α -helical content in the shear-denatured product while a (4:1) copolymer was still largely α -helical in the coagulated state. One might reasonably speculate that the increased hydrophobic character, due to increased alanine content, may alter the nature of the shear-induced coagulation mechanism. Interactions and structural changes of this sort may be responsible

for the unusual viscometric and denaturation properties observed when concentrated (up to 40%) protein solutions are subjected to flow experiences relevant to food processing (Pradipasena and Rha, 1977). These observations may also be relevant to the work of Riihimaki and Middleman (1974) who have shown that increasing shear rate increases the rate of gelation of gelatin (collagen fold formation) as well as modifying the structure of the gel, formed in solutions of concentration around 0.65% by weight gelatin.

There is evidence in the medical literature for plasma protein denaturation in flow through extracorporeal devices. If a blood-gas interface exists in the device, the strong intermolecular forces acting at the interface may produce denaturation in the absence of flow; however, it seems clear that there is also measurable denaturation, even in the absence of a gas interface, which must be due to hydrodynamic effects (Lee et al., 1961a, b; Wright et al., 1962; Dobell et al., 1965). Sheared plasma seems to be toxic in itself; furthermore, it interacts with platelets to delay ADP-, epinephrine- or collagen-stimulated platelet aggregation (Wallace et al., 1975). This indicates the potential for a shear induced clotting deficiency. Since surfaces of some sort are present in most flow situations, the investigator must be aware of the separate, and perhaps synergistic, hydrodynamic and surface effects. The importance of these two types of effects seems to be well sorted out in studies

of flow induced erythrocyte damage. In the presence of solid boundaries, shear stresses of 1.0 Pascal or less can cause erythrocyte lysis (Bernstein et al., 1967), however, in the absence of walls, the critical shear stress for lysis has been estimated to be 6000 Pascals in liquid-into-liquid jet experiments (Blackshear et al., 1966; Bernstein et al., 1967) and 400 to 560 Pascals for lysis due to an ultrasonically pulsating bubble (Rooney, 1970, 1972) or an ultrasonically vibrating wire (Williams et al., 1970; Nyborg, 1973). One method of distinguishing the two types of effects is by ascertaining whether shear stress or shear rate determines the magnitude of the flow induced effect (Nevaril et al., 1969). The former is characteristic of a purely hydrodynamic effect, while the latter may indicate that wall collision frequency is most important. Clearly, the complex interaction between flowing cells and artificial walls dominates the low stress lysis. This should be kept in mind in studies of shear on protein solutions, as well. Similar remarks apply with regard to gas-fluid surfaces. Interactions here are also poorly understood. Proteins are known to adsorb at gas-fluid interfaces. In fact, measurements of the surface viscosity (Scriven, 1960), of protein layers adsorbed at gas-fluid interfaces, have been used to study protein-protein interactions at these interfaces (Watterson et al., 1974) and to learn something about the conformational flexibility of protein molecules (Joly, 1968).

Some parenthetical remarks about the use of ultrasonics in this sort of research are relevant here. Ultrasonic irradiation has been used to degrade all types of macromolecules and cells. The vast literature on this has been reviewed by ÉI'Piner (1964) and Casale and Porter (1977) and there are some interesting applications to biological macromolecules. More to the point, noncavitational ultrasound has recently been applied to study mechanochemical effects in solutions of the enzyme malate dehydrogenase (Rooney, 1977). The sonically generated viscous shear stresses can be calculated for known geometries, although they often have large spatial variations (Rooney, 1972). This technique would seem to be potentially very useful to study small amplitude, forced mechanical deformations. Cavitational ultrasonic irradiation in aqueous solutions of enzymes produces hydrogen and hydroxyl radicals which in turn produce drastic chemical effects not directly of interest here (Berezin et al., 1974a, 1975; Klibanov et al., 1974, 1976).

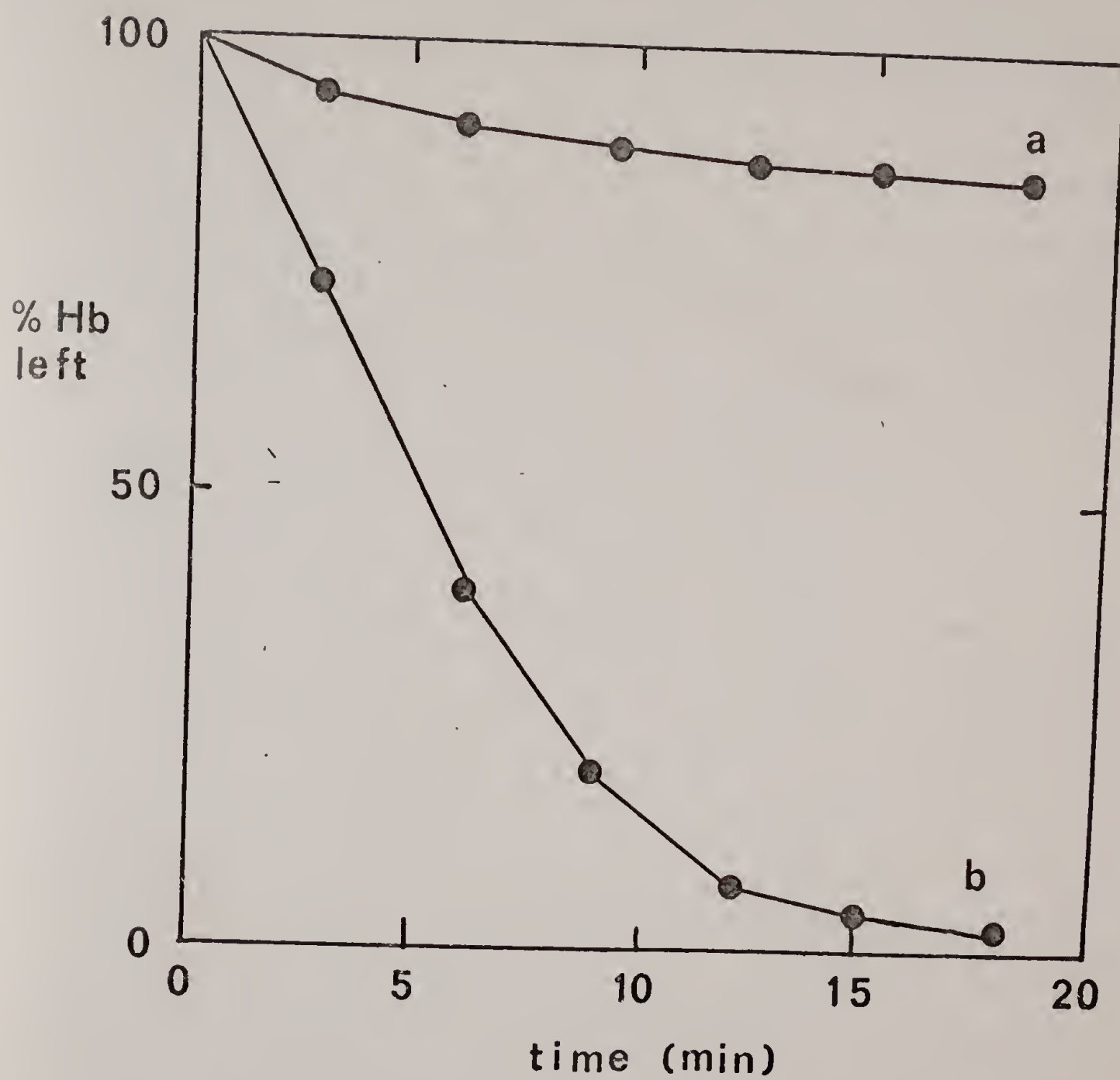
Returning to the discussion of flow induced structure and activity changes in blood components, Charm and Wong (1970a) have observed decreasing clottability of fibrinogen with prolonged shear exposure. They applied very low values of shear stress (as low as 0.1 Pascal) and found that the decline in clottability correlates with the total shear strain (shear rate x exposure time) experienced by the mole-

cule. They suggest that shear disruption of molecular structure may be a mechanism for fibrinogen turnover in the circulation. Unfortunately, the group at Rice University has been unable to confirm these experimental results (McIntire, 1977). There is however some other evidence that distortions of protein structures, such as those produced by small molecule binding, to higher energy states, has physiological significance in protein turnover (Grisolia and Hood, 1972; Silverstein and Grisolia, 1972).

Studies of the effects of plasma proteins on blood rheology have shown that molecules such as fibrinogen, β -globulin and albumin interact with cells in flowing blood in ways that suggest shear-induced conformational change (Wells, 1964; Merrill et al., 1964). For example, fibrinogen is responsible for the yield stress shown by normal blood (Merrill et al., 1964). Shear stresses at levels of about 40.0 Pascals have also been shown to irreversibly damage the endothelial layer of blood vessels (Fry, 1968, 1969; Caro et al., 1969; Texon, 1968).

Asakura and coworkers (Asakura et al., 1973, 1974a, b; Adachi and Asakura, 1974; Ohnishi et al., 1974) have reported on a very interesting effect of mechanical forces on hemoglobins. They found that the oxy form of sickle hemoglobin (HbS) is readily denatured and precipitated by application of mechanical forces. (Unfortunately, they did not quantify this aspect.) They applied the mechanical forces

Figure I-4. Kinetics of mechanical denaturation of (a) HbA, and (b) HbS (after Asakura et al., 1974a).



both by turbulent shaking and laminar mixing. The former was more rapid, but laminar mixing without a large gas-fluid interface still produced very significant denaturation. Increasing oxygen concentration in solution increased the denaturation, indicating an oxidative process, and suggesting that turbulent shaking produced the fastest denaturation by enhancing the oxygen supply as well as by creating gas-fluid interface. Normal hemoglobin (HbA) denatures under the same conditions but more slowly (see Figure I-4). Deoxy HbS on the other hand is extremely stable compared to other forms of Hb. They found, by separating the subunits, that it is the conformational instability of the β^S subunit which is responsible for the mechanical instability of HbS. Binding of molecules containing phosphate groups completely protected HbS against mechanical disruption. They speculate that this mechanical instability may be related to the increased incidence of Heinz bodies (intraerythrocytic aggregations of denatured Hb) observed in erythrocytes obtained from patients with sickle cell disease. Since intraerythrocytic denaturation increases erythrocyte rigidity, the abnormal instability of HbS may be of importance in the pathogenesis of vaso-occlusive crisis. Rigid erythrocytes may be impeded in their passage through smaller blood vessels; furthermore, erythrocyte deformation at some point in the circulation may initiate the formation of Heinz bodies in erythrocytes containing HbS.

An area of biophysical research where the role of mechanochemistry has long been realized is the study of muscle contraction. Muscle is a mechanochemical system which normally functions to convert chemical energy into mechanical energy. In muscle, the mechanical work of contraction is closely coupled with a chemical reaction (myosin ATP-ase catalyzed dephosphorylation of adenosine triphosphate (ATP)). It is also possible to show that mechanical forces applied to muscle fibers will alter the ATP-ase activity. It has been demonstrated that the rate of cleavage of ATP by glycerinated muscle fiber (Szent-Györgyi, 1949) is increased when the fibers are stretched (Vorob'ev and Ganelina, 1963) or oscillated or subjected to sonic vibrations (Ohnishi and Ohnishi, 1963). This effect was hypothesized previously by Vol'kenshtein (1962) in his theory of muscle contraction. It was subsequently shown that larger strains will decrease the ATP-ase activity (Vorob'ev, 1965). If myosin in solution is subjected to increasing concentrations of urea or guanidine-HCl (Brahms et al., 1962) or changing ionic strength (Tonomura et al., 1961), expansion of its molecular conformation can be detected by physical chemical methods, such as viscometry. Each of these studies correlated ATP-ase activity with molecular expansion and found, like the direct mechanical studies, that activity first rose and then fell with extension. Myosin in solutions, subjected to shearing in a Couette device (Vorob'ev and Kukhareva, 1965), in the pres-

ence of ATP, showed increasing rates of ATP cleavage (up to 120% increase over zero shear rates) with increasing shear rate up to 4500 s^{-1} ; above which activity began to fall. Interestingly, if myosin is sheared in the absence of ATP it loses activity; for example, a 25% loss in activity was observed after 10 min at 3100 s^{-1} (Vorob'ev and Kukhareva, 1965). This suggests that small molecule binding can prevent molecular deformation. Similar results were reported for actomyosin.

Enzymes may be immobilized on deformable supports (Berezin, 1974b, c; Klibanov, 1974b, 1976b). It has been shown that trypsin and chymotrypsin show a completely reversible activity loss of up to about 75% with about 1% macroscopic tensile strain of the support. If the deformation is affine down to the molecular level, this corresponds to a molecular deformation of only 0.5\AA . These results suggest the possible importance of mechanical-chemical coupling in vivo, not only in muscle, but in dynamic membranes as well, for example in the movement of sperm flagella and cilia, where a dynein ATP-ase activity is important (Gibbons and Gibbons, 1976).

Biotechnological processing conditions may also lead to changes in the structure and activity of proteins. Extrusion of a protein solution through a finely porous membrane causes a globular \longrightarrow fibrous transition with some proteins (Kargin et al., 1960). Less drastic treatment seems

to have significant effects as well. Increasing the wall shear rate at the interior of a hollow fiber where a saccharide polymerizing enzyme is immobilized seems to alter both the activity and specificity of the enzyme (Drew, 1976). Shear inactivation of enzymes has been observed in stirred tank reactors (Foster et al., 1976; Krishnaswamy and Tirrell, 1975) and in ultrafiltration apparatus (Charm and Lai, 1971; Bowski and Rya, 1974). Charm and Wong (1970b) recognized early the biotechnological significance of this phenomenon and published a study which is probably most directly related to the work of this dissertation. They subjected three enzyme preparations, catalase, carboxypeptidase and rennet to shearing stresses from 0.02 to 2.6 Pascals in a concentric cylinder viscometer. They reported irreversible inactivation of all three enzymes under these conditions, the extent of which correlated with the shear strain parameter discussed previously in connection with their work on fibrinogen (Charm and Wong, 1970a).

This completes our survey of results relevant to the work presented in this dissertation. The broad spectrum of examples of, interest in, and applicability of research on the effects of imposed mechanical forces on enzyme molecules is quite apparent. However, the individual pieces of work that have been done are rather isolated from one another. There is no common or generally accepted methodology. Little in the way of general results on mechanism or critical stress

levels has emerged.

The bulk of the work of this dissertation has been an attempt to obtain this information for at least one enzyme system, with the idea in mind that a thorough understanding for one system may serve as a starting point from which to build a more general understanding. The methodology and underlying philosophy of this work has been to study the kinetics of enzyme catalyzed reactions occurring in media subjected to uniform shear rate. When a rate-modifying effect of shearing has been observed, ancillary methods have been brought to bear to determine the molecular changes responsible for the effect. The enzyme which was the object of most of the work reported herein was, urease; selected primarily because of the previous experience in our laboratory (Lewis and Middleman, 1974; Bollmeier and Middleman, 1974; Bollmeier, 1977) with it and the appropriate analytical techniques. The next chapter discusses the relevant information on urease catalysis and structure.

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C H A P T E R I I

BACKGROUND ON UREASE CATALYSIS AND STRUCTURE

The present state of understanding of the structure and function of urease has evolved by a series of spasmodic advances, dramatic breakthroughs followed by periods of relatively little advancement. In 1926, Sumner isolated from jack beans (Canavalia ensiformis), the first crystalline enzyme, urease, thereby establishing the now commonplace fact that enzymes are chemically distinct protein molecules (Sumner, 1926). It was twelve years later that the molecular weight of urease was reported to be 483,000 from ultracentrifugation measurements (Sumner et al., 1938). Most of the work from 1935 to 1955 involved more or less routine studies, obtaining kinetic parameters and activation energies.

Sizer and coworkers studied the temperature dependence of urease activity and demonstrated a variation of the activation energy with temperature (Sizer, 1939, 1940; Sizer and Tytell, 1941a, b). As pointed out by Kistiakowsky and Lumry (1949), their data could not support their claims for a sharp transition between two activation energies at 23°C but a gradually decreasing activation energy with increasing temperature was confirmed by Hoare and Laidler (1950b). The extensive kinetic studies done by Laidler and coworkers

(Laidler and Hoare, 1949; Hoare and Laidler, 1950a, b; Wall and Laidler, 1953a, b) during that period contributed much of the present information on the general features of urease kinetics. They showed that urease is inhibited by substrate urea at concentrations greater than about 250 mM (Laidler and Hoare, 1949; Wall and Laidler, 1953a) and that urease is inhibited by product ammonia, with a noncompetitive inhibition constant of 475 liters/mole (Hoare and Laidler, 1950a). Their temperature studies (Hoare and Laidler, 1950b) showed that the entropy of activation for formation of a urease-urea-water complex is positive, suggesting that there is an increase in entropy in forming the activated state from the initial reactants. This is consistent with a reversible structural change in the enzyme molecule, in their words, an "opening out" of the molecule and perhaps is related to the substrate inactivation phenomenon. They reported Michaelis-Menten parameters of $V_{\max} \approx 9 \text{ } \mu\text{mole/min/SU}$ and $K_m \approx 4.0 \text{ mM}$, which are in reasonable agreement with other more recently reported values, for example: $V_{\max} \approx 35 \text{ } \mu\text{mole/min/SU}$ and $K_m = 7 \text{ to } 10 \text{ mM}$ (Fishbein et al., 1965). (SU stands for Sumner unit; 1 SU will produce 1.0 mg of ammonia from urea in 5 minutes at pH 7.0 and 30°C.) Variations among buffers must be recognized. The only result of Laidler's group which is not in agreement with more recent results is their report of a rather sharp optimum in the pH-activity curve at pH 8.0 (Wall and Laidler, 1953a); a broad pH-activity optimum cen-

tered at pH 6.5 to 7.0 with only about 30% activity loss at pH 8.5 seems to be more correct (Fishbein et al., 1965; Tirrell and Bollmeier, unpublished).

Inactivation of urease by metal containing compounds was reported early (Hellerman et al., 1943; Ambrose et al., 1951; Evert, 1953; Shaw, 1954) and studied for more than 25 years (Gorin et al., 1962; Gorin and Chin, 1965; Hill and Elliot, 1966; Hughes et al., 1969). These studies have shown that inhibition by heavy metals (i.e., Cd, Co, Hg, Zn, Ag) is noncompetitive and correlates well with metal sulfide insolubility. From this type of work, it has become clear that urease has essential sulfhydryl groups. The species of molecular weight 483,000 contains approximately 47 sulfhydryl groups (Gorin and Chin, 1965) and various workers have estimated that 4 to 8 of these are essential to activity, from measurements of the stoichiometry of silver ion (Ambrose et al., 1951) or mercurial inactivation (Gorin et al., 1962). If the highest of these reported values is taken, this suggests 8 subunits of molecular weight approximately 60,000. While physical evidence exists for urease fragments of molecular weight 17,000 (Hand, 1939), 75,000 (Bailey and Boulter, 1969), 83,000 (Reithel et al., 1964) and 120,000 (Stewart and Craig, 1966), there is no conclusive evidence for urease activity in a species of molecular weight this low.

Improvements in Sumner's (1926) method for isolating crystalline urease have been reported over the years (Dounce,

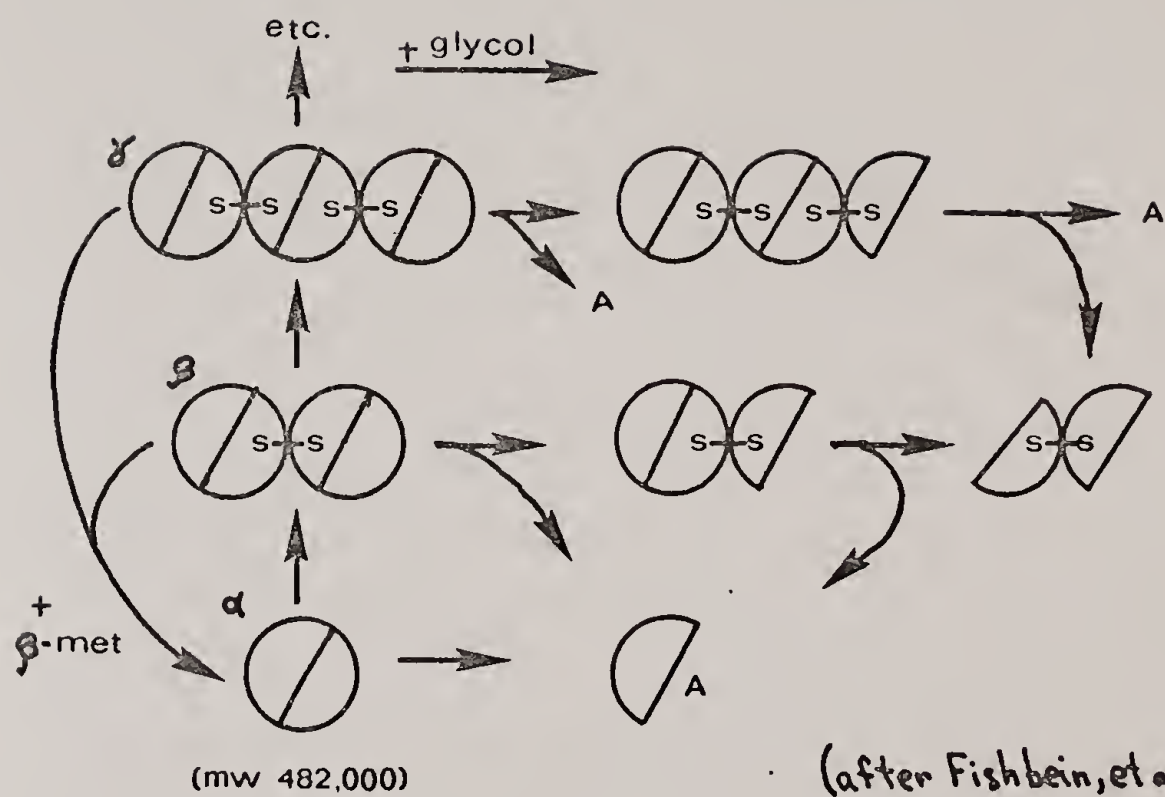
1941; Mamiya and Gorin, 1965), the former introducing a recrystallization procedure and the latter demonstrating the improvements in yield and specific activity possibly by including 0.25 M β -mercaptoethanol ($\text{HOCH}_2\text{CH}_2\text{SH}$) in the crystallization liquor. The specific activities (SA) of preparations used by workers have varied from about 10 SU/mg to a maximum reported value of 500 SU/mg (Lynn, 1967), with a generally accepted average maximum SA of 130-150 SU/mg (Fishbein, 1969). The beneficial effect of β -mercaptoethanol is related to the importance of sulfhydryl groups for urease activity. This thiol-reducing agent also affects the quaternary structure of urease in a manner which will be discussed presently.

Creeth and Nichol (1960) first demonstrated that the 483,000 molecular weight species of urease aggregates. They attributed this aggregation or oligomerization to intermolecular (or intermonomer as we shall henceforth refer to it) disulfide bonding, due to the fact that the aggregates could be dissociated by addition of thiol reducing sulfite ion. In a very insightful and thorough exposition on the multiple molecular forms of urease, Fishbein (1969) reviewed the work of his own and other laboratories on the aggregates (he applied the term "isozymes") found in various urease preparations. His group (Fishbein, 1969) has identified more than a dozen urease isozymes, up to a pentamer of 2,500,000 molecular weight. Subsequent publications (Fishbein et al.,

1969, 1970, 1971, 1973, 1976a), using combined analyses of electrophoretic mobility and sedimentation coefficient, have shown that high molecular weight species are spatially extended (as opposed to compact) oligomers of the 483,000 mol. wt. species. The monomer was found to have a radius of 6.2 nm (Fishbein et al., 1970). In the presence of 1 mM or greater β -mercaptoethanol (β -met), only the monomer exists; if β -met is removed by gel filtration or dialysis, a full series of oligomers forms over the course of several hours. In view of the dynamics of urease conformations, it is no wonder that several groups have been led to invoke interacting and interconvertible active sites to explain their own kinetic data as well as variations between groups (see for example: Kistiakowsky and Rosenberg, 1952; Lynn and Yankwich, 1962, 1964).

A significant discovery came in 1967 (Blattler et al., 1967) when it was found that solutions of urease in mixtures of glycerin or glycols and water (9/1) gave molecular weights of a fully active (Contaxis and Reithel, 1971) urease subunit of 240,000 molecular weight. This was confirmed by Fishbein and coworkers (Fishbein et al., 1973, 1976) who also demonstrated the existence of urease hemipolymers (odd integral multiples of 240,000 molecular weight) in glycerin treated preparations. Figure II-1 illustrates the formation of these isozymes as well as some other conformational possibilities. It is clear that glycerol halves the molecular

Figure II-1. Schematic models of the urease isozymes.



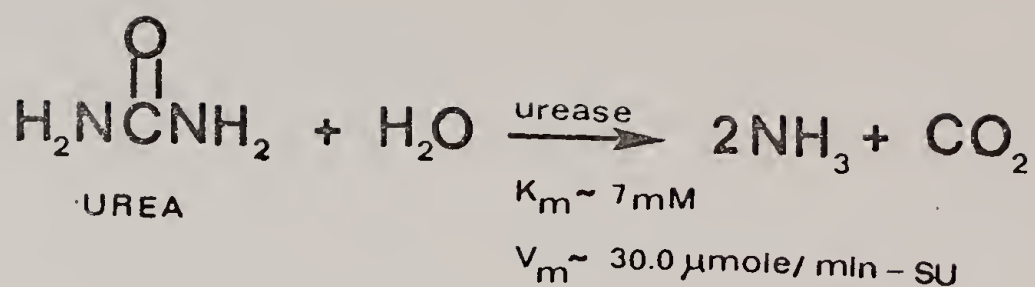
(after Fishbein, et al, 1976a)

weight of the 480,000 mol. wt. species by dissociating a non-covalently bound interface. The nature of this interface is not yet understood.

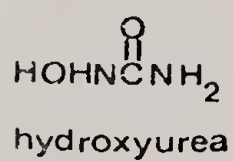
Urease was thought to be absolutely specific for urea until 1965 when Fishbein and coworkers (Fishbein et al., 1965; Fishbein and Carbone, 1965; Fishbein, 1969) showed that hydroxy- and dihydroxyurea were also substrates. The chemistry and kinetic parameters of all these urease catalyzed reactions are given in Figure II-2. Fishbein and Carbone (1965) also discovered the strong inhibiting action of the related compounds, hydroxylamine and acetohydroxamic acid.

During a study of urease denaturation, Fishbein et al. (1976b) noted that inactivation at pH 7-10 by urea, guanidine and heat was markedly delayed by EDTA (ethylenediaminetetraacetate) and other metal chelating agents. This suggested that a metal ion, at the time thought to be adventitious, could catalyze thiol oxidation in the unfolding urease protein, if not chelated. This evidence (Fishbein et al., 1976b), as well as a closer examination of inhibition by acetohydroxamic acid (Dixon et al., 1975a, b), known to be a strong nickel chelater, led to the independent discovery by these two groups that urease is a nickel metalloenzyme, the first natural example. Six to eight nickel atoms (note consistency with subunit estimations mentioned previously) per 483,000 molecular weight seem to be necessary for activity and maintaining the native structure. Indications are (Fish-

Figure II-2. Chemistry of urease catalyzed reactions and the kinetic parameters.

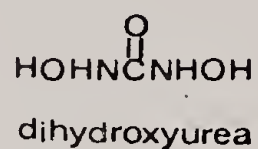


other substrates:



$$K_m = 1.6 \text{ mM}$$

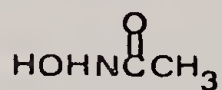
$$V_m = 0.03 \mu\text{mole} / \text{min} - \text{SU}$$



$$K_m = 12.5 \text{ mM}$$

$$V_m = 0.24 \mu\text{mole} / \text{min} - \text{SU}$$

inhibitor:



acetoxyhydroxamic acid

bein et al., 1976b; Nagarajan and Fishbein, 1977) that sulfhydryl and ionized carboxyl groups are involved in nickel coordination.

To sum up, urease has been shown to be an enzyme of catalytic and structural complexity. None of the crystal structure or primary structure sequencing has been accomplished as yet (although several amino acid analyses have been performed [Reithel and Robbins, 1967; Milton and Taylor, 1969]). This is a disadvantage in the present work, in that it makes interpretation of any shear-induced conformational changes more difficult. The large size, labile quaternary structure, conformation-related effects such as substrate inhibition and ease of assay are other features which make urease a very interesting and amenable protein for the studies of enzyme mechanochemistry to be reported.

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[NOTE: The references for this chapter only are arranged chronologically to facilitate following the historical development of the present understanding of urease catalysis and structure.]

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CHAPTER III

EXPERIMENTAL METHODS AND MATERIALS

A. Kinetic Experiments

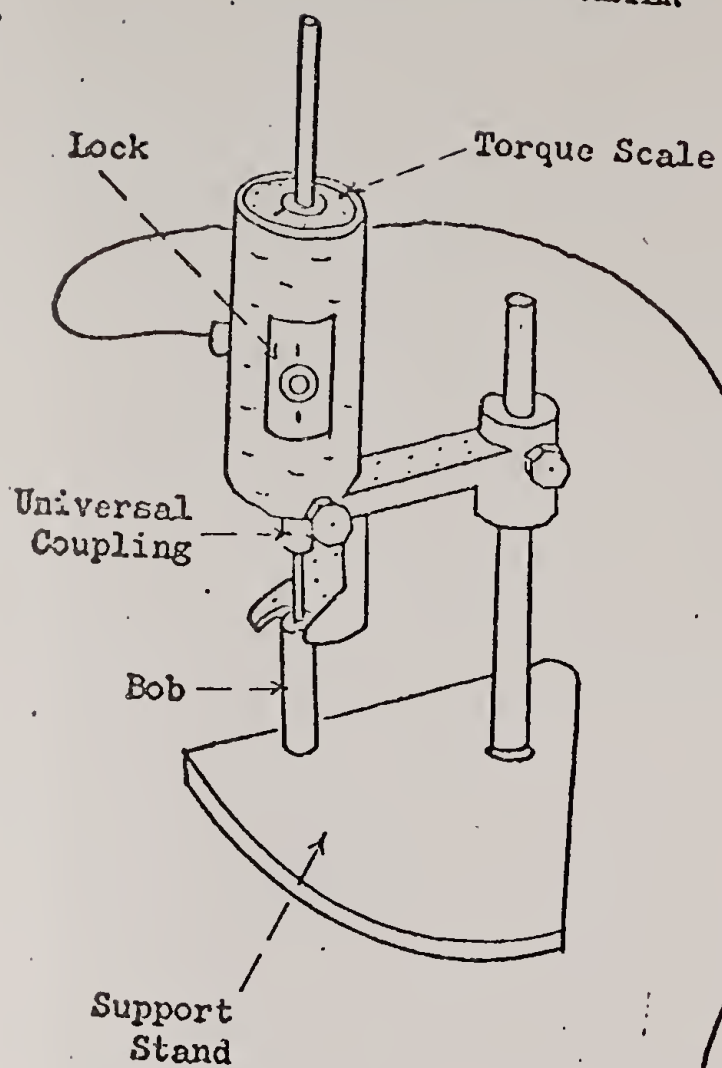
The principal experiment on which this dissertation is based is the measurement of the rate of the urease catalyzed hydrolysis of urea in a (nearly) homogeneous steady simple shear flow. The flow system used to generate the shearing will be discussed first, followed by the manipulative procedures for the basic kinetic experiments and finally the variations used for the several additives and pretreatments will be described.

1. Flow System

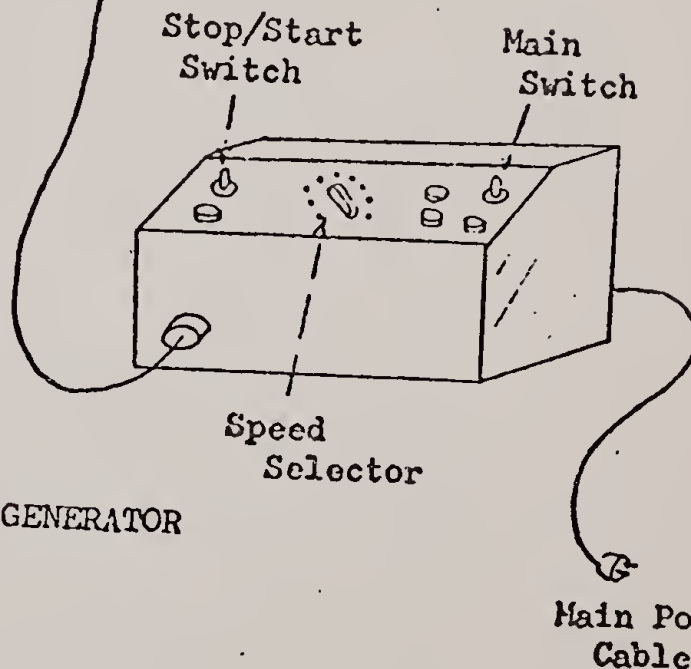
The instrumentation for the flow system was that of the Epprecht Rheometer-Rheomat 15 manufactured by Contraves A.G., Ltd., Zurich. It has been designed primarily for viscosity measurement of Newtonian and nonNewtonian liquids. A representation of this system is shown in Figure III-1. It consists of two main components: (1) a measuring head mounted on a stand, and (2) a control unit. A precision spring assembly housed in the measuring head measured 3.88×10^{-5} newton-m of torque per scale division or 3.88×10^{-3} newton-m

Figure III-1. Sketch of Epprecht rheometer system.

RHEOMETER

Rheometer Connection
CableStop/Start
SwitchMain
SwitchSpeed
Selector

FREQUENCY GENERATOR

Main Power
Cable

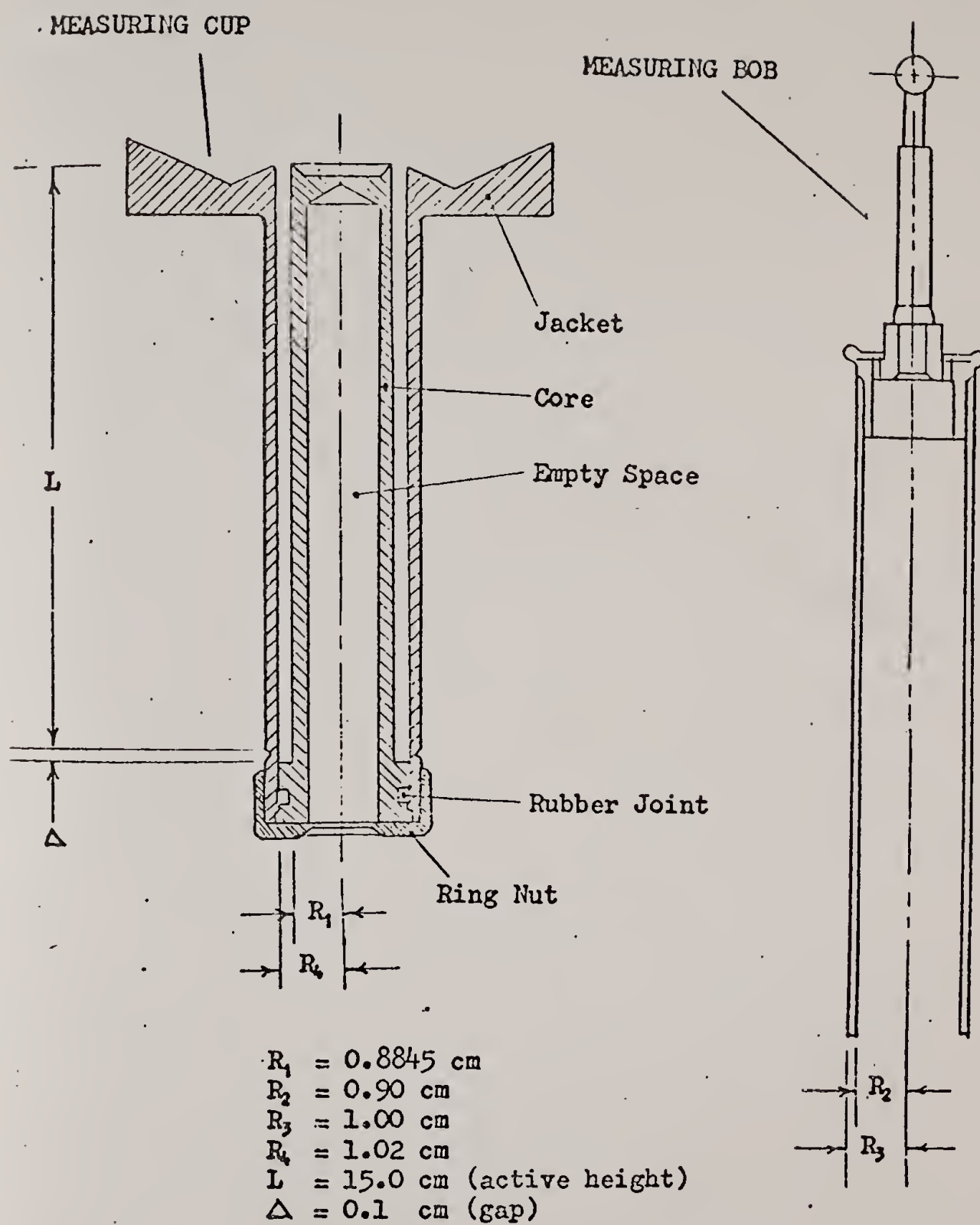
for full scale deflection. For Newtonian liquids, this torque reading is directly proportional to the viscosity of the liquid being sheared. The control unit contains the electronics for speed control, including a 15 position frequency selector which inputs 15 possible angular velocity settings to the measuring head from 5.6 to 352 rpm.

The measuring head may be attached to any of a number of rotational devices, which hold the sample, the most common being cone and plate and concentric cylinder geometries. In this work, a specially made, double concentric cylinder geometry was used exclusively. This system, designated by the letters MS-0, consists of a hollow rotor with holes in the top to allow air to escape when the rotor is inserted into an annular cup channel. A schematic diagram of this device is shown in Figure III-2. With the MS-0 unit and the Epprecht drive, shear rates from 27 to 1717 s⁻¹ can be obtained with shear stresses varying from 0.223 to 22.3 Pascals, the latter producing the largest measurable torque. It is simple to show (Moore and Davies, 1956) that the shear stress distributions are identical in the inner and outer gaps, provided that $R_1/R_2 = R_3/R_4$. The shear rates for Newtonian fluids in the inner ($\dot{\gamma}_i$) and outer ($\dot{\gamma}_0$) gaps are given by:

$$\dot{\gamma}_0 = \frac{2\Omega}{1 - s_0^2} \cdot \frac{R_3^2}{r^2} \quad (1)$$

$$\dot{\gamma}_i = \frac{2\Omega}{1 - s_i^2} \cdot \frac{R_1^2}{r^2} \quad (2)$$

Figure III-2. Schematic diagram of MS-O unit.



where Ω is the angular velocity of the rotor, $s_0 = R_3/R_4$ and $s_i = R_1/R_2$. As can readily be calculated from Equations (1) and (2), this provides a quite homogeneous simple shearing flow throughout the entire reaction medium. The highest Ω attainable is well below the transition Reynolds number to a secondary or turbulent flow, for the Newtonian fluids composing the reaction media in these experiments, aqueous buffers (0.0013 Pascal-s.) and mixtures of these with glycerin (up to 0.012 Pascal-s.).

The stand supporting the measuring head is constructed in such a way as to allow the MS-O system to be fully temperature controlled by immersion in a circulating water bath (Haake) controlled to $\pm 0.05^\circ\text{C}$. The MS-O system has considerable thermal inertia. So, to ensure operation at the proper temperature throughout the entire course of the reactions, the viscometer (as we shall henceforth refer to the MS-O system) was allowed to sit in the water bath at the proper temperature for 1 hour prior to initiating any kinetic run on a given day. Throughout the day, when many consecutive experiments were being done, the viscometer was removed from the bath only briefly for sampling and cleaning.

2. Basic Procedure for Obtaining Kinetic Data During Shearing

Urease was dissolved in buffer at a concentration to give the desired zero shear activity. The exact procedure by

which this was accomplished varied somewhat according to the urease preparation used. Three different commercial urease preparations were used: (A) Fisher (no further designation, found to have 0.4 SU/mg); (B) Sigma, Type III (a lyophilized preparation, average about 5.0 SU/mg); and (C) Sigma, Type C-3 (crystalline, 70.0 SU/mg). For ureases A and B the proper amount of urease was simply weighed out from the bottle (1.0 mg/ml for A; 0.05 mg/ml for B) and dissolved in buffer. Urease A contained much water insoluble material and therefore was allowed to dissolve for 2 hours prior to starting any kinetic runs. With urease C, the odor of mercaptan was readily detectable in the bottle as supplied. Therefore, it was dissolved and dialyzed against buffer prior to use. To accomplish this, the urease was dissolved at up to 10 mg/ml, placed inside 1/4" (pencil) dialysis tubing and dialyzed 12 hours against 400 volumes of well stirred pure buffer at room temperature. After 12 hours the exterior buffer was changed to fresh buffer and the dialysis continued for 12 more hours at 4°C. The stock urease solution was prepared then from this dialyzed Urease C. Average concentration of the stock urease C solution was 0.01 mg/ml. Fifty to one hundred milliliters of these stock urease solutions would be prepared, depending on the number of experiments planned in the next 24 to 48 hour period. All data was taken with urease solutions not more than 48 hours old and maintained at 4°C while in solution. A separate 50 to 100 ml of

solution of urea in the same buffer was prepared. These solutions were prepared twice as concentrated as the desired initial reaction concentration. The nominal initial concentration of urea at the start of each reaction after combining with urease solution was 10 mg/ml (160 mM) in experiments with the Fisher urease and 5 mg/ml in all subsequent experiments. All urea concentrations were determined using the colorimetric method of Levine et al. (1969) summarized at the end of this chapter.

