ABSTRACT

CHARACTERIZATION OF THE 3.5S ALDOLASE INTERMEDIATE AS A DIMER AND ANALYSIS FOR DIMER CATALYTIC ACTIVITY

by Stanley P. Blatti

The first major aim of this research was to study the mechanism of subunit association of native rabbit muscle aldolase. Two initial problems had to be resolved: (1) the subunit structure of aldolase had to be clarified; the native enzyme was thought to have either 3 or 6 subunits, and (2) the 3.5S obligate intermediate in the acid dissociation and reassociation of aldolase had to be characterized by physical techniques.

A detailed subunit molecular weight analysis of rabbit muscle aldolase using the dissociation medium of 5.6M guanidine HCl and 0.1M β-mercaptoethanol produced, upon extrapolation to zero protein concentration, a weight-average molecular weight (M^{o}_{w}) of 42,000 for the subunits. This value, together with the characterization of the 3.5S intermediate as a dimer of aldolase subunits, provided support for the four subunit model.

In the pH dissociation of rabbit muscle aldolase, the best conditions for production of the 3.5S intermediate were found to be pH 3.35, low salt (0.01M NaCl), and 0^{o}-2^{o}. The extrapolated sedimentation coefficients and diffusion coefficients under these conditions were calculated to be $s^{o}_{20,w} = 3.45S$ and $D^{o}_{20,w} = 3.73 \times 10^{-7}$ cm^2/second, respec-

tively. These values yielded a molecular weight of 83,000-86,000. However, some aggregate was found to be present; correction for this would reduce the molecular weight by 4% to 80,000-83,000, a value which is most consistent with a dimer of aldolase subunits (42,000). This was further supported by the fact that the intrinsic viscosity of 14.0 cc/g for the intermediate was found to be almost mid-way between that for the native enzyme (4 cc/g) and that for the acid dissociated subunits (24.0 cc/g).

Thus the most likely mechanism for subunit association into tetrameric aldolase was expected to involve two consecutive bimolecular association reactions: (1) monomer association into dimers, followed by (2) dimer association into tetramers.

The discovery and proof that the 3.5S intermediate was a dimer, together with the fact that aldolase contained at least 3 catalytic sites, led to the second major aim of this research: to answer the question of whether aldolase dimers could possess catalytic activity independent of their 'partner dimer' in the native tetramer. But before this question could be examined, a study was conducted to find the proper conditions for complete reversibility of activity from pH 3.35 dimers. The following conditions were found to give 100% recovery of activity: (1) pH 7.9, (2) 0.1M β-mercaptoethanol, (3) 1 hour incubation at 0° followed by a 2 hour incubation at 20°, and (4) reversal concentrations between 0.013 mg/ml and 0.065 mg/ml. The rate of activity

recovery was found to be second-order with respect to dimer concentration (0.09 mg/ml to 0.18 mg/ml), and the second-order rate constant was calculated to be $k = 1.35 \times 10^4$ liters/mole-second at 16°. These results suggested that the pH 3.35 dimer was inactive and had to associate into tetramers before a catalytic activity was regained.

In the course of these studies, it was discovered that FDP inhibited dimer association at pH 5.0 and inhibited activity reversal in the assay at pH 7.5. At 50% inhibition of activity recovery, the dissociation constant for FDP was found to be $k_{FDP} = 4 \times 10^{-3}$M; this was 500 times the binding constant of FDP for the native enzyme.

Since dimers at pH 3.35 were inactive, the possibility of producing active dimers under conditions of higher pH values was investigated. When the pH of pH 3.35 dimers was raised to pH 4.0 or pH 5.0, association was inhibited; however, the reactivation which occurred in the assay showed essentially zero initial rates so that dimers at these pH values must have also been inactive. In contrast, the pH 5.5 dimers, which were incubated and separated by sucrose density centrifugation sedimentation velocity (SDSV), gave activity immediately with little or no lag times. For this reason all subsequent studies were completed at pH 5.5. Dimer association was found to be concentration dependent as expected; only about 3% dimer association had occurred in a 20 hour period at very low protein concentrations (0.03 mg/ml) and -6°. On the other hand, incubation at

higher temperatures (0°, 4°, and 12°) stimulated dimer association. Sucrose concentrations of 4%, 16%, and 20% could effectively inhibit dimer association. In conclusion to this section, pH 5.5 dimers subjected to SDSV separation exhibited immediate activity upon assaying with essentially no lag time; therefore, it was under these conditions (except for the SDSV analysis) that the final analysis for active dimers was investigated.

