69-16,653

GREEN, Robert William, 1932-
THE BEHAVIOR OF HORSE LIVER ALCOHOL DEHYDROGENASE IN GUANIDINE HYDROCHLORIDE SOLUTIONS.

University of Hawaii, Ph.D., 1969
Biochemistry

University Microfilms, Inc., Ann Arbor, Michigan

THE BEHAVIOR OF HORSE LIVER ALCOHOL DEHYDROGENASE IN GUANIDINE HYDROCHLORIDE SOLUTIONS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE
UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN BIOCHEMISTRY
JANUARY 1969

By

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PLEASE NOTE: Appendix pages are not original copy. Print is indistinct on many pages. Filmed in the best possible way.

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ACKNOWLEDGMENTS

The author wishes to express appreciation to Dr. Roy A. Scott and Dr. Hannah O. Green for their helpful advice. He is grateful to the National Institutes of Health for providing financial support during graduate study at the University of Hawaii.

ABSTRACT

The Z average molecular weight of horse liver alcohol dehydrogenase (LADH) in dilute neutral buffer was found to be 78,200 by sedimentation equilibrium. One molar guanidine hydrochloride (GuHCl) reversibly inhibited LADH activity. The inhibition was competitive with limiting nicotinamide adenine dinucleotide and mixed with limiting ethanol. When EDTA was added to LADH in 1 M GuHCl, zinc was removed followed by aggregation. Sedimentation velocity experiments indicated that no subunit was formed under these conditions. Higher concentrations of GuHCl resulted in dissociation of LADH. In 3 M GuHCl, the sedimentation equilibrium data indicated the presence of a reversible association-dissociation system involving subunit, dimer, and trimer. EDTA removed zinc but had no effect on the molecular weight of LADH in 3 M GuHCl. Reduction and alkylation of LADH in 3 M GuHCl resulted in almost complete dissociation into two subunits. Apparently, sulfhydryl residues are involved in subunit association, while zinc plays little or no role. The Z average molecular weight of LADH in 5 M GuHCl containing mercaptoethanol was found to be 45,700, a value higher than expected. The discrepancy may be attributed to incomplete dissociation, a small error in apparent partial specific volume, or solvent binding.

Measurements of molecular weight, sedimentation coefficient, and intrinsic viscosity of LADH in 5 M GuHCl indicate that the structure of LADH is composed of two subunits.

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ABBREVIATIONS

a	Radius of meniscus
A	Angstrom
b	Radius of cell bottom
B	Second virial coefficient
c	Concentration
C	Centigrade
cm	Centimeter
CTogen	Chymotrypsinogen A
D	Diffusion coefficient
DTE	Dithioerythritol
DTT	Dithiothreitol
EDTA	Disodium ethylenediaminetetraacetic acid
EtSH	Mercaptoethanol
F	Fick
gm	Gram
GuHCl	Guanidine hydrochloride
IA	Iodoacetate
k	Huggins constant
K	Michaelis constant
LADH	Horse liver alcohol dehydrogenase
M	Molar
M^o	Molecular weight at zero concentration
M_{app}	Apparent molecular weight
mg	Milligram

min	Minute
ml	Milliliter
mm	Millimeter
mμ	Millimicron
n	Index of refraction
N	Number of residues per chain
NaCl	Sodium chloride
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide reduced
obs	Observed
OP	Orthophenanthroline
PCMB	p-chloromercuribenzoate
ppm	Parts per million
r	Radius
R	Gas constant
rpm	Revolutions per minute
S	Sedimentation coefficient, Svedberg
sec	Second
SDS	Sodium dodecylsulfate
t	Time
T	Absolute temperature
tris	Trishydroxymethylaminomethane
$\bar{v}$	Apparent partial specific volume
V_{max}	Maximum specific activity
w	Water
ZAMW	Z average molecular weight

η	Viscosity
$[\eta]$	Intrinsic viscosity
μ	Micron
ρ	Density
ω	Angular velocity
$^{\circ}$	Degree

INTRODUCTION

Dehydrogenases are a class of enzymes which catalyze many metabolic oxidation-reduction reactions. Horse liver alcohol dehydrogenase (LADH), Enzyme Commission number 1.1.1.1, with the cofactor nicotinamide adenine dinucleotide (NAD) catalyzes the conversion of primary and secondary alcohols to aldehydes and ketones. Since the initial isolation of LADH by Bonnichsen and Wassen in 1948 (Bonnichsen and Wassen, 1948), much kinetic data has accumulated which substantiate the compulsory order mechanism proposed by Theorell and Chance (Theorell and Chance, 1951) in which the cofactor must add to the enzyme before the substrate.

$$\text{LADH} + \text{NAD} \rightleftharpoons \text{LADH NAD}$$

$$\text{LADH NAD} + \text{alcohol} \rightleftharpoons \text{LADH NADH} + \text{aldehyde}$$

$$\text{LADH NADH} \rightleftharpoons \text{LADH} + \text{NADH}$$

This mechanism and supporting experimental evidence is thoroughly reviewed by Theorell (Theorell, 1967). Other reviews of LADH have been published by Sund and Theorell (Sund and Theorell, 1963) and by McKinley-McKee (McKinley-McKee, 1964).

The molecular weight of LADH has been measured by several laboratories and found to be about 80,000 (Ehrenberg, 1957; Ehrenberg and Dalziel, 1958; Hamburg, 1966; Drum _et al_., 1967; Castillino and Barker, 1968). There are

26 sulfhydryl groups (Witter, 1960; Oppenheimer *et al.*, 1967) and 4 zinc atoms per molecule (Akeson, 1964; Oppenheimer *et al.*, 1967). Each molecule has two cofactor binding sites and presumably two active centers (Theorell and Bonnichsen, 1951; Ehrenberg and Dalziel, 1958; Oppenheimer *et al.*, 1967).

Recent work has been devoted toward determining which of the functional groups of the enzyme are involved in the catalytic mechanism. The fact that all dehydrogenases have a high sulfhydryl content suggests that sulfhydryl groups are involved in the catalytic process. The sulfhydryl reagents p-chloromercuribenzoate (PCMB) and iodoacetate have been used to substantiate this hypothesis. When PCMB is added to LADH, there is a linear loss of enzymatic activity and cofactor binding (Witter, 1960). The presence of cofactor retards the reaction of PCMB with LADH. Iodoacetate appears to be more discriminating than PCMB toward the sulfhydryl groups in LADH. Only two moles of iodoacetate per mole of enzyme are needed to completely inactivate the enzyme (Li and Vallee, 1964; Harris,1964). Iodoacetate reactivity is also hindered by the presence of cofactor. Although these experiments seem to directly implicate sulfhydryl residues in the catalytic mechanism of LADH, the modification of these residues may cause conformational changes of catalytically important regions which are distant from sulfhydryl loci.

When LADH was reacted with radioactive iodoacetate, followed by tryptic digestion and peptide mapping, only one radioactive peptide was found (Li and Vallee, 1964; Harris, 1964). When the radioactive peptide was isolated and its sequence determined, it was found to be homogeneous. The total number of peptides was about half the number expected. These data indicate that LADH is composed of two identical subunits, each containing an unusually reactive sulfhydryl group.

The role of the four zinc atoms in LADH is also of considerable interest. The kinetic and structural functions of zinc have been investigated by means of chelating agents. Orthophenanthroline (OP) and 2,2-bipyridine have two binding sites per molecule of LADH (Vallee and Coombs, 1959; Yonetani, 1963; Sigman, 1967). They competitively inhibit activity by interfering with cofactor binding (Vallee _et al._, 1959). The spectrum of the chelate-enzyme complex is characteristic of a chelate-zinc complex. When zinc is removed at low pH, there is a linear loss of cofactor binding (Oppenheimer _et al._, 1967). Apparently the cofactor binds at or near zinc atoms. On the other hand, when zinc is removed from LADH by lowering the pH in the presence of chelating agent, activity decreases faster than zinc is removed (Akeson, 1964; Drum _et al._, 1967). Furthermore, two zinc atoms per molecule of LADH seem to be more easily exchanged with Zn^{65} than the other two zinc

atoms (Drum *et al.*, 1967; Akeson, 1964; Druyan and Vallee, 1964). When LADH is denatured with 8 M urea, dissociation into two subunits occurs with zinc retention. Subsequent addition of EDTA causes zinc removal and further dissociation to a molecular weight of 20,000 (Drum *et al.*, 1967). It is probable that the four zinc atoms are nonequivalent in their catalytic effectiveness. The suggestion has been made that the two zinc atoms which are more easily exchanged are involved in catalysis while the other two zinc atoms are buried and have a structural role (Akeson, 1964; Drum *et al.*, 1967).

Recent phosphorescence studies indicate that OP is not complexing directly with the zinc atoms in LADH (Piette and Rabold, 1967). When zinc ion is added to an OP solution, there is a doubling of the OP triplet life time when the zinc to OP ratio is 1 : 1. However, when OP is added to an LADH solution, the triplet life time does not increase, indicating that all four zinc atoms are buried in the enzyme. Thus the precise structural and catalytic roles of zinc in LADH remain unclear.

Subunit content is an important factor in enzyme structure. If subunits are identical, structural analysis is considerably simplified. Often one active center is associated with each subunit (Sund and Weber, 1966). Sometimes subunits interact with each other forming an allosteric system (Monod *et al.*, 1963).

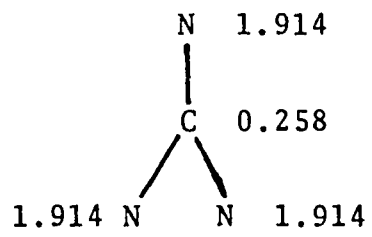
The number of subunits in LADH is in dispute. Some laboratories have reported two subunits and others have reported four. Dissociation of LADH in 7 M urea containing reducing agent produced subunits of 42,000 weight average molecular weight, determined by high speed and low speed sedimentation equilibrium (Hamburg, 1966). The detergent, sodium dodecyl sulfate (SDS), was also used successfully in dissociating LADH into two subunits (Hamburg, 1966; Blomquist et al., 1967). Osmotic pressure measurements of LADH in 6 M guanidine-HCl (GuHCl) with reducing agent presented indicated dissociation into two subunits with a number average molecular weight of 41,000 (Castillino and Barker, 1968). This result was confirmed by high speed sedimentation equilibrium, resulting in a weight average molecular weight of 40,000 (Castillino and Barker, 1968). Light scattering measurements indicated dissociation to four subunits in low pH, in 8 M urea, and in 4 M GuHCl (Cheng et al., 1968). The ultracentrifuge data of Vallee and coworkers indicated that the structure of LADH contains four subunits (Drum et al., 1967).

Several derivatives of LADH have been successfully crystallized for x-ray analysis. The results at 6 A resolution indicate that LADH is composed of two identical subunits (Branden, 1965).

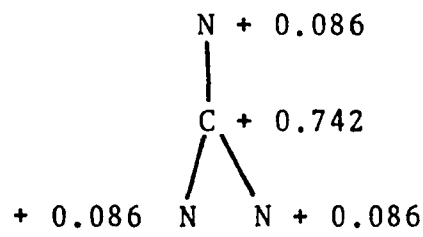
The following work was undertaken to determine the number of subunits in LADH and to help clarify the structural role of zinc in the enzyme. The approach used was

stepwise disruption of structure with GuHCl while measuring molecular weight, sedimentation, diffusion, viscosity, and zinc content at each step.

GuHCl was chosen because of its strong denaturing ability. The mechanism of denaturation of proteins by GuHCl is not completely understood. The guanidinium ion consists of three equivalent amino groups arranged with a planar trigonal symmetry around a central carbon atom. The carbon atom has an empty p orbital where most of the positive charge is located. Each nitrogen of the amino groups has three planar, trigonally hybridized atomic orbitals engaged in sigma bonds with hydrogen and carbon. The lone pair of electrons are in a p orbital which is involved in pi-pi conjugation with the carbon p orbital. Six electrons are involved in this conjugated system (Dewar and Paoloni, 1957). The distribution of pi like electrons is shown below together with the charge distribution. This charge distribution is thought to play an important role in denaturation (Joly, 1965).



Pi Electron Distribution



Charge Distribution

Tanford and coworkers observed that proteins in concentrated GuHCl with a reducing agent present are completely dissociated and behave like random coils (Tanford *et al.*, 1967). Their data for sedimentation coefficient and intrinsic velocity of several proteins fit the following equations, which were derived assuming a random coil.

$$S^o = (1 - \bar{v}\rho)\ AN^B$$

$$[\eta] = CN^D$$

where S^o = sedimentation coefficient at zero concentration

$\bar{v}$ = apparent partial specific volume

ρ = solvent density

N = number of residues per chain

$[\eta]$ = intrinsic viscosity

A, B, C, and D are constants

In the following study, measurements of molecular weight, sedimentation velocity, diffusion, viscosity, and zinc content were made at 1 M, 3 M, and 5 M GuHCl concentrations. From these data the stepwise denaturing effect of GuHCl on LADH could be observed. The Tanford equations

were applied to LADH in 5 M GuHCl. The minimum size of subunits in LADH was then determined and conclusions were drawn regarding the structural role of zinc and of sulfhydryl in the enzyme.

EXPERIMENTAL PROCEDURES

MATERIALS

Proteins

Horse liver alcohol dehydrogenase was obtained as a crystalline suspension from C. F. Boehringer and Son, Manheim, Germany.

Chymotrypsinogen A from bovine pancreas (CGC grade) was purchased from Worthington Biochemical Corporation, Freehold, New Jersey. This protein was obtained as a lyophilized salt free powder after being recrystallized five times.

Reagents

Acetylpyridine adenine dinucleotide, C. F. Boehringer and Son, Manheim, Germany.

Disodium ethylene diaminetetraacetic acid (EDTA), Reagent grade, J. T. Baker Chemical Co., Phillipsburg, New Jersey.

Disodium hydrogen phosphate, Reagent grade, Allied Chemical Corporation, New York, New York.

Dithioerythritol, Grade I, Cyclo Chemical Corporation, Los Angeles, California.

Dithiothreitol, A grade, Calbiochem, Los Angeles, California.

Ethanol, U. S. P. grade, Commercial Solvents Corporation, Los Angeles, California.

Guanidine-HCl, Ultrapure, Mann Research Laboratories, New York, New York.

Hydrochloric acid, Reagent grade, J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Iodoacetic acid, Sigma Chemical Company, St. Louis, Missouri.

Mercaptoethanol, Calbiochem, Los Angeles, California.

Nicotinamide adenine dinucleotide, C. F. Boehringer and Son, Manheim, Germany.

Sephadex G-100, Pharmacia Fine Chemicals, Inc., Piscataway, New Jersey.

Sodium Carbonate, Reagent grade, J. T. Baker Chemical Company, Phillipsburg, New Jersey.

Sodium Chloride, Reagent grade, Matheson Company, Inc., Norwood, Ohio.

Sodium succinate, Matheson Company Inc., Norwood, Ohio.

Starch, Connaught Medical Research Laboratories, Toronto, Canada.

Thioglycolic acid, Purified, Mann Research Laboratories, New York, New York.

Tris (trishydroxymethylaminomethane), Reagent grade, Sigma Chemical Company, St. Louis, Missouri.

Whatman CM-52, Reeve Angel, Clifton, New Jersey.

EXPERIMENTAL PROCEDURES

METHODS

Preparation of LADH Stock Solution

The LADH suspension, obtained from C. F. Boehringer and Son, was dialyzed against three changes of 0.05 M tris, pH 7.5 at 4° C for approximately 24 hours. Subsequent filtering through a 0.45μ Millipore filter resulted in a stock solution of LADH which was stable for about one month. Turbidity in this stock solution, which appeared after several weeks, could be removed by heating at 40° C for 15 minutes and filtering.

Protein Concentration Determination

Protein concentrations were determined spectrophotometrically by absorbance at the wave length of maximum absorptivity in the 280 mμ region. Absorbance was also measured at 400 mμ to correct for light scatter. Scatter corrections were previously determined empirically by measuring absorbance at 400 mμ and 280 mμ before and after centrifuging down the aggregate causing turbidity (McKay, 1962). LADH solutions having an absorbance higher than 0.006 at 400 mμ were heated to 40° C for 15 minutes and then filtered through a 0.45 μ Millipore filter. Table 1 gives the absorptivity of LADH and chymotrypsinogen A in tris buffer and in 3 M GuHCl (Ehrenberg and Dalziel,

TABLE 1

Absorptivity of LADH and CTogen

Enzyme	Solvent	Wave Length (mμ)	Absorptivity ($mg^{-1}cm^2$)	Reference
LADH	tris buffer, pH 7.4	280	0.420	Ehrenberg and Dalziel, 1958
LADH	3 M GuHCl, pH 7.2	277	0.426*	
CTogen	tris buffer, pH 7.4	280	2.00	Schwert, 1951
CTogen	3 M GuHCl, pH 7.2	280	2.06*	

*Absorbance was measured after concentrated GuHCl solution was added to solutions of known protein concentration in tris buffer. Absorptivity in 3 M GuHCl solution is based on absorptivity in dilute buffer.

1958; Schwert, 1951).

LADH Activity Determination

LADH activity was determined spectrophotometrically by measuring the rate of formation of NADH in a solution containing 1.7×10^{-3} M NAD, 1.6×10^{-2} M ethanol and 0.06 M tris, pH 8.8 at 25° C. This environment gave optimum LADH activity.

Sephadex G-100 Chromatography of LADH

Sephadex chromatography of LADH was done with a G-100 column having a diameter of 1.5 cm and a height of 21 cm. This column was washed with 0.05 M tris, pH 7.1 until the effluent had the same pH, conductivity, and 280 mμ absorbance as the starting tris buffer. 1.00 ml of LADH (7.79 mg/ml) was added to the column and then eluted with tris buffer at a flow rate of 1.1 ml/min. The temperature was approximately 24° C. Protein eluted from the column was measured by absorbance at 280 mμ.

Carboxy Methyl Cellulose Chromatography of LADH

A column of Whatman CM-52 having a diameter of 1.5 cm and a height of 26.5 cm was washed with 0.01 M tris, pH 7.1 until the effluent had the same pH and conductivity as the starting tris buffer. 1.00 ml of LADH (5.52 mg/ml) was added to the column. After the first peak was eluted,

the buffer was changed to 0.05 M tris, pH 7.5. The flow rate was 0.67 ml/min. The temperature was approximately 25° C. Eluted protein was measured by absorbance at 280 mμ.