To initiate a kinetic run, equal volumes (typically 5 ml of each) of the urease solution and the urea solution were mixed and 5 ml of the mixture placed in the viscometer cup. The viscometer was assembled in the water bath and connected to the Epprecht drive unit which could then be switched on at any of the 15 available shear rates. These manipulations require approximately 20 seconds. This did not produce a measurement lag, however, since the initial concentration was determined after this assembly process was complete. So, time = 0 was at the point of initial sampling and the entire reaction was performed at constant shear rate. The reaction was allowed to run for the required amount of time. For experiments where a period of shearing was followed by a period of zero shear, the shearing was stopped at the required point but the viscometer left otherwise undisturbed until sampling. Sampling was performed by disassembling the viscometer and withdrawing 1 to 2 ml from the an-

nular cup with a 25 cm #20 hypodermic needle and syringe. One milliliter of this was then taken for the urea assay. One sample only was taken from a given kinetic run; thus, one experimental run gave one point in the concentration-time space. This was done because multiple sampling in an individual run gave poorer reproducibility and the sampling itself seemed to produce deactivation, possibly because of the introduction of oxygen by withdrawal and reinsertion of the rotor.

Whenever fresh urease and urea solutions were prepared, a zero shear kinetic curve was first obtained, in exactly the same manner as described above except that the viscometer drive was never switched on. Once an appropriate urease activity level was established in the initial experiments with a given grade of urease, subsequent solutions of that grade urease were prepared to match the zero shear activity. So, when a fresh urease solution was prepared, it was first checked to see if it gave activity which matched the established zero shear activity level for that series of experiments. If not, the urease concentration was adjusted appropriately. As noted above, all zero shear data was obtained from reactions in the viscometer; but, several preliminary experiments indicated that there was only a small difference in apparent activity observed in the stainless steel viscometer or in nitric acid washed glass.

After each individual run, that is, after obtaining

each individual concentration-time data point, the viscometer was rinsed three or four times with distilled water, brushed with a wet nylon-bristled brush and rinsed several more times. The viscometer was then dried and reimmersed in the water bath. The effect of preexposing the clean viscometer surface to a concentrated urease solution and then allowing it to air dry prior to starting a run was investigated and no effect on the resultant kinetic data was found.

3. Variations

a. EDTA. In the series of experiments which included EDTA in the reaction medium, both the urease and urea solutions were made up to the appropriate concentration in EDTA. It usually took about 1/2 hour for this amount of EDTA to dissolve into the buffer and an additional 2 hours incubation time was allowed before starting any kinetic experiments.

b. Chelex 100. The urease and urea solutions were prepared in the basic way. Each was then exposed to one-fifth their volume of wet Chelex and slurried with a small magnetic stirrer for 2 hours. After this period, the Chelex was separated from the solution by filtration. This treatment caused a pH rise from 7.0 to about 7.75; therefore, before kinetic experiments, pH was readjusted to 7.0 by addition of the required amount of citric acid monohydrate.

c. β -Mercaptoethanol. In the series of experiments

which included β -mercaptoethanol (β -met) in the reaction mixture both the urease and urea solutions were made up to the appropriate concentrations in β -met. It was found they could be used immediately without changing the results.

d. Parachloromercuribenzoate (PCMB). For the experiments including PCMB in the reaction mixture, the urease solution was made somewhat more concentrated than usual (see Chapter IV for exact concentration). The amount of PCMB required to give the desired final concentration, after combining the urease and urea solution, was all dissolved in the otherwise normally prepared urea solution. Thus, when the two were mixed, the reaction of PCMB with urease proceeded simultaneously with the urease-catalyzed urea hydrolysis.

e. Glycerin-buffer experiments. In this set of experiments, the urease and urea solutions were prepared as usual in aqueous buffer, except that: (1) 5 mM EDTA was included in both, and (2) it was found necessary to use twice the normal urease concentration to give the desired zero shear rate constant. Only urease C was used. To each of these solutions was added the same required proportion of pure glycerin to give the desired final viscosity of the reaction medium. These proportions were in the range 50 to 60% by volume. This gave viscosities of 0.0078 Pascal-s to 0.0120 Pascal-s as measured in the Epprecht MS-O viscometer. Since it was tedious to duplicate the viscosities of the stock solutions of urease and urea, they were prepared in

sufficient quantities so that enough data could be taken without replenishing the stock solutions. Still, no stock solution more than 48 hours old was used for any kinetic experiments. Otherwise, the procedure for obtaining kinetic data in these media was exactly as described in Section 2. A change in the calibration curve for the urea assay was necessary.

f. Miscellaneous. A few kinetic experiments including other additives were performed as well. These additives were 8-hydroxyquinoline, glycine, N,N-[bis(2-hydroxyethyl)]-glycine (also known as bicine), ferric nitrate and nickel nitrate. These were all added to the reaction mixture in the same manner as for EDTA, except that for the last two, the solutions received Chelex treatment prior to the addition of the metal salts. The desired amounts of these substances were added to the stock solutions of urease and urea and allowed to incubate for 2 hours prior to initiating the kinetic experiments. Several attempts were made to run kinetic experiments in the absence of oxygen. The entire viscometer, necessary glassware and chemicals were placed in a glove bag purged with high purity nitrogen bubbled through an alkaline pyrogallol solution. This purge lasted 24 hours. The stock urease and urea solutions were bubbled with nitrogen for 1 hour each before starting the purge. Otherwise, the manipulations were as usual.

B. Size Exclusion Chromatography Experiments

1. General Considerations

In 1965, Siegel and Monty reported the separation of the oligomeric urease isozymes by size exclusion chromatography on a Sephadex G-200 column. This finding has been disputed by Fishbein (1969, 1976) who claims that urease activity peaks occur only at smaller elution volumes than the exclusion limit of the column. Blakeley et al. (1969) found that urease was included in the pore volume of G-200 but did not report molecular weight separation. It is not clear what the resolution of this contradiction is. It is obvious, however, even from the paper of Siegel and Monty (1965), that resolution of the higher molecular weight isozymes is impossible on a G-200 column. Fishbein and Nagarajan (1971) also reported unsuccessful attempts to separate the isozymes on Sepharose-6B. It was found in preliminary experiments of the present work, that urease oligomers were not totally excluded from a Sepharose-6B column, as indicated by the peak elution volume of Blue Dextran 2000 marker, but that good molecular weight resolution was not possible.

A system whose urease isozyme separating capabilities had not previously been investigated and which seemed to have potential were the CPG-10 porous glasses. This became the size exclusion chromatography column packing for the separations to be described in this work.

2. Column Set-up

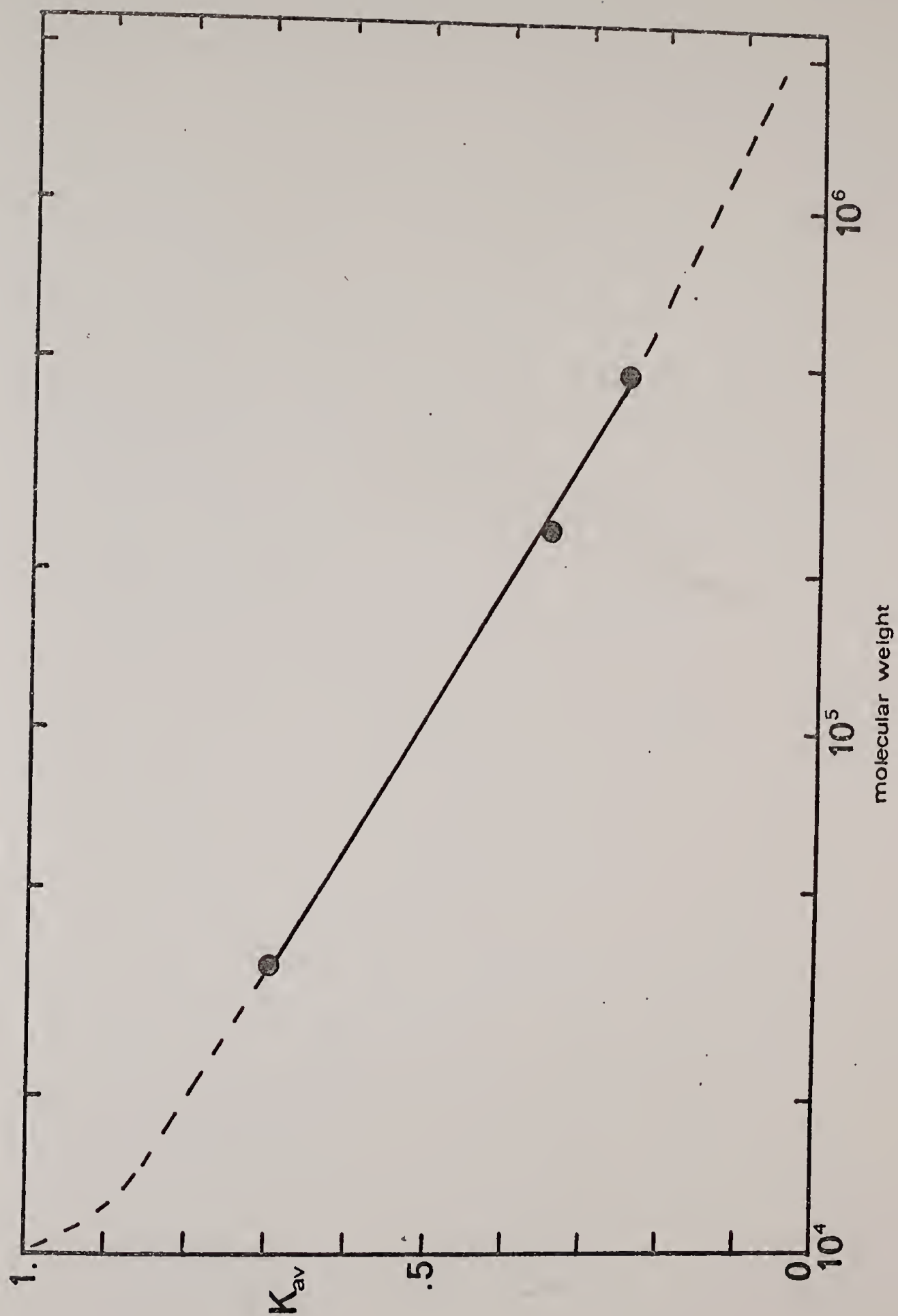
Since the distribution of urease isozymes has a relatively broad (discrete) distribution of molecular sizes it seemed probable that several separations on consecutively arranged columns would provide the best separation. Accordingly, the following design was implemented. A column of 5.6 mm radius by 450 mm length was packed from the bottom with the following CPG-10 porous glasses: (1) 100 mm, 2000 Å; (2) 100 mm, 1000 Å; (3) 100 mm, 500 Å; (4) 100 mm, 350 Å; and (5) 50 mm, 240 Å. The manufacturer's specification is that 80% of the pore volume be in pores with diameters within $\pm 10\%$ of the nominal value. The minimum specific pore volume is 0.8 cc/g and efficient packing produces a bulk density of the empty bed of 0.5 g/cc leading to a figure of 40% of the column bed volume available for separation. Surface activity of these materials can cause adsorption. In this work, the relatively high ionic strength of the buffer worked against adsorption. To further reduce the possibility of adsorption, the porous glasses were exposed overnight to a 1% solution of Carbowax 20-M, before packing the column. Contamination of the eluent with Carbowax was not detected after the column was equilibrated with buffer. After every 30 or so column runs the packed column was again exposed to the Carbowax solution. The column was operated at ambient temperatures between 23°C and 27°C. Gravity elution was em-

ployed; flow rates of 0.20 to 1.00 ml/min were used. The entire bed volume (45 cm^3) could be eluted in 2 hours. The column was connected to a Beckman model 132 fraction collector. The dead volume of the tubing connecting the column and fraction collector was 4.5 cm^3 .

The packed column was calibrated by measuring the peak elution positions of β -lactoglobulin (36,000), catalase (240,000), urease monomer, Type C-3 (482,000; eluted with 5 mM β -met) and Blue Dextran 2000 (nominally 2,000,000). Protein concentration in the eluent was determined by the Lowry procedure (Lowry et al., 1951). The plot of $K_{av} = (V_{elution} - V_{bed}) / (V_{exclusion} - V_{bed})$ vs. molecular weight, shown in Figure III-3, is seen to be fairly linear.

Urease solutions for chromatography were prepared in the same manner as for kinetic experiments, including pre-dialysis for urease C. Concentrations were somewhat higher, however, typically 10 mg/ml with urease B and 0.5 mg/ml with urease C. One half milliliter of sample was applied to the column in all cases. Fractions of either 0.5 or 1.0 ml were collected and analyzed for protein concentration by the method of Lowry et al. (1951) or by measuring optical density at 278 nm. Analyses for urease activity were done according to the method of Gorin and Chin (1966).

Figure III-3. Calibration curve for size exclusion chromatography column.



C. Spectroscopy

1. Circular Dichroism (CD)

CD spectra were recorded on a Cary 60 spectropolarimeter with the model 61 CD attachment. The spectra are plotted as molar ellipticities $[\theta] = \theta \times \text{MRW}/10 \times d \times c$ vs. wavelength, where θ is the measured ellipticity in degrees, MRW is the mean residue molecular weight taken as 115 for urease, d is the pathlength of the cell in cm and c is the concentration of the sample in g/ml. The buffer for the recording of the CD spectra was not the usual but rather a 0.01 M Na_2HPO_4 - KH_2PO_4 pH 7.0 buffer. In the case of sheared samples, the buffer was exchanged by dialysis from the usual buffer used for shearing to the 0.01 M phosphate buffer used for recording of CD spectra.

2. Ultraviolet-visible

The measurements of optical density required for determining the protein concentrations were done on a Beckman Acta M-VI double-beam spectrophotometer.

3. Atomic Absorption

The analyses for metal contents reported were obtained from the Microanalytical Laboratory. The results reported are averages of duplicates analyzed by atomic absorption spectroscopy.

4. Electron Paramagnetic Resonance (EPR)

A Varian E-9 x-band EPR spectrometer was used in an attempt to record an EPR signal from urease or a nitrosyl derivative. The urease sample was prepared as 25 mg/ml in 0.050 M Tris-H₂SO₄, pH 7.0. No EPR signal was detected. Exposure to gaseous NO, for 24 hours, of this sample, in an attempt to form a paramagnetic nitrosyl derivative, also failed to produce any detectable EPR signal.

D. Recipes, Materials and Suppliers

1. Buffer

The buffer used throughout all these experiments except where otherwise noted was prepared in 2 liter batches according to the following recipe: 85.7824 g of Na₂HPO₄·7H₂O; 8.404 g of citric acid monohydrate; make up to 2000 ml with distilled water; adjust pH to 7.00±0.05 if necessary with small amount of citric acid monohydrate. This buffer is variously referred to throughout this dissertation as the "usual" buffer, the "basic" buffer or the "0.16 M phosphate" buffer.

2. Analysis for Urea

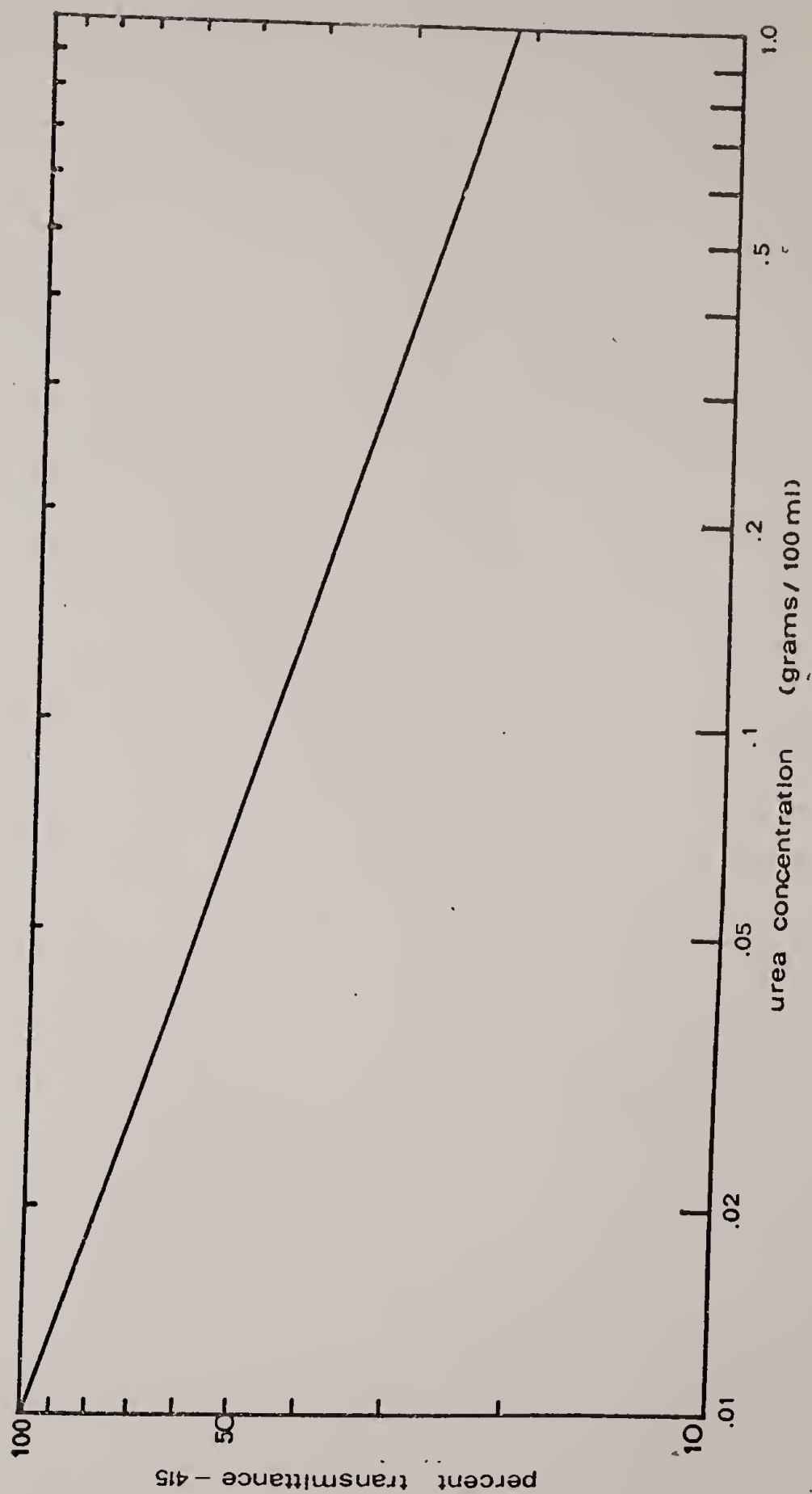
A modification of the method of Levine et al. (1961) was used: (1) take 1 ml sample of reaction mixture; (2) add 4 ml trichloroacetic acid solution (10% w/v); (3) add 1 ml

modified Ehrlich reagent (see below for recipe); (4) filter through Whatman #1 filter paper (only necessary for sample containing greater than 0.1 mg/ml protein); (5) adjust Leitz Model M Photometer to read 100% transmittance at 415 nm through a blank containing 1 ml distilled water treated as in steps (1)-(4); (6) measure % transmittance of sample and obtain urea concentration from calibration curve. Calibration curve is shown in Figure III-4. Modified Ehrlich reagent: 5 g of paradimethylaminobenzaldehyde, 20 ml of concentrated HCl, 80 ml distilled water.

3. Lowry Procedure (Lowry et al., 1951)

Reagents: 1. 3.0% Na_2CO_3 in 0.1 M NaOH; 2. Alkaline Cooper Reagent. Add 1.0 ml 2.0% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 1.0 ml 4.0% NaK Tartrate to 50 ml volumetric flask. Make up to 50 ml with Reagent 1. 3. Folin-Phenol Reagent. Dilute 1 part with 2 parts distilled water immediately before use. Procedure: To 0.5 ml sample, add 5 ml of Reagent 2. Mix, let stand 10 minutes. Add 0.5 ml Reagent 3 and rapidly mix. After 30 minutes, measure absorbance at 660 nm relative to 0.5 ml pure buffer treated as above. Obtain protein concentration from calibration curve. Calibration curve was linear for urease from 0.0 to 0.5 mg/ml; equation of calibration line: [urease] in mg/ml = $1.15 A_{660}$.

Figure III-4. Calibration curve for urea analysis.



4. Gorin and Chin Assay for Urease Activity (Gorin and Chin, 1966)

The following modification of the Gorin and Chin procedure was used to analyze for urease activity in fractions from exclusion chromatography and other samples. To 1.0 ml of sample, add 0.5 ml 1 mM EDTA (in pH 9.0, 0.1 M Tris-HCl buffer). Let stand at least 2 hours. Add 0.5 ml urea solution (6% by weight in above Tris buffer). After exactly 5.0 minutes, the reaction is stopped by addition of 1.0 ml of 0.2 M HCl. The excess HCl is then back titrated with 0.05 M NaOH to the methyl orange endpoint. V_s is the volume of NaOH (in ml) required. One ml of pure buffer is treated in the same way. V_b is the volume (in ml) of NaOH required for the blank.

$$\text{Activity (in SU)} = 14(V_b - V_s)[\text{NaOH}] \text{ (in moles/l)} \quad (3)$$

Accurate measurement of V_s and V_b is required; a 5.00 ml burette was employed. Accurate knowledge of [NaOH] is also required to get accurate absolute numbers for the urease activity. Most often the present work required only relative activities, as in the assay of chromatographic fractions. When this was the case, $V_b - V_s$ was taken as a measure of the relative activity, and absolute activities were not determined.

5. Sources of Materials

<u>Material</u>	<u>Source</u>
Water	Distilled, Deionized with Barnstead Standard Blue Demineralizing Column, resistance 1 megaohm at issue.
Urease A	Fisher Scientific Co. (no other designation)
Urease B	Sigma Chemical Co., Type III (lot #105C-7350)
Urease C	Sigma Chemical Co., Type C-3 (lot #95C-7110)
Urea	Fisher Scientific Co. Certified ACS (lot #754710)
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	Fisher Scientific Co. Certified ACS (several lots)
Citric Acid, monohydrate	Fisher Scientific Co. Certified ACS (lot #731388)
Ethylenediaminetetra acetate, disodium (EDTA)	Fisher Scientific Co. Certified ACS (lot #722800)
Chelex 100, sodium form	Bio-Rad Laboratories, Inc. Analytical grade (lot #13857)
β -Mercaptoethanol	Sigma Chemical Co., Type I (lot #35C-0104-1)
p-Chloromercuribenzoic acid (PCMB)	Sigma Chemical Co. (lot #17B-5460)
Glycerin	Fisher Scientific Co. Laboratory grade (lot #763894)
8-Hydroxyquinoline	Sigma Chemical Co. (lot #95C-0045)
Glycine	Sigma Chemical Co.
Ferric Nitrate	Fisher Scientific Co. Certified ACS (lot #744569)

<u>Material</u>	<u>Source</u>
Nickel Nitrate	Matheson, Coleman and Bell
Porous Glasses, 80/120 mesh	Electro-Nucleonics, Inc. (240 Å - lot #P1040) (350 Å - lot #L1039) (500 Å - lot #156) (1000 Å - lot unknown) (2000 Å - lot #147)
Blue Dextran 2000	Pharmacia (lot #6567)
KH_2PO_4	Fisher Scientific Co.
Trichloroacetic acid, 10% w/v solution	Fisher Scientific Co. (several lots)
p-Dimethylaminobenzaldehyde	Fisher Scientific Co. Certified ACS (several lots)
HCl , H_2SO_4 (concentrated)	Fisher Scientific Co. Reagent Grade (several lots)
Na_2CO_3	Baker Chemical Co. (lot #38231)
NaOH	Fisher Scientific Co. Certified ACS (lot #752823)
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	Fisher Scientific Co.
NaK Tartrate	Fisher Scientific Co.
Folin-Phenol Reagent	Will Corporation
Tris(hydroxymethyl)amino- methane (Tris)	Sigma Chemical Co. (several lots)
Methyl Orange, 0.1%	Fisher Scientific Co. (lot #722347)
2,3-Dimercaptopropanol (British Anti-Lewisite; BAL)	Sigma Chemical Co.
1,2-Ethanedithiol	Sigma Chemical Co.
Dialysis tubing	Arthur H. Thomas Co.
Carbowax 20-M (polyethylene oxide)	Union Carbide Corp.

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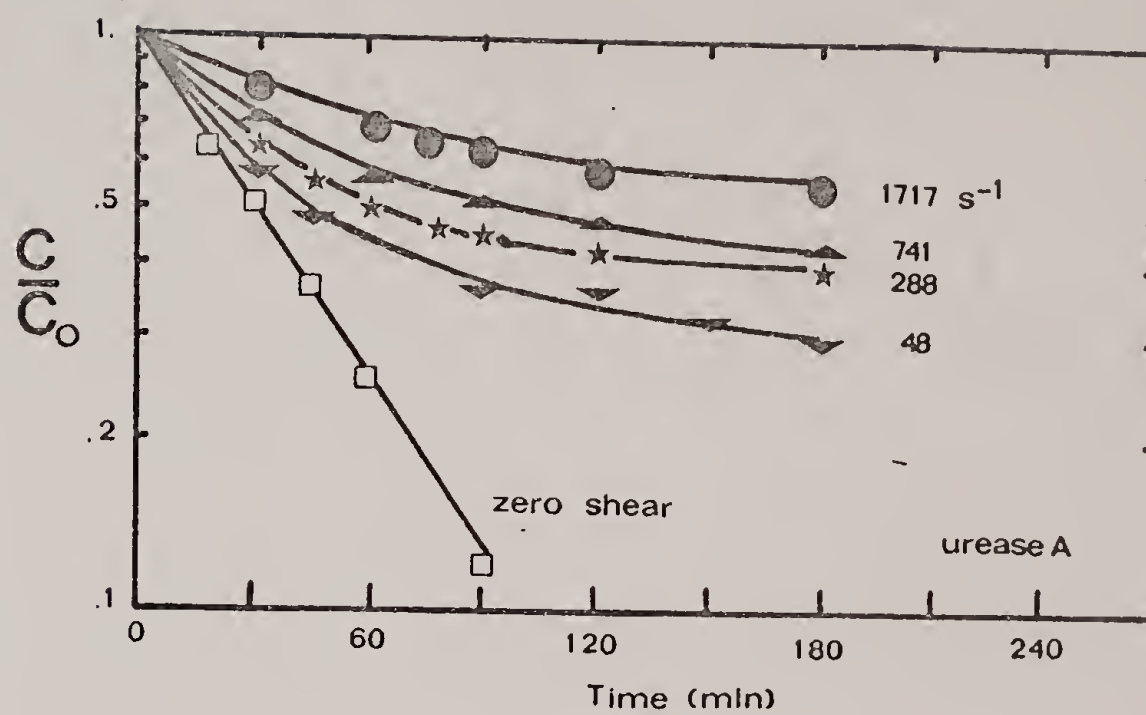
CHAPTER IV

RESULTS ON UREASE

A. Kinetic Results in Aqueous Buffers

The preliminary experimental work of this dissertation, done in an effort to see what effects may be possible, was a study of the kinetics of urea hydrolysis, in the viscometer, catalyzed by the crude urease preparation, urease A. In Figure IV-1, a comparison of data obtained at zero shear and at various shear rates is seen. The rate of urea hydrolysis at zero shear is apparently first order in urea concentration. This may seem surprising since the initial concentration, 160 mM, is well above the concentration for half-maximal reaction rate, K_m , the Michaelis constant. Reported values of K_m range from 4 to 10 mM (see Chapter II). The reason for this apparent first order behavior is product inhibition as will be conclusively demonstrated in Chapter V. The apparent first order rate constant is still directly proportional to urease activity. The data of Figure IV-1 show that urease is inactivated during exposure to shearing forces and that this inactivation occurs more rapidly with increasing shear rate. The first question these data brought to mind was, "Is this effect reversible?" So, a series of ex-

Figure IV-1. Urea concentration as a function of time under varying rates of shear.



periments was performed in which the shearing was stopped at various points in time and the remaining zero shear activity observed. These data are shown in Figure IV-2 where it is seen that apparent first order kinetics are resumed in all cases upon stopping the shearing, with a slope greater than the instantaneous slope of the data at the point in time where the shearing was stopped but less than the slope of the data from urease with no shear history. This means that there is both reversible and irreversible inactivation due to shearing. If the ratio of the slope of the data obtained after stopping the shearing to the slope of the data from urease with no shear history is taken, it is found that all the data at different shear rates correlate with the product of shear rate \times exposure time, a total shear strain. This correlation of the shear inactivation data is shown in Figure IV-3, curve a. Note that this is the same correlation used by Charm and Wong (1970a, b) for several other biologically active proteins, but their data were obtained from a different type of experiment. In their experiments, the enzyme preparations were subjected to shearing in the absence of substrate and subsequently assayed for activity. Here, the activity is observed during shearing. If that type of Charm-style experiment is performed with this urease A, it is seen in Figure IV-3, curve b, that the same correlation works but that activity is lost much less rapidly.

The next piece of information uncovered is shown in

Figure IV-2. Urea concentration as a function of time at zero shear after variable periods of steady shear.

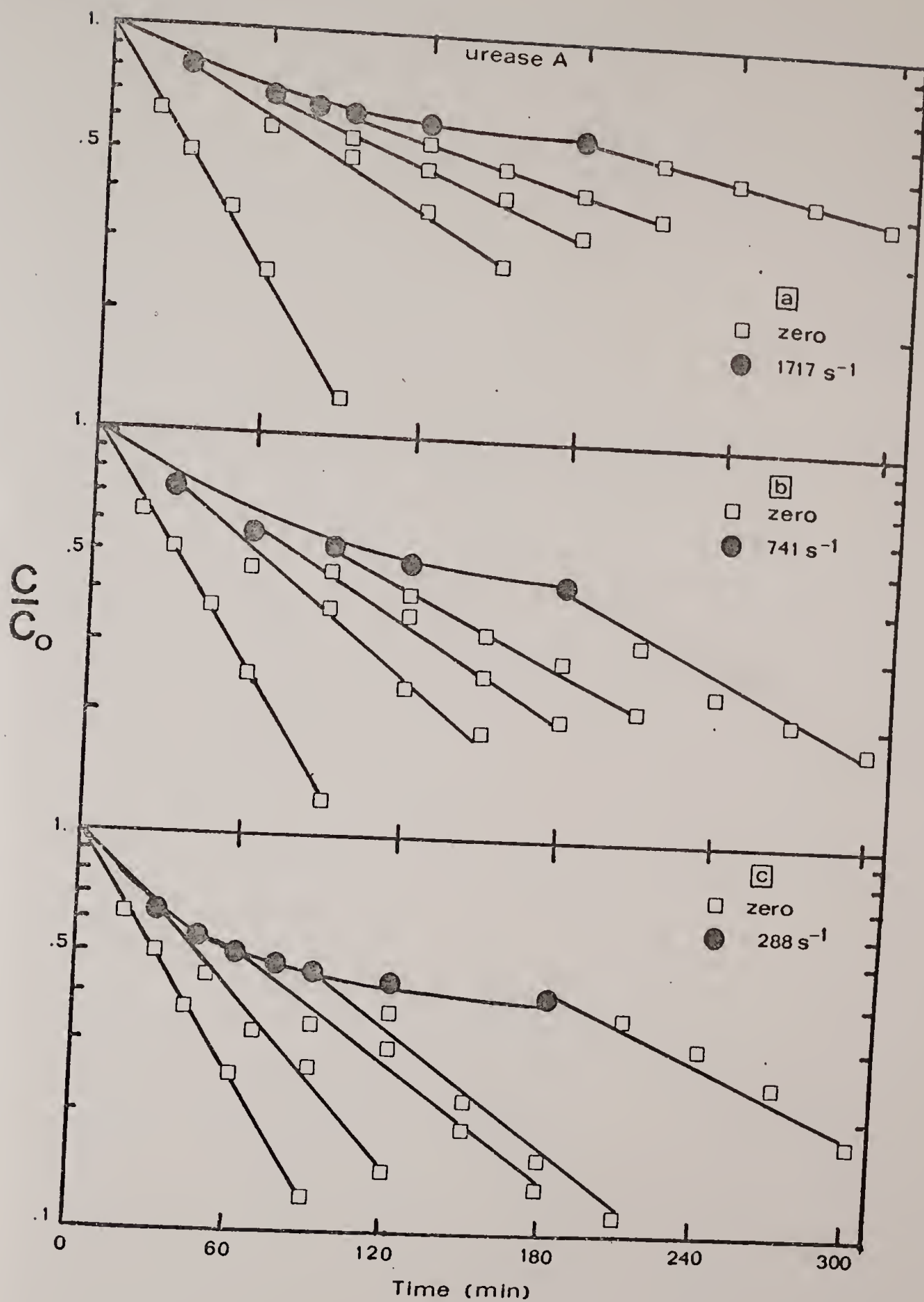


Figure IV-3. Percent urease activity after shearing as a function of shear strain in the (a) presence and (b) absence of urea. Urease A.

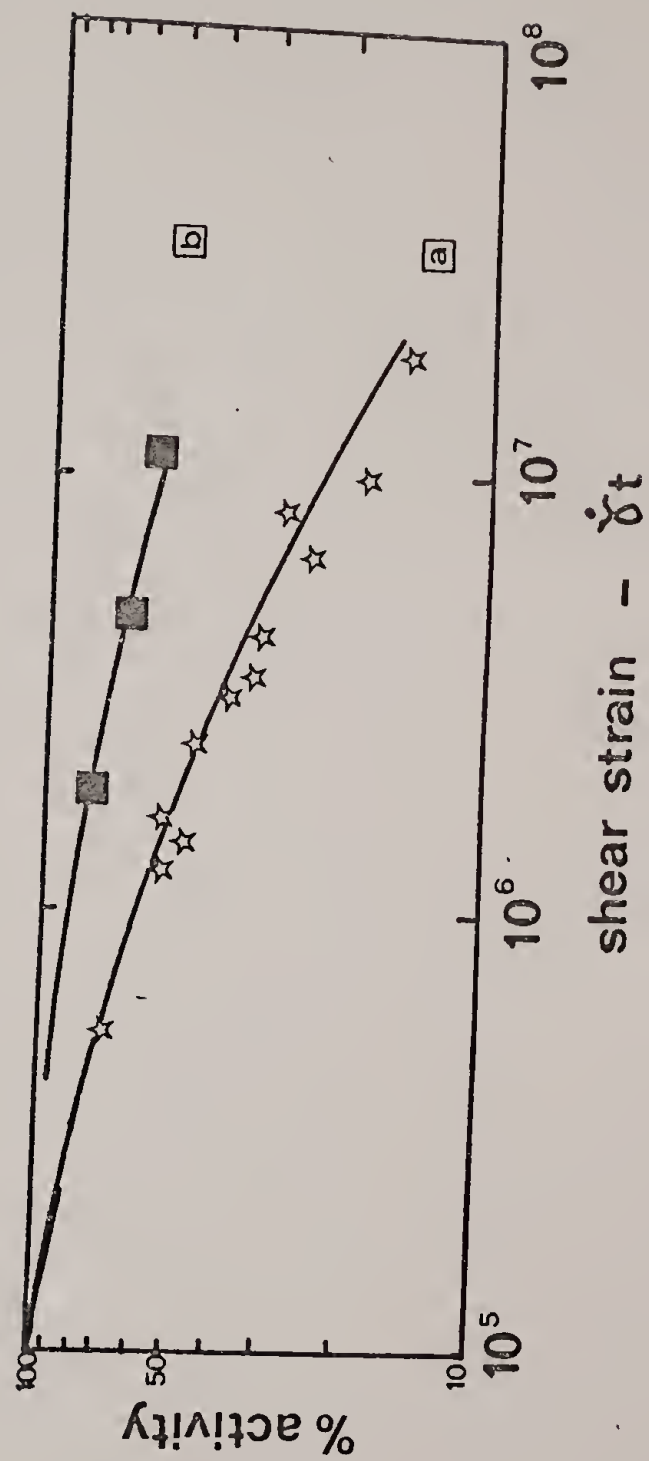


Figure IV-4. Urea concentration as a function of time in the presence of 1.0 mM EDTA. \square - zero shear; \bullet - 1717 s^{-1} ; \blacktriangle - 288 s^{-1} . Urease A.

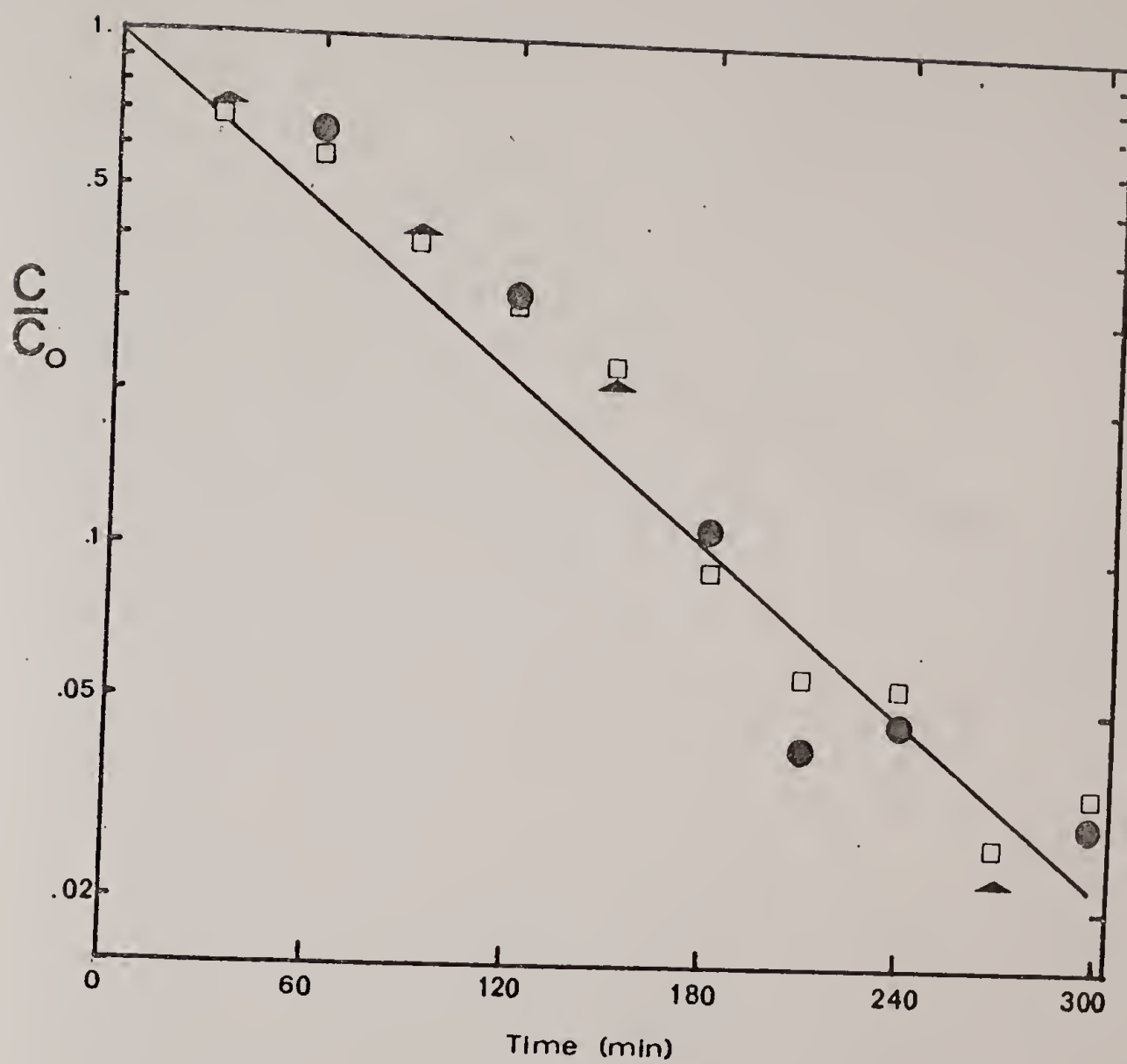


Figure IV-4. The shear inactivation of urease is drastically reduced by the inclusion in the reaction medium of 1.0 mM EDTA.

These experiments establish some general features of the effect of shearing on the kinetics of the urease catalyzed hydrolysis of urea and were the subject of a preliminary communication (Tirrell and Middleman, 1975). Study of the behavior of some more pure urease preparations was then undertaken in order to elucidate the molecular basis of this phenomenon of shear inactivation, and to try to understand the protective effect of EDTA. Figure IV-5 shows the data on kinetics during and after shear obtained with urease B and Figure IV-6 shows the data obtained with the best commercially available grade of urease, urease C. Again, a shear rate dependent inactivation is seen. The inactivation seems to be less drastic and less rapid with urease B than with the other two preparations. The inactivation of ureases B and C seems to be less reversible than that of urease A. In fact, no reversibility at all seems to be the most accurate assessment of the data of Figure IV-6b, taken at 288 s^{-1} . Some slight reversibility after shearing at 1717 s^{-1} is seen for urease C in Figure IV-6a. Since urease C, a crystalline urease as supplied, is the best commercially available grade of urease, roughly 50 to 75% pure, most of the remainder of the results to be reported in this dissertation, aimed at a molecular understanding of the shear inactivation phenomenon,

Figure IV-5. Urea concentration as a function of time during and after variable periods of steady shear.
Urease B.