The last section then of this work was devoted entirely to proving whether the pH 5.5 dimer at -6° was active when assayed at pH 7.5 and 25°. To examine this question, a kinetic analysis of activity reversal, together with the physical analysis of the dimer-tetramer distribution in the assay, were performed to exclude all reaction mechanisms but one.

The physical analysis of the dimer-tetramer distribution in the assay at various times during the reversal process showed increased tetramer formation. The kinetics of the reversal process in the assay was shown to be first-order at relatively high dimer concentrations; however, at lower dimer concentrations and under improved reversal conditions in the assay of 1 mg/ml BSA and 0.1M β-mercaptoethanol, the half-times for reversal increased, indicating a shift towards a second-order process. The rate constants for the first-order and second-order reactions were calculated to be $k_1 = 2.4 \times 10^{-3}$/second and $k_2 = 6.4 \times 10^{4}$ liters/mole-second, respectively. The only mechanism which was consistent with

this data was the following: (1) inactive dimer association into an inactive tetramer followed by (2) a first-order folding reaction of the inactive tetramer into an active tetramer. In conclusion under all the conditions studied here, aldolase dimers were found to be inactive--only the tetramers had activity.

CHARACTERIZATION OF THE 3.5S ALDOLASE INTERMEDIATE AS A DIMER AND ANALYSIS FOR DIMER CATALYTIC ACTIVITY

By

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A THESIS

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Dedicated

to my wife, Jane

and to my son, Todd.

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VITA

Stanley P. Blatti was born in Kasson, Minnesota, on April 28, 1942. He graduated from Kasson-Mantorville High School on June 6, 1960, and then attended St. Olaf College where his interest in chemistry was first stimulated by Dr. Finholt. Mr. Blatti continued his undergraduate studies at the University of Minnesota where his interest in chemistry was further stimulated by Dr. Kreevoy and Dr. Noland. He once was asked to leave his apartment because fumes from his makeshift laboratory were causing some discomforts to his upstairs neighbors. He married Jane M. Kirkwood in 1962 and a year later a son, Todd, was born. They moved into a house where Mr. Blatti built another laboratory of a more permanent nature equipped with steam baths, gas, and even a hood. He received a B.A. degree in 1964 from the University of Minnesota and then worked as a research assistant for Dr. L. M. Henderson, chairman of Biochemistry Department at the University of Minnesota. In his work there, he synthesized 5 and 6 carbon fatty acid pantothiel anhydrides using microtechniques, the results of which were eventually published. Mr. Blatti then attended Michigan State University to continue his education in the field of biochemistry under the direction of Dr. W. C. Deal, Jr. In August of 1968 he will have received the degree of Doctor of Philosophy with a

biochemistry major.

Upon completion of his work at Michigan State University the author plans to attend the animal microbiology course offered by Dr. Phillip Marcus at the Cold Spring Harbor Laboratory of Quantitative Biology during the late summer of 1968. He then plans to continue his research training with Dr. Marcus at Albert Einstein College of Medicine on the general problem of regulation of protein synthesis by translational inhibitory proteins in such cellular systems as the viral interferon-induced cells, the mitotic phase of cell division, and eventually the unfertilized egg.

Mr. Blatti was awarded a National Defense Education Act, Title IV, Predoctoral Fellowship in 1965 to complete his graduate work and a National Institutes of Health Post-doctoral Fellowship to continue his training in biochemistry with Dr. Marcus. Mr. Blatti was also accepted for membership to the American Chemical Society.

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INTRODUCTION

One of the central problems of biochemistry is to achieve an understanding of protein biosynthesis. It is convenient to divide protein biosynthesis into two steps: (a) the synthesis of the primary sequence and (b) the folding and association of subunits into the native, polymeric enzyme. It is presently impossible to study protein conformational changes in cell free extracts during protein biosynthesis; however, *in vitro* reassociation of subunits provides a model system which in many respects may simulate this process.