Starch Gel Electrophoresis of LADH

Starch gel electrophoresis was done by the method of Smithies (Smithies, 1955) as modified by Ashton (Ashton and Braden, 1961). Sixty-two gm of Connaught starch were mixed with 500 ml of hot buffer to form the gel. Whatman filter paper was saturated with LADH solution and inserted into the starch gel of 3 mm thickness. Electrophoresis was done for 4 to 12 hours at 4° C with approximately 7 volts/cm. Protein was detected by nigrosin stain. Dehydrogenase activity was detected by spraying the starch gel with a solution of ethanol and acetylpyridine adenine dinucleotide at pH 8.8 and observing green fluorescence when the gel was irradiated with ultraviolet light.

Preparation of LADH and Chymotrypsinogen A Solutions in GuHCl

One M GuHCl solutions were made by adding concentrated GuHCl to LADH stock solutions. Measurements were carried out immediately since LADH is unstable in this environment and tends to aggregate even when reducing agent is present. Three molar and 5 M GuHCl solutions were prepared in

three different ways: (1) Enzyme stock solution was dialyzed for 48 hours against GuHCl in 0.05 M tris buffer, containing 0.1 M mercaptoethanol. (2) Enzyme stock solution was added to solid GuHCl and then dialyzed for 48 hours against GuHCl-tris buffer solution containing 0.1 M mercaptoethanol. (3) Enzyme stock solution was added to solid GuHCl. This solution was made 2.5×10^{-3} M in dithiothreitol. After an hour, an excess of iodoacetate over the total sulfhydryl content was added and the pH adjusted to 8.0. Four hours later, the alkylated LADH was dialyzed for 48 hours against GuHCl-tris buffer solution. Solutions of chymotrypsinogen A in 5 M and 6 M GuHCl were prepared in the same way. In addition to the above procedures, some solutions of LADH in 3 M GuHCl containing no reducing agent were prepared by dialysis against GuHCl-tris buffer solutions with and without EDTA.

Apparent Partial Specific Volume of LADH

Densities of enzyme and dialysate solutions were measured with 3 ml and 5 ml pycnometers, designed by Lipkin (Lipkin *et al.*, 1944), obtained from Lab Glass, Inc., Vineland, New Jersey. LADH concentrations ranged from 6 to 15 mg/ml. Weighings were to the nearest 0.1 mg with a Mettler type H16 analytical balance. Volumes were measured in a constant temperature viscosity bath (Precision Scientific Company, Chicago, Model S) which was

maintained at 20.00° C and regulated to ± 0.005° C. Apparent partial specific volume was calculated by the following equation.

$$\bar{v} = 1/\rho_o - 10^2/c \left[\frac{\rho - \rho_o}{\rho\rho_o} \right]$$

ρ_o = density of dialysate

ρ = density of enzyme solution

c = enzyme concentration in mg/ml

Zinc Analysis

Zinc was determined by atomic absorption using the method of Fuwa (Fuwa *et al.*, 1964). A Westinghouse type WL hollow cathode source was directed through a horizontal hydrogen-air flame into a Zeiss Model PMQ-II spectrophotometer. With this arrangement, zinc aspirated into the flame could be accurately measured in the 0.1 to 0.5 ppm range. In the concentrations used, tris and GuHCl had no enhancing or quenching effect on the zinc analysis.

Kinetics of Inhibition of LADH with GuHCl

Lineweaver-Burk plots (Lineweaver and Burk, 1934) were made for limiting ethanol and limiting NAD concentrations. These kinetic measurements were done with and without 0.5 M GuHCl present in the activity assay mixture.

Kinetics of Denaturation of LADH with GuHCl

LADH was dissolved in 1 M, 2 M, and 3 M GuHCl at 0° C. Aliquots were taken at different time intervals and activity was measured in an assay mixture containing the same concentration of GuHCl. The decrease of activity as a function of time was recorded. At the end of each experiment, activity was determined in the absence of GuHCl to measure reversibility. This procedure caused a 1 to 250 dilution of the GuHCl.

Sedimentation Velocity

Measurements of sedimentation velocity were done at 20.0° C with a Spinco Model E analytical ultracentrifuge using a Schlieren optical system. The change of index of refraction with radial distance in the cell was recorded on Kodak metallographic plates at different times during a run. The radial distance of the maximum at each time was measured with a Gaertner microcomparator. Between four and ten pictures were used for the calculation of the sedimentation coefficient (Schachman, 1957). Densities and relative viscosities of solvents were measured for correction to water as solvent. The reciprocal sedimentation coefficient was extrapolated to zero concentration by the least squares method. Because of radial dilution, concentration was corrected to the concentration of the plateau region at the half time of the run (Kegeles

and Gutter, 1951). A standard aluminum centerpiece (4°, 12 mm) was used for LADH in tris buffer and 1 M GuHCl. An aluminum filled epon double sector synthetic boundary centerpiece (2.5°, 12 mm) was used for LADH and chymotrypsinogen A in 3 M and 5 M GuHCl so that early measurements could be made prior to large diffusion and so that measurements could be made at the plateau region of GuHCl. Thus a correction for GuHCl sedimentation was not necessary.

Diffusion of LADH

The diffusion coefficient of LADH was obtained by measuring the spreading of a synthetic boundary formed by layering dialysate on enzyme solution. A double sector synthetic boundary cell (2.5°, 12 mm) was used at 20.0° C and 8000 rpm. No sedimentation was observed at this speed. The diffusion coefficient was calculated by the height-area method after reading Schlieren pictures by a Gaertner microcomparator (Ehrenberg, 1957). Correction to water as solvent was done by the procedure outlined by Schachman (Schachman, 1957).

Sedimentation Equilibrium

The method used for sedimentation equilibrium permitted an extrapolation of molecular weight to zero concentration after one overnight experiment. Four

millimeter columns of enzyme solution and dialysate were inserted into a cell containing an aluminum filled epon double sector centerpiece (2.5°, 12 mm). Rotor speeds were used that gave approximately a ten fold difference in concentration between the top and bottom of the solution column after equilibrium had been reached. In cases where concentration differences were less than eight fold, extrapolation to zero concentration would involve an undesirably small concentration interval. When concentration differences were greater than twelve fold, the molecular weight measurements near the meniscus would be subject to large errors. Making an initial assumption of molecular weight, the following integrated sedimentation equilibrium equation was used to calculate the rotor speed which would give the desired concentration gradient (Van Holde, 1967).

$$\ln \frac{c\ (b)}{c\ (a)} = \frac{M\ (1 - \bar{v}\ \rho)\ \ \omega^2\ (b^2 - a^2)}{2\ RT}$$

c	=	protein concentration
a	=	radius of meniscus
b	=	radius of cell bottom
M	=	molecular weight
$\bar{v}$	=	apparent partial specific volume
ρ	=	solvent density
ω	=	rotor speed
R	=	gas constant
T	=	absolute temperature

By using the rpm step down procedure of Hexner (Hexner *et al.*, 1961), equilibrium could be reached within a day, thus saving considerable time. This procedure consists of sedimenting the protein solution at a higher rpm for a short time until a distribution is reached which approximates the equilibrium distribution at a lower rpm. Then the rotor speed is decreased to the lower rpm and allowed to rotate overnight. Pictures of the protein distribution at equilibrium were taken on Kodak metallographic plates using the Schlieren optical system. The differences in change of index of refraction with distance between enzyme and dialysate solutions, at 0.04 cm observed radial intervals from meniscus to the cell bottom, were measured with a Gaertner microcomparator. The actual intervals were approximately 0.02 cm when corrected for optical magnification. By measuring data in this way, the Schlieren readout is effectively divided into about 20 small trapezoids. Concentration at each radial interval was calculated by the following equation, assuming a conservation of mass.

$$c = \frac{\text{area} \cdot c_o \, (r_b^2 - r_a^2)}{\sum_a^b [\text{area} \cdot \Delta \, (r^2)]}$$

c = concentration in the interval

c_o = initial concentration

area = trapezoid area in the interval

r_a = radius of the meniscus

r_b = radius of the cell bottom

Δr^2 = maximum difference of radius squared in the interval

Calculation of Z average molecular weight was based on the differential logarithmic form of the equilibrium equation derived by Lamm (Lamm, 1929).

$$\frac{\Delta \ln(1/r \cdot dn/dr)}{\Delta r^2} = \frac{M\ (1 - \bar{v}\rho)\omega^2}{2\ RT}$$

n = index of refraction

r = radius

M = molecular weight

$\bar{v}$ = apparent partial specific volume

ρ = solvent density

ω = rotor speed

R = gas constant

T = absolute temperature

The reciprocal square roots of molecular weight calculated at each radial interval were extrapolated to zero concentration by the least squares method.

Intrinsic Viscosity

Flow times of enzyme solutions and dialysates were measured in a 1 ml Ubbelohde semi-micro dilution type viscometer of 0.004 centistokes per second (Cannon Instrument Company, State College, Pennsylvania). Constant temperature was maintained at 25.00° C and regulated to ± 0.005° C with a viscosity bath (Precision Scientific Company, Chicago, Model S). Intrinsic viscosity was calculated by a least square extrapolation of the following equation.

$$\frac{\eta/\eta_o - 1}{c} = [\eta] + k[\eta]^2 c$$

$$\eta/\eta_o = t/t_o \cdot \rho/\rho_o$$

t = solution flow time

t_o = solvent flow time

ρ = solution density

ρ_o = solvent density

c = concentration

$[\eta]$ = intrinsic viscosity

k = Huggins constant

Calculations

Molecular weight and sedimentation coefficient calculations were done by an IBM 360 computer. Other calculations

were done by a Digital PDP-8/S computer. All straight lines were computed by the least squares method. Error analyses are standard deviations (Baird, 1962). The computer program listings and descriptions will be found in the Appendix.

RESULTS

Sephadex G-100 Chromatography of LADH

Chromatography of LADH with a Sephadex G-100 column resulted in a major and a minor component. See Figure 1. Recovery of protein was 104 per cent. The major component contained 97 per cent of the total protein recovered. The minor component, apparently of low molecular weight since it was somewhat retarded by Sephadex G-100, was 3.2 per cent of the total protein recovered and had no enzyme activity. Table II gives the specific activity of fractions in the major peak. The small increase of specific activity over that of unchromatographed LADH and the high values of specific activity throughout the major peak indicate that this preparation of LADH has a high degree of homogeneity.

Carboxy Methyl Cellulose Chromatography of LADH

Figure 2 shows a chromatogram of LADH after elution from a column of carboxy methyl cellulose (Whatman CM-52). One initial minor component and one major component were observed. Recovery of protein was 93 per cent. The major component contained 93 per cent of the total protein recovered and the minor component contained 7.5 per cent. Table III gives the specific activity of fractions in the major and minor peaks. Both peaks had approximately the

Figure 1

Elution diagram for Sephadex G-100 chromatography of LADH. Absorbance at 280 mμ is plotted against fraction number. Each fraction contained 2.0 ml. 7.8 mg of LADH were applied to the column and eluted with 0.05 M tris, pH 7.1. Temperature was 24° C. Recovery of protein was 104 per cent.

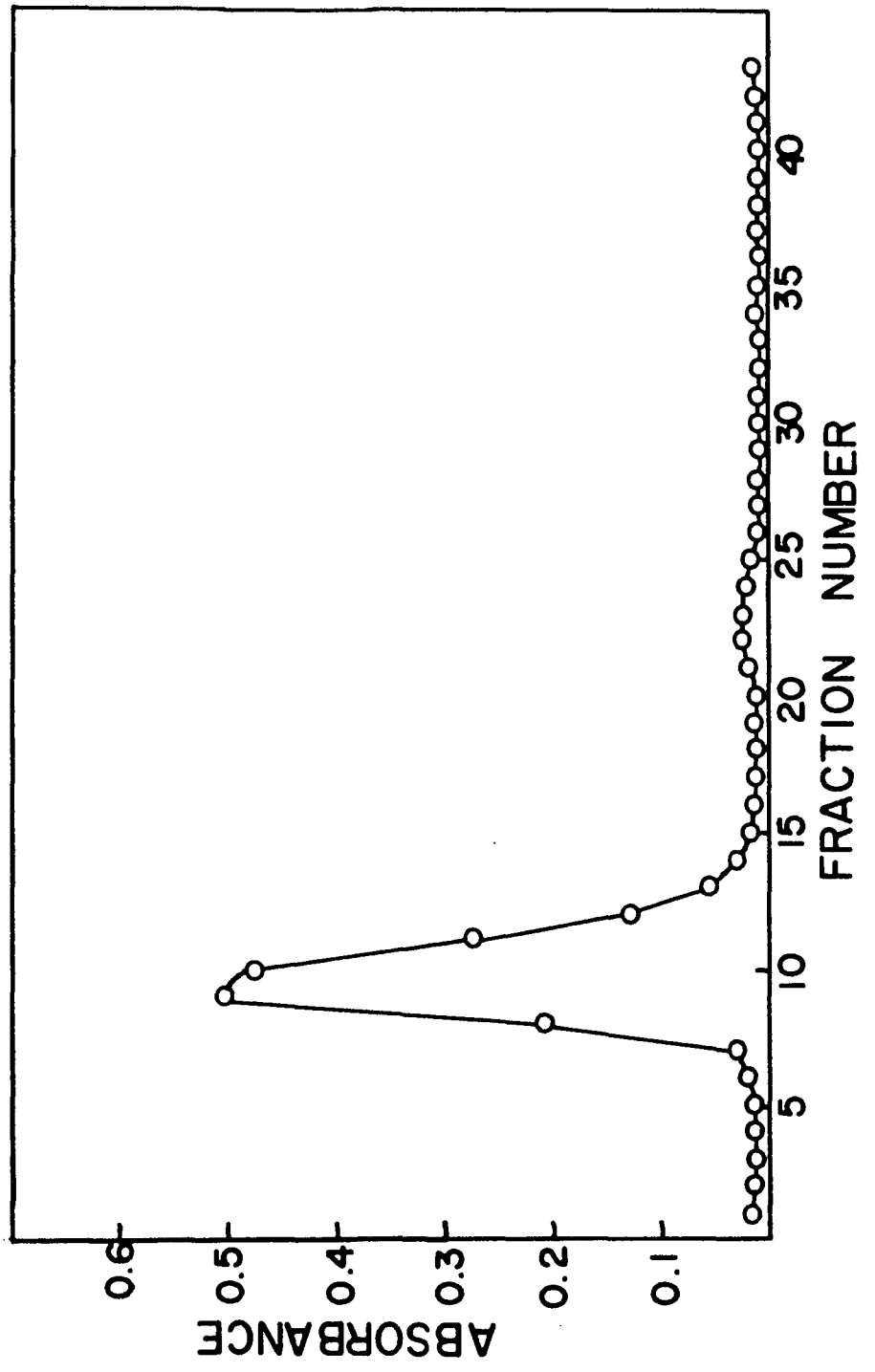

ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
5
10
15
20
25
30
35
40
FRACTION NUMBER

TABLE II

Specific Activity of Fractions in Major Peak from Sephadex G-100

Fraction Number	Specific Activity (μmole min^{-1} mg^{-1})
8	8.13
9	8.42
10	8.42
11	8.57
12	8.04
13	7.45
original enzyme	8.23

Figure 2

Elution diagram for carboxy methyl cellulose (Whatman CM-52) chromatography of LADH. Absorbance at 280 mμ is plotted against fraction number. Each fraction contained 1.0 ml. 5.5 mg of LADH were added to the column with 0.01 M tris, pH 7.1. After the first peak was eluted, the buffer was changed to 0.05 M tris, pH 7.5 to elute the second peak. Temperature was 25° C. Recovery of protein was 93 per cent.

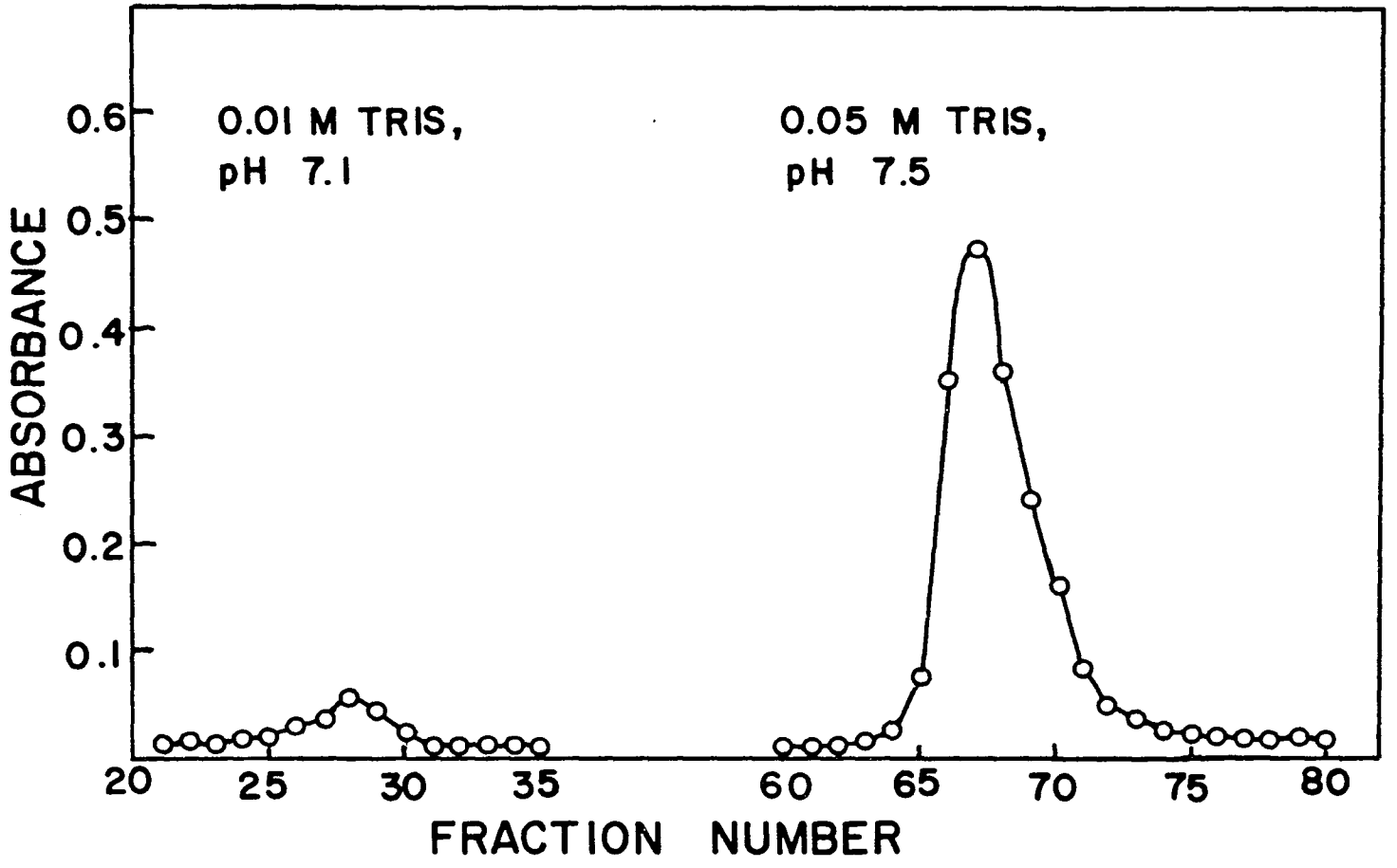

0.01 M TRIS,
pH 7.1
0.05 M TRIS,
pH 7.5
ABSORBANCE
0.6
0.5
0.4
0.3
0.2
0.1
20
25
30
35
60
65
70
75
80
FRACTION NUMBER

TABLE III

Specific Activity of Fractions From Whatman CM-52

Fraction Number	Specific Activity (μmoles min^{-1} mg^{-1})
27	8.57 ± 0.12*
28	8.23 ± 0.00
65	8.42 ± 0.00
66	8.08 ± 0.07
67	8.28 ± 0.08
68	8.18 ± 0.27
69	7.94 ± 0.03
70	7.31 ± 0.05
71	7.85 ± 0.33
original enzyme	8.23 ± 0.23

*Average deviation of two determinations.

same specific activity as the unfractionated enzyme. These results suggest that the two components are isozymes and that a high degree of homogeneity is present. The difference between the two components may be a difference in amino acid content which apparently does not affect the active center.

