12-1969

The Purification, Properties and Subunit Structure of Glycerol Dehydrogenase

Michael James Barrett
University of Tennessee Health Science Center

The Purification, Properties and Subunit Structure of Glycerol Dehydrogenase

Abstract

The inducible, NAD-linked glycerol dehydrogenase (E.C. 1.1.1.6) of a guanine requiring mutant of A. aerogenes has been purified to homogeneity. The molecular weight of the pure enzyme was found to be 3.4×10^5 daltons. The sedimentation coefficient of the enzyme was 10.7×10^{-13} sec.$^{-1}$. The diffusion constant was 3.07×10^{-7} cm^2/sec.

The partial specific volume calculated from the amino acid composition was 0.72 ml/g. The following kinetic parameters were determined; K_m glycerol, 2.4×10^{-3} m, K_m NAD, 2.8×10^{-4} M, K_m dihydroxyacetone, 5.1×10^{-4} M, K_m NADH, 2.3×10^{-5} M.

During the course of the purification of the enzyme it was found that multiple peaks of enzyme activity were eluted from ion-exchange chromatography experiments. Ultracentrifugation experiments with the enzyme treated with urea and dithiothreitol, and with dithiothreitol and p- hydroxymercuribenzoate in the presence of guanidinium chloride indicated that the isolated enzyme is composed of subunits. The untreated enzyme, with a molecular weight of 3.4×10^5 daltons, is a hexamer of a species of molecular weight 5.6×10^4 daltons. This species in turn is a dimer of a species of molecular weight 2.8×10^4 daltons. Disulfide bonds between the smallest species are responsible for the stability of the dimer, while the untreated hexamer of the dimers is stabilized by ionic and hydrophobic forces Thirteen major peaks and one minor peak of enzyme activity were consistently eluted during ion-exchange experiments with the native enzyme. Polyacrylamide gel electrophoresis of the enzyme treated with dithiothreitol and urea indicated the presence of two species of protein. The amino acid composition of these two proteins and of the thirteen major peaks are consistent with the hypothesis that these two proteins represent the proteins of molecular weight 2.8×10^4 daltons observed in the ultracentrifuge and that the stiochiometry of the thirteen major peaks may be represented by the formula (A (12-n) B_n), n = 0, 1,..., 12, where A and B represent the two proteins separated by polyacrylamide gel electrophoresis.

Document Type

Dissertation

Degree Name

Doctor of Philosophy (PhD)

Program

Biochemistry

Research Advisor

Charles Walter

Subject Categories

Analytical, Diagnostic and Therapeutic Techniques and Equipment | Chemicals and Drugs | Enzymes and Coenzymes | Investigative Techniques | Medical Biochemistry | Medical Sciences | Medicine and Health Sciences

THE PURIFICATION, PROPERTIES AND SUBUNIT STRUCTURE OF GLYCEROL DEHYDROGENASE

A Dissertation

Presented to the

Graduate Medical Sciences Council

of

The University of Tennessee

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

---

by

Michael James Barrett

December 1969

ACKNOWLEDGEMENT

I am indebted to my major professor, Dr. Charles Walter, for his patience, encouragement and his many helpful criticisms during the time the research reported here was carried out.

I also wish to express my sincere appreciation to the members of my graduate faculty committee, Dr. William Purcell, Dr. Ronald Quintana, Dr. Nathan Sloane and Dr. William Jefferson for the time and effort they extended on my behalf.

Many members of the Department of Biochemistry took an active interest in some portions of my research, as evidenced by their stimulating discussions and by the use of their equipment and expertise. In this regard, I acknowledge Dr. N. H. Sloane, Dr. W. E. Jefferson, Dr. A. F. S. A. Habeeb, Dr. W. E. Groves, Dr. A. J. Sophianopolis and my student colleagues, past and present.

I owe special thanks to the late Dr. M. P. Drake for his many helpful discussions and for assistance in performing the amino acid analyses.

Dr. Robert Macleod gave my invaluable help in the use of the ultracentrifuge.

I am compelled to recognize Dr. Robert J. Hill for giving me more of his time than someone not his student has

a right to ask, free access to the equipment of his laboratory and, most important, an image of the complete scientist.

I acknowledge the financial support of the Department of Biochemistry in the form of a teaching assistantship and a training grant and of the Public Health Service in the form of a predoctoral fellowship.

I dedicate this dissertation to April, whose help and understanding made it possible.

ABSTRACT

The inducible, NAD-linked glycerol dehydrogenase (E.C. 1.1.1.6) of a guanine requiring mutant of A. aerogenes has been purified to homogeneity. The molecular weight of the pure enzyme was found to be 3.4×10^5 daltons. The sedimentation coefficient of the enzyme was 10.7×10^{-13} sec.$^{-1}$. The diffusion constant was 3.07×10^{-7} cm^2/sec. The partial specific volume calculated from the amino acid composition was 0.72 ml/g. The following kinetic parameters were determined; K_m glycerol, 2.4×10^{-3} M, K_m NAD, 2.8×10^{-4} M, K_m dihydroxyacetone, 5.1×10^{-4} M, K_m NADH, 2.3×10^{-5} M.

During the course of the purification of the enzyme it was found that multiple peaks of enzyme activity were eluted from ion-exchange chromatography experiments. Ultracentrifugation experiments with the enzyme treated with urea and dithiothreitol, and with dithiothreitol and p-hydroxymercuribenzoate in the presence of guanidinium chloride indicated that the isolated enzyme is composed of subunits. The untreated enzyme, with a molecular weight of 3.4×10^5 daltons, is a hexamer of a species of molecular weight 5.6×10^4 daltons. This species in turn is a dimer

of a species of molecular weight 2.8×10^4 daltons. Disulfide bonds between the smallest species are responsible for the stability of the dimer, while the untreated hexamer of the dimers is stabilized by ionic and hydrophobic forces.

Thirteen major peaks and one minor peak of enzyme activity were consistently eluted during ion-exchange experiments with the native enzyme. Polyacrylamide gel electrophoresis of the enzyme treated with dithiothreitol and urea indicated the presence of two species of protein. The amino acid composition of these two proteins and of the thirteen major peaks are consistent with the hypothesis that these two proteins represent the proteins of molecular weight 2.8×10^4 daltons observed in the ultracentrifuge and that the stiochiometry of the thirteen major peaks may be represented by the formula $(A_{(12-n)}B_n)$, $n = 0, 1, \ldots, 12$, where A and B represent the two proteins separated by polyacrylamide gel electrophoresis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

FIGURE PAGE

CHAPTER I

REVIEW OF THE LITERATURE

Quaternary Structure of Proteins

Early work. The work of Fisher (1907) and Bergmann (1932) established that the primary covalent link in proteins was the peptide bond and that proteins were for the most part linear polymers of the amino acids. The similarity of behavior of protein solutions and colloidal suspensions, e.g., slow diffusion, membrane impermeability and sensitivity to salt, led early workers to believe that proteins, like the well-studied gold hydrosols, were clusters of small molecules of undefined mass. The work of Svedberg (1926) with the ultracentrifuge and of Sumner (1926) on the crystallization of enzymes made it clear that proteins are well defined entities of unique molecular weight. Svedberg (1938) after studying the molecular weights of a large number of respiratory proteins concluded that these molecular weights could be expressed as integral multiples of a unit of mass of 1.7×10^4 daltons. The fact that the annelid respiratory proteins studied were of high molecular weight coupled with the fact that only a few small proteins (lactalbumin, hemoglobin and cytochrome c) were studied would make almost any divisor appear in integral multiples.

This is especially true since there was considerable error in these early studies. Nevertheless, the basic concept that large proteins were assemblies of proteins of smaller size appearing in integral multiples was established.

Renewed emphasis on the characterization of the subunit structure of proteins has developed since the observation of two related phenomena. The first concerns the appearance of multiple molecular forms of a single enzyme activity (isozymes); the second is related to the observations of Yates and Pardee (1956) and Umbarger (1956) that the activity of certain biosynthetic enzymes is modified i.e., either inhibited or activated, by compounds unrelated structurally to the substrates of the enzymes, but which are intermediates or end-products of the biosynthetic path in which the enzyme participates. In the first case the most common basis for the appearance of isozymes was found to be due to the association in different stoichiometries of two or more subunits of the enzyme to yield a set of electrophoretically or otherwise distinct forms of the enzyme. Lactic acid dehydrogenase (LDH) was the first and most thoroughly documented example of this type (Apella and Markert, 1961; Markert, 1963). In the second case the theoretical (Monod, et al., 1965; Gerhart and Pardee, 1962) and experimental (Gerhart and Schachman,

1965) development of the basis for this type of behavior led to the postulate that the fact that such proteins were composed of subunits was intimately related to the observed behavior of these enzymes.

Since the initial reports of enzyme systems of the two types, an extensive literature describing new enzymes of either kind and attempting to assign metabolic significance to them has developed. Reviews of the literature on both these topics are available (Stadtman, 1966; Vessel, 1968). This discussion will limit itself to a description of an enzyme of each kind in order to illustrate the features of the respective types.

Lactic acid dehydrogenase. Meister (1950) and Nielands (1952) demonstrated that an apparently pure preparation of beef heart LDH was in fact composed of two catalytically active components. Wieland Pfleiderer (1957) and Markert and Moller (1959) showed that there were as many as five forms of LDH in most animal tissues and that the relative amount of each form varied in different tissues from the same animal. Kaplan and Ciotti (1961), indicated that these five forms originated from only two main forms of the enzyme. One form is designated M since it occurs largely in skeletal muscle while the second form, type H,

is usually found in heart muscle. Each major form was found to have a molecular weight of 1.4-1.5 x 10^5 daltons and was dissociable into four proteins each of molecular weight 3.5 x 10^4 daltons (Apella and Markert, 1961). When mixtures of the two forms of the enzyme were subjected to dissociating conditions and then allowed to recombine, five forms of LDH activity were observed after electrophoresis or ion-exchange chromatography (Markert, 1963). These hybrids were shown (DiSabato and Kaplan, 1964) to be composed of subunits of the two main forms in the stoichiometry M_nH_{4-n}, n = 0, 1... 4, thus accounting for the natural occurrence of five forms of activity.

The evidence relating the physiological significance of the isozymes to the sensitivity of each form to pyruvate inhibition has been reviewed by Kaplan (Kaplan et al., 1968). The H-type enzyme is strongly inhibited by pyruvate at low concentrations. It was postulated that this form should be present in aerobically functioning tissues, e.g., brain and heart, because there is not much pyruvate accumulation under aerobic conditions. The M-type enzyme is much less sensitive to pyruvate inhibition. Thus the presence of this form in anaerobically functioning tissue such as skeletal muscle would allow reoxidation of the NADH necessary for energy metabolism under anaerobic conditions.

The greater levels of accumulated pyruvate in rapidly exercising muscle would inhibit the H-form of the enzyme if it were present, preventing the further oxidation of lactate to pyruvate and thus depressing the level of NADH.

On the basis of this hypothesis a predominance of H-type LDH should be found in those tissues which function aerobically while the opposite should be true for the M-form of the enzyme. This has been found to be the case. Wilson et al. (1963) demonstrated that the M-type enzyme is present in the breast muscles of chicken and pheasant which require energy for short flight while birds which are capable of long flight contain mostly H-LDH in their breast muscles. Similar correlations have been found for enzymes from different parts of kidney (Leonhardt and Landes, 1963) and in chicken muscles functioning under varying degrees of oxygen availability (Kaplan and Cahn, 1962).

Aspartic acid transcarbamylase (ATCase). A common type of metabolic control seems to involve the regulation of the substrate affinity of an enzyme catalyzing the first reaction after a metabolic branch point. This regulation aims to control the relative effectiveness with which enzymes having a common intermediate at the branch point compete with each other for the intermediate. In addition such regulation serves to conserve a substrate when adequate

amounts of the end product are present.

Yates and Pardee (1956) observed the inhibition of ATCase from E. coli by derivatives of cytosine. This enzyme catalyzes the condensation of carbamyl phosphate with aspartic acid to yield carbamyl aspartate, the latter being the initial step in the synthesis of the pyrimidines. Subsequently Gerhart and Pardee (1962) reported that various treatments, e.g., heating, destroyed the sensitivity of ATCase to cytosine while at the same time leaving the catalytic activity of the enzyme unaffected. On the basis of these observations Gerhart and Pardee (1962) and Monod and Jacob (1961) proposed the existence of two separate but interacting sites for substrate and inhibitor. Later it was found that ATCase could be dissociated into two types of subunits. Only one subunit bound cytosine derivatives while the other contained the catalytic activity (Gerhart and Schachman, 1965). In addition it was found that treatment which destroyed cytosine sensitivity also altered the typical sigmoid shape of plots of initial steady state rate versus initial substrate concentration into the usual rectangular hyperbola commonly observed in classical enzyme kinetics. Initial estimates of the molecular weight of the two types of subunits were in error because conditions used to dissociate the native enzyme were inadequate. Weber (1968)

finally reported that the catalytic portion of the ATCase protein had a molecular weight of 1.7×10^4 daltons and that the molecular weight of the regulatory protein was 3.3×10^4 daltons. The undissociated enzyme has a molecular weight of 3.1×10^5 daltons and is composed of six of each type of subunit. Kinetic models to account for the observed sigmoid shape of plots of initial rate *versus* initial substrate concentration have usually required the existence of separate regulatory and catalytic sites (Monod *et al.*, 1965) although the necessity of such a distinction has been challenged (Walter and Frieden, 1963). Nevertheless, it appears that those enzymes which seem to function as control enzymes do have separate binding sites for substrate and effector molecules. It is also generally true that these same enzymes have been found to be composed of subunits.

Subunit structure and function. There does not appear to be any *a priori* reason why enzymes of both kinds are found to be composed of subunits. In the case of isozymic proteins it may be that an organism can achieve a wider variety of enzymes each with similar activity but with slightly different properties (e.g., sensitivity to substrate inhibition) by mutations in polymeric enzymes rather than by mutations in an enzyme consisting of a single polypeptide. Thus by mutation

of the subunit peptide of an original LDH the organism generated five different isozymes rather than merely two. In any case the most significant genetic reason for the existence of isozymes appears to be the combination in various stoichiometries of two or more subunits.

A common feature emerging from the study of control enzymes is that the quaternary structure as well as the kinetic parameters of the enzyme are altered by binding of the specific effector molecule. One of the earliest reports of this phenomenon was the observation by Haurowitz (1938) that crystals of oxyhemoglobin break up when they are deoxygenated, showing that the two crystal forms are not isomorphous. The conformational differences between the two types of hemoglobin have also been observed from nuclear magnetic resonance studies (Lumry et al., 1961), from X-ray studies (Muirhead and Perutz, 1963) and from the rates of reaction of the two hemoglobins with various ligands (Antonini et al., 1963). Other control enzymes display similar changes. Thus deoxycytidylate deaminase was shown to undergo aggregation in the presence of deoxycytidine-5'-triphosphate. The aggregation reaction paralleled the increase in the affinity of the enzyme for the substrate (Maley and Maley, 1964). Similarly, acetylcoenzyme-A carboxylase is activated by citrate with a concomitant increase in the molecular

weight (Vagelos et al., 1963). Frieden (1959) found that the presence of both reduced and oxidized di- and tri-phosphopyridine nucleotides caused an association of glutamate dehydrogenase. The presence of these compounds also resulted in increasing rates of reaction. Mitochondrial isocitric acid dehydrogenase, inactive in the aggregated form, is activated by citrate, isocitrate, Mg^{2+}, and Mn^{2+}. These compounds also cause a decrease in the molecular weight of the enzyme (LeJon et al., 1969). The fructose 1,6-diphosphatase of E. coli is reversibly dissociated in acid pH to a form whose molecular weight is one-half that of the native enzyme. The smaller molecule has diminished catalytic activity and is substantially less sensitive to AMP inhibition. The presence of fructose 1,6-diphosphate tends to maintain the native state whereas high concentrations of AMP permit dissociation into the less active form (Rosen et al., 1966). The homoserine dehydrogenase of Rhodospirillum rubrum is inhibited by threonine, which also causes aggregation of the enzyme into a catalytically inactive form. Homoserine, isoleucine and methionine are activators of the enzyme and can reverse this aggregation (Datta et al., 1964). A correlation exists between the catalytic activity and physical state of formyltetrahydrolfolate synthetase. The inactivity of the enzyme in the absence of certain cations is due to the

fact that the enzyme is dissociated in their absence into catalytically inactive subunits (Scott and Rabinowitz, 1967). The threonine-sensitive aspartokinase of E. coli appears to undergo a decrease in molecular weight in the presence of this compound (Wampler and Westhead, 1968).

Although these examples illustrate the structural changes which occur in control enzymes upon binding of specific effector molecules, it is not certain that the physical association-dissociation relationships are the only means by which these molecules regulate enzyme activity. Thus extensive alteration in the state of aggregation of many enzymes of this type are not always associated with the action of regulator metabolites. The dissociation of glutamate dehydrogenase into subunits which occurs upon dilution is not accompanied by a loss of enzyme activity (Fisher et al., 1962). However, the absence of observed changes in the state of aggregation of some of these regulatory enzymes may be due "to differences in the strength of subunit interactions which may govern the activity of the catalytic site and which incidentally may or may not allow association and dissociation to occur." (Stadtman, 1966).

The distinction between isozymic enzymes and regulatory enzymes made here may be more logical than real.

Thus E. coli elaborates three different aspartokinases. One, the synthesis of which is repressed by lysine, is also strongly inhibited by lysine. A second is inhibited by threonine, while a third, present in small amounts, is inhibited by homoserine. In turn each aspartokinase is unaffected by compounds which inhibit the other two. These enzymes are clearly control enzymes catalyzing the formation of a product required in the synthesis of each of the amino acids mentioned above, and these enzymes also represent multiple molecular forms of the same enzyme activity although they do not seem to be related to each other in the way the isozymes of LDH are related (Stadtman et al., 1961).

These results indicate that although no necessary relation may exist between the functional role of these enzymes and the fact that they are also composed of subunits, the characterization of the subunit stoichiometry is an essential requirement in the accurate description of new enzymes. This information is even more essential in the case of enzymes which catalyze a reaction at metabolic branch points or which control the energy state of the organism.

Glycerol Dehydrogenase

Occurrence. The oxidation of glycerol to dihydroxyacetone (DHA) by micro-organisms is well known (Bertrand,

1896) and is the basis for the commercial preparation of DHA (Underkofler and Fulmer, 1937).

Enzymes which oxidize glycerol to DHA or glyceraldehyde have been found in a wide variety of organisms. NAD-dependent glycerol dehydrogenases have been found in E. coli (Asnis and Brodie, 1953), Bacillus subtilis (Wiame et al., 1954a, 1954b), Pseudomonas salinaria and Vibrio costicolos (Baxter and Gibbons, 1955), Acetobacter suboxydans (Hauge et al., 1955), Aerobacter aerogenes (Burton and Kaplan, 1953), Streptococcus faecalis (Jacobs and Vandemark, 1960), and Mycobacterium smegmantis (Widner et al., 1961). Both NAD- as well as NADP-dependent glycerol dehydrogenases have been found in rat liver (Leuthardt and Wolfe, 1954; Moore, 1959). In this case the oxidation product was found to be dl-glyceraldehyde. A strictly NADP-dependent enzyme was found in rat skeletal muscle (Toews, 1966), the oxidation product being dl-glyceraldehyde.

Glycerol dehydrogenases which are NADP dependent have been found in Candida albicans (Rao et al., 1960), Aspergillus niger (Baliga et al., 1962), in the conidia of Aspergillus oryzae (Horikoshi and Ikeda, 1965), and in Penicillium chrysogenum (Chiang and Knight, 1959).

Metabolic role. The principal metabolic role of this enzyme in organisms in which it has been studied is to

provide an alternate pathway of glycerol metabolism which does not require phosphorylation of glycerol prior to its metabolism. Thus in strain S_1^- of B. subtilis, glycerol is initially oxidized to DHA by an NAD-linked glycerol dehydrogenase. Subsequent metabolism of DHA requires phosphorylation to dihydroxyacetone phosphate in a specific kinase reaction (Wiame et al., 1954a). The oxidation of glycerol in cell-free extracts of Acetobacter suboxydans (Hauge et al., 1955) has been found to proceed by two pathways. One is independent of ATP and NAD and proceeds at pH 6 to the formation of DHA; the second, with a pH optimum of 8.5, requires ATP and Mg^{2+} in a kinase reaction to yield α-glycerolphosphate. The absence of an NAD requirement in the first case suggested that the glycerol dehydrogenase may be coupled directly to a bacterial cytochrome as the immediate hydrogen acceptor. Cell-free extracts of S. faecalis (Jacobs and Vandemark, 1960) grown on glucose display two different glycerol oxidizing systems, depending on whether the original cells were grown aerobically or anaerobically. In the case of extracts of cells grown aerobically, glycerol is first phosphorylated by a glycerol kinase and subsequently oxidized by a flavine adenine dinucleotide-linked α-glycerolphosphate oxidase. Anaerobically grown cells are deficient in these two enzymes, and glycerol is first

oxidized by an NAD-dependent glycerol dehydrogenase which is coupled with a flavine-linked NADH oxidizing system.

Magasanik (Magasanik et al., 1953) has described two strains of A. aerogenes. Glycerol is metabolized in the usual fashion in the first strain (1041); in the second (1033), glycerol induces a glycerol dehydrogenase in cells grown either aerobically or anaerobically. The presence of DHA in the aerobic and anaerobic metabolism of glycerol was demonstrated in the second case.

Glycerol dehydrogenase from a guanine-requiring mutant of A. aerogenes. Lin (Lin and Magasanik, 1960; Lin et al., 1960) described the isolation, partial purification and some properties of an inducible NAD-linked glycerol dehydrogenase from a guanine-requiring mutant of strain 1033 of A. aerogenes (AGDH) (Ushiba and Magasanik, 1952). Unlike the parent strain, AGDH is induced in the mutant only when the bacteria are grown anaerobically or under low oxygen tension. When cells are grown under 95 per cent oxygen, glycerol induces a glycerol kinase and an α-glycerol phosphate dehydrogenase which is not NAD-dependent.

Of greater interest from the viewpoint of metabolic control, however, is the fate of AGDH in bacteria exposed to high oxygen tension after initial growth on glycerol under low oxygen tension. The rate of inactivation of AGDH is

greater than can be accounted for by simple dilution of existing enzyme protein during the course of growth on glycerol and under high oxygen tension. Both an energy source (i.e., glycerol or glucose) and oxygen are required for inactivation to take place. Thus when cells grown under low oxygen tension are transferred to a medium with no carbon source but under high oxygen tension or to a medium with a carbon source but kept under nitrogen, inactivation of AGDH does not occur. In addition, both 2,4-dinitrophenol and arsenite inhibit inactivation in cells grown in the presence of both a carbon source and oxygen.

On the other hand, protein synthesis is not required for inactivation of AGDH since the addition of chloramphenicol to a culture with an energy source and high oxygen tension does not prevent inactivation. Furthermore, growth of the transferred bacteria is not necessary for inactivation since enzyme activity is lost even when a nitrogen source (ammonium sulfate) or guanine is absent from an otherwise complete growth medium. Finally, evidence was obtained which indicated that inactivation did not occur by breakdown of enzyme protein into its constituent amino acids.

While experiments on an impure preparation of the enzyme did not indicate a mechanism for inactivation, the results did show that AGDH was activated by monovalent

cations and especially by NH_4^+ (Lin and Magasanik, 1960). Work by Strickland and Miller (1968) demonstrated that AGDH from an impure preparation was inhibited by high concentrations of Li^+ and Na^+ as well as by DHA at a substrate concentration above 0.4 millimolar.

CHAPTER II

STATEMENT OF THE PROBLEM

It is to the benefit of an organism to be able to eliminate unnecessary or redundant metabolic pathways as conditions of growth are changed. The data of Lin (Lin et al., 1960) suggest that the inactivation of AGDH is the point at which this control is exercised in the case of A. aerogenes. Further, the data imply that the inactivation of the enzyme is related to the presence of metabolites normally absent during growth under anaerobic conditions but present during metabolism under high oxygen tension.

In view of the interesting role of this enzyme it was felt that further characterization would be useful. This dissertation therefore describes the results of the purification to homogeneity of the hitherto only partially purified glycerol dehydrogenase from this bacterium and describes some of the properties of the pure enzyme. In addition, the role of this enzyme as a possible metabolic control point coupled with the emerging significance of the quaternary structure of such enzymes prompted an investigation into the subunit structure of the enzyme. The results of that investigation are also described.

CHAPTER III

EXPERIMENTAL SECTION

Source of Materials

Phenazine sulfate, nitro blue tetrazolium, NAD, NADH, dithiothreitol (DTT), p-hydroxymercuribenzoate (PMB), iodoacetic acid, protamine sulfate, and guanine were obtained from the Sigma Chemical Co.; acrylamide, N,N'-methylenebisacrylamide and N,N,N',N'-tetramethylenediamine (TMED) from the Eastman Kodak Co.; dihydroxyacetone from Mann Research Laboratories and glycerol from the Matheson, Coleman and Bell Company. Urea was purchased from the Fisher Chemical Co., and all urea solutions were passed through a Dowex AG-501-X8(D) (Dow Chemical Co.) mixed-bed, ion-exchange resin before use. Guanidinium chloride (GDC) was a gift of Dr. E. P. K. Hade, prepared from the carbonate by the method of Nozaki and Tanford (1967). Tris (2-amino-2-(hydroxymethyl)-1,3-propanediol) was the Trizma Base grade of the Sigma Chemical Co. This material was found to have an ultraviolet absorbing impurity which was concentrated along with the enzyme during the concentration step of the enzyme purification procedure. Therefore, solutions of 0.1 M Tris-HCl, routinely used in the column chromatography of the enzyme, were first passed through a Sephadex DEAE A-50 (Pharmacia, Inc.) ion-exchange column before use.

Water was prepared by passing distilled water through a Barnstead Standard Demineralizing column and then redistilling in a glass still before use. All other chemicals were reagent grade.

Aerobacter aerogenes, strain 1033, mutant P-14 (guanine-requiring) described by Ushiba and Magasanik (1952) was a gift of Dr. Magasanik and was maintained by monthly transfers on Yeast-Dextrose Agar (Difco Laboratories).

Methods

Growth of the bacteria. The growth medium was composed of the following constituents:

KH_2PO_4	12.6 g/liter
K_2HPO_4	4.5 g/liter
$(NH_4)_2SO_4$	2.0 g/liter
$MgSO_4 \cdot 7H_2O$	0.2 g/liter
$CaCl_2$	0.01 g/liter
guanine	40.0 mg/liter
glycerol	10.0 g/liter

A solution containing all of the components except glycerol was prepared and adjusted to pH 6.8 with 1.0 N NaOH, and autoclaved for 2 hours at 15 pounds per square inch (p.s.i.) and 115° (centigrade). A separate solution of 10 per cent glycerol was autoclaved for 15 minutes at

p.s.i. and 115° and the correct amount of this solution was added aseptically to the sterile salt plus guanine solution to give the desired concentration of glycerol.