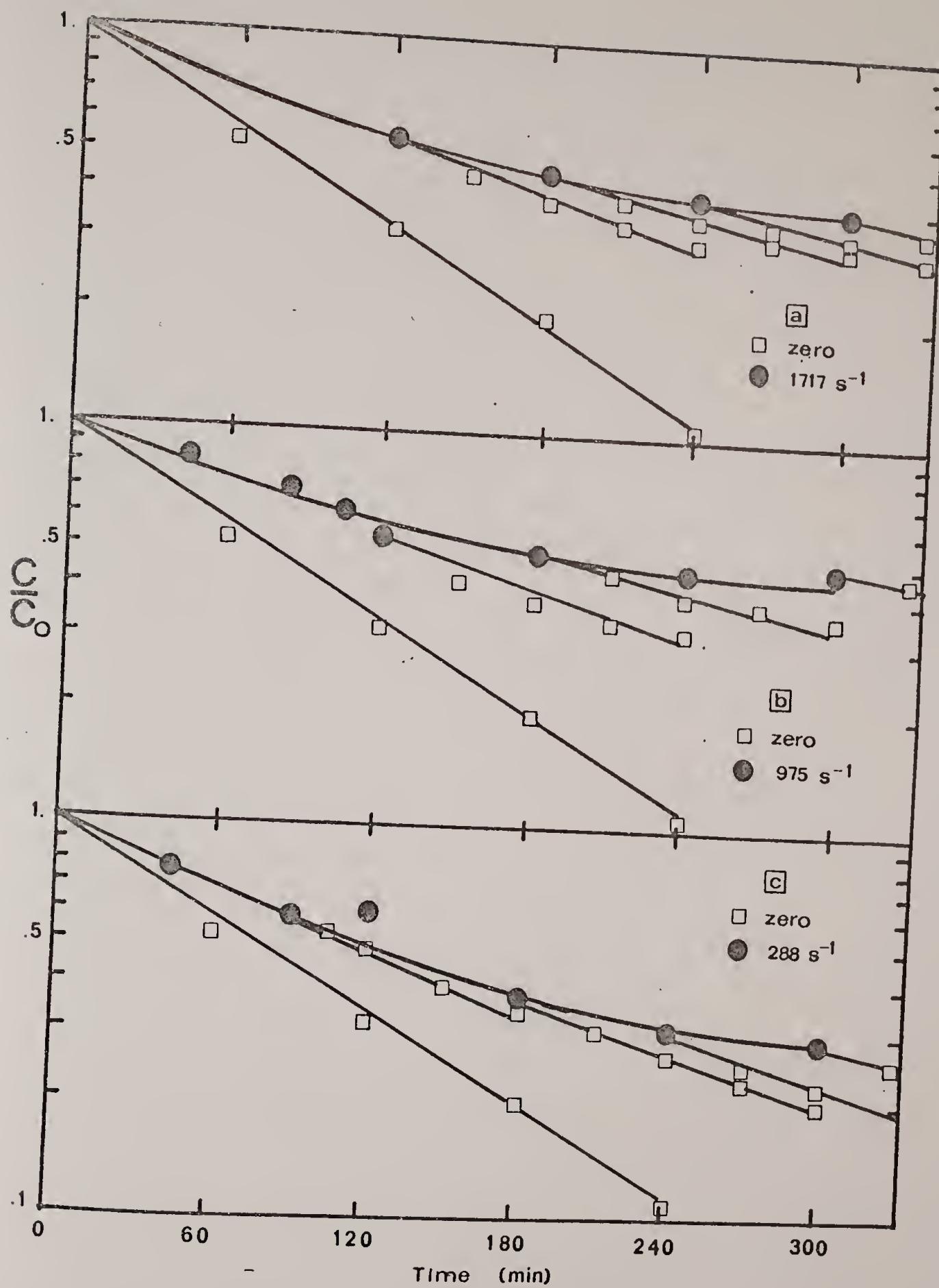
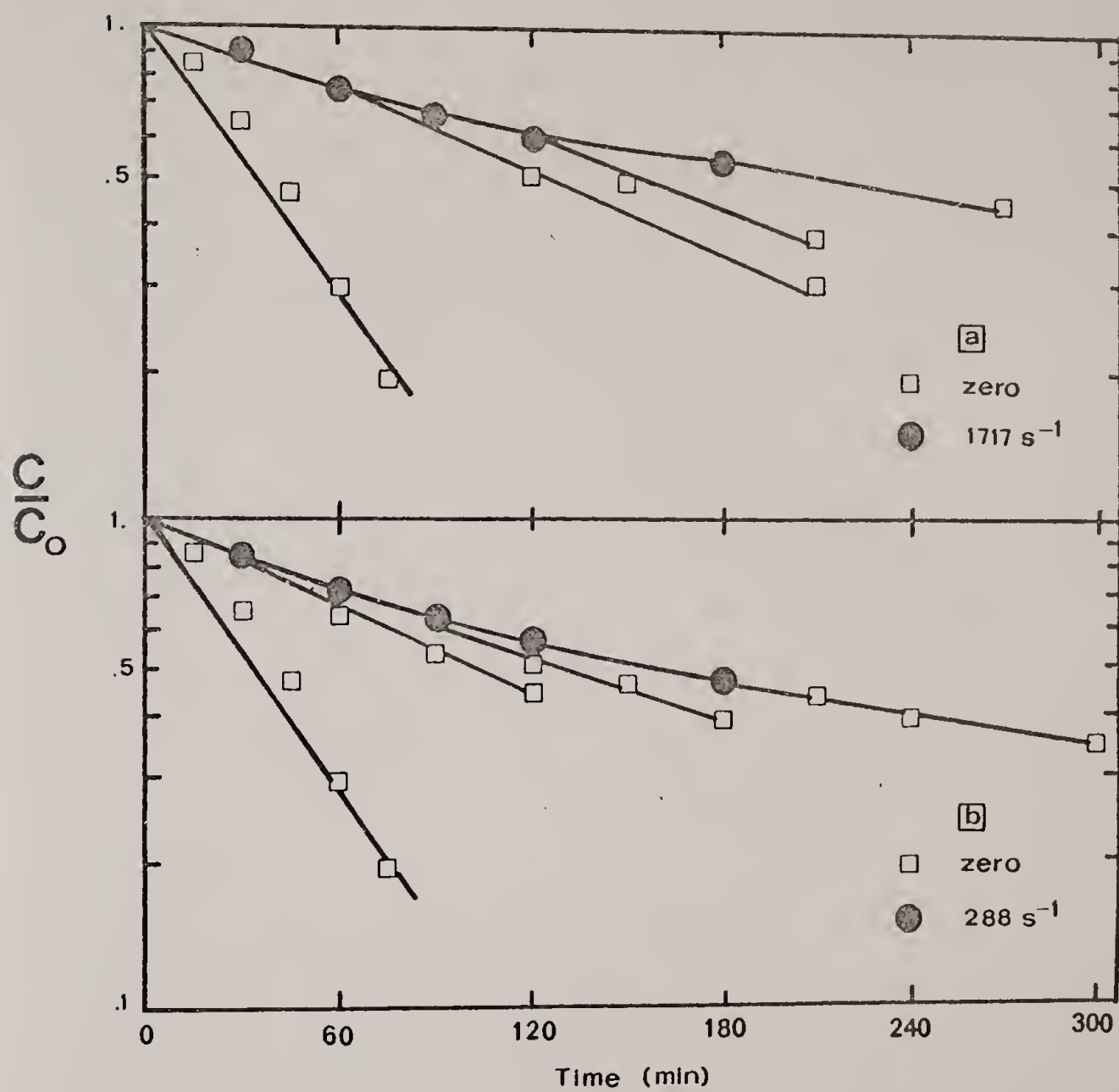


Figure IV-6. Urea concentration as a function of time during and after variable periods of steady shear.
Urease C.



unless otherwise specified, have utilized urease C.

The first step in this mechanistic investigation was to examine the influence of EDTA on urease C and its shear inactivation behavior. Some zero shear rate kinetic data in the presence and absence of EDTA is shown in Figure IV-7. EDTA is seen to have an activating effect, roughly 30% rate enhancement, in the buffer system used in this work. When shearing forces are applied, the data of Figure IV-8 are obtained. Slight inactivation due to shearing is seen at 1717 s^{-1} , which seems to be completely reversible, within experimental error, upon removal of the shearing. No inactivation at all is seen at 288 s^{-1} . Note the correspondence between these results and the relative amounts of reversible inactivation seen in the data of Figure IV-6. It seems that in the presence of EDTA, the irreversible inactivation is filtered out of the total inactivation seen in Figure IV-6, leaving only the reversible component.

If EDTA reduces the shear inactivation, it means one of two things: (1) EDTA binds directly to urease to protect its structure from the hydrodynamic forces in some way, or (2) metal ions play some role in the apparent shear inactivation and EDTA, a well known metal chelating agent, sequesters them. Number (1), EDTA-protein binding, is not without precedent; for example, EDTA is known to bind to fibrinogen (Bithell, 1964; Blomback et al., 1966). Number (2) has two conceivable variations: (a) A metal atom could be responsible

Figure IV-7. Zero shear urèa hydrolysis by urease with and without EDTA.

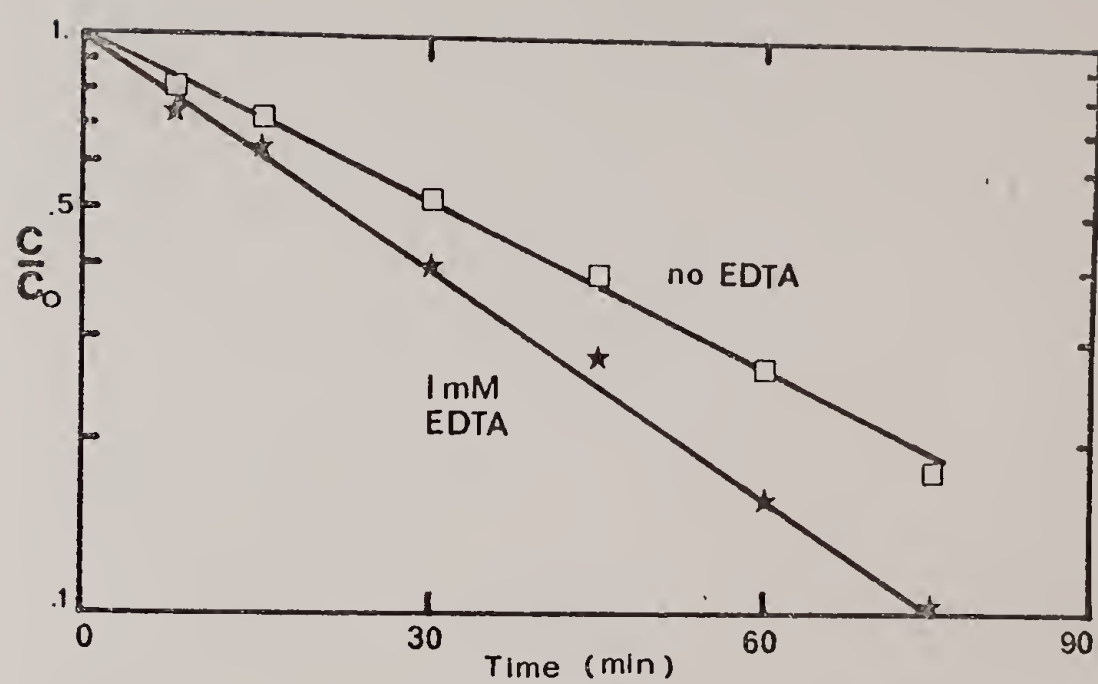
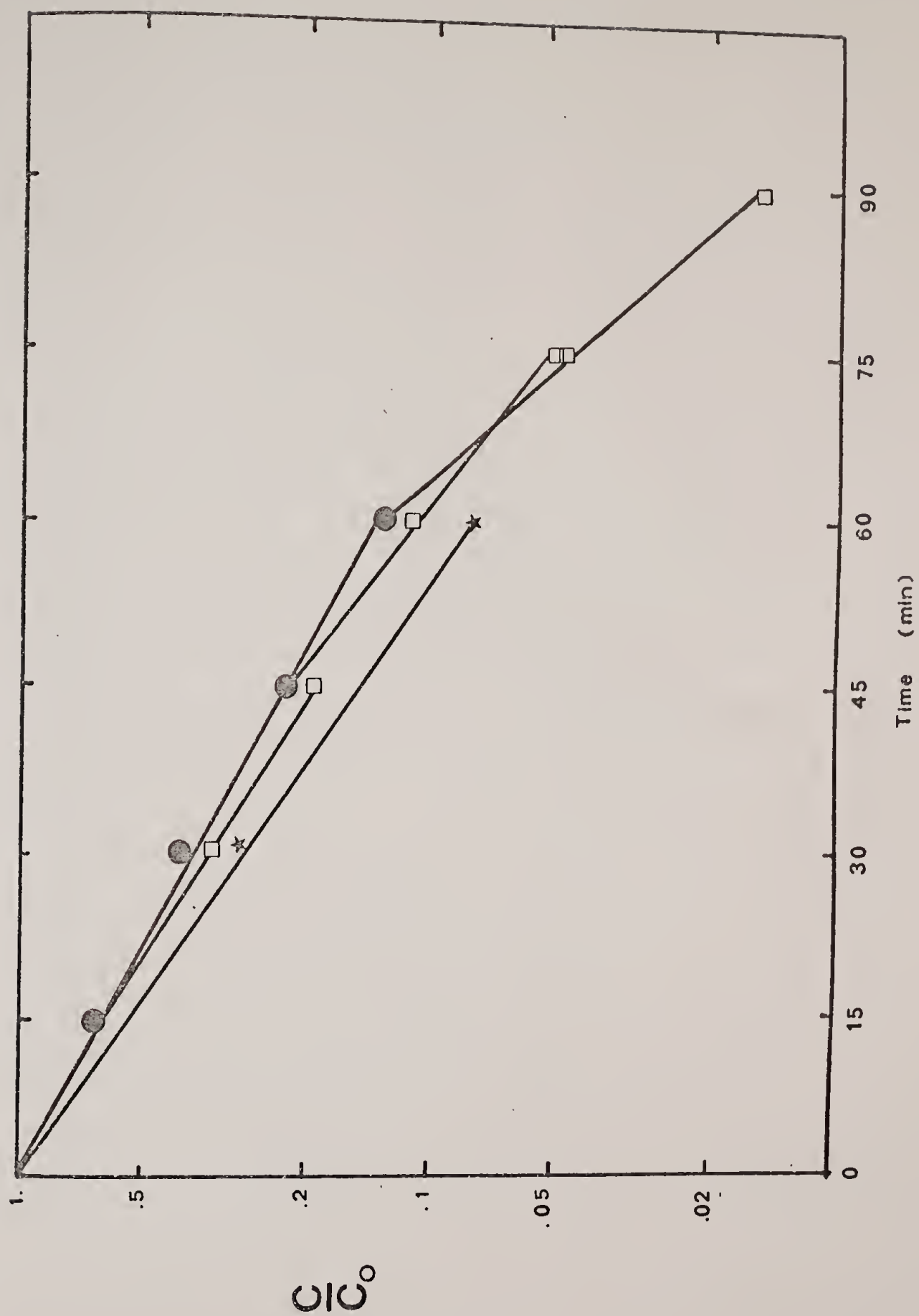
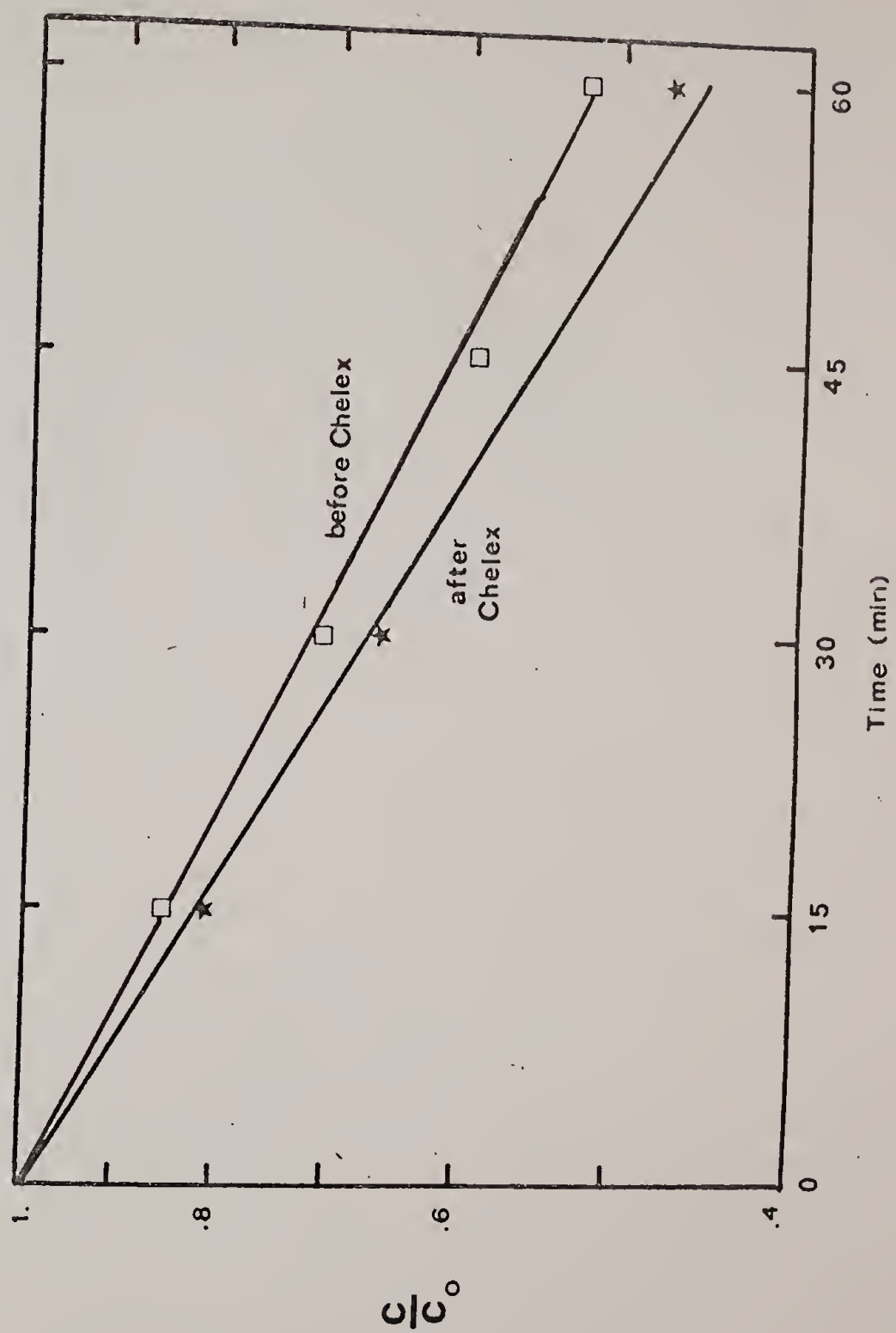


Figure IV-8. Urea concentration as a function of time in the presence of 1.0 mM EDTA. ★ - no shear; ● - 1717 s^{-1} ; □ - stop shear. Urease C.



for physically holding together some parts of the urease quaternary structure. EDTA may bind the metal more strongly than urease, remove the metal and break the urease structure down to smaller less shear susceptible species, although without greatly altering the urease activity. (b) Metal ions in solution act upon the urease molecules, whose structure is deformed by shearing, in such a way to greatly enhance the apparent shear inactivation. Alternative (a) may be rejected on the basis of previously known evidence. Many chemical and physical-chemical studies have examined urease in the presence and absence of EDTA. No changes in structure correlating with the presence of EDTA in the urease solution can be detected. Further evidence for this will be mentioned in section B of this chapter. To distinguish between possibilities (1) and (2b), experiments were performed where the urease preparations were exposed to a chelating resin, Chelex 100, prior to shearing. Chelex 100 is essentially one half of the EDTA molecule immobilized on polystyrene beads. These beads could then easily be separated from the urease, except for that portion of the urease which may be bound to the resin. Figure IV-9 shows zero shear kinetic data obtained before and after treatment with Chelex. Comparable enhancement in rate to that seen with EDTA is noted, 22% in this case, strongly indicating that no significant fraction of the urease has been removed from the solution with the Chelex. Furthermore, when the Chelex-pretreated urease was subjected

Figure IV-9. Zero shear urea hydrolysis by urease before and after Chelex treatment.



to shearing, kinetic data is obtained which is qualitatively identical to that obtained in the presence of EDTA (Figure IV-10; compare with Figure IV-8). Slight inactivation due to shearing is seen at 1717 s^{-1} ; no inactivation at all is seen at 288 s^{-1} . Kinetic data with and without applied shearing forces, after Chelex treatment, have also been obtained with urease B as shown in Figure IV-11. Similar results are seen, with reversibility indicated as well. Taken together, the data of Figures IV-9 and IV-10 clearly indicate that metal ions play a role in the shear inactivation of urease. A further implication is that the metal species responsible is an adventitious one, coming from the water or buffer salts or loosely bound (easily removed by chelating agent) to the protein, rather than being an essential metal atom or one released during the shearing process, since it is not necessary to have the chelating moiety present during shearing to provide protection.

The speculation on the possible role of metal ions focused on their potential for catalyzing the oxidation of sulfhydryl groups in proteins (Patai, 1974; Meens and Feeney, 1971). Elsewhere, independently, remarkably similar speculations were being made. Recall from Chapter II that while this work was in progress, Fishbein et al. (1976) reported that inactivation of urease at pH 7 to 10 by concentrated urea, guanidine and heat was markedly delayed by EDTA and other chelating agents. He tentatively attributes this to a

Figure IV-10. Urea concentration as a function of time after Chelex treatment. \square - zero shear; \bullet - 1717 s^{-1} ; \blacktriangle - 288 s^{-1} . Urease C.

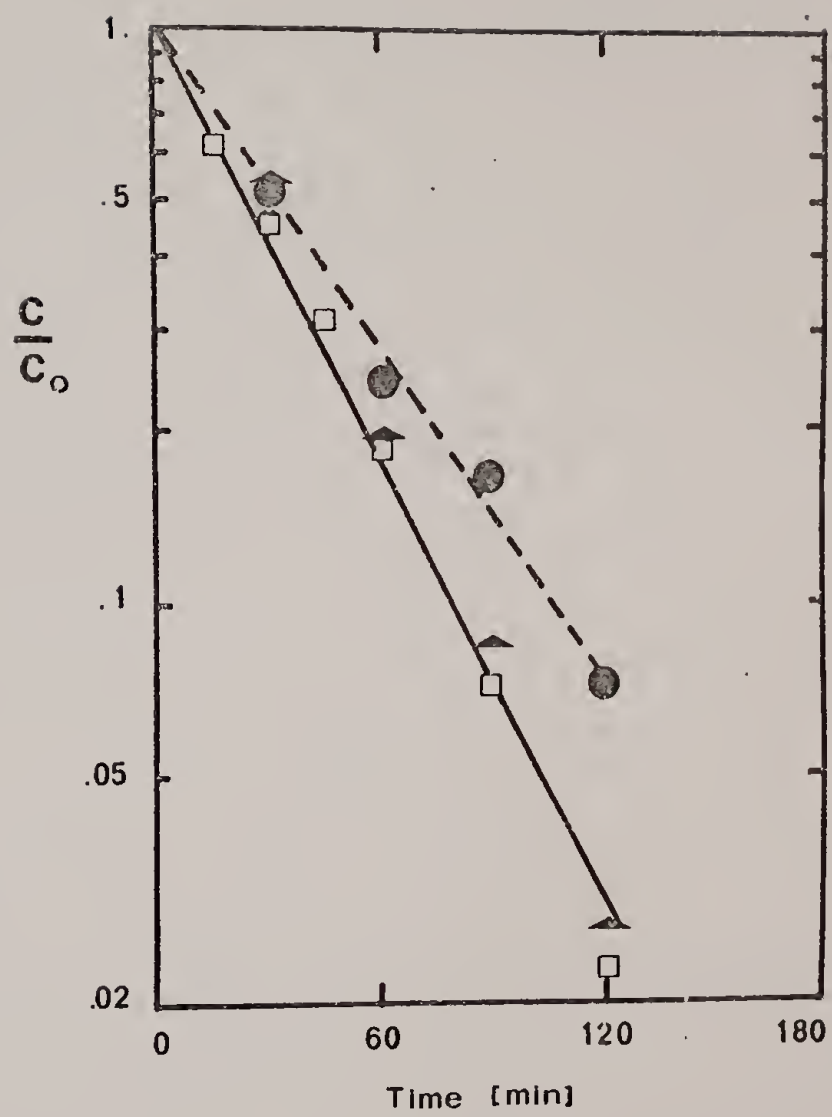
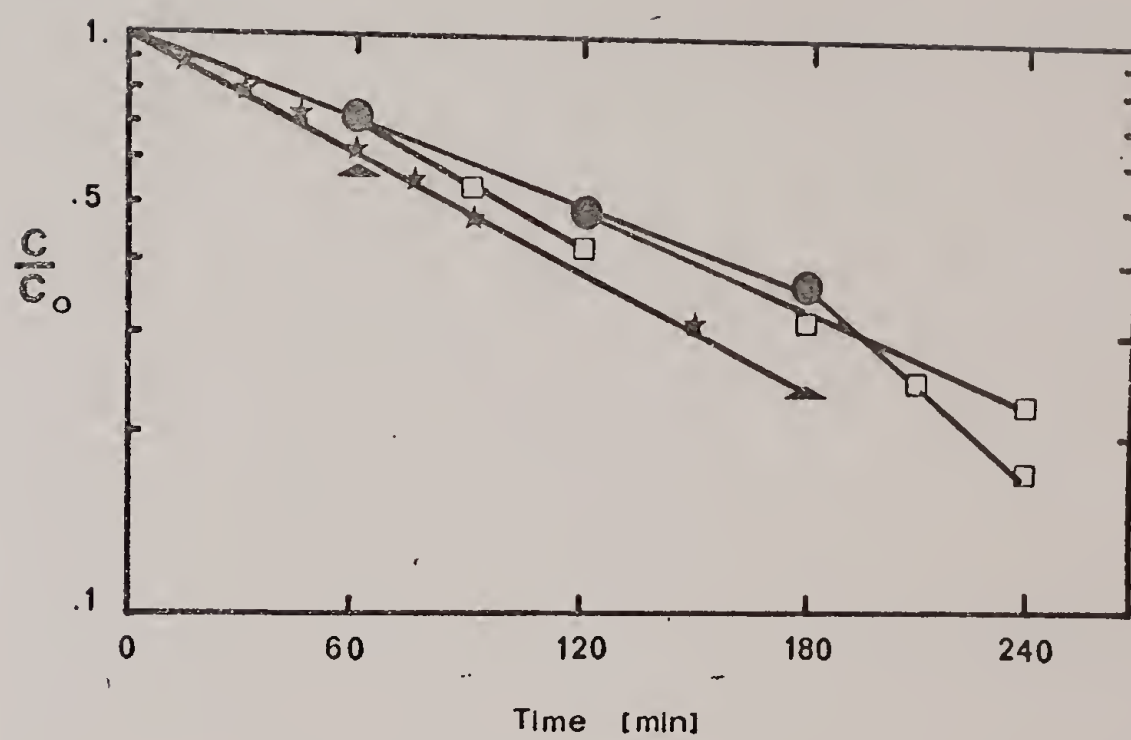


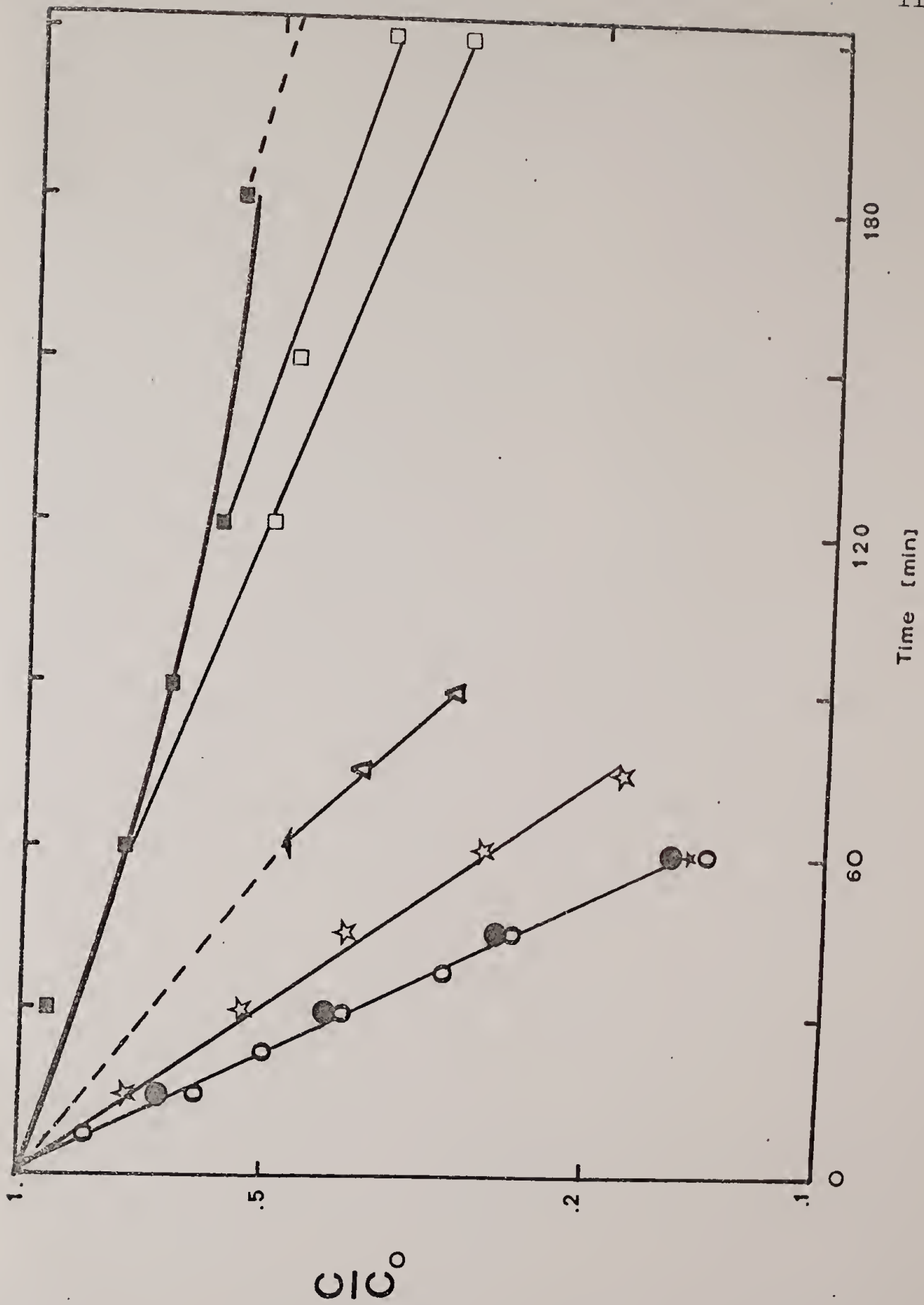
Figure IV-11. Urea concentration as a function of time after Chelex treatment. \star - no shear; \bullet - 1717 s^{-1} ; \blacktriangle - 288 s^{-1} ; \square - stop shear. Urease B.



metal ion catalyzed oxidation of an essential thiol group(s) rendered more labile when the protein unfolds due to the action of the denaturing agent (Fishbein, 1976). To test whether shear promoted, metal ion catalyzed thiol oxidation could be related to the shear inactivation phenomenon, the kinetic reactions in the viscometer were performed in the presence of a thiol reducing agent, β -mercaptoethanol ($\text{HOCH}_2\text{CH}_2\text{SH}$). The results of these experiments are shown in Figure IV-12. β -Mercaptoethanol is seen to have an enhancing effect on the zero shear activity of urease under the conditions of these experiments. When the maximum hydrodynamic shearing force possible in the purely aqueous buffer is applied at 1717 s^{-1} , in the presence of 1.0 or 0.1 mM β -mercaptoethanol, the kinetics are unaltered from the zero shear kinetics. If the β -met concentration is further reduced to 0.001 mM, inactivation similar in character to that of Figures IV-2, IV-5, and IV-6 is seen.

There is a qualitative difference in the protection of urease from shearing inactivation afforded by EDTA and β -met. This is that at 1717 s^{-1} , with EDTA there is small, totally reversible inactivation whereas with β -met there is no inactivation at all. This could be due to the fact that, as a thiol reducing agent, β -met not only protects against metal ion catalyzed thiol oxidation but also disaggregates oligomeric urease isozymes to the 482,000 molecular weight monomer. This species, being smaller, is probably less shear

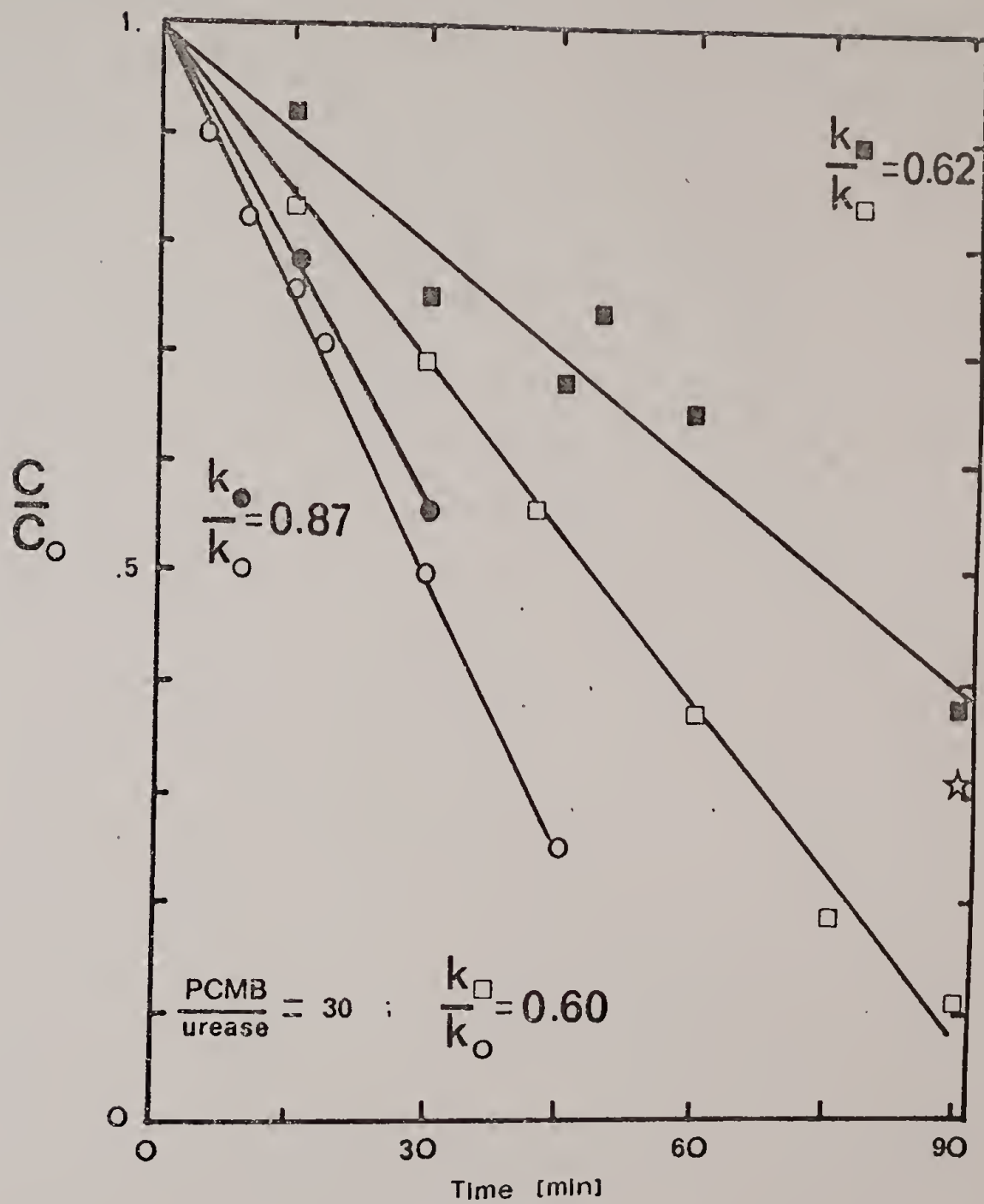
Figure IV-12. Urea concentration as a function of time with various levels of β -mercaptoethanol.
☆- zero shear, no additive; ○- zero shear, 1 mM β -mercaptoethanol; ●- 1717 s⁻¹, 1 mM β -mercaptoethanol; ★- 1717 s⁻¹, 0.1 mM β -mercaptoethanol; ▲- 1717 s⁻¹, stop shear, 0.001 mM β -mercaptoethanol; ■- 1717 s⁻¹, stop shear, no additive (data of Figure IV-6 reproduced for comparison).



susceptible and therefore the protective effect observed in these experiments may be partially or totally due to the effect of β -met on urease molecular size. It seems likely that this could at least explain the qualitative difference in the effects of EDTA and β -met.

So, the β -met protection observed is consistent with and some positive evidence for the shear inactivation being related to a shear promoted metal ion catalyzed thiol oxidation. However, because of its effect on molecular size, the evidence is not clear-cut. Some direct evidence of a shear promoted reaction with urease thiol group(s) was sought. To accomplish this, the kinetics, during shear, of Chelex pretreated urease were studied in the presence of p-chloromercuribenzoate (PCMB). PCMB reacts very specifically with sulfhydryl groups, although with widely varying rates depending on the reaction conditions and the location of the sulfhydryl group(s) within the protein structure (Means and Feeney, 1971). Figure IV-13 shows the results of the reactions in the presence of PCMB. Note that the metal ion effect has been eliminated by Chelex pretreatment; also the amount of urease has been increased somewhat over the usual level for kinetic runs to provide a convenient activity level for these deactivation experiments. It is seen that in the absence of PCMB, shearing at 1717 s^{-1} produces about 13% inactivation whereas in the presence of $1 \text{ }\mu\text{M}$ PCMB, shearing at 1717 s^{-1} produces about 38% inactivation. Shearing forces of

Figure IV-13. Urea concentration as a function of time, effect of PCMB. ○ - zero shear, no additive; ● - 1717 s^{-1} , no additive; □ - zero shear, $1 \text{ } \mu\text{M}$ PCMB; ■ - 1717 s^{-1} , $1 \text{ } \mu\text{M}$ PCMB; ☆ - 288 s^{-1} , $1 \text{ } \mu\text{M}$ PCMB.

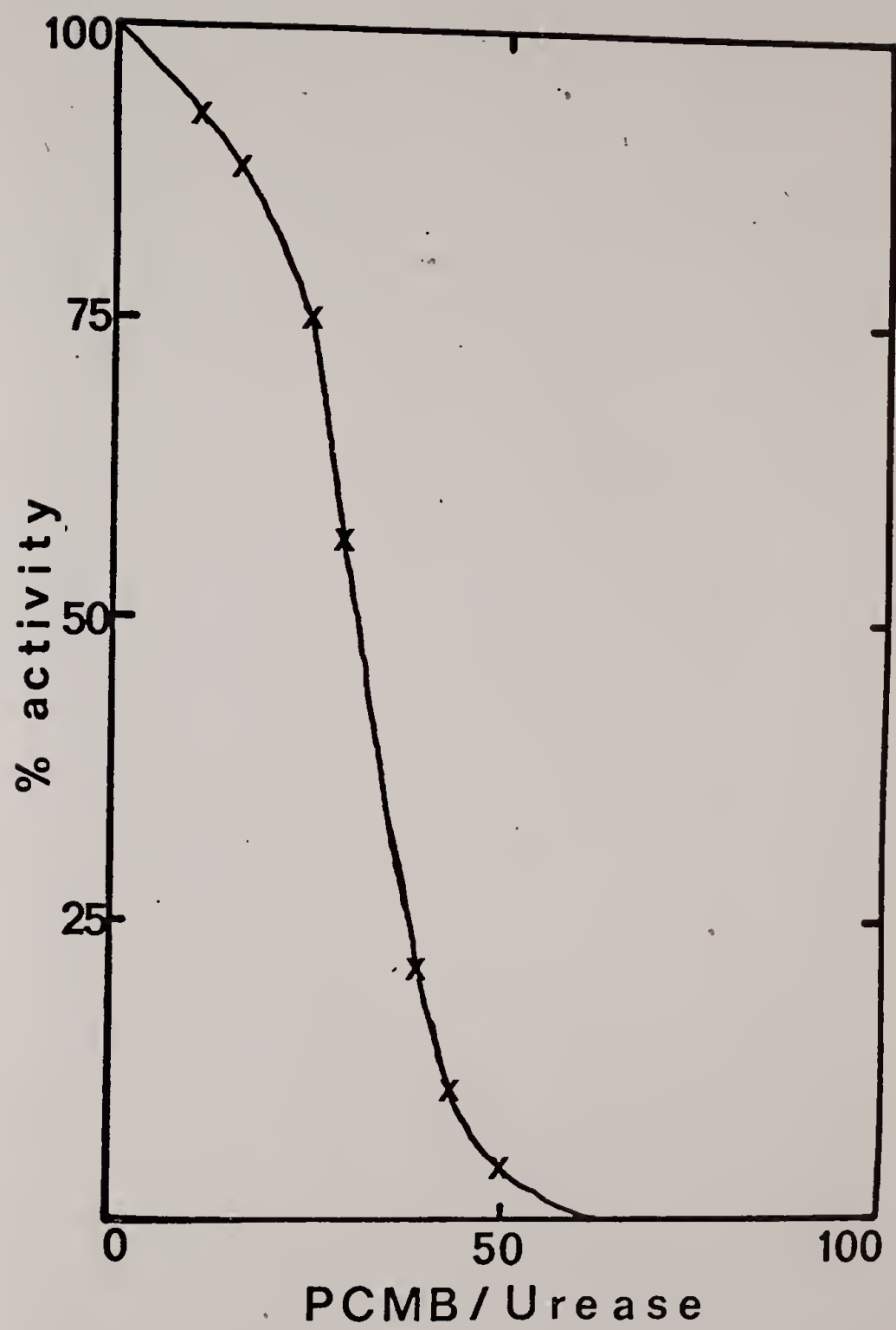


this magnitude therefore are seen to be roughly three times as effective in promoting inactivation of urease in the presence of 1 μ M PCMB as in its absence. Even at 288 s^{-1} , PCMB seems to produce markedly enhanced shear inactivation.