Starch Gel Electrophoresis of LADH

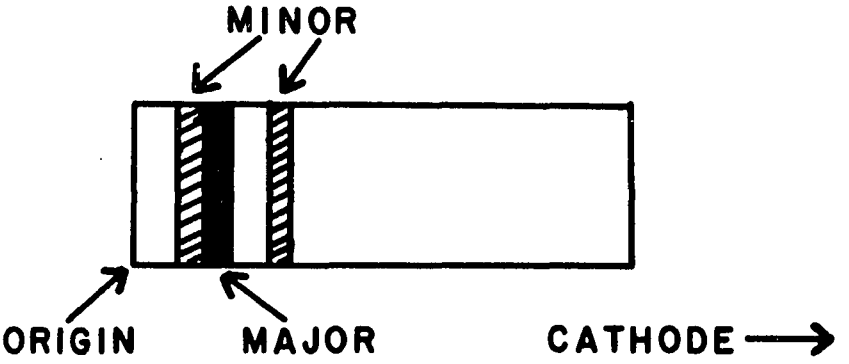
In starch gel electrophoresis at pHs below 9, LADH moved toward the cathode. See Figures 3a and 3b. When electrophoresis was done at pHs 7 to 9 in 0.05 M tris buffer or 0.05 M phosphate buffer, two minor bands and one major band were observed. The major band appeared to be about 90 per cent of the total protein. The leading minor band had almost twice the mobility of the major band. The other minor band was a trailing shoulder of the major band. All bands had dehydrogenase activity. At pH 5.0 in 0.05 M succinate buffer, only one cathode migrating band was observed. At pH 10.0, in 0.05 M carbonate buffer, only one anode migrating band appeared. The minor bands detected after electrophoresis in the pH 7 to 9 range are presumed to be isozymes. Apparently, functional groups in the minor components are titrated at pH 5.0 and pH 10.0 so that they display the same mobility as the major component at these pHs. Since all the protein bands observed had alcohol dehydrogenase activity, the starting enzyme

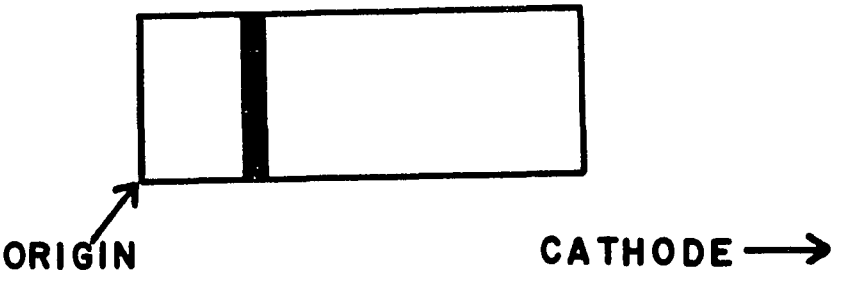
Figure 3a

Starch gel electrophoresis of LADH in 0.05 M phosphate buffer, pH 7.0. This run was done at 4° C and 7 volts/cm for 12 hours. Protein was detected with nigrosine stain.

Figure 3b

Starch gel electrophoresis of LADH in 0.05 M succinate buffer, pH 5.0. This run was done at 4° C and 7 volts/cm for 4 hours. Protein was detected with nigrosine stain.


MINOR
ORIGIN
MAJOR
CATHODE


ORIGIN
CATHODE

appears to be homogeneous in enzymatic activity.

Kinetics of Inhibition of LADH by GuHCl

Kinetic measurements were made with limiting ethanol concentrations (4.0×10^{-4} M to 1.6×10^{-2} M) and also with limiting NAD concentrations (6.8×10^{-5} M to 1.7×10^{-3} M). Each case was examined with and without 0.5 M GuHCl. Lineweaver-Burk plots of reciprocal specific activity *versus* reciprocal concentration of substrate or cofactor are shown in Figures 4 and 5. These data indicate that there is competitive inhibition of the guanidinium ion with NAD and mixed inhibition with ethanol. The kinetic constants, V_{max} and K, were calculated after a least squares treatment of the data according to the following equation (Eadie, 1952). See Table IV.

$$v = V_{max} - K \frac{v}{[S]}$$

v = specific activity

V_{max} = maximum specific activity

K = Michaelis constant

$[S]$ = substrate or cofactor concentration

Kinetics of Denaturation of LADH by GuHCl

The effects of GuHCl on the activity of LADH when

Figure 4

Reciprocal specific activity is plotted against reciprocal ethanol concentration (Lineweaver-Burk plot). Specific activity was measured in 0.06 M tris, pH 8.8, containing 1.7×10^{-3} M NAD and 6.9×10^{-8} M LADH at 25° C with and without 0.5 M GuHCl.

Δ 0.05 M GuHCl in assay.

O No GuHCl in assay.

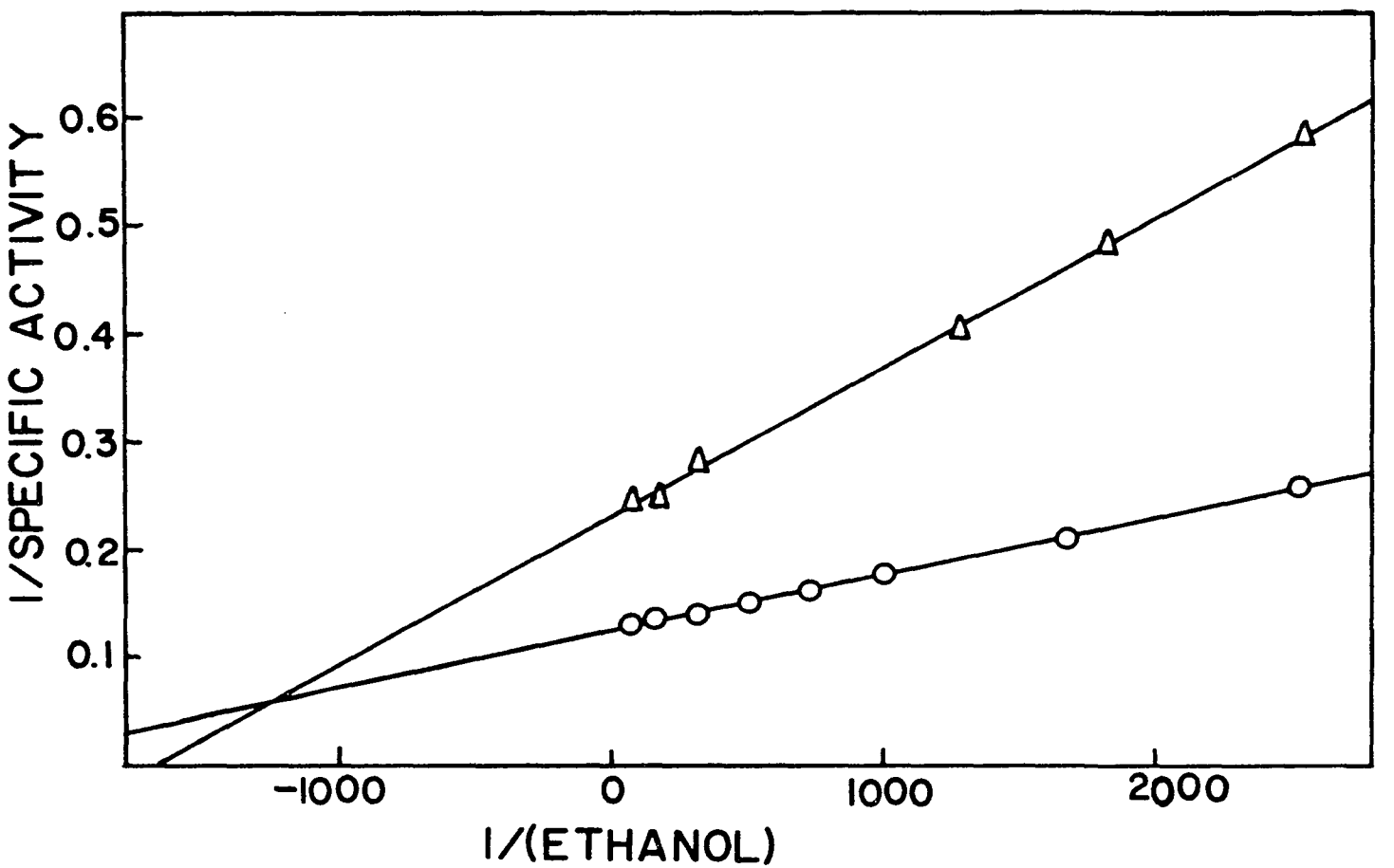

1/SPECIFIC ACTIVITY
0.6
0.5
0.4
0.3
0.2
0.1
-1000
0
1000
2000
1/(ETHANOL)

Figure 5

Reciprocal specific activity is plotted against reciprocal NAD concentration (Lineweaver-Burk plot). Specific activity was measured in 0.06 M tris, pH 8.8, containing 1.6×10^{-2} M ethanol and 7.5×10^{-8} M LADH at 25° C with and without 0.5 M GuHCl.

Δ 0.05 M GuHCl in assay.

O No GuHCl in assay.

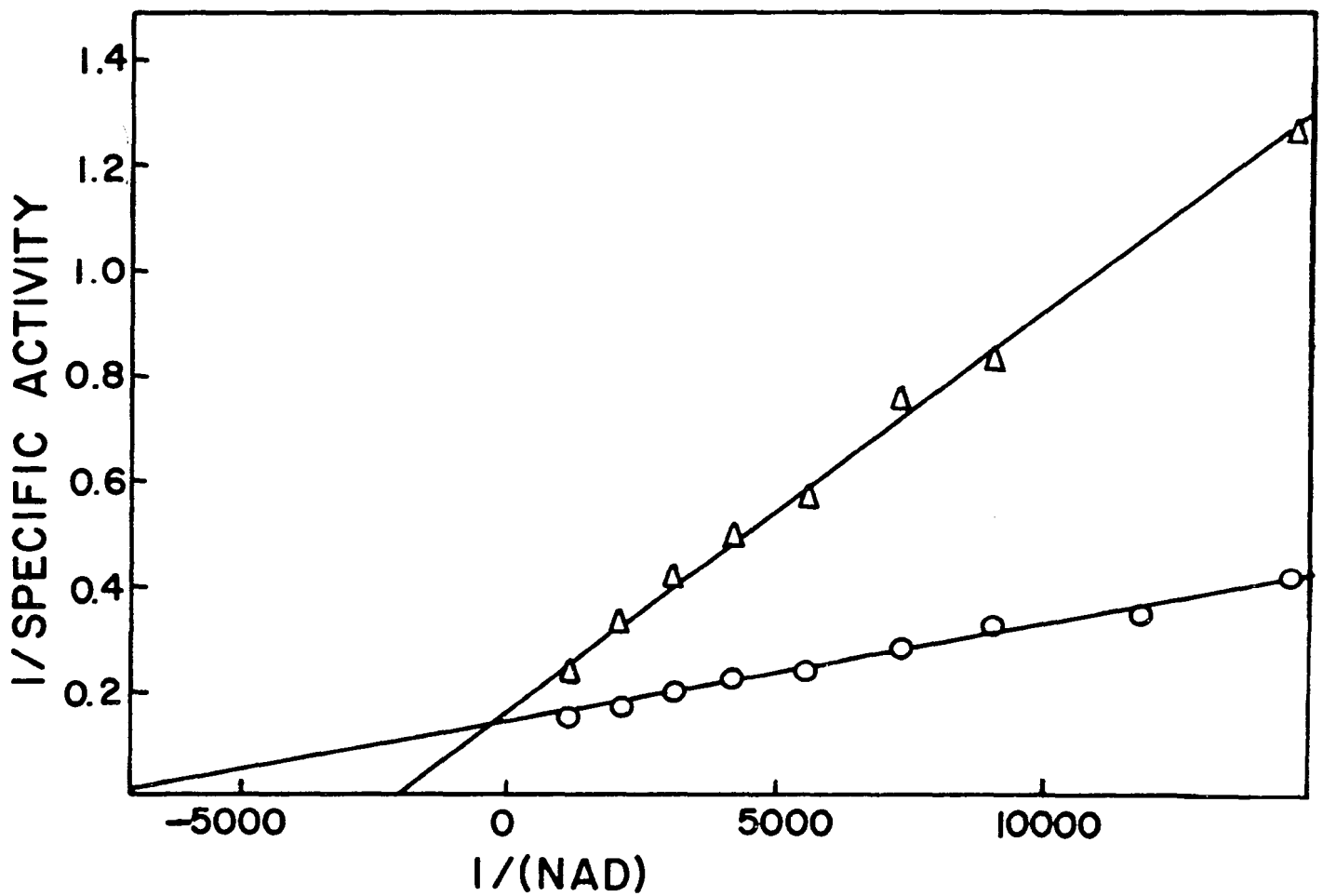

1.4
1.2
1.0
0.8
0.6
0.4
0.2
1/SPECIFIC ACTIVITY
-5000
0
5000
10000
1/(NAD)

TABLE IV

Kinetic Constants for LADH

Solvent	V_{max} (μmoles min^{-1} mg^{-1})	K x 10^4 (M)	Number of Points
0.06 M tris, pH 8.8 1.7 x 10^{-3} M NAD ethanol limiting	7.82 ± 0.06	4.07 ± 0.12	8
0.06 M tris, pH 8.8 1.7 x 10^{-3} M NAD ethanol limiting 0.50 M GuHCl	4.23 ± 0.04	5.82 ± 0.17	8
0.06 M tris, pH 8.8 1.6 x 10^{-2} M ethanol NAD limiting	7.58 ± 0.15	1.44 ± 0.10	12
0.06 M tris, pH 8.8 1.6 x 10^{-2} M ethanol NAD limiting	7.46 ± 0.30	1.46 ± 0.13	9
0.06 M tris, pH 8.8 1.6 x 10^{-2} M ethanol 0.50 M GuHCl NAD limiting	6.32 ± 0.43	4.85 ± 0.48	8

treated with 1 M, 2 M, and 3 M GuHCl at 0° C and assayed in the presence of the same concentration of GuHCl are shown in Figure 6. These data show the transition from reversible inhibition by 1 M GuHCl to rapid and totally irreversible denaturation by 3 M GuHCl. At the end of each experiment, activity was determined in the absence of GuHCl in order to indicate the reversibility of guanidine treatment. This procedure caused a 1 to 250 dilution of the GuHCl. The effect of 1 M GuHCl was found to be completely reversible after one hour (96 per cent recovery of activity). On the other hand, dilution after 2 M GuHCl treatment resulted in only 1 per cent restoration of activity after 75 minutes. Activity loss after 3 M GuHCl treatment was irreversible within a short time.

When a solution of LADH in 2 M GuHCl was examined by ultracentrifugation, two Schlieren peaks were observed (2.5 S and 5.0 S). See Figure 7. The untreated enzyme displayed only one peak (5.0 S). The appearance of two peaks in GuHCl denatured LADH suggests an irreversible interaction of LADH with GuHCl. Thus, a new structure seems to be formed in 2 M GuHCl which cannot return to its former state under the conditions of these experiments. Whether this new structure is the result of dissociation or the result of a large structural change without dissociation, can be determined by molecular weight measurements.

Figure 6

Per cent activity remaining in GuHCl treated LADH is plotted against time. LADH was reacted with 1 M, 2 M, and 3 M GuHCl at 0° C. The same concentration of GuHCl was present in the activity assay mixtures. At the end of each experiment, activity was measured without GuHCl present in the assay mixture.

○ LADH in 1 M GuHCl.

Δ LADH in 2 M GuHCl.

● LADH in 3 M GuHCl.