In the laboratory, the volume of growth medium used was 32 liters (l.). The growth temperature was maintained at 37° by placing two 16-l. carboys in a thermostated box. The medium was inoculated with a 100 ml solution of the bacteria which had been grown overnight. Growth was allowed to proceed for 18 hours, beyond which time there was no increase in the optical density of the bacteria suspension measured at 360 nanometers (nm). The growth medium was agitated during the growth period by gently bubbling filtered air through a gas tube immersed in the medium.

The bacteria were harvested by centrifuging the suspension in a continuous-flow Sharples Centrifuge operating at maximum speed. The flow rate through the centrifuge was 500 ml per minute and at this flow rate there was no significant rise in the temperature of the out-flowing solution. The yield of bacteria from this volume of growth medium was about 200 g wet weight.

Isolation and purification of glycerol dehydrogenase (AGDH). The following procedures were carried out at 0-4° in a cold room or ice bath except where indicated. Volumes of the various buffers used are based on the yield of

bacteria from 32 l. of growth medium.

The bacteria paste was suspended in 400 ml of cold, distilled water and centrifuged at 10,000 x gravity (g) for ten minutes, then resuspended in 320 ml of 0.01 M sodium phosphate buffer pH 7.4. The suspension was then sonically disrupted with a Brownwill Sonifier operated at maximum power in three 100-ml portions in a stainless steel beaker immersed in an ice bath. Each portion was treated for two minutes with alternate cooling for two minutes for a total sonicating time of six minutes. The cellular debris was removed by centrifuging the sonically treated preparation at 10,000 x g for 15 minutes. The supernatant was adjusted to pH 7.0, and 0.23 mg of protamine sulfate (Sigma Chemical Co., Grade II) per mg of protein in the crude extract was added as a one per cent aqueous solution (also adjusted to pH 7.0) to the crude extract with continuous stirring. This suspension was then centrifuged at 10,000 x g for 15 minutes and the supernatant decanted and assayed. The solution was made 50 per cent saturated with $(NH_4)_2SO_4$ by adding a sufficient quantity of the solid reagent, and the precipitate which formed was collected by centrifuging the suspension at 10,000 x g for 15 minutes. In this case the supernatant was discarded and the precipitate which contained the enzyme activity was dissolved in 80 ml of cold

water and assayed for protein and enzyme activity. The solution of dissolved precipitate was adjusted to pH 7.0 with 1.0 N NaOH, immersed in a boiling water bath until the temperature of the solution was 60°, and then maintained at that temperature for four minutes in a thermostated water bath. The coagulated protein was removed by centrifuging the suspension for 15 minutes at 10,000 x g and then the supernatant was decanted and assayed. At this point the preparation was lyophilized and stored in the freezer until needed.

For gel filtration chromatography, 200 mg of the lyophilized preparation was dissolved in 10 ml of a solution of 0.1 M Tris·HCl, pH 7.6, and applied to a 5-cm by 100-cm column filled with Agarose A-0.5 M (Bio-Rad Co.) previously equilibrated with this same buffer. The protein was eluted with the starting buffer at a flow rate of 20 ml per hour and collected in 15-ml fractions. A typical elution pattern is shown in Figure 1.

Ion exchange chromatography of the peak fractions from the gel filtration procedure was performed using either Sephadex DEAE A-50 or DEAE A-25. In both cases the experimental conditions were the same. The ion-exchange material was equilibrated with a solution of 0.1 M Tris·HCl, pH 8.2, and a 2.5-cm by 45-cm column was filled

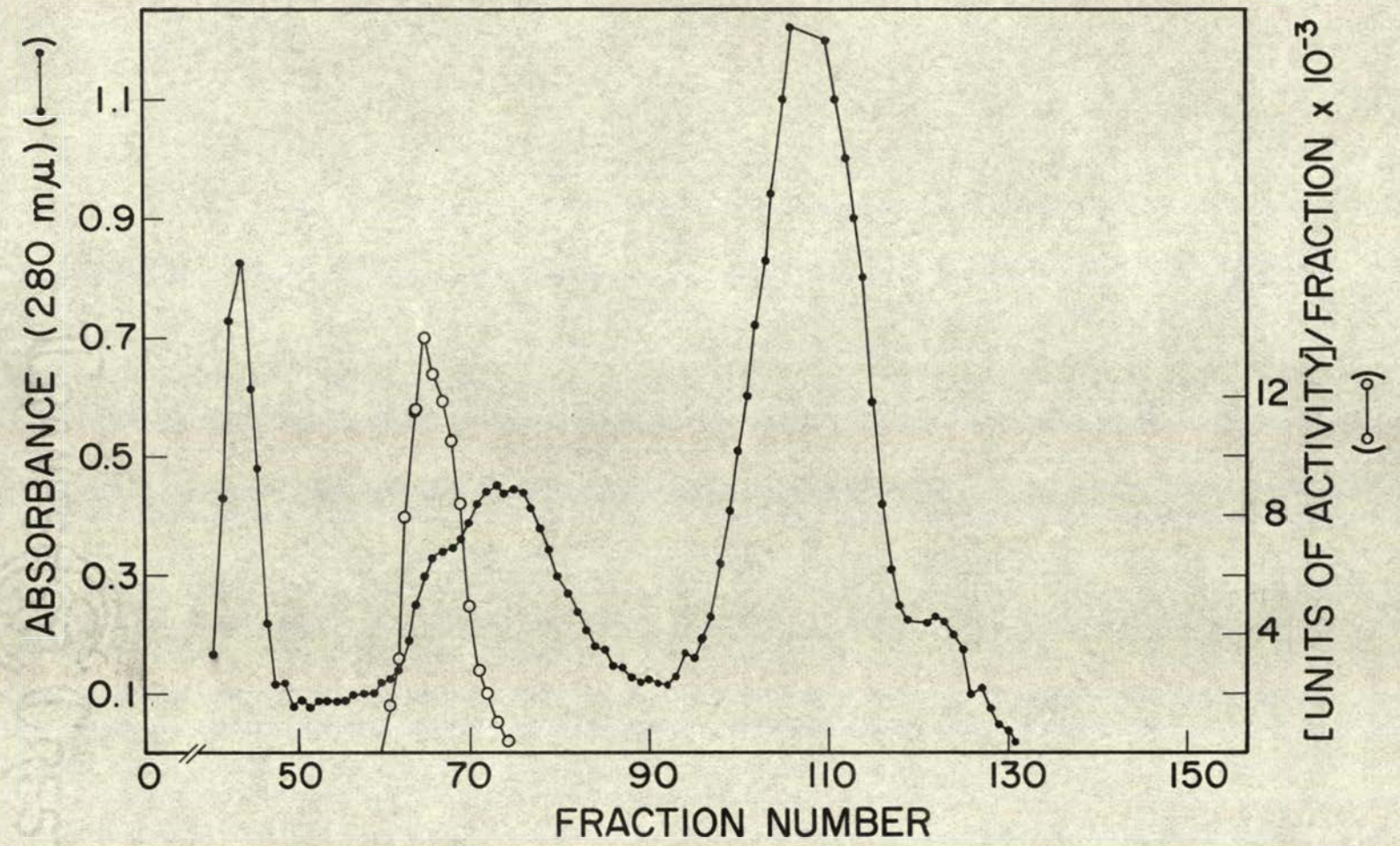

Figure 1. Gel filtration chromatography of glycerol dehydrogenase.

with the resin. The peak fractions from the gel-filtration chromatography experiment were pooled and applied to the ion-exchange column. The protein was eluted with a linear gradient of KCl concentration formed from a reservoir containing 2 l. of a solution of 0.1 M Tris·HCl, pH 8.2, and 0.5 M KCl, and a mixing chamber which contained 2 l. of a solution of 0.1 M Tris·HCl, pH 8.2. The flow rate was 10 ml per hour for the experiments with the A-50 material and 60 ml per hour for the A-25 material. The results of a typical experiment with the A-25 material are shown in Figure 2 when 15 ml fractions were collected. Figure 3 illustrates the elution pattern obtained when the A-50 material was used. In this case, 20-ml fractions were collected. The significance of these results with respect to the structure of the protein will be discussed later (Chapter VI, page 125).

In both cases, the protein was concentrated by pooling the peak fractions, diluting with an equal volume of water and applying the solution to a 1-cm by 5-cm column filled with DEAE A-25 equilibrated as before. The enzyme was eluted from the column with a solution of 0.1 M Tris·HCl, pH 8.2, and 0.5 M KCl. Most of the activity was recovered in a volume of 5 to 8 ml. The concentrated enzyme was dialyzed against a saturated solution of $(NH_4)_2$

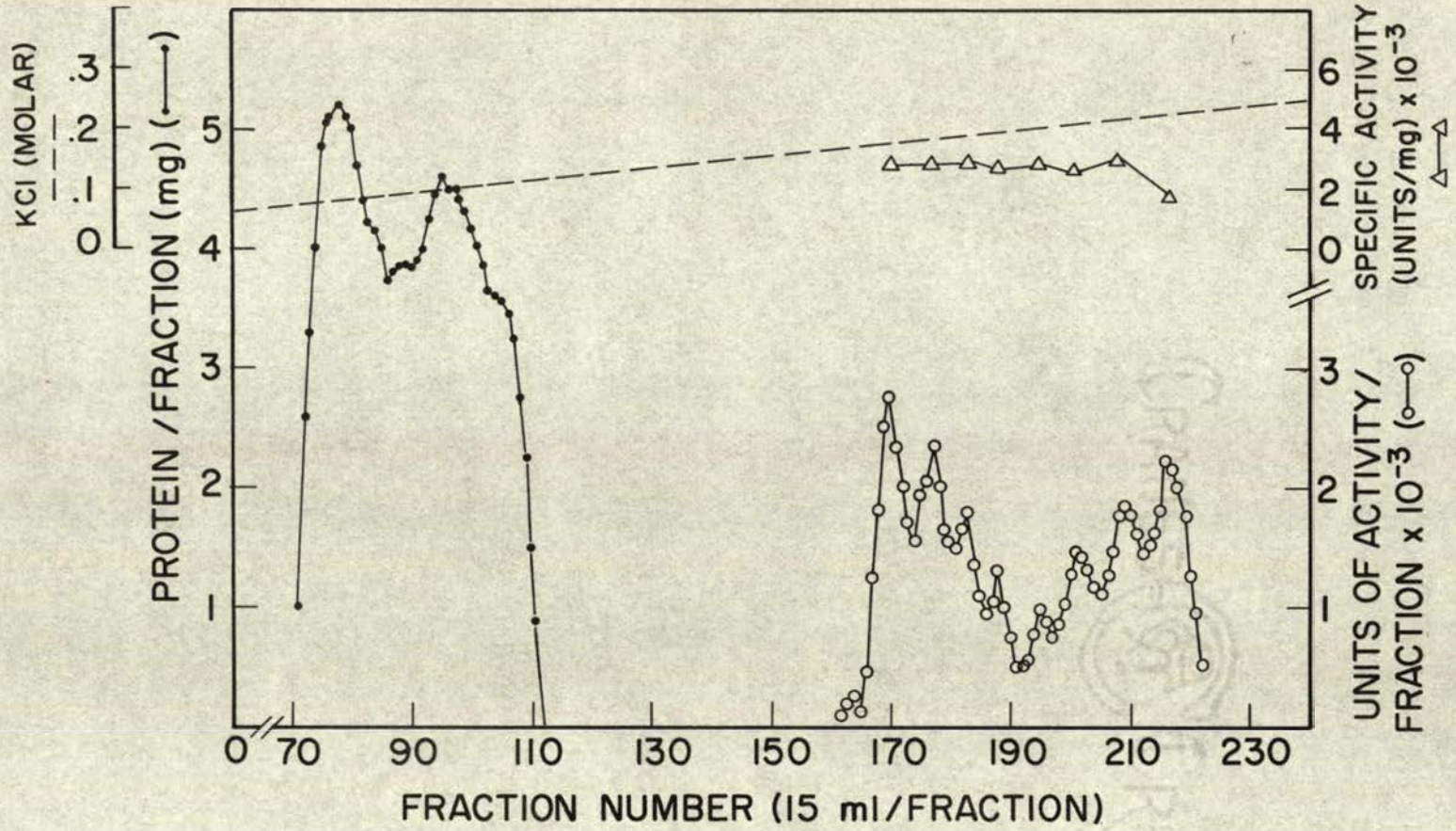

Figure 2. Ion-exchange chromatography of glycerol dehydrogenase on Sephadex DEAE A-25.

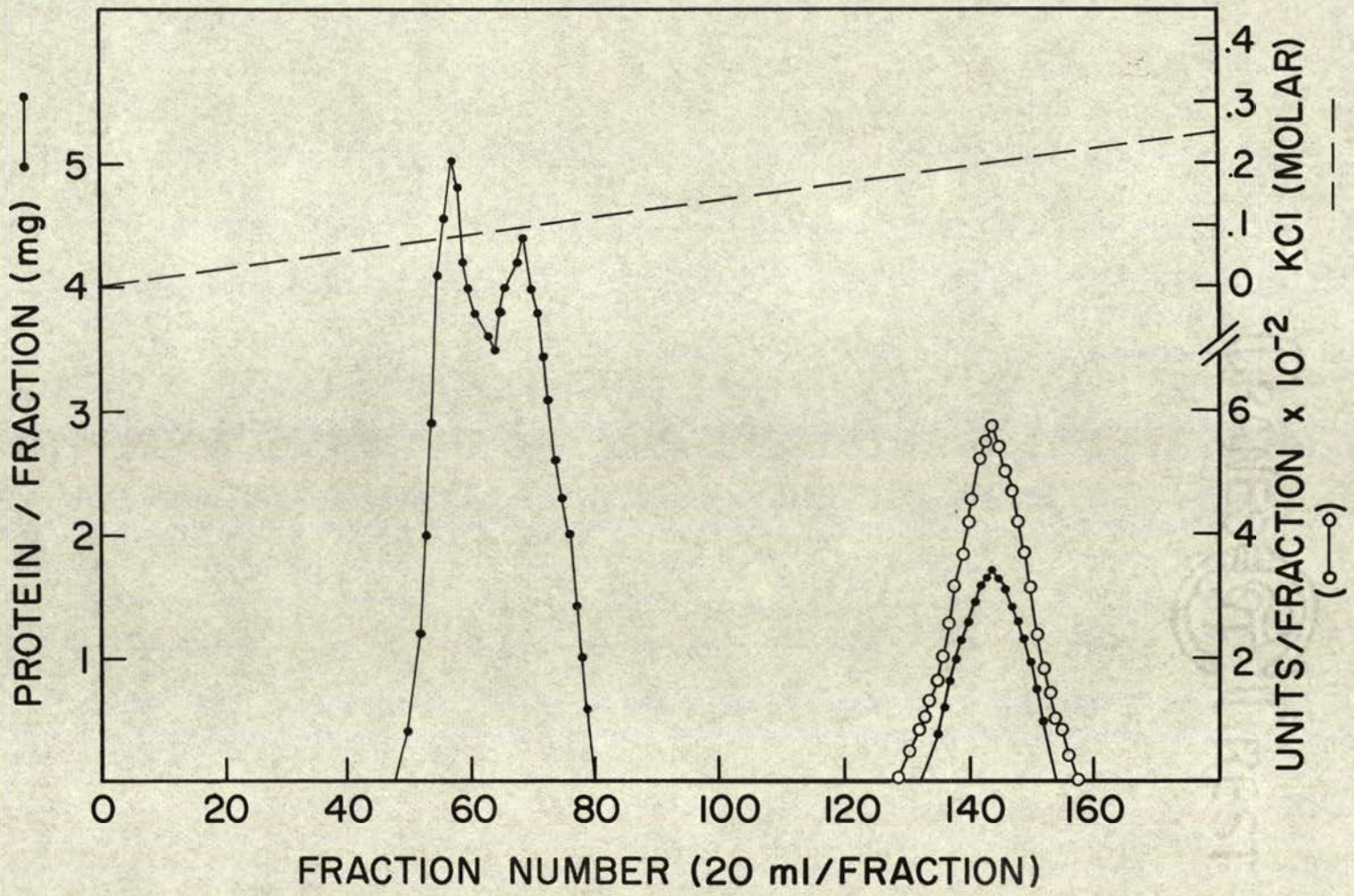

Figure 3. Ion-exchange chromatography of glycerol dehydrogenase on Sephadex DEAE A-50.

SO_4 and the precipitated enzyme collected by centrifugation at 5,000 x g for five minutes and stored as a precipitate in saturated $(NH_4)_2SO_4$ at 0°-4°. Stored in this manner, the preparation loses about ten per cent of the initial total activity per month. The results of a typical laboratory scale preparation (32 l. growth medium) are summarized in Table I.

Determination of protein. The Lowry (Lowry et al., 1951) modification of the Folin-Ciocalteau method was used to assay protein concentration during the purification of AGDH. This method has the advantages that it is rapid and that assays may be performed in the presence of nitrogenous buffers. The method is not suitable for the determination of absolute amounts of protein, however, because it is specific for the aromatic amino acid residues which vary among proteins. In addition, the reactivity of these residues may not be constant due to differences in their environment in the protein. For these reasons a modification of the nitrogen procedure of Ballentine (1963) was employed when it was necessary to determine absolute amounts of protein.

Lowry method. The following solutions were required for this method: solution A, 2.7 g $CuSO_4 \cdot 8H_2O$/100 ml H_2O;

TABLE I

PURIFICATION OF GLYCEROL DEHYDROGENASE

Step	Volume (ml)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)
Crude extract[a]	320	6,145	8.5×10^5	1.4×10^2
Protamine sulfate supn.	330	4,750	8.3×10^5	1.8×10^2
Ammonium sulfate ppt.	80	1,340	9.3×10^5	6.9×10^2
Heat supernatant	76	400	6.9×10^5	1.7×10^3
Gel filtration[b]	100	41	1.7×10^5	4.0×10^3
Ion-exchange A-50[c]	5	13	0.44×10^4	3.2×10^2
Ion-exchange A-25[c]	6	15	0.42×10^5	2.8×10^3[d]

[a]Based on 32 l. growth medium.
[b]Based on 200 mg lyophilized extract.
[c]After concentration and precipitation.
[d]For pooled material or any of the 13 peaks indicated on page 81.

solution B, 1.0 g Na-K tartrate/100 ml H_2O; solution C, 2.0 g Na_2CO_3/100 ml H_2O. One milliliter each of solutions A and B was added to 100 ml of solution C, and 5.0 ml of this solution was added to 1.0 ml of a solution of protein to be assayed. The mixture was allowed to incubate for fifteen minutes, then 0.5 ml of Folin-Ciocalteau reagent (Sargent Chemical Co., Inc.) was added with rapid mixing, and the solution was allowed to incubate at room temperature for thirty minutes. The optical density of the solution was read at 660 nm against a blank prepared as above except that 1.0 ml of water was substituted for the protein solution. A one per cent solution of three-times crystallized bovine serum albumin was used as the protein standard.

Nitrogen. The solution containing the protein to be analyzed was exhaustively dialyzed against water to remove any nitrogenous buffers, and a volume of this solution corresponding to approximately two micrograms of protein nitrogen was pipetted into a test tube. The solution was then made slightly basic with NaOH and evaporated to dryness in an oven at 100°. The residue was taken up in 0.3 ml of concentrated H_2SO_4 and 0.1 ml of water, and the test tubes were placed in a heating block at 375° for six hours. Finally the digestion mixture was taken up in 7 ml of

0.144 N H_2SO_4.

One volume of stock Nessler's reagent (Fisher Scientific Company, Stock Number So-N-24) was diluted with two volumes of water. The color reagent was then prepared by mixing, in the ice bath, 10 ml of diluted Nessler's reagent, 2.5 ml of a 0.05 per cent (w/v) solution of methyl cellulose and 10 ml of 10 N NaOH. The color reagent was then filtered through glass wool. Finally 3.0 ml of color reagent was added to the acid digest solution and the optical density read at 425 nm in a one-cm Pyrex cuvette. Blanks were prepared as above except no protein was used, and standard protein solutions were prepared from three-times crystallized bovine serum albumin.

Determination of enzyme activity. The activity of glycerol dehydrogenase was measured by following the reduction of NAD at 340 nm in a Bausch and Lomb Model 600 spectrophotometer equipped with a strip chart recorder. The reaction was carried out in 1-cm light-path cuvettes. The assay solution was of the following composition: 0.1 M glycerol, 3.3×10^{-4} M NAD, and 0.033 M $(NH_4)_2SO_4$ in 0.5 M sodium carbonate buffer, pH 9.0. The reaction was started by adding 0.1 ml of the solution to be assayed to 2.9 ml of the assay solution. One unit of activity is defined as that amount of enzyme which causes an initial rate of

reduction of NAD of 1.0 micromole per unit.

Polyacrylamide gel electrophoresis. The general procedure outlined by Ornstein (1964) and Davis (1964) was employed with the modification that no sample gel was used. Instead, a solution of the protein in ten per cent sucrose was layered over the resolving gel. An apparatus was constructed according to the method of Davis (1964).

The following solutions were required in the preparation of the resolving gel. The final volume of each solution was 100 ml.

Solution A	30 g Acrylamide
Solution B	0.8 g N,N'-Methylenebisacrylamide
Solution C	2.08 g Tris, and 0.1 ml TMED adjusted to pH 9.5 with glycine
Solution D	0.18 g Ammonium persulfate

For experiments in the absence of urea all solutions were prepared in water. Equal volumes of the four solutions were mixed and degassed with the water aspirator. Gels for experiments in 2.5 M urea were prepared as described above except that the ammonium persulfate (Solution D) was dissolved in 10 M deionized urea. Gels for experiments in 6.0 M urea were prepared by dissolving the constituents

of solutions A through D (above) in 40 ml of H_2O and diluting the solutions to 100 ml with 10 M urea. Gels for experiments at pH 4.0 were prepared using solutions A, B, and D described above and preparing a solution of 0.192 ml glacial acetic acid and 0.1 ml TMED in 100 ml H_2O and adjusting to pH 4.0 with glycine. The acrylamide content of the gels was varied by changing the concentration of acrylamide in solution A. The upper tray buffer for experiments at alkaline pH consisted of a solution of 0.0425 M Tris-glycine, pH 8.9, while for experiments at pH 4.0 the buffer used was a solution of 0.0425 M acetic acid adjusted to pH 4.0 with glycine. The lower tray buffer for the runs at pH 9.5 was a 0.12 M solution of Tris adjusted to pH 8.1 with 1.0 N HCl, and for experiments at pH 4.0, a solution of 0.12 M acetic acid adjusted to pH 4.0 with glycine. These same buffers were used for experiments with gels containing urea.

The sample buffer used for experiments at pH 9.5 was a 0.008 M solution of Tris adjusted to pH 8.9 with glycine while the sample buffer used at pH 4.0 was a solution of 0.008 M acetic acid again adjusted to pH 4.0 with glycine. In both cases the sample buffers contained 10 per cent (w/v) sucrose. The sample buffers for experiments in urea were the same as described above except that they were

prepared in urea of the desired concentration.

General procedure. Glass columns 7.0 cm long and 0.5 cm inner diameter were cut from the same length of glass tubing. The columns were cleaned in chromic acid, then rinsed in tap water, then in distilled water, then soaked in a 0.5 per cent solution of Photo-Flo (Eastman Kodak Co.) and allowed to drain dry before each experiment. During the preparation of the gels the glass columns were inserted in holders made from Vacutainer tops (Becton-Dickson Co.) which were cemented to a block of wood. The gels were prepared in an inverted position, i.e., with the top end of the column in the column holder. The other end of the column, i.e., eventually the bottom, was wrapped with Parafilm to form a column which extended 2 to 3 cm beyond the end of the glass.

A solution of 20 per cent (w/v) sucrose was pipetted into the glass column to a height of 1 cm. The gel solution of the desired composition, prepared just prior to use, was carefully layered on top of the sucrose solution and the glass column filled beyond the end of the glass into the column formed by the Parafilm. The Parafilm column was then pinched closed below the level of the gel solution with a hemostat. The properties of Parafilm are such that when

the hemostat was released the end of the column was sealed air-tight by the Parafilm. These precautions were necessary since the polymerization process is inhibited by molecular oxygen and if the ends of the column were not completely sealed, gels of non-uniform properties resulted. Gel formation was usually complete in forty-five minutes to an hour.

The glass columns were then removed from the holder and that portion of the gel which extended into the Parafilm column was removed by cutting the Parafilm and gel inside with a razor blade. This resulted in the end of the gel being flush with the glass column.

The gels were pre-run in order to remove unreacted acrylamide as well as any other artifacts from the polymerization process. The pre-run buffer consisted of the Solution C characteristic of the particular system of pH and urea which was used to prepare the gel with the modification that the TMED was not added and the solution was diluted 1:4 with water for experiments without urea, 1:4 with 3.3 M urea for experiments in 2.5 urea and 1:4 with 6.0 M urea for experiments in 6.0 M urea. The gels were inserted in the upper buffer tray and the tray was filled with the proper pre-run solution. The lower buffer tray was also filled with the pre-run solution and the

bottom of the gels inserted into the solution. The tops of the gels were rinsed with pre-run solution, the electrodes connected (anode at the top) and a current of 2 milliamperes per gel applied for two hours.

Samples of protein were prepared by dialyzing an aliquot of the enzyme suspension in saturated $(NH_4)_2SO_4$ against the sample buffer to be used. The concentration of protein used in these experiments was between 1 mg/ml and 5 mg/ml. When dithiothreitol was used it was added as the solid reagent to the protein sample just prior to the experiment. Finally 0.05 ml of a tracking dye solution, (0.1 per cent bromphenol blue for experiments at pH 9.4 and 0.1 per cent methyl green for runs at pH 4.0) was added to each 0.1 ml of protein solution.

The pre-run solution was removed from the buffer trays and the trays filled with the upper and lower buffers required for the particular set of experimental conditions. The tops of the gels were rinsed with the upper tray buffer, and 0.1 ml of the protein solution carefully layered on top of the resolving gel. The electrodes were attached to obtain the proper polarity (anode at the top for experiments at pH 4.0, and at the bottom at pH 9.5), and a current of 0.5 milliamperes per gel applied until the tracking dye penetrated into the gel, then 2 milliamperes per gel for the

remainder of the experiment. When the tracking dye was about 1 cm from the bottom of the gel column the current was turned off and the glass columns removed from the apparatus. The gel columns were extracted from the glass columns by injecting cold water between the glass tubing and the gel with a syringe and hypodermic needle, starting from the bottom.

The gels were stained for protein by first fixing the gels in a solution of 10 per cent sulfosalicylic acid for 10 hours, then in a one per cent solution of coomassi brilliant blue (Colabs, Inc.) in 7 per cent acetic acid for 18 hours, then washing out the excess stain in a 7 per cent solution of acetic acid. Enzyme activity was located on the gels by incubating them in a solution made by dissolving 50 mg NAD, 20 mg nitro blue tetrazolium, 0.6 mg phenazine sulfate and 4.5 g glycerol in 50 ml of a solution of 0.5 M sodium carbonate buffer, pH 9.0, and 0.033 M $(NH_4)_2SO_4$. Staining was carried out in the dark for fifteen minutes and the gels subsequently washed with several changes of water.