It is worth digressing slightly here to discuss the inactivation of urease by PCMB in the absence of shearing. Figure IV-14 shows some data previously published by Gorin et al. (1962) on the stoichiometry of the reaction of PCMB with urease. Their data was obtained via the method of Boyer (1954). They report no results on the kinetics of the mercurial-urease reaction but recorded the final activity vs. $[\text{PCMB}]/[\text{urease}]$ values after 3 hours reaction. In contrast, the data of Figure IV-13 were obtained in the presence of saturating concentrations of substrate. The value of 60% remaining activity at $[\text{PCMB}]/[\text{urease}] = 30$ agrees very well with the data of Gorin et al. (1962) but here, the mercurial-urease reaction seems to be quite fast in the presence of urea. Similarly, under the conditions of these experiments, $[\text{PCMB}]/[\text{urease}] = 60$ produced complete inactivation of urease within 5 minutes. This enhancement of the rate of the PCMB-urease reaction by urea may be related to the substrate inhibition phenomenon discussed earlier in Chapter II.

In any case, the data of Figure IV-13 provide decisive evidence that a shear-promoted reaction of a low molecular weight moiety and urease thiol group(s) takes place.

Figure IV-14. Urease activity remaining vs. PCMB/urease molar ratio (after Gorin et al., 1962).



It is thus quite clear, from all the data presented so far, that a shear-promoted metal ion catalyzed oxidation is responsible for the irreversible inactivation of urease under shearing conditions. A further bit of evidence to support this conclusion was sought by attempting to perform the kinetic reactions in shear in the absence of oxygen in the normal buffer medium, without additives, the idea being that oxygen exclusion may prevent thiol oxidation. Figure IV-15 shows the results of a typical experiment of this sort. It is seen that reducing the oxygen level has not been successful in preventing inactivation during shearing. It is not certain what the oxygen level was in this experiment since no convenient oxygen measuring technique was available. It is known that very low levels of oxygen will support metal ion catalyzed thiol oxidation (Patai, 1974). In the work of Fishbein et al. (1976) similar attempts at excluding oxygen were unsuccessful; the metal ion catalyzed thiol oxidation persisted and residual oxygen could be detected (Fishbein, 1976). So, it is felt that this result, while disappointing, does not argue convincingly against the mechanism of irreversible shear inactivation being a shear promoted metal ion catalyzed thiol oxidation.

Attention was next directed toward ascertaining what metal ion was responsible, under the conditions of these experiments. The field can be rapidly narrowed down by examining the potential of various metal ions for thiol oxidation

Figure IV-15. Urea concentration as a function of time in nitrogen atmosphere. \square - zero shear; \bullet - 1717 s^{-1} . Urease B.

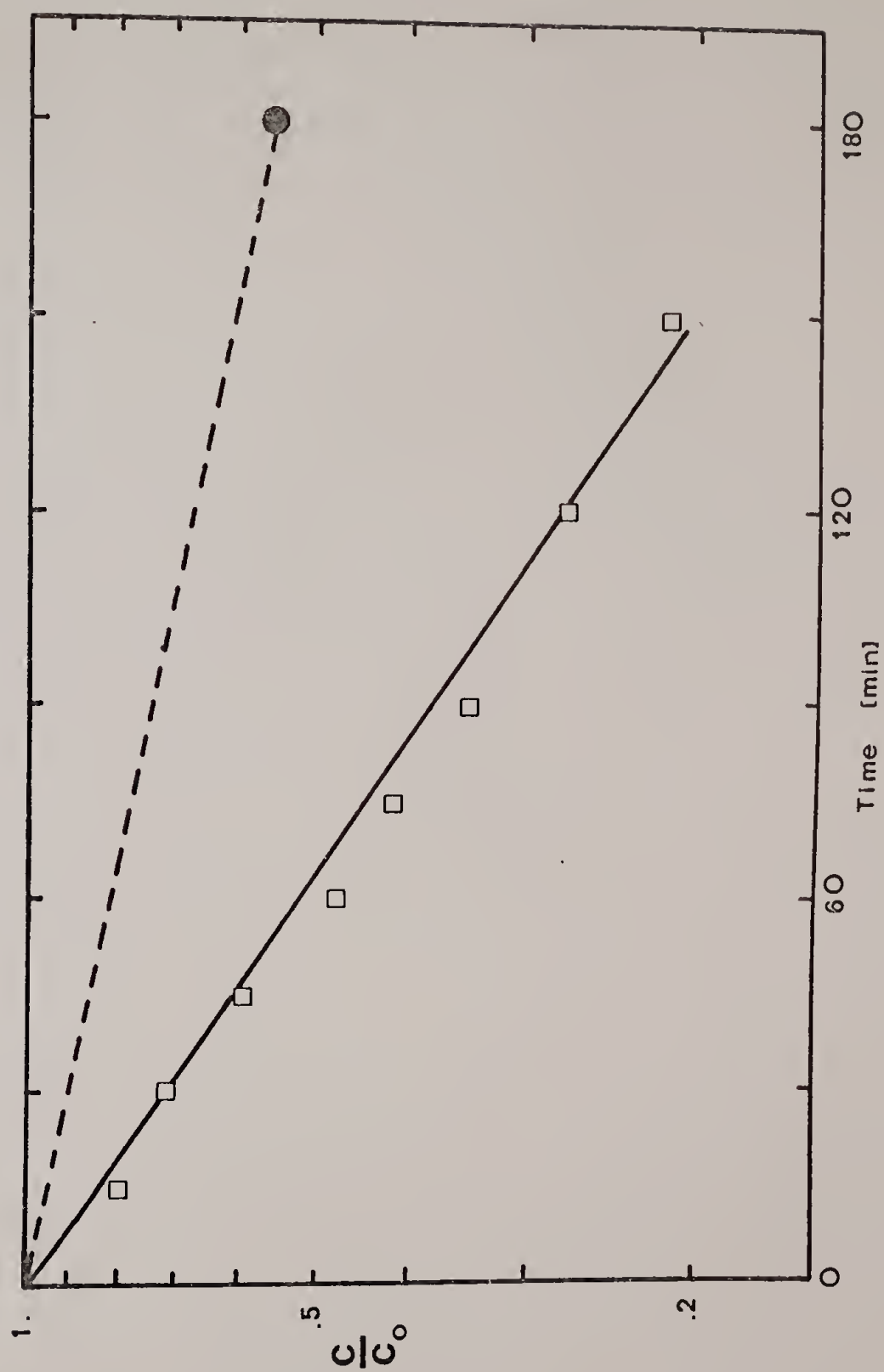


TABLE IV-1
RESULTS OF ATOMIC ABSORPTION ANALYSES

SAMPLE	Fe	Ni
Urease/buffer (0.129 mg/ml)	15.6 μ M	22.6 μ M
Urease/buffer (treated)* [‡] (0.126 mg/ml)	9.8 μ M	19.6 μ M
Buffer alone (treated)* [‡]	8.4 μ M	16.5 μ M

*Treated means exposed to Chelex 100.

[‡]Note that $\Delta\text{Ni}_{2-3} \longrightarrow 12$ atoms Ni/482,000 daltons.

(Patai, 1974). The most potent metal ion catalysts for thiol oxidation are Cu^{2+} and Fe^{3+} ; Ni^{2+} is less than one-half as potent and Co^{2+} a bit less than Ni^{2+} ; many other ions, while showing definite activity, are all an order of magnitude or more weaker than these. Atomic absorption spectroscopic analyses for Cu, Fe and Ni found no detectable Cu but substantial amounts of Fe and Ni as shown in Table 1. Most of this metal content comes from the buffer salts and water, not from the urease; the nickel bound by the urease is a little more than twice the iron apparently bound by urease. To determine which of these two metal ions is responsible for the shear promoted metal ion catalyzed thiol oxidation, experiments were performed in which all reaction ingredients and buffers were pretreated with Chelex, previously shown to eliminate the irreversible inactivation. Various levels of the nitrate salts of Fe^{3+} and Ni^{2+} were then added to the reaction media. The kinetics of urea hydrolysis in and out of shear were then observed. Figure IV-16 shows the results of addition of $5\ \mu\text{M}\ \text{Fe}^{3+}$. Compare Figures IV-6a, IV-10, and IV-16. The irreversible inactivation seen in IV-6a, is eliminated by Chelex treatment in IV-10 and is nearly reproduced by adding in $5\ \mu\text{M}\ \text{Fe}^{3+}$ in IV-16. The effects of various levels of Fe^{3+} and Ni^{2+} are illustrated in Table 2. In those experiments fractional conversion after 1 hour was measured. It is seen that $5\ \mu\text{M}\ \text{Ni}^{2+}$ gives no shear effect; in fact, very little effect with Ni^{2+} is seen until concen-

Figure IV-16. Urea concentration as a function of time during and after variable periods of steady shear in presence of $5 \mu\text{M Fe}(\text{NO}_3)_3$.
 \square - zero shear; \bullet - 1717 s^{-1} . Urease C.

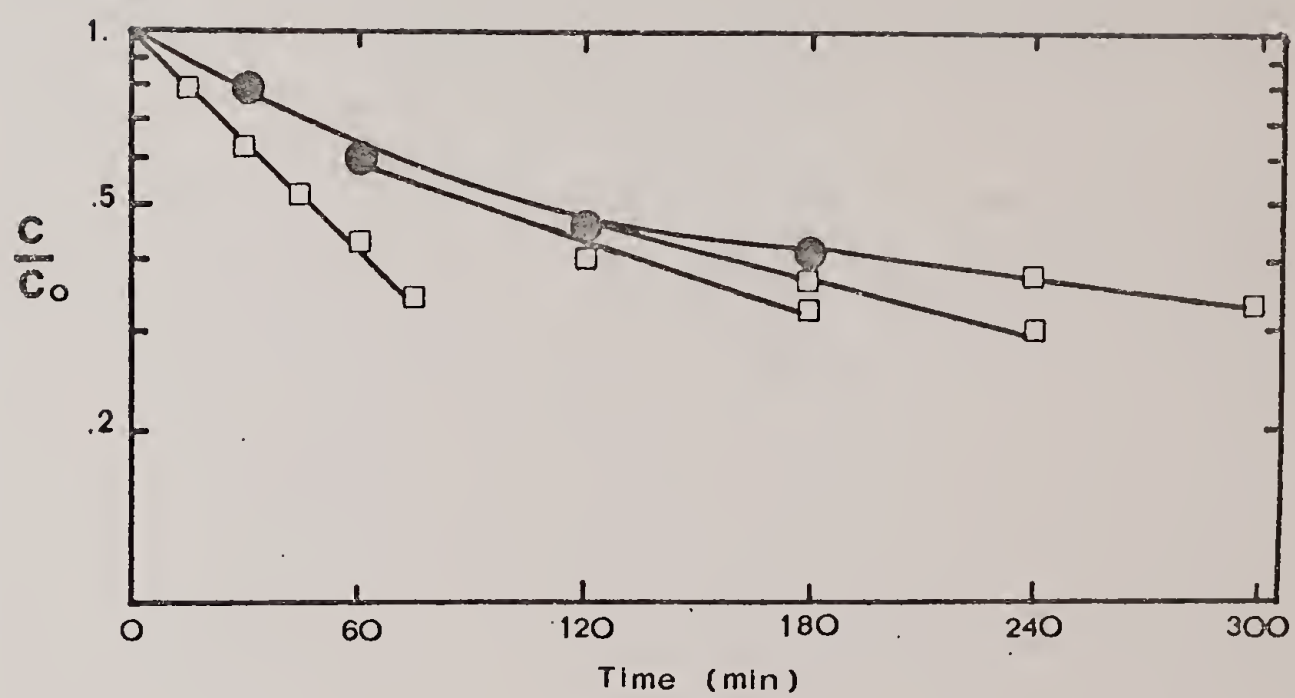


TABLE IV-2
EFFECT OF ADDING METAL IONS AFTER
CHELEX TREATMENT

METAL CONCENTRATION	$(X_{\text{shear}}/X_{\text{no shear}})^{*}\text{metal}$
	$(X_{\text{shear}}/X_{\text{no shear}})_{\text{no metal}}$
5 μM Fe^{3+}	0.81
10 μM Fe^{3+}	0.79
5 μM Ni^{2+}	1.00
45 μM Ni^{2+}	0.86
90 μM Ni^{2+}	0.91

*X is fractional conversion after 1 hour.

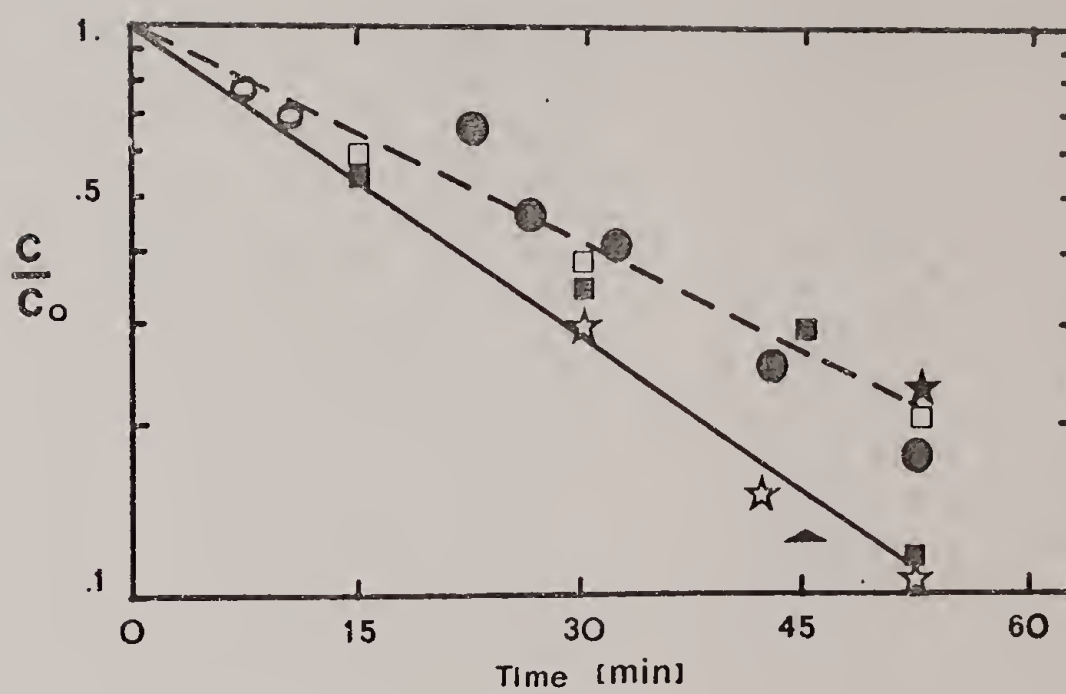
Shear rate = 1717 s^{-1} .

trations 2 to 5 times higher than those measured by AA are reached and even then the effects are much less pronounced than with Fe^{3+} . In contrast, Fe^{3+} produces comparable degrees of inactivation during shearing to that seen in Figure IV-6 at precisely the concentrations of Fe measured by AA. Further proof that Fe^{3+} is responsible is shown in Figure IV-17. Two Fe^{3+} specific chelating agents, N,N-[bis(2-hydroxyethyl)glycine] and 8-hydroxyquinoline are seen to afford protection against the shearing forces. In the case of the former, the protection is quite strong, whereas the kinetic behavior in the presence of the latter more closely resembles that in the presence of EDTA. Very limited data with glycine indicates that it, too, affords some protection, which gives cause for wonder whether previous reports of its activating effect on urease (Wall and Laidler, 1953; Choi and Perlmutter, 1976) may have to do with its metal chelating ability.

The evidence is thus decisive that the irreversible inactivation by shearing of urease seen previously in these reaction media is due to a shear promoted metal ion catalyzed oxidation of a urease sulfhydryl group(s), rendered more labile by the hydrodynamic shear. Further, it has been shown that the monomeric urease isozyme, in the presence of β -met, is absolutely unaffected by this magnitude of shearing force, that is, up to about 2.3 Pascals.

The connection of this work with the results of Fish-

Figure IV-17. Urea concentration as a function of time at varying shear rates with several different chelating agents. 8-hydroxyquinoline (1 mM): ○ - zero shear; ☆ - 48 s^{-1} ; ▲ - 288 s^{-1} ; ● - 1717 s^{-1} ; bicine (0.1 mM): □ - zero shear, ■ - 1717 s^{-1} ; glycine (1 mM): ★ - 1717 s^{-1} .



bein et al. (1976) on the chemical and thermal deactivation of urease is worth discussing briefly. Their hypothesis on the mechanism of EDTA protection was that it, likewise, prevented metal ion catalyzed thiol oxidation on the thermally or chemically deformed urease molecule. The work of this dissertation certainly supports the plausibility of that hypothesis. However, they have also speculated that the metal ion responsible in their case is Ni (Fishbein, 1976), the idea being that a Ni atom, released as a urease molecule unfolds, catalyzes this oxidation on a different urease molecule. This may be the case in their work. However, the fact that Chelex pretreatment affords protection against deactivation in the present work, argues against the essential Ni atoms of urease being responsible for the effects seen here. In this connection, it is also relevant to point out that some evidence was obtained in the course of this work which, although peripheral to the main thrust of this dissertation, corroborate the recent reports that urease is a nickel metalloenzyme (Dixon et al., 1975; Fishbein et al., 1976a). The first is the atomic absorption data shown in Table 1. The difference in Ni content between Chelex treated urease + buffer and Chelex treated buffer alone represents the nickel bound to urease. From this, it can be calculated that urease has 12 atoms of Ni per 482,000 molecular weight; this is slightly higher than, but in reasonable agreement with, the previous reports: 8 to 11 (Dixon et al., 1975) and 6 to 8

(Fishbein et al., 1976a). The other more novel evidence for the crucial role played by nickel in urease catalysis and structure is the effect on urease in solution of 10 mM quantities of 1,2-ethanedithiol ($\text{HSCH}_2\text{CH}_2\text{SH}$) and 2,3-dimercaptopropanol (HOCHCH_2SH ; BAL; British antilewisite). These com-
 SH

These compounds, in addition to the obvious structural analogies to β -mercaptoethanol ($\text{HOCH}_2\text{CH}_2\text{SH}$), are very powerful Ni^{2+} chelating agents with log (stability constant) of 47 (Leussing, 1959, 1960). Evert (1953) had previously used BAL in concentrations of about 0.1 mM, two orders of magnitude lower than the present level, as a thiol reducer, to reverse mercurial inhibition of urease. They were exposed to urease more or less as a curiosity, to see if their thiol reducing capabilities or their nickel binding capabilities would produce any unusual effects. What was found with both reagents was a rapid development of turbidity in the solution, becoming opaque within 10 minutes. Protein precipitation was obvious visually. This is most likely due to removal of Ni from urease. A similar removal can be accomplished at pH 3.5 to 4.0 with EDTA (Fishbein et al., 1976a), also accompanied by precipitation.

B. Size Exclusion Chromatography Results

While the chemical evidence of the previous section is convincing support of the proposed mechanism for the irre-

versible inactivation of urease, some attempts were made to ascertain the nature of the structural changes, if any, accompanying the inactivation. The size exclusion chromatographic technique described in Section III-B was used toward this end. Figure IV-18 shows urease activity vs. elution volume profiles of urease C before and after dialysis to remove any thiol reagent in the crystalline urease as supplied. The smaller proportion of oligomers in evidence before dialysis is evidence that the thiols which were present initially were separated from the urease by the column and aggregation of urease has begun. If thiol is removed prior to chromatography, aggregation is more advanced. Chromatograms virtually the same as Figure IV-18b were obtained if 1 to 5 mM EDTA was included in the otherwise identical buffer. This precludes the EDTA-induced depolymerization mechanism discussed earlier. The peaks correspond, based on the calibration curve of Figure III-3, to integral multiples of 482,000 molecular weight. Figure IV-19 shows the urease activity vs. elution volume and protein concentration vs. elution volume profiles of urease B. It can be readily seen that this urease preparation consists largely (about 95%) of inactive protein. Figure IV-20 shows how the protein concentration vs. elution volume profile changes as a function of shear exposure time at 1717 s^{-1} . No trends are discernible. Obviously, this urease preparation B is too impure to be useful in deriving any molecular information. For this reason,

Figure IV-18. Size exclusion chromatography of urease C
(a) before and (b) after dialysis.

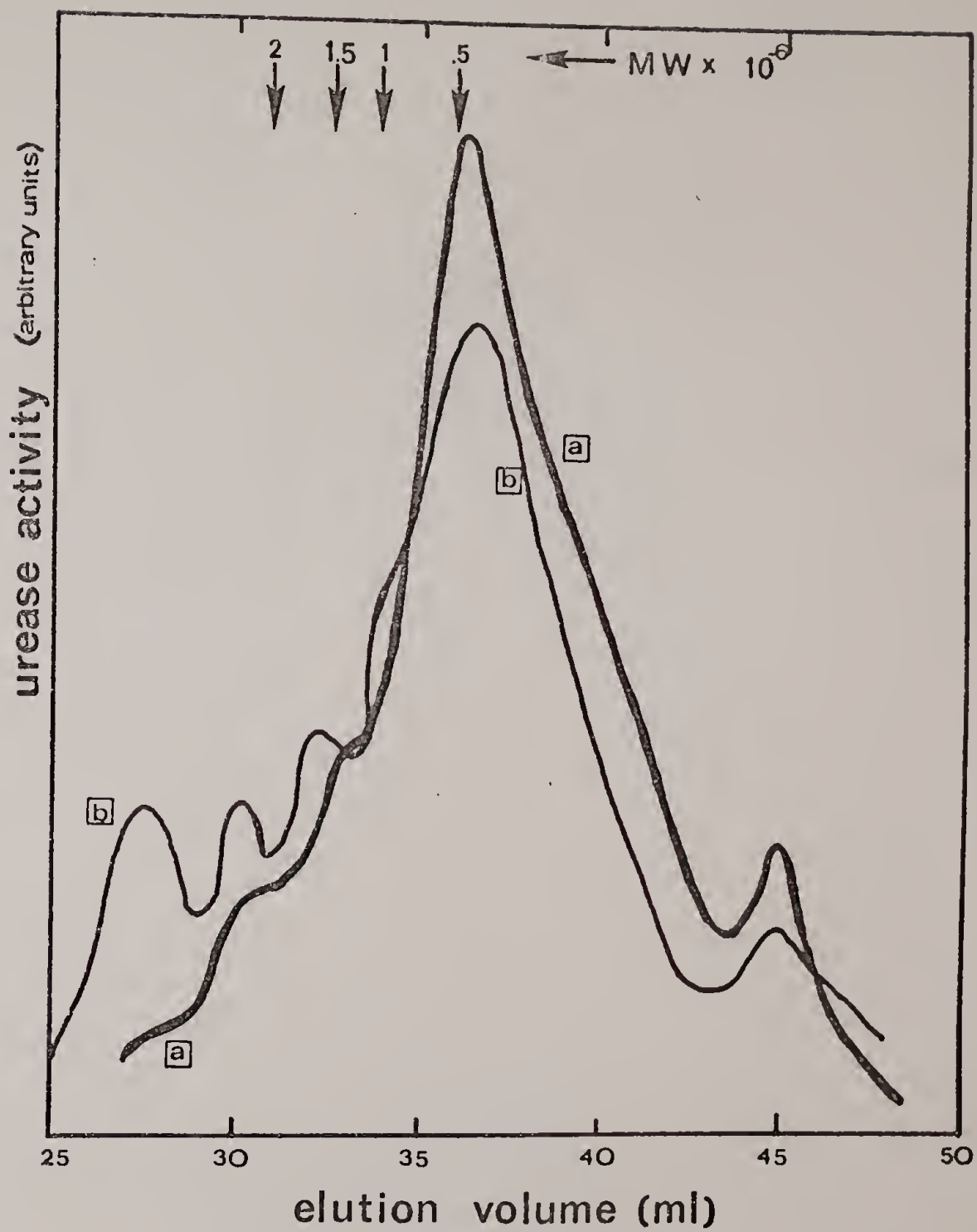


Figure IV-19. Urease activity and protein concentration vs. elution volume for Urease B.

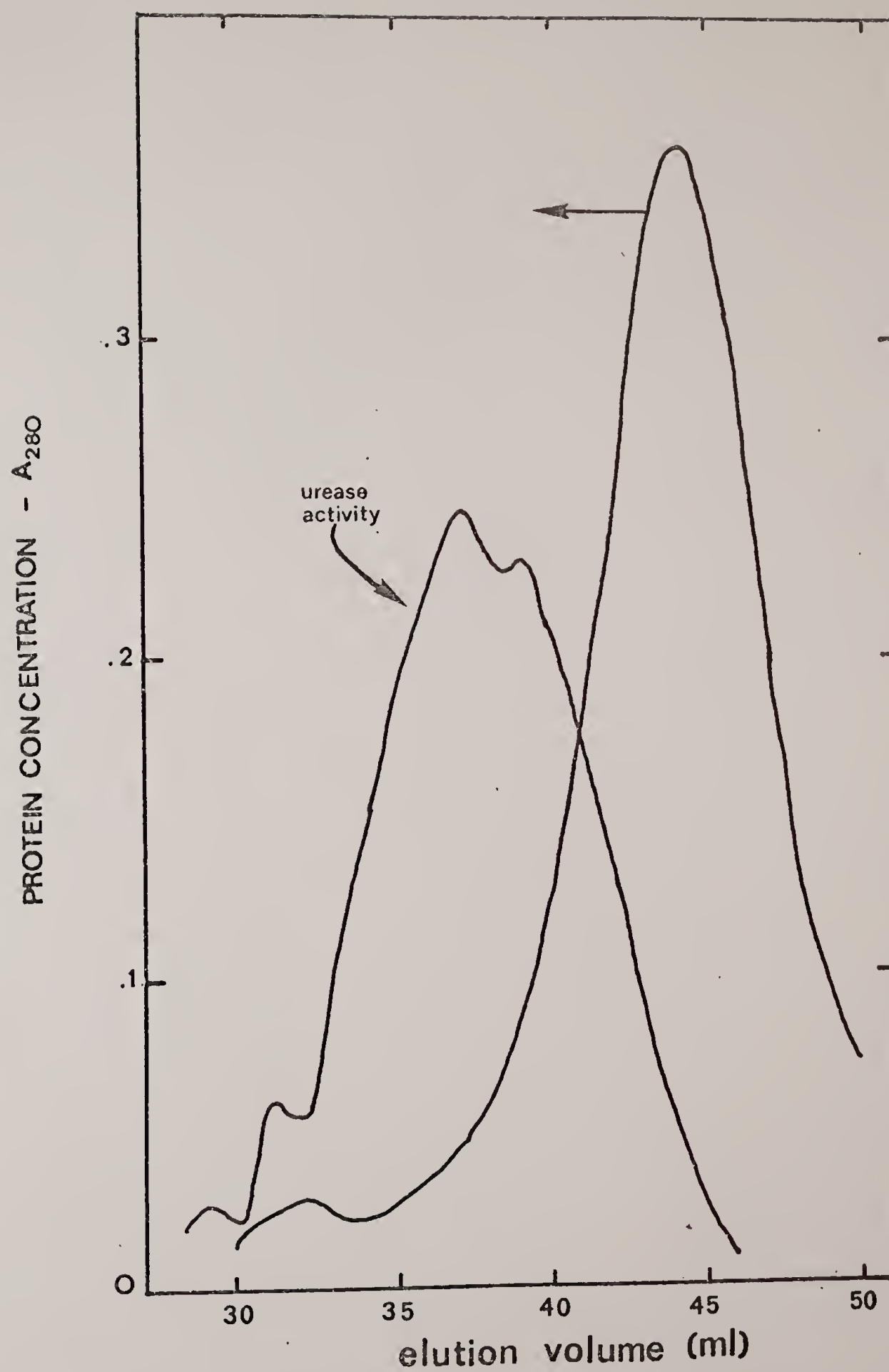
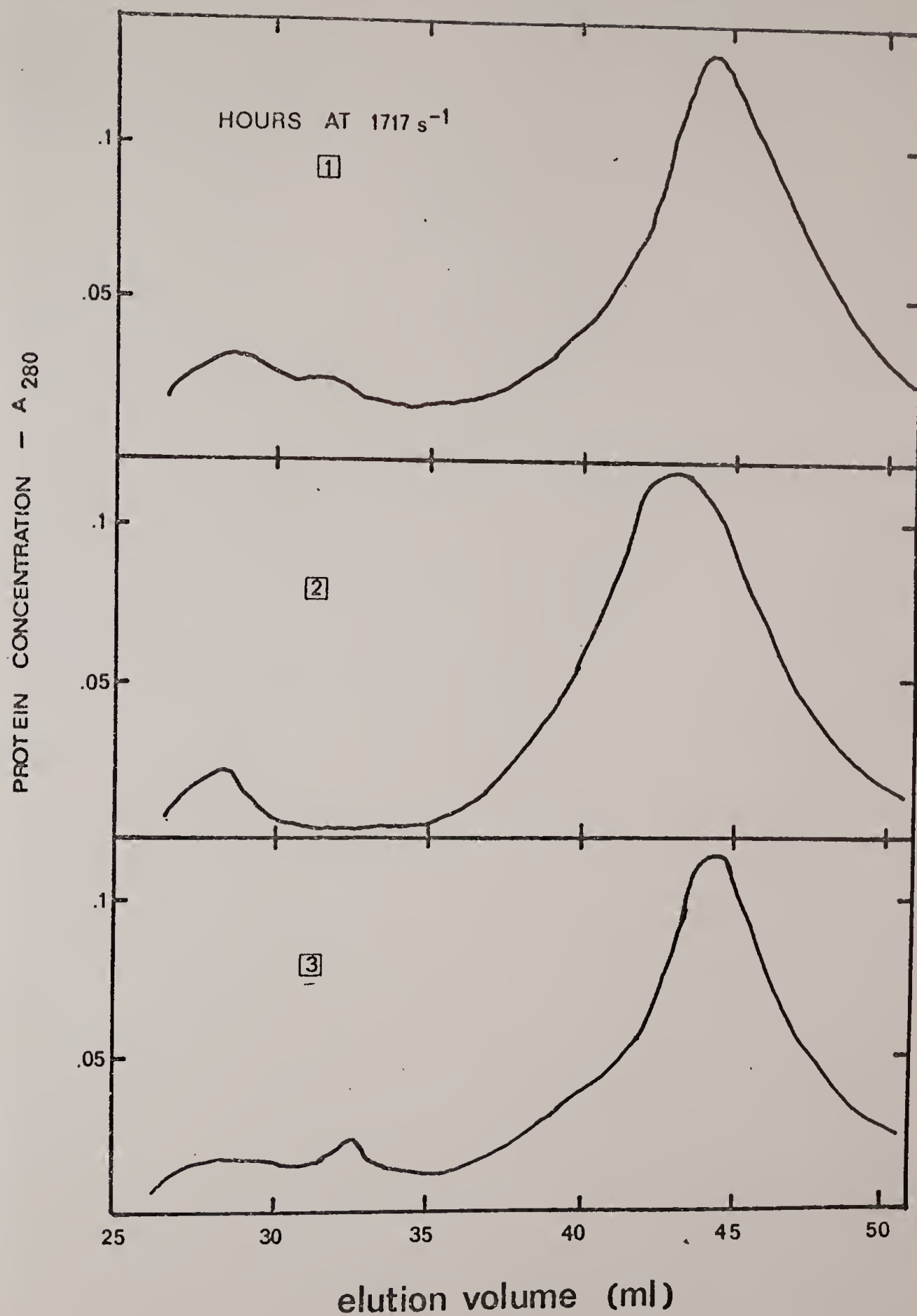


Figure IV-20. Protein concentration vs. elution position as a function of shear exposure time at 1717 s^{-1} . Urease B.



it was not studied further in any kinetic, chromatographic or spectroscopic experiments.

Urease activity vs. elution volume for urease C as a function of shear exposure time at 1717 s^{-1} is shown in Figure IV-21. Protein concentration vs. elution volume for this same urease preparation subjected to the same deformation histories is shown in Figure IV-22. In Figure IV-21, activity disappearing from the higher molecular weight isozymes is seen. These species are not degraded or broken down in molecular weight by the shearing forces however, as Figure IV-22 shows. There is actually an increase in the relative concentration of the high molecular weight species, implying a shear induced aggregation is occurring simultaneously with the loss in activity of the aggregated isozymes. The effect of β -mercaptoethanol in preventing the shear inactivation may now be fully understood as a dual function: (1) preventing metal ion catalyzed thiol oxidation in the shear distorted urease molecules, and (2) keeping all the urease in the more shear stable monomeric form. The increase in concentration of the higher molecular weight species during shearing accounts for the narrowing of the 482,000 molecular weight activity peak during shearing seen in Figure IV-21. The utility of these chromatographic results in interpreting the results of the kinetic reactions in shear is compromised somewhat by the fact that the shearing for the chromatography samples took place in urease solutions fifty times as concen-

Figure IV-21. Urease activity vs. elution position as a function of shear exposure time at 1717 s^{-1} . Urease C.

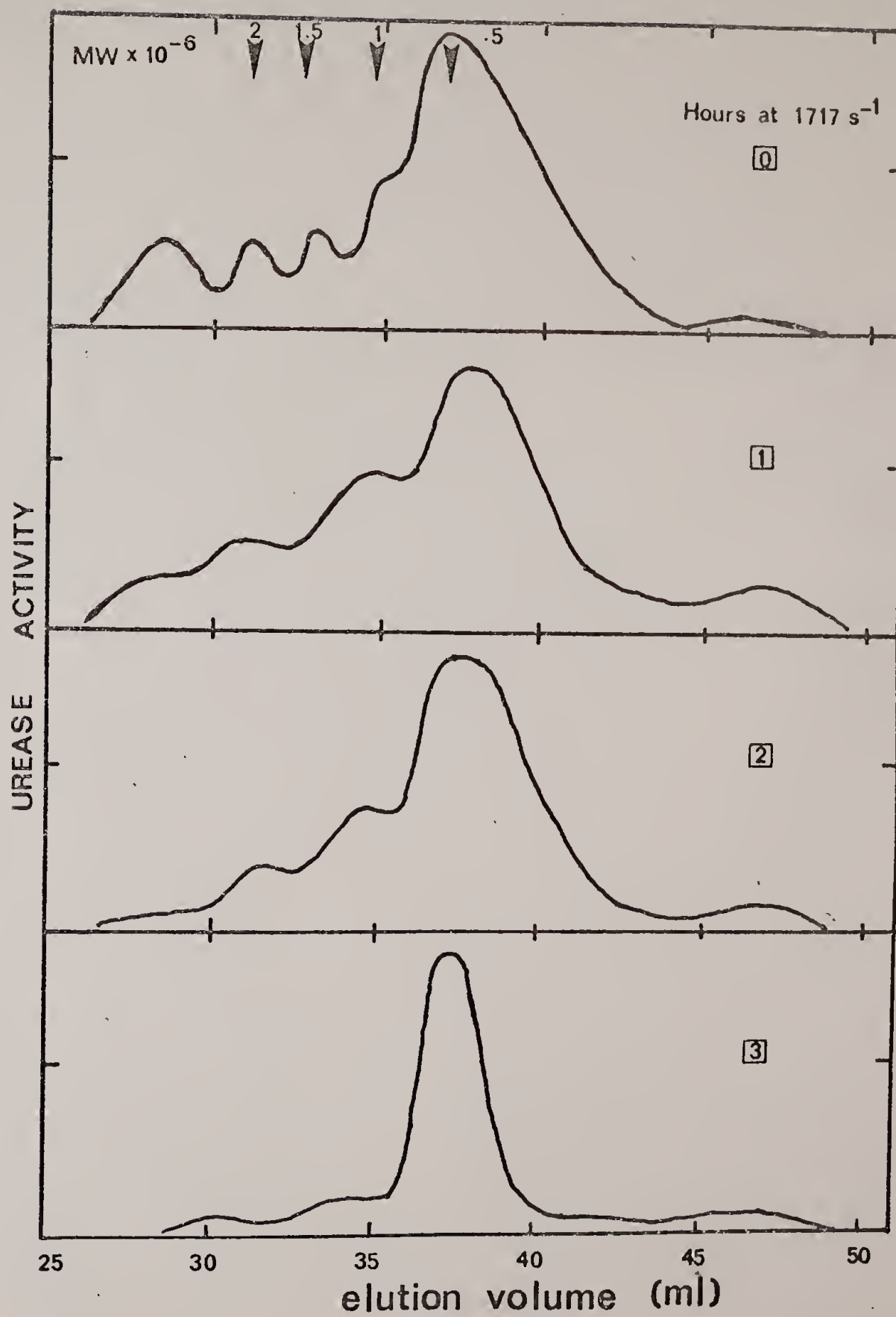
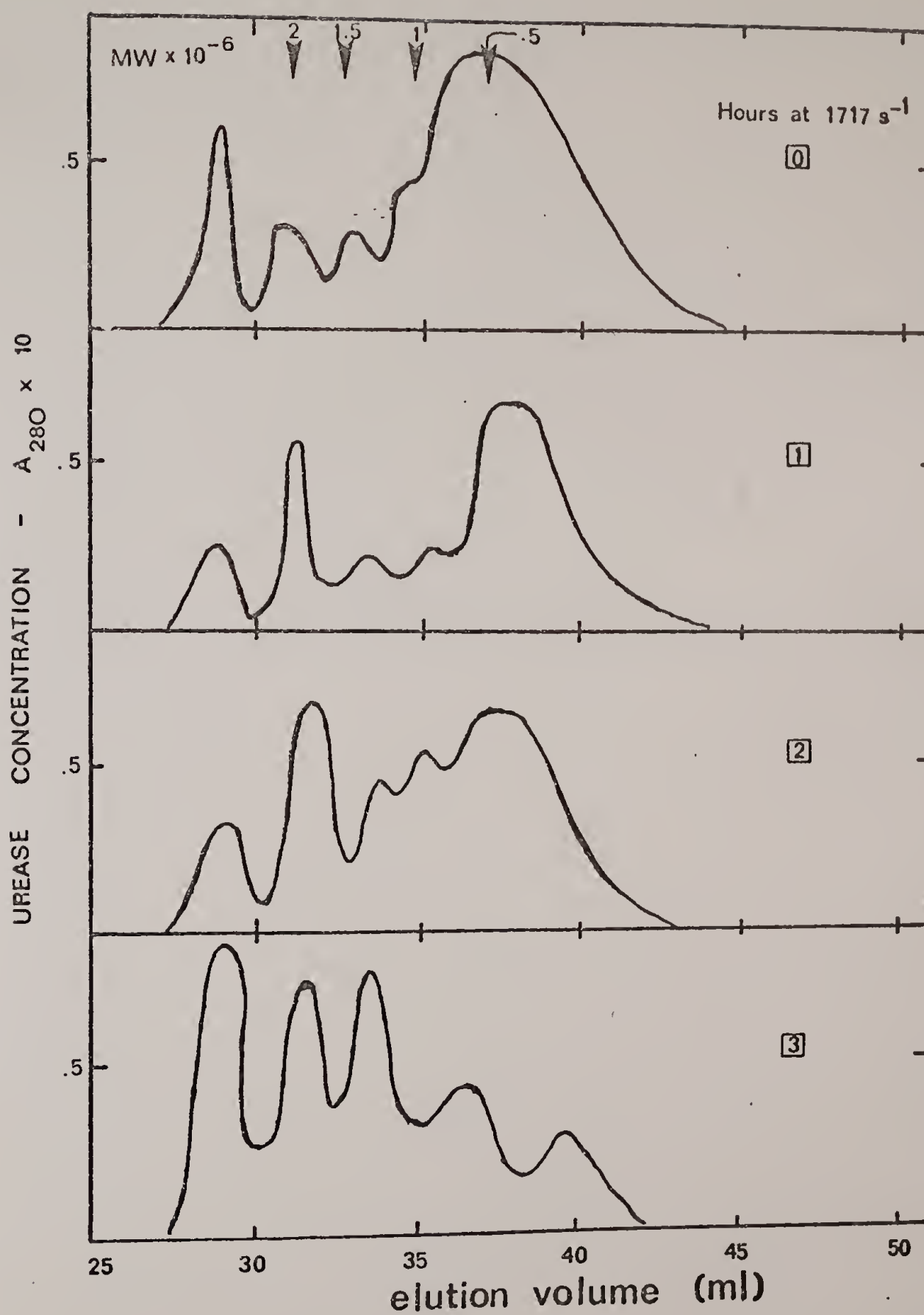


Figure IV-22. Protein concentration vs. elution position as a function of shear exposure time at 1717 s^{-1} .
Urease C.



trated as in the kinetic experiments. This is necessary to have sufficient urease in the fractions to perform a sensitive activity assay. Shear induced aggregation could definitely be altered with such a concentration variation. Still, the notion that the high molecular weight, disulfide-bonded urease isozymes are most susceptible, to whatever shear-induced conformational distortion promotes the metal ion catalyzed thiol oxidation, is reinforced by these chromatographic results.