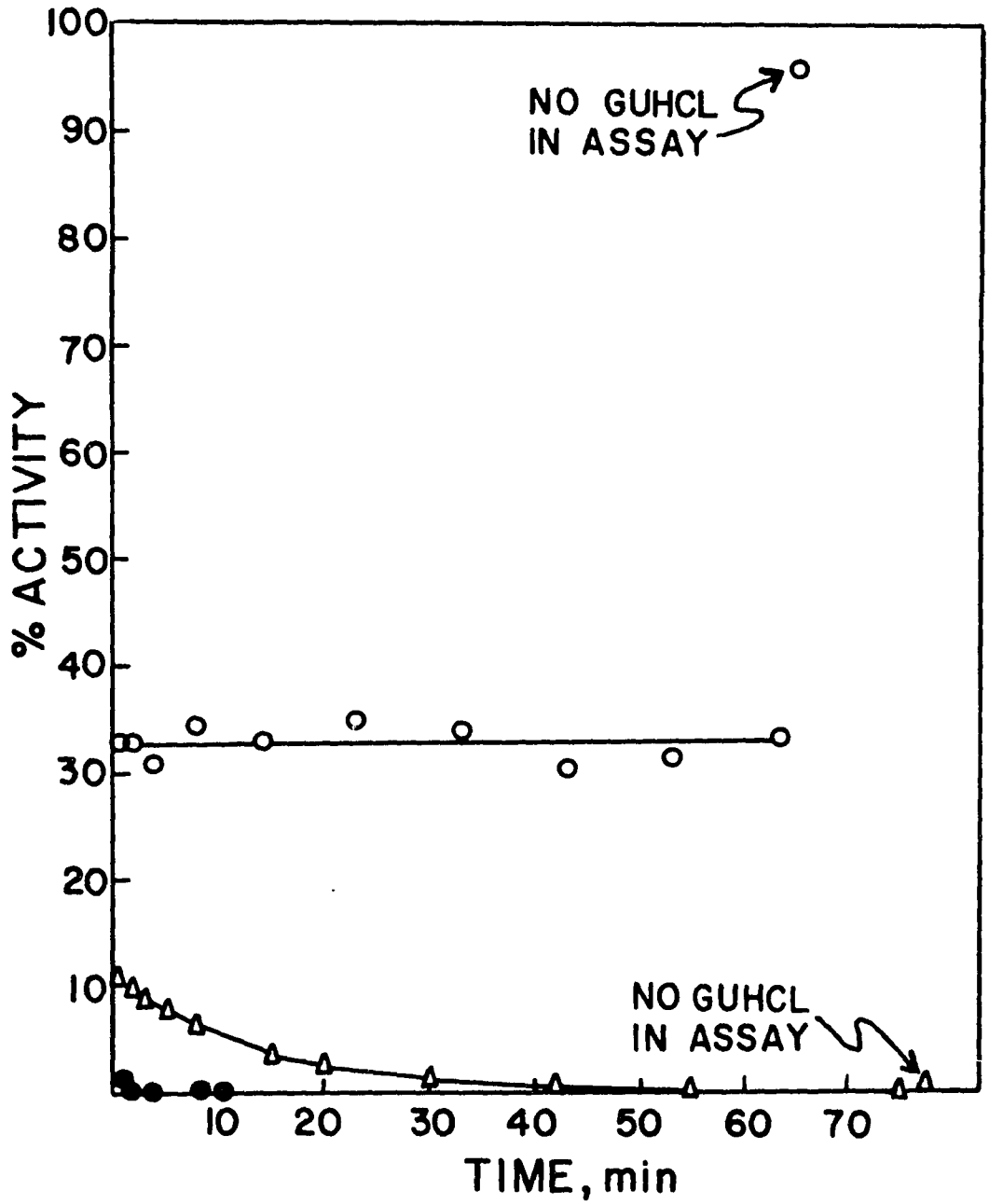
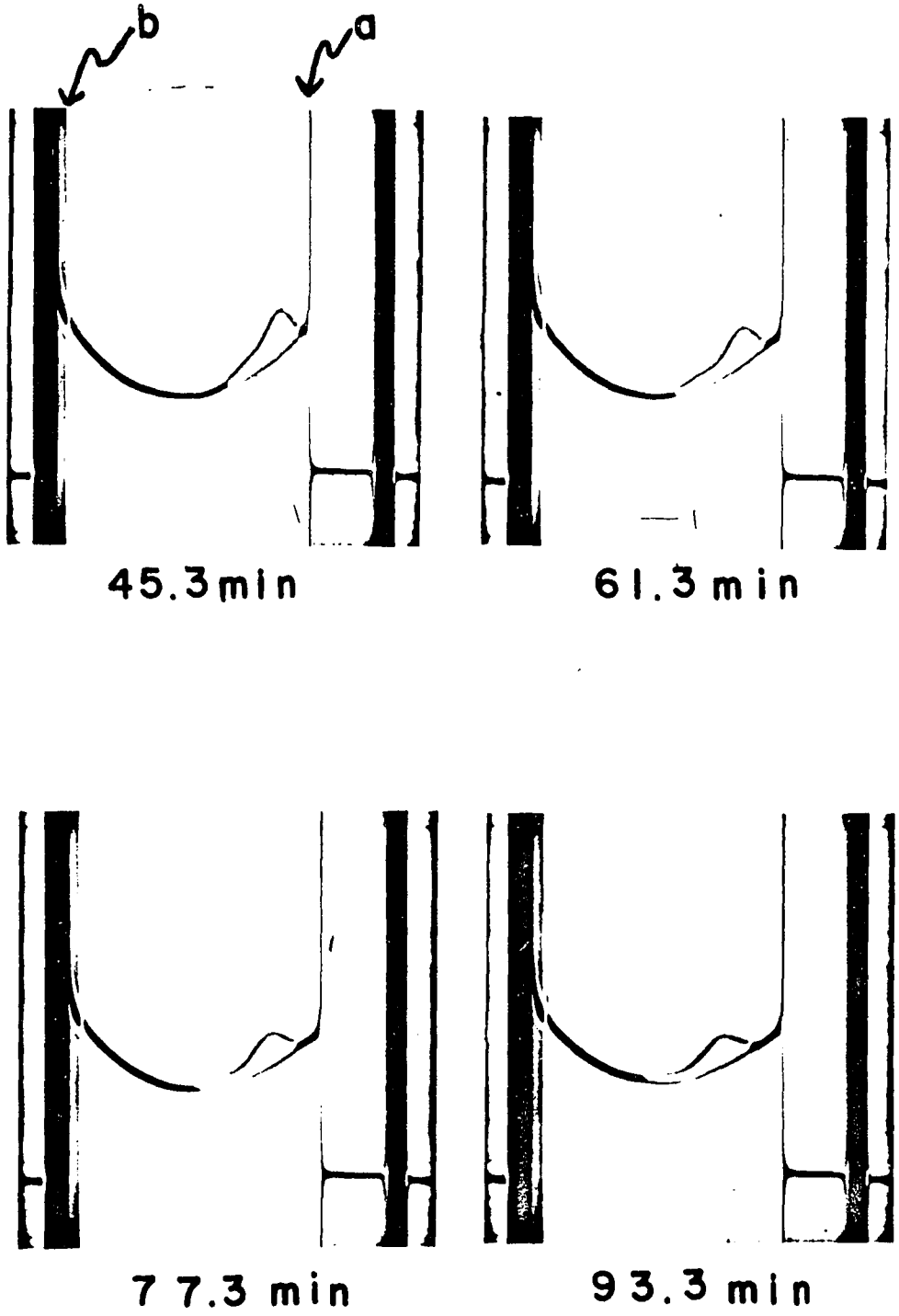

100
90
80
70
60
50
40
30
20
10
% ACTIVITY
NO GUHCL
IN ASSAY
NO GUHCL
IN ASSAY
10
20
30
40
50
60
70
TIME, min

Figure 7

Sedimentation velocity of LADH in 2 M GuHCl was observed by a Schlieren optical system. The solvent contained 0.05 M tris, 0.02 M thioglycolate, and 2.0 M GuHCl at pH 7.2. LADH dissolved in solvent was centrifuged at 59,780 rpm in a cell containing a double sector centerpiece (2.5°, 12 mm). Pictures were taken at 16 minute intervals with a bar angle of 60° C. Zero time was when top speed was reached. In this figure, a and b represent the meniscus and cell bottom.


b
a
45.3min
61.3 min
77.3 min
93.3 min

Apparent Partial Specific Volume of LADH in 5 M GuHCl

Apparent partial specific volumes ($\bar{v}$) of LADH at 20° C in tris buffer and in 5 M GuHCl are given in Table V. The values of $\bar{v}$ in tris buffer agree quite well with the $\bar{v}$ of 0.750 ml/gm obtained by Ehrenberg and Dalziel in phosphate buffer (Ehrenberg and Dalziel, 1958). The slight increase of $\bar{v}$ from 0.750 in tris buffer to 0.754 in 5 M GuHCl indicates little or no change of $\bar{v}$ when LADH is denatured in GuHCl solution. Other laboratories have found no change or a slight decrease in $\bar{v}$ for other proteins dissolved in GuHCl (Seery *et al.*, 1967; Kielley and Harrington, 1960; Reithel and Sakura, 1963; Woods *et al.*, 1963; Marler *et al.*, 1964). A $\bar{v}$ of 0.750 ml/gm was used for calculating the molecular weight and sedimentation coefficient of LADH in tris buffer and in 1 M GuHCl. For calculations when LADH was in 3 M or 5 M GuHCl, a $\bar{v}$ of 0.754 was used.

Sedimentation Coefficient of LADH and Chymotrypsinogen A

Table VI gives observed and corrected sedimentation coefficients after extrapolation of the reciprocal sedimentation coefficients to zero concentration to correct for nonideality. The slopes of the extrapolations, also listed in Table VI, are a measure of the nonideality of each system. Solutions containing mercaptoethanol gave significantly smaller slopes than solutions containing

TABLE V

Apparent Partial Specific Volume of LADH at 20° C

Solvent	Concentration (mg ml^{-1})	Pycnometer Volume (ml)	$\bar{v}$ (ml gm^{-1})
0.05 M tris, pH 7.5	9.85	5	0.750
0.05 M tris, pH 7.5	9.60	5	0.749
5 M GuHCl, pH 7.0 0.1 M EtSH	14.52	3	0.757
5 M GuHCl, pH 7.0 0.1 M EtSG	4.80	5	0.752

TABLE VI

Enzyme	Solvent	Sedimentation Coefficients $S^o_{20,obs}$ (S)*	$S^o_{20,w}$ (S)*	Slope of $1/S_{20,obs}$ (ml mg^{-1} S^{-1})	Points in Extra-polation
LADH	0.05 M tris, pH 7.5	4.93 ± 0.04	5.08 ± 0.04	0.00217	5
LADH	1 M GuHCl, pH 7.4 1.1×10^{-3} DTE	4.52 ± 0.03	5.24 ± 0.04	0.00355	4
LADH	3 M GuHCl, pH 7.2 0.1 M EtSH	1.47 ± 0.10	2.29 ± 0.15	0.00348	5
LADH	3 M GuHCl, pH 7.2 DTT, IA	1.43 ± 0.04	2.25 ± 0.06	0.00458	8
LADH	5 M GuHCl, pH 7.0 0.1 M EtSH	0.87 ± 0.09	1.98 ± 0.21	0.00607	4
LADH	5 M GuHCl, pH 7.0 DTT, IA	0.94 ± 0.02	2.15 ± 0.04	0.00813	8
CTogen	5 M GuHCl, pH 7.0 0.1 M EtSH	0.87 ± 0.06	1.83 ± 0.12	0.00658	6
CTogen	5 M GuHCl, pH 7.0 DTT, IA	0.87 ± 0.02	1.87 ± 0.05	0.00864	8

* One Svedberg unit (S) = 10^{-13} seconds.

dithiothreitol, dithioerythritol, or no reducing agent. Since nonideality is minimized by the presence of mercaptoethanol, this reducing agent is preferred in experiments where calculations require an extrapolation to zero concentration.

For the purpose of comparing the results obtained for LADH with a well characterized protein, the sedimentation coefficient was determined for chymotrypsinogen A in 5 M GuHCl solution containing reducing agent. Subsequent determinations of the molecular weight and intrinsic viscosity of LADH were also compared to measurements of these parameters for chymotrypsinogen A.

Diffusion Coefficient of LADH

The diffusion coefficient of LADH in tris buffer was precise enough to extrapolate to zero concentration. See Table VII. When diffusion experiments were done in GuHCl, however, the forming of symmetrical and undisturbed synthetic boundaries became increasingly difficult as the GuHCl concentration increased. This difficulty has been observed by others (Creeth, 1967). Consequently the precision was poor, and extrapolation to zero concentration was not justified. Diffusion coefficients of LADH in GuHCl represent average values for the concentration range of 4 to 8 mg/ml.

TABLE VII

Diffusion Coefficients of LADH

Solvent	$D_{20,obs}$** (F)*	$D_{20,w}$** (F)*	Number of Points
0.05 M tris, pH 7.5	6.11 ± 0.12	6.23 ± 0.12	4
1 M GuHCl, pH 7.4 1.1 x 10^{-3} M DTE	5.84	6.16	2
3 M GuHCl, pH 7.2 0.1 M EtSH	3.22 ± 0.20	3.80 ± 0.23	11
5 M GuHCl, pH 7.0 0.1 M EtSH	2.57 ± 0.12	3.67 ± 0.16	4

*One Fick unit (F) = 10^{-7} cm^2 sec^{-1}

**The diffusion coefficients in tris buffer were extrapolated to zero concentration. In GuHCl solutions the diffusion coefficients were averaged for the concentration range of 4 to 8 mg/ml.

Molecular Weight of LADH from Sedimentation and Diffusion Coefficients

Table VIII gives molecular weight calculations based on the Svedberg equation.

$$\text{Molecular Weight} = \frac{S \quad RT}{D\ (1 - \bar{v}\rho)}$$

S = sedimentation coefficient

D = diffusion coefficient

$\bar{v}$ = apparent partial specific volume

ρ = solvent density

R = gas constant

T = absolute temperature

The sedimentation and diffusion coefficients used in these calculations are average values from Tables VI and VII. The apparent slight increase of molecular weight for LADH in 1 M GuHCl may be due to a small amount of aggregation since LADH is somewhat unstable in this environment. However, the error analysis indicates that the difference is not significant. Even though the diffusion coefficient is unreliable in 3 M and 5 M GuHCl (Creeth, 1967), the molecular weight of LADH in these solvents does appear to be significantly decreased. These results suggest that LADH dissociates into subunits in concentrated GuHCl.

TABLE VIII

Molecular Weight of LADH from Sedimentation and Diffusion Coefficients

Solvent	$S^{o}_{20,w}$	$D_{20,w}$	$\bar{v}$ (ml gm^{-1})	Molecular Weight
0.05 M tris, pH 7.5	5.08 ± 0.04	6.23 ± 0.12	0.750	79100 ± 2100
1 M GuHCl, pH 7.4 1.1×10^{-3} M DTE	5.24 ± 0.04	6.16	0.750	82500
3 M GuHCl, pH 7.2 0.1 M EtSH	2.29 ± 0.15	3.80 ± 0.23	0.754	59400 ± 7500
5 M GuHCl, pH 7.0 0.1 M EtSH	1.98 ± 0.21	3.67 ± 0.16	0.754	53200 ± 7900

Molecular Weights of LADH and Chymotrypsinogen A by Sedimentation Equilibrium

Table IX gives Z average molecular weights after extrapolation of reciprocal square roots of Z average molecular weight to zero concentration. In order to demonstrate the absence of charge effects which may be present in dilute buffer, some LADH solutions were dialyzed against 0.2 M NaCl in tris buffer. When reducing agent was not present in 3 M GuHCl solutions, with and without EDTA, nonlinear plots were obtained. See Figures 8 and 9. This nonlinearity indicates reversible association-dissociation. In these experiments, only the points near the meniscus of the solution column were used for extrapolation to zero concentration. Typical plots of reciprocal square root of Z average molecular weight *versus* concentration in GuHCl containing reducing agent, with and without alkylation, are given in Figures 10, 11, and 12. The second virial coefficient was calculated from the slope of the extrapolation by the following equation (Marler *et al.*, 1964).

$$M_{app}^{-1/2} = M^{o\ -1/2} + 2BM^{o\ 1/2}c$$

M_{app} = apparent molecular weight

M^o = molecular weight at zero concentration

B = second virial coefficient

TABLE IX

Z Average Molecular Weight

Enzyme	Solvent	Molecular Weight	$B \times 10^5$ (ml mole gm^{-2})	No. of Points
LADH	0.05 M tris, pH 7.5	78500 ± 1100	0.03	13
LADH	0.05 M tris, pH 7.5, 0.2 M NaCl	77800 ± 600	0.48	10
LADH	0.05 M tris, pH 7.5, 0.2 M NaCl	77900 ± 1100	1.23	13
LADH	3 M GuHCl, pH 7.2	68100 ± 1800	-58.1	10
LADH	3 M GuHCl, pH 7.2	61100 ± 1900	-65.3	10
LADH	3 M GuHCl, pH 7.2, 1.9×10^{-3} M EDTA	61100 ± 1300	-63.8	13
LADH	3 M GuHCl, pH 7.2, 1.9×10^{-3} M EDTA	69500 ± 2200	-39.9	13
LADH	3 M GuHCl, pH 7.2, DTT, IA	56400 ± 1100	44.2	16
LADH	3 M GuHCl, pH 7.2, DTT, IA	54400 ± 700	40.4	17
LADH	3 M GuHCl, pH 7.2, DTT, IA	52600 ± 600	34.4	18

TABLE IX (Cont'd.)

Enzyme	Solvent	Molecular Weight	B x 10^5 (ml mole gm^{-2})	No. of Points
LADH	5 M GuHCl, pH 7.0, DTT, IA	50500 ± 1000	54.8	15
LADH	5 M GuHCl, pH 7.0, DTT, IA	47700 ± 1200	51.3	14
LADH	5 M GuHCl, pH 7.0, 0.1 M EtSH	45700 ± 700	24.0	13
LADH	5 M GuHCl, pH 7.0, 0.1 M EtSH	45700 ± 600	25.6	20
CTogen	5 M GuHCl, pH 7.0, DTT, IA	23900 ± 300	56.7	15
CTogen	5 M GuHCl, pH 7.0, DTT, IA	23600 ± 400	48.2	11

Figure 8

Reciprocal square root of Z average molecular weight is plotted against LADH concentration. Enzyme solution was dialyzed against 3 M GuHCl for 48 hours. Four mm columns of enzyme solution and dialysate were put in a double sector cell (2.5°, 12 mm) and centrifuged at 8225 rpm. When equilibrium was reached, pictures were taken of the Schlieren optical system with a bar angle of 54°.