In order to perform amino acid analyses on the proteins which were stained on the gels, slices were cut from identical unstained gels at locations indicated by a gel which was stained. The gel slices were crushed with

a glass stirring rod in a 1-cm by 5-cm test tube and the protein extracted with several 1-ml aliquots of 1 N HCl. The gel extract was centrifuged at 5,000 x g for ten minutes to remove any gel particles and the supernatant was dialyzed against several changes of distilled water.

Ultracentrifuge Experiments

General procedure. A Beckman Model E analytical ultracentrifuge equipped with schlieren optics was used for all experiments. The centrifuge cell used was a 12 mm double sector cell with sapphire windows. The solution side of the cell was filled first with 0.02 ml FC-43 fluorocarbon (Spinco, Inc.), then with 0.1 ml of protein solution for sedimentation equilibrium and synthetic boundary experiments or with 0.3 ml of solution for sedimentation velocity experiments. The solvent side was filled with 0.01 ml of the fluorocarbon, then with 0.3 ml of solvent for sedimentation velocity and synthetic boundary experiments or with 0.12 ml for sedimentation equilibrium experiments.

Sedimentation equilibrium experiments require that sufficient time elapse for equilibrium between sedimentation and diffusion to be established. Therefore, for each solvent system used for the first time, photographs were

taken and evaluated 14 and 24 hours after speed had been reached. In each case there was no difference in the molecular weights determined at the two times. Experiments were allowed to proceed for at least 18 hours and usually for 24 hours. Speeds were chosen for the meniscus depletion technique (Chapter IV, p. 64) so that the displacement of the schlieren pattern was flat for at least half the solution column height. Photographs were evaluated using a Nikon Shadowgraph microcomparator (Nippon-Kogaku, Tokyo, Japan) at 0.5 cm intervals (on the plate) until the displacement was at least 0.01 cm, then at 0.01 cm intervals for the remainder of the column. Displacements at the cell bottom, and at the meniscus for low-speed experiments were determined by graphical extrapolation of plots of displacement versus X-comparator co-ordinate. The meaning of the schlieren displacements and other details concerned with the estimation of hydrodynamic properties from ultracentrifuge experiments are developed in the next chapter.

Preparation of glycerol dehydrogenase for ultracentrifugation. The native[1] enzyme was prepared by dialyzing an aliquot of the enzyme precipitate in saturated

---

[1]The enzyme as isolated from the bacteria is defined as the native enzyme during this discussion. This does not imply that such is the state of the enzyme in the bacteria.

ammonium sulfate against a solution of 0.2 M NaCl in 0.1 M Tris·HCl, pH 7.6. The concentration of the enzyme was usually between 3 to 4 mg/ml. The solution of native enzyme was centrifuged at 10,000 x g for 10 minutes just prior to ultracentrifugation to remove any precipitate.

For experiments in 2.5 M urea, a solution of the native enzyme was dialyzed against a solution of 2.5 M urea and 0.2 M NaCl and then diluted with the dialyzing solution to give a final protein concentration of 1 mg/ml.

The enzyme was prepared for experiments in 6.0 M guanidinium chloride (GDC) by dissolving an amount of solid GDC equal to the weight of an aliquot of solution of the native enzyme in 0.2 M NaCl and 0.1 M Tris·HCl, pH 7.6. Typically a 0.5-ml aliquot of a solution of the native enzyme was used.

Dithiothreitol (DTT) was introduced into the solution of enzyme in 6.0 M GDC by diluting one volume of a stock solution of DTT in 6.0 M GDC ten times the final concentration (of DTT) desired with nine volumes of the enzyme solution in 6.0 M GDC.

The S-carboxymethyl (CM) derivative of AGDH was prepared by first dialyzing a solution of the native enzyme in the Tris-saline buffer against a solution of 8 M urea and 10^{-3} M EDTA. The enzyme concentration was 5 mg/ml.

One milliliter of this solution was pipetted into a 1-cm by 5-cm test tube, and the pH adjusted to 8.0 with methylamine. The test tube was sealed with a sleeve-type serum stopper and alternately degassed by applying vacuum from a water aspirator and flushed with nitrogen several times to remove oxygen. Then 0.1 ml of a 0.1 M solution of DTT in 8 M urea was added with a syringe and hypodermic needle to the reaction vessel and the mixture allowed to incubate for one hour.

After reduction of the protein, 0.1 ml of a 1.0 M solution of iodoacetic acid adjusted to pH 8.0 with methylamine was added to the reaction vessel with a syringe and the reaction was allowed to proceed in the dark for one hour. The preparation was then exhaustively dialyzed against water, and the carboxymethyl (CM) derivative of the protein which was insoluble in water was recovered by centrifuging the aqueous suspension of protein at 10,000 x g for 15 minutes. The precipitate was subsequently resuspended in 0.5 ml of water and this suspension was made 6.0 M in GDC by adding an equal weight of the solid reagent. A portion of the suspension of CM-protein in water was subjected to amino acid analysis. The analysis showed that all the cysteine had been converted to the S-carboxymethyl derivative, and in addition no significant amounts of other

carboxymethylated amino acids were detected.

Enzyme kinetic experiments. The apparent Michaelis constant (K_m) and maximal velocity (V_m) were determined for all four substrates of AGDH. Measurements of initial rates were carried out at 25° using a Bausch and Lomb Spectronic 600 double-beam spectrophotometer. All substrate solutions were prepared in a solution of a 0.5 M sodium carbonate buffer, pH 9.0, and 0.033 M ammonium sulfate. Solutions of NAD, NADH and DHA were prepared fresh each day. The concentration of NAD solutions was checked by measuring the optical denisty (O.D.) at a wavelength of 258 nm in a 1-cm path-length quartz cuvette using the known extinction coefficient of NAD of 18.3×10^3 O.D./M. The concentration of the NADH solutions was also checked by measuring the O.D. at a wavelength of 340 nm and using the known extinction of 6.22×10^3 O.D./M. Solutions of DHA were observed to darken during the course of preliminary experiments; therefore, these solutions were kept in a brown reagent bottle. Stock solutions of glycerol in the carbonate buffer were stored at 4°.

A stock solution of enzyme was prepared by dissolving an aliquot of the enzyme suspension (in the saturated ammonium sulfate solution) in a solution of 0.1 M Tris·HCl pH 7.6 and 0.1 M KCl. The stock solution was dialyzed

against this buffer and stored at 4°. The final protein concentration was 0.63 mg/ml, and 0.025 ml was used for each experiment. This solution was assayed each day in the standard assay solution and no loss of activity was observed during the time required to perform these experiments (seven days). The concentration of NADH was followed at 340 nm and a tracing of NADH concentration versus time was obtained with a Sargent Model SLR strip chart Recorder. Initial steady-state rates (v(0)) were determined by estimating the initial slopes of these tracings. Experiments were performed in a 1-cm-path-length quartz cuvette. The total volume of substrate solution plus buffer in each experiment was 3.0 ml. The reaction was initiated by adding 0.025 ml of the stock enzyme solution which was pipetted onto a mixing spoon. The spoon served both to add the enzyme and to stir the solution quickly.

Amino acid analysis. Amino acid analyses were carried out according to the general procedure of Moore and Stein (1963) on a Beckman Model 190 Automatic Amino Acid Analyzer. Samples of protein for analysis were exhaustively dialyzed against water. Analyses of protein from the A-25 ion-exchange column chromatography experiments were performed by first concentrating the protein by lyophilization,

redissolving in 2 ml of water and then dialyzing against water to remove buffer. The aqueous solution of protein was transferred to an ampoule, evaporated to dryness and then redissolved in 2 ml of glass-distilled, constant-boiling HCl. The solution was purged of dissolved oxygen by alternate degassing with a vacuum formed with a water aspirator and flushing with nitrogen. The ampoule was then sealed under vacuum and placed in a temperature-regulated over at 105° for the appropriate time. After hydrolysis, the ampoule was opened and the solution evaporated to dryness. The hydrolyzate was then redissolved in 1.00 ml of water and a 0.50 ml aliquot removed for nitrogen analysis. The remainder of the hydrolyzate was again evaporated to dryness and redissolved in the citrate buffer of the amino acid analyzer.

Titration of AGDH with PMB. The general procedure outlined by Benesch and Benesch (1962) was employed to determine the number of titratable sulfhydryl groups both before and after complete reduction of AGDH with dithiothreitol.

The method is based on the fact that the absorption maximum of PMB shifts from 330 nm to 350-355 nm upon formation of the mercaptide. The extinction coefficient of

the mercaptide, however, depends upon the nature of compound to which the thiol group is attached. Instead of a single measurement with an excess of PMB, therefore, the analysis was carried out in the form of a spectrophotometric titration.

Procedure. An aqueous solution of approximately 10^{-3} M PMB was prepared and standardized against a known concentration of DTT. A Bausch and Lomb Model 600 double-beam spectrophotometer was employed and increments of the PMB solution were added to the test solution and a corresponding blank and the change in O.D. recorded and corrected for dilution by the PMB solution. The end-point was determined from a plot of O.D. versus volume of PMB added in a manner illustrated in Figure 4.

It was not practical to titrate the protein in dilute salt solutions since the addition of the first increment of PMB caused the formation of a precipitate which interferred with the spectrophotometric determination of the mercaptide. Therefore, titrations were carried out in 6.0 M GDC since the protein-PMB complex was soluble in this reagent.

The protein solution in 6.0 M GDC was prepared in the same manner as that for experiments in the ultracentrifuge and the concentration of protein was determined from

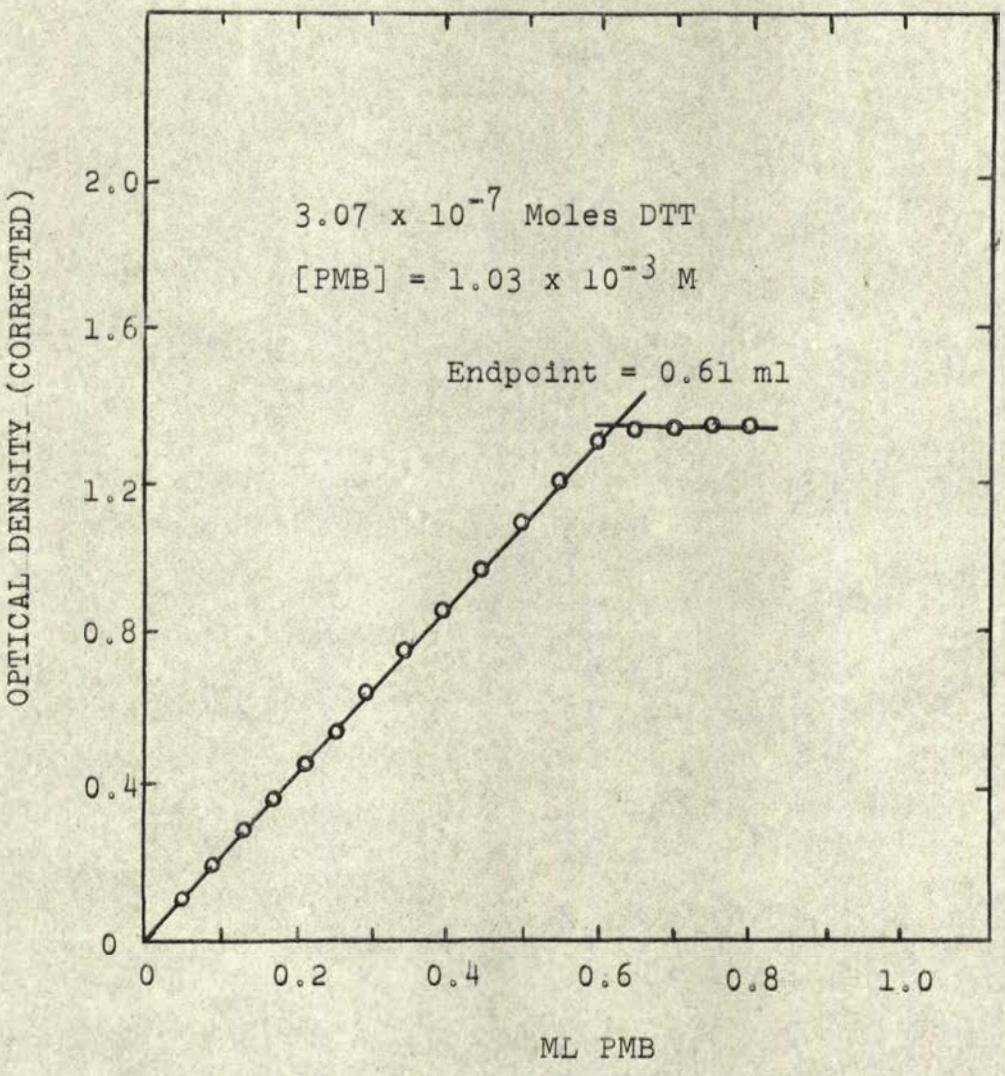

Figure 4. Standardization of p-hydroxymercuribenzoate.

The endpoint of the titration is indicated by the intersection of the straight lines.

a nitrogen analysis of an aliquot of enzyme solution prior to the addition of GDC. The titration was performed using 1.0 ml of a 0.10 per cent solution of protein.

The protein was pre-reduced by adding solid DTT to a solution of the protein in 6.0 M GDC and 10^{-3} M EDTA to give a final concentration of 0.05 M DTT. The mixture was allowed to incubate at room temperature for one hour and then exhaustively dialyzed against water which had been purged of dissolved oxygen by repeated degassing and flushing with nitrogen. The reduced protein was insoluble in water and the precipitate was recovered by centrifuging the suspension at 5,000 x g for ten minutes. The precipitate was resuspended in 0.5 ml of water and an equal weight of solid GDC added to make the solution 6.0 M in that reagent. Protein concentration was determined from the O.D. at 280 nm and the extinction coefficient which was determined from the preceding experiment.

Ultracentrifuge determinations of the molecular weight were performed on the titrated protein solutions immediately after the titration experiment was completed.

CHAPTER IV

THEORETICAL SECTION

Derivation of Fundamental Equations of the Ultracentrifuge

The theoretical basis on which molecular weights and other hydrodynamic properties of particles (proteins) dissolved in a viscous fluid (e.g., water) will be derived in order to illustrate the assumptions and approximations which were made in determining the molecular weights of glycerol dehydrogenase treated with various reagents. Since the aim of this section is to relate the theoretical requirements for successful use of the ultracentrifuge to the experimental limitations imposed when working with real materials and thereby to evaluate the uncertainties in the numbers determined, it is neither the most sophisticated nor complete treatment available but rather is adequate for its purpose.

It is assumed that the flow of matter is a linear function of the forces which cause the flow. The constant which relates the force to the flow is known as a phenomenological coefficient. In the ultracentrifuge the forces which cause the flow act along a single direction

perpendicular to the axis of rotation of the rotor and therefore the phenomenological equations may be expressed in terms of a single direction, x. These equations are of the form

$$J_i = \sum_k L_{ik} X_k \qquad (1)$$

where J_i is the flow per unit area of a component i across a plane perpendicular to x and the X_k are the forces which act in the x direction. The L_{ik} are the coefficients which relate force to flow, i.e., the flow of the i^{th} component due to the k^{th} force. These coefficients are independent of the X_k when the X_k are small and are functions of the thermodynamic variables temperature, pressure and composition. It is further assumed that the viscous liquid is incompressible, that matter and energy are conserved, that there are no chemical reactions between components, that no temperature gradients exist within the centrifuge cell, that the particle is non-ionic and there are no solvent-solute interactions.

Forces in ultracentrifuge experiments can be separated into two kinds: forces which act in the positive x-direction, i.e., centrifugal forces due to the spinning of the rotor, and forces which act in the negative x-direction. The second set of forces arises from concentration gradients which are set up in the cell and these

forces are therefore related to the chemical potential of the particle. Equation 1 can then be written for a two component system as

$$J = LF - L \cdot (\partial \mu / \partial x)_T \qquad (2)$$

where F is now the external force and μ the chemical potential.

The coefficient L cannot be related to the properties of the particle from thermodynamic considerations alone. To do this a mechanical model is required.

In free space the motion of a particle is described by the equation F = ma where m is the mass of the particle and a = dv/dt, its acceleration. In a viscous fluid an opposing frictional force is set up as soon as the velocity of the particle becomes finite. For small velocities this force is directly proportional to the velocity so that the equation of motion becomes m dv/dt = F - fv where f is the frictional coefficient. Therefore as v increases, the acceleration, dv/dt, decreases until fv equals F and no further change in velocity occurs. The steady-state velocity which is then reached is given by equation 3.

$$v = F/f \qquad (3)$$

The flow of particles across a unit area perpendicular to the x-axis in the time interval dt is the number of particles in a volume vdt. Since the force, F, in equation 3 is the force per particle, then J in equation 2

must be expressed as particles per square centimeter per second. If N is the number of particles per cubic centimeter the flow in time dt is vdtN or, per unit time

$$J = vN = NF/f \tag{4}$$

L in equation 2 is therefore N/f. If flow is expressed as moles per cubic centimeter per second and F as force per mole, equation 4 becomes

$$J = LF = CF/\underline{N}f \tag{5}$$

where $\underline{N}$ is Avagadro's number and C is the concentration of the particle in moles per cubic centimeter.

Combining equations 2 and 5 and recognizing that the external force in equation 2 is the centrifugal force due to rotation, we obtain

$$J = (C/\underline{N}f)\cdot(M\omega^2 r) - (C/\underline{N}f)\cdot(\partial\mu/\partial r)_T \tag{6}$$

where M is the molecular weight, ω the angular velocity of the rotor in radians per unit time, and r the distance from the axis of rotation.

Since at constant temperature

$$\mu = F(P,C)_T \tag{7}$$

then by Euler's rule

$$(\partial\mu/\partial r)_T = (\partial\mu/\partial P)_C(\partial P/\partial r)_T + (\partial\mu/\partial C)_P(\partial C/\partial r)_T \tag{8}$$

From elementary thermodynamics it may be shown (Daniels and Alberty, 1961) that

$$(\partial\mu/\partial P)_{T,C} = CV = M\bar{v} \tag{9}$$

where M is the molecular weight, $\bar{v}$ is the partial specific volume, and V is the partial molal volume. It is true that

$$(\partial \mu/\partial C)_{T,P} = RT/C + RT(\partial \ln y_C/\partial C)_{T,P} \quad (10)$$

where y_C is the activity coefficient of species, C, R is the gas constant and T the temperature in °K. Finally,

$$(\partial P/\partial r)_{T,C} = \rho\omega^2 r \quad (11)$$

where ρ is the density of the solution. Combining equations 9, 10, and 11, equation 8 becomes

$$(\partial \mu/\partial r)_T = M\bar{v}\rho\omega^2 r + [RT/C](1+C(\partial \ln y_C/\partial C)_{T,P}(\partial C/\partial r)_T) \quad (12)$$

Combining equation 12 with equation 6 results in equation 13, the fundamental equation of the ultracentrifuge.

$$J = [CM\omega^2 r/\underline{N}f](1-\bar{v}\rho) -$$
$$- [RT/\underline{N}f][(1+C(\partial \ln y_C/\partial C)_{T,P}]\cdot(\partial C/\partial r)_T \quad (13)$$

Sedimentation equilibrium. When the sedimenting particle is at equilibrium in the ultracentrifuge there is no net transport of the particle at any point in the centrifuge cell. The left-hand side of equation 13 may be set equal to zero. Upon rearranging, equation 13 becomes[2]

$$(RT/C)\cdot dC/dr = M(1-\bar{v}\rho)\omega^2 r/(1 + C(\partial \ln y_C/\partial C)_{T,P})) \quad (14)$$

---

[2] $(\partial C/\partial r)_T$ is treated as the total derivative, dC/dr, in the remainder of the derivative by Tanford (1961).

If the non-ideality term, $\partial \ln y_c / \partial C)_{T,P}$ in equation 14 may be neglected, then equation 14 becomes

$$(RT/C)\cdot(dC/dr) = M(1 - v\rho)\omega^2 r \qquad (15)$$

If the molecular weight expressed by equation 15 is defined as the apparent molecular weight (M_{App}) then equation 14 becomes

$$M_{App} = M/(1 + C(\partial \ln y_C / \partial C)_{T,P})) \qquad (16)$$

Therefore, in a two-component non-electrolyte system the apparent molecular weight approaches the true molecular weight as the concentration of the sedimenting particle approaches zero.

The above analysis is incomplete since the experiments reported below were done in the presence of electrolyte or dissociating reagent. In addition the protein itself is an ampholyte. The general equation describing the behavior of a charged macromolecule at equilibrium in a gravitational field in the presence of a third ionic component has been derived by Johnson (Johnson et al., 1954) and is reproduced below as equation 17

$$(RT/C_2)\, dC_2/dr\, [1 + C_2 Z^2/(C_3 + ZC_2) + C_2(\partial \ln y_p + y_X^Z - /\partial C_2)_{T,P}] = M_2(1-\bar{v}\rho)\omega^2 - dC_3/dr\, [ZRT/(C_3 + ZC_2) + RT(\partial \ln y_p + y_X^Z - /\partial C_3)_{T,P}] \qquad (17)$$

where the terms are defined as:

C_2, C_3, the molal concentration of macromolecule and supporting electrolyte, respectively,

Z, the net charge on the macromolecule (taken to be positive),

X^-, the molal concentration of the counter-ion, e.g., chloride,

Y_p+, Y_X-, the activity coefficient of the protein and counter-ion, respectively,

M_2, $\bar{v}_2$, the molecular weight and partial specific volume of the macromolecule respectively.

In the absence of supporting electrolyte, equation 17, at the limit C_3, $dC_3/dr \to 0$ becomes

$$(RT/C_2)\ (dC_2/dr)\ [1 + (C_2/(Z + 1))$$

$$\partial \ln\ y_p+y_X^Z- C_2)_{T,P}] = (M_2/(Z + 1))\ (1-\bar{v}\rho)\omega^2 r \qquad (18)$$

If the derivative of the activity coefficient in equation 18 is neglected, then equation 18 becomes identical to equation 14 except that M is replaced by M/(Z + 1). Therefore in the absence of supporting electrolyte the observed molecular weight is a function of the charge, Z, on the protein. In general Z cannot be determined in solutions of low ionic strength because of the tendency of the protein to associate, and unless Z can be determined then molecular weight determinations must be performed experimentally in the presence of supporting electrolyte.

In order to evaluate the effect of the concentration of supporting electrolyte on the observed molecular weight

the concentration distribution of this component, dC_3/dr, in equation 17 must be evaluated. This equation has also been derived by Johnson and ignoring derivatives of activity coefficients, is reproduced as equation 19.

$$(RT/C_3)\cdot dC_3/dr[2 - (ZC_2/(C_3 + ZC_2))] = M_3(1 - \bar{v}_3\rho)\omega^2 r \quad (19)$$

In a typical experiment the concentration of protein is about 0.1 per cent or if the protein has a molecular weight of 10,000 daltons, the solution is about 10^{-4} molar in protein. A reasonable value for the net charge on such a protein near its isoelectric point is four. Typically, electrolyte is present at a concentration of 0.2 molar. Therefore $C_2 << C_2 >> ZC_2$ and the second term inside parentheses in equation 19 and the term $(C_2\cdot Z^2/(C_3 + ZC_2)$ in equation 17 may be neglected. In addition, the term $ZRT/(C_3 + ZC_2)$ in equation 17 is approximately equal to ZRT/C_3. With these simplifications and again ignoring derivatives of activity coefficients in equation 17, the expression for dC_3/dr in equation 19 may be substituted into equation 17. The result of this procedure is equation 20.

$$(RT/C_2)\cdot(dC_2/dr) = M_2(1 - \bar{v}_2\rho)\omega^2 r - ZM_3(1 - \bar{v}_3\rho)\omega^2 r/2 \quad (20)$$

Equation 20 is identical to equation 15 except that M_{App} has been replaced by

$$M_2 - ZM_3(1 - \bar{v}_3\rho)/2(1 - \bar{v}_2\rho) \qquad (21)$$

Thus experiments in the presence of a third ionic component do not give the true molecular weight even when M_{App} is extrapolated to zero protein concentration, which is required in order to justify neglecting the activity coefficients. However, if the typical experimental conditions described above are used and with NaCl as the third component (M_3 = 58 and $\bar{v}_3$ = 0.3) and assuming $\bar{v}_2$ = 0.75, then the negative term of expression 21 is equal to 320 daltons or about 3 per cent of M_2.

In order to neglect derivatives of activity coefficients the assumption that no specific interactions are present between the protein and supporting electrolyte must be made. For dilute solutions of protein and electrolyte this assumption is justified. However, this assumption has recently been evaluated for the case when guanidinium chloride is the supporting electrolyte at concentrations (e.g., 6.0 M) which also are effective in eliminating protein-protein interactions (Hade and Tanford, 1968). These authors concluded that "molecular weight [determinations] in 6.0 M guanidinium chloride without taking preferential binding into account will be somewhat too large, typically 5 to 10 per cent." The experimental determinations of this interaction requires amounts of materials

not available in this study and this uncertainty must be taken into account in evaluating experiments performed in the presence of this reagent.

Sedimentation velocity. The experimental determination of the sedimentation coefficient of a protein is performed under conditions such that diffusion is negligible compared to sedimentation. The second term on the right hand side of equation 13 may therefore be set equal to zero. Equation 22 then is the expression for the flow of material due to sedimentation alone.

$$J = CM\omega^2 r(1 - \bar{v}\rho)/\underline{N}f \qquad (22)$$

Defining s, the sedimentation coefficient, as

$$s = M(1 - \bar{v}\rho)/\underline{N}f \qquad (23)$$

and substituting into equation 22 we obtain

$$J = sC\omega^2 r \qquad (24)$$

Generally the sedimentation velocity of a protein is determined by recording the position of the boundary between the protein solution and the protein-depleted solvent as the boundary moves towards the bottom of the centrifuge cell. If the protein preparation is homogeneous, the concentration of protein is constant at every point below the sedimenting boundary, i.e., $dC/dr = 0$. If the variables in this plateau region are designated with a

subscript p, then equation 24 becomes

$$J_p = s_p C_p \omega^2 r_p \tag{25}$$

In a wedge-shaped cell the area of a plane at any point r parallel to the axis of rotation is proportional to r. If the constant of proportionality is designated as α then the number of moles of C crossing the plane at r_p per unit time is $\alpha r_p J_p$. This quantity is also equal to the decrease per unit time in the number of moles of C present in the volume between the plane at r_p and the plane at the meniscus, r_m. Since the number of moles between a plane at r and a plane at r + dr is $\alpha r C dr$ then

$$\alpha J_p r_p = - d(\int_{r_m}^{r_p} \alpha r C dr)/dt \tag{26}$$

A new variable r_z is defined such that at any time

$$\int_{r_z}^{r_p} \alpha r C_p dr = \int_{r_m}^{r_p} \alpha r C dr \tag{27}$$

From equation 27 it is clear that r_z denotes the position of the sedimenting boundary if this boundary were infinitely sharp. Integrating the left-hand side of equation 27 gives

$$\int_{r_z}^{r_p} \alpha r C dr = \alpha C_p (r_p^2 - r_z^2)/2 \tag{28}$$

Substituting equation 28 into equation 26 and differentiating gives

$$J_p r_p = -(r_p^2 - r_z^2)(dC_p/dt)/2 + C_p r_z (dr_z/dt) \tag{29}$$

For a wedge-shaped cell (radial dilution)

$$dC_p/dt = -(d(Jr)/dr)/r \tag{30}$$

From equation 25 and equation 30

$$dC_p/dt = -(d(s_p C_p \omega^2 r^2)/dr)/r \tag{31}$$

or

$$dC_p/dt = -2s_p \omega^2 C_p \tag{32}$$

Using equation 32 for dC_p/dt and equation 25 for J_p and substituting into equation 29 gives

$$s_p = (dr_z/dt)/r_z\omega^2 \tag{33}$$

which relates the sedimentation coefficient to r_z.