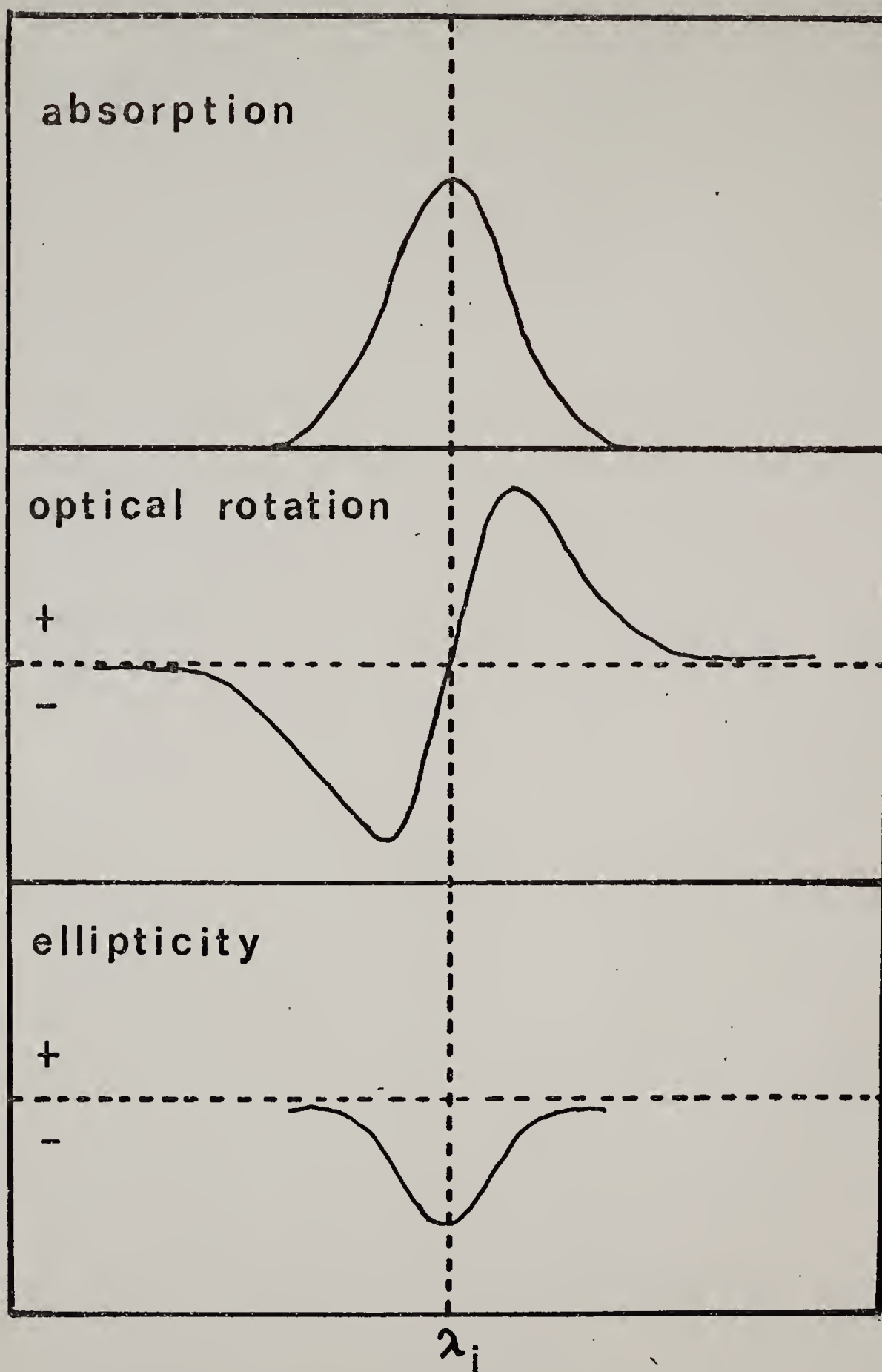
C. Circular Dichroism Results

With size exclusion chromatography giving some insight into the quaternary structure of urease, circular dichroism spectroscopy was employed in an effort to learn something about any changes in secondary structure that may occur upon shearing. A very brief summary of the use of optical rotation in general and circular dichroism in particular for the study of protein conformation is called for (see Kauzmann, 1957, and Haschemeyer and Haschemeyer, 1973). Optical activity refers to the ability of asymmetric carbon atoms to rotate plane polarized light. In all amino acid residues (except glycine) in proteins, the α -carbon atom is asymmetric. The variation of rotation with wavelength of incident light is known as optical rotatory dispersion (ORD). The rotation observed at any wavelength is due to contributions from various electronic transitions occurring in the

molecule. These transitions have inherent rotational strengths and the observed rotation at wavelength λ is a function of these rotational strengths as well as the difference $\lambda^2 - \lambda_i^2$, where λ_i is the wavelength corresponding to the absorption maximum for the associated electronic transition. The inherent rotational strength of electron transitions in proteins in the wavelength range 270-180 nm is a strong function of environment, i.e., of protein conformation, often even when the ultraviolet absorption spectrum is not. At the transition, or absorptive maximum, wavelength the optical rotation changes sign, producing what is known as a Cotton effect. Circular dichroism (CD) is the absorptive counterpart of ORD. CD refers to the unequal absorptivity, near an electronic transition with finite rotational strength, for right and left circularly polarized light. This difference is referred to as ellipticity. Figure IV-23 illustrates the absorption, Cotton effect and the ellipticity associated with a hypothetical electronic transition. In optical rotatory studies of protein conformations, CD is somewhat preferable to ORD because of the relative ease in isolating a contribution for a specific chromophore transition, i.e., the ellipticity band is less spread out than the Cotton effect.

Means of quantifying CD results are still in the research stage. A measured CD spectrum of a native protein is usually thought of as a weighted average of contributions

Figure IV-23. Idealized absorption band, Cotton effect and ellipticity band of a hypothetical electronic transition.



from a purely helical spectrum, a purely β -structure spectrum and purely random spectrum. The weighting factors are the fractions of residues in the native structure which adopt the particular conformation. Characteristic spectra for these three conformations are illustrated in Figure IV-24. Based on these, several empirical methods of estimating, for example, fractional helix content, have been proposed (Greenfield and Fasman, 1969; Chen et al., 1972; Chen et al., 1974; Haschemeyer and Haschemeyer, 1973; Baker and Isenberg, 1976). Several of these will be discussed somewhat further in the course of presenting the results of the present work.

Figure IV-25 shows the CD spectrum of native urease. Tabular values of the molar ellipticities are given in Table 3. Two methods were used to estimate the helical content of urease from this spectrum. Chen et al. (1972) proposed the following relation:

$$f_H = \frac{[\theta]_{222} + 2340}{-30,300} \quad (1)$$

From this, the fractional helical content may be estimated at 9.5%. Greenfield and Fasman (1969) proposed (correcting an obvious misprint in their paper):

$$f_H = \frac{[\theta]_{208} + 4000}{-29,000} \quad (2)$$

This leads to $f_H = 5.8\%$. No previously published CD spectrum of urease exists. Contaxis and Reithel (1971) published

Figure IV-24. Circular dichroism spectra of poly-L-lysine in (1) 100% α -helix form; (2) 100% β -form; and (3) 100% random form (after Greenfield and Fasman, 1969).

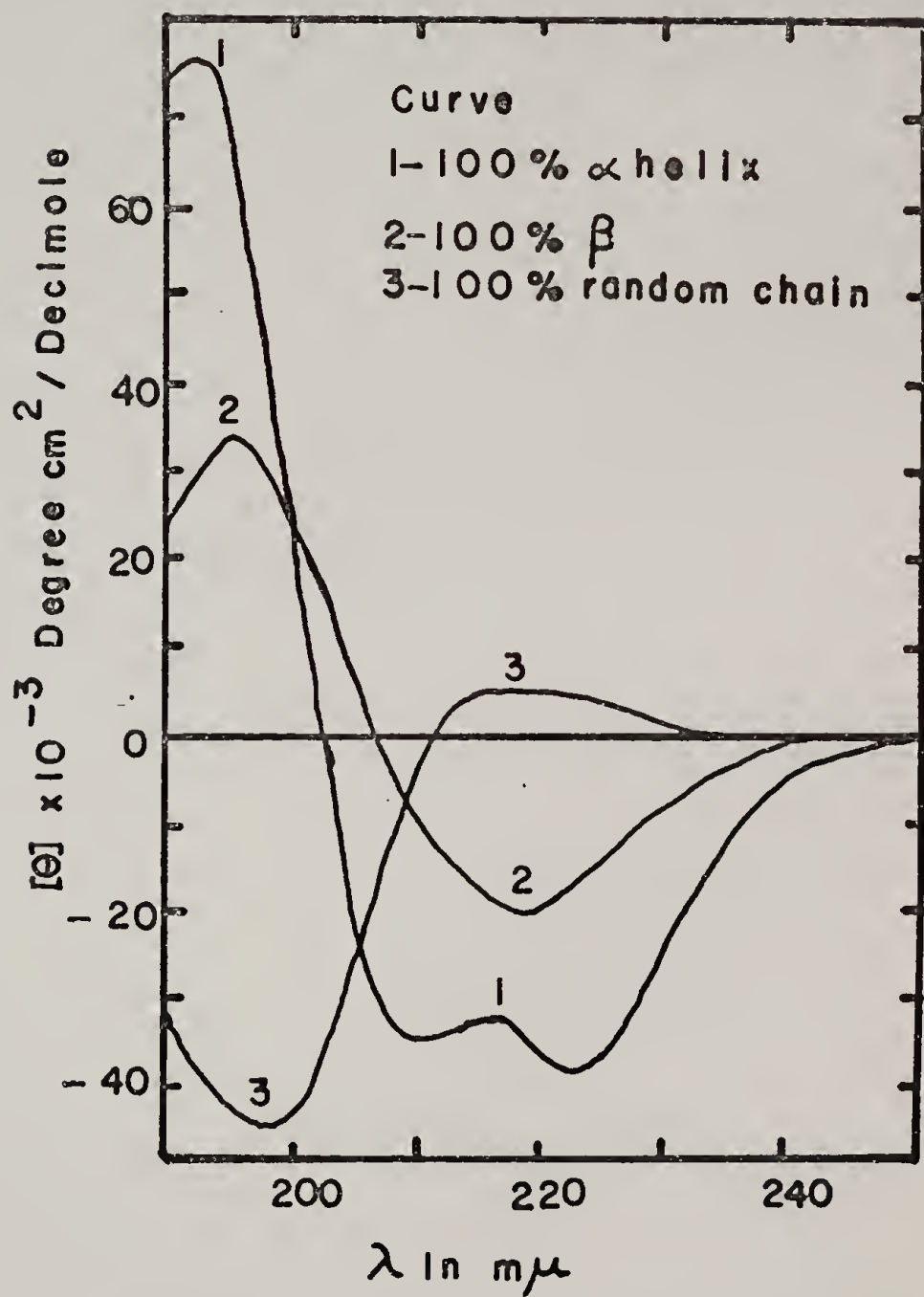


Figure IV-25. Circular dichroism spectrum of native urease.

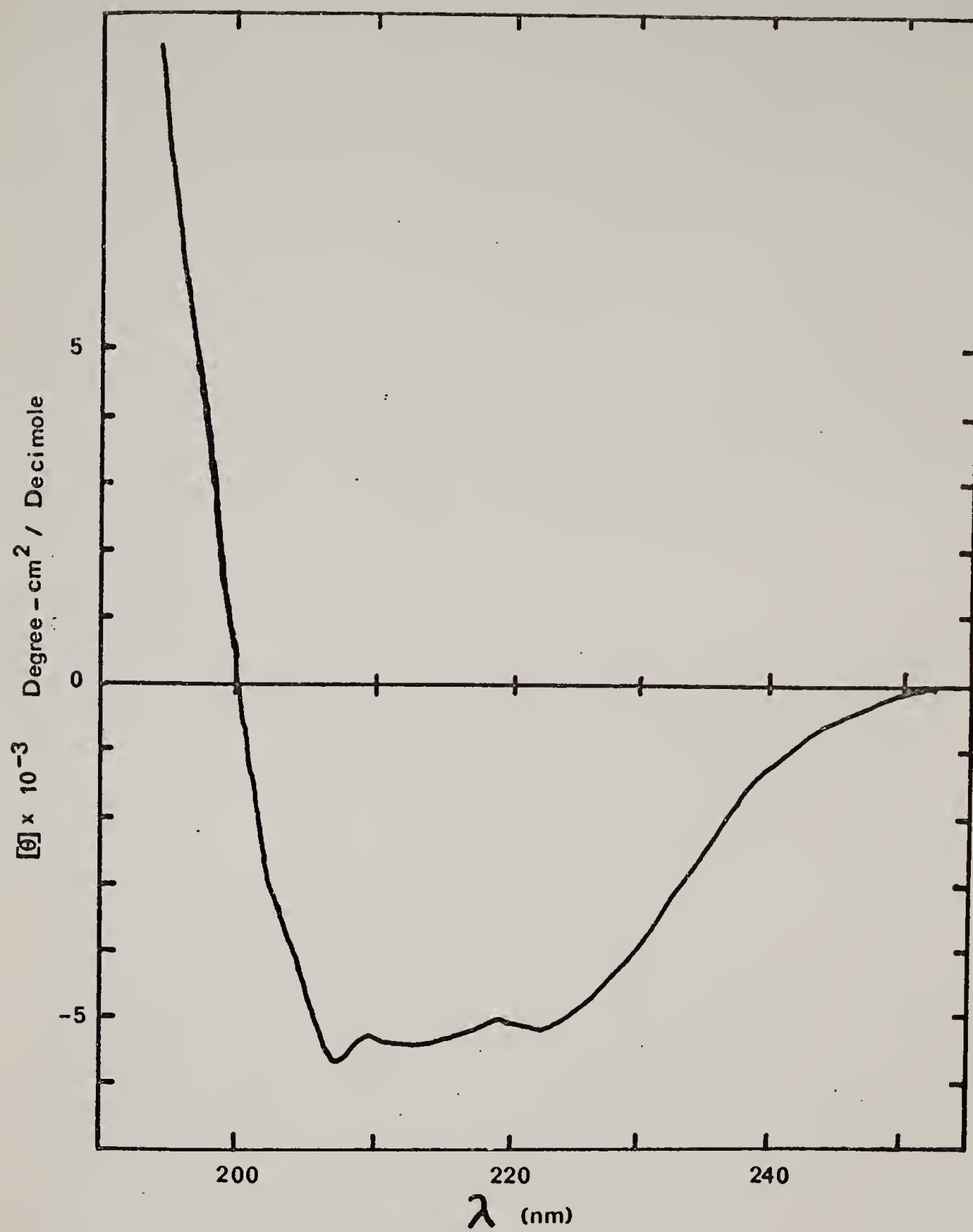


TABLE IV-3

TABULATION OF MEAN RESIDUE ELLIPTICITIES
 DERIVED FROM CD SPECTRUM (FIGURE IV-25)
 OF NATIVE UREASE

λ (nm)	$[\theta]$ ($\frac{\text{deg-cm}^2}{\text{dmole}}$)
260.0	0.000
257.5	0.000
255.0	- 27.52
252.5	- 55.04
250.0	- 110.1
247.5	- 316.5
245.0	- 509.1
242.5	- 839.3
240.0	-1225.
237.5	-1706.
235.0	-2504.
232.5	-3316.
230.0	-3963.
227.5	-4541.
225.0	-4981.
222.5	-5215.
220.0	-5022.
217.5	-5215.
215.0	-5311.
212.5	-5394.
210.0	-5269.
207.5	-5669.
205.0	-4031.
202.5	-2972.
200.0	+ 178.0
197.5	+4197.
195.0	+9439.

an ORD curve of urease showing the characteristic α -helical negative Cotton effect. This curve is illustrated in Figure IV-26. They made no estimate of helical content. Two methods of estimating the helical content of urease from their data were therefore employed in an effort to obtain a quantitative comparison with the present work. A Moffitt-Yang (1956) plot was constructed by interpolation from Figure IV-26, utilizing $[m']_{\lambda} = 0.9[\alpha]_{\lambda}$ and $\lambda_0 = 216$ nm, good approximations for many proteins in this wavelength range (Haschemeyer and Haschemeyer, 1973). The derived parameters are $a_0 = -41^\circ$ and $b_0 = -130^\circ$. Helical content is estimated from:

$$f_H = \frac{b_0}{-630} \quad (3)$$

at 20.7%. Chen et al. (1972) proposed the following relationship to estimate helical content from ORD data:

$$f_H = \frac{[m']_{233} + 2520}{-12,700} \quad (4)$$

This gives an estimate of 1% helical content in urease. The data for the Moffitt-Yang plot are shown in Table 4 and the estimates of helical contents based on Equations (1)-(4) summarized in Table 5.

For the reasons mentioned previously, the CD results probably give the more accurate conformation information. In spite of the discrepancies among the various estimates, it is

Figure IV-26. Optical rotatory dispersion curve of native urease (after Contaxis and Reithel, 1971).

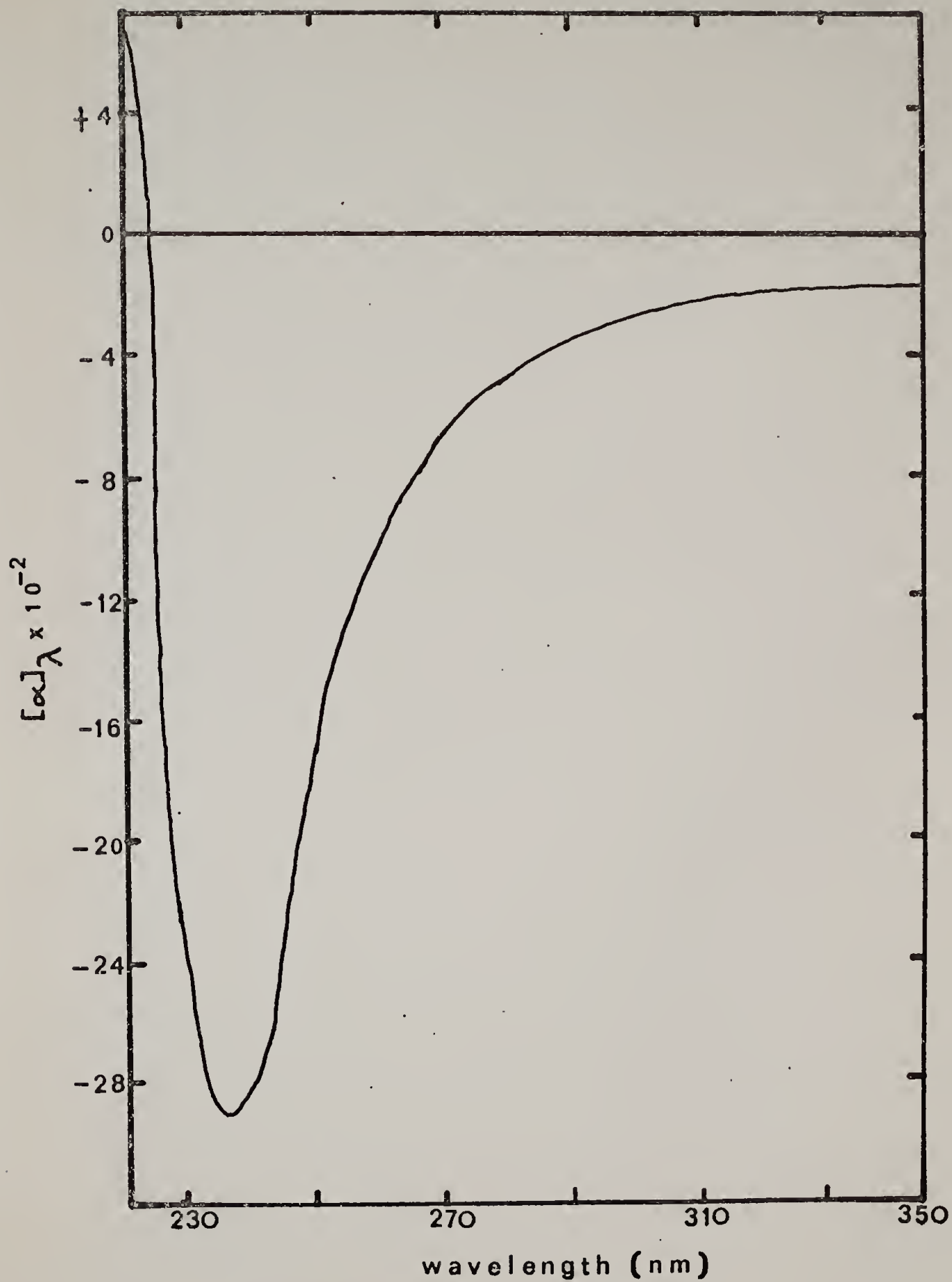


TABLE IV-4

NUMERICAL VALUES FOR MOFFITT-YANG PLOT
CALCULATED FROM FIGURE IV-26

λ (nm)	$[m']_{\lambda} (\lambda^2 - \lambda_0^2)$	$(\lambda^2 - \lambda_0^2)^{-1}$
290	8.99×10^6	2.67×10^{-5}
280	11.28×10^6	3.15×10^{-5}
270	12.75×10^6	3.81×10^{-5}
260	15.08×10^6	4.77×10^{-5}
250	20.25×10^6	6.31×10^{-5}
240	27.58×10^6	9.14×10^{-5}

$$\lambda_0 = 216 \text{ nm}; [m']_{\lambda} = 0.9[\alpha]_{\lambda};$$

$$a_0 = -40.6^\circ; b_0 = -130^\circ.$$

TABLE IV-5
SUMMARY OF ESTIMATES OF UREASE HELICAL CONTENT
FROM THIS AND PREVIOUS WORK

ESTIMATED PERCENT HELIX	SPECTRUM USED FOR ESTIMATE	PROCEDURE* USED FOR ESTIMATE
9.5%	CD spectrum from this work	$[\theta]_{222}$
5.8%	CD spectrum from this work	$[\theta]_{208}$
20.7%	ORD spectrum from Contaxis and Reithel (1971)	Moffitt-Yang b_0
1.0%	ORD spectrum from Contaxis and Reithel (1971)	$[m']_{233}$
[Average = 9.3%]		

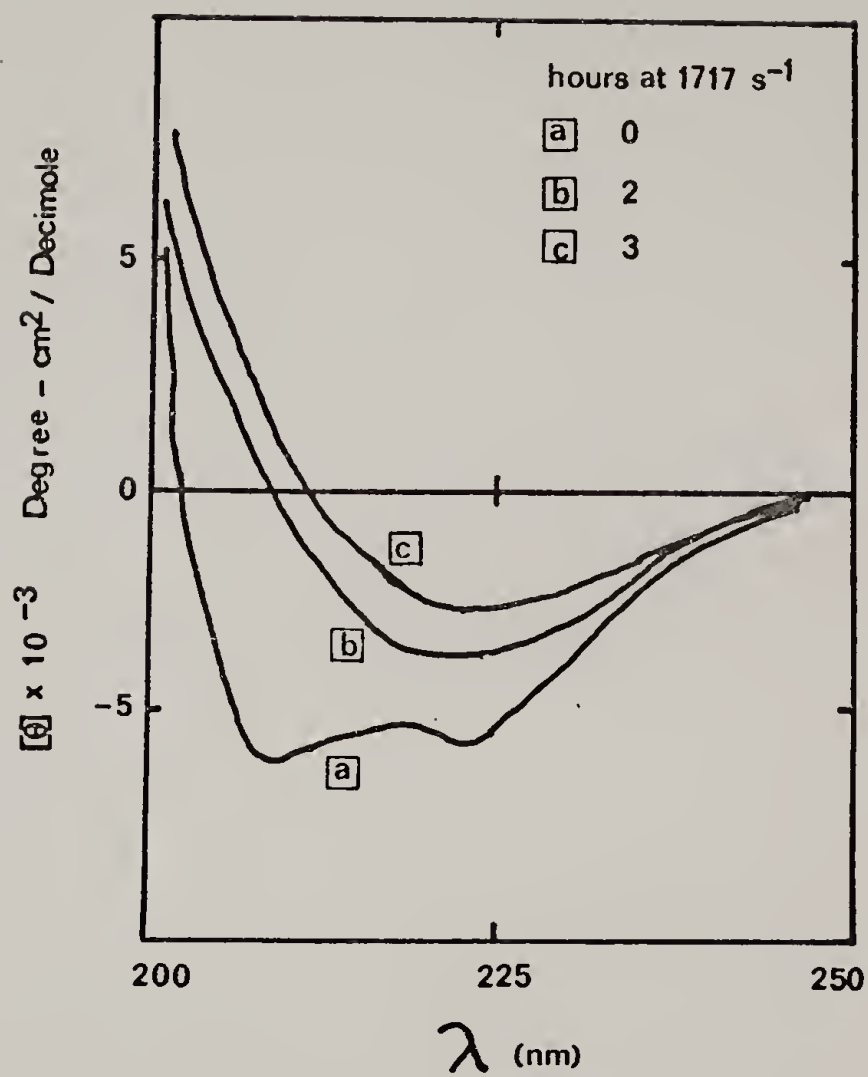
*Equations used for these estimates given in text.

seen that native urease is quite low in helical content, certainly less than 20%. By comparison with the computed curves of Greenfield and Fasman (1969), a crude estimate that the remainder of the urease structure is roughly equally shared between random and β -structure is offered.

The changes in the CD spectrum that occur when urease is exposed to shearing forces are illustrated in Figure IV-27. First of all, no significant change in the CD spectrum is seen after 1 hour at 1717 s^{-1} . Note that measurable inactivation has occurred, however. Whatever changes in structure have occurred, they have not affected the secondary structure sufficiently to be seen in the CD spectrum. This could be the case with a localized thiol oxidation. After longer periods of time changes are noticeable, but difficult to interpret. There seems to be a change toward a more β -like structure, perhaps related to the aggregation phenomenon observed chromatographically. The concentrations here are the same as those in the chromatography experiments.

In summary, these spectroscopic experiments are seen to provide some interesting information, but suffer from two inadequacies for the present purposes. First, they are difficult to interpret in terms of the structural changes which are occurring, partly due to the fact that the urease structure is so poorly understood. Secondly, and this applies to the chromatographic experiments as well, they can only be applied to study irreversible shear-induced conformational

Figure IV-27. Circular dichroism spectrum of urease as a function of shear exposure time at 1717 s^{-1} .



changes, that is, after removing the shearing force. Thus, the only insight we have into the conformational changes actually happening during shear comes from the kinetic experiments, which fortunately are very sensitive (Teipel and Koshland, 1971a, b), albeit rather nondescriptive indicators. This situation may change, however. Recently, Paladini and Weber (1977) have constructed a device capable of measuring protein fluorescence emission while the protein is subjected to shear in a Couette device. This may open the door to a direct observation of protein conformational change due to hydrodynamic forces.

D. Kinetic Results in Glycerin-Buffer Mixtures

The results presented in Section A are limited to shear stresses below 2.5 Pascals. In those results, it was shown that very little inactivation of urease results directly from the shearing force. The significant effects there were caused by a chemical, although shear promoted, phenomenon. In order to explore the effects of higher shear stresses, within the same range of accessible shear rates, it was necessary to increase the viscosity of the buffer medium. This is readily accomplished by the addition of glycerin. Since in this series of experiments, the emphasis is strictly on hydrodynamic effects, any possibility of a metal ion effect was eliminated by inclusion of 5 mM EDTA in

the reaction medium. The urease catalyzed hydrolysis of urea proceeds similarly in glycerin/buffer mixtures, up to at least 60% glycerin.

The effects of shear stresses up to 21.0 Pascals on the kinetics of urease catalyzed urea hydrolysis are shown in Figures IV-28 to IV-32. The rheological conditions and parameters of the buffer medium are given in each case. The inactivation seen in each case is of the same character as that seen in EDTA-containing, low viscosity buffers. Shearing produces a modified, lower first order rate constant; removal of shearing reverses the effect, completely, within the precision of the data. The first order rate constants derived from these data are tabulated in Table 6. $k_{\text{no shear}}$ refers to data obtained from a urease sample with no shear history; k_{τ} refers to data obtained during shearing at shear stress equal to τ (Pascals); $k_{\text{zero shear}}$ refers to the mean first order rate constant from data obtained after stopping the shearing at various point. Errors given represent 95% confidence limits derived from linear regression analyses performed on an SR-52 pocket calculator. As can be seen in Figure IV-33, the ratio $k_{\tau}/k_{\text{no shear}}$ decreases in a non-linear fashion with increasing shear stress. Much of this data, showing different $k_{\tau}/k_{\text{no shear}}$, was obtained at the same shear rate, implying that purely hydrodynamic stress and not shear dependent collision frequency is the dominant mode of inactivation in this range of stress. As noted

Figure IV-28. Urea concentration as a function of time in glycerin-buffer mixture. ★ - no shear; □ - zero shear; ● - 1717 s^{-1} , $\eta = 0.0120$ Pascal-s, $\tau = 21.0$ Pascals.

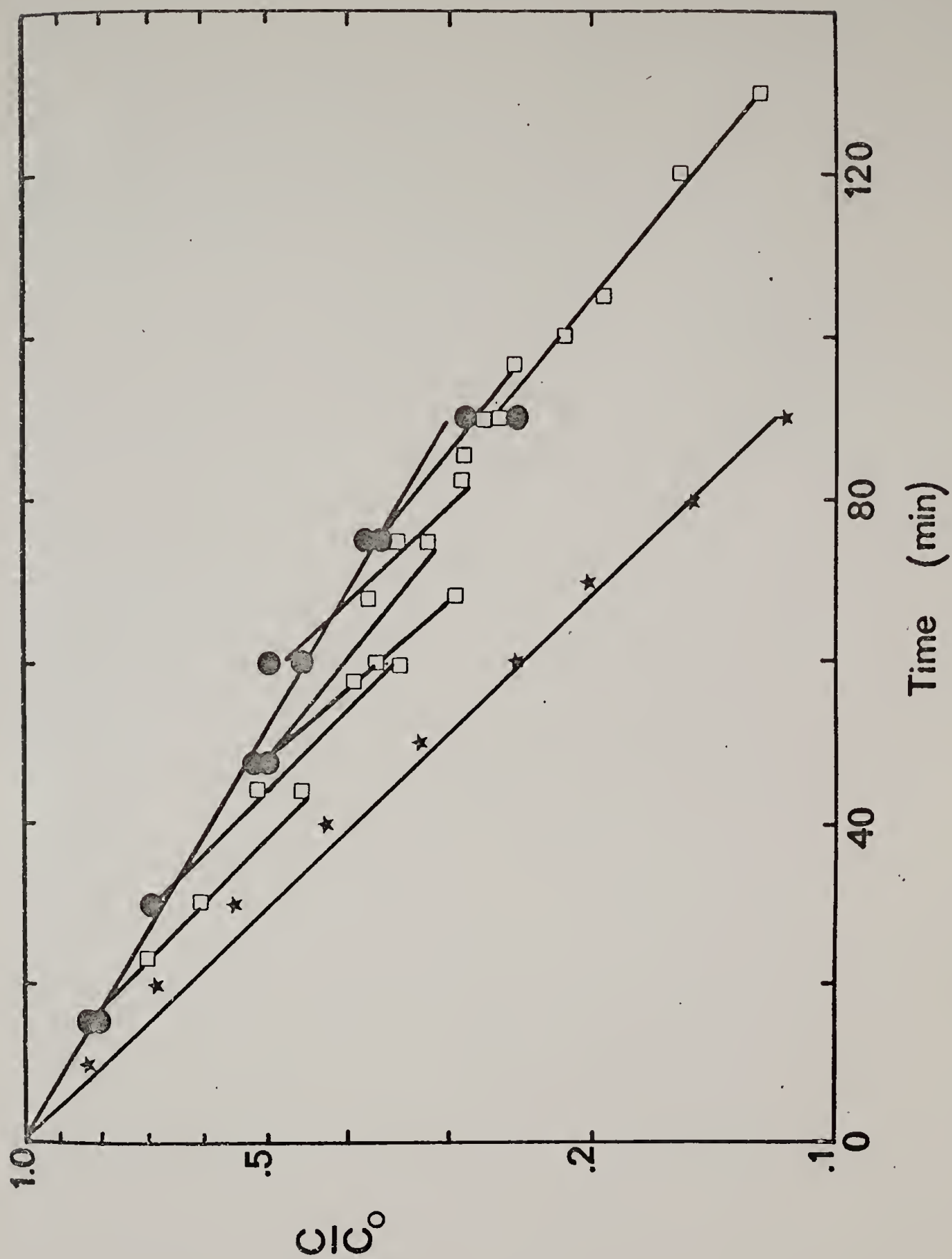


Figure IV-29. Urea concentration as a function of time in glycerin-buffer mixture. ★- no shear; □ - zero shear; ● - 1717 s^{-1} , $\eta = 0.0078$ Pascal-s, $\tau = 13.4$ Pascals.

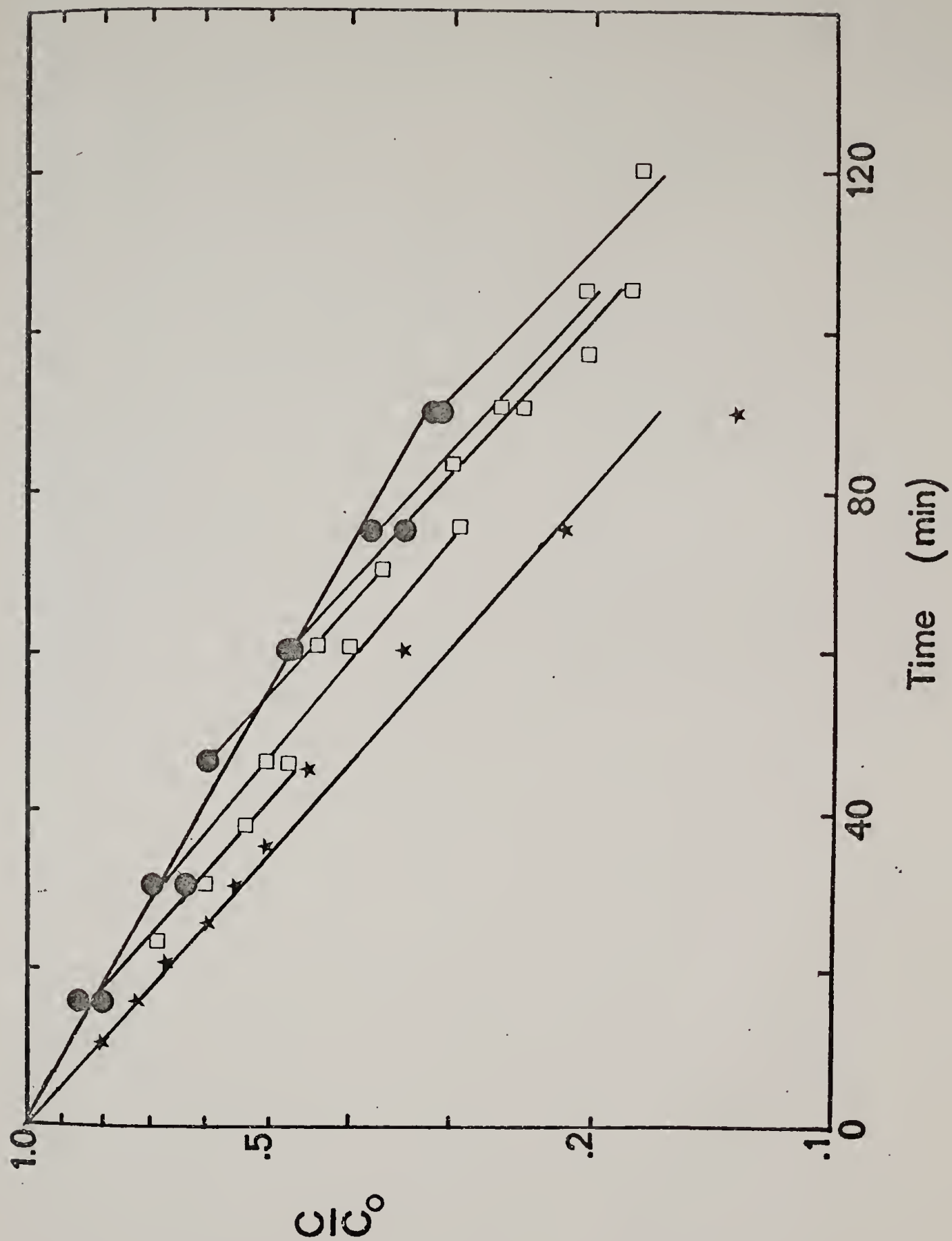


Figure IV-30. Urea concentration as a function of time in glycerin-buffer mixture. ★ - no shear; □ - zero shear; ● - 1301 s^{-1} , $\eta = 0.0078$ Pascal-s, $\tau = 10.4$ Pascals.

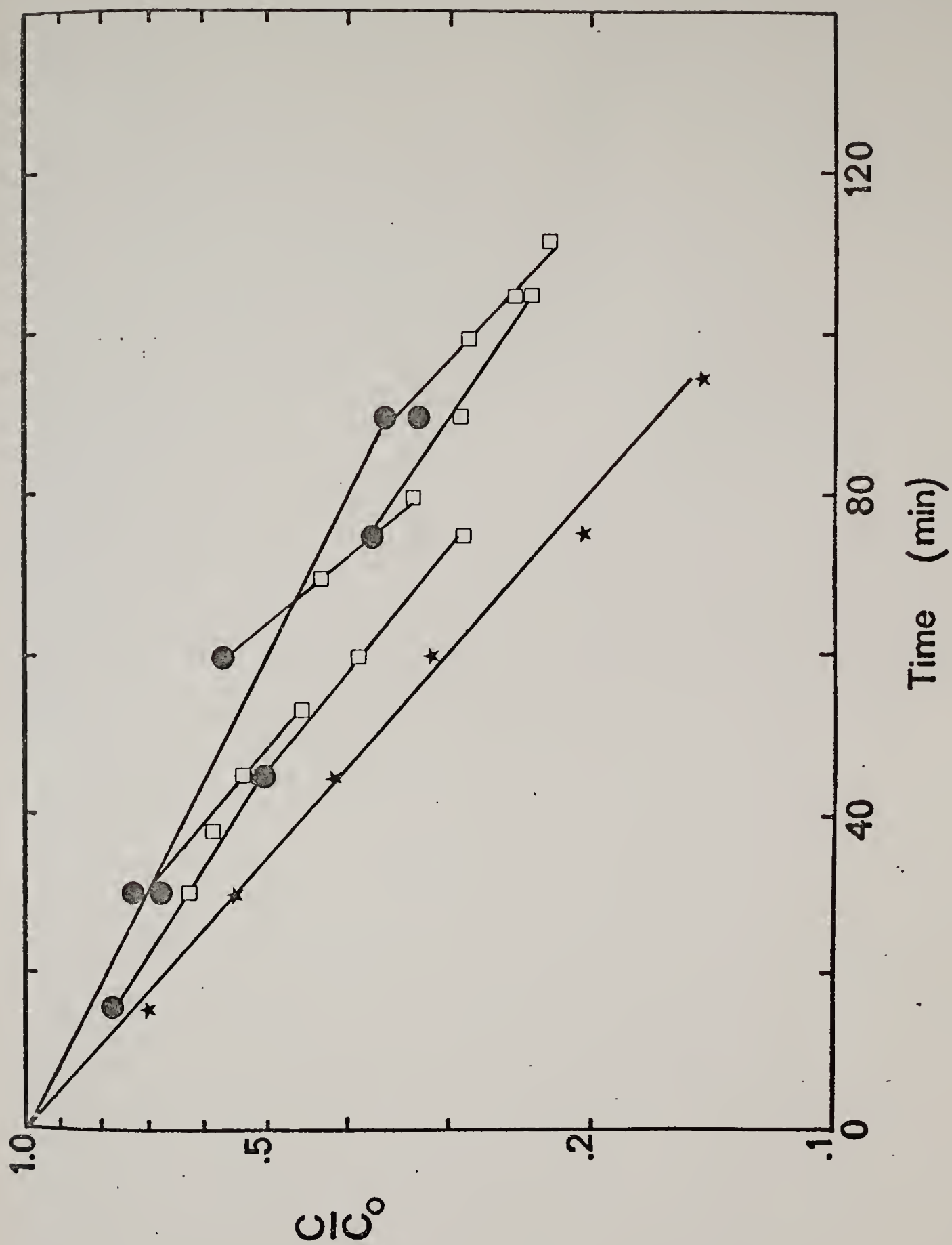


Figure IV-31. Urea concentration as a function of time in glycerin-buffer mixture. ★ - no shear; ● - 741 s^{-1} , $\eta = 0.0120 \text{ Pascal-s}$, $\tau = 8.9 \text{ Pascals}$.