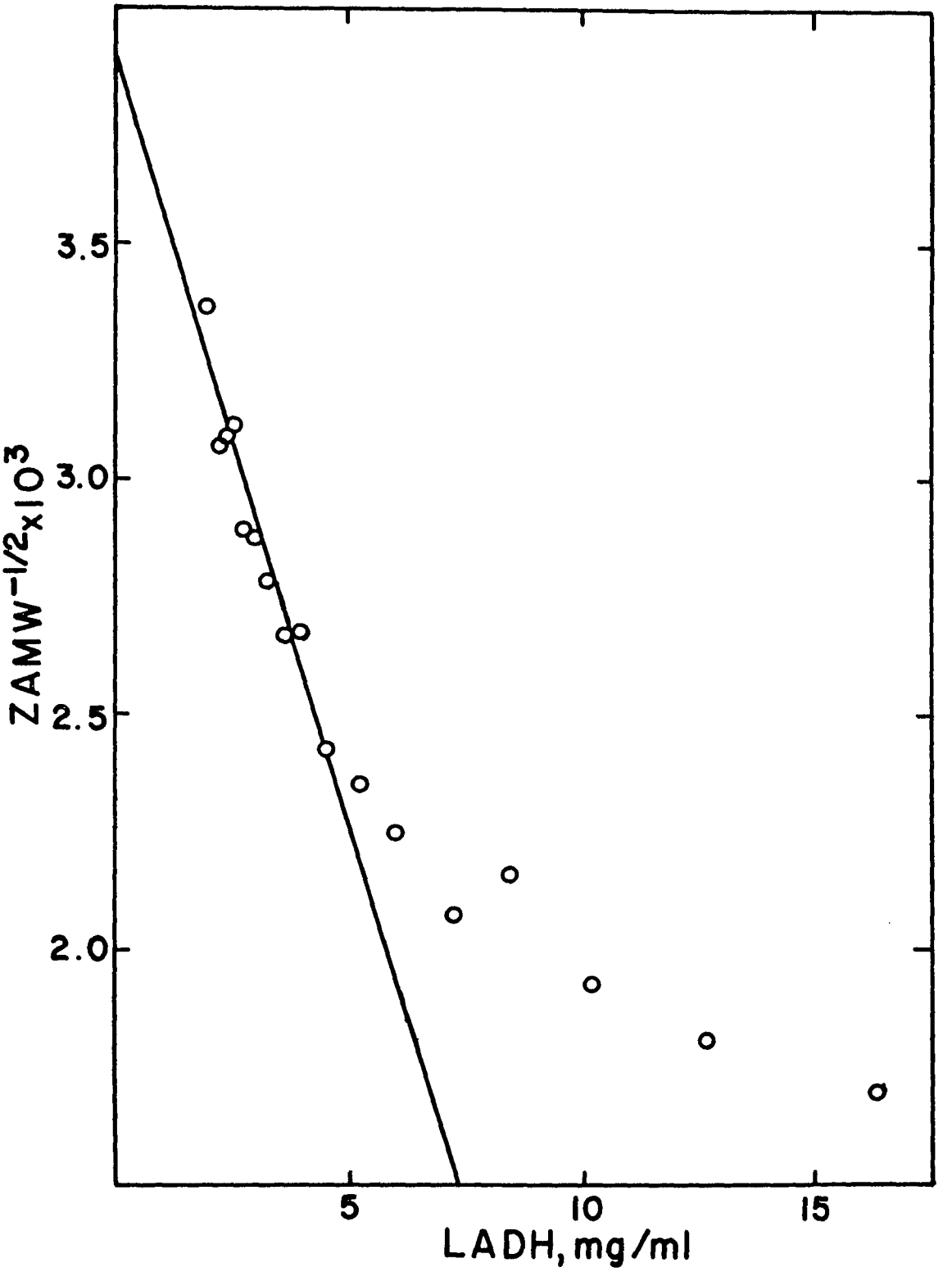

3.5
3.0
2.5
2.0
ZAMW$^{-1/2}$x10^3
5
10
15
LADH, mg/ml

Figure 9

Reciprocal square root of Z average molecular weight is plotted against LADH concentration. The enzyme solution was dialyzed against 3 M GuHCl containing 1.9×10^{-3} M EDTA for 48 hours. Four mm columns of enzyme solution and dialysate were put in a double sector cell (2.5°, 12 mm) and centrifuged at 11,272 rpm. At equilibrium, pictures were taken of the Schlieren optical system with a bar angle of 65°.

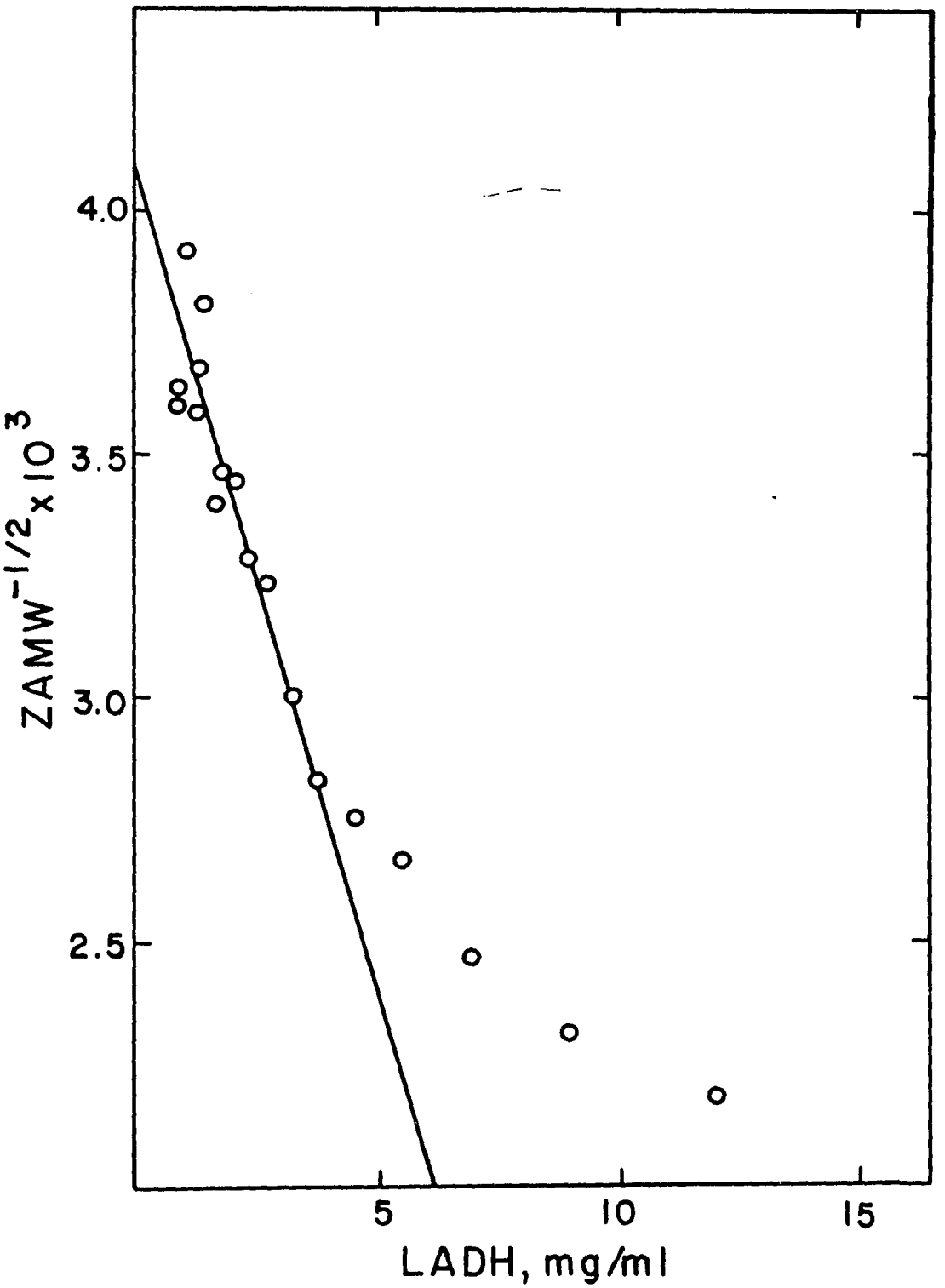

4.0
3.5
3.0
2.5
ZAMW$^{-1/2}$ x 10^3
5
10
15
LADH, mg/ml

Figure 10

Reciprocal square root of Z average molecular weight is plotted against LADH concentration. Enzyme in 3 M GuHCl solution was treated with DTT and excess iodoacetate and then dialyzed against 3 M GuHCl for 48 hours. Four mm columns of enzyme solution and dialysate were put in a double sector cell (2.5°, 12 mm) and centrifuged at 17,250 rpm. At equilibrium, pictures were taken of the Schlieren optical system with a bar angle of 55°.

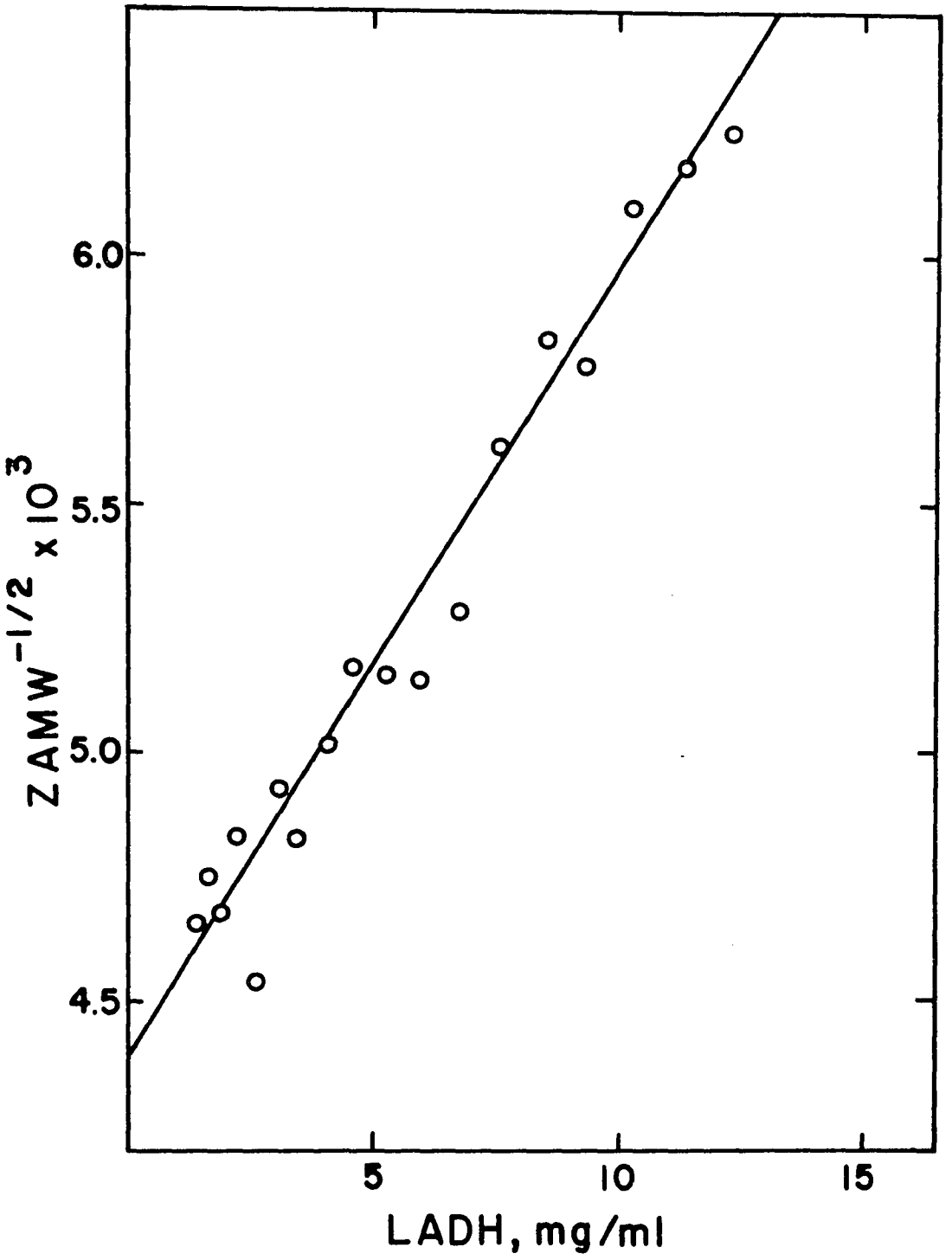

6.0
5.5
5.0
4.5
ZAMW$^{-1/2}$ x 10^3
5
10
15
LADH, mg/ml

Figure 11

Reciprocal square root of Z average molecular weight is plotted against LADH concentration. Enzyme in 5 M GuHCl solution was treated with DTT and excess iodoacetate and then dialyzed against 5 M GuHCl for 48 hours. Four mm columns of enzyme solution and dialysate were put in a double sector cell (2.5°, 12 mm) and centrifuged at 19,160 rpm. At equilibrium, pictures were taken of the Schlieren optical system with a bar angle of 42°.

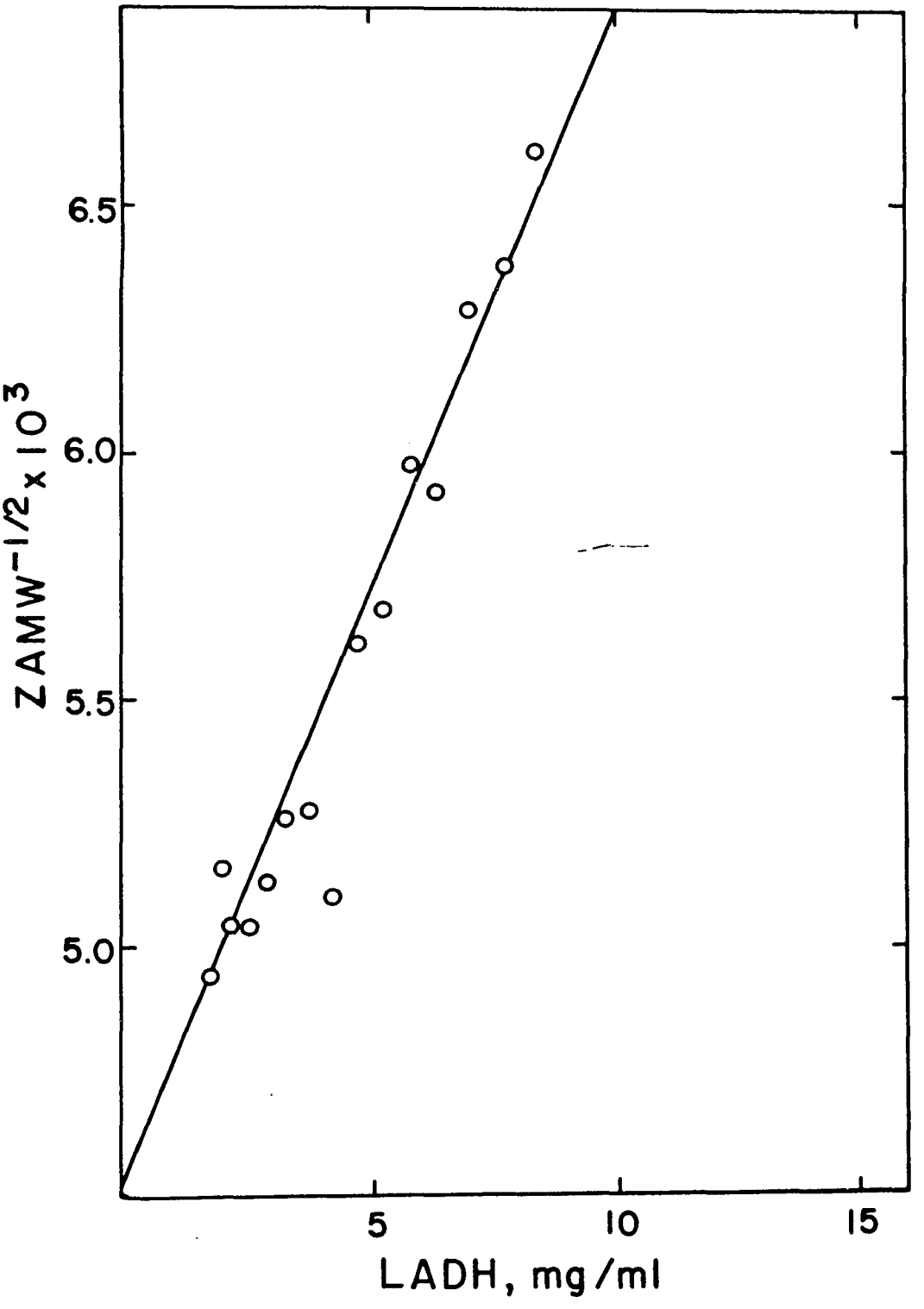
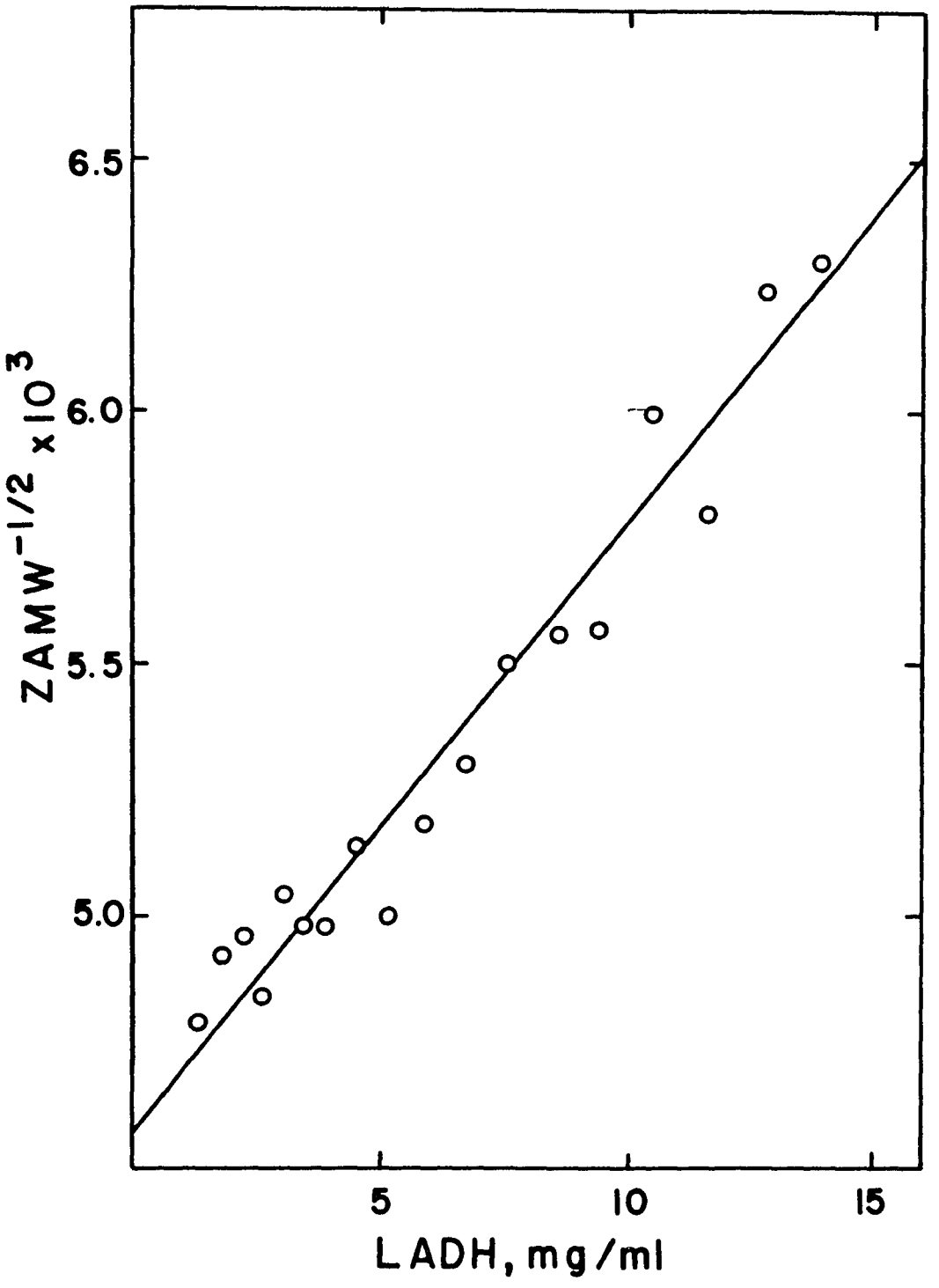

6.5
6.0
5.5
5.0
ZAMW$^{-1/2}$ x 10^3
5
10
15
LADH, mg/ml

Figure 12

Reciprocal square root of Z average molecular weight is plotted against LADH concentration. Enzyme in 5 M GuHCl was dialyzed against 5 M GuHCl containing 0.1 M mercaptoethanol for 48 hours. Four mm columns of enzyme solution and dialysate were put in a double sector cell (2.5°, 12 mm) and centrifuged at 19,160 rpm. At equilibrium, pictures were taken of the Schlieren optical system with a bar angle of 45°.