Diffusion. Experimentally the diffusion constant of a protein is determined by layering a solution of the protein over a layer of solvent and observing the time-dependent distribution of solute. Conditions are such that, during the course of the experiment, transport by sedimentation is small compared with transport due to diffusion and thus the first term on the right-hand side of equation 13 may be neglected. The transport of solute due to diffusion may then be expressed by equation 34.

$$J = -(RT/\underline{N}f)\cdot[1 + C(\partial \ \ln y_C/\partial C)]\ C/\partial r \tag{34}$$

Fick's law (equation 35) states that the flow of particles per unit time, J, across a plane perpendicular to the direction x of a concentration gradient is propor-

tional to the concentration gradient.

$$J = -D(\partial C/\partial x)_T \qquad (35)$$

From equation 34 and 35 the diffusion constant may then be defined as

$$D = (RT/\underline{N}f)\cdot(1 + C(\partial \ln y_C/\partial C)_{T,P}) \qquad (36)$$

Equation 36 is correct for a two-component, non-ionic, non-interacting system at equilibrium except for the presence of a concentration gradient. In the three-component systems used in this study, however, both the protein and supporting electrolyte are ionic. The effect of charged species on equation 36 has been developed by Schachman (1959). It may be shown that such effects are minimized by performing experiments (1) under conditions which also minimize the effects of charge on the molecular weight determined by sedimentation equilibrium (i.e., neutral pH) and (2) at low concentration of protein and moderate concentration of electrolyte.

Experimental Determination of Hydrodynamic Properties

The purpose of this section is to demonstrate how the equations derived in the preceding section were used experimentally to determine the hydrodynamic properties of AGDH.

Optical systems. From the equations of the previous section it is clear that evaluation of the hydrodynamic properties of proteins using the analytical ultracentrifuge requires determination of the concentration distribution of protein with respect to either time (sedimentation velocity), radius (sedimentation equilibrium) or as will be shown, with respect to both (diffusion).

The Beckman Model E Analytical Ultracentrifuge provides a photographic record of $K\Sigma_i dC_i(r)/dr$ versus r where K is a constant which depends on the optical arrangement of the ultracentrifuge and on the refractive index increment of the solute, and the subscript i refers to each species present.

It will be shown that experimental evaluation of the equations of the preceding section requires determination of ratios of concentrations and therefore the optical constant K need not be evaluated in order to use the experimental results.

Figure 5 illustrates the record obtained from a typical sedimentation equilibrium experiment. Inspection of equation 37 shows that

$$C(r) = \int_{r_m}^{r} (dC(r)/dr)dr \qquad (37)$$

the concentration at any point r may be determined from such data.

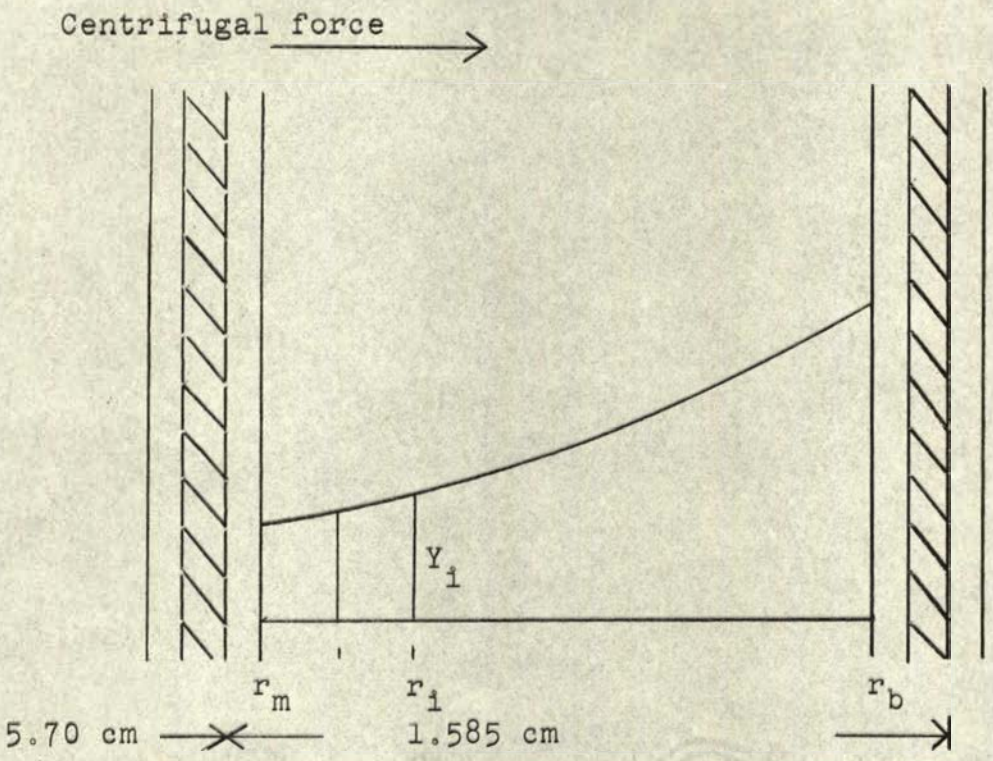

Figure 5. Illustrative sedimentation equilibrium pattern.

$Y_i = K\, dC(r)/dr$
r_m = solution meniscus
r_b = cell bottom
$\Delta r_i = r_i - r_{i-1}$
K = optical constant
$C(r) = K\Sigma_i(\Delta r_i/2)(Y_i + Y_{i-1})$

In Figure 5, let Y_i be the net displacement, $K\sum_i dC_i(r)/dr$, at any point r and let

$$\Delta r_i = r_i - r_{i-1} \tag{38}$$

Then by the trapazoid rule of integration the area (concentration) between any two points r_i and r_{i-1} is

$$\Delta C_i = (\Delta r_i/2)(Y_i - Y_{i-1}) + \Delta r_i Y_{i-1} \tag{39}$$

The total concentration at any point, r, is therefore

$$C(r_i) = \sum_{i=0}^{i} (\Delta r_i/2)(Y_{i-1} + Y_i) \tag{40}$$

If the concentration gradient is established by layering the solvent over the solution in a synthetic boundary cell (page 66) rather than by redistribution of solute by sedimentation then the parameter evaluated by equation 40 will be the initial concentration of solute, C(0).

Sedimentation equilibrium. The fundamental equation for the experimental determination of molecular weights from sedimentation equilibrium experiments is equation 14 of the preceding section. Two rearrangements of this equation are reproduced below. In what follows it is assumed that the non-ideality term in equation 14 may be neglected. The first form of this equation is

$$dC(r)/dr = M(1 - \bar{v}p)\omega^2 rC(r)/RT \tag{41}$$

The integral of this equation from $r = r_a$ (the meniscus), to $r = r_b$ (the cell bottom), is

$$(C(r_b) - C(r_a))/C(0) = M(1 - \bar{v}\rho)\omega^2(r_b^2 - r_a^2)/2RT \quad (42)$$

where $C(r_a)$, $C(r_b)$ are the protein concentrations at the meniscus and cell bottom respectively. The second form of equation 15 is

$$dC(r)/C(r) = M(1 - v\rho)\omega^2 r dr/RT \quad (43)$$

The integrated form of equation 43 between the limits $r = r_a$ to r is

$$\ln C(r) - \ln C(r_a) = M(1 - v\rho)\omega^2(r^2 - r_a^2)/2RT \quad (44)$$

Each of the terms in equation 42 is known or may be determined from plots of $KdC(r)/dr$. The initial concentration $C(0)$ was determined from a separate synthetic boundary experiment. A second method of determining molecular weights directly from equation 41 is from the slope of a plot of $(dC(r)/dr)/r$ versus $C(r)$. This method does not require a separate determination of $C(0)$ but, as will be shown, gives a different value for the molecular weight than does equation 42.

When sedimentation equilibrium experiments are carried out in the presence of high concentrations of dissociating reagents, e.g., 6.0 M guanidinium chloride, the matching of solvents necessary to eliminate all concentration gradients except that due to protein concentration is difficult even after extensive dialysis. This makes the determination of

C(0) subject to considerable uncertainty. Analysis of data according to equation 44 eliminates the necessity of determining C(0). An additional difficulty arises from the use of equation 44, however, since the concentration at the meniscus $C(r_a)$ must be determined. This difficulty may be overcome by choosing a rotor speed that depletes the meniscus of protein so that the left-hand side of equation 44 becomes independent of meniscus concentration (Yphantis, 1964). The molecular weight of the protein is now proportional to the slope of a plot of ln C(r) versus r^2.

Sedimentation velocity. The derivation of equation 33 results in an expression which relates the sedimentation coefficient to the change in the distance from the axis of rotation to the center of the sedimenting boundary with time. In fact, the sedimenting boundary is not infinitely sharp because as soon as the boundary between solvent and solution is established, diffusion will occur. However, it may be shown (Tanford, 1961) that for a symmetrical boundary, r_z is essentially identical to the maximum in the concentration gradient curve. At the center of this boundary, dC(r)/dr is a maximum and experimentally the peak of the photographic record of KdC(r)/dr versus r is easily determined.

Equation 33 may be integrated by separation of variables.

$$\omega^2 s(t - t_0) = \ln r_z - \ln r_a \qquad (45)$$

From equation 45 it is clear that the seimentation coefficient is proportional to the slope of a plot of $\ln r_z$ *versus* t.

Diffusion. Although equation 37 provides a definition of the diffusion constant, it does not provide an experimental method for determining its value.

If a solution of protein is layered over the solvent in which the protein is dissolved, then, in the absence of other forces the time course of the distribution of solute on either side of the solute-solvent interface is given by equation 46 (Schachman 1959).

$$dC/dr = C(0)/(2\pi Dt)^{1/2} \cdot e^{-r^2/4Dt} \qquad (46)$$

If at the maximum of a plot of dC(r)/dr *versus* r, r is defined as zero then equation 46 becomes

$$|dC/dr|_{max} = C(0)/(2\pi Dt)^{1/2} = h_{max} \qquad (47)$$

At any time t the area, A, under the plot of dC(r)/dr *versus* r will be proportional to C(0). Therefore at r = 0 equation 47 may be written as

$$h_{max} = A/(2\pi Dt)^{1/2} \qquad (48)$$

Rearranging equation 48 gives

$$A^2/h^2 = 4\pi Dt \qquad (49)$$

Therefore the slope of a plot of A^2/h^2 versus t is proportional to the diffusion constant.

In the ultracentrifuge the layering process is accomplished by using a synthetic boundary cell. This cell consists of two chambers connected by a very thin capillary tube halfway up the wall separating the chambers. One chamber is filled with protein solution to a point below the capillary tube while the second chamber is filled with solvent to a point somewhat above the tube. The capillary tube is thin enough so that flow of solvent into the solution chamber occurs only when the centrifugal force on the cell exceeds 1,000 x g. This force does not cause appreciable sedimentation during the course of the experiment while at the same time the optical system of the ultracentrifuge provides a photographic record of dC(r)/dr versus r at several time intervals.

Molecular Weight Averages

The molecular weight averages with which this discussion will be concerned are the weight-average (M_w), and z-average (M_z) molecular weights. These averages are defined as

$$M_w = \sum_i M_i C_i / \sum_i C_i \qquad (50)$$

$$M_z = \sum_i C_i M_i^2 / \sum_i M_i C_i \qquad (51)$$

Treatment of a protein with dissociating reagents may not completely eliminate all specific protein-protein interactions. Sedimentation equilibrium studies on such preparations or on preparations which are otherwise polydisperse will result in the estimation of a molecular weight average. The purpose of this section is to determine which moments of the molecular weight are determined when sedimentation equilibrium data are treated as described previously.

For a heterogeneous preparation of protein, the behavior of each species must be described by equation 15

$$dC_i/dr = C_i M_i (1 - \bar{v}\rho)\omega^2 r/RT \qquad (52)$$

where terms with subscripts refer to the i^{th} species. The optical system will measure $K\sum_i dC_i(r)/dr$ at each point in the cell.

Integrating equation 52 between r_a and r_b gives

$$[2RT/\omega^2(1 - \bar{v}\rho)]\cdot[(C_i(r_b)-C_i(r_a)] = C_i(0)(r_b^2-r_a^2)M_i \qquad (53)$$

Summing over all components and dividing by $\sum_i C_i(0)$ gives

$$2RT/\omega^2(1 - \bar{v}\rho)\cdot[\sum_i (C_i(r_b)-C_i(r_a))/\sum_i C_i(0)] = M_w(r_b^2-r_a^2) \qquad (54)$$

Equation 54 is identical to equation 42 except that the molecular weight which is determined by treating data from sedimentation equilibrium experiments according to

equation 42 is the weight-average molecular weight.

It is clear from equation 41 that the slope of a plot of $(dC(r)/dr)/r$ versus $C(r)$ is proportional to the weight. Rearranging equation 53 and summing over all components gives

$$RT/r \cdot \sum_i dC_i(r)/dr = (1 - \bar{v}\rho)\omega^2 \cdot$$

$$\cdot [\sum_i M_i C_i(r) \cdot \sum_i C_i(r) / \sum_i C_i(r)] \qquad (55)$$

A polydisperse preparation of protein will thus result in experimental data from sedimentation equilibrium experiments which when plotted according to equation 41 will give a non-linear curve. The slope of this plot at any point is proportional to the weight-average molecular weight of the preparation at each point r.

The slope of a line joining the terminal points of such a plot can be expressed by equation 56.

$$\text{slope} = (1/r_b \sum_i dC_i(r)/dr)_{r=r_b} - (1/r_a \sum_i dC_i(r)/dr)_{r=r_a} /$$

$$/(\sum_i C_i(r_b) - \sum_i C_i r_a) \qquad (56)$$

Each parameter in equation 56 may be determined directly from experimental data. Using equation 52 for each $(dC_i(r)/dr)/r$ in the numerator of equation 56, and using equation 53 for each $C_i(r_b)-C_i(r_a)$ and summing over all components, equation 56 becomes

$$\text{slope} = (((1 - \bar{v}\rho)\omega^2)/2RT) \cdot \sum_i M_i^2 C_i(0) / \sum_i M_i C_i(0) \qquad (57)$$

The slope of such a line is therefore proportional to the Z-average molecular weight of the heterogeneous protein sample.

In order to determine which molecular weight average the meniscus depletion technique yields it is convenient to write equation 44 in the exponential form.

$$C(r) = C(a) \cdot \exp[[M(1 - \bar{v}\rho)\omega^2 r^2/2RT]] \cdot \cdot [-M(1 - \bar{v}\rho)\omega^2 r_a^2/2RT]] \qquad (58)$$

From the definition of M_w and using the substitution that $m_i = M_i(1 - \bar{v}\rho)\omega^2/RT$, then

$$m_w(r) = \sum_i m_i(r) C_i(r) / \sum_i C_i(r) \qquad (59)$$

substituting equation 59 into equation 58 yields

$$m_w(r) = \sum_i m_i(r) C_i(r) \cdot \exp[[m_i(r) r^2/2] \cdot [-m_i(r_a) r_a^2/2]] / / \sum_i C_i(a) \exp[[m_i(r) r^2/2] \cdot [-m_i(r_a) r_a^2/2]] \qquad (60)$$

The denominator of equation 60 is C(r) and the numerator is $dC(r)/d(r^2/2)$. Therefore equation 60 may be written as

$$m_w(r) = (1/C(r)) \cdot dC(r)/d(r^2/2) = d \ln C(r)/d(r^2/2) \qquad (61)$$

From the definition of m_i and equation 61 it is seen that the meniscus-depletion technique yields the weight-average molecular weight when the data from such experiments are plotted according to equation 44.

Molecular Shape

The molecular weight of a protein may be determined from sedimentation equilibrium experiments or from the sedimentation coefficient and diffusion constant. In the latter case, if the constants are determined under identical conditions, then the frictional coefficient in equations 36 and 23 are identical and may be eliminated from the two equations to give

$$M = sRT/(D(1-\bar{v}\rho)) \tag{62}$$

Equations 23 and 36 are more useful, however, in providing information concerning the shape of the macromolecule.

Stokes (1880) has shown that the frictional coefficient of a spherical particle of radius R_o in a solvent of viscosity η is given by equation 63.

$$f_0 = 6\pi\eta R_0^3 \tag{63}$$

A protein particle is considered to occupy a volume which depends on its size and its degree of hydration. If the protein particle is assumed to be a sphere then its radius can be shown (Oncley, 1943) to be

$$R_0 = [3M(v_2 + \delta\bar{v}_1)/4\pi\underline{N}]^{1/3} \tag{64}$$

where $\bar{v}_2$ is the partial specific volume of the protein, $\bar{v}_1$ the partial specific volume of the solvent and δ the

degree of hydration, i.e., grams of water per gram of protein. Combining the definition of the diffusion constant (extrapolated to zero protein concentration) with equations 63 and 64 results in an expression for the frictional coefficient of the protein in terms of its molecular weight and hydrodynamic volume.

$$f = f_0 kT/D = 6\pi\eta f/f_0 \, [3M(\bar{v}_2 + \delta v_1)/4\pi\underline{N}]^{1/3} \qquad (65)$$

The term f/f_0, the ratio of the frictional coefficient of the molecule to the coefficient the molecule would have if it were a perfect sphere, provides information concerning the shape of the protein, i.e., the closer the ratio is to unity, the more spherical is the molecule under consideration. However, the terms δ and f/f_0 cannot both be determined from measurements of the diffusion constant. Nevertheless, the minimum frictional coefficient, f_{min}, as well as the maximum diffusion constant compatible with the known molecular weight, D_{max}, can be estimated by setting δ equal to zero and f/f_0 equal to unity. Equation 65 then becomes

$$f_{min} = kT/D_{max} = 6\pi\eta(3Mv_2/4\pi\underline{N})^{1/3} \qquad (66)$$

Combining equation 66 with the definition of the diffusion constant yields

$$f/f_{min} = D_{max}/D \qquad (67)$$

Since D_{max} may be computed from known parameters and an independently determined value of the molecular weight, the frictional ratio f/f_{min} can also be determined. The calculated ratio may then be compared to the same ratio obtained for proteins of known shape, and an indication of the asymmetry of the protein under study may be obtained.

In addition, by assuming reasonable values for δ in equation 65, Oncley (1943) has related the ratio f/f_0 to the ratio of the major to minor axes of prolate and oblate ellipsoids. These equations will not be derived here since extensive use of them will not be made. However, the procedure outlined above will be used to obtain an estimate of the assymmetry of AGDH.

Experimental Errors in the Estimation of Molecular Weights

The preceding sections indicate that estimation of molecular weights of ionic macromolecules in the presence of supporting electrolyte involves an uncertainty of about 5 per cent if there are no specific interactions between any of the components in the system. These uncertainties result from the inability to determine the net charge on the macromolecule at or near the isoelectric pH (Equation 21). While these inaccuracies may be minimized by performing the experiments at neutral pH and moderate ionic strength,

nevertheless in the absence of data on the net charge of the protein, the values determined from sedimentation equilibrium must be assigned an uncertainty of 5 per cent on the basis of theoretical considerations alone even if non-ideality terms are neglected.

A second possible source of error arises when the derivatives of activity coefficients are neglected (equation 17). At the low protein concentrations used experimentally and in the presence of moderate electrolyte concentrations, this assumption is probably valid. However, as has been pointed out by Hade and Tanford (1968), this assumption introduces significant error when experiments are performed in the presence of high concentrations of guanidinium chloride.

A final source of error arises from determinations of K dC/dr and r from the photographic record obtained from the ultracentrifuge. The K dC/dr at r (the Y_i in Figure 5, page 61) were measured by placing the photographic plate in a Nippon Kogzku microcomparator. It was found that the variance in the measurement of the Y_i was independent of the value of the Y_i except for small values of Y_i. This is due to the fact that the plots of K dC/dr versus r recorded photographically are not sharp lines but are recorded as bands (page 108). The overlap between the

baseline (solvent) curve and the curve due to protein concentration changes at low protein concentration causes greater uncertainty in the estimation of Y_i than at points where the two curves are well separated. However these points do not greatly affect the results. The statistical errors involved in measuring the schlieren displacements and in the concentrations derived from such measurements may be evaluated as follows.

From elementary statistics (Worthing and Geffner, 1943) if $A = F(Y_i)$ then

$$\mathrm{Var}(A) = \sum_i (\partial A/\partial Y_i)^2 \, \mathrm{Var}(Y_i) \qquad (67a)$$

where A is the area under the plot of K dC/dr versus r. From equation 41:

$$\mathrm{Var}(A) = \sum_i (\partial A/\partial Y_i)^2 \cdot \mathrm{Var}(Y_i) + \sum_i (\partial A/\partial Y_{i-1})^2 \cdot \mathrm{Var}(Y_{i-1}) \qquad (68)$$

Also from equation 40

$$\partial A/\partial Y_i = \Delta r_i/2 \; ; \; \partial A/\partial Y_{i-1} = \Delta r_i/2 \qquad (69)$$

Since $\mathrm{Var}(Y_i) = \mathrm{Var}(Y_{i-1})$ and since the Y_i were measured at equal intervals of r_i equation 69 becomes

$$\mathrm{Var}(A) = 2N \, \mathrm{Var}(Y_i) \, (\Delta r/2)^2 \qquad (70)$$

where N is the number of increments of Δr_i.

The relative error in the estimate of any area is therefore

$$\mathrm{Var}(A)/A = 2N\ \mathrm{Var}(Y_i)(\Delta r/2)^2/\Sigma_i(\Delta r/2)(Y_i + Y_{i-1}) \qquad (71)$$

In typical experiments the $\mathrm{Var}(Y_i)$ is about .002 cm, N is 20, $\Delta r/2$ is 2.3×10^{-3} cm and A is 2×10^{-2} cm^2. The relative error in the estimate of a typical area is therefore about 0.1 per cent. This is considerably less than the uncertainty which arises from the assumptions which were made in the derivations of the equations in the preceding sections.

Inspection of equation 71 shows that the relative error in the estimate of an area depends on the area. Therefore, when functions of the area are plotted against functions of the radius it is necessary to apply a weighting factor. Use of the meniscus depletion technique requires estimation of the slope of a plot of ln C(r) versus r^2. From elementary statistics the proper weighting factor is defined as

$$1/w_i = (\partial F(A_i)/\partial Y_i)^2 \qquad (72)$$

Since $F(A_i) = \ln A_i$ and from equation 40

$$A_i = A_{i-1} + (\Delta r/2)(Y_i + Y_{i-1}) \qquad (73)$$

then

$$\partial F(A_i)/\partial Y_i = d \ln A_i/dY_i = (\Delta r/2)/\ln A_i \qquad (74)$$

and

$$w_i = (2 \ln A_i/\Delta r)^2 \qquad (75)$$

In practice, the slopes of plots of ln C(r) versus

r^2 were determined by a weighted least-squares procedure. This technique also gives an estimate of the variance of the slopes so determined.

In typical experiments the uncertainty in the estimate of the slopes was about one to two per cent, again less than the uncertainty arising from the assumptions and approximations made in the derivation of the equations.

CHAPTER V

RESULTS

Enzyme Kinetic Experiments

The apparent Michaelis constant (K_m) and maximal velocity (V_m) were estimated for each substrate of AGDH and are listed in Table II. Estimates of these parameters obtained by two other workers using an impure preparation of the enzyme are also listed in Table II. Figures 6a-d are the plots of v(0) *versus* v(0)/S(0) based on the data obtained from these experiments. The values of K_m and V_m were determined by fitting initial rate data to the Michaelis-Menten equation (Henri, 1903) using the method of least squares. Each initial rate was assigned a statistical weight proportional to $(v(0))^4$ (Wilkinson, 1961). The standard deviations (S.D.) of the data points from the fitted line are also reported in these figures.

Ion-exchange Chromatography

The results of ion-exchange chromatography of AGDH on DEAE A-25 indicated that as many as 8 peaks of enzyme activity could be eluted in these experiments. Since the

TABLE II

KINETIC PARAMETERS OF GLYCEROL DEHYDROGENASE

Substrate	Concentration of Saturating Substrate M	K_m (M) a	b	c	V_m Moles/Min./ Mole Enz.
NAD	(Glycerol) 5×10^{-2}	2.8×10^{-4}	1.5×10^{-4}	d	2.7×10^{-5}
Glycerol	(NAD) 3×10^{-3}	2.4×10^{-3}	1.3×10^{-2}	1.3×10^{-2}	1.4×10^{-5}
NADH	(DHA) 5×10^{-3}	2.3×10^{-5}	1.4×10^{-5}	d	2.3×10^{-5}
DHA	(NADH) 1×10^{-4}	5.1×10^{-4}	1.3×10^{-3}	1.3×10^{-4}	1.9×10^{-5}

[a]This study; DHA, dihydroxyacetone.
[b]Burton (1963).
[c]Strickland and Miller (1968).
[d]Not reported.