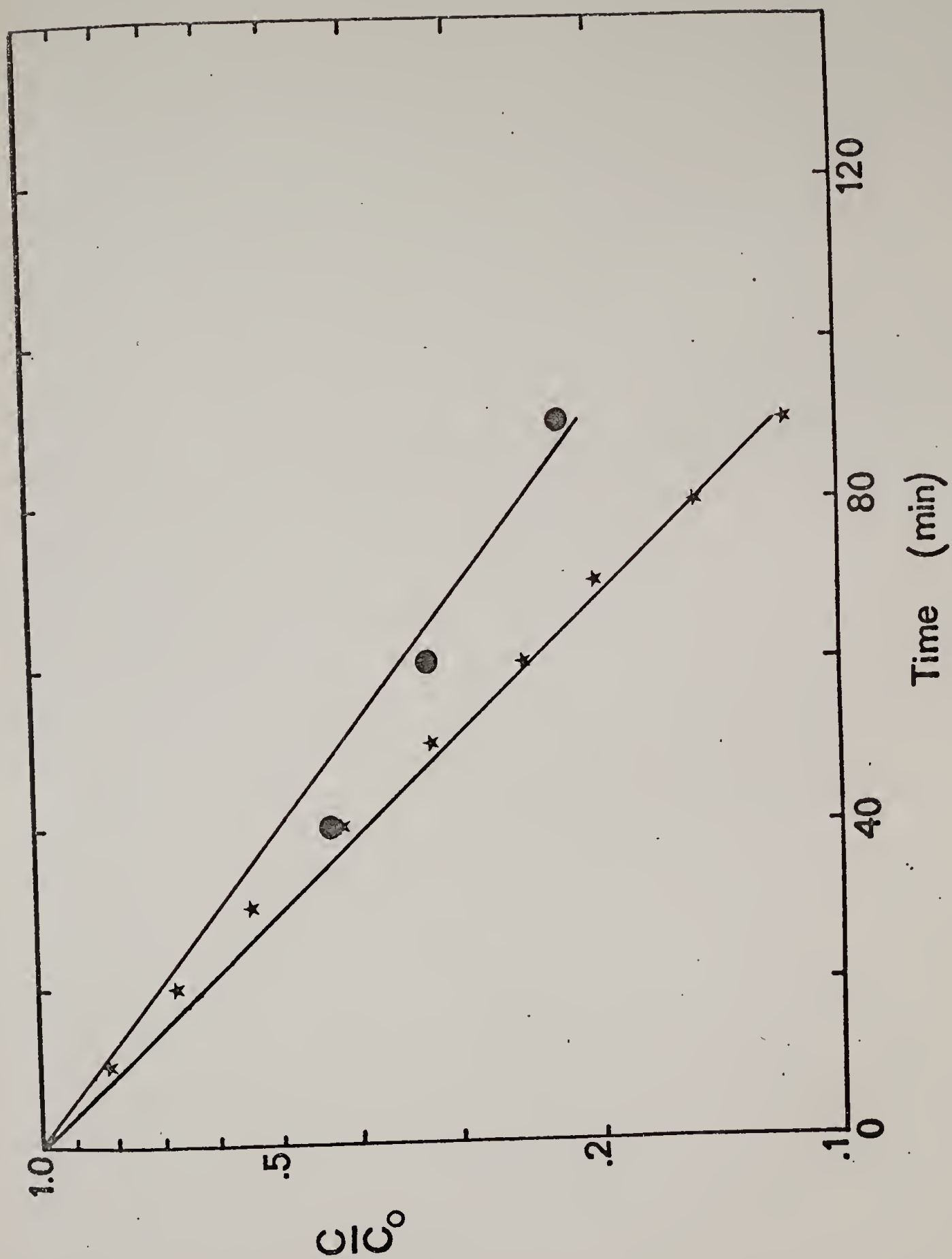


Figure IV-32. Urea concentration as a function of time in glycerin-buffer mixture. ★ - no shear; □ - zero shear; ● - 522 s^{-1} , $\eta = 0.0095$ Pascal-s, $\tau = 5.0$ Pascals.

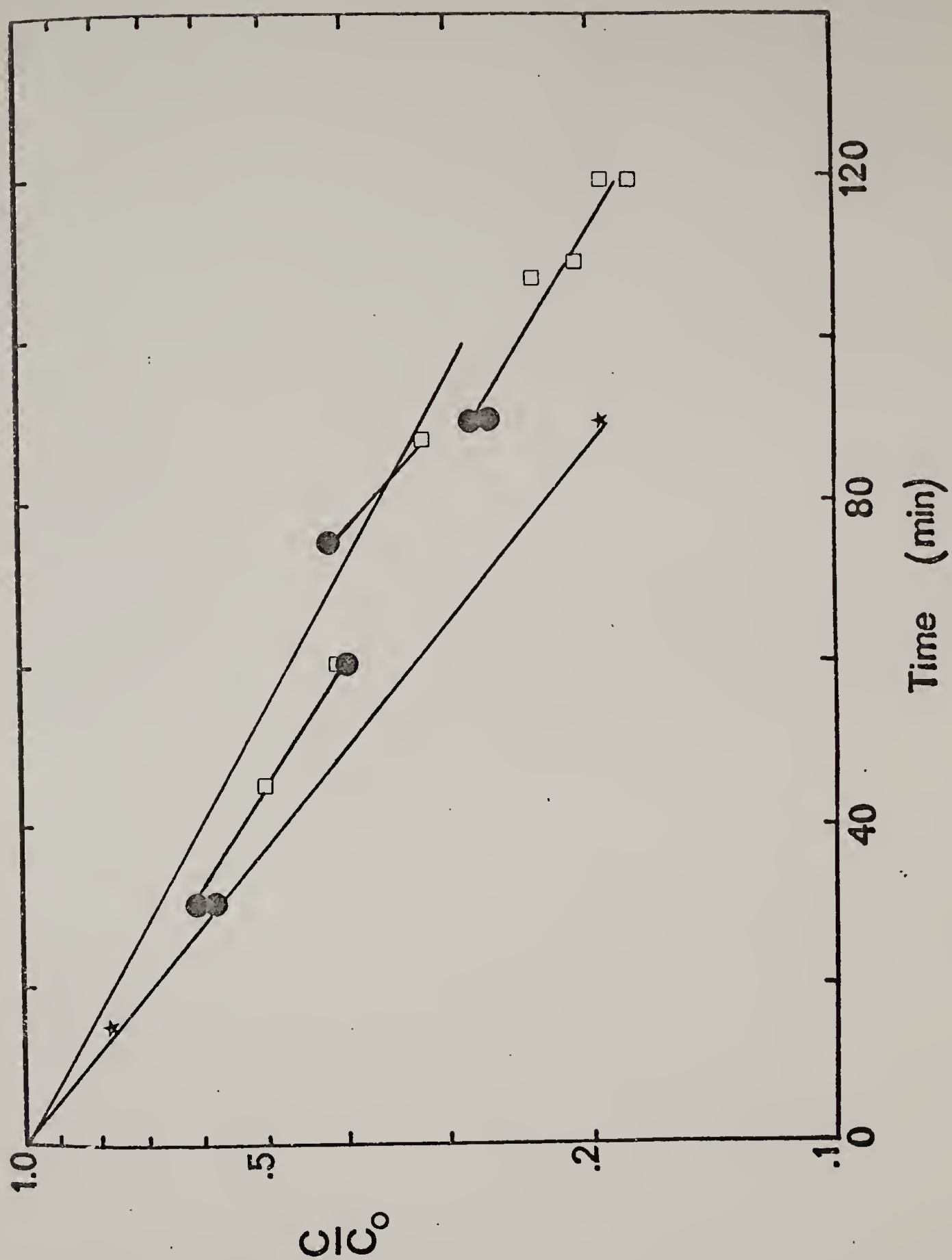


Figure IV-33. Urease activity during shearing as a function of shear stress.

Figure IV-33. Urease activity during shearing as a function of shear stress.

TABLE IV-6
SUMMARY OF GLYCERIN/WATER DATA

τ (Pascals)	k (min^{-1})
5.0	0.018 \pm 0.001 (no shear)
	0.012 \pm 0.003 (τ)
	0.014 \pm 0.005 (zero shear)
8.9	0.024 \pm 0.001 (no shear)
	0.017 \pm 0.003 (τ)
10.4	0.021 \pm 0.001 (no shear)
	0.012 \pm 0.002 (τ)
	0.016 \pm 0.005 (zero shear)
13.4	0.024 \pm 0.004 (no shear)
	0.013 \pm 0.001 (τ)
	0.019 \pm 0.002 (zero shear)
21.0	0.024 \pm 0.001 (no shear)
	0.014 \pm 0.002 (τ)
	0.021 \pm 0.009 (zero shear)

above, all this inactivation is reversible within experimental error, however, the mean $k_{\text{zero shear}}$ is always lower than the mean $k_{\text{no shear}}$ which may mean some amount of irreversible inactivation below the precision of the experiments. In any case, it is clear that a stress regime has been reached where a direct effect of applied hydrodynamic force on the molecule alters the activity rather than enhancing a chemical modifying effect.

An interesting final observation on these results is that if the urease was not dialyzed prior to preparing the stock solutions in glycerin/buffer, no inactivation at all was observed, similar to the results in Figure IV-12 in the presence of β -mercaptoethanol. This leads one to believe that not only are larger stresses, 10 to 20 Pascals, necessary to significantly influence urease conformation, it is also necessary for the intermonomer disulfide bonds to be intact. Glycerin is known to modify the urease quaternary structure, in the manner described previously (Contaxis and Reithel, 1971), by cleaving a noncovalent interface in the urease monomer. It will not affect previously formed disulfide bonds (Fishbein et al., 1976b) such as those present in these experiments. So, while the molecular weight of urease is uncertain under these glycerin-buffer shearing conditions, the necessity of having intermonomer disulfide bonds to observe shear inactivation of urease is still suggested by this and several other previously described experiments in this dissertation.

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C H A P T E R V
MODELLING OF ENZYME REACTIONS AFFECTED
BY DEFORMATION

A. Introduction

In attempting to describe physical or chemical phenomena, one often chooses between a rather superficial phenomenological description, which may return great predictive capability for the effort invested in formulation, or a more "microscopic" mechanistic description, which contains more rigorous, detailed information for a comparatively high level of invested effort. Both approaches are valid, depending on the goals and expected uses of the formulated model. The overall rate equations derived from analysis of reaction networks (Boudart, 1968) are examples of the former, whereas the absolute reaction rate calculations of Eyring and coworkers (see: Glasstone, Laidler and Eyring, 1941) are examples of the latter. As will presently be shown, a phenomenological description of the effect of hydrodynamic stresses on the kinetics of urease catalyzed reactions can be evolved relatively simply. Incorporating more detailed information to arrive at a theory relating: enzyme activity \longleftrightarrow enzyme structure \longleftrightarrow hydrodynamic stress is a much more formidable task.

B. Phenomenological Kinetic Description of Urease Catalyzed Reactions Subject to Deformation

1. Zero Deformation Rate Kinetics

The first point to be addressed before proceeding with the modelling discussion, is whether the kinetic behavior at zero shear, reported in previous chapters, is consistent with the results of other work on the kinetics of urease catalyzed urea hydrolysis. Unlike most previous studies of urease, or other enzymes for that matter, the reactions studied here were carried to high conversion. The more usual technique in enzyme kinetic studies is to obtain and analyze initial rate data, that is, data obtained at very low fractional conversion. This is done so that a one-to-one correspondence between a reaction rate and a substrate concentration (i.e., the initial substrate concentration) exists, to avoid having to differentiate rate vs. $[S]$ data, and also to avoid complications such as inhibition of the enzyme by products of the reaction. In the present work, primary interest is in modification of the kinetics of urease catalyzed urea hydrolysis during a deformation experience. As shown in previous chapters, under some conditions, the deactivation process occurs over a period of up to 3 hours. Therefore, it was necessary to study the reaction kinetics over this time period as well. This, in turn, necessitates carrying the reaction to high conversion in order to see significant varia-

tions in reaction rate with shear exposure.

A discussion of the kinetics of urea hydrolysis by urease at high conversion must include the phenomenon of inhibition by product ammonia. The starting point will be the fundamental Michaelis-Menten expression, appropriate for urease in the absence of product and substrate inhibition:

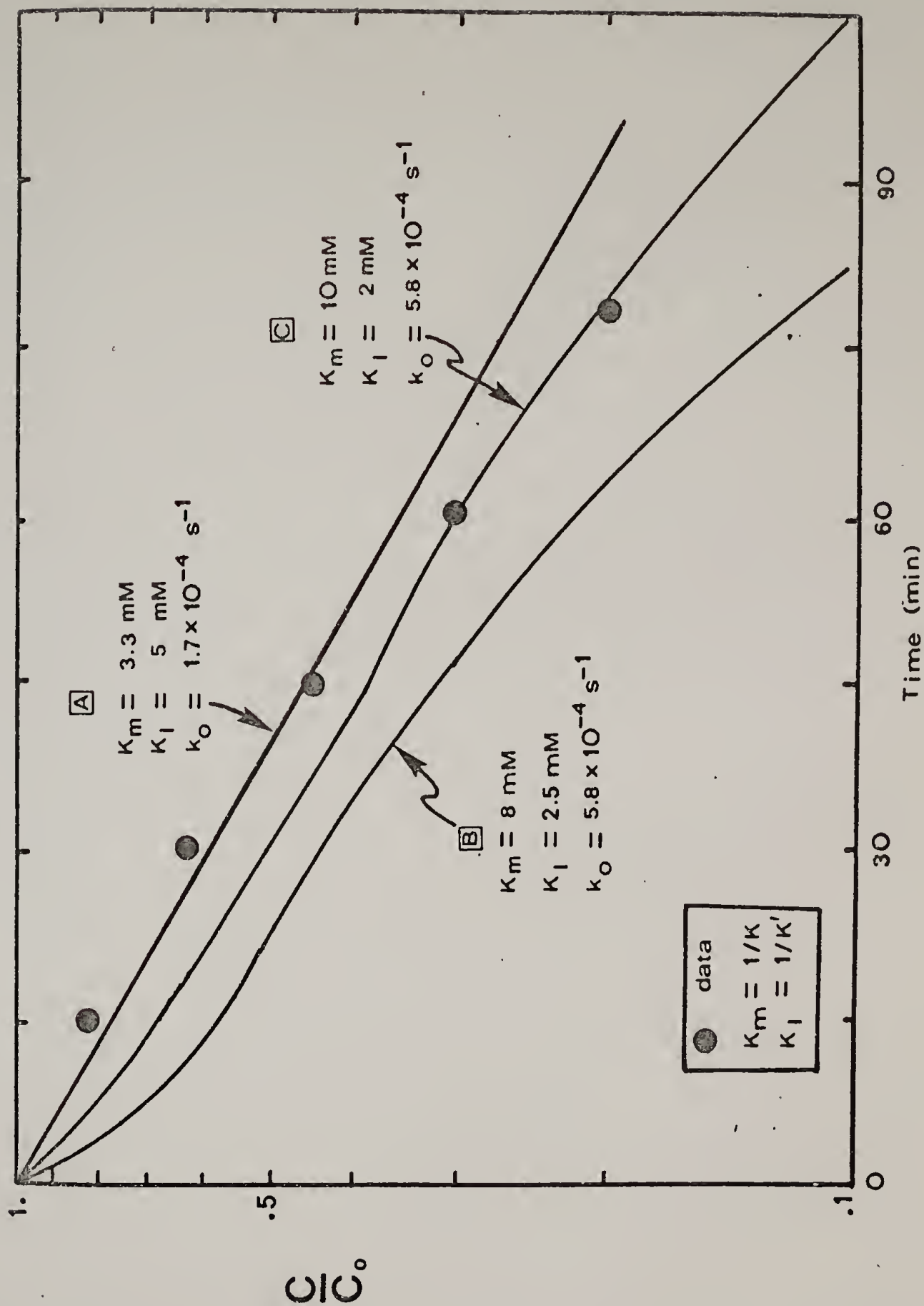
$$\left[\frac{dc}{dt} \right]_{\text{uninhibited}} = \frac{-k_0 E_0 K c}{1 + K c} \quad (1)$$

where c is urea concentration, k_0 is the turnover number, E is the urease concentration, K is the reciprocal of the Michaelis constant. Since, all experimental conditions of the present work are well below the onset of substrate inhibition ($[S] \gtrsim 250$ mM), only product inhibition will be considered. Hoare and Laidler (1950) have shown inhibition of urease by ammonium ion to be noncompetitive. This means, conceptually at least, that NH_4^+ alters the rate of the catalytic function of urease without specifically competing with urea for binding to urease. Mathematically, this modification is made in the following way:

$$\frac{dc}{dt} = \frac{-k_0 E_0 K c}{(1+K c)(1+K' I)} \quad (2)$$

where I is the concentration of noncompetitive inhibitor (see for example Bernhard, 1968), in this case ammonium, and K' is the inhibition constant. The stoichiometry of urea hydrolysis dictates that two moles of ammonium are produced

Figure V-1. Comparison of predictions from product inhibition model with zero shear data from Figure IV-6.



for each mole of urea cleaved. This allows the rewriting of Equation (2) entirely in terms of c :

$$\frac{dc}{dt} = \frac{-k_0 E_0 K c}{(1+K c)[1+2K'(c_0-c)]} \quad (3)$$

where c_0 is the initial urea concentration. Rewriting this in terms of the dimensionless concentration, $r = c/c_0$, gives:

$$\frac{dr}{dt} = \frac{-k_0 E_0 K r}{1+x_0 K r+2K'x_0+2K'Kx_0^2 r-2K'x_0 r-2K'Kx_0^2 r^2} \quad (4)$$

Solving, subject to $r = 1$ at $t = 0$ gives:

$$t = -\left(\frac{1+2K'x_0}{k_0 E_0 K}\right) \ln r - \left(\frac{Kx_0+2K'Kx_0^2-2K'x_0}{k_0 E_0 K}\right) (r-1) + \left(\frac{K'x_0^2}{k_0 E_0}\right) (r^2-1) \quad (5)$$

So, the time to reach a given r may be calculated from Equation (5) if values for the three parameters k_0 , K and K' are specified. Predicted curves for three different groups of the above parameters are compared to the data in Figure V-1. All the values of each parameter are quite consistent with those previously reported. Turnover numbers of order of magnitude 10^4 s^{-1} are reported for urease (Bernhard, 1968) or can be estimated from published data (Hoare and Laidler, 1950). Typical reported K_m values range from 4 to 10 mM (Hoare and Laidler, 1950; Fishbein et al., 1965). A single

reported ammonium inhibition constant of 2.1 mM (Hoare and Laidler, 1950) is close to the values used in Figure V-1.

Several features of the theoretical curves in Figure V-1 are very interesting. Curve A illustrates the highly linear, apparent first order behavior possible with some choices of parameters; it is even more linear than the data. Curve B is an example of the, perhaps intuitively unexpected, downward curvature on a semilog plot at higher conversions, possible with product inhibition. Curve C is the closest to the data obtained in this rather hit-or-miss fitting procedure. It fits the data at higher conversions better than at low concentration, perhaps partly because the analytical technique used for urea analysis (see Levine et al., 1961, and Figure III-4) is more sensitive in this concentration range. This is not to say that a better fit could not be obtained with a more rigorous fitting procedure; but five data points do not warrant exact fitting of a three-parameter model.

The main points of this section are these: (1) it is clear that the behavior represented by any of the curves could be mistaken for strictly first order behavior with sparse experimental data; (2) Equation (5) shows that the time to achieve a given conversion is inversely proportional to the urease activity in solution, $k_0 E_0$, so that it is justifiable to take changes in the slopes of the data under these conditions as directly proportional to changes in ac-

tivity.

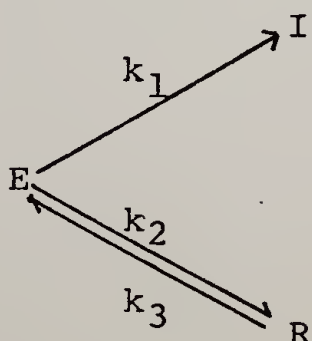
Finally, in this connection, it is noted that studies of the full time course of enzyme catalyzed reactions are beginning to be (Bates and Friedan, 1973a, b), and could be more frequently, used to obtain mechanistic information not available from initial rate studies. Analyses of the present type are expected to be relevant in these cases.

2. Models for Kinetics During Shear

The next question is, "How does one formulate a model for kinetic reactions modified by shearing?" The first step in this direction is to postulate a reaction scheme for the, in this case, inactivation process. Ignoring the details of the product inhibition mechanism, it is assumed that the zero shear reaction is described by a first order model:

$$\frac{dc}{dt} = -k^0 E(t)c \quad (6)$$

where k^0 is the apparent zero shear first order rate constant and the native enzyme concentration, E , is a function of time. Since both irreversible and reversible inactivation occur during shearing, a plausible inactivation model is:



(7)

where I is irreversibly deactivated enzyme and R is reversibly deactivated enzyme. This leads to the following pair of equations:

$$\frac{dE}{dt} = -(k_1 + k_2)E + k_3R \quad (8)$$

$$\frac{dR}{dt} = k_2E - k_3R$$

The secular equation of this pair leads in a straightforward manner to eigenvalues which are algebraically surprisingly complex. The solution, obtained illustratively by Laplace transforms in Appendix A, is:

$$E(t) = \frac{1+c}{2} e^{-\lambda_1 t} + \frac{1-c}{2} e^{-\lambda_2 t} \quad (9)$$

where

$$\lambda_1 = A - B;$$

$$\lambda_2 = A + B;$$

$$A = 1/2 (k_1 + k_2 + k_3);$$

$$B = [1/2 (k_1 k_2 - k_1 k_3 + k_2 k_3) + 1/4 (k_1^2 + k_2^2 + k_3^2)]^{1/2}$$

$$C = -1/2 (k_1 + k_2 - k_3) / B.$$

In order to account for the shear rate dependence of the inactivation, it is necessary to take the inactivation rate constants k_1 , k_2 and k_3 to be functions of the state of deformation of the reaction medium. In this endeavor there is no formally correct way to proceed. What was done here was to take the rate constants to be given by the Arrhenius expression, with activation energies modified by an empirical

deformation related function:

$$k_i = A_i \exp[\Delta G_i^\ddagger - \tau v]/RT \quad (10)$$

where $i = 1, 2, 3$; A_i is the appropriate frequency factor, ΔG_i^\ddagger the free energy of activation and τv the deformation function. It is written in this form to show that if the shear stress, τ , is included explicitly, it must be multiplied by a parameter, v , with the units of volume, which may be called the activation volume, after Eyring (see Glasstone et al., 1941). For guidance as to appropriate values of activation energies to use, a survey of literature values for activation energies of protein unfolding and refolding was made (see Tanford, 1961, and references on protein denaturation contained therein). Suitable representative values chosen were: $\Delta G_{1,2}^\ddagger = 25$ kcal/mole and $\Delta G_3^\ddagger = 22.5$ kcal/mole; there seemed to be little basis on which to make a distinction between the ΔG^\ddagger values appropriate to the reversible and irreversible reactions. The frequency factors were arbitrarily set at 10^{13} sec^{-1} . Given these values, native enzyme concentration as a function of time may be predicted from Equation (9) with τv as a parameter. τv was taken to be equal in magnitude for all three reactions, but positive for 1 and 2 and negative for 3. Conceptually, this means that the stress is considered to lower the activation barrier for inactivation, while raising the activation barrier for reactivation. If the results of Equation (9) are substituted into Equation (6), urea concentration vs. time behavior may be

simulated. Results of these predictions for several values of the deformation function are shown in Figure V-2. Very close resemblance to the data is seen. In Figure V-3, the predictions of this model for $\tau v = 11.4$ RT are compared directly to the data of Figure IV-1c. The zero shear lines after various shear exposures are obtained by setting $\tau v = 0$ at the appropriate points in time. The predicted behavior seems to be qualitatively and quantitatively in good agreement with the data. The stress levels in these experiments lead to values of the activation volume of 10^5 nm^3 which is an order of magnitude larger than any of the urease species in solution. This same type of model has been applied by Adam and Zimm (1976) to DNA shear breakage kinetics. They do not report any values of parameters but one can estimate that the activation dimensions derived from their experimental conditions and results must also be much larger than any molecular entity in the system.

This model is fairly useful in illustrating the relative magnitudes of the various effects influencing the kinetics of urea hydrolysis and urease deactivation during shearing. A sort of matrix of experiments could be designed, varying temperature on the one hand and shear stress on the other. Temperature variation of the k_i at constant stress would give ΔG^\ddagger plus the contribution from the stress related term; shear stress variation of the k_i would give τv and could then be used to separate temperature and stress ef-

Figure V-2. Simulated urea concentration vs. time behavior for several values of the parameter τ_v .
(Values of other parameters given in text.)

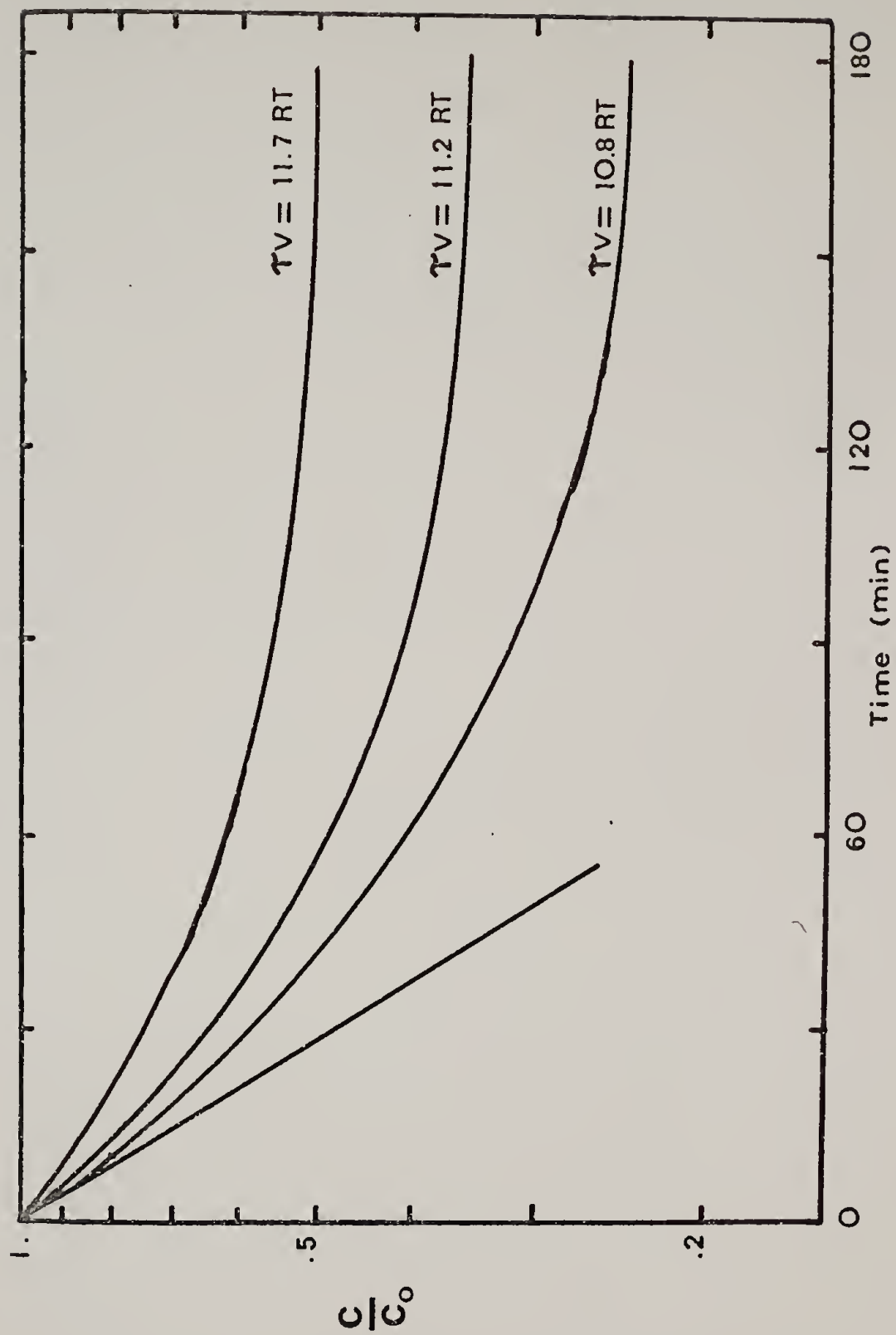
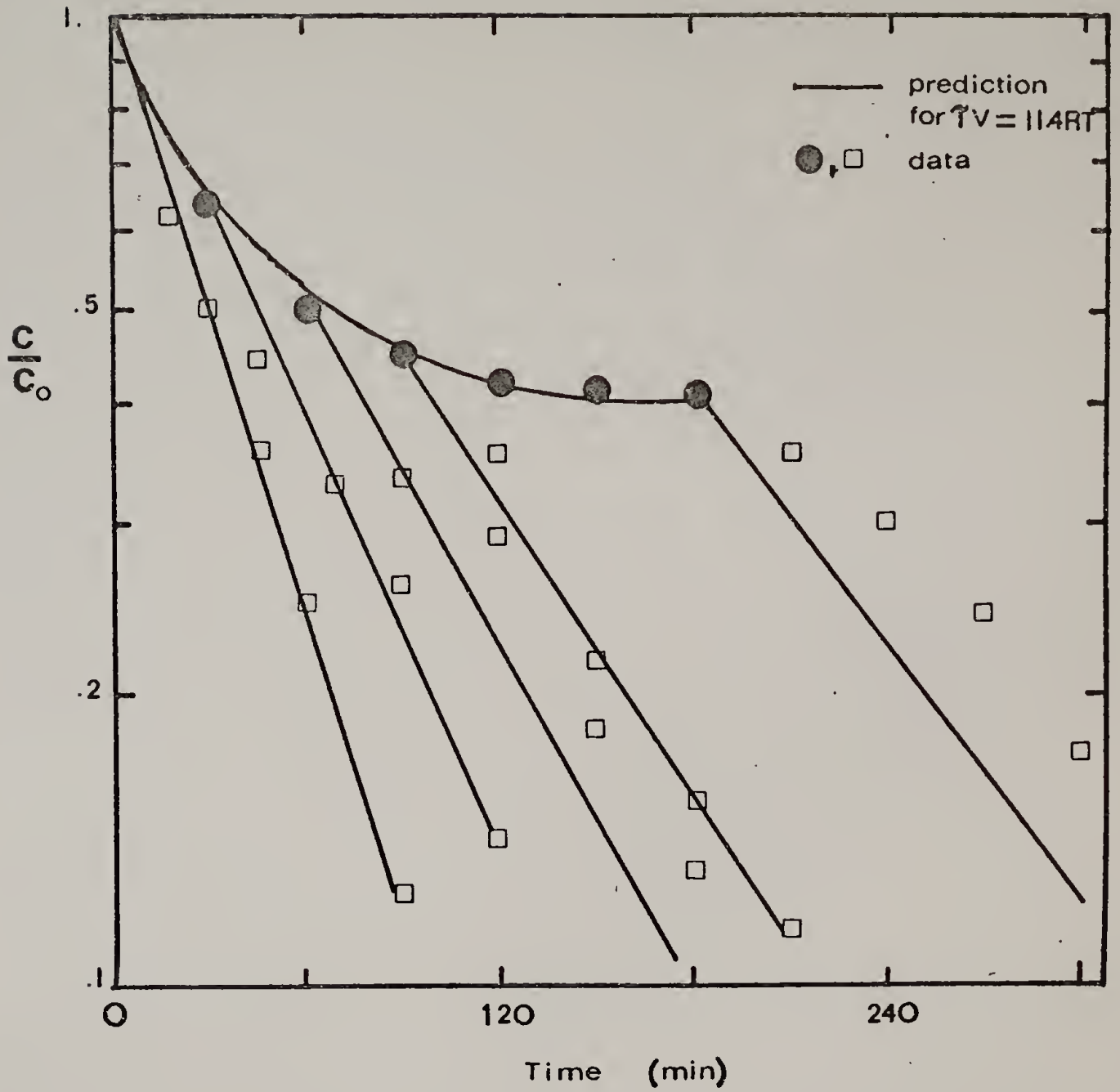


Figure V-3. Comparison of predicted kinetic behavior for $\tau_v = 11.4$ RT with data of Figure IV-2c.



fects. This procedure assumes constant activation energy over the temperature range studied and requires obtaining three kinetic constants from a nonlinear fit to rather sparse experimental data.

Skepticism over whether this could be done with reasonable precision provided the incentive for formulating a somewhat simpler model. Suppose it is assumed that the reversible deactivation reaction is always at equilibrium, so that, $k_2E = k_3R$, or, defining an equilibrium constant, $K = k_2/k_3 = E/R$. The experimental observation that recovery of activity on stopping shear is instantaneous is consistent with this assumption; beyond that, the validity of the assumption is difficult to assess. From a mass balance on total enzyme concentration, E_0 , it is seen that:

$$E_0 = E + I + R, \quad (11)$$

or

$$E_0 = E + I + KE. \quad (12)$$

Since E_0 is constant:

$$\frac{dI}{dt} = -(1+K)\frac{dE}{dt}. \quad (13)$$

But from Equation (7):

$$\frac{dI}{dt} = k_1E. \quad (14)$$

Equating these two expressions gives a first order deactivation model:

$$\frac{dE}{dt} = \frac{-k_1}{1+K}E \quad (\text{parallel deactivation}) \quad (15)$$

with a solution considerably simpler than Equation (9).

Another inactivation model, which may be more consistent with the mechanism of irreversible inactivation proposed in Chapter IV, is the following sequential mechanism:



This model can easily be shown, with the same equilibrium assumption, to lead to a form equivalent to Equation (15):

$$\frac{dE}{dt} = \frac{-k_1 K}{1+K} E \text{ (sequential deactivation)} \quad (17)$$

So, both models may be expected to fit the data equally well (or poorly). The difference is in the physical interpretation of the mechanism and of the derived constants.

These models have two constants, an irreversible inactivation constant and an equilibrium constant. Both lead to a deactivation model of the form $dE/dt = -k_d E$. There is an obvious conceptual scheme for extracting k_d and k_1 from the data. The overall or total inactivation exhibited by the data obtained during shear are governed by a first order rate law with first order catalyst activity decay (Levenspiel, 1962); fitting this exponential decay will yield k_d . The decline in slope of the zero shear data, obtained after stopping the shear at various points, reflects only the irreversible inactivation, that is, k_1 . Both of these constants can be obtained from appropriate linear plots. This procedure for obtaining the constants is given in Appendix B. Values

of the constants obtained in this way are tabulated for all experimental data taken under conditions of partial irreversible inactivation in Table 1. The one conclusion that can be drawn from this modelling effort is that, interpreted in the light of the physically more reasonable sequential model, the $E \xrightleftharpoons{k} R$ equilibrium lies far to the right for all shear rates, that is, most of the enzyme molecules are in a shear modified conformation. The problem with the predictions based on these constants is that they are inaccurate quantitatively. Even with some empirical improving adjustments, the disagreement is as large as 27% in some parts of the concentration vs. time space, as illustrated in Figure V-4. The reason for this is partly the difficulty, discussed in more detail in Appendix B, in the fitting procedure and, even more importantly, in the constraints placed by the model on physically reasonable values of the constants. From Equations (15) and (17) it is seen that:

$$K_{\text{parallel}} = \frac{k_1}{k_d} - 1; K_{\text{sequential}} = \frac{1}{\frac{k_1}{k_d} - 1}, \quad (18)$$

so that $k_d > k_1$ is not reasonable for these models, even though, were this constraint ignored, very near perfect fits could be obtained in many cases.

This problem probably arises: (1) because of the difficulty to assess equilibrium assumption, and (2) because of the need to include more steps in the deactivation reaction

Figure V-4. Comparison of predicted kinetic behavior from simplified deactivation model with data of Figure IV-2a.

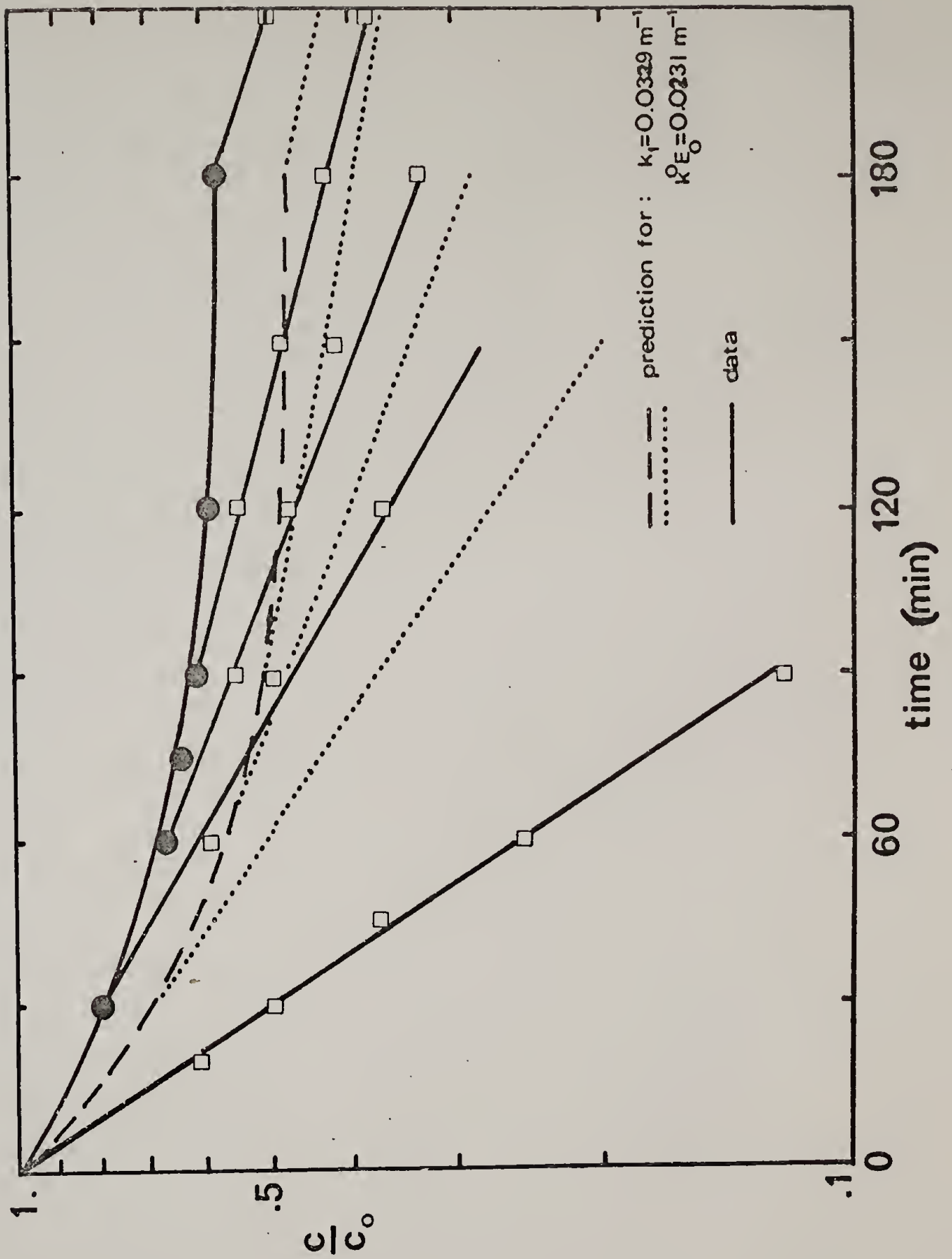


TABLE V-1
RATE CONSTANTS DERIVED FROM SIMPLIFIED
DEACTIVATION MODELS

UREASE	SHEAR RATE (s^{-1})	k_1 (m^{-1})	k_d (m^{-1})	$K_{parallel}$	$K_{sequential}$
A	1717	0.0329	0.0234	0.406	2.46
A	741	0.0229	0.0199	0.151	6.63
A	288	0.0213	0.0183	0.164	6.10
B	1717	0.00766	0.00735	0.042	23.7
B	975	0.00802	0.00773	0.037	26.6
B	288	0.00742	0.00793	---	---
C	1717	0.0228	0.0211	0.081	12.4
C	288	0.00433	0.00122	2.55	0.392

scheme, such as a multiple step sequential process. Physical understanding of what is happening to urease subjected to shearing does not justify such a formulation at this point. This section does serve to outline the possibilities (and impossibilities) of a group of very simple kinetic models. More on the molecular basis of this phenomenon follows directly.

C. Toward a Molecular Theory of Shear Modification of Enzyme Kinetics

The purpose of this section is to discuss the changes in protein conformation possible under the hydrodynamic conditions of these experiments and speculate on how they might be related to the changes in activity observed. This must begin with an examination of the urease enzyme protein macromolecule as a mechanical system.

First, the motion of the molecule as a whole in the shear field will be considered, ignoring the subtle conformational changes, as the motion of a rigid body. Evidence has been described in the literature (Fishbein et al., 1970, 1973, 1976) and further conclusive evidence will be provided in Chapter VII of this dissertation that the urease monomer is roughly a sphere of radius 6.2 nm and that the higher molecular weight isozymes are linearly arranged oligomers, a sort of peas-in-a-pod model. Assuming that no major conformational changes occur during shearing, the spherical

monomer will translate with the local velocity and rotate with the vorticity, experiencing the shear stress which obtains in the bulk fluid. The assymmetric isozymes on the other hand will also rotate, but not at constant angular velocity. The higher the aspect ratio and shear rate, the larger proportion of time will be spent aligned with the flow direction. The distribution of orientations has been obtained analytically by Peterlin (1938) and computed numerically by Scheraga (1955). Orientation is governed by the aspect ratio of the molecule and by the rotary Peclet number, $\theta/\dot{\gamma}$, where θ is the rotary diffusion coefficient and $\dot{\gamma}$ is the shear rate. At low enough rotary Peclet number this orientation effect will cause nonNewtonian viscosity. It is readily verified, for any of the urease isozymes, that the aspect ratios are too small and the rotary Peclet number too large to produce any shear rate dependence of viscosity (see Scheraga, 1961, for a further discussion of this point).

For any species in any orientation, the maximum tension experienced is of the order of magnitude of the shear stress multiplied by the macromolecular cross-section. The question which must be addressed is, "How does this force modify the catalytic activity?" Assuming that it is by changing the shape of the protein molecule it must then be asked, "How does this force modify the shape of the protein macromolecule?" As mentioned in an earlier chapter, this question, which can be answered satisfactorily in a very

general way with randomly coiling macromolecules, is exceedingly difficult to answer with any generality for a protein molecule.

Several quantitative estimates of the possible deformation of the urease structure will be made below based on its known quaternary structure and hopefully plausible assumptions about the "mechanical" properties of proteins. The cross-sectional area of the urease monomer is 121 nm^2 which means that for every 1 Pascal of applied shear stress, the molecule experiences a maximum tension of 1.21×10^{-16} newtons. In these experiments, significant effects have been observed at stresses from 0.5 to 21 Pascals, forces from 10^{-16} to 10^{-14} newtons.

a. Estimate based on elastic moduli of proteins.