6.5
6.0
5.5
5.0
ZAMW$^{-1/2}$ x 10^3
5
10
15
LADH, mg/ml

c = concentration

In 5 M GuHCl with mercaptoethanol as the reducing agent, the second virial coefficient was about half the value obtained from solutions treated with dithiothreitol and iodoacetate. As in the case of the sedimentation velocity experiments, mercaptoethanol is the preferred reducing agent when the calculations require an extrapolation to zero concentration to correct for nonideality.

Intrinsic Viscosity of LADH and Chymotrypsinogen A

Intrinsic viscosities and Huggins constants are listed in Table X. The Huggins constant is somewhat inaccurate since it includes the error of the slope plus twice the error of the intrinsic viscosity. The Huggins constant for LADH in 5 M GuHCl is 1.03, an average of three values. This value is higher than those calculated for most other proteins (Tanford, 1967). For a random coil, the expected value of the Huggins constant is 0.35 and 0.40 (Huggins, 1942; Flory, 1953). The high Huggins constant calculated for LADH suggests that LADH is not completely a random coil in 5 M GuHCl.

Zinc Content of LADH in GuHCl Solutions

After LADH was dissolved in 1 M, 2 M, or 3 M GuHCl and dialyzed against three changes of GuHCl dialysate (volume

TABLE X

Intrinsic Viscosity of LADH and CTogen

Enzyme	Solvent	Intrinsic Viscosity (ml gm^{-1})	Huggins Constant	No. of Points
LADH	3 M GuHCl, pH 7.2, DTT, IA	26.5 ± 0.5	-	3
LADH	3 M GuHCl, pH 7.2, DTT, IA	17.4 ± 0.8	-	4
LADH	3 M GuHCl, pH 7.2, 0.1 M EtSH	26.4 ± 0.4	-	3
LADH	3 M GuHCl, pH 7.2, 8 x 10^{-3} M DTT	17.0 ± 0.1	-	3
LADH	5 M GuHCl, pH 7.0, DTT, IA	30.8 ± 0.4	0.99	4
LADH	5 M GuHCl, pH 7.0, 0.1 M EtSH	28.8	-	2
LADH	5 M GuHCl, pH 7.0, 0.1 M EtSH	26.2 ± 1.0	1.24	4
LADH	5 M GuHCl, pH 7.0, 0.1 M EtSH	25.7 ± 0.4	0.87	4
CTogen	5 M GuHCl, pH 7.0, DTT, IA	23.1 ± 0.2	0.32	4
CTogen	5 M GuHCl, pH 7.0, 0.1 M EtSH	22.0 ± 0.3	0.25	4

TABLE X (Cont'd.)

Enzyme	Solvent	Intrinsic Viscosity (ml gm^{-1})	Huggins Constant	No. of Points
CTogen	6 M GuHCl pH 6.9 0.1 M EtSH	25.9 ± 0.3	0.40	4
CTogen	6 M GuHCl pH 6.9 0.1 M EtSH	26.3 ± 0.6	0.36	4

ratio of dialysate to enzyme solution was 250), zinc analyses presented in Table XI were obtained. Dialysis against 1 M GuHCl caused a loss of only 11 per cent of the zinc from LADH. However, all zinc was removed when exhaustive dialysis was carried out in 2 M or 3 M GuHCl.

The effect of EDTA on zinc removal from LADH in 1 M GuHCl was measured by sedimentation experiments, followed by zinc analysis of the supernatant and the bottom layer after all the protein had sedimented to the bottom of the cell. See Table XII. Under the conditions of these experiments, about 65 per cent of the zinc was removed. Eventually aggregation of LADH was observed, even in the presence of reducing agent. Since DTT and DTE are quite reactive and consequently unstable (Cleland, 1964), their effectiveness decreases as a function of time. Calculated sedimentation coefficients indicate that no subunit was formed during these experiments.

When LADH in 3 M GuHCl was dialyzed to equilibrium against GuHCl dialysate (volume ratio of dialysate to enzyme solution was 8), 80 per cent of the zinc remained bound to the enzyme. An analogous experiment with the addition of EDTA resulted in complete zinc removal from LADH. See Table XIII. These results indicate that zinc is weakly bound to LADH in 3 M GuHCl and that the addition of EDTA or exhaustive dialysis removes all zinc. Similar data were obtained by Drum and coworkers for LADH in 8 M

TABLE XI

Zinc Removal from LADH By Exhaustive Dialysis

Solvent	% Zinc Bound
0.05 M tris, pH 7.5	100
1 M GuHCl, pH 7.4	89
2 M GuHCl, pH 7.3	0
3 M GuHCl, pH 7.2	0

TABLE XII

Zinc Removal from LADH in 1 M GuHCl Containing EDTA

	Time (Hr)	% Zinc Bound	$S_{20,w}$
Experiment A*	0	100	-
	2	80	4.68
	21	48	4.67
	49	37	4.81, 14.4, 37.9
Experiment B**	0	100	-
	2	70	4.65
	20	48	4.70
	49	34	4.71, 46.3

*Solution composition: LADH, 3.85 mg/ml; enzyme zinc, 2.02×10^{-4} M; EDTA, 2.49×10^{-3} M; DTE, 1.08×10^{-2} M; tris, 0.05 M, pH 7.4; GuHCl, 1.0 M.

**Solution composition: LADH, 4.51 mg/ml; enzyme zinc, 2.20×10^{-4} M; EDTA, 4.98×10^{-3} M; DTT, 2.16×10^{-2} M; tris, 0.05 M, pH 7.4; GuHCl, 1.0 M.

TABLE XIII

Zinc Removal from LADH By Equilibrium Dialysis

Solvent	% Zinc Bound
0.05 M tris, pH 7.5	100
3 M GuHCl, pH 7.2	80
3 M GuHCl, pH 7.2, 1.9×10^{-3} M EDTA	0

urea (Drum *et al*., 1967).

DISCUSSION

Though the kinetics of LADH have been thoroughly studied (Theorell, 1967), relatively little is known about LADH structure. The number of subunits in LADH is in dispute, and only preliminary investigations have been made concerning the role of zinc in structure and catalysis. The object of the present work was to determine the number of subunits in LADH and to further elucidate the function of zinc in the enzyme.

The LADH prepared by Boehringer exhibited a high degree of homogeneity as shown by chromatography and starch gel electrophoresis. Sephadex G-100 chromatography revealed about 3 per cent of low molecular weight material. See Figure 1. Carboxymethyl cellulose chromatography indicated a minor component (7 per cent) which had the same specific activity as the major component. See Figure 2. Starch gel electrophoresis (Figure 3) confirmed the presence of minor components presumed to be isozymes. Since the LADH prepared by Boehringer appeared to be well above 90 per cent homogeneous by all criteria, it was used without further purification.

The molecular weight of Boehringer LADH was determined by equilibrium sedimentation in tris buffer and in tris buffer containing 0.2 M NaCl. See Table IX. Since no significant change was observed when NaCl was

present, charge effects on measurements of LADH in dilute neutral tris buffer appear to be negligible. Averaging the results for the two conditions gives a Z average molecular weight of 78,200 for LADH in dilute neutral buffer. This value is in good agreement with Hamburg who found 78,700 and with Vallee and coworkers who found 80,000 (Hamburg, 1966; Drum *et al.*, 1967).

Figure 6 shows that the effect of 1 M GuHCl on the kinetics of LADH is reversible. When LADH was assayed for activity in the presence of 1 M GuHCl, its activity was 33 per cent of activity measured when no GuHCl was present in the assay mixture. After being treated with 1 M GuHCl at 0° C for one hour, enzymatic activity of LADH was measured in the absence of GuHCl. This condition represents a 1 to 250 dilution of the GuHCl. Ninety-six per cent of the enzymatic activity was recovered indicating complete reversibility of the effect of 1 M GuHCl. Lineweaver-Burk plots (Figure 5) indicate that guanidinium ion competitively inhibits LADH activity by interfering with NAD binding. With limiting ethanol, the inhibitory effect of GuHCl is mixed (Figure 4). Such results suggest that the primary kinetic event is the addition of NAD or NADH to the enzyme and that the rate limiting step may be the dissociation of the cofactor-enzyme complex. Similar conclusions have been made by others (Theorell and Chance, 1951; Theorell and

Bonnichsen, 1951; Dalziel, 1962).

Addition of GuHCl to the 2 M level resulted in irreversible denaturation. After 2 M GuHCl treatment, enzymatic activity was not recovered when LADH was assayed in the absence of GuHCl (Figure 6). Although the GuHCl was diluted 1 to 250, recovery of activity was only 1 per cent. The two components observed by sedimentation velocity (Figure 7) also suggest irreversibility. If the system were reversible, the Schlieren pictures would be expected to show only one peak. When compared to LADH in tris buffer, this peak should have a lower sedimentation coefficient in the case of reversible denaturation or dissociation and a higher sedimentation coefficient in the case of aggregation. Although reversibility was not observed in these experiments, some reversibility should be possible if suitable conditions were found. One laboratory has reported reversibility of activity after 8 M urea denaturation of LADH when reducing agent and excess zinc were present (Drum et al., 1967).

A molecular weight calculation from sedimentation and diffusion coefficients gave a value of 82,500 for LADH in 1 M GuHCl. See Table VIII. Although this result is slightly higher than the molecular weight of 79,100 found for LADH in tris buffer by the same method, the error analysis shown in Table VIII shows that both results are within experimental error. The apparent small increase

of molecular weight in 1 M GuHCl is not surprising, since LADH is unstable in this environment and tends to aggregate. Clearly, LADH does not dissociate in 1 M GuHCl, though there appears to be some loosening of the structure which results in instability and leads to aggregation. The formation of intermolecular disulfide bonds may be a factor in LADH aggregation.

The effect of EDTA on LADH in 1 M GuHCl is shown in Table XII. About 65 per cent of the zinc in LADH was removed in 48 hours. At that time, some aggregation was observed which may or may not be due to zinc removal. Sedimentation velocity calculations indicate that no subunits were formed at any time during these experiments. The sedimentation coefficient ($S_{20,w}$) did not drop below 4.7, the value for native LADH. Since exhaustive dialysis of LADH in tris buffer, pH 7.5, against EDTA solution causes no zinc removal (Akeson, 1964), the loss of zinc from LADH in 1 M GuHCl when EDTA is present again suggests that the enzyme structure is loosened in this environment. Zinc does not seem to play an important role in subunit association, because the removal of zinc in 1 M GuHCl, a structure-loosening environment, does not result in dissociation.

Sedimentation equilibrium studies of LADH in 3 M GuHCl, with and without EDTA, revealed information about subunit association. The curvature of molecular weight

data shown in Figures 8 and 9 suggests a reversible association-dissociation system (Adams, 1967). Irreversible aggregation, a possible alternative hypothesis, does not explain the large negative slope near the meniscus. If aggregation of subunit were the only process occurring in 3 M GuHCl, the molecular weight data should be concave down rather than concave up as shown in Figures 8 and 9. The best explanation for this data is a multiple equilibrium between subunit, dimer, and trimer with the possibility of some irreversible aggregation at the bottom of the cell. The extrapolated molecular weight values for LADH in 3 M GuHCl, with and without the presence of EDTA, are shown in Table IX. These values (61,000 to 69,000) are somewhat artificial since points near the meniscus were arbitrarily chosen for the extrapolation. Curvature near the meniscus would cause the molecular weight at zero concentration to be lower than the extrapolated values and would result in a molecular weight approaching that of the subunit. Figures 8 and 9 indicate that the presence or absence of EDTA has no effect on the molecular weight distribution of LADH in 3 M GuHCl. Table XIII shows that the addition of EDTA to a solution of LADH in 3 M GuHCl causes all bound zinc to be removed. Since zinc removal had no effect on the molecular weight distribution, zinc appears to have little or no role in subunit association. This conclusion is at variance with

the results of Vallee and coworkers who found that the molecular weight of LADH in 8 M urea, when treated with EDTA or mercaptoethanol, decreased from 40,000 to 20,000 (Drum et al., 1967).

Quite different sedimentation equilibrium data were obtained for alkylated LADH in 3 M GuHCl. The straight line relationship with a large positive slope shown in Figure 10 is typical for a homogeneous protein subunit in concentrated GuHCl. The molecular weight of alkylated LADH in 3 M GuHCl is 54,500, an average of three determinations. See Table IX. Apparently alkylation destroyed the reversible system observed previously and resulted in the presence of subunit and perhaps some dimer. The large difference in sedimentation equilibrium behavior of LADH before and after alkylation indicates that sulfhydryl groups have a role in subunit association. This role may include hydrogen bonding between sulfhydryl groups and other residues or ionic sulfur bonding to regions of positive charge in adjacent peptide chains. The presence of mercaptoethanol or the alkylation of cysteine residues would destroy such associations.

The data in Table IX indicate a slight decrease in molecular weight for alkylated LADH when the environment was changed from 3 M to 5 M GuHCl. This decrease in molecular weight from 54,500 in 3 M GuHCl to 49,100 in 5 M GuHCl suggests that some additional dissociation has

occurred. The best molecular weight results were obtained when LADH was dissolved in 5 M GuHCl containing mercaptoethanol. The average value for the Z average molecular weight of LADH in 5 M GuHCl containing 0.1 M mercaptoethanol is 45,700. This value is in reasonable agreement with the result of Hamburg who reported a molecular weight of 42,000 in 7 M urea containing reducing agent. He assumed a $\bar{v}$ of 0.735 (Hamburg, 1966). Castillino and Barker have reported a molecular weight of 41,000 for LADH dissolved in 6 M GuHCl containing reducing agent. They assumed that $\bar{v}$ is 0.74 (Castillino and Barker, 1968). If a $\bar{v}$ of 0.735 is assumed for LADH in 5 M GuHCl containing 0.1 M mercaptoethanol, the molecular weight calculated from data of this thesis would be lowered to 40,200. The fact that the molecular weight, calculated with the measured $\bar{v}$ of 0.754, was about 10 per cent higher than expected may be due to incomplete dissociation, to solvent binding to the enzyme, or to an error in the measurement of $\bar{v}$. It is tempting to question $\bar{v}$, since its value measured in 5 M GuHCl is slightly higher than the value observed in dilute buffer solution. See Table IV. Other laboratories have reported no change or a slight decrease of $\bar{v}$ when proteins were dissolved in concentrated GuHCl (Kielley and Harrington, 1960; Woods _et al_., 1963; Reithel and Sakura, 1963; Marler _et al_., 1964; Seery _et al_., 1967). On the other hand, binding of solvent can account for 5

to 10 per cent of the molecular weight according to the results obtained when myosin was dissolved in 5 M GuHCl (Kielley and Harrington, 1960).

The molecular weight of LADH decreases approximately 10 per cent when the GuHCl concentration is increased from 3 M to 5 M. Such a small decrease when the denaturing agent was nearly doubled implies that further increase in denaturant will not significantly change the molecular weight of LADH. Consequently, these data indicate that there are no more than two subunits in LADH. This conclusion does not agree with the results of other workers who have reported that LADH is composed of four subunits (Drum *et al.*, 1967; Cheng *et al.*, 1968).

The constants of Tanford's equations for sedimentation velocity and viscosity were determined empirically in 6 M GuHCl and 0.1 M mercaptoethanol at 25° C by measuring the sedimentation coefficient and intrinsic viscosity of twelve proteins (Tanford *et al.*, 1967). These equations are given below.

$$S^o = (1 - \bar{v}\rho)\ 0.286\ N^{0.473}$$

$$[\eta] = 0.716\ N^{0.66}$$

S^o = sedimentation coefficient at zero concentration

ρ = solvent density

$\bar{v}$ = apparent partial specific volume

N = number of residues per chain

$[\eta]$ = intrinsic viscosity

The average sedimentation data from Table VI for LADH and chymotrypsinogen A in 5 M GuHCl were corrected to 6 M GuHCl containing 0.1 M mercaptoethanol at 25° C by the method outlined by Schachman (Schachman, 1957). The calculated number of residues per chain is compared with the expected chain lengths in Table XIV. Both LADH and chymotrypsinogen A appear to be longer than the theoretical chain lengths. Table XV shows calculations of the number of residues per chain from the average intrinsic viscosity data obtained for the two proteins in 5 M GuHCl (Table X). These results indicate chain lengths less than expected for LADH and chymotrypsinogen A. In both sedimentation velocity results and viscosity results, the difference between expected and calculated number of residues per chain for LADH and chymotrypsinogen A may be attributed to a small conformational change when the solvent containing 0.1 M mercaptoethanol, is changed from 5 M to 6 M GuHCl. Table X shows that the intrinsic viscosity of chymotrypsinogen A increases from 22.0 to 26.1 for this solvent change reflecting a difference in protein conformation.