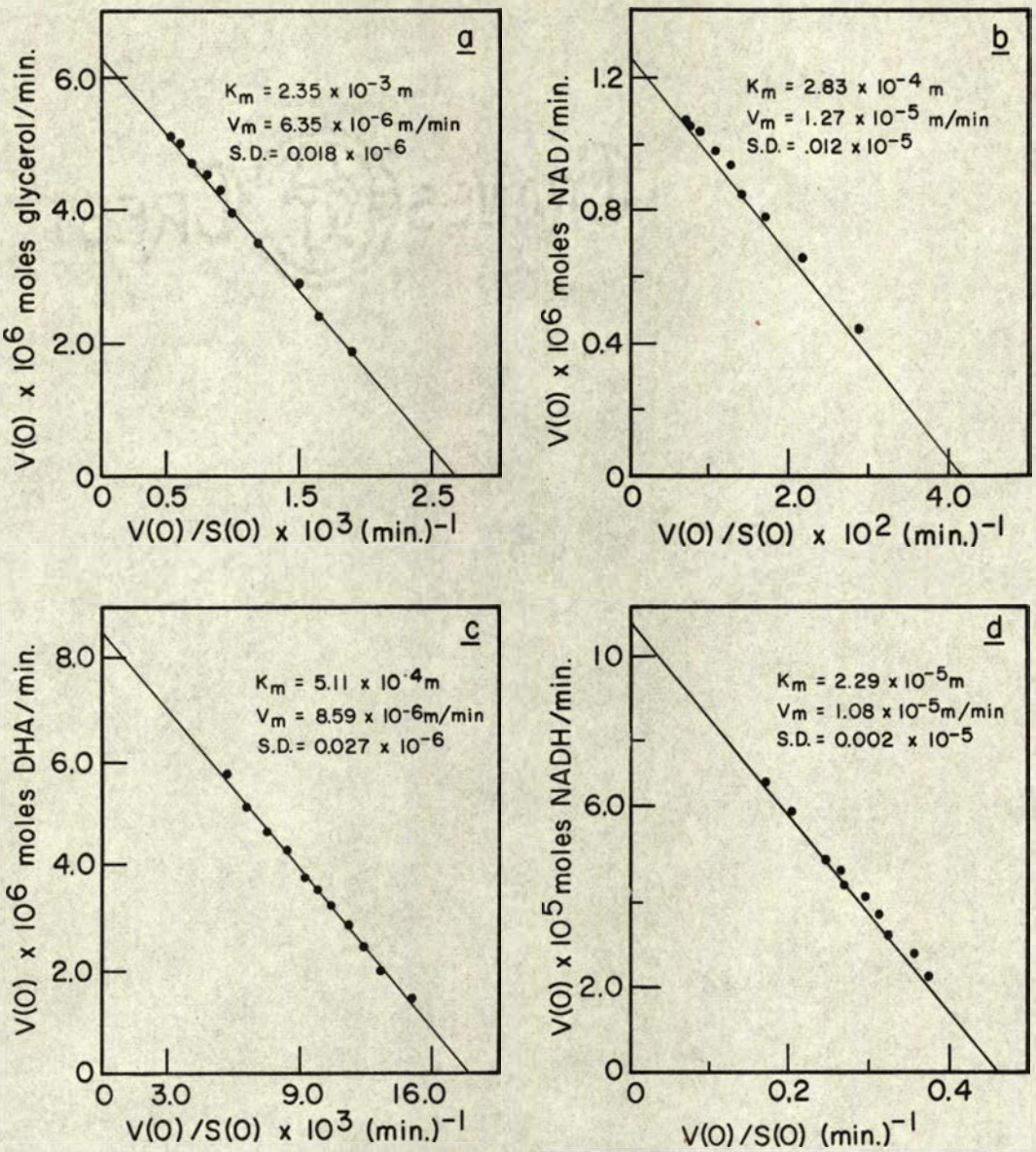

Figure 6. Plots of v(0) versus v(0)/S(0) from kinetic data obtained with glycerol dehydrogenase.

(a) Glycerol varies.
(b) NAD varies.
(c) Dihydroxyacetone varies.
(d) NADH varies.

possibility exists that the number of peaks is greater than eight but that some were missed because the fractions which were collected were too large (15 ml), these experiments were repeated and 5-ml fractions were collected. A typical elution pattern obtained from these experiments is shown in Figure 7. Note that some peaks are the result of a single measurement. These peaks did not appear consistently throughout several experiments. The thirteen major peaks and one minor peak (peak zero in Figure 7) which did appear consistently are indicated by bars over the peaks.

Polyacrylamide Gel Electrophoresis

Figure 8a is an illustration of the results obtained when an apparently pure preparation of the enzyme was subjected to electrophoresis on polyacrylamide gel at pH 9.5. Each band of protein in Figure 8a also gave a positive stain for enzyme activity although not in proportion to the amount of protein present.

Treatment of the enzyme with 2.5 M urea gave the results illustrated in Figure 8b. Under these conditions distinct bands of protein were not observed even though experiments with bovine serum albumin under identical conditions gave sharp distinct bands of protein.

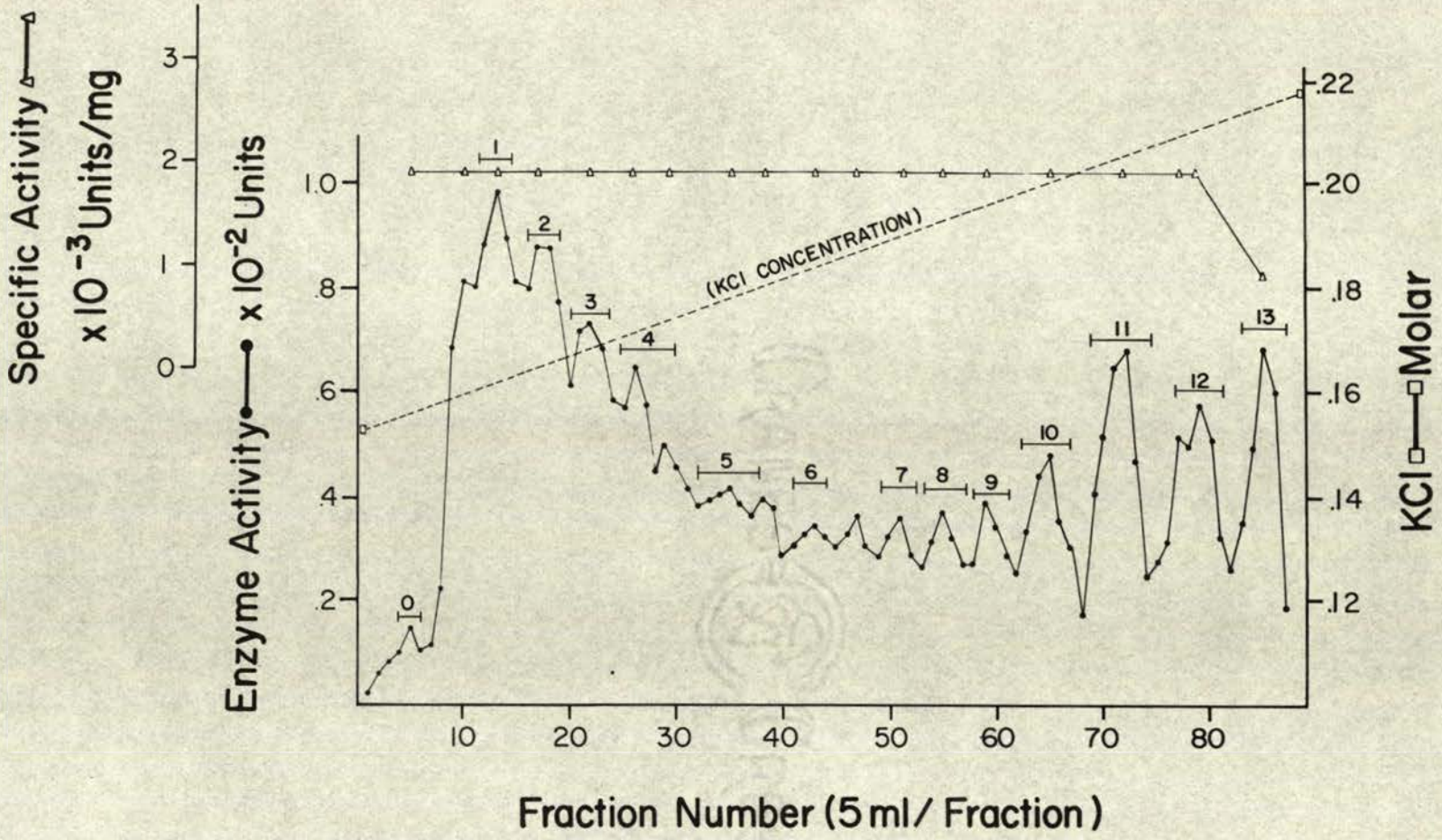

Figure 7. Elution pattern of glycerol dehydrogenase from DEAE A-25.

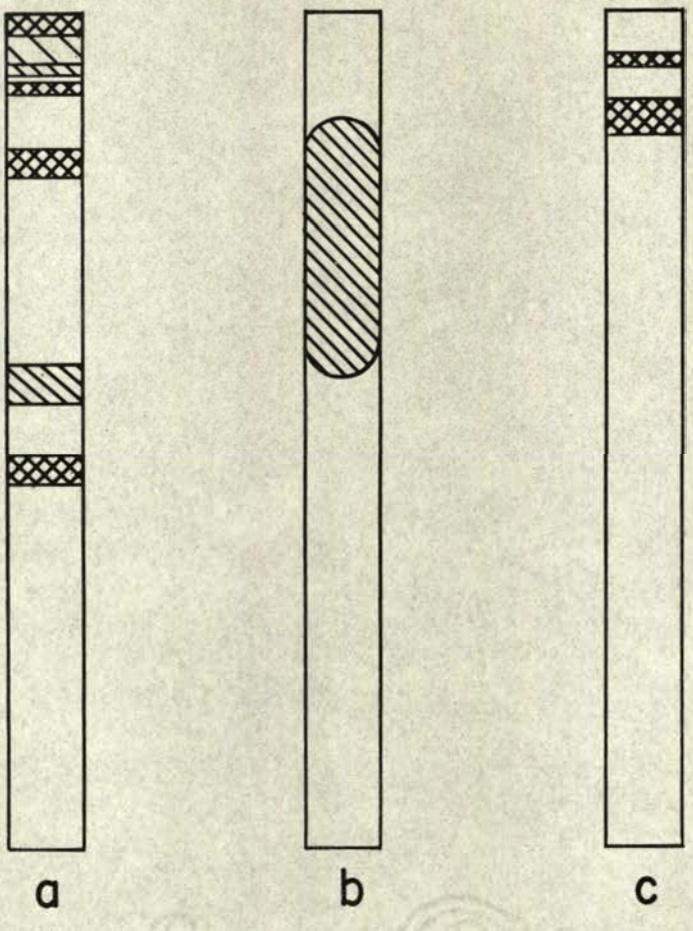

Figure 8. Polyacrylamide gel electrophoresis of glycerol dehydrogenase stained for protein.

(a) Native enzyme.
(b) Enzyme treated with urea.
(c) Enzyme treated with urea and dithiothreitol.

The slower component in c is designated as protein A.

Treatment of the enzyme with 2.5 M urea and 10^{-3} M dithiothreitol at pH 9.5 gave the results illustrated in Figure 8c. In this case only two distinct bands of protein were observed. Similar results were obtained at pH 4.0. When gels from the experiments in urea were stained for enzyme activity, no activity was found.

Amino Acid Analysis

Table III lists the results of amino acid analysis of a pooled preparation of the enzyme eluted from DEAE A-50. Table IV lists the amino acid analysis of each of the two proteins which appeared on polyacrylamide gel electrophoresis of the enzyme treated with urea and dithiothreitol.

Table V summarizes the amino acid composition of each of the thirteen major peaks and one minor peak of activity eluted from Sephadex DEAE A-25. Oxidation of the cysteine and methionine residues during the course of hydrolysis requires that hydrolyses be carried out for increasing lengths of time and the results extrapolated to zero time. Since these samples were hydrolyzed for twenty-four hours only, the cysteine and methionine content are not reported. However, the results did indicate that these two residues were present in approximately the same amounts

TABLE III

AMINO ACID COMPOSITION OF GLYCEROL DEHYDROGENASE

AMINO ACID	RESIDUES PER[a] 340,000 g PROTEIN
Lysine	203
Histidine	66
Arginine	103
Aspartate[b]	277
Threonine	166
Serine	400
Glutamate[b]	326
Proline	158
Glycine	476
Alanine	359
Cysteine	61
Valine	289
Methionine	105
Isoleucine	117
Leucine	214
Tyrosine	81
Phenylalanine	109

[a]24-, 36-, and 48-hour hydrolyses extrapolated to zero time.

[b]Includes Glutamine and Asparagine.

TABLE IV

AMINO ACID COMPOSITION OF PROTEINS ISOLATED FROM POLYACRYLAMIDE GEL

AMINO ACID	RESIDUES PER 28,000 G	
	Protein A	Protein B
Lysine	17	17
Histidine	4	7
Arginine	8	9
Aspartate[a]	20	26
Threonine	14	14
Serine	36	29
Glutamate[a]	26	32
Proline	15	15
Glycine	45	33
Alanine	30	30
Cysteine	5	5
Valine	31	16
Methionine	3	4
Isoleucine	8	10
Leucine	14	22
Tyrosine	7	7
Phenylalanine	8	9

[a]Includes Glutamine and Asparagine.

TABLE V

AMINO ACID COMPOSITION OF PEAKS ELUTED FROM ION-EXCHANGE CHROMATOGRAPHY

Peak Number	(0)	1	2	3	4	5	6	7	8	9	10	11	12	13
Amino Acid	Moles of Each Residue per 3.4×10^5 g Protein													
Lysine	205	211	206	198	209	204	206	212	205	199	197	206	204	209
Histidine	45	50	50	53	56	59	62	68	69	73	74	78	84	85
Arginine	105	110	107	102	109	108	102	107	102	102	108	105	108	102
Aspartate[a]	235	250	246	258	258	263	272	291	291	289	308	300	309	309
Threonine	170	176	175	164	167	173	167	165	178	176	171	167	177	170
Serine	437	429	414	418	405	401	389	381	366	371	370	359	349	351
Glutamate[a]	314	319	330	338	336	347	347	368	370	376	374	385	391	386
Glycine	537	526	507	504	485	475	461	446	439	415	425	415	389	390
Alanine	363	360	364	371	364	351	360	367	357	362	368	359	357	362
Valine	370	355	336	319	321	291	272	255	256	246	237	214	202	194
Isoleucine	100	105	104	108	106	109	113	113	120	120	122	125	127	129
Leucine	168	174	183	189	189	197	207	216	220	225	233	240	254	268
Tyrosine	79	79	80	76	78	79	84	78	79	78	83	77	79	80
Phenylalanine	102	99	103	97	107	104	101	97	104	102	102	105	107	102

[a]Includes Glutamine and Asparagine.

as in the pooled material.

Ultracentrifuge experiments

Native enzyme. Figure 9a-b shows plots of the sedimentation and diffusion constants respectively versus the protein concentrations at which they were determined. When these plots were extrapolated to zero protein concentration, a value of 10.7×10^{-13} sec^{-1} was obtained from the sedimentation constand and 3.07×10^{-7} cm^2/sec as the value for the diffusion constant.

The partial specific volume of the native enzyme was calculated from the amino acid composition of the pooled enzyme preparation given in Table III (page 84), and the partial specific volume of each amino acid residue (Schachman, 1963). Table VI illustrates the manner in which the value of 0.72 ml/g was determined. The molecular weight calculated from these constants and equation 62 was 3.45×10^5 daltons.

Sedimentation equilibrium experiments were performed with the native enzyme at speeds which did not deplete the meniscus of protein. Initial concentrations were determined from synthetic boundary experiments and calculations from these experiments were based on equation 42. Figure 10 is a plot of the observed molecular weight of AGDH versus

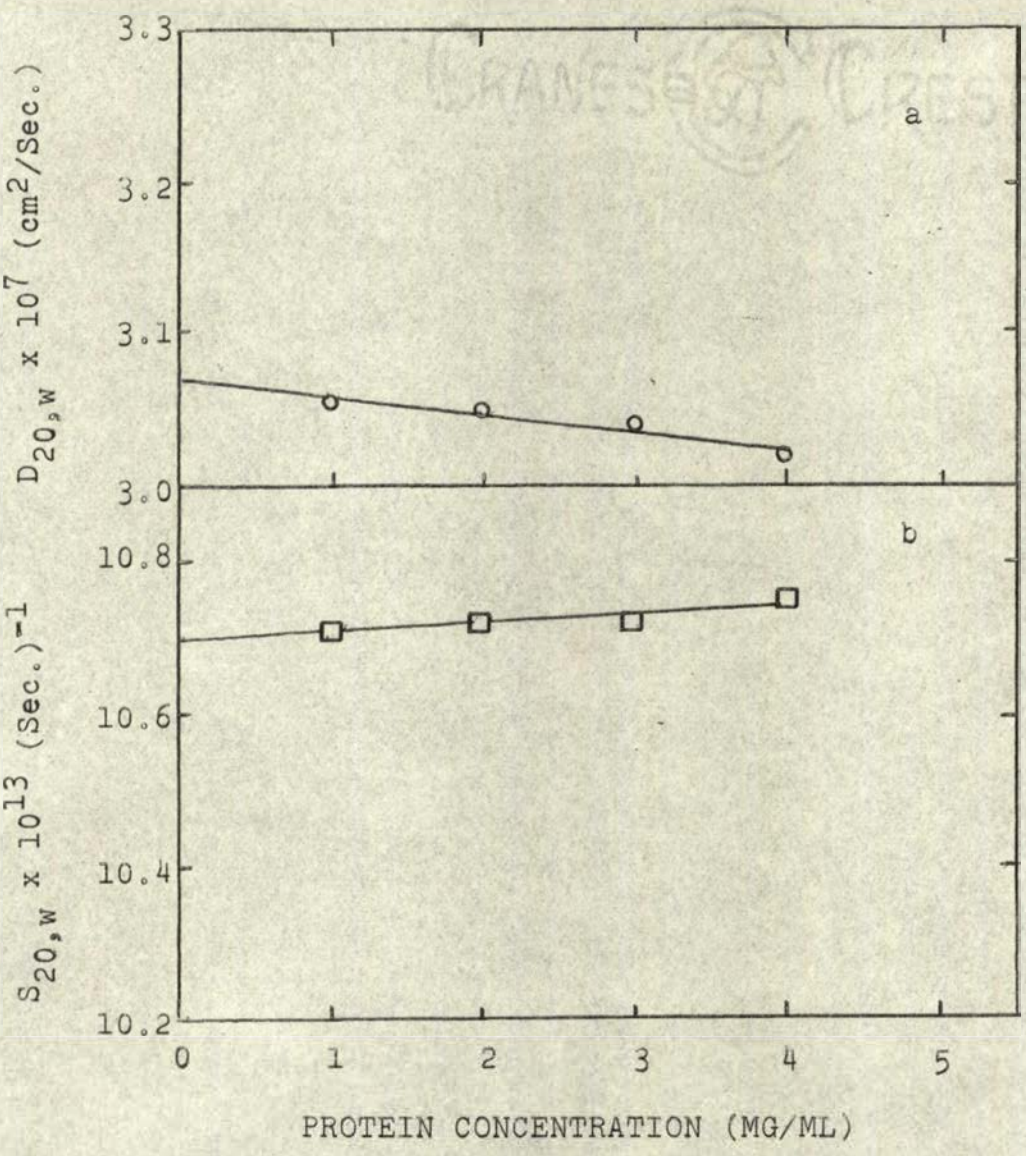

Figure 9 Sedimentation and diffusion constants of glycerol dehydrogenase extrapolated to zero protein concentration.

(a) Diffusion constant.
(b) Sedimentation constant.

TABLE VI

DETERMINATION OF PARTIAL SPECIFIC VOLUME OF GLYCEROL DEHYDROGENASE FROM AMINO ACID COMPOSITION

Amino Acid	Weight Percent[ab]	Residue Partial Specific Volume[c]	Residue Volume Percent[d]
Lysine	6.83	0.82	5.60
Arginine	2.36	0.67	1.58
Histidine	4.21	0.70	2.95
Aspartate	8.57	0.69	5.06
Threonine	4.89	0.70	3.42
Serine	9.75	0.63	6.14
Glutamate	12.30	0.66	8.12
Proline	4.84	0.74	3.68
Glycine	8.29	0.64	5.31
Alanine	7.71	0.74	5.71
Cysteine	1.78	0.63	1.12
Valine	7.85	0.86	6.75
Methionine	3.53	0.75	2.65
Isoleucine	3.55	0.90	3.20
Leucine	6.52	0.90	5.87
Tyrosine	3.33	0.71	2.36
Phenylalanine	3.57	0.77	2.75
		$\sum_i w_i$ = 100 g	$\sum_i w_i v_i$ = 72.2 ml

[a]Calculated from amino acid composition of pooled protein given in Table

[b]Units: g of each residue per 100 g protein.

[c]Units: ml per g of each residue.

[d]Units: ml per residue per 100 g protein.

$\bar{v} = \sum_i w_i v_i / \sum_i w_i$ = 0.722 ml/g protein

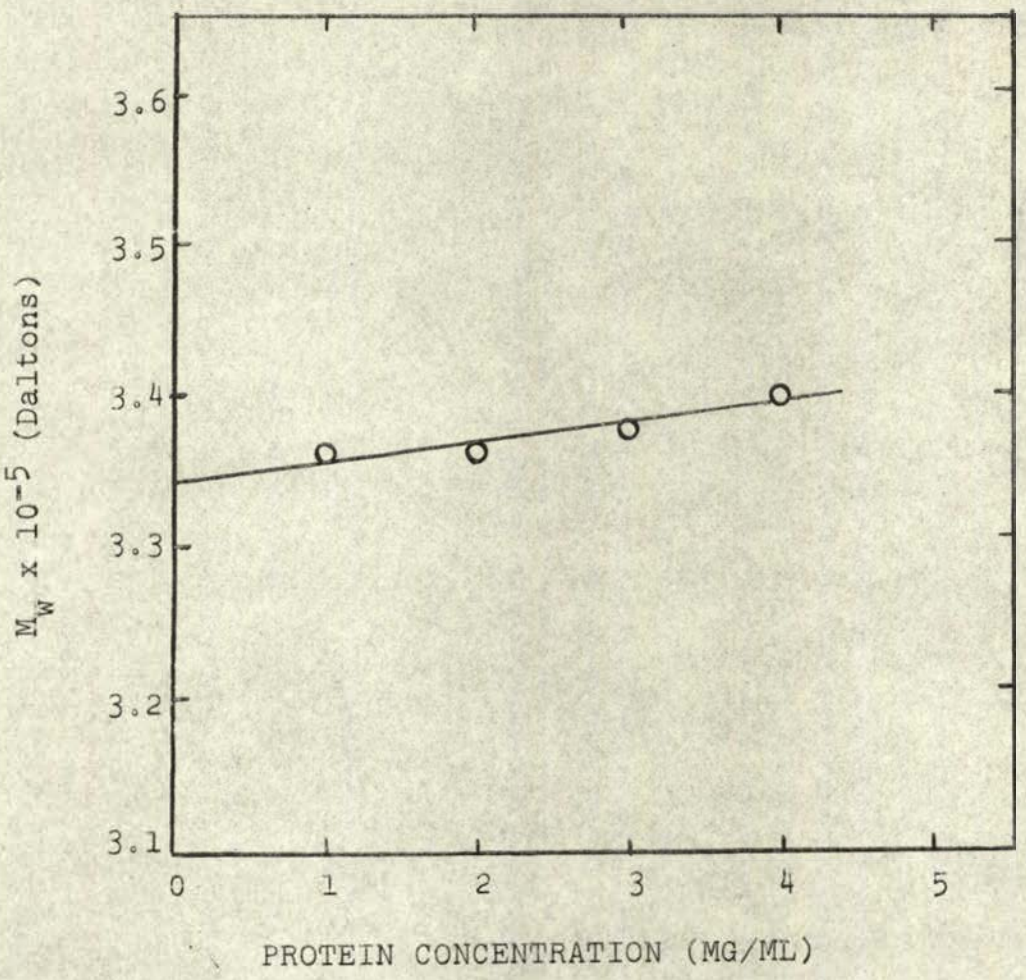

Figure 10. Molecular weight of glycerol dehydrogenase *versus* protein concentration.

the protein concentration at which the experiment was performed. When this plot was extrapolated to zero protein concentration, a value of 3.34×10^5 daltons was obtained for the weight-average molecular weight. An estimate of the z-average molecular weight of the preparation was obtained by plotting the data obtained at a protein concentration of 3 mg/ml according to equation 41 (Figure 11). The slight non-linearity of this plot indicates that the preparation is poly disperse. The z-average molecular weight calculated from equation 57 was 4.9×10^5 daltons. The weight-average molecular weight determined from equation 55 was estimated at two points to illustrate dispersity ($M_w(1)$, $M_w(2)$; Figure 11). Thus at the meniscus the weight-average molecular weight approaches 3.4×10^5 daltons while at the cell bottom it approaches 4.5×10^5 daltons. A frictional ratio (f/f_{min}) of 1.5 was calculated using equation 66 and the values of $S^\circ_{20,w}$, $D^\circ_{20,w}$, and the weight-average molecular weight which was estimated from sedimentation equilibrium experiments. For convenience the hydrodynamic properties of native AGDH are summarized in Table VII.

Enzyme treated with urea. The weight average molecular weight of AGDH treated with 2.5 M urea, estimated from a low-speed sedimentation equilibrium experiment,

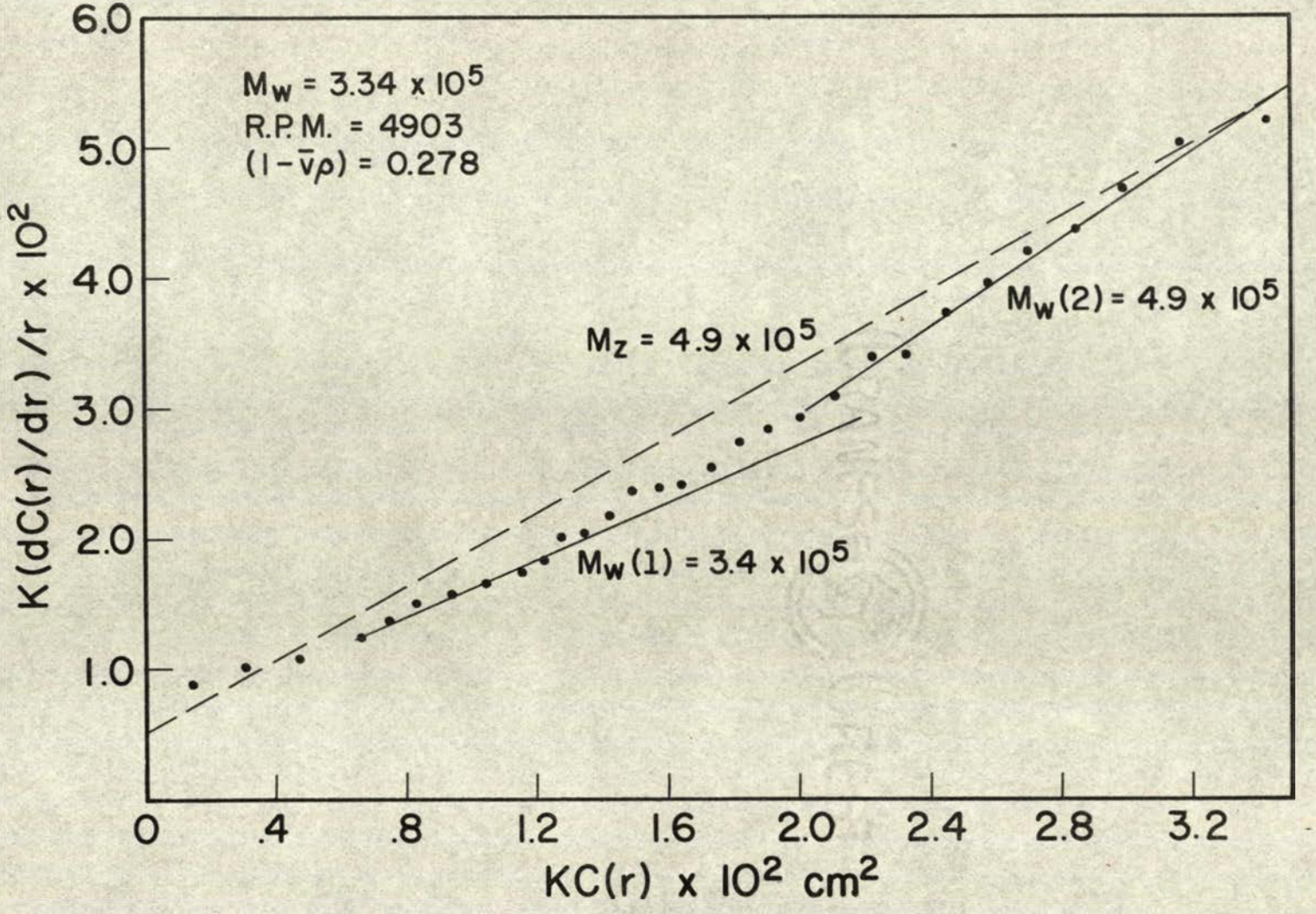

Figure 11. Z-average molecular weight of native glycerol dehydrogenase.