The elastic or Young's moduli of several protein materials have been measured: 10^{10} Pascals is typical of wool (Mizushima, 1954), 10^9 to 10^7 Pascals for F-actin and some other unspecified proteins (Oosawa and Asakura, 1975; Khurgin et al., 1967) and 10^4 Pascals has been reported for muscle (see Riseman and Kirkwood, 1948). Comparison of these values of modulus with the applied stresses gives estimates of the strain from a minimum of 0.00000001% to a maximum of 0.1%. This latter strain would produce a deformation of approximately 0.01 nm in the urease monomer. It is not certain how the effective "elastic modulus" of an isolated globular protein molecule in solution compares to the above

values for solid proteins.

b. Estimate based on bond force constants. The force constants of various important bonds in protein structures have been estimated (Mizushima, 1954): for covalent bond stretching, 10^2 to 10^3 newtons/m; for covalent bond angle distortion, 10 to 10^2 newtons/m; for hydrogen bond stretching, 1 to 10 newtons/m. Comparison of these values with the applied forces leads to estimates of the deformation of any individual bond from a minimum of 10^{-10} nm to a maximum of 10^{-5} nm. For a molecule containing a linear array of, say, 100 hydrogen bonds all experiencing a force of 10^{-14} newtons, the deformation of the entire macromolecule could conceivably reach 0.01 nm. No estimates of force constants of hydrophobic or apolar bonds are known to this author. This information is quite important to the present study since it has been estimated that the contribution of apolar bonds to the conformational stability is 10 to 15 greater than that of hydrogen bonds (Haschemeyer and Haschemeyer, 1973). The strength of an individual apolar bond (0.5 to 2.0 kcal per mole of bonds) is known to be somewhat less than that of a hydrogen bond (2.0 to 5.0 kcal per mole of bonds) (Nemethy and Scheraga, 1962).

c. Estimate based on the intrinsic rigidity of randomly coiling macromolecules. The so-called intrinsic rigidity, Q , of a randomly coiling macromolecule is the coefficient of resistance to the rate of conformational change by

an applied force:

$$\dot{r} = \frac{F_{\text{applied}}}{Q} \quad (19)$$

where r is some molecular dimension (Cerf, 1968). Q has been estimated as 10^{-8} newton-s/m. Comparison of this number with the applied forces shows that very large conformational changes could occur rapidly in synthetic macromolecules at these stress levels. For example, Cottrell et al. (1969) measured, by light scattering, 43% elongation of a high molecular weight polyisobutylene, at an applied stress of about 10^{-15} newtons, one-tenth the maximum used in the present work. The relevance of this to conformational distortion of proteins is not clear. Perhaps it indicates that if the protein contains any relatively large, "loose" or "floppy," "random" structure, this portion may readily be distorted by stress.

Additional types of stress felt by the enzyme under these conditions are torques and/or bending moments. It is difficult to even begin to quantify their possible effects. In any case, these calculations show that a molecular understanding of the shear modification of enzyme kinetics is still rather remote but that it is intimately related to the conformational stability and dynamics of proteins. It is known, for example, from deuterium-hydrogen exchange experiments (Hvidt and Nielsen, 1966) and low frequency laser Raman spectroscopy (Brown et al., 1972) on proteins, that conformations are dynamic rather than static, and that simultaneous

coherent displacement of the atomic nuclei occurs continually throughout all or many of the residues of the molecule. In addition, it is known that large amplitude conformational changes in proteins occur on substrate binding; for example, Lipscomb and coworkers (Reeke et al., 1967) have shown that a tyrosine oxygen atom moves 1.4 nm on binding of a substrate analog! It seems quite certain that effects demonstrated in this dissertation must be the result of a complex interaction between the conformational dynamics and the flow field; perhaps a bias is introduced by shearing forces among the various conformations assumed naturally by the protein.

D. Conclusion

This lack of understanding should serve as an incentive for further well designed experiments on the interactions of flow fields with kinetic processes. These results, coupled with adequate phenomenological or molecular models, would be of interest in a broad range of problems in macromolecular science and engineering. Among these are polymer crystallization kinetics, polymerization kinetics and stress induced diffusion, all of which may be modified by deformation. It is interesting to note that this area of kinetic-kinematic interaction is now beginning to be studied in a purely formal, and rather elegant, way by mathematicians and continuum mechanicians (Kelly, 1964; Eringen and Ingram, 1965; Ingram and Eringen, 1967; Bowen, 1968, 1969a, b; Doria

and Bowen, 1970; Gurtin and Vargas, 1971; Gurtin, 1971).

Those results may be expected to complement investigations of the present sort.

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CHAPTER VI

DISCUSSION OF RESULTS ON UREASE CATALYZED REACTIONS IN DEFORMING MEDIA

The purpose of this chapter is to make some further general remarks on the interpretation and significance of the experimental results of Chapter IV and the calculations of Chapter V. A detailed summary of the specific results of this dissertation is postponed until Chapter IX.

It has been demonstrated that, for shearing forces up to about 2.5 Pascals, urease is inactivated only if sulfhydryl destroying moieties, such as metal ions (most likely ferric) or PCMB are present. The basic mechanism proposed is that urease is rendered more susceptible to these damaging reactions by the applied hydrodynamic stresses. The stresses necessary to promote this metal ion effect, i.e., the irreversible part of the inactivation, are of the order of 1.0 Pascals. Somewhat higher stresses, 2.0 to 2.5 Pascals are necessary to produce a direct effect on the activity, i.e., any reversible inactivation. More potent inactivation, apparently totally reversible in the absence of metal ions, is seen in reactions in more viscous media with stresses up to 21.0 Pascals. This represents the first attempt to quantify the critical stress for measurable inactivation of any enzyme

system. In this regard, it is reiterated that no statistically significant permanent inactivation of urease has been observed up to 21.0 Pascals. The dependence of the reversible inactivation on shear stress rather than shear rate, indicates that wall effects, i.e., collisions, are not the dominant force in this mode of inactivation. The possible role of wall effects in irreversible inactivation has not been thoroughly investigated. Indications are that urease is not strongly absorbed on the surface of the viscometer so, if there is a surface effect it is a shear induced one.

It is not clear at this point why the stresses necessary for significant inactivation in this work should be so much higher, 2 to 3 orders of magnitude, than those used by Charm and Wong (1970a, b) who reported permanent inactivation at as low as 0.1 Pascals. In light of the present work, an undetected role of metal ions in their results seems plausible.

A correlation scheme for the irreversible inactivation has been shown to apply to urease. For biomedical or biotechnological reactors or flow devices where the chemical environment is similar to the present experimental conditions, this type of correlation may be useful in defining nondeactivating operating conditions. A rule-of-thumb is that shear exposures (shear rate x exposure time) below 10^5 can be tolerated by urease with no loss of activity, sheared in the presence of micromolar quantities of unchelated metal

metal ions.

The reversible inactivation has been correlated with shear stress and seems to be leveling off at 55 to 60% of zero shear activity between 10.0 and 21.0 Pascals. It is tempting to think that perhaps at this stress level the molecule has been pushed to some physical limit and that at some higher critical shear stress irreversible inactivation due directly to a hydrodynamic effect may begin.

These results may imply that shear stress effects may be present in some of the experiments designed to study very rapid reactions (see Eigen and de Maeyer, 1963, for a discussion of those techniques). Tanford (1961) claims that linear velocities, V , of up to 10 m/sec obtain in some of the continuous flow and stop-flow devices. If a conservatively large estimate of the tube diameter, D , at 0.01 m is made, a nominal shear rate can be calculated from $\dot{\gamma}_{\text{nominal}} = 8 V/D$. This means a nominal shear rate of 8000 s^{-1} may exist in these apparatus, corresponding to shear stresses in aqueous media of 8.0 Pascals. Based on the present work, stress related effects certainly seem possible. It could be that the rate of shear induced conformational changes prevent them from happening on the microsecond time scale of some rapid reaction techniques. In any event, a continuous flow device seems to have potential for studying these stress effects themselves. A proposed design for such a device is discussed in Chapter IX.

It is made clear in Chapter V that description of the shear modification effects is hampered by the lack of detailed structural knowledge of proteins in general and urease in particular. That is why some preliminary shear-kinetic experiments have been done with an enzyme that is thoroughly understood structurally, lactic dehydrogenase. These results are reported in Chapter VIII after a digression in Chapter VII to discuss some intrinsic viscosity experiments designed to further elucidate the urease structure, in particular, the quaternary structure; it will be seen that the information from the intrinsic viscosity studies may aid in understanding the shear-kinetic results.

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CHAPTER VII

INTRINSIC VISCOSITY STUDIES OF UREASE

A. Introduction

Calculation of the hydrodynamic properties of rigid, multisubunit protein assemblies, or plausible models thereof, utilizing variations of the Oseen tensor (Oseen, 1927) to account for the hydrodynamic interaction between the subunits, has properly drawn the attention of many authors (a representative sample of the scope of this work is: Kirkwood and Auer, 1951; Kirkwood, 1954; Bloomfield et al., 1967a, b; Paul and Mazo, 1969; Yamakawa and Tanaka, 1972; Yamakawa and Yamaki, 1972, 1973; McCammon and Deutch, 1976). Some hydrodynamic properties of interest in protein structural studies are sedimentation coefficient, translational and rotational diffusion coefficients and intrinsic viscosity. The above theories lead to relatively simple expressions for the diffusion tensors, from which the sedimentation coefficients and diffusion coefficient may readily be obtained (Yamakawa, 1971). These may be interrelated by the molecular friction coefficient, f , which is related to the translational diffusion coefficient by the Einstein equation (Einstein, 1905):

$$D_T = kT/f_T \quad (1)$$

(an identical relation exists between D_R and f_R) and to the sedimentation coefficient, S , by the Svedberg equation (Yamawaka, 1971):

$$S = \frac{M(1-\bar{v}\rho)}{\tilde{N}f_T} \quad (2)$$

where k is Boltzmann's constant, T is absolute temperature, M is molecular weight, \bar{v} is the partial specific volume of the protein molecule, ρ the solvent density and \tilde{N} is Avogadro's number. On the other hand, explicit expressions for the intrinsic viscosity of an arbitrarily arranged array of subunits have often not been obtained or are computationally quite complex.

Recently, Abdel-Khalik and Bird (1975) have presented a theory which is especially well suited to calculating intrinsic viscosities of large, irregular assemblies of non-identical subunits. This theory utilizes shielding coefficients to account for the hydrodynamic interaction between subunits, these being determined from exact hydrodynamic calculations on well defined geometries. The purpose of this chapter is to show how intrinsic viscosity measurements of urease under various conditions can be combined with the AK-B theory to elucidate the geometrical arrangement of the urease quaternary structure. The relationship of the AK-B theory to the approaches utilizing the Oseen tensor is then discussed. This is done to show the connections between the present viscosity measurements and previous ultracentrifugal studies of

urease by Fishbein and coworkers (1970, 1973). We begin with brief sketches of the Kirkwood and AK-B formalisms.

B. Theoretical Treatments

In the Kirkwood theory (1954), the macromolecule is treated as a collection of point centers of frictional resistance, held together by links which constrain the relative positions of the N centers to be invariant. These point centers of resistance are immersed in a hydrodynamic continuum, and the i^{th} center exerts a force on the fluid given by:

$$\underline{\underline{F_i}} = -\xi(\underline{\underline{v_i}}^0 - \underline{\underline{u_i}}) - \xi \sum_{j \neq i}^N \underline{\underline{T_{ij}}} \cdot \underline{\underline{F_j}}, \quad (3)$$

where T may be the Oseen tensor:

$$\underline{\underline{T_{ij}}} = \frac{1}{8\pi\eta_0 R_{ij}} [\underline{\underline{I}} + (\underline{\underline{R_{ij}}}\underline{\underline{R_{ij}}}/R_{ij}^2)]. \quad (4)$$

or some modification thereof (see below). Here, ξ is the friction coefficient of the point center or subunit, $\underline{\underline{v_i}}^0$ is the velocity of the fluid at the position of center i , which would have obtained if the macromolecule were not present, $\underline{\underline{u_i}}$ is the velocity of the center i , η_0 is the solvent viscosity, $\underline{\underline{R_{ij}}}$ is the vector between centers i and j , R_{ij} is the magnitude of this vector and $\underline{\underline{I}}$ is the unit tensor. (Note that the subscripts do not refer to components in some coordinate system.) Other, more correct tensorial expressions for calculating the hydrodynamic interaction have been proposed by Rotne and Prager (1969) and Yamakawa (1970).

From simple energy dissipation arguments, it can be shown (Yamakawa, 1971, pg. 259) that:

$$[\eta] = - \frac{\tilde{N}}{Mn_0g} \sum_i^N \langle F_{ix} y_i \rangle \quad (5)$$

where F_{ix} is the x-component of the frictional force of the i^{th} center and y_i is the y-component of position of the i^{th} center in a velocity field defined by $\underline{v}_i^0 = (gy_i, 0, 0)$. Therefore, what is needed is a solution for the force F_i from Equation (3). The 3N Equations (3), for the components of the force F_i , can be rewritten as a single matrix equation, following McCammon and Deutch (1976):

$$\underline{\underline{H}} \cdot \underline{F} = \underline{U} \quad (6)$$

where $\underline{\underline{H}}$ is a $3N \times 3N$, hydrodynamic interaction hypermatrix containing the 3×3 blocks:

$$\underline{\underline{h}}_{ij} = \underline{\underline{I}} + \xi \underline{\underline{T}}_{ij} \quad (7)$$

and \underline{F} and \underline{U} are $3N \times 1$ column vectors: $\underline{F} = (\underline{F}_1, \underline{F}_2, \dots, \underline{F}_N)$ and $\underline{U} = (\underline{u}_1 - \underline{v}_1^0, \underline{u}_2 - \underline{v}_2^0, \dots, \underline{u}_N - \underline{v}_N^0)$. The formal solution is, of course:

$$\underline{F} = \underline{\underline{H}}^{-1} \cdot \underline{U}. \quad (8)$$

Computation of $\underline{\underline{H}}^{-1}$ is the major hurdle to overcome in applying this method. If the Oseen tensor is used in constructing $\underline{\underline{H}}$, Zwanzig et al. (1968) have pointed out that zero eigenvalues (singularities) of $\underline{\underline{H}}^{-1}$ can exist for some strengths of the hydrodynamic interaction. This, in part, prompted the modifications suggested by Rotne and Prager (1969) and

Yamakawa (1970). Nevertheless, exact analytical expressions for the transport coefficients can be derived for many of the variations of geometry and hydrodynamic interaction tensor (Kirkwood, 1954; Bloomfield et al., 1967a; Paul and Mazo, 1969). On the other hand, only approximate or numerical solutions exist for the intrinsic viscosity (Riseman and Kirkwood, 1950; McCammon and Deutch, 1976). For large assemblies, these latter must be done by computer.

Abdel-Khalik and Bird (1975) have taken an entirely different approach. They have considered each subunit in a multisubunit assembly to itself be constructed from a tightly packed array of v identical, spherical beads, of diameter d , small compared to the subunit dimensions. They have built their work upon the results of Curtiss et al. (1976). Curtiss et al. have combined a molecular theory expression for the stress tensor, given in terms of a bead friction coefficient, ξ_b , with an expression for the stress tensor suggested by continuum mechanics, the so-called retarded motion expansion (see Bird et al., 1977), to relate the viscosity contribution of the i^{th} subunit to the bead friction coefficient:

$$(\eta - \eta_0)_i = \frac{n_i}{6} \sum_{s=1}^v \sum_{p=x,y,z} \xi_b R_{sp(i)}^2 \quad (9)$$

where n_i is the number of i subunits per unit volume and $R_{sp(i)}$ is the p^{th} component of the distance from bead s to the center of resistance of subunit i . Abdel-Khalik and Bird then take the bead friction coefficient to be given by

Stokes' law, modified by a shielding coefficient, C_b , to account for the hydrodynamic interaction between the tightly packed beads:

$$\xi_b = 3\pi\eta_0 d C_b. \quad (10)$$

The intrinsic viscosity of the entire multisubunit assembly is then given by:

$$[\eta] = \frac{\pi \tilde{N}}{2M} \sum_{i=1}^N \sum_{s=1}^v \sum_p C^{(i)} d R_{sp}^{(i)2} \quad (11)$$

The subunit shielding coefficients $C^{(i)}$ are determined by substituting Equation (10) for ξ_b into Equation (9) and requiring the latter to give the correct analytical results for isolated units of well-defined geometries, such as spheres, rods and disks, and where $R_{sp}^{(i)}$ is now the p^{th} component of distance from bead s to the center of resistance of the entire assembly. $C^{(i)}$ is actually the C_b appropriate for the packing arrangement of spheres required to form the subunits of the above shapes. Equation (11) can be split into two parts:

$$[\eta] = \frac{\pi \tilde{N}}{2M} \sum_{i=1}^N (A + B R_C^{(i)2}), \quad (12)$$

A being dictated by the internal geometry of the subunit and $B R_C^{(i)2}$ given by the arrangement of subunits in the total assembly. $R_C^{(i)}$ is the distance from the center of resistance of the assembly to the center of resistance of the subunit. A and B for different subunit geometries have been derived by

TABLE VII-1
VALUES OF A AND B FOR AK-B THEORY

GEOMETRY	A	B
Sphere (diameter D)	$\frac{10}{3} D$	$\frac{5}{6} D^3$
Rod (length L, diameter D)	$\frac{16 L^3}{15 [\ln(L/D)] (3D^2 + L^2)}$	$\frac{4}{45} \frac{L^3}{\ln(L/D)}$
Disk (thickness H, diameter D)	$2H\nu$ [Where ν is a function of D/H given by Scheraga, J. Chem. Phys., <u>23</u> , 1526 (1955).]	$\frac{1}{2} \nu D^2 H$

This table is abstracted from Abdel-Khalik and Bird, 1975. It may also be found in Bird et al., 1977, along with a more complete tabulation of their results.

Abdel-Khalik and Bird (1975); some of their results are tabulated in Table 1.

So, Equation (12) is the principal working formula of the AK-B theory. To use it, one needs only to find the center of resistance of the assembly under study (Bird et al., 1977), specify the distances from it to the centers of resistance of the subunits (this can often be done by inspection, as will soon be demonstrated), look up A and B for the desired subunit geometries (this theory handles nonidentical subunits as easily as identical ones) and use Equation (12). The ease of using Equation (12) relative to Equations (5) and (8) is apparent on considering, for example, a four subunit assembly; some simple arithmetic is required with the former as opposed to inverting a 12 x 12 matrix with the latter.

C. Calculations on Urease Monomer and Oligomers with AK-B Theory

The intrinsic viscosities of urease isozymes, up to the pentamer, have been calculated using Equation (12). Based on the best available evidence (Fishbein et al., 1970, 1973), the radius and molecular weight of the spherical urease monomer, the oligomerizing unit, have been taken to be 6.2 nm and 482,000 daltons respectively. Four different geometrical oligomerization patterns were assumed. These are illustrated in Figure VII -1, along with all the values of $R_C^{(i)}$ for each model. The fraction of each oligomer present

Figure VII-1. Illustrations of various hypothetical geometrical urease oligomerization patterns.





















AGGREGATION PATTERN	I	II	III	IV	V
linear					
R'_C s \rightarrow (all in nm)		6.2	0, 12.4	6.2, 18.6	0, 12.4, 24.8
polygonal					
R'_C s \rightarrow			7.16	8.77	10.55
polyhedral					
R'_C s \rightarrow				7.60	7.16, 10.13
staggered					
R'_C s \rightarrow				6.2, 10.74	3.58, 9.47, 12.91

TABLE VII-2
INTRINSIC VISCOSITIES OF HYPOTHETICAL UREASE ISOZYMES
AND MIXTURES THEREOF PREDICTED BY AK-B THEORY

AGGREGATION PATTERN*	monomer	dimer	trimer	tetramer	pentamer	PREDICTION FOR MIXTURE
	67.1%**	10.1%	7.3%	6.2%	9.1%	
Linear	3.11***	6.23	10.39	18.70	27.42	7.13
Polygonal	3.11	6.23	7.27	9.35	12.14	4.93
Polyhedral	3.11	6.23	7.27	7.79	8.94	4.54
Staggered	3.11	6.23	7.27	9.35	11.63	4.88

*See Figure VII-1 for illustrations.

**Estimated from Figure VII-2.

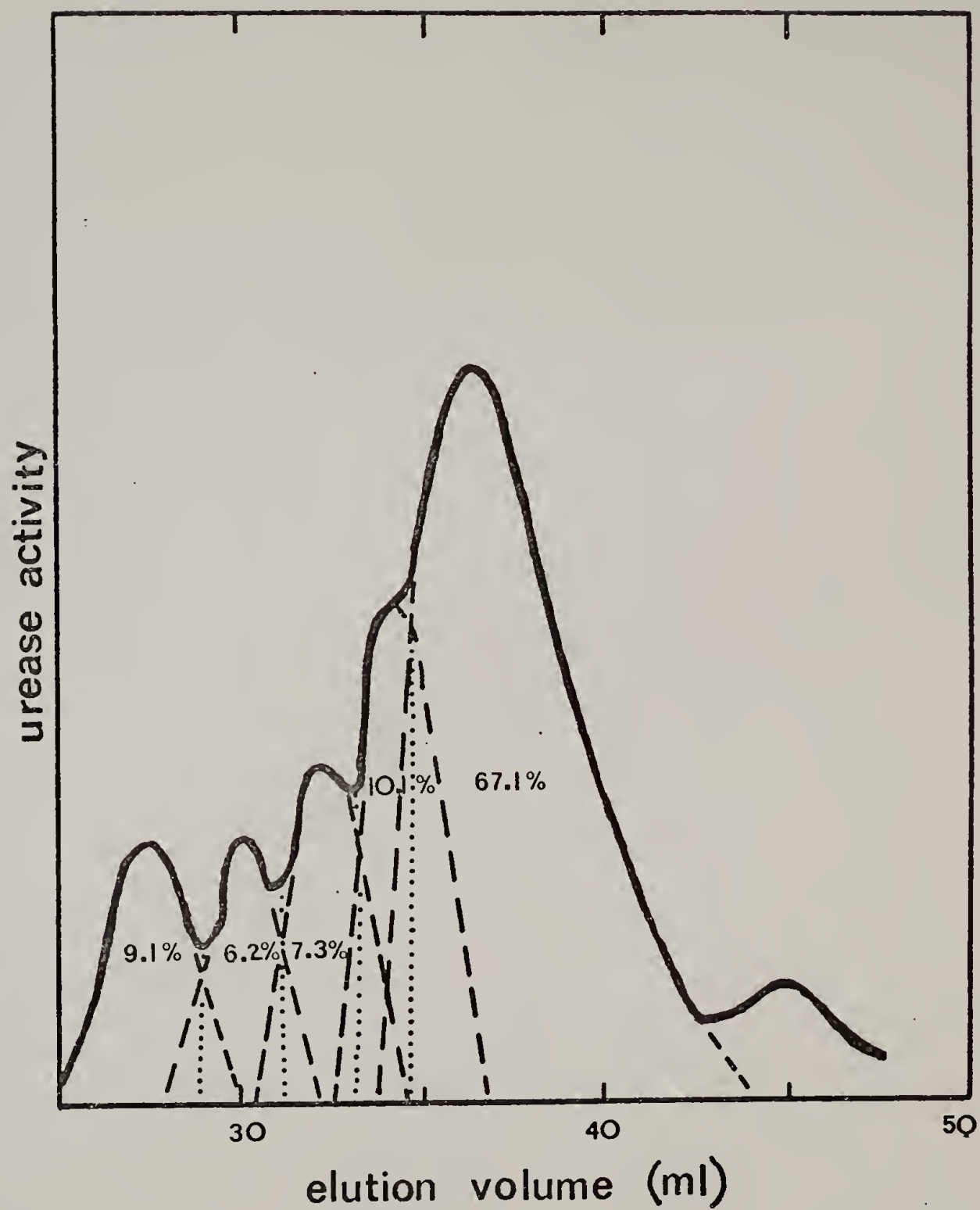
***Intrinsic viscosity predicted by AK-B theory for this species (cm³/g).

during the intrinsic viscosity measurement was determined chromatographically (see Experimental Section below). The fractions were then used as weighting factors to calculate the intrinsic viscosities for the mixtures of oligomers based on the four geometrical patterns. The results of these calculations are tabulated in Table 2. It is seen that the linear aggregation pattern gives a prediction of $[\eta] = 7.13 \text{ cm}^3/\text{g}$, whereas the more compact patterns all lead to predictions in the range $4.79 \pm 0.25 \text{ cm}^3/\text{g}$.

D. Experimental

The size exclusion chromatography was performed as described in Chapter III. Specifically, for this determination, 55 mg of Sigma, type C-3, crystalline urease, was dissolved in 8 ml of 0.16 M phosphate buffer, pH 7.00 ± 0.05 . This mixture was allowed to stir gently for 2 hours, then was dialyzed against 400 volumes of buffer for 24 hours at room temperature, with one change to 400 volumes of fresh dialyzate after 12 hours. The solution was then centrifuged for 1 hour at speed 70, on a Precision Scientific Co., Centricone, centrifuge to remove any insoluble material. The supernatant was found by the Lowry procedure to have a urease concentration of 3.40 mg/ml. A small aliquot was diluted to 0.5 mg/ml and applied to the size separation column described in Chapter III, section B.2. The elution profile is shown in Figure VII-2. The remainder of the solution was used for

Figure VII-2. SEC of oligomerized urease sample illustrating determination of fraction of each isozyme.



the intrinsic viscosity measurements. The preparation of a solution containing urease strictly in the monomeric form, proceeded in exactly the same way except that the solution was made 5 mM in β -mercaptoethanol following dialysis. The urease in solution was found to be 4.1 mg/ml after centrifuging. No chromatography was done with this particular sample since there is abundant proof that only the 482,000 molecular weight species exists under these conditions (see Chapter IV, section B; also Creeth and Nichol, 1960; Fishbein et al., 1973, 1976).

The percentages of the total urease content contributed by each of the isozymes was determined by graphical integration of Figure VII-2. These percentages are included in Table 2.

The intrinsic viscosities of these two samples were determined using a Cannon-Ubbelohde E796-50 viscometer. The flow time for buffer alone exceeded 200 sec. The viscometer was immersed in a constant temperature bath controlled to $25.00 \pm 0.05^\circ\text{C}$. Centrifugation, then Lowry concentration determination preceded measurement of the flow time at each concentration. Solvent density was determined using a pycnometer.

E. Results and Discussion

The apparent reduced viscosity, v_{sp}/c , was determined from the flow times t by $v_{sp}/c = (t-t_0)/t_0c$, where t_0

is the pure solvent flow time. The results are plotted in Figure VII-3. Linear regression analysis of the data gives for the mixture of oligomers $[\nu] = 7.11 \pm 0.27 \text{ cm}^3/\text{g}$ and for the monomer $[\nu] = 3.57 \pm 0.41 \text{ cm}^3/\text{g}$. Errors reported here are 1 standard deviation. The true intrinsic viscosity is obtained after making a buoyancy correction (Tanford, 1955):

$$[\eta] = [\nu] + (1 - \bar{\nu}\rho)/\rho. \quad (13)$$

The measured buffer density is 1.0126 g/cm^3 . The partial specific volume of urease was taken as $0.73 \text{ cm}^3/\text{g}$ from Reithel and Robbins (1966). So, the results for intrinsic viscosity are:

$$[\eta] = 7.37 \pm 0.27 \text{ cm}^3/\text{g} \text{ for the mixture of oligomers,}$$

and

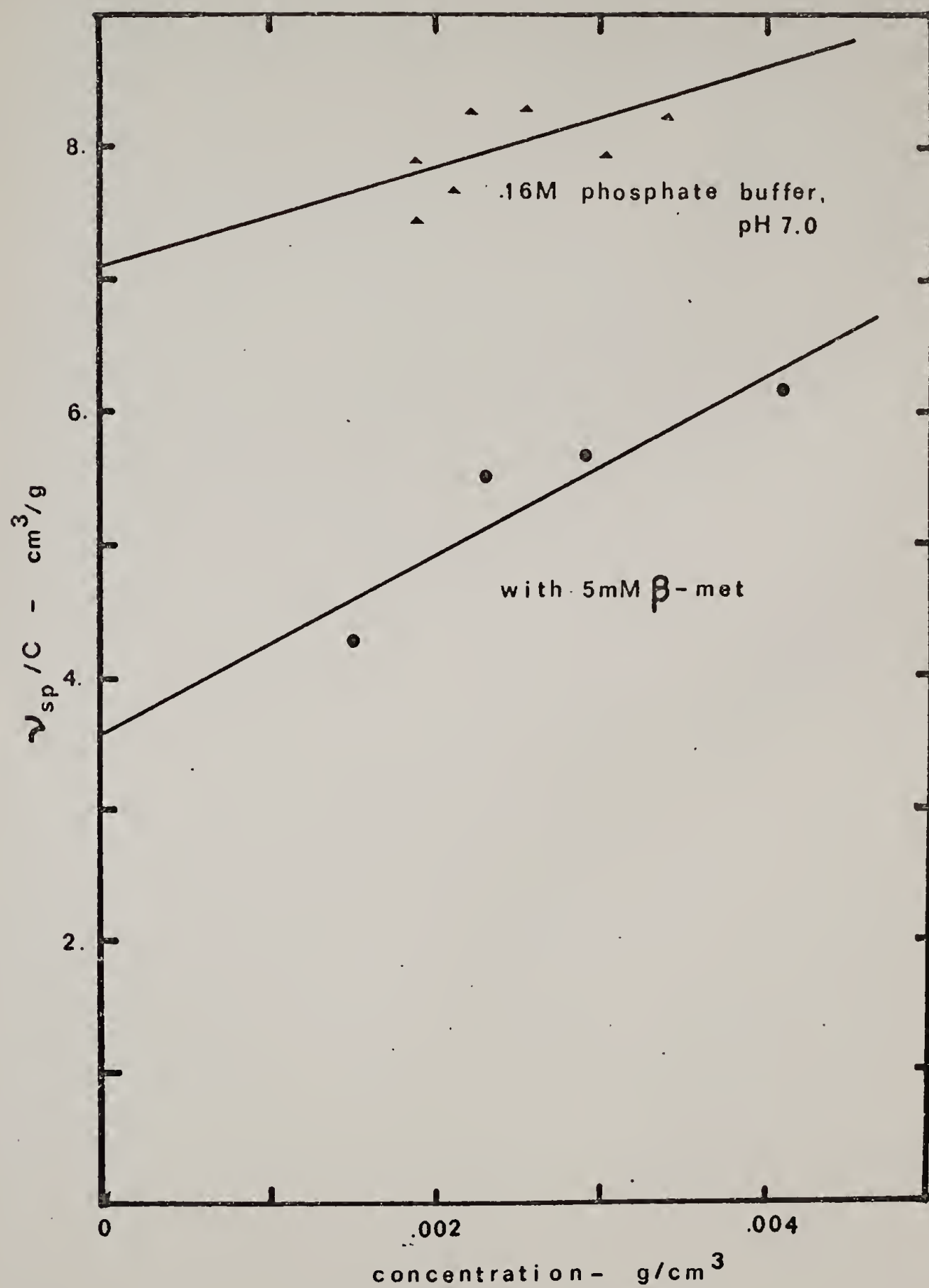
$$[\eta] = 3.83 \pm 0.41 \text{ cm}^3/\text{g} \text{ for the monomer.}$$

Comparison of the measured value for the mixture of oligomers and the results of calculations shown in Table 2 show the clear indication that urease aggregates in a linear fashion. The measured value for the monomer gives an estimate for the equivalent hydrodynamic radius from the Einstein viscosity equation:

$$R_e = \left(\frac{3[\eta]M}{10\pi \tilde{N}} \right)^{1/3}, \quad (14)$$

of $R_e = 6.6 \pm 0.3 \text{ nm}$. This is 6% higher than what seems to be the best value of 6.2 nm (Fishbein et al., 1973); the reason for this slight discrepancy is not clear. Naturally, the measured value for $[\eta]$ also exceeds the value calculated

Figure VII-3. Apparent reduced viscosities of urease samples plotted against urease concentration.



from the AK-B theory since the AK-B equation is identical to the Einstein viscosity equation for spherical molecules.

The sedimentation coefficients of the urease isozymes have been determined by Fishbein and coworkers (1970, 1973). They have utilized a Kirkwood type calculation for the sedimentation coefficients and proposed a linear aggregation pattern based on those calculations. So, in a sense, this study is complementary and corroborative to theirs. In some situations, a simple calculation based on the AK-B theory and an intrinsic viscosity determination may be preferable to a Kirkwood type analysis and an ultracentrifuge experiment. The present work demonstrates the ability to obtain similar information from the two methods.

The results of Fishbein's group for the sedimentation coefficients may be combined with the computed values of $[\eta]$ for the individual species to calculate the Scheraga-Mandelkern (1953) β factor:

$$\beta = \frac{\tilde{N} \eta_0 S[\eta]^{1/3}}{(1-\bar{v}_p)(100)^{1/3} M^{2/3}} \quad (15)$$

Table 3 shows the sedimentation coefficients, the intrinsic viscosities and the β 's for monomer through pentamer. For comparison, β 's calculated from an expanding prolate ellipsoid model (Scheraga and Mandelkern, 1953), of axial ratio equal to the degree of oligomerization, is shown. It is seen that β for the urease species grows much more rapidly than

TABLE VII-3
SCHERAGA-MANDELKERN β FACTORS

	s^*	$[\eta]^{1/3}_{**}$	β^{***}	β^{****} (for ellipsoid of aspect ratio = degree of poly- merization)
monomer	18.3	1.46	2.17	2.12
dimer	27.2	1.84	2.56	2.13
trimer	32.7	2.18	2.78	2.16
tetramer	39.8	2.65	3.40	2.20
pentamer	44.4	3.02	3.72	2.23

*From Fishbein et al. (1970).

**From present calculations.

***From Equation (15).

****From Scheraga and Mandelkern (1953).

the ellipsoid model, consistent with the conclusion reached by Fishbein et al. (1970) by another route. The β 's for the tetra- and pentamer are so high that they may reflect the need for some revision of the $[\eta]$ and/or s values. The s values for these are the least precise (Fishbein et al., 1970); and the individual $[\eta]$ values, except for the monomer, have only been inferred from measurements on the mixture.

The results here do present a totally consistent picture of the geometrical aggregation pattern in the urease oligomers. A new avenue in protein structure determination has been opened by Abdel-Khalik and Bird and exploited in the present work. One final remark is to note that the AK-B theory may be connected with the Kirkwood theory by using the latter to compute C_b in Equation (10). The crucial step of relating $[\eta]$ directly to the friction coefficient is achieved by Curtiss et al. (1976) and used by Abdel-Khalik and Bird. It may now be possible to improve the AK-B theory by using a properly constructed hydrodynamic interaction tensor. These calculations may be done once for the various subunit geometries, then tabulated in the AK-B style. This will be pursued in future work.

It is highly probable that this extended, linear aggregation pattern of urease causes it to experience larger hydrodynamic stresses in this type of Couette shear flow than some more compact protein conformations. This may in part

be responsible for the dramatic effects of shearing forces on urease catalysis demonstrated in previous chapters.

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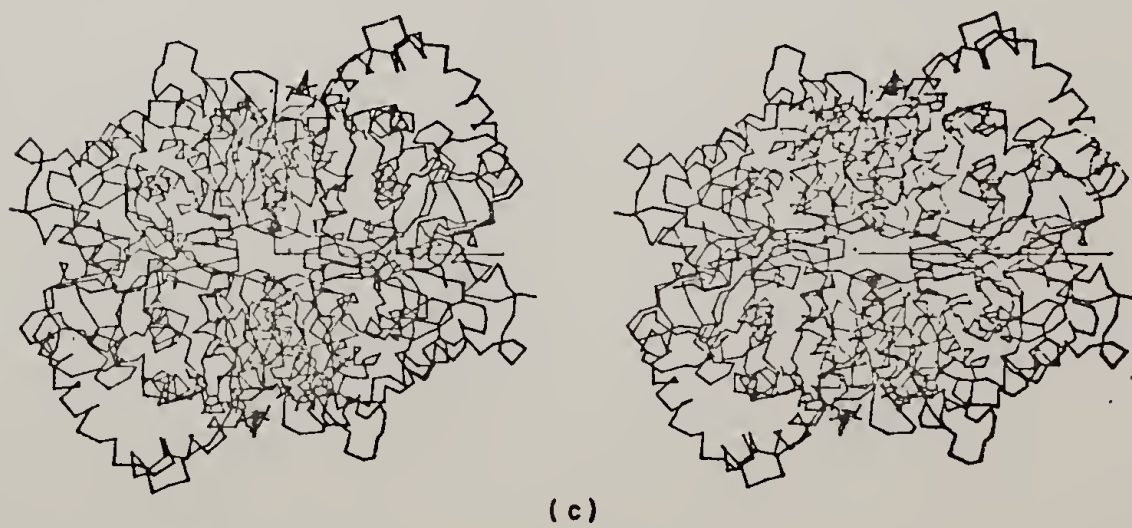
C H A P T E R V I I I
PRELIMINARY EXPERIMENTAL RESULTS ON
LACTIC DEHYDROGENASE

A. Introduction

In order to begin to formulate some general ideas on the causes and characteristics of modifications of enzyme catalyzed reactions by hydrodynamic forces, it is necessary to work with more than one enzyme. Since generalizations about protein structural details are difficult, understanding the effects of hydrodynamic forces on these structures will not be easy. Some kinetic reactions in sheared media catalyzed by lactic dehydrogenase (LDH) have been studied in order to see what, if any, of the conclusions obtained with urease can be extended to at least one other enzyme and what generalizations may be possible with some knowledge of two enzymes.

The reasons for the selection of LDH are primarily that: (1) its complete three-dimensional structure is known (Adams et al., 1970; see also the review by Holbrook et al., 1975); and (2) it has a number of superficial structural similarities to urease. Among these are a very labile quaternary structure consisting of four identical subunits and six

Figure VIII-1. Three different stereo views of the tetrameric lactic dehydrogenase (after Holbrook et al., 1975).



B. Experimental

The reaction medium for all these experiments was a standard solution of sodium pyruvate available from Sigma Chemical Co. (stock no. 500 L-1). This consists of a 0.75 mM solution of sodium pyruvate in pH 7.5 Tris buffer. Prior to a series of reactions, 100 to 200 ml of this substrate solution was made 1 mg/ml in β -nicotinamide adenine dinucleotide, reduced form, disodium (NADH). All reactions with this solution were performed within 12 hours subsequent to its preparation. To initiate each reaction, 50 μ l of a 0.080 mg/ml stock solution of LDH (Sigma, Type V, M_4 isozyme) was added to 8 ml of the substrate-NADH solution. Exact LDH concentrations were determined spectrophotometrically where necessary from: $[\text{LDH}] (\text{mg/ml}) = 1.13 A_{280}$ (Decker, 1977).

Pyruvate concentration was determined after the desired time interval by analyzing a 1 ml sample according to a modification of the method of Berger and Broida (1975). To the 1 ml sample was added 1 ml, 2,4-dinitrophenylhydrazine, 20 mg/100 ml (Sigma Color Reagent, Stock no. 505-2). This terminated reaction in the sample. After mixing and waiting 20 minutes, 10 ml of 0.4 M NaOH was added and mixed well. After 5 minutes, the percent transmittance at 415 nm was determined relative to that of a blank of 1 ml distilled water treated in the same manner. Pyruvate concentration was then determined from the calibration curve shown in Figure VIII-2.

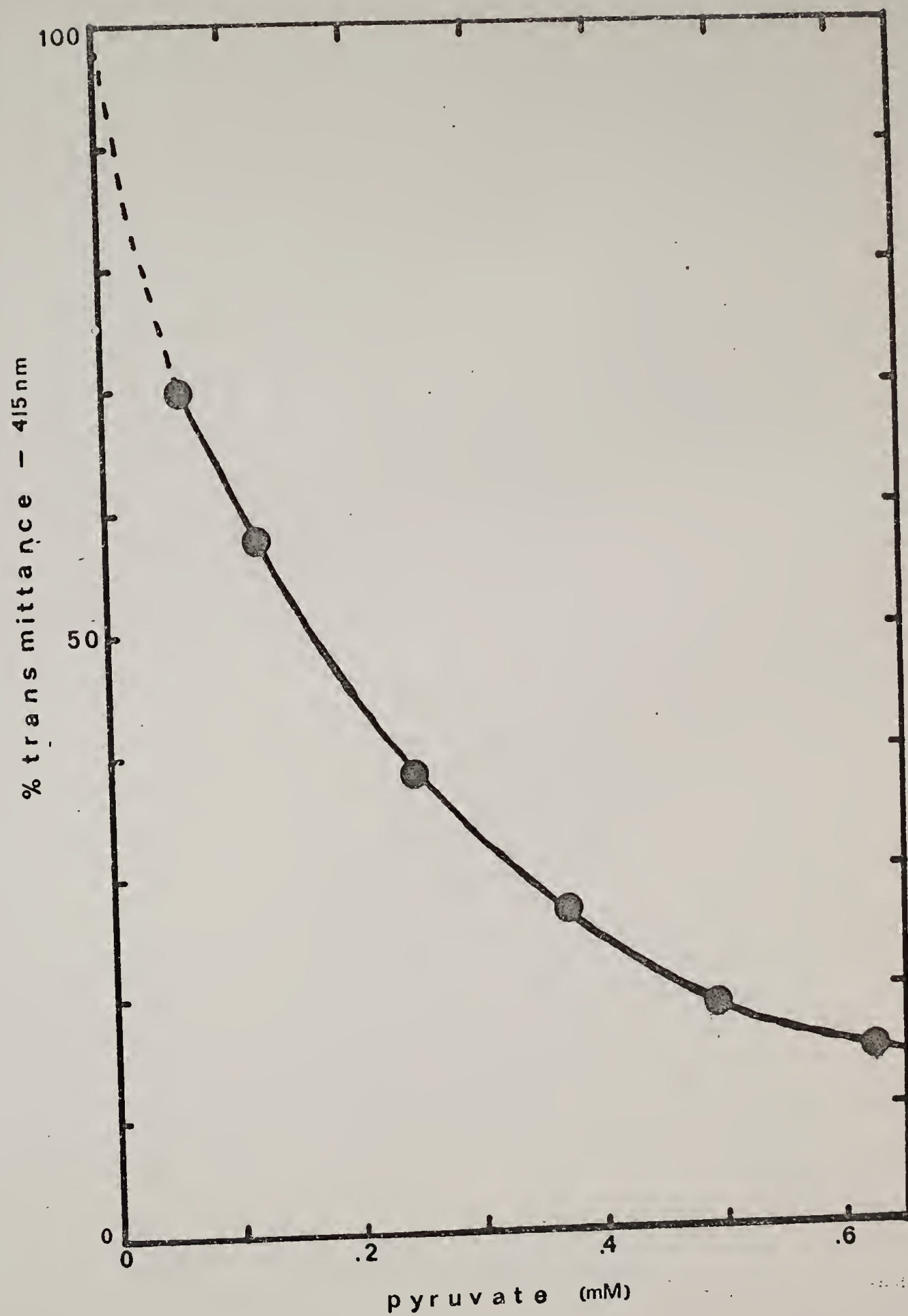


Figure VIII-2. Calibration curve for pyruvate analysis.