TABLE XIV

Number of Residues per Chain from Sedimentation Coefficient

Protein	$S^{o}_{25,6M}$ Calculated	$S^{o}_{25,6M}$ Observed*	Chain Length Calculated	Chain Length Theoretical
LADH	0.794	-	465	368**
CTogen	0.779	0.65	290	245

* Tanford *et al.*, 1967

** Calculated from the amino acid analysis of Hamburg (Hamburg, 1966), assuming a molecular weight of 39,000.

TABLE XV

Number of Residues per Chain from Intrinsic Viscosity

Protein	Intrinsic Viscosity in 5 M GuHCl (ml gm^{-1})	Intrinsic* Viscosity in 6 M GuHCl (ml gm^{-1})	Chain Length Calculated	Chain Length Theoretical
LADH	27.9	-	257	368**
CTogen	22.5	26.8	186	245

* Tanford *et al.*, 1967

** Calculated from the amino acid analysis of Hamburg (Hamburg, 1966), assuming a molecular weight of 39,000.

The latter value agrees with the result obtained by Tanford and coworkers for chymotrypsinogen A in 6 M GuHCl containing 0.1 M mercaptoethanol (Tanford, 1967). If the calculated number of residues per chain for LADH are corrected for the apparent conformational change due to different GuHCl concentrations, the molecular weight of LADH in 5 M GuHCl would be about 40,000. From sedimentation equilibrium, sedimentation velocity, and viscosity data, the molecular weight of LADH in 5 M GuHCl is between 40,000 and 45,000. Thus from three criteria, LADH has a structure composed of two subunits.

APPENDIX

FORTRAN Programs for Calculations

Programs 1 and 2 are for use on an IBM 360 computer. The other programs are for use on a Digital Equipment Company PDP-8/S computer.

1. Sedimentation Coefficient - The first data card should have only one number which corresponds to the number of groups of data. For example, if five sedimentation velocity runs were made, then the first data card should have the number 05. The second data card should have the ten numbers indicated in statement #12 of the program with the format of statement #13, where CONC is the initial concentration, EN is the number of photographic frames read, RO is the distance from the center of rotation to the first reference hole (approximately 5.7 cm), REFH is the distance between the left and right reference holes in the counterweight (approximately 1.6 cm), RPM is the revolutions per minute of the rotor, VBAR is the apparent partial specific volume, VISS is the relative viscosity of the solvent to water, DENS is the relative density of the solvent to water, VISW is the ratio of viscosity of water at the temperature of the run to the viscosity of water at 20° C., DENW is the density of water at the temperature of the run. Following the second data card there should be N cards each containing the four numbers indicated in statement #15 of the

program with the format of statement #16, where T is the time in minutes of each set of variables after the rotor has reached the rpm of the run, ELF is the left reference line, XM is the observed position in the x direction of the Schlieren maximum, and RT is the right reference line. The last data card of each group should have the three numbers indicated in statement #36 of the program with the format of statement #37, where EL is the left reference line, XONE is the meniscus, and R is the right reference line. It would be best to measure these numbers on the first and last photographic frames, then calculate XONE minus EL of both frames to see if the meniscus did not change. Average the R's, XONE's, and EL's of the first and last frames for the data of this card. If a synthetic boundary cell was used, use the left reference line, the Schlieren maximum, and the right reference line of the first frame for the three numbers of this data card. Then put in the data cards of the second group of data with one card like statement #12, N cards like statement #15, and one card like statement #36, and so forth until all groups of data are inserted.

Readout will be S observed, 1/S observed, S_{20w}, $1/S_{20w}$, the delta y's (residuals) of each set of data for the least squares straight line plot of log radius versus time, the standard deviation of the residuals, the standard deviation of S_{20w}, the radius of the meniscus, the radius of the Schlieren maximum in the

first frame, and the average concentration during the run (the concentration of the plateau region at the half time of the run).

```
              C      SEDIMENTATION COEFFICIENT  7-6-68
0001                 DIMENSION T(20),XA(20),Y(20),RA(20),ELF(20),XM(20),RT(20),
                    1DY(20),DRA(20)
0002                 READ 4,M
0003               4 FORMAT(I2)
0004                 DO 6 J=1,M
0005                 SUMT=0.0
0006                 SUMY=0.0
0007                 TY=0.0
0008                 TSQ=0.0
0009                 SDSQ=0.0
0010                 SURA=0.0
0011                 RASQ=0.0
0012                 READ 2,CONC,EN,RO,REFH,RPM,VBAR,VISS,DENS,VISW,DENW
0013               2 FORMAT(F5.3,1X,F2.0,1X,F3.2,1X,F5.4,1X,F6.1,1X,F4.4,
                    11X,F6.5,1X,F6.5,1X,F6.5,1X,F6.5)
0014                 N=EN
0015                 READ(5,3) (T(K),ELF(K),XM(K),RT(K),K=1,N)
0016               3 FORMAT(F6.3,1X,F5.4,1X,F5.4,1X,F5.4)
0017                 H=1.51982/(RPM*RPM)
0018                 DO 5 K=1,N
0019                 Y(K)=H*ALOG(RO+(XM(K)-ELF(K))*REFH/(RT(K)-ELF(K)))
0020                 SUMT=T(K)+SUMT
0021                 SUMY=Y(K)+SUMY
0022                 TY=T(K)*Y(K)+TY
0023               5 TSQ=T(K)*T(K)+TSQ
0024                 C=EN*TSQ-SUMT*SUMT
0025                 A=(EN*TY-SUMT*SUMY)/C
0026                 B=(TSQ*SUMY-SUMT*TY)/C
0027                 D=1.0/A
0028                 S20W=A*VISW*VISS*(1.0-VBAR*.99823)/(1.0-VBAR*DENS*DENW)
0029                 G=1.0/S20W
              C      STANDARD DEVIATION OF RESIDUALS AND SED. COEF.
0030                 DO 8 K=1,N
0031                 DY(K)=A*T(K)+B-Y(K)
```

```
      C=EN*TSQ-SUMT*SUMT
      A=(EN*TY-SUMT*SUMY)/C
      B=(TSQ*SUMY-SUMT*TY)/C
      D=1.0/A
      S20W=A*VISW*VISS*(1.0-VBAR*.99823)/(1.0-VBAR*DENS*DENW)
      G=1.0/S20W
C     STANDARD DEVIATION OF RESIDUALS AND SED. COEF.
      DO 8 K=1,N
      DY(K)=A*T(K)+B-Y(K)
    8 SDSQ=DY(K)*DY(K)+SDSQ
      SY=SQRT(SDSQ/(EN-1.0))
      F=C*(EN-2.0)
      SA=SQRT(EN*SDSQ/F)*S20W/A
      READ 20,EL,XONE,R
   20 FORMAT(F5.4,1X,F5.4,1X,F5.4)
      AVRA=RO+(XONE-EL)*REFH/(R-EL)
      RB=RO+(XM(1)-ELF(1))*REFH/(RT(1)-ELF(1))
      AVER=EXP(A*T(N)/H+B/H)
      AVEC=CONC*AVRA*AVRA/(AVER*AVER)
      PRINT 1
    1 FORMAT(T3,'SEDIMENTATION COEFFICIENT',/)
      PRINT 7,A,D
    7 FORMAT(T3,'SOB=',T8,E15.7,T24,'1/SOB=',T31,E15.7,/)
      PRINT 12,S20W,G
   12 FORMAT(T3,'S20W=',T9,E15.7,T25,'1/S20W=',T33,E15.7,/)
      WRITE(6,9) (DY(K),K=1,N)
    9 FORMAT(T3,'DELTA Y=',T12,E15.7,/)
      PRINT 10,SY
   10 FORMAT(T3,'SD OF Y=',T12,E15.7,/)
      PRINT 11,SA
   11 FORMAT(T3,'SD OF S20W=',T15,F15.7,/)
      PRINT 21,AVRA,RB
   21 FORMAT(T3,'RA=',T7,E15.7,T23,'R1=',T27,E15.7,/)
      PRINT 16,AVEC
   16 FORMAT(T3,'AVE CONC=',T13,E15.7,//)
    6 CONTINUE
      STOP
      END
```

2. Z Average Molecular Weight From Sedimentation Equilibrium - After sedimentation equilibrium has been reached and the Schlieren optical system has been photographed, read a frame having good contrast by a microcomparator. That is, place the photographic frame on the table of the microcomparator so that the reference lines are parallel to the y direction. Read the x position of the left and the right reference lines. Then read the solvent line and the solution line at 0.04 cm intervals in the x direction from meniscus to cell bottom. Each set of numbers will correspond to x, yo, and y in the program. There should be about 20 sets of these numbers for a column of 4 mm. Two adjacent sets will be equivalent to one trapezoid. The total number of trapezoids will be the number of sets plus one if the meniscus and cell bottom are included.

The first data card should have only one number which corresponds to the number of groups of data. For example, if five frames were read each giving about 20 sets of x, yo, and y, then the first data card should have the number 05. The second data card should have the eight numbers indicated in statement #6 of the program with the format of statement #7, where N is the number of trapezoids plus one, CO is the initial concentration, ELF is the left reference line, RT is the right reference line, REFH is the distance between reference

holes in the counterbalance (approximately 1.6 cm), VBAR is apparent partial specific volume, RPM is the revolutions per minute of the rotor, and T is the absolute temperature. Following the second data card there should be N cards each containing the four numbers indicated in statement #8 of the program with the format of statement #9 (D is the solvent density of each trapezoid which is usually constant). The second group of data will follow the N'th card where its first card has the eight numbers described on statement #6 of the program followed by N cards as described on statement #8 of the program and so forth until all groups of data are inserted.

Readout will be concentration, Z ave. M.W., and reciprocal square root of Z ave. M.W. for each trapezoid.

The following equations are used in the calculations, where r is radius, n is index of refraction, M is molecular weight, ρ is density of solvent, w is the angular velocity, R is the gas constant, T is absolute temperature, c_o is initial concentration, c is concentration of each trapezoid, A is area of trapezoid, r_a and r_b are meniscus and cell bottom respectively.

$$\frac{\Delta \ln (1/r \cdot dn/dr)}{\Delta r^2} = \frac{M (1 - \bar{v} \rho) w^2}{2 RT}$$

$$c = \frac{A \cdot c_o (r_b^2 - r_a^2)}{\sum_a^b [A \cdot \Delta(r^2)]}$$

```
C     Z AVE MW  8-20-68
      DIMENSION X(50),YO(50),Y(50),D(50),AR(50),ZA(50),ZR(50),C(50)
      READ(5,1) M
    1 FORMAT(I2)
      DO 2 J=1,M
      SARS=0.0
      READ(5,3) N,CO,ELF,RT,REFH,VBAR,RPM,T
    3 FORMAT(I2,1X,F5.3,1X,F5.4,1X,F5.4,1X,F5.4,1X,F4.4,1X,F6.1,1X,F5.2)
      READ(5,4) (X(K),YO(K),Y(K),D(K),K=1,N)
    4 FORMAT(F5.4,1X,F4.3,1X,F4.3,1X,F5.4)
      F=(RT-ELF)/REFH
      A=(T*1.51684E+10)/(RPM*RPM)
      L=N-1
      DO 5 K=1,L
      R1=(X(K)-ELF)/F+5.7
      R2=(X(K+1)-ELF)/F+5.7
      DRSQ=R2*R2-R1*R1
      ELN1=ALOG((Y(K)-YO(K))/R1)
      ELN2=ALOG((Y(K+1)-YO(K+1))/R2)
      ZA(K)=(ELN2-ELN1)*A/((1.0-VBAR*D(K))*DRSQ)
      ZR(K)=1.0/SQRT(ZA(K))
      AR(K)=(Y(K)-YO(K)+Y(K+1)-YO(K+1))/2.0*(X(K+1)-X(K))/F
    5 SARS=SARS+AR(K)*DRSQ
      RA=(X(1)-ELF)/F+5.7
      RB=(X(N)-ELF)/F+5.7
      B=CO*(RB*RB-RA*RA)/SARS
      DO 6 K=1,L
    6 C(K)=B*AR(K)
      WRITE(6,7)
    7 FORMAT(T3,'Z AVE MOLECULAR WEIGHT',/)
      WRITE(6,8) (C(K),ZA(K),ZR(K),K=1,L)
    8 FORMAT(T3,'CONC=',T9,E15.7,T25,'ZAMW=',T31,E15.7,T47,'ZRSR=',
     1T53,E15.7,/)
    2 CONTINUE
      STOP
      END
```

3. <u>Standard Deviation</u> - The average and standard deviation can be determined for up to 50 numbers by the following equation.

$$\sigma = \{[\Sigma x_i^2 - (\Sigma x_i)^2/n]/(n^{-1})\}^{1/2}$$

Enter n, then the numbers.

Readout will be the average and standard deviation, then each number with its deviation from the average.

```
?
P
C;STANDARD DEVIATION PROGRAM  2-26-68
DIMENSIONX(50)
1;FORMAT(/,"STANDARD DEVIATION PROGRAM",/)
2;FORMAT("ENTER N, THEN THE NUMBERS",/)
3;FORMAT(/)
4;FORMAT(E)
SUMX=0.0
XSQ=0.0
TYPE 1
TYPE2
ACCEPT4,EN
N=EN
DO5K=1,N
TYPE3
ACCEPT4,X(K)
SUMX=X(K)+SUMX
XSQ=X(K)*X(K)+XSQ
5;CONTINUE
AVEX=SUMX/EN
SD=SQTF((XSQ-SUMX*SUMX/EN)/(EN-1.0))
TYPE6,AVEX,SD
6;FORMAT(/,"AVE NUM=",E,"SD=",E,)
7;FORMAT(/,"NUM=",E,"D=",E,)
DO8K=1,N
D=X(K)-AVEX
TYPE7,X(K),D
8;CONTINUE
STOP
END
```

```
DO8K=1,N
D=X(K)-AVEX
TYPE7,X(K),D
8CONTINUE
STOP
END
```

```
STANDARD DEVIATION PROGRAM
ENTER N, THEN THE NUMBERS
6
2
3
2
3
2
3
AVE NUM=+0.250000E+1      SD=+0.547722E+0
NUM=+0.200000E+1.     D=-0.500000E+0
NUM=+0.300000E+1.     D=+0.500000E+0
NUM=+0.200000E+1.     D=-0.500000E+0
NUM=+0.300000E+1.     D=+0.500000E+0
NUM=+0.200000E+1.     D=-0.500000E+0
NUM=+0.300000E+1      D=+0.500000E+0
```

4. <u>Least Squares Straight Line</u> - The calculation of slope and intercept (A and B in the equation $y = Ax + B$) can be determined by the least squares method for up to 70 points.

Enter the number of points (N), then each x and y.

Readout will be the slope and intercept, then the individual x's and y's with their Δy's (the distance between entered y and calculated y for each x), then the average deviation of y based on the equation:

$$\sigma = \{[\Sigma(\Delta y)_i^2 - \Sigma(\Delta y)_i \cdot \Sigma(\Delta y)_i/n]/(n^{-1})\}^{1/2},$$

then the standard deviation of A and B.

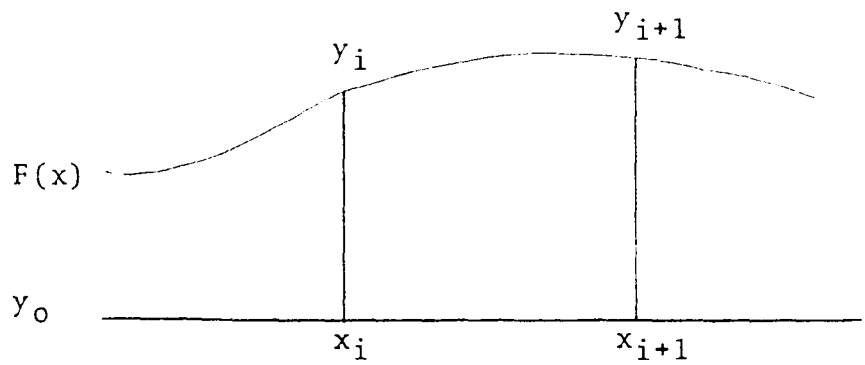
```
P
C;LEAST SQUARES STRAIGHT LINE 5-23-68
C;CALCULATION FO SLOPE AND INTERCEPT
1;FORMAT(/,"LEAST SQUARES STRAIGHT LINE",/)
2;FORMAT("ENTER N, THEN X, Y",/)
3;FORMAT(/)
4;FORMAT(E)
5;FORMAT(/,"A=",E,"B=",E,/)
DIMENSION X(70),Y(70)
11;SUMX=0.0
SUMY=0.0
XY=0.0
XSQ=0.0
SDSQ=0.0
TYPE 1
TYPE2
ACCEPT4,EN
N=EN
DO6K=1,N
TYPE3
ACCEPT4,X(K),Y(K)
SUMX=X(K)+SUMX
SUMY=Y(K)+SUMY
XY=X(K)*Y(K)+XY
XSQ=X(K)*X(K)+XSQ
6;CONTINUE
C=EN*XSQ-SUMX*SUMX
A=(EN*XY-SUMX*SUMY)/C
B=(XSQ*SUMY-SUMX*XY)/C
TYPE5,A,B
C; CALCULATION OF STANDARD DEVIATION OF A AND B
DO7K=1,N
DY=A*X(K)+B-Y(K)
TYPE8,X(K),Y(K),DY
8;FORMAT(/,"X=",E,"Y=",E,"DY=",E,)
SDSQ=DY*DY+SDSQ
7;CONTINUE
SY=SQTE(SDSQ/(EN-1.0))
TYPE9,SY
```

```
TYPE8,X(K),Y(K),DY
8;FORMAT(/,"X=",E,"Y=",E,"DY=",E,)
SDS0=DY*DY+SDS0
7;CONTINUE
SY=SQTF(SDS0/(EN-1.0))
TYPE9,SY
9;FORMAT(/,"SIGMA OF Y=",E,)
D=C*(EN-2.0)
SA=SQTF(EN*SDS0/D)
SB=SQTF(XS0*SDS0/D)
TYPE10,SA,SB
10;FORMAT(/,"SIGMA OF A=",E,"SIGMA OF B=",E,//)
GOTO11
END
LEAST SQUARES STRAIGHT LINE
ENTER N, THEN X, Y
10
7.13 1.74
2.6 1.89
5.51 1.73
2.6 1.85
4.22 1.72
2.6 1.82
7.13 1.68
5.17 1.7
3.45 1.87
6.9 1.66
A=-0.376053E-1     B=+0.194391E+1

X=+0.713000E+1     Y=+0.174000E+1     DY=-0.642166E-1
X=+0.260000E+1     Y=+0.189000E+1     DY=-0.438652E-1
X=+0.551000E+1     Y=+0.173000E+1     DY=+0.670337E-2
X=+0.260000E+1     Y=+0.185000E+1     DY=-0.386524E-2
X=+0.422000E+1     Y=+0.172000E+1     DY=+0.652156E-1
X=+0.260000E+1     Y=+0.182000E+1     DY=+0.261354E-1
X=+0.713000E+1     Y=+0.168000E+1     DY=-0.421715E-2
X=+0.517000E+1     Y=+0.170000E+1     DY=+0.494899E-1
X=+0.345000E+1     Y=+0.186999E+1     DY=-0.558281E-1
X=+0.690000E+1     Y=+0.166000E+1     DY=+0.244326E-1
SIGMA OF Y=+0.437476E-1
SIGMA OF A=+0.813431E-2     SIGMA OF B=+0.411859E-1
```

5. <u>Area by the Trapezoid Rule</u> - For integration purposes, the area, A, under a curve, F(x), can be determined by dividing this region into trapezoids, determining the area of each, and then adding up the individual areas.

$$A = \sum_{i=1}^{n-1} [(y_{i+1} + y_i)/2 - y_o] \; [x_{i+1} - x_i]$$



In this program, up to a hundred trapezoids can be added by entering the x_i, x_{i+1}, y_o, y_i, and y_{i+1} of each trapezoid.