TABLE VII

HYDRODYNAMIC PROPERTIES OF GLYCEROL DEHYDROGENASE

PARAMETER	VALUE
$s^{\circ}_{20,w}$	10.7 S
$D^{\circ}_{20,w}$	[a] 3.07×10^{-7} $cm^2/sec.$
$f/f_{min.}$	1.5
$\bar{v}$	[b] 7.2×10^{-1} ml/g
Molecular weight	[c] 3.45×10^5 daltons
	[d] 3.34×10^5 daltons

[a] From synthetic boundary experiment.
[b] From amino acid composition.
[c] From $s^{\circ}_{20,w}$ and $D^{\circ}_{20,w}$.
[d] From sedimentation equilibrium.

was $5.8 \pm 0.2 \times 10^4$ daltons. An estimate of the dispersity of the preparation was obtained by plotting the data from one of these experiments according to equation 41. The non-linearity of this plot suggests that the preparation at this stage of dissociation is polydisperse (Figure 12). The z-average molecular weight of this preparation was 2.8×10^5 daltons, while the weight-average molecular weight at the meniscus approaches 5.8×10^4 daltons and at the cell bottom approaches 2.0×10^5 daltons.

Enzyme treated with guanidinium chloride. Figure 13 is a plot of log C(r) versus r^2 obtained from a high-speed sedimentation equilibrium experiment in which AGDH was treated with 6.0 M guanidinium chloride and 5.0×10^{-3} M dithiothreitol. The inset in this and other plots based on data obtained from the meniscus depletion technique is a plot of C(r) versus r^2 throughout the entire cell. The range of the data points is the maximum expected uncertainty in log C(r). The weight-average molecular weight estimated from this experiment was 3.2×10^4 daltons.

Since the presence of forms of AGDH in which all disulfide bonds are not reduced will cause the observed weight-average molecular weight to be greater than the smallest species present, a series of sedimentation equilibrium experiments was carried out at increasing

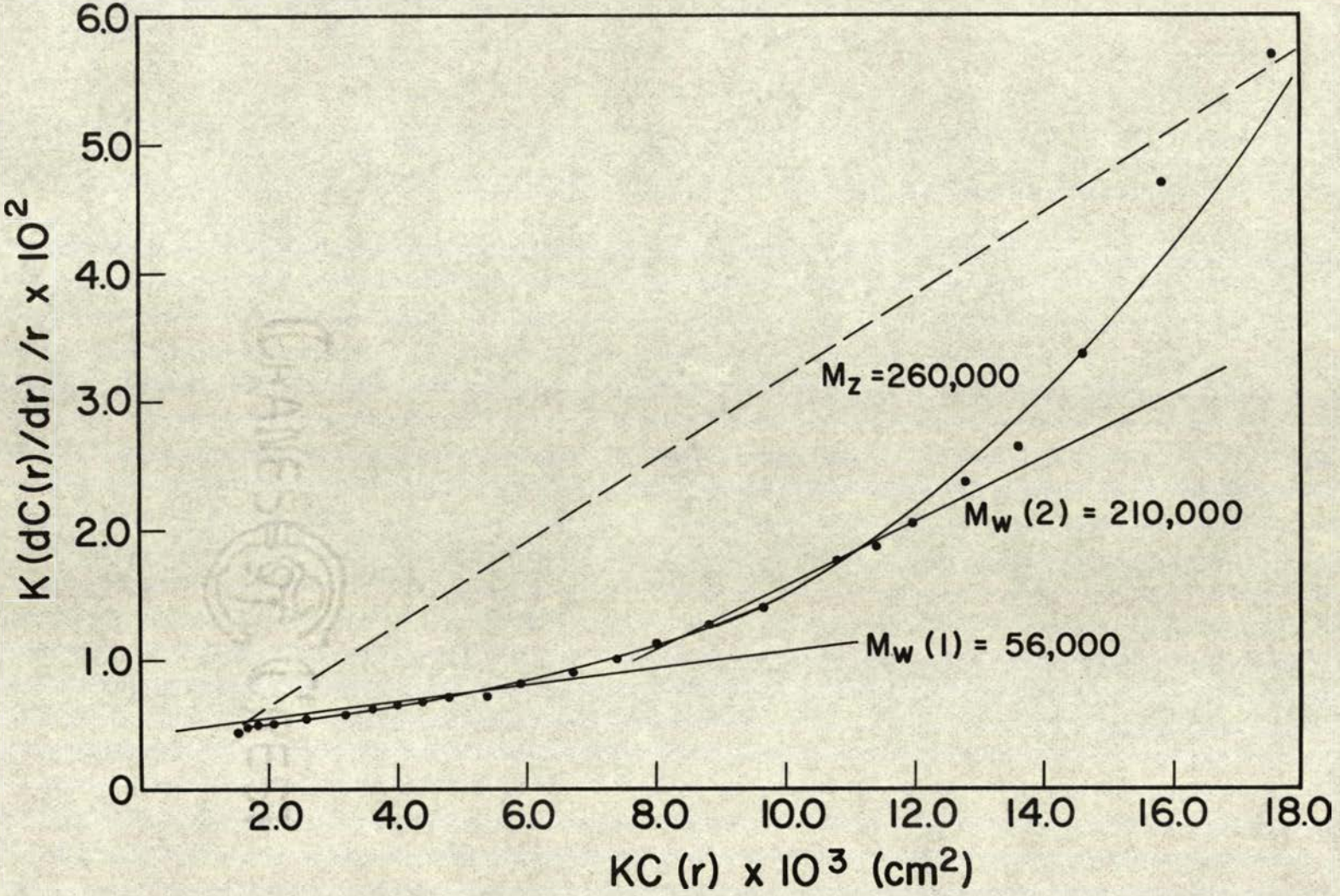

Figure 12. Z-average molecular weight of glycerol dehydrogenase treated with urea.

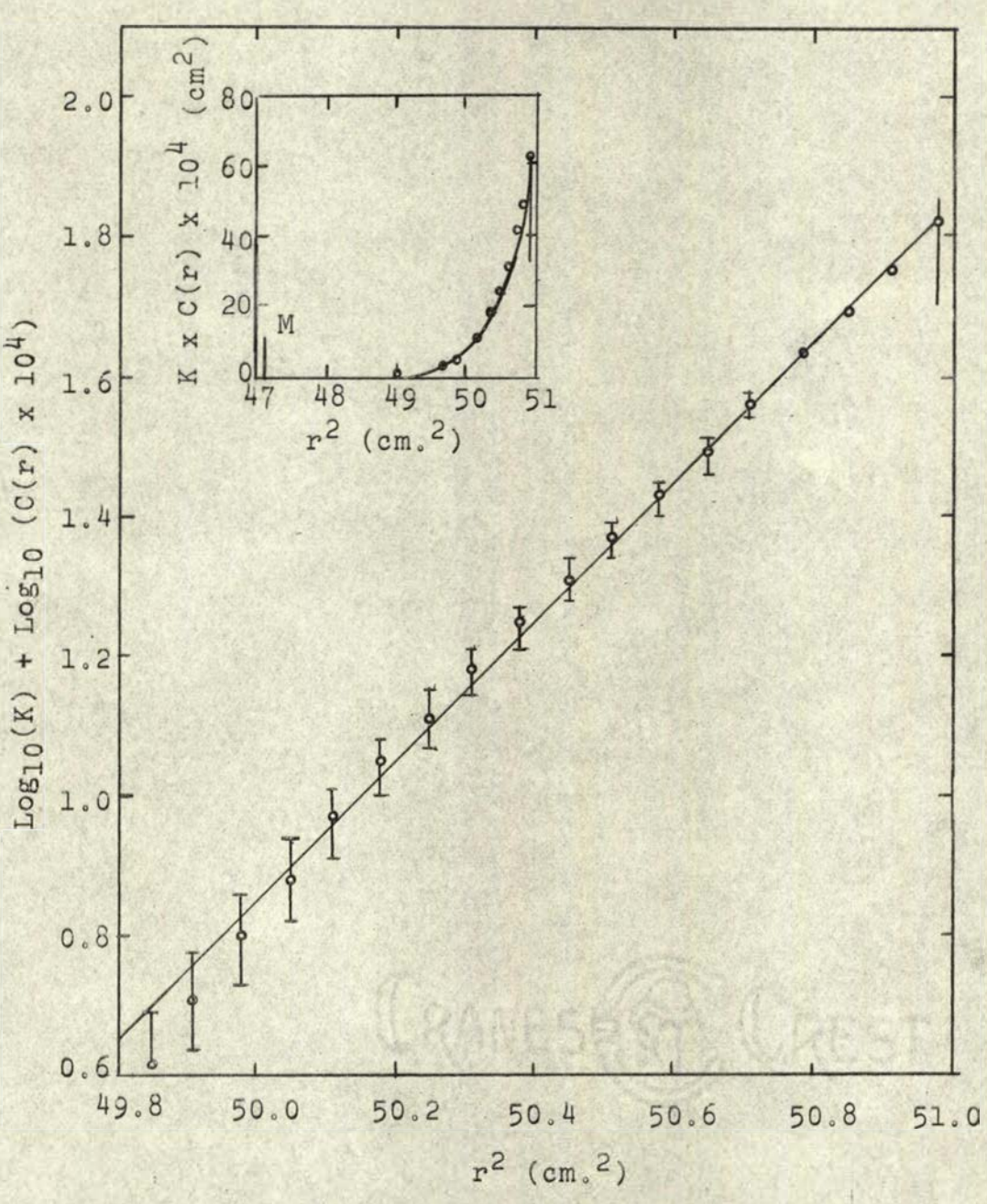

Figure 13. Meniscus depletion experiment with glycerol dehydrogenase treated with 5 x 10^{-3} M dithiothreitol and 6.0 M guanidinium chloride.

rotor speeds on the enzyme treated with 6.0 M guanidinium chloride and 10^{-2} M dithiothreitol.

The results of an experiment at 21,000 r.p.m. are shown in Figure 14. At this rotor speed the meniscus was not depleted and the data were plotted in the form (dC(r)/dr)/r versus C(r) (equation 55). The z-average molecular weight calculated from the slope of the line connecting the end points of the plot (equation 57) was 4.3×10^4 daltons. The weight-average molecular weight at the meniscus was 2.8×10^4 daltons while at the cell bottom it approached 4.3×10^4 daltons.

Increasing the rotor speed to 31,000 r.p.m. was sufficient to deplete the meniscus. The plot of log C(r) versus r^2 based on the data from this experiment is presented in Figure 15. The weight-average molecular weight calculated from this plot was 3.6×10^4 daltons. When the rotor speed was increased to 39,000 r.p.m., the plot in Figure 16 was obtained. In this case the observed weight-average molecular weight was 2.8×10^4 daltons. The weight-average molecular weight of the carboxymethyl derivative of the enzyme estimated from a meniscus depletion experiment in the presence of 6.0 M guanidinium chloride was 2.8×10^4 daltons. Table VIII summarizes the results obtained from sedimentation equilibrium experiments in which the enzyme

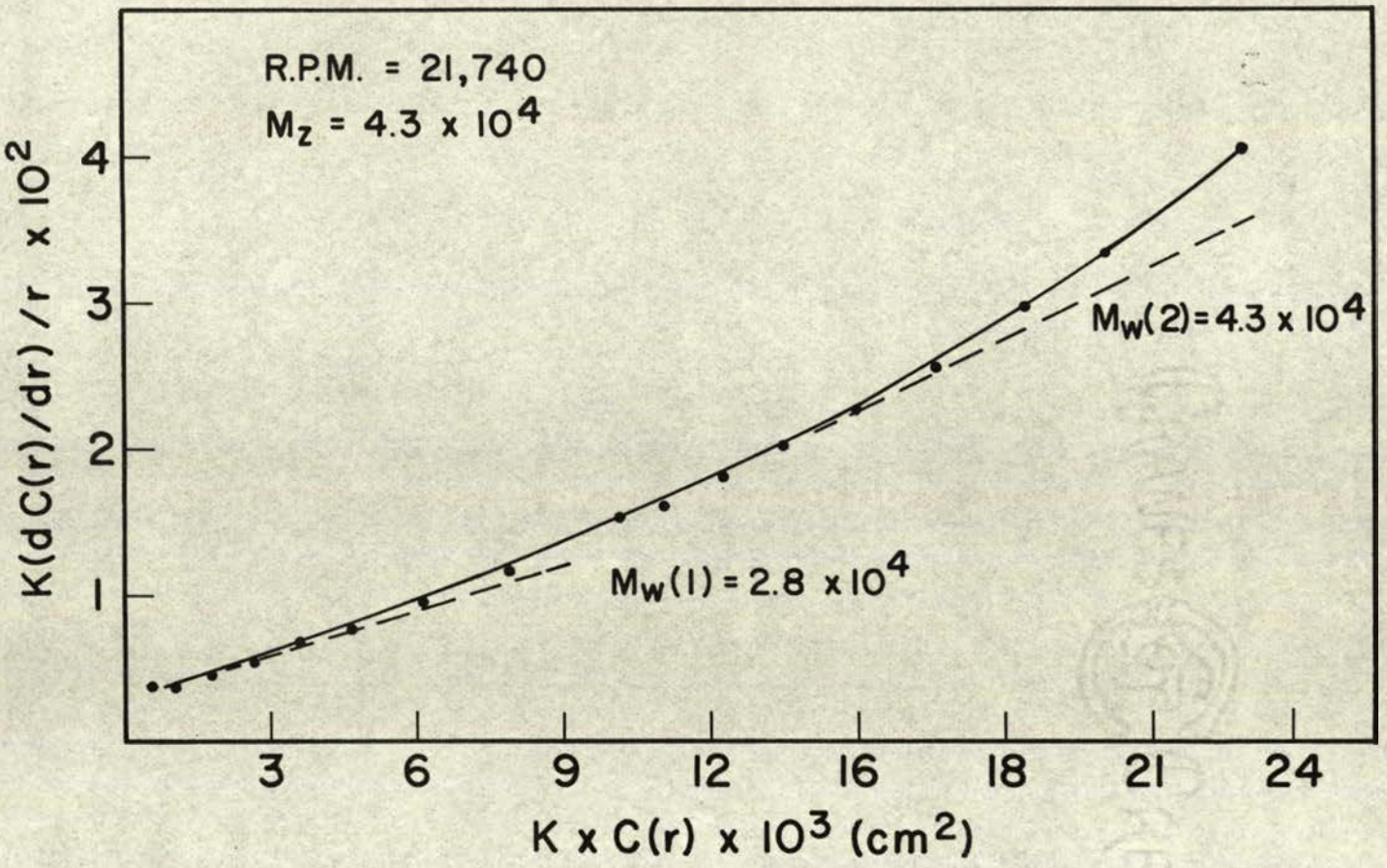

Figure 14. Z-average molecular weight of glycerol dehydrogenase treated with 0.01 M dithiothreitol and 6.0 M guanidinium chloride.

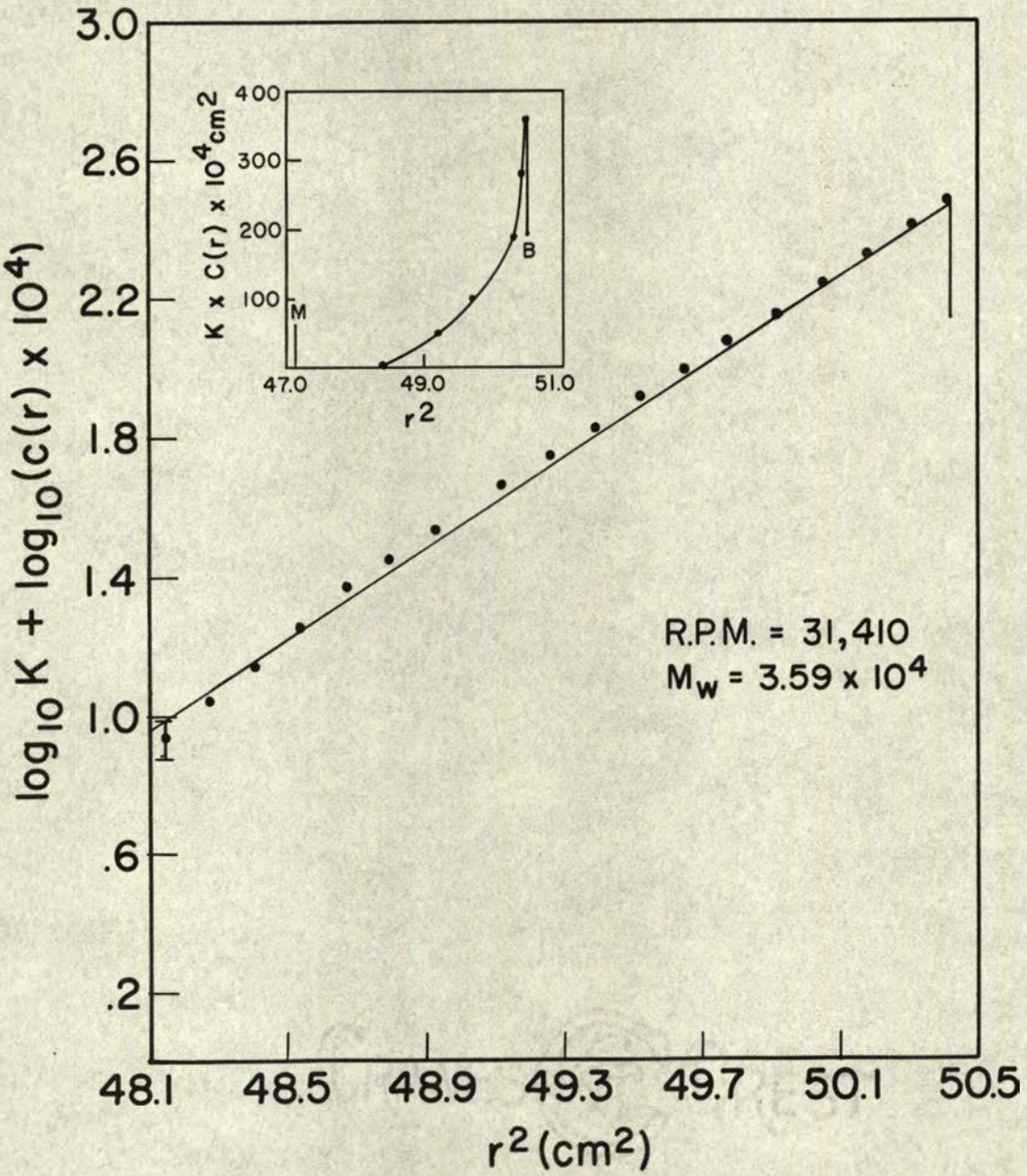

Figure 15. Meniscus depletion experiment with glycerol dehydrogenase treated with 0.01 M dithiothreitol and 6.0 M guanidinium chloride at 31,000 revolutions per minute.

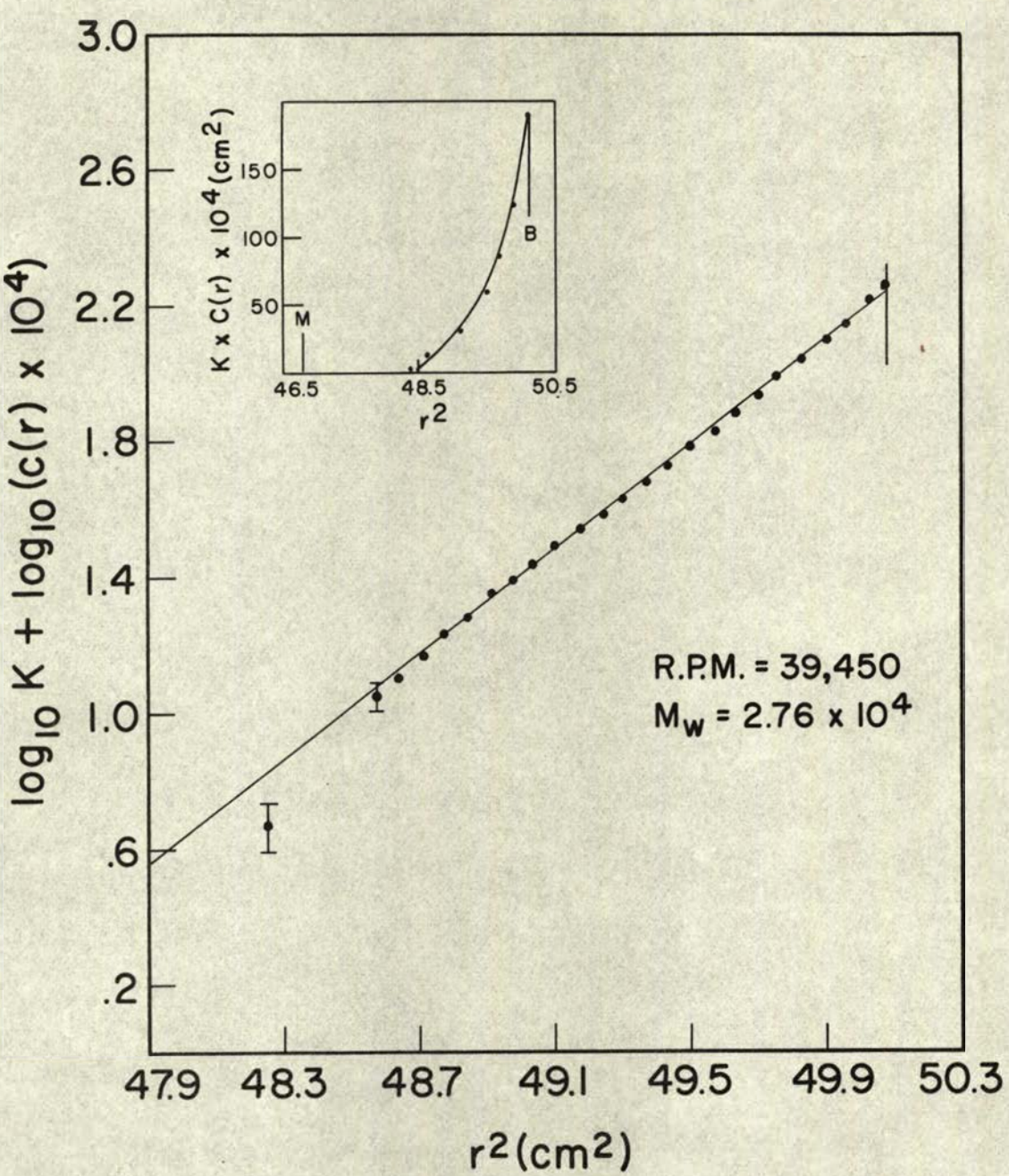

Figure 16. Meniscus depletion experiment with glycerol dehydrogenase treated with 0.01 M dithiothreitol and 6.0 M guanidinium chloride at 39,000 revolutions per minute.

TABLE VIII

APPARENT MOLECULAR WEIGHT OF GLYCEROL DEHYDROGENASE TREATED WITH THE INDICATED REAGENTS

EXPT.	CONDITIONS		
	Solvent	Rotor Speed rpm	Molecular Weight Daltons
1	0.2 M NaCl + 0.1 M Tris-HCl pH 7.6	5,650	3.34×10^{5}[a]
2	2.5 M urea + 0.2 M NaCl	10,980	6.0×10^{4}[b]
3	6.0 M guanidine-HCl + 5.0×10^{-3} M dithiothreitol	39,460	3.15×10^{4}[d]
4	6.0 M guanidine-HCl + 10^{-2} M dithiothreitol	21,740	2.8×10^{4}, 4.3×10^{4}[c]
5	Same as expt. 4	31,410	3.6×10^{4}[d]
6	Same as expt. 4	39,450	2.78×10^{4}[d]
7	S-carboxymethyl protein in 6.0 M guanidine-HCl	39,450	2.84×10^{4}[d]

[a] Low speed method.
[b] Low speed method.
[c] Weight average molecular weight at the meniscus and cell bottom from a plot of the data in the form $(dC(r)/dr)/r$ versus $C(r)$.
[d] Meniscus depletion experiment.

was treated with the various dissociating and sulfhydryl-active reagents.

Treatment of enzyme with PMB. The results of the spectrophotometric titration of AGDH with PMB both before and after reduction with dithiothreitol are listed in Table IX. The data are consistent with the hypothesis that there are, in the completely reduced protein, five moles of titratable sulfhydryl groups per 28,000 g of protein. Three of these residues in the unreduced (native) enzyme are present as disulfide bonds.

The results of meniscus depletion experiments on the PMB-titrated protein are illustrated in Figure 17 for the unreduced protein and in Figure 18 for the protein which had been reduced before titration. In the first case the protein preparation had a molecular weight of 5.6×10^4 daltons while in the second case the molecular weight was 3.1×10^4 daltons.

When experiments with the pre-reduced, PMB-titrated protein were allowed to proceed for an additional thirty-six hours, the observed weight-average molecular weight increased to 4.2×10^4 daltons. The increase in the observed weight-average molecular weight may be due to the reformation of interchain disulfide bonds, since the reaction between thiols and p-hydroxymercuribenzoate is

TABLE IX

TITRATION OF GLYCEROL DEHYDROGENASE WITH p-HYDROXYMERCURIBENZOATE

Expt.	Conditions	Moles PMB Per 3.4×10^5 g Protein	Molecular Weight Daltons
1	6.0 M Guanidine-HCl	24.1	[b] 5.6×10^4
2	Enzyme pre-reduced with 10^{-2} M dithiothreitol; 6.0 M guanidine HCl.	60.1[a]	[c] 3.4×10^4
3	Same as 2 except ultracentrifuge run proceeded for additional 24 hours.	---	[c] 4.2×10^4
4	Material from 2 made 10^{-2} M in dithiothreitol; other conditions the same.	---	[c] 3.3×10^4

[a] When this preparation was allowed to reoxidize for 3 days then 25.4 moles PMB reacted per 3.4×10^5 g protein.
[b] Rotor speed was 25,980 rpm.
[c] Rotor speed was 34,960 rpm.