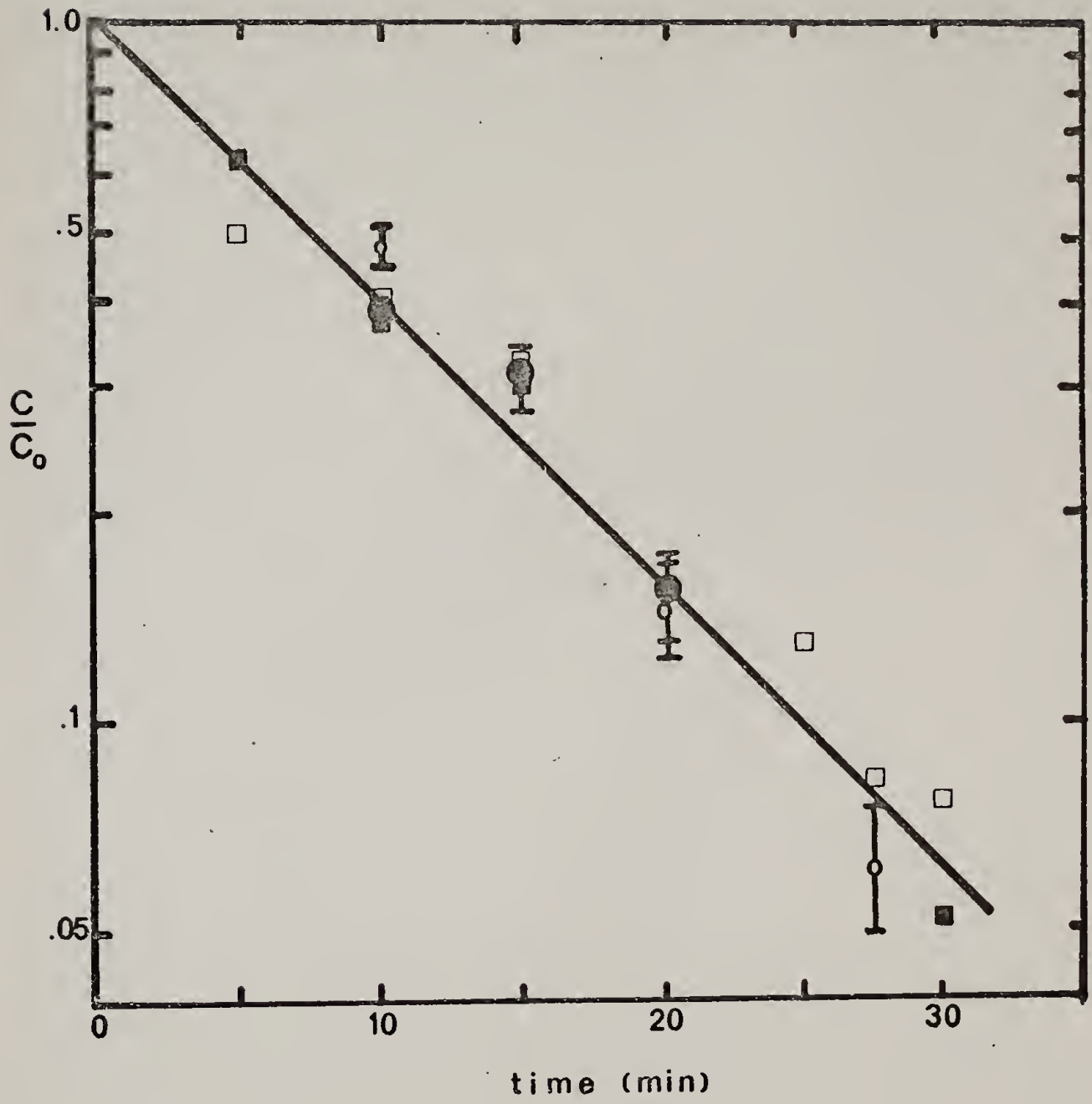
Experiments with LDH were conducted in two ways: (1) kinetic reactions were performed in the viscometer, and (2) kinetic reactions were performed in glassware after subjecting the stock LDH solutions to shearing forces. According to the former procedure the reaction mixture was added to the viscometer and shearing begun immediately after adding the enzyme to the substrate-NADH solution. In the latter type of experiment, stock LDH solutions were prepared in both pure distilled water and glycerin-water mixture (viscosity 0.0096 Pascal-s). These were subjected to shearing in the viscometer for the appropriate time interval, then removed. A reaction was then done in glassware, adding the sheared LDH to the substrate-NADH solution.

Ultraviolet absorption spectra of these sheared stock LDH solutions were recorded on a Beckman Acta M-VI double-beam spectrophotometer; the circular dichroism spectra of the same samples were determined on a Cary 60 spectropolarimeter with the Model 61 CD attachment.

C. Results and Discussion

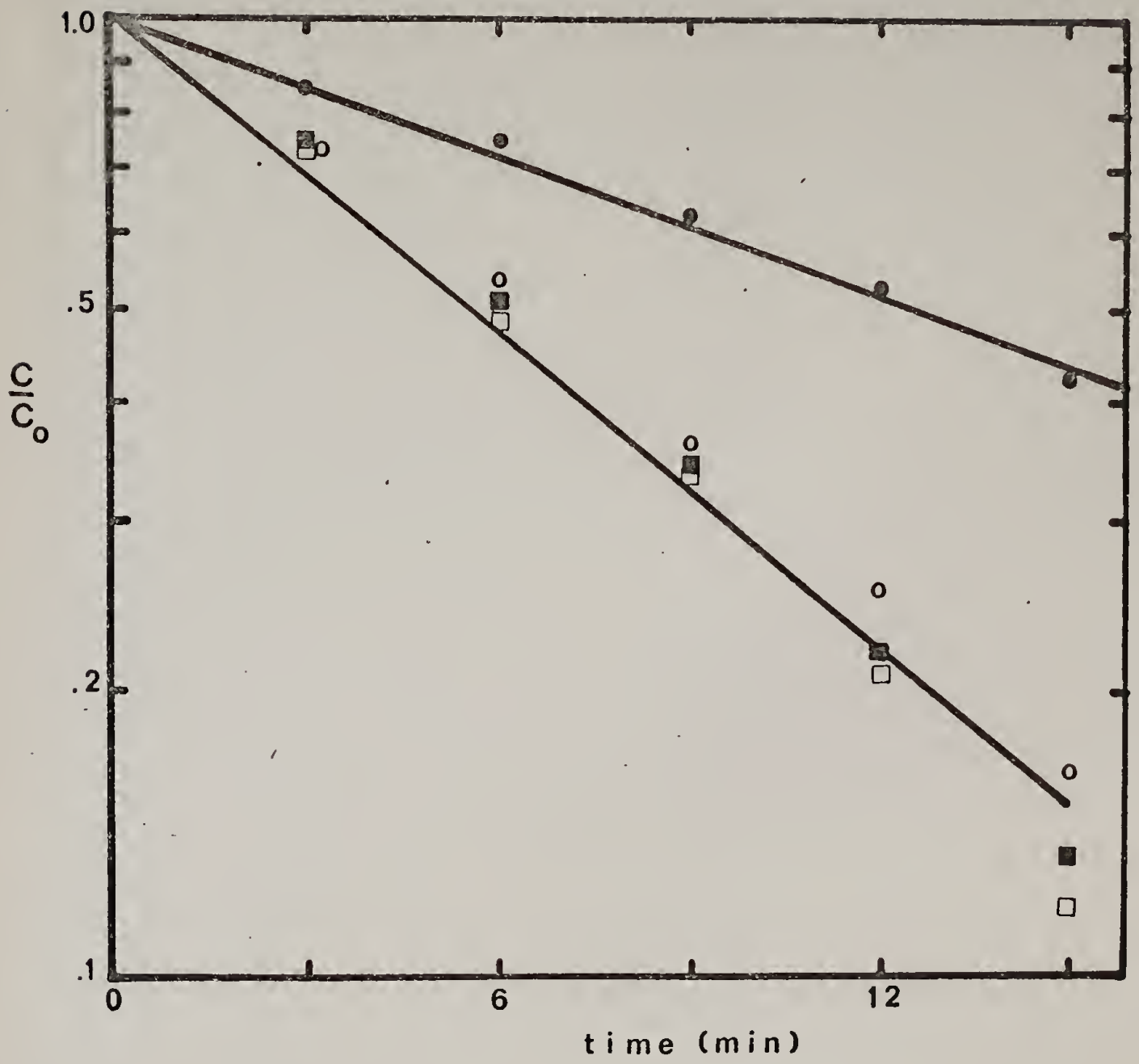
The results of performing the LDH catalyzed reduction of pyruvate in the viscometer are shown in Figure VIII-3. It is seen there that, within the precision of these experiments, the rate of LDH catalyzed reduction of pyruvate to lactate is unaltered by shear rates up to 1717 s^{-1} . This corresponds to shear stresses up to 2.1 Pascals.

Figure VIII-3. Pyruvate concentration as a function of time.
○ - 48 s^{-1} , run LDH-510; □ - 48 s^{-1} , run LDH-517; ● - 1717 s^{-1} , run LDH-510; ■ - 1717 s^{-1} , run LDH-517. (Error bars where given indicate standard deviation of repetitive trials.)



The results of shearing the LDH prior to the reaction are shown in Figure VIII-4. Two shearing conditions are illustrated: (1) shearing done for 1 hr in purely aqueous medium, viscosity 0.001 Pascal-s, shear rate 1717 s^{-1} , shear stress 1.72 Pascal; (2) shearing done for 1 hr in glycerin/water mixture, viscosity 0.0096 Pascal-s, shear rate 975 s^{-1} , shear stress 9.36 Pascal. For each set of kinetic results from the sheared LDH there is a control experiment run with LDH which was exposed to the viscometer for 1 hour under the same chemical condition but no applied shearing. This was necessary since mere exposure to the viscometer under quiescent conditions seems to alter the activity and structure (see Figures VIII-5 and VIII-6) of LDH somewhat. The results in Figure VIII-4 show that under these conditions, in aqueous (low viscosity) reaction media, the kinetics of pyruvate reduction are still unaltered by preexposure to shear rates up to 1717 s^{-1} . This indicates that the presence of pyruvate and NADH, and the concomittal conformational changes caused by them, are not necessarily providing any protection against shearing at this stress level. Stresses of this magnitude, that is, up to 2.1 Pascals, simply do not affect LDH activity. On the other hand, increasing the shear stress by roughly a factor of five, even while simultaneously reducing the shear rate, produces very significant irreversible inactivation, to roughly 50% of zero shear activity. This is clear evidence that hydrodynamic shear stress, as opposed to

Figure VIII-4. Pyruvate concentration as a function of time at zero shear with LDH samples with various shear histories. \square - zero shear, aqueous buffer; \blacksquare - $\tau = 1.7$ Pascals, $\dot{\gamma} = 1717 \text{ s}^{-1}$, aqueous buffer; \circ - zero shear, g-w mixture; \bullet - $\tau = 9.4$ Pascals, $\dot{\gamma} = 975 \text{ s}^{-1}$, g-w mixture. All in viscometer at above deformation conditions for 1 hour.



shear rate, is the controlling factor in the shear inactivation of LDH. The significance of this is that a direct molecular deformation due to stress is indicated as the mechanism rather than flow induced wall collisions, which depend on shear rate (Levich, 1962).

Some spectroscopic results on the LDH solutions before and after shearing are shown in Figures VIII-5 and VIII-6. Looking first at the UV spectra, it is seen that there is virtually no difference in the two purely aqueous samples which were exposed to the viscometer one at 1717 s^{-1} and one at zero shear. There is a pronounced difference, however, between these two and the spectrum prior to exposure to the viscometer. This indicates that there is some alteration induced in LDH by mere exposure to the viscometer. This difference exists also in the samples sheared in glycerin and water, but there is also a pronounced spectral difference between the unsheared and sheared samples, with the same viscometer exposure. The blue shifting of the peak originally at 280 nm and the hyperchromicity at 250 nm, which appears on exposure to the viscometer, is significantly enhanced by the applied shear stress. This same pattern of effects is repeated in the CD spectra of Figure VIII-6. No difference between the aqueous samples is seen up to 1717 s^{-1} , although major disruption of the structure on exposure to the viscometer is evident. On the other hand, there are significant stress-induced alterations evident in the LDH exposed to

Figure VIII-5. Ultraviolet absorption spectra of lactic dehydrogenase samples with various shear histories. ☆ - no viscometer exposure, aqueous buffer; ★ - no viscometer exposure, g-w mixture. Other symbols have same meaning as in Figure VIII-4.

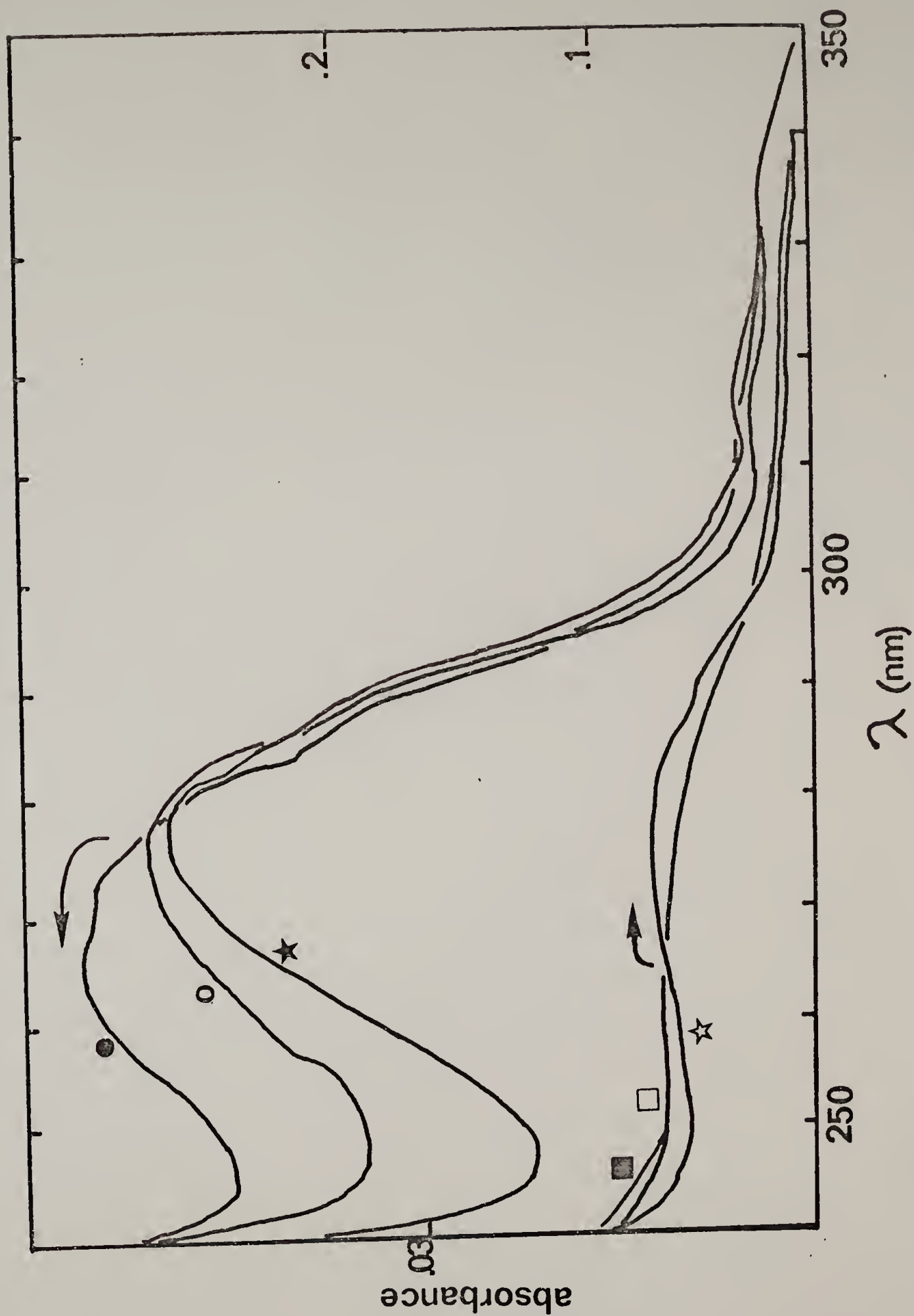
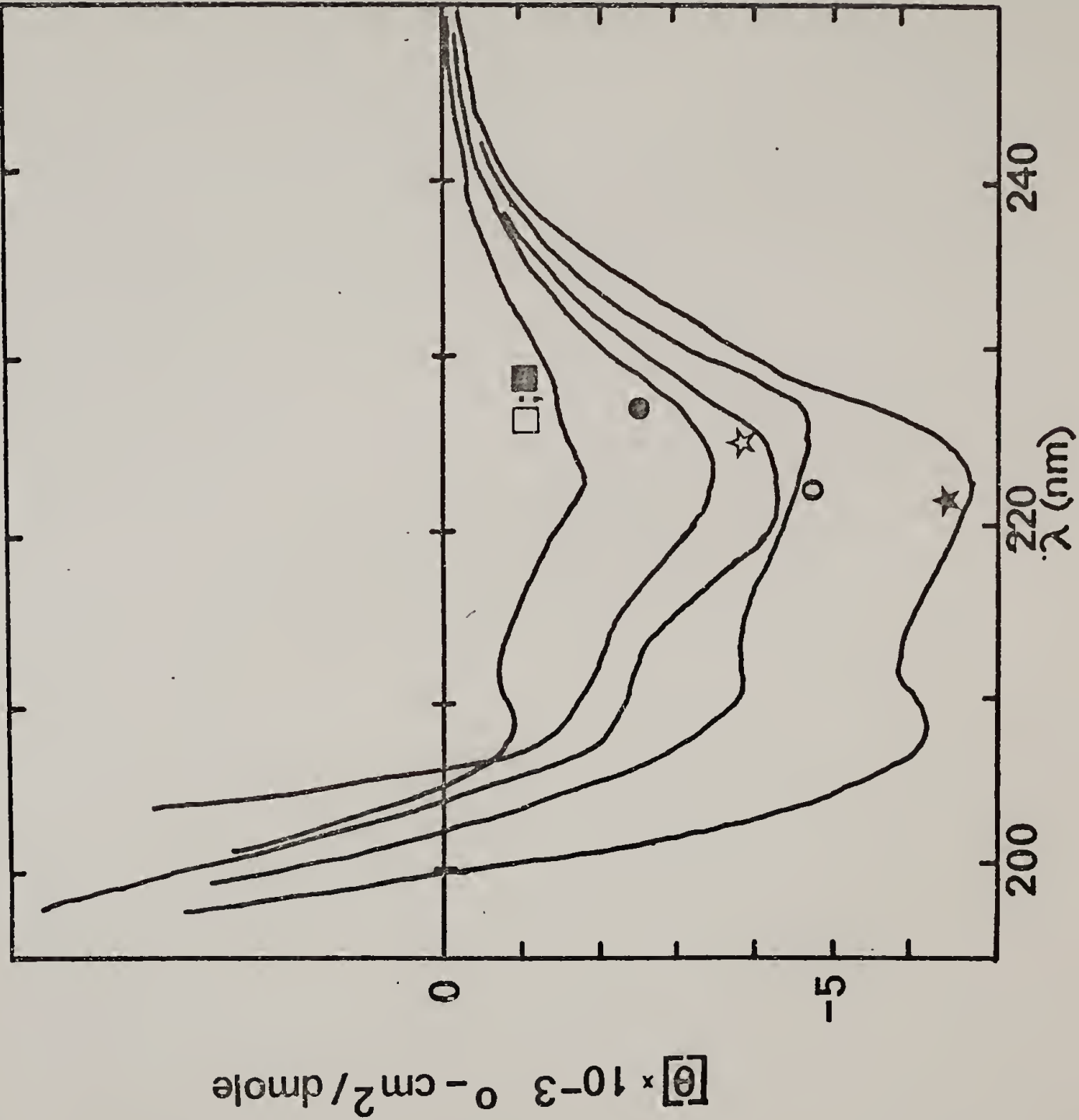


Figure VIII-6. Circular dichroism spectra of lactic dehydrogenase samples with various shear histories. Symbols have same meaning as in Figure VIII-5.



9.36 Pascals. Glycerin also seems to somehow protect the native LDH structure under quiescent conditions. These CD results may be interpretable in terms of the known helical structure of LDH but that will not be attempted here.

To briefly summarize, it has been found that LDH, either alone or with bound substrate and cofactor, is unaffected by shearing forces up to 2.1 Pascals. At higher stress levels, there is inactivation which is shear stress dependent, as in the case of urease, however in the case of LDH the inactivation is at least partially irreversible. A high viscosity reaction medium (as opposed to a preshearing medium) has not yet been devised. Therefore, no results are presently available on modifications of LDH kinetics during shear nor are there any results on the reversibility of any modifications. Efforts in that direction may be expected to be fruitful; likewise, further results on the effects of shear on LDH kinetics and structure may produce a structural interpretation of the shear modification phenomena. It is clear now that it is not necessary to have the huge (for a protein) linearly aggregated structure found in urease in order for shearing forces to modify the catalytic behavior of an enzyme.

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CHAPTER IX

CONCLUSION

Since this work began with the rather vague objective of simply trying to find out what effects, if any, are possible when enzyme catalyzed reactions occur in deforming media, it is not surprising that the information gathered during the course of it covers a rather broad range. The purpose of this concluding chapter is to concisely enumerate the most important discoveries of this work so that it may serve as well as possible as a groundwork for future research. This enumeration is done in section A followed by some thoughts on significant future work in section B.

A. Summary of Results

1. An experimental methodology of conducting enzyme catalyzed reactions in deforming media has been developed.
2. In this type of experiment, urease is inactivated, partially irreversibly, if subjected to shearing stresses in the range 0.5 to 2.5 Pascals, in the presence of micromolar quantities of metal ions and in the absence of chelating or thiol reducing agents.
3. The extent of irreversible inactivation occurring under these conditions can be correlated with the total shear

strain (shear rate x exposure time) experienced by the enzyme. A rule-of-thumb is that shear strains below 10^5 can be tolerated by urease with no loss of activity.

4. A shear-promoted metal ion catalyzed thiol oxidation on a urease cysteine is responsible for the irreversible inactivation.

5. Evidence that ferric ion is responsible has been obtained.

6. This irreversible inactivation due to shearing is decreased when shearing takes place in the absence of urea. Substrate urea binding to urease is thus seen to cause a conformational change, detected by these experiments as enhanced susceptibility to shearing forces.

7. EDTA completely eliminates any irreversible inactivation. Some reversible inactivation is still seen at stresses of 2.0 Pascals or greater. Other chelating agents, especially ferric specific, produce similar effects.

8. β -Mercaptoethanol, at levels greater than 0.01 mM, completely eliminates all inactivation. Parachloro-mercuribenzoate reaction with urease is enhanced both by shearing and by presence of urea.

9. Size exclusion chromatography (SEC) indicates that the high molecular weight urease isozymes are more readily inactivated by shearing. This means that β -met not only protects against the metal ion thiol oxidation but also reduces urease to a more shear stable form. SEC also shows

some shear induced aggregation in urease solutions sheared at [urease] of 0.5 mg/ml or greater. CD spectra of these same solutions show conformational changes consistent with some aggregation.

10. Urease catalyzed reactions have been conducted in high viscosity media in the absence of metal ions. In this regime, stresses from 5.0 to 21.0 Pascals, inactivation seems to be totally reversible, within experimental error. The extent of reversible inactivation, clearly depends on shear stress, not on shear rate. The extent of inactivation reaches about 55% at 21.0 Pascals. β -Mercaptoethanol eliminates this inactivation, also, indicating the necessity of having an intact intermonomer disulfide bond in urease to see any inactivation.

11. Some new structural information on urease has been obtained in the course of this work: (a) CD spectra of native urease have been used to estimate the percentage of α -helical residues in urease at about 8%; (b) intrinsic viscosity measurements have been combined with calculations based on the Abdel-Khalik-Bird theory to demonstrate that urease aggregates are linearly arranged; (c) atomic absorption indicates 12 nickel atoms per urease monomer; (d) some vicinal dithiol compounds, extremely strongly bound nickel ligands, have been shown to irreversibly denature urease, indicating that nickel is essential to urease structural integrity, as well as activity; and (e) urease shows no EPR

signal nor can the urease nickels be made paramagnetic by a 24 hour nitrous oxide exposure.

12. Lactic dehydrogenase catalyzed reactions unaffected by shearing forces up to 2.1 Pascals.

13. LDH is irreversibly inactivated 50% by preexposure to 9.4 Pascals. Inactivation clearly depends on shear stress not on shear rate.

14. Structural changes in LDH are seen by UV and CD spectra to accompany shear inactivation.

15. A good quantitative model of urease shear inactivation has been developed empirically. The physical significance of some of the parameters is obscure, however. A molecular theory of structural deformation in globular proteins is still a task for the future.

B. Possibilities for Future Work

The most direct and potentially fruitful extension of this work seems to be a more detailed study of lactic dehydrogenase. Experiments outlining the critical stress necessary for inactivation could be done with the present apparatus and procedure. A thorough study of activity and structural changes due to stress should be done in order to correlate the two. The problem here will be to interpret in detail the structural changes seen through UV and CD spectra. One path to this objective may be a simultaneous study of the changes in UV and CD spectra caused by some other denaturing

agents. The type of data potentially obtainable from the fluorescence-in-shear device designed by Paladini and Weber (1977) would be especially useful in this endeavor.

It would be advisable to build any future apparatus for this sort of work from some nonmetallic material, given the major metal ion effects demonstrated in this work. A device capable of generating high stresses, say, up to 200 Pascals, in 0.001 Pascal-s fluids is necessary, as well. A capillary flow device, similar to those employed to study rapid enzyme reactions, seems to meet this requirement. The sort of device envisioned would have a substrate solution reservoir and an enzyme solution reservoir. These two solutions would be brought together by a dual syringe pump (or possibly gas pressure on the solution reservoirs may be necessary to achieve high enough flow rate) in a mixing chamber. The design should be such that the stresses experienced by the solutions up to and in the mixing chamber are minimized. The controlled shearing would be done in a set of interchangeable capillaries inserted after the mixing chamber. This set would be composed of capillaries of varying length and diameter, to vary the deformation experience. Analytical procedures to follow the reaction would be applied to the effluent, by appropriate quenching of the reaction, or applied directly, i.e., spectrophotometrically, to the flowing reacting solution. Reversibility of any hydrodynamic effects may be studied by obtaining concentration vs. residence

time data at constant flowrate and cross-section in capillaries of varying length and comparing this to concentration vs. real time data obtained when shearing in any of the capillaries is instantaneously stopped.

So, it is seen that this type of device would be as convenient for every purpose as a Couette device. In addition, higher stresses may be obtained, sampling is easier and large volumes of enzyme samples subjected to identical stress experiences may be prepared for subsequent study, for example, determination of structural changes. Also, very short time behavior may be examined which is impossible with the Couette device. One drawback to the capillary device may be the inhomogeneous velocity field generated, but some averaging of the stress history can probably be applied.

Another type of experiment which may be useful in understanding the molecular basis of these phenomena is one in which an oscillatory strain is applied to an enzyme catalyzed reacting medium. Oscillations may be applied sonically or directly mechanically. Study of the frequency dependence of any modification in the enzyme kinetics may provide a different sort of insight into the conformational dynamics responsible than the stress dependence studies in the present work.

The list of potential future projects could be extended to more speculative areas. It is hoped that this dissertation has been as worthwhile for its thought provoking

character and stimulation for future work as it has for any of the definite results obtained.

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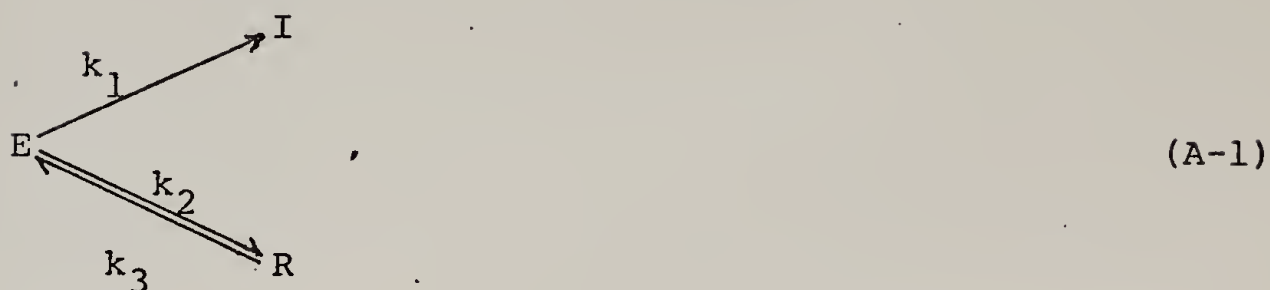
A. Paladini and G. Weber, personal communication, Urbana, Illinois, January, 1977.

A P P E N D I C E S

APPENDIX A

SOLUTION OF EQUATIONS FOR PARALLEL DEACTIVATION MODEL OF CHAPTER V

For the proposed reaction scheme:



the following differential equations result, assuming all first order reactions:

$$\frac{dE}{dt} = -(k_1 + k_2)E + k_3R \quad (A-2)$$

$$\frac{dR}{dt} = k_2E - k_3R.$$

Algebraic equations are obtained on taking the Laplace transforms, using the initial conditions $E = 1$ and $R = 0$ @ $t = 0$:

$$s\varepsilon - 1 = -(k_1 + k_2)\varepsilon + k_3\rho \quad (A-3)$$

$$s\rho = k_2\varepsilon - k_3\rho,$$

where s is the transform variable and $\varepsilon = L(E)$ and $\rho = L(R)$.

Solving for ε gives:

$$\varepsilon = \frac{s+k_3}{(s+k_3)(s+k_1+k_2)-k_2k_3}. \quad (A-4)$$

The algebraic complexity arises on manipulating this to an invertible form. Several steps lead to:

$$\epsilon = \frac{s + k_3}{\left(s + \frac{k_1 + k_2 + k_3}{2}\right)^2 + k_1 k_3 - \frac{1}{4}(k_1 + k_2 + k_3)^2} \quad (\text{A-5})$$

which may be inverted to give the result in Chapter V on consulting any table of Laplace transform pairs.

APPENDIX B

PROCEDURE FOR OBTAINING KINETIC CONSTANTS FOR SIMPLIFIED DEACTIVATION MODEL

For a first-order reaction with first-order catalyst decay, it is easily shown that reactant concentration, C , as a function of time is given by:

$$\ln C/C_0 = \frac{k^0}{k_d} (1 - e^{-k_d t}) \quad (B-1)$$

(other symbols have same meanings as in Chapter V). For an equation of the form:

$$y = y_\infty - Ae^{-Bt}, \quad (B-2)$$

it can easily be shown that:

$$y_\infty = \frac{y_1 y_4 - y_2 y_3}{y_1 + y_4 - y_2 - y_3}, \quad (B-3)$$

where y_1, y_2, y_3, y_4 are the function y evaluated at four equally spaced time intervals t_1, t_2, t_3, t_4 . This is simply verified by substituting the appropriate expressions for y_i from (B-2) into (B-3) and realizing that since the time intervals are equally spaced, $\exp(t_1+t_4) = \exp(t_2+t_3)$.

In Equation (B-1), k^0/k_d may be designated $[\ln C/C_0]_\infty$. This quantity was estimated by application of the above procedure. The composite deactivation constant was then es-

estimated from the slope of a plot of $\ln[\ln(C/C_0) - \ln(C/C_0)_\infty]$ vs. t . The irreversible inactivation constant was estimated by taking the slope at the first stop shear point, calculating a first order zero shear rate constant at this point in time, dividing by k^0 , taking the natural log of this and dividing by the time elapsed to the first stop shear point. This is given as k_1 . The equilibrium constants are then given by the relations in Chapter V.

This technique proved to be very difficult to use to obtain good values of constants from data as sparse as these. Specifically, the entire procedure is very sensitive to the value of $C/C_0|_\infty$ which is not estimated accurately when using as few data points as were obtained here.

APPENDIX C

BASIC SHEAR INACTIVATION DATA IN
NORMAL 0.16 M PHOSPHATE BUFFER

Introduction

This appendix presents in tabular form all the data obtained on urea hydrolysis by urease during and after shearing in the normal phosphate buffer used throughout this research. By "normal," is it meant that the data here are without any additives (EDTA, etc.) present. The format is as follows: (a) each set of data is identified with a Figure number from the main text for cross referencing purposes; (b) within each set the data is tabulated in groups; (c) each group is headed by a data point obtained at the indicated shear rate; and (d) this is followed by any zero shear data obtained on stopping the shear at the point in time indicated with the data point heading the group.

TABLE C-1
 DATA OF FIGURE IV-2a
 Urease A (1 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
0	0	1.000
0	20	0.632
0	30	0.502
0	45	0.364
0	60	0.252
0	90	0.123
1717	30	0.800
0	60	0.590
0	90	0.505
0	120	0.375
0	150	0.277
1717	60	0.685
0	90	0.555
0	120	0.480
0	150	0.415
0	180	0.330
1717	75	0.653
1717	90	0.628
0	120	0.555
0	150	0.486
0	180	0.430
0	210	0.373
1717	120	0.604
1717	180	0.580
0	210	0.530
0	240	0.487
0	270	0.430
0	300	0.385

TABLE C-2

DATA OF FIGURE IV-2b
Urease A (1 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
741	30	0.712
0	60	0.465
0	90	0.385
0	120	0.242
0	150	0.197
741	60	0.565
0	90	0.470
0	120	0.370
0	150	0.260
0	180	0.200
741	90	0.521
0	120	0.415
0	150	0.330
0	180	0.285
0	210	0.210
741	120	0.491
741	180	0.445
0	210	0.320
0	240	0.233
0	270	0.210
0	300	0.175

TABLE C-3
DATA OF FIGURE IV-2c
Urease A (1 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
288	30	0.645
0	50	0.450
0	70	0.333
0	90	0.265
0	120	0.140
288	45	0.553
288	60	0.504
0	90	0.335
0	120	0.300
0	150	0.210
0	180	0.125
288	75	0.472
288	90	0.459
0	120	0.360
0	150	0.210
0	180	0.150
0	210	0.110
288	120	0.429
288	180	0.410
0	210	0.355
0	240	0.315
0	270	0.250
0	300	0.175

TABLE C-4

DATA OF FIGURE IV-5a
Urease B (0.025 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
0	0	1.000
0	60	0.661
0	120	0.368
0	180	0.186
0	240	0.117
1717	120	0.547
0	150	0.470
0	180	0.413
0	210	0.381
0	240	0.355
1717	180	0.459
0	210	0.404
0	240	0.372
0	270	0.360
0	300	0.331
1717	240	0.419
0	270	0.373
0	300	0.348
0	330	0.314
1717	300	0.398
0	330	0.355
0	360	0.322
0	390	0.300

TABLE C-5

DATA OF FIGURE IV-5b
Urease B (0.025 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C ₀
975	45	0.819
0	75	0.710
0	105	0.622
0	135	0.547
0	165	0.472
975	90	0.702
0	105	0.595
0	135	0.529
0	165	0.471
975	120	0.538
0	150	0.415
0	180	0.383
0	210	0.350
0	240	0.328
975	180	0.505
0	210	0.462
0	240	0.413
0	270	0.393
0	300	0.370
975	240	0.452
975	300	0.488
0	330	0.475
0	360	0.463

TABLE C-6
 DATA OF FIGURE IV-5c
 Urease B (0.025 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
288	45	0.761
288	90	0.600
0	105	0.547
0	120	0.502
0	150	0.403
0	180	0.359
288	120	0.605
288	180	0.372
0	210	0.320
0	240	0.262
0	270	0.234
0	300	0.199
288	240	0.340
0	270	0.255
0	300	0.219
0	360	0.177
288	300	0.299
0	330	0.258
0	360	0.232
0	390	0.220
0	420	0.181

TABLE C-7

DATA OF FIGURE IV-6a
Urease C (0.005 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
0	0	1.000
0	15	0.858
0	30	0.640
0	45	0.459
0	60	0.292
0	75	0.192
1717	30	0.920
1717	60	0.750
0	120	0.510
0	210	0.309
1717	90	0.668
1717	120	0.590
0	150	0.487
0	210	0.379
1717	180	0.570
0	270	0.469

TABLE C-8

DATA OF FIGURE IV-6b
Urease C (0.005 mg/ml)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
288	30	0.838
0	60	0.678
0	90	0.570
0	120	
288	60	0.718
288	90	0.640
0	120	0.542
0	150	0.485
0	180	0.402
288	120	0.585
288	180	0.478
0	210	0.442
0	240	0.396
0	300	0.340

APPENDIX D
SHEAR INACTIVATION DATA IN GLYCERIN-BUFFER
MIXTURES

Introduction

This appendix presents in tabular form all the data of Figures IV-28 through IV-32 which were used to obtain the constants in Table IV-6. Format is the same as in Appendix C.

TABLE D-1

DATA OF FIGURE IV-28
Flow Conditions: $\dot{\gamma} = 1717 \text{ s}^{-1}$,
 $\eta = 0.0120 \text{ Pascal-s}$, $\tau = 21.0 \text{ Pascals}$

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
0	0	1.000
0	10	0.831
0	20	0.680
0	30	0.543
0	40	0.423
0	50	0.320
0	60	0.243
0	70	0.200
0	80	0.149
0	90	0.114
1717	15	0.781
1717	15	0.803
0	22.5	0.694
0	30	0.591
0	45	0.457
1717	30	0.680
0	45	0.522
0	60	0.345
1717	45	0.485
1717	45	0.485
0	52.5	0.377
0	60	0.368
0	67.5	0.296
0	75	0.326
1717	60	0.446
1717	60	0.505
0	67.5	0.365
0	75	0.351
0	82.5	0.295
0	90	0.274

TABLE D-1--Continued

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
1717	75	0.318
1717	75	0.342
0	82.5	0.290
0	90	0.261
0	97.5	0.249
0	105	0.211
1717	90	0.231
1717	90	0.243
0	105	0.194
0	120	0.162
0	135	0.128

TABLE D-2--Continued

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
1717	60	0.458
1717	60	0.440
0	75	0.346
0	90	0.248
0	97.5	0.204
1717	75	0.370
1717	75	0.334
0	82.5	0.300
0	90	0.259
0	97.5	0.256
0	105	0.187
1717	90	0.306
1717	90	0.316
0	105	0.206
0	120	0.176
0	150	0.130

TABLE D-3

DATA OF FIGURE IV-30
 Flow Conditions: $\dot{\gamma} = 1301 \text{ s}^{-1}$,
 $\eta = 0.0078 \text{ Pascal-s}$, $\tau = 10.4 \text{ Pascals}$

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
0	0	1.000
0	15	0.772
0	30	0.563
0	45	0.421
0	60	0.313
0	75	0.200
0	90	0.147
1301	15	0.760
0	30	0.640
0	45	0.535
1301	30	0.735
1301	30	0.666
0	37.5	0.590
0	45	0.544
0	52.5	0.490
1301	45	0.497
0	60	0.413
0	75	0.298
1301	60	0.575
0	75	0.446
0	90	0.335
1301	75	0.378
0	90	0.290
0	105	0.241
1301	90	0.365
1301	90	0.331
0	97.5	0.295
0	105	0.245
0	112.5	0.230

TABLE D-4

DATA OF FIGURE IV-31
 Flow Conditions: $\dot{\gamma} = 741 \text{ s}^{-1}$,
 $\eta = 0.0120 \text{ Pascal-s}$, $\tau = 8.9 \text{ Pascals}$
 (no shear data same as in Table D-1)

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
741	0	1.000
741	30	0.532
741	60	0.339
741	90	0.221

TABLE D-5

DATA OF FIGURE IV-32
 Flow Conditions: $\dot{\gamma} = 522 \text{ s}^{-1}$,
 $\eta = 0.0095 \text{ Pascal-s}$, $\tau = 5.0 \text{ Pascals}$

SHEAR RATE (s^{-1})	TIME (min)	C/C_0
0	0	1.000
0	15	0.760
0	90	0.196
522	30	0.625
0	60	0.425
522	75	0.425
0	90	0.335
0	105	0.251
522	90	0.275
522	90	0.286
0	105	0.250
0	112.5	0.220
0	120	0.200
0	120	0.181

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