Readout is A_i and ΣA_i.

```
P
C;AREA BY THE TRAPEZOID RULE  2-28-68
SUMA=0.0
TYPE1
1;FORMAT(/,"AREA BY TRAPEZOID RULE",/)
TYPE2
2;FORMAT("ENTER X1,X2,Y0,Y1,Y2",/)
3;FORMAT(E)
DO4K=1,100
ACCEPT 3,X1,X2,Y0,Y1,Y2
A=((Y1+Y2)/2.0-Y0)*(X2-X1)
SUMA=A+SUMA
TYPE5,A,SUMA
5;FORMAT(/,"A=",E,"QA=",E,/)
4;CONTINUE
STOP
END
```

```
AREA BY TRAPEZOID RULE
ENTER X1,X2,Y0,Y1,Y2
1,2,0,3,4
A=+0.350000E+1      QA=+0.350000E+1
2,3,0,4,5
A=+0.450000E+1      QA=+0.800000E+1
```

6. Calculator Program - If constant, first number, and second number are C, 1, and 2 respectively, then the following operation symbols will give the following readout.

Operation Symbol	Readout
1	sum and cumulative sum
2	product and quotient
3	C x prod. and C x quot.
4	C x 1 and C x 2
5	1/C and 2/C
6	Log 1 and Log 2
7	Sq. root 1 and Sq. root 2
8	sine 1 and sine 2
9	cosine 1 and cosine 2
10	exponential 1 and exponential 2

```
P
C,CALCULATOR PROGRAM  4-6-68
QSUM=0.0
TYPE12
12;FORMAT(/,"CALCULATOR PROGRAM",/)
TYPE13
13;FORMAT("ENTER CONSTANT, THEN TWO NUMBERS AND OPERATION SYMBOL",/)
14;FORMAT(E)
ACCEPT14,C
TYPE15
15;FORMAT(/)
16;ACCEPT14,A,B
ACCEPT11,MODE
11;FORMAT(I)
GOTO(1,2,3,4,5,6,7,8,9,10),MODE
1;SUM=A+B
QSUM=QSUM+SUM
TYPE17,SUM,QSUM
17;FORMAT(/,"SUM=",E,"QSUM=",E,/)
GOTO16
2;PROD=A*B
QUOT=A/B
TYPE18,PROD,QUOT
18;FORMAT(/,"PROD=",E,"QUOT=",E,/)
GOTO16
3;CPRO=C*A*B
CQUO=C*A/B
TYPE19,CPRO,CQUO
19;FORMAT(/,"CXPROD=",E,"CXQUOT=",E,/)
GOTO16
4;CXA=C*A
CXB=C*B
TYPE20,CXA,CXB
20;FORMAT(/,"CX1=",E,"CX2=",E,/)
GOTO16
5;QUAC=A/C
QUBC=B/C
TYPE21,QUAC,QUBC
21;FORMAT(/,"1/C=",E,"2/C=",E,/)
GOTO16
6;ELNA=LOGF(A)
ELNB=LOGF(B)
TYPE22,ELNA,ELNB
```

```
TYPE17,SUM,QSUM
17;FORMAT(/,"SUM=",E,"QSUM=",E,/)
GOTO16
2;PROD=A*B
QUOT=A/B
TYPE18,PROD,QUOT
18;FORMAT(/,"PROD=",E,"QUOT=",E,/)
GOTO16
3;CPRO=C*A*B
CQUO=C*A/B
TYPE19,CPRO,CQUO
19;FORMAT(/,"CXPROD=",E,"CXQUOT=",E,/)
GOTO16
4;CXA=C*A
CXB=C*B
TYPE20,CXA,CXB
20;FORMAT(/,"CX1=",E,"CX2=",E,/)
GOTO16
5;QUAC=A/C
QUBC=B/C
TYPE21,QUAC,QUBC
21;FORMAT(/,"1/C=",E,"2/C=",E,/)
GOTO16
6;ELNA=LOGF(A)
ELNB=LOGF(B)
TYPE22,ELNA,ELNB
22;FORMAT(/,"LOG1=",E,"LOG2=",E,/)
GOTO16
7;SQRA=SQTF(A)
SQRB=SQTF(B)
TYPE23,SQRA,SQRB
23;FORMAT(/,"SQRT 1=",E,"SQRT 2=",E,/)
GOTO16
8;SINA=SINF(A)
SINB=SINF(B)
TYPE26,SINA,SINB
26;FORMAT(/,"SINE 1=",E,"SINE 2=",E,/)
GOTO16
9;COSA=COSF(A)
COSB=COSF(B)
TYPE24,COSA,COSB
24;FORMAT(/,"COS 1=",E,"COS 2=",E,/)
GOTO16
10;EXPA=EXPF(A)
EXPB=EXPF(B)
TYPE25,EXPA,EXPB
25;FORMAT(/,"EXP1=",E,"EXP2=",E,/)
GOTO16
```

```
CALCULATOR PROGRAM
ENTER CONSTANT, THEN TWO NUMBERS AND OPERATION SYMBOL
3
1 2 1
SUM=+0.300000E+1      QSUM=+0.300000E+1
1 2 1
SUM=+0.300000E+1      QSUM=+0.600000E+1
1 2 2
PROD=+0.200000E+1     QUOT=+0.500000E+0
1 2 3
CXPROD=+0.600000E+1     CXQUOT=+0.150000E+1
1 2 4
CX1=+0.300000E+1      CX2=+0.600000E+1
1 2 5
1/C=+0.333333E+0      2/C=+0.666667E+0
1 2 6
LOG1=+0.000000E+0     LOG2=+0.693147E+0
1 2 7
SQRT 1=+0.100000E+1     SQRT 2=+0.141421E+1
1 2 8
SINE 1=+0.841470E+0     SINE 2=-0.909297E+0
1 2 9
COS 1=+0.540302E+0     COS 2=-0.416146E+0
1 2 10
EXP1=+0.271828E+1     EXP2=+0.738906E+1
```

7. <u>Apparent Partial Specific Volume</u> - The pycnometric calculation of $\bar{v}$ is by the following equation where ρ_o and ρ are the densities of solvent and solution respectively, and c is concentration in mg/ml.

$$\bar{v} = 1/\rho_o - 1000/c\ [1/\rho_o - 1/\rho]$$

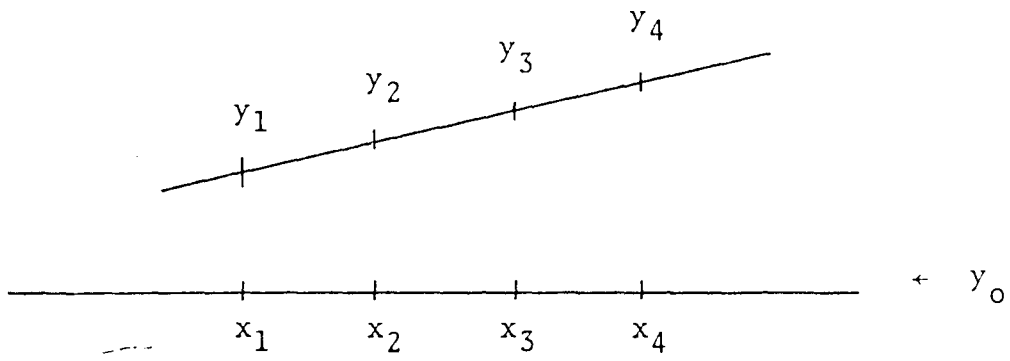
Enter concentration, gms of solvent, mls of solvent, gms of solution, and mls of solution.

Readout is ρ_o, ρ , and $\bar{v}$.

```
P
C;APPARENT SPECIFIC VOLUME 4-6-68
TYPE 1
1;FORMAT(/,"APPARENT SPECIFIC VOLUME",/)
TYPE2
2;FORMAT("ENTER CONC, GMØ, MLØ , GM, ML",/)
7;TYPE3
4;FORMAT(E)
3;FORMAT("DATA:")
ACCEPT 4,CONC,GMO,VOLO,GM,VOL
RHOO=GMO/VOLO
RHO=GM/VOL
TYPE5,RHOO,RHO
5;FORMAT(/,"RHOØ=",E,"RHO=",E,/)
PHI=1.0/RHOO-(1000.0/CONC)*(1.0/RHOO-1.0/RHO)
TYPE6,PHI
6;FORMAT("PHI=",E,/)
GOTO7
END
```

```
APPARENT SPECIFIC VOLUME
ENTER CONC, GMØ, MLØ , GM, ML
DATA:7.66 3.3245 3.09707 3.3303 3.09817
RHOO=+0.107343E+1      RHO=+0.107492E+1
PHI=+0.763916E+0
DATA:7.66 3.3267 3.09897 3.3315 3.09867
RHOO=+0.107348E+1      RHO=+0.107513E+1
PHI=+0.744546E+0
DATA:
```

8. <u>R square and ln (y/R) from Sedimentation Equilibrium</u> - Enter two identification numbers (e.g. date and concentration of sample), then the observed left reference line (EL), the magnification factor (Rt-EL/1.6), then observed x, y_o, and y from Schlieren readout.

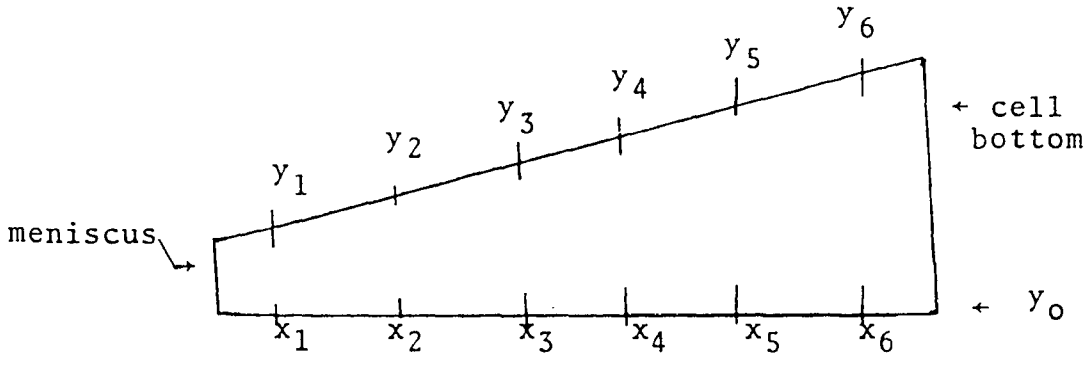


The typeout will be ln (y/R) and R^2.

```
100;FORMAT(/,"MW-ZAVG-LAMM",/)
101;FORMAT("TO CALC LOG&RSQ, ENTER DATE,CONC,EL, F, X,YO,Y",/)
102;FORMAT("DATA: ")
103;FORMAT(E)
104;FORMAT(/,"LOG= ",E,"RSQ= ",E,/)
   ;TYPE 100
   ;TYPE 101
105;TYPE 102
   ;ACCEPT 103, DATE, CONC, EL, F
106;ACCEPT 103, X, YO, Y
   ; R= (X-EL)/F + 5.70
   ;ALOG=LOGF((Y-YO)/R)
   ;RSQ=R**2
   ;TYPE 104, ALOG, RSQ
108;TYPE 102
   ;GO TO 106
   ;END
```

9. Z Average Molecular Weight from Sedimentation Equilibrium (Constant Solvent Density) - (NOTE: The program for varying solvent density is the same as the program below except that the density of each trapezoid is entered as a variable).

After sedimentation equilibrium has been reached, and the Schlieren optical system has been photographed, read a frame having good contrast by a microcomparator. At observed intervals of 0.04 cm in the radial direction from meniscus to cell bottom, read both the solvent line and the solution line. These three numbers will correspond to x, y_o, and y in the program. There should be about 20 sets of these numbers for a column of 4 mm. Two adjacent sets will be equivalent to one trapezoid. The total number of trapezoids will be the number of sets plus one if the meniscus and cell bottom are included.



Into the program, enter the following constants: the number of trapezoids (N), the macromolecular concentration, the left reference line (LF), the meniscus (XA), the cell bottom (XB), the distance between reference holes in the counter weight which is approximately 1.6 cm (REFH), $\bar{v}$ (VBAR), solvent density (D), the rpm (RPM), and absolute temperature (T). Then enter the variables for each trapezoid including the first and last trapezoids formed by the meniscus and the cell bottom. These variables must be entered in the form x_i, x_{i+1}, y_o, y_i, y_{i+1}. Then the program will loop for x_{i+1}, x_{i+2}, y_o, y_{i+1}, y_{i+2} and so forth until N trapezoids are entered.

Readout after each loop will be Z ave. M.W., reciprocal sq. root of Z ave. M.W., and the area of the trapezoid (A). At the end of the program a constant (D) will be printed out which when multiplied by the area of each loop (each trapezoid) will give the macromolecular concentration of each trapezoid. Thus molecular weight versus concentration can be plotted for extrapolation to zero concentration.

The following equations are used in the calculations, where r is radius, n is index of refraction, M is molecular weight, ρ is density of solvent, w is the angular velocity, R is gas constant, T is absolute temperature, c_o is initial concentration, c is concentration of each trapezoid, A is area of trapezoid, r_a and r_b are meniscus and cell bottom respectively.

$$\frac{\Delta \ln\ (1/r \cdot dn/dr)}{\Delta r^2} = \frac{M(1-\bar{v}\rho)w^2}{2\ RT}$$

$$c = \frac{A \cdot c_o\ (r_b^2 - r_a^2)}{\sum_a^b [A \cdot \Delta(r^2)]}$$

```
P
C;ZAMM     (constant solvent density)
SARS=0.0
TYPE1
1;FORMAT(/,"Z AVERAGE MW",/)
TYPE2
2;FORMAT("ENTER N,CONC,LF,XA,XB,RT,REFH,VBAR,D,RPM,T",/)
TYPE3
3;FORMAT("THEN X1,X2,Y0,Y1,Y2",/)
4;FORMAT(E)
ACCEPT4,EN,CONC,ELF,XA,XB,RT,REFH,VBAR,D,RPM,T
S(T*1.51684E+10)/((1.0-VBAR*D)*RPM*RPM)
N=EN
F=(RT-ELF)/REFH
DO9K=1,N
TYPE5
5;FORMAT(/,"DATA:")
ACCEPT4,X1,X2,Y0,Y1,Y2
R1=(X1-ELF)/F+5.7
R2=(X2-ELF)/F+5.7
DRSQ=R2*R2-R1*R1
ELN1=LOGE((Y1-Y0)/R1)
ELN2=LOGE((Y2-Y0)/R2)
ZAMW=((ELN2-ELN1)*C)/DRSQ
ZRSR=1.0/SQTF(ZAMW)
A=((Y1+Y2)/2.0-Y0)*(X2-X1)/F
TYPE7,ZAMW,ZRSR,A
7;FORMAT(/,"ZAMW=",E,"ZAMWRSR=",E,"A=",E,)
SARS=SARS+A*DRSQ
9;CONTINUE
RA=(XA-ELF)/F+5.7
RB=(XB-ELF)/F+5.7
D=CONC*(RB*RB-RA*RA)/SARS
TYPE11,D
11;FORMAT(/,"D=",E,)
END
?
```

```
P
C;ZAMM      (variable solvent density)
SARS=0.0
TYPE1
1;FORMAT(/,"Z AVERAGE MW",/)
TYPE2
2;FORMAT("ENTER N,CONC,LF,XA,XB,RT,REFH,VBAR,RPM,T",/)
TYPE3
3;FORMAT("THEN X1,X2,Y0,Y1,Y2,D",/)
4;FORMAT(E)
ACCEPT4,EN,CONC,ELF,XA,XB,RT,REFH,VBAR,RPM,T
C=(T*1.51684E+10)/(RPM*RPM)
N=EN
F=(RT-ELF)/REFH
DO9K=1,N
TYPE5
5;FORMAT(/,"DATA:")
ACCEPT4,X1,X2,Y0,Y1,Y2,D
R1=(X1-ELF)/F+5.7
R2=(X2-ELF)/F+5.7
DRSQ=R2*R2-R1*R1
ELN1=LOGE((Y1-Y0)/R1)
ELN2=LOGE((Y2-Y0)/R2)
ZAMW=((ELN2-ELN1)*C)/((1.0-VBAR*D)*DRSQ)
RZR=1.0/SQTF(ZAMW)
A=((Y1+Y2)/2.0-Y0)*(X2-X1)/F
TYPE7,ZAMW,ZRSR,A
7;FORMAT(/,"ZAMW=",E,"ZAMWRSR=",E,"A=",E,)
SARS=SARS+A*DRSQ
9;CONTINUE
RA=(XA-ELF)/F+5.7
RB=(XB-ELF)/F+5.7
D=CONC*(RB*RB-RA*RA)/SARS
TYPE11,D
11;FORMAT(/,"D=",E,)
END
```

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