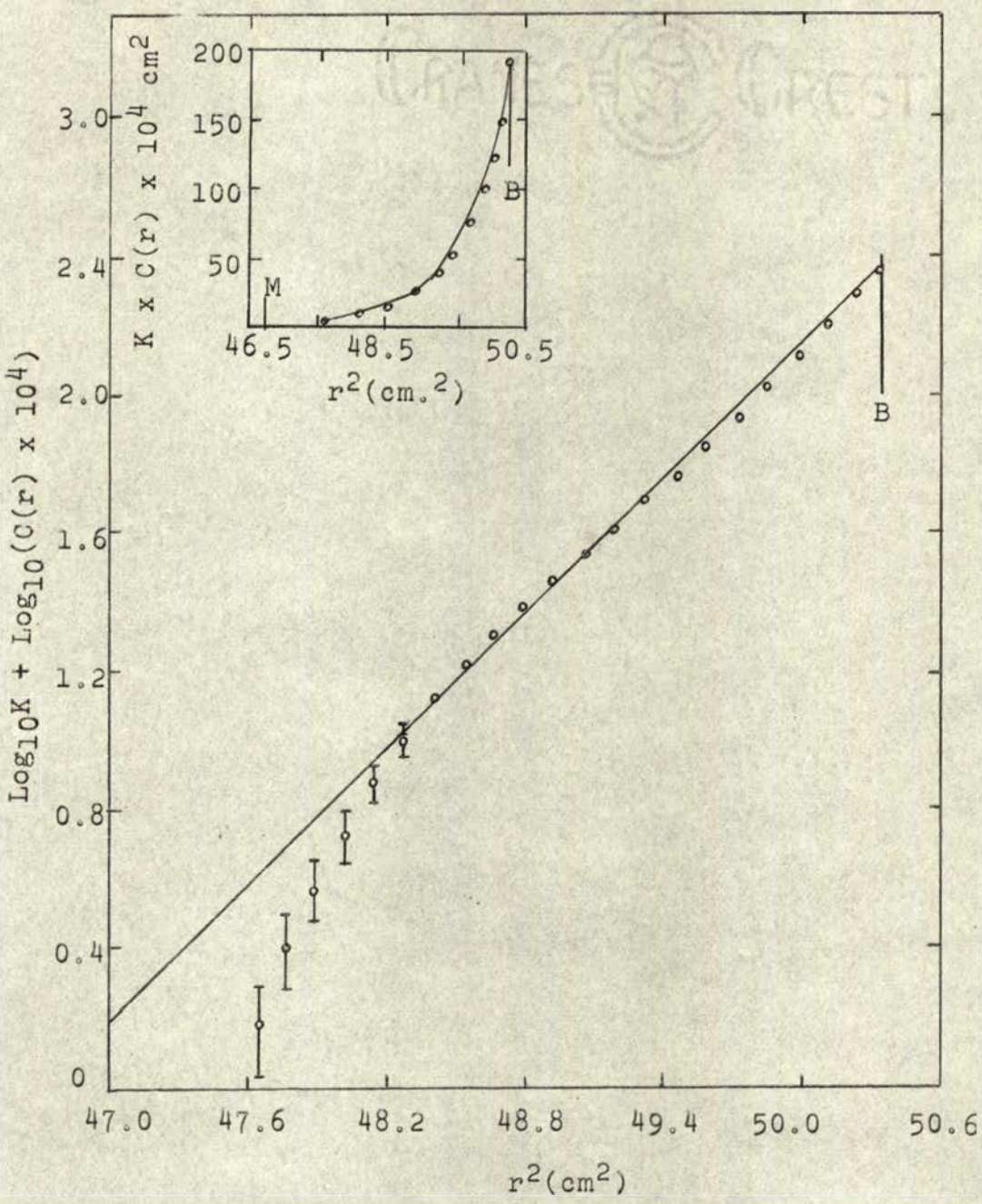

Figure 17. Meniscus depletion experiment with glycerol dehydrogenase titrated with p-hydroxymercuribenzoate.

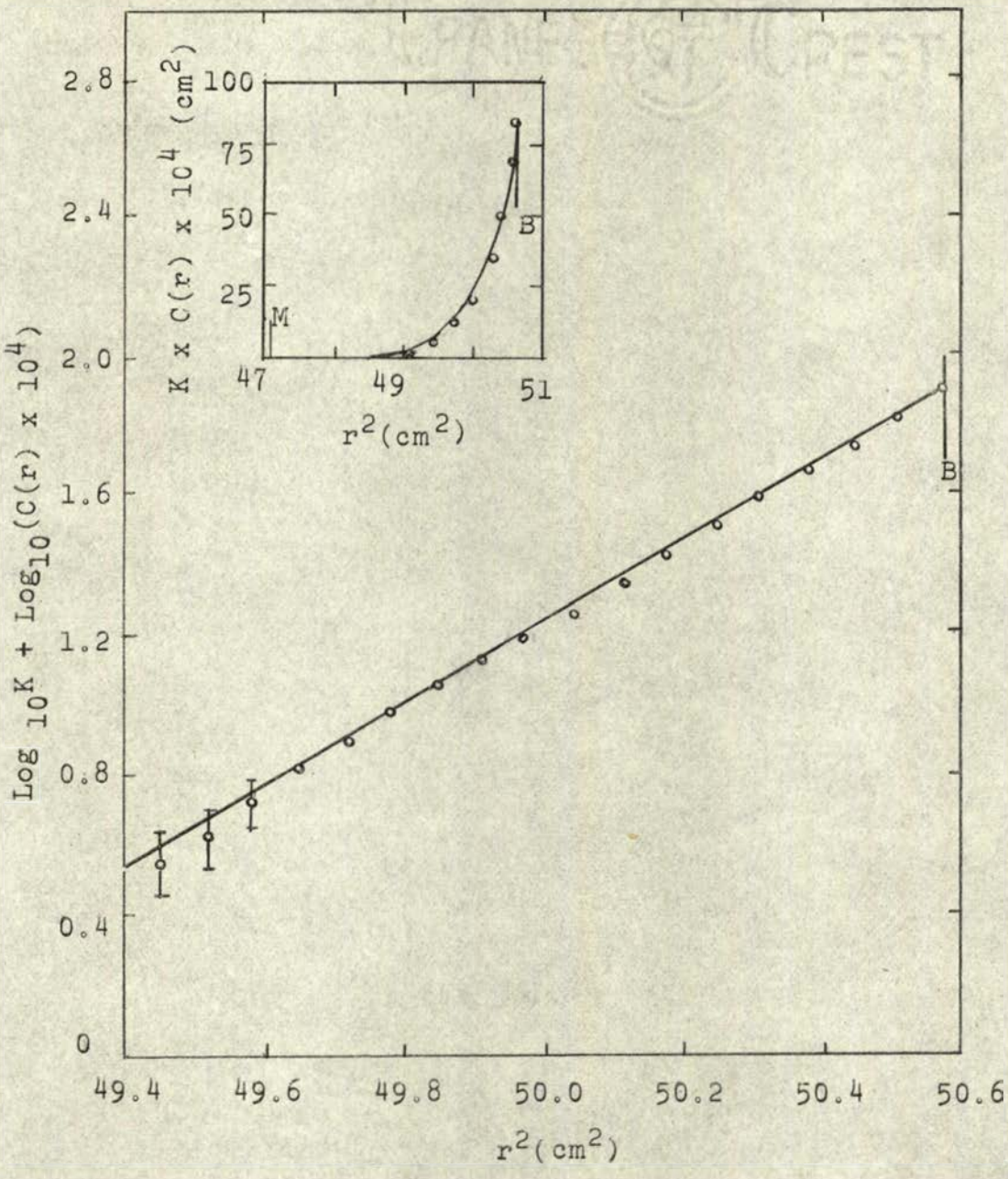

Figure 18. Meniscus depletion experiment with glycerol dehydrogenase titrated with p-hydroxymercuribenzoate.

known to be reversible (Benesch and Benesch, 1962). Addition of excess dithiothreitol to this preparation (10^{-2} M final concentration) caused the observed weight-average molecular weight to decrease to 3.4×10^4 daltons. The results of these experiments are also summarized in Table IX.

CHAPTER VI

DISCUSSION

Purity of the Enzyme Preparation

The results of the sedimentation velocity experiments (Figure 19) indicate that AGDH prepared according to the procedures outlined here is homogeneous with respect to molecular weight. Attempts to estimate the electrophoretic homogeneity of an apparently pure preparation of the enzyme gave the results of Figure 8a (page 82). The appearance of several bands of protein (each of which gave a positive stain for enzyme activity) indicates that each band of protein is related to the enzyme activity and does not represent a contaminating impurity. The ultimate resolution of the complex pattern of Figure 8a into two bands of protein (Figure 8c) when the enzyme is treated with urea and dithiothreitol suggests that the proteins of Figure 8a represent multiple molecular forms of AGDH. The absence of any sharp protein bands in the experiments in the presence of urea alone is suggestive evidence that the preparation is free from extraneous protein. The constant specific activity of the eluate from the ion-exchange chromatography experiments as well as the consistent amino acid analyses

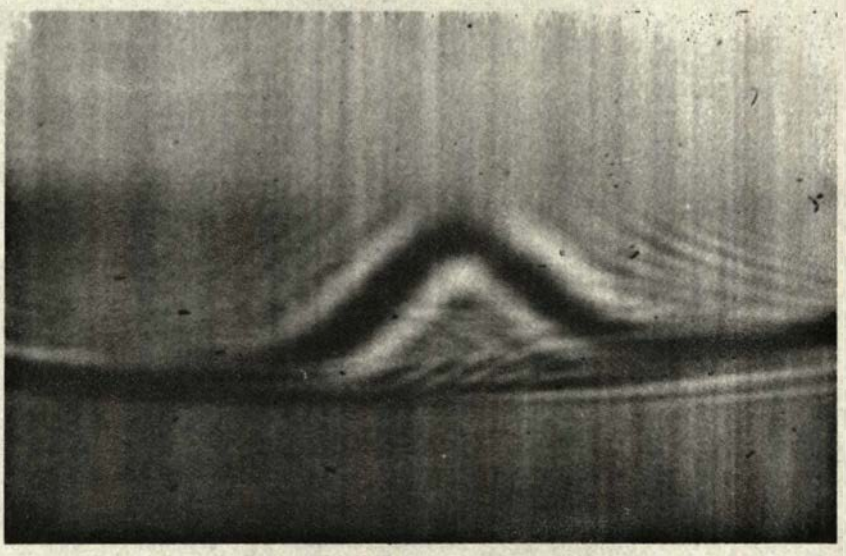

Figure 19. Sedimentation velocity pattern of glycerol dehydrogenase.

The photograph was taken 32 minutes after reaching speed. The bar angle was 50° and the rotor speed was 59,000 revolutions per minute.

of different preparations of the enzyme also suggest that the enzyme preparation is reasonably homogeneous.

The results of sedimentation equilibrium experiments are usually ambiguous with respect to the purity of a protein preparation. This is true in the present case. If an impurity with a molecular weight equal to the molecular weight of the native enzyme were present in small amounts, the high speeds used in the meniscus depletion technique would force this material to the bottom of the centrifuge cell. In addition, the impurity would probably precipitate, considering the extended times which sedimentation equilibrium experiments require. In such a case the impurity would not be detected nor would it have any significant effect on the results.

In the experiments conducted at lower speeds with the native enzyme and with the enzyme treated with urea, the presence of proteins of higher molecular weight as indicated by differences between the weight- and z-average molecular weights can be explained, in the former case, by a slow aggregation of the native enzyme, and in the latter case by incomplete dissociation of the enzyme. However, the data from the various experiments indicate the absence of any significant amount of impurity having the same molecular weight as the native enzyme. Thus ninety-five

per cent homogeneity is a reasonable estimate for the minimum purity of the preparation.

It is important to note, especially where an enzyme is dissociable into subunits, that the complex ion-exchange and electrophoretic patterns make definition of purity ambiguous. In these cases the presence of protein which is enzymatically inactive does not necessarily mean that the protein is unrelated to the enzyme.

Ion-exchange Chromatography

The decrease in the specific activity of the enzyme after elution from a column of Sephadex DEAE-50 contrasts markedly with the results obtained when Sephadex DEAE A-25 was the resin. In the former case the extended elution times may account for a part of the observed inactivation. The possibility exists that the loss of activity may be due to the removal of a small, non-covalently bound molecule during the course of chromatography. Fluorometric studies on the enzyme eluted from the A-25 material did not indicate the presence of bound pyridine nucleotide (Walter and Singer, 1968) which in the case of some dehydrogenases is necessary for the preservation of activity during purification (Murdock and Koeppe, 1964). The addition of Zn^{2+}, Cu^{2+}, Fe^{2+}, Ca^{2+}, and Mg^{2+} was without effect in

restoring activity as was the addition of boiled crude extract and aliquots of eluate which did not contain enzyme activity.

The elution of multiple peaks of enzyme activity, each with essentially identical specific activity, from the ion-exchange experiments using DEAE A-25 differs from the results obtained with the A-50 material from which a single symmetrical peak of constant specific activity was eluted. The different behavior of the enzyme on the two gels may be due to physical differences between them. The properties of these ion-exchange materials depend on the degree of cross-linking in the dextran polymers from which they are made. The extent of cross linking is reflected in the size of the molecule which is excluded from the interior of the hydrated gel bead. The dextran from which the A-50 material is made has an exclusion limit for globular proteins of about 70,000, while the gel from which the A-25 material is made has an exclusion limit of 2,500. Moreover, the size of the hydrated beads is greater for the A-50 material than for the A-25 material (although the dry beads have the same size). Thus the ion binding capacity per unit volume of A-50 gel is less than that of the A-25 gel (Determan, 1968).

Enzyme Kinetics

Judging from the linearity of the plots of v(0) versus

v(0)/S(0), the initial rate may be described by a rectangular hyperbola over the range of substrate concentrations used in this study (Frieden and Walter, 1963). Thus, no evidence was found of the sigmoidal shape plots characteristic of many control enzymes, nor was there any indication that AGDH is subject to substrate inhibition by DHA, as reported by Strickland and Miller (1968). However in the latter case the experimental conditions were different from those used in the present study. The buffer used in the experiments discussed here was 0.5 M sodium phosphate, pH 9.0, while in the other study the buffer was glycine-KOH, pH 7.7.

The reported reaction between NAD and DHA to form a stable, enzymatically inactive adduct (Burton and Kaplan, 1953; Burton et al., 1957) requires that caution by used in carrying out and interpreting experiments with this enzyme and these two substrates.

Dalziel (1957) has derived a relationship between the equilibrium constant of a reaction and the steady-state parameters for a class of two-substrate, two-product reactions. This relationship is expressed by equation 76

$$K_{eq.} = \frac{V_{1f}V_{2f}K_{3r}K_{4r}}{V_{3r}V_{4r}K_{1f}K_{2f}}, \qquad (76)$$

where maximum velocities and apparent Michaelis constants with the subscript f are for the forward reaction and parameters with a subscript r are for the reverse reaction.

Parameters with subscripts 1 and 2 refer to the substrates, and those with 3 and 4 to the products.

The equilibrium constant for the reaction

$$NAD + glycerol \quad NADH + H^+ + DHA$$

was reported as 5.1×10^{-12} (Burton, 1963). This constant determined in the present study was 14×10^{-12}. The precise source of this discrepancy is not clear. The statement that $(NH_4)_2SO_4$ increased the equilibrium constant, which appeared in the report by Burton, casts some doubt on the value which was reported. The reaction between NAD and DHA also complicates measurement of this parameter directly (i.e., rather than by separate measurement of the reduction potential of the two sets of oxidation-reduction pairs). The equilibrium constant calculated from equation 76 and the parameters reported in Table II (page 78) was 21×10^{-12} for the reaction as written above. This value is in reasonable agreement with the value determined directly.

Polyacrylamide Gel Electrophoresis

The experiments reported here were carried out with a system which did not employ a sample gel or a concentrating gel as described in the original paper describing this technique (Davis, 1964). The advantages of using the continuous system arise because it is not necessary, as it is in the case

of the discontinuous system, to polymerize the protein in the sample gel. This avoids possible reactions between the protein and the free radicals which are generated during the polymerization process. Artifacts which arise from this source have been reported (Mitchell, 1967).

The ammonium persulfate which is used as the catalyst in the formation of the resolving gel has also led to the formation of artifacts (Brewer, 1967). These artifacts disappeared when the gels were subjected to electrophoresis prior to using them with protein samples. The discontinuous system of Davis does not lend itself readily to pre-electrophoresis, while the continuous system used in these studies permits pre-electrophoresis without changing the pH or ionic strength of the buffer inside the gel matrix. Thus this technique avoids exposing the protein to the free radicals of the gelling process, thereby preventing the formation of artifacts and, in the case of enzymes, eliminating a possible source of inactivation.

Therefore, the appearance of several bands of enzymatically active protein when an apparently pure preparation of the enzyme is subjected to electrophoresis in the absence of dissociating reagents (Figure 8a, page 82) cannot be accounted for by the action of free radicals. The precise nature of each protein and its relation to the native enzyme

is not clear. The experiments in the presence of urea indicate that the native enzyme is dissociated by this reagent. Thus the bands of Figure 8a may represent species of the enzyme in various stages of dissociation (or association). The presence of protein at the top of the gel in Figure 8a suggests that some of the protein did not penetrate the gel or migrated only slightly.

A relationship of the ratio of the distance moved by the protein to the distance moved by the tracking dye, the acrylamide content of the gel, and the molecular weight of the protein has been established by Hendrick and Smith (1968). This report indicated that catalase (molecular weight 2.4×10^5 daltons) migrates one-sixth of the distance of the tracking dye in a 7 per cent acrylamide gel while apoferritin (molecular weight 4.5×10^5 daltons) migrated only one-twentieth of the distance of the tracking dye. The protein at the top of the gel in Figure 8a may thus represent dimers or higher associations of the native enzyme, while proteins further into the gel may represent dissociated species of the native enzyme. The fact that all the protein enters the gel when treated with urea is consistent with the view that urea is effective in dissociating higher polymers of the enzyme.

Exposure of the enzyme to dithiothreitol in the

presence of urea resulted in the appearance of two bands of protein on electrophoresis. Since urea alone does not cause complete dissociation of the enzyme but urea and dithiothreitol resolve the pattern of Figure 8b page 82 into distinct bands of protein, it is suggested that reduction of disulfide bonds in the enzyme decreases the strength of protein-protein interactions probably by causing significant changes in the structure of the enzyme.

The results of the electrophoresis experiments do not provide evidence concerning the actual role of disulfide bonds in the protein, i.e., whether they are inter- or intra-chain, but they do indicate that both covalent and non-covalent bonds are present in the enzyme and that disruption of these bonds causes profound changes in the conformation of the enzyme.

Ultracentrifuge Experiments

Native enzyme. The molecular weight of the native enzyme estimated from the sedimentation and diffusion constants and from sedimentation equilibrium experiments was $3.4 \pm .17 \times 10^5$ daltons. This value is not in agreement with the value of 1.4×10^5 daltons reported by Andrews (1964, 1965), obtained from gel filtration experiments with Sephadex P-100 using a commercially available impure

preparation of the enzyme. Estimation of molecular weights from gel filtration experiments requires a linear relationship between the elution volume of the protein and the logarithm of the molecular weight of the protein. Inspection of the data of Andrews (1964) indicates that, for the particular column used in that study, this linear relation no longer exists for proteins with a molecular weight greater than 1.3×10^4 daltons. Thus the elution volume of the bovine serum albumin dimer (molecular weight 1.3×10^5 daltons) was about 80 ml while for bovine thyroglobulin (molecular weight 6.7×10^5 daltons) the elution volume was about 70 ml. A reported variability of as much as 4 ml in the elution volumes of the proteins used as molecular weight markers in those experiments can easily account for the difference in the value reported here and the value obtained by Andrews.

Early attempts, during this investigation, to purify the enzyme using Sephadex P-200 indicated that the enzyme had a molecular weight in the range of 3×10^5 daltons. This result suggests that estimations of the molecular weights of proteins using gel filtration should be carried out with gels of different exclusion limits. The exclusion limit of the P-100 gel is 150,000 while that for the P-200 material is 800,000 (Determan, 1968). Thus the error in the original value obtained by Andrews would have been readily apparent

had the experiments been repeated using a gel of greater exclusion limit.

The minimum frictional coefficient of AGDH calculated from equation 66 is 8.7×10^{-8} g/sec. This is the coefficient the protein would have were it an unhydrated sphere. The actual frictional coefficient may be calculated from either equation 23 or equation 36. The value obtained from the sedimentation constant and the molecular weight estimated from sedimentation equilibrium experiments is 1.4×10^{-7} g/sec, while the value obtained from the diffusion constant is 1.3 c 10^{-7} g/sec. The difference in the values obtained from the two equations arises from the uncertainty in the value of the partial specific volume which was a calculated value rather than one determined experimentally. If the value calculated from the diffusion constant is assumed to be the more accurate of the two, then a frictional ratio, f/f_{min}, of 1.5 is obtained. Comparison of this value with that obtained for other proteins suggests that AGDH is relatively assymmetric, the values for most globular proteins usually being below about 1.3.

If a typical value of 0.2 g of water per gram of dry protein is assumed as the water of hydration (Tanford, 1961), then from equation 65 a ratio f/f_0 of 1.4 is obtained. This corresponds to a prolate ellipsoid with 6.3 as the ratio of

the major to minor axes (Oncley, 1943). It must be pointed out that although these estimates of the shape of AGDH indicate that the protein in solution is relatively asymmetric, the actual numbers, since they were obtained from some calculated or typical constants rather than from experimentally determined estimates of these constants, must be taken more as a qualitative rather than quantitative estimate of the asymmetry.

The difference in the weight average and z-average molecular weight of the native enzyme (Figure 11, page 92) implies that the preparation is poly disperse. The higher value of the M_z is probably due to aggregation of the protein. This view is consistent with the results of the electrophoresis experiments (Figure 8a, page 82) on the native enzyme since the proteins which penetrated the gel only slightly or not at all are also most likely aggregations of the native enzyme.

Enzyme treated with dissociating and thiol reagents. The dispersity of the preparation treated with 2.5 M urea, suggested by the polyacrylamide gel electrophoresis experiments (Figure 8b-c), was confirmed by data from the sedimentation equilibrium experiments under the same conditions (Figure 12, page 95). Thus the weight-average molecular weight of this preparation was found to be 6.0×10^4 daltons while the z-average molecular weight was 2.2 x

10^5 daltons.

It has been shown that guanidinium chloride is effective in replacing the hydrophobic and ionic forces responsible for the stability of protein-protein interactions (Tanford et al., 1967). Thus, treatment with this reagent exposes all regions of the protein molecule, thereby facilitating the reduction of disulfide bonds in those areas of the protein normally inaccessible to attack by reducing agents. For these reasons a series of sedimentation equilibrium experiments was carried out on the enzyme treated with 6.0 M guanidinium chloride and dithiothreitol. The molecular weight obtained from an experiment with the enzyme in the presence of 6.0 M guanidinium chloride and 5×10^{-3} M dithiothreitol was 3.4×10^4 daltons. The possible presence of forms of AGDH in which all disulfide bonds are not reduced will cause the apparent weight-average molecular weight to be greater than that of the smallest component present in the preparation. One advantage of the meniscus depletion technique is to concentrate species whose molecular weight is greater than that of the bulk of the preparation in a small region at the bottom of the centrifuge cell.

As an illustration of the extent of fractionation which can be achieved with this method let us assume that in

a typical experiment, at a rotor speed of 39,000 r.p.m. and a solution column height of 2 cm, that the cell bottom is 7.1 cm from the axis of rotation. In this case ninety per cent of a protein of molecular weight of 6.0×10^4 daltons will be present in the bottom 0.36 cm of the cell while the same fraction of a protein of molecular weight 3.0×10^4 daltons will be contained in the bottom 0.76 cm of the cell. Since the distribution of protein is an exponential function of the radius (at constant speed) and if the higher molecular weight species is present as a minor component, the effect of this component should be apparent only in a small region at the cell bottom.

To exploit this advantage, a series of experiments was carried out at increasing rotor speeds on the protein treated with 6.0 M guanidinium chloride and 0.01 M dithiothreitol (experiments 4, 5, and 6, Table VIII, page 101). The presence of higher molecular weight species is confirmed by the difference between the weight- and z-average molecular weight estimated from data obtained at a rotor speed which did not deplete the meniscus (21,000 r.p.m.). The results of these experiments indicate that the smallest component present in the preparation treated with dithiothreitol and guanidinium chloride is about 2.8×10^4 daltons.

Titration with p-hydroxymercuribenzoate. The preceding experiments suggested the presence of interchain disulfide bonds in the enzyme. If this is the case then there should be a difference in the number of titratable sulfhydryl groups in the protein before and after reduction of the enzyme with dithiothreitol. This was indeed found to be the case (Table IX, page 103). The molecular weight of the titrated preparation before reduction was found to be 5.6×10^4 daltons, while after reduction and titration the molecular weight decreased by (about) one-half. The molecular weights observed for the reduced, titrated preparation were greater than those observed with the preparation treated with dithiothreitol and guanidinium chloride. These experiments also indicated a time-dependent increase in the apparent molecular weight (Table IX). These observations, coupled with the fact that excess dithiothreitol is effective in lowering the molecular weights of these preparations, suggests that some reformation of interchain disulfide bonds has occurred. This is consistent with the known reversibility of the reaction between p-hydroxymercuribenzoate and thiols (Benesch and Benesch, 1962). The molecular weight of the S-carboxymethyl derivative of the enzyme (in which formation of disulfide bonds is impossible) is consistent with the results obtained with

the preparation treated with guanidinium chloride and dithiothreitol.

Subunit Stoichiometry and Amino Acid Composition

The derivation of the equations used to evaluate the photographic data as well as the treatment of the random error involved in reading the photographs require that molecular weights determined using the ultracentrifuge be assigned an uncertainty of about five per cent. The greatest source of error arises from the ionic nature of the protein, dissociating reagent, and electrolyte, and the assumptions used to derive equations which are experimentally useful in evaluating data obtained from such systems. In the present case the data on net charge and on the magnitude of protein-protein and protein-electrolyte interactions which might have allowed a more complete evaluation of the ultracentrifuge data could not be obtained. Evaluation of the two parameters of charge and protein-protein interaction require adequate quantities of dry material. Attempts to prepare AGDH in suitable form were unsuccessful since the enzyme coagulates as soon as electrolyte is removed. Also, enzyme activity is rapidly lost when the concentration of electrolyte is below about 0.1 M. However the approximations which are made in the derivation and use of these equations may be minimized (see Chapter V).

With these limitations in mind, the molecular weight of the smallest protein observed in these studies may be taken to be $2.8 \pm 0.14 \times 10^4$ daltons (five per cent). The molecular weight of the dimer of this protein formed by interchain disulfide bonds is then $5.6 \pm 0.28 \times 10^4$ daltons. A dodecamer of the smallest species (or a hexamer of the dimer) will then have a molecular weight of $3.36 \pm 0.17 \times 10^5$ daltons.

The experimentally determined molecular weights of the native enzyme and the enzyme treated with urea and with guanidinium chloride and PMB fall within the ranges postulated on the basis of a smallest species of molecular weight 2.8×10^4 daltons and a stoichiometry of the native enzyme of $6(S_2)$, where S represents the smallest species observed in the ultracentrifuge. The existence of disulfide bonds between two species of molecular weight 2.8×10^4 daltons is confirmed by the results obtained from the experiments with the reduced enzyme and with the carboxymethyl derivative of the enzyme. The existence of a stable dimer of the smallest species is indicated by the experiments on the protein titrated with PMB before reduction.

The multiple molecular forms of AGDH which appeared on ion-exchange chromatography may arise from a wide variety of factors. For example, multiple forms of alcohol

dehydrogenase from Drosophila melanogaster containing 0, 1 and 2 moles of bound pyridine nucleotide were separated electrophoretically (Jacobson, 1968). Other, non-genetic sources have also been observed. Multiple forms of yeast hexokinase were found to be derived from the action of a protease (Gazith et al., 1965). The removal of a C-terminal tyrosine from chicken muscle aldolase led to the appearance of additional forms of the enzyme (Masters, 1967). Forms of cytochrome c from Neurospora in which the dibasic amino acids were aminated to different extents have been observed (Kaplan et al., 1968). The glutamine synthetase of E. coli exists in various forms differing from each other by their content of covalently bound adenyl groups (Shapiro et al., 1967). Some of the serum cholinesterases appear to exist as aggregates which differ in the rate of electrophoretic migration (LaMotta et al., 1965). Finally, forms of chicken mitochondrial malate dehydrogenase were shown to differ only in conformation which resulted in different electrophoretic behavior (Kitto et al., 1966).

The most significant genetic cause for the existence of isozymes is in cases where the protein is composed of subunits. Associations of the subunits of the enzyme in different stoichiometries result in the appearance of isozymes having the same molecular weight but different net

charge (if the same is true of the subunits). If it is assumed that the A and B proteins which appear on polyacrylamide gel electrophoresis represent the species of molecular weight 2.8×10^4 daltons which were observed in the ultracentrifuge, then there are three possible combinations of these two subunits to form a dimer. There are 28 combinations of these dimers taken six ways, if it is assumed that forms with identical dimer composition are degenerate with respect to net charge. Once the hexamers of the dimers are formed, if it is assumed that the net charge of each hexamer depends only on the number of each kind of subunit present in the hexamer and not on which of the three types of dimers contributed the subunit, then the number of charge distinct isozymes is reduced to thirteen.

On the basis of this model the isozyme stoichiometry of AGDH may be represented by the formula $(A_{(12-n)}B_n)$, $n = 0, 1, \ldots, 12$. The isozyme with the lowest net negative charge at pH 8 would therefore be composed of 12 A subunits (the subunit with the lower number of glutamate plus aspartate residues) and should therefore be eluted first from an anionic ion-exchange column. The net negative charge on subsequent isozymes should increase as one B subunit replaces one A subunit. On this basis the isozymes should also be eluted in this order.

This model for the isozyme stoichiometry may be tested by comparing the amino acid composition of each of the thirteen peaks eluted from the A-25 ion-exchange column with the amino acid composition predicted for each hypothetical isozyme based on the proposed subunit stoichiometry and the known amino acid composition of each subunit. Thus the first peak eluted from the ion-exchange column should have an amino acid composition similar to that of the A subunit while the last peak should have an amino acid composition similar to that of the B subunit. Inspection of Tables III and IV (pages 84 and 85) shows good agreement between the amino acid composition of the first and last major peaks and the two subunits.

This relationship between the amino acid composition of each peak and the composition predicted from the proposed stoichiometry is further illustrated in Figures 20a-d. In these figures are plotted peak number versus the amino acid content of the protein in each peak of seven amino acids which are present in different amounts in the two subunits and of one (alanine) which is present in equal amounts. The number of moles of each amino acid is based on the assumption that the protein in each peak has a molecular weight of 340,000. The straight line in each figure is the least-squares line fitted through the plot of composition versus

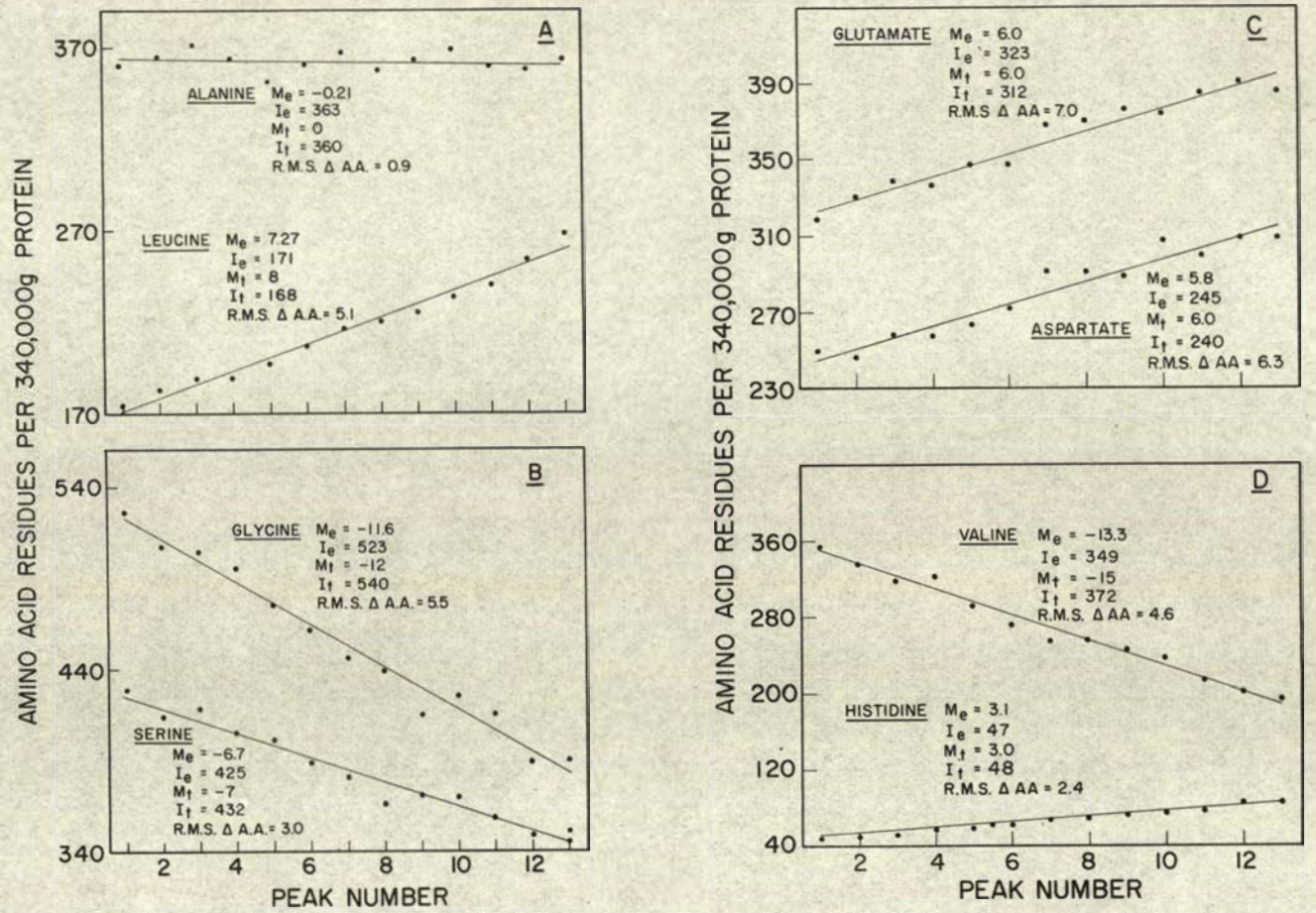

Figure 20. Relationship between the proposed and experimentally determined amino acid composition of each isozyme of glycerol dehydrogenase.

peak number. The slope and intercept (at peak one) are indicated by M_e and I_e respectively, while the root mean square of the deviations about the fitted line is designated as R. M. S. ΔA.A.

The change in amino acid content with peak number for the hypothetical isozymes is the difference in the number of moles of each amino acid in the two subunits. This theoretical slope is designated as M_t in Figure 20a-d while the theoretical intercept at peak one (i.e., the expected amino acid composition of that peak) is designated as I_t.

The agreement between the experimental and proposed amino acid composition of each peak suggests that the origin of the isozymes of AGDH is indeed due to the association of the two subunits in the proposed stoichiometry. However, it must be pointed out that other stoichiometries could account for the appearance of at least some of the isozymes. Most notable is the possibility that the molecular weight of the isozymes eluted from the ion-exchange column may not all be the same. As an example, the relative amino acid composition (i.e., the number of residues per 100 moles of amino acids) of an isozyme of the form (A_2B_2) would be the same as that for an isozyme of the form (A_6B_6) although the molecular weights of the two would be quite different.

The course of inactivation of this enzyme in A. aerogenes (as described earlier) strongly suggests that the enzyme may function as a control point during the metabolism of glycerol. The properties of control enzymes outlined earlier suggest that subunit structure is at least an incidental property of such enzymes. It would thus be of interest to determine whether AGDH is specifically sensitive to compounds formed during the highly aerobic metabolism of the organism but not related structurally to the substrates of the enzyme.

SELECTED BIBLIOGRAPHY

SELECTED BIBLIOGRAPHY

Andrews, P. 1964 Estimation of the molecular weights of proteins by sephadex gel filtration. Biochem. J. 91: 222-223.

Andrews, P. 1965 The gel filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. 96: 595-606.

Antonini, E., J. Wyman, R. Moretti, and A. Rossi-Fanelli. 1963 The interaction of bromthymol blue with hemoglobin and its effect on the oxygen equilibrium. Biochem. Biophys. Acta 71: 124-138.

Apella, E., and C. L. Markert. 1961 Dissociation of lactate dehydrogenase into subunits with guanidine hydrochloride. Biochem. Biophys. Res. Comm. 6: 171-176.

Asnis, R. E., and A. F. Brodie. 1953 A glycerol dehydrogenase from Escherichia coli. J. Biol. Chem. 203: 153-159.

Baliga, B. S., G. M. Bhatnagar, and V. Jagannathan. 1962 Triphosphopyridine nucleotide- (TPN-) specific glycerol dehydrogenase from Aspergillus niger. Biochem. Biophys. Acta 58: 384-385.

Ballentine, R. 1963 Determination of total nitrogen and ammonia. Methods Enzymol. 3: 984-995.

Baxter, R. M., and N. E. Gibbons. 1955 Effects of homologus and heterologus antisera on the glycerol dehydrogenase of halophilic and non-halophilic bacteria. Can. J. Microbiol. 1: 262-265.

Benesch, R., and R. Benesch. 1962 Determination of -SH groups in proteins. Methods Biochem. Anal. 10: 44-70.

Bergmann, M. and L. Zervas. 1932 A general method of peptide synthesis. Ber. 65 B: 1192-1201.

Bertrand, G. 1896 Préparation biochemique du sorbose. Acad. Sci. Paris Compt. Rend. 122: 900-903.

Brewer, J. M. 1967 Artifact produced in disc electrophoresis by ammonium persulfate. Science 156: 256-257.

Burton, R. M. 1963 Glycerol dehydrogenase from Aerobacter aerogenes. Methods Enzymol. 1: 397-400.

Burton, R. M., and N. O. Kaplan. 1953 A DPN-specific glycerol dehydrogenase from Aerobacter aerogenes. J. Am. Chem. Soc. 75: 1005-1006.

Burton, R. M., A. SanPietro, and N. O. Kaplan. 1957 Preparation of pyridine analogues by the carbonyl addition reaction. Arch. Biochem. Biophys. 70: 87-106.

Chiang, C., and S. G. Knight. 1959 d-Xylose metabolism by cell-free extracts of Penicillium chrysogenum. Biochem. Biophys. Acta 35: 454-463.

Dalziel, K. 1957 Initial steady state velocities in the evaluation of enzyme-coenzyme-substrate reaction mechanisms. Acta Chem. Scand. 11: 1706-1723.

Danials, F., and R. Alberty. 1961 Physical Chemistry. John Wiley and Sons, New York.

Datta, P., H. Gest, and H. Segal. 1964 Effects of feedback modifiers on the state of aggregation of homoserine dehydrogenase of Rhodospirillum rubrum. Proc. Natl. Acad. Sci. 51: 125-130.

Davis, B. J. 1964 Disk electrophoresis. II. Method and application to human serum proteins. Ann. N. Y. Acad. Sci. 121: 404-427.

Determan, H. 1968 Gel Chromatography. Springer-Verlag, New York.

DiSabato, G., and N. O. Kaplan. 1964 The denaturation of lactate dehydrogenases I. The effect of sodium dodecyl sulfate. J. Biol. Chem. 239: 438-443.

Fisher, E. 1907 Synthese von polypeptides. Ber. 40: 1754-1768.

Fisher, H. F., D. G. Cross, and L. L. McGregor. 1962 Catalytic activity of subunits of glutamate dehydrogenase. Nature 196: 895-896.

Frieden, C. 1959 Glutamine dehydrogenase I. The effect of coenzyme on the sedimentation velocity and kinetic behavior. J. Biol. Chem. 234: 809-814.

Frieden, E., and C. Walter. 1963 The prevalence and significance of the product inhibition of enzymes. Nature 198: 834-837.

Gazith, J., I. Schulze, R. Gooding, F. Womak, and S. Colowick. 1968 Multiple forms and subunits of yeast hexokinase. Ann. N. Y. Acad. Sci. 151: 307-331.

Gerhart, J. C., and A. B. Pardee. 1962 The enzymology of control by feedback inhibition. J. Biol. Chem. 237: 891-896.

Gerhart, J. C., and H. K. Schachman. 1965 Distinct subunits for the regulation and catalytic activity of aspartate transcarbamylase. Biochem. 4: 1054-1062.

Hade, E. P. K., and C. Tanford. 1967 Isopiestic compositions as a measure of preferential interactions of macromolecules in two-component solvents. Application to proteins in concentrated aqueous cesium chloride and guanidine hydrochloride. J. Am. Chem. Soc. 89: 5034-5040.

Hague, J. G., T. E. King, and U. T. Cheldelin. 1955 Alternate conversions of glycerol to dihydroxyacetone in Acetobacter suboxydans. J. Biol. Chem. 214: 1-9.

Haurowitz, F. 1938 Das gleichwicht zwischen Hamoglobin und Sauerstoff. Z. Physiol. Chim. 254: 266-274.

Hendrick, J. L., and A. J. Smith. 1968 Size and charge isomer separation and estimation of molecular weights by disc gel electrophoresis. Arch. Biochem. Biophys. 126: 155-164.

Henri, V. 1903 Lois Generales de l'action des diastases. Herman, Paris.

Horikoshi, K., and Y. Ikeda. 1965 Glycerol and glycerol dehydrogenases in the conidida of Aspergillus oryzae. J. Gen. Appl. Microbiol. (Tokyo) 11: 303-312.

Jacobs, N. J., and P. J. Vandemark. 1960 Comparison of the mechanism of glycerol oxidation in aerobically and anerobically grown Streptococcus faecalis. J. Bacteriol. 79: 532-538.

Jacobson, K. B. 1968 Alcohol dehydrogenase of Drosophila. Interconversion of isoenzymes. Science 159: 324-325.

Johnson, J. S., K. A. Kraus, and G. Scatchard. 1954 Distribution of charged polymers at equilibrium in a centrifugal field. J. Phys. Chem. 58: 1034-1039.

Kaplan, N. O., and R. D. Cahn. 1962 Lactate dehydrogenases and muscular distrophy in the chicken. Proc. Natl. Acad. Sci. 48: 2123-2130.

Kaplan, N. O., and M. N. Ciotti. 1961 Evolution and differentiation of lactate dehydrogenases. Ann. N. Y. Acad. Sci. 94: 701-720.

Kaplan, N. O., J. Everse, and J. Adimiraal. 1968 Significance of substrate inhibition of dehydrogenases. Ann. N. Y. Acad. Sci. 151: 400-412.

Kitto, G., P. Wasserman, and N. O. Kaplan. 1966 Enzymatically active conformers of mitochondrial malate dehydrogenase. Proc. Natl. Acad. Sci. 56: 578-585.

LaMotta, R., B. McComb, and P. Wetstone. 1965 Isozymes of serum cholinesterase. A new polymerization sequence. Can. J. Physiol. Pharm. 43: 313-318.

LeJon, H. B., B. McCrea, I. Suzuki, and S. Jackson. 1969 Association dissociation reactions of mitochondrial isocitric dehydrogenase induced by protons and various ligands. J. Biol. Chem. 244: 2484-2493.

Leonhardt, K. O., and R. R. Landes. 1963 Oxygen tension of urine and renal structures: Preliminary report of clinical findings. New Eng. J. Med. 269: 115-121.

Leuthart, F., and H. P. Wolfe. 1954 The glycerol dehydrogenase of liver. Helv. Chim. Acta 37: 1732-1733.

Lin, E. C. C., and B. Magasanik. 1960 The activation of glycerol dehydrogenase from Aerobacter aerogenes by monovalent cations. J. Biol. Chem. 235: 1820-1823.

Lin, E. C. C., A. P. Levin, and B. Magasanik. 1960 The effect of aerobic metabolism on the inducible glycerol dehydrogenase of Aerobacter aerogenes. J. Biol. Chem. 235: 1824-1829.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lumry, R., H. Matsumiya, F. A. Bovey, and A. Kowalsky. 1961 The study of the structure and denaturation of heme proteins by nuclear magnetic resonance. J. Phys. Chem. 65: 837-843.

Magasanik, B., M. S. Brooke, and D. Karibian. 1953 Metabolic pathways of glycerol dissimilation. A comparative study of two strains of Aerobacter aerogenes. J. Bacteriol. 66: 611-619.

Maley, G. F., and F. Maley. 1964 The purification and properties of deoxycytidylate deaminase from chick embryo extracts. J. Biol. Chem. 239: 1168-1176.

Markert, C. L., and F. Moller. 1959 Multiple forms of enzymes: Tissue, ontogenetic, and species specific patterns. Proc. Natl. Acad. Sci. 45: 753-763.

Markert, C. L. 1963 Lactate dehydrogenase isozymes: Dissociation and recombiation of subunits. Science 140: 1329-1330.

Masters, C. J. 1967 Characteristics of aldolase variformity. Biochem. Biophys. Res. Comm. 28: 978-984.

Meister, A. 1950 Reduction of α,γ-diketo and α-keto acids catalyzed by muscle preparations and by crystalline lactate dehydrogenase. J. Biol. Chem. 184: 117-129.

Mitchell, W. M. 1967 A potential source of artifacts in polyacrylamide gels. Biochem. Biophys. Acta 147 171-174.

Monod, J., and F. Jacob. 1961 General conclusions: Teleonomic mechanisms in cellular metabolism, growth, and differentiation. Cold Spring Harbor Symp. Quant. Biol. 26: 389-401.

Monod, J., J. Wyman, and J. P. Changeux. 1965 On the nature of allosteric transitions: A plausible model. J. Mol. Biol. 12: 88-118.

Moore, B. W. 1959 A triphosphopyridine nucleotide specific glycerol dehydrogenase from liver. J. Am. Chem. Soc. 81: 5837-5838.

Moore, S., and W. Stein. 1963 Chromatographic determination of amino acids by the use of automatic recording equipment. Methods Enzymol. 6: 819-831.

Muirhead, H., and M. F. Perutz. 1963 Structure of haemoglobin. A three-dimensional Fourier synthesis of reduced human haemoglobin at 5.5 A resolution. Nature 199: 633-638.

Murdock, A. L., and O. N. Koeppe. 1964 The content and action of diphosphopyridine nucleotide in triose-phosphate dehydrogenase. J. Biol. Chem. 239: 1983-1988.

Nielands, J. B. 1952 Studies on lactate dehydrogenase of heart. I. Purity, kinetics, and equilibria. J. Biol. Chem. 199: 373-381.

Nozaki, Y., and C. Tanford. 1967 Acid-base titrations in concentrated guanidine hydrochloride. Dissociation constants of the guanidinium ion and of some amino acids. J. Am. Chem. Soc. 89: 736-742.

Oncley, J. L. 1943 The electric moments and the relaxion times of proteins as measured from their influence upon the dielectric constants of solutions in E. J. Cohn and J. T. Edsall, Proteins, Amino Acids, and Peptides. Reinhold Publishing Corp., New York, ch. 22.

Ornstein, L. 1964 Disk electrophoresis. I. Background and theory. Ann. N. Y. Acad. Sci. 121: 321-349.

Rao, G. R., T. Ramakrishnan, and M. Sirsi. 1960 Enzymes in Candida albicans: Pathways of glucose dissimilation. J. Bacteriol. 80: 654-658.

Rosen, O., P. Copeland, and S. Rosen. 1966 The subunit structure of fructose 1,6-diphosphatase. Proc. Natl. Acad. Sci. 56: 1810-1816.

Schachman, H. K. 1959 Ultracentrifugation in Biochemistry. Academic Press, New York.

Schachman, H. K. 1963 Ultracentrifugation, diffusion, and viscometry. Methods Enzymol. 4: 32-103.

Scott, J. M., and J. Rabinowitz. 1967 The association and dissociation of formyltetrahydrofolate synthtase and its relation to monovalent cation activation of catalytic activity. Biochem. Biophys. Res. Comm. 29: 418-423.

Shapiro, B. M., H. S. Kingdon, and E. R. Stadtman. 1967 Regulation of glutamine synthetase. VII. Adenylglutamine synthetase; a new form of the enzyme with altered regulatory and kinetic properties. Proc. Natl. Acad. Sci. 58: 642-649.

Stadtman, E. R., G. N. Cohen, and G. LeBras. 1961 Feedback inhibition and repression of aspartokinase activity in Escherichia coli and Saccharomyces cerevisiae. J. Biol. Chem. 236: 2033-2038.

Stadtman, E. P. 1966 Allosteric regulation of enzyme activity. Adv. Enzymol. 28: 41-154.

Stokes, G. 1880 Mathametical and Physical Papers. Cambridge University Press.

Strickland, J. E., and O. N. Miller. 1968 Inhibition of glycerol dehydrogenase from Aerobacter aerogenes by dihydroxyacetone, high ionic strength, and monovalent cations. Biochem. Biophys. Acta. 159: 221-226.

Sumner, J. B. 1926 The isolation and crystallization of the enzyme urease. J. Biol. Chem. 69: 435-441.

Svedbert, T., and O. Fahraeus. 1926 A new method for the determination of the molecular weights of proteins. J. Am. Chem. Soc. 48: 430-438.

Svedbert, T. 1938 A discussion on the protein molecule. Proc. Roy. Soc. London B. 127: 1-17.

Tanford, C. 1961 Physical Chemistry of Macromolecules. John Wiley and Sons, New York.

Tanford, C., K. Kawahara, and S. Lapanje. 1967 Proteins as random coils. I. Intrinsic viscosities and sedimentation coefficients in concentrated guanidine hydrochloride. J. Am. Chem. Soc. 89: 729-736.

Toews, C. J. 1966 Evidence for the metabolism of glycerol by skeletal muscle and the presence of a muscle nicotinamide adenine dinucleotide phosphate dependent glycerol dehydrogenase. Biochem. J. 98: 27c-29c.

Umbarger, H. E. 1956 Evidence for a negative feedback mechanism in the biosynthesis of isoleucine. Science 123: 848-849.

Underkofler, L. A., and E. I. Fulmer. 1937 The production of dihydroxyacetone by the action of Acetobacter suboxydans upon glycerol. J. Am. Chem. Soc. 59: 301-302.

Ushiba, D., and B. Magasanik. 1952 Effects of auxotrophic mutations on the adaption to inositol degradation in Aerobacter aerogenes. Proc. Soc. Exptl. Biol. Med. 80: 626-632.

Vagelos, P. R., A. W. Alberts, and D. B. Martin. 1963 Studies on the mechanism of activation of acetyl coenzyme A carboxylase by citrate. J. Biol. Chem. 238: 533-540.

Vessel, E. S. 1968 Multiple molecular forms of enzymes. Ann. N. Y. Acad. Sci. 151: 1-698.

Walter, C., and E. Frieden. 1963 The prevalence and significance of the product inhibition of enzymes. Adv. Enzymol. 25: 167-274.

Walter, C., and J. Singer. 1968 Unpublished results.

Wampler, D. E., and E. W. Westhead. 1968 Two aspartokinases from Escherichia coli. Nature of the inhibition and molecular changes accompanying inactivation. Biochem. 7: 1661-1671.

Weber, K. 1968 New structural model of E. coli aspartate transcarbamylase and the amino acid sequence of the regulatory polypeptide chain. Nature 218: 1116-1119.

Wiame, J. M., S. Bourgeois, and R. Lambion. 1954a Oxidation of glycerol by variants of Bacillus subtillis. Arch. Intern. Physiol. 62: 155-156.

Wiame, J. M., S. Bourgeois, and R. Lambion. 1954b Oxidative dissimilation of glycerol studied with variants of B. subtillis. Nature 174: 37-38.

Widner, F. G., C. O'Hara, and C. Ratledge. 1961 Levels of some enzymes in normal, in iron deficient and in Zn-deficient Mycobacterium smegmantis. Biochem. J. 80: alp.

Wieland, T. O., and G. Pfleiderer. 1957 Nachweis der heterogenitat von milchsauer-dehydrogenasen verschiedenen ursprungs durch tragerelektrophorese. Biochem. A. 329: 112-116.

Wilkinson, G. N. 1961 Statistical estimations in enzyme kinetics. Biochem. J. 80: 324-332.

Wilson, A. C., R. D. Cahn, and N. O. Kaplan. 1963 Functions of the two forms of lactate dehydrogenase in the breast muscle of birds. Nature 197: 331-334.

Worthing, A. G., and J. Goffner. 1943 Treatment of Experimental Data. John Wiley and Sons, New York.

Yates, R. A., and A. B. Pardee. 1956 Control of pyrimidine biosynthesis in Escherichia coli by a feedback mechanism. J. Biol. Chem. 221: 757-770.

Yphantis, D. A. 1964 Equilibrium ultracentrifugation of dilute solutions. Biochem. 3: 297-317.

VITA

Michael James Barrett was born in Bridgeport, Connecticut on November 12, 1942. In June 1964 he was graduated with the Bachelor of Science degree from Boston College, Chestnut Hill, Massachusetts. He entered the Department of Biochemistry of the University of Tennessee, Memphis, Tennessee, in September, 1964.

From January 1964 to July 1964 he held a teaching assistantship in the Department of Biochemistry and in July, 1964 was awarded a training grant from the same department. In February, 1968 he was awarded a Predoctoral Fellowship from the General Medical Institute of the National Institutes of Health.

He is married to the former April Marianna Purcell of Newton, Massachusetts and has two sons, Andrew Jerome and James Purcell.