Previous studies (Deal *et al.*, 1963; Stellwagen *et al.*, 1962) on rabbit muscle aldolase had demonstrated reversible dissociation by mild acid. More importantly, the studies by Deal *et al.*, (1963) have indicated an obligate 3.5S intermediate in both the dissociation and reassociation processes. The physical and chemical data at that time indicated that rabbit muscle aldolase was composed of three polypeptide chains. Assuming, when this study was begun, this model to be correct (it has subsequently been revised to a four subunit native enzyme, (Penhoet *et al.*, 1966)) the 3.5S intermediate was expected to be either: (1) very unfolded trimers, (2) folded monomers, or (3) a rapid equilibrium mixture consisting of monomers and dimers.

The original purpose of this research was to determine which of these possibilities was correct. The approach was to physically characterize the intermediate. This 3.5S aldolase intermediate is of special interest for its significance to protein biosynthesis since the intermediate may also occur in the process of subunit association in living cells.

During this investigation it became evident that none of the previously mentioned structural models for the intermediate was consistent with the physical data. This necessitated a consideration of the accuracy of the values of the molecular weight of the native enzyme and subunits. Using the values for the native enzyme from the literature and a subunit molecular weight determined in this research, the number of subunits was calculated to be mid-way between three and four subunits. Thus, the subunit molecular weight analysis, together with physical analysis of the 3.5S intermediate as a dimer, necessitated a consideration of the tetramer model.

The discovery and proof that the 3.5S in aldolase intermediate was a dimer from this research, together with the fact that aldolase contained at least 3 catalytic sites (Horecker et al., 1963; Lai et al., 1964; Castellino and Barker, 1966; Ginsburg and Mohler, 1966) led to the question of whether aldolase dimers could possess catalytic activity independently of their "partner dimer" in the native tetramer. Answering this question was the second major aim of this thesis. The question of whether the "individual subpolymers of multi-subunit, multi-active site enzymes are active" is a

fundamental question in biochemistry which needs to be answered. The strength of the dimer-dimer interaction is most important in this regard; if it is too strong, the conditions required for breaking the interaction would probably also unfold the resulting dimer molecules. But since aldolase is so easily dissociated, it might be expected to have an active dimer.

The effect of breaking this dimer bond on the other parts of the molecule, especially the active site, could be tested by the analysis of possible catalytic activity of the dimers. For example, if the integrity of the active site had remained intact after dissociation, as if the dimers were hard spheres, then the dimers would be catalytically active. However, any small displacement of a side chain resulting from dissociation could conceivably destroy the catalytic site, and the test for activity would be negative.

But how does one approach this problem? The approach of this investigator was to try to produce dimers under conditions in which some native enzyme can remain as a tetramer and can retain its catalytic activity. Obviously, there are many complicating problems in this kind of approach: (1) The tetramers may lose activity as the solvent conditions are altered. (2) The dimers may tetramerize and the activity assays may be complicated by this activity in the tetramer form. (3) The dimers may associate into aggregates of higher order which will further complicate this problem. Since nonspecific aggregation is usually a major problem in subunit

reassociation studies, a thorough study of the reactivation of the pH 3.35 dimers into tetramers was first conducted. After this study, conditions were investigated which affected dimer association and reversal of activity at higher pH values (pH 5.5 and pH 7.5). Finally, the question of dimer catalytic activity was examined, and an answer to this question was obtained.

LITERATURE REVIEW

I. General Properties of Aldolases

Fructose diphosphate aldolases have been divided into two classes, I and II; this division is based upon their respective physio-chemical properties, catalytic properties, and their biological origins (for a detailed review, see Rutter, 1964). What follows is a general description of class I aldolases and class II aldolases, in that order.

Class I aldolases have been isolated from animals, plants, protozoans, and green algae. This class of aldolase, until recently, was thought to be composed of three subunits--in fact, it was thought to be the only well-documented case of a three subunit enzyme. But it now appears that the enzyme has four subunits (Tanford and Kawahara, 1966; Rajkumar *et al.*, 1966; Penhoet *et al.*, 1966). Three isozymes, A, B, and C, have been discovered in mammals (Rajkumar, 1966). The enzymes of this class (I) are inhibited by metals, whereas class II aldolases are stimulated by monovalent or divalent metals. Removal of the C-terminal tyrosine from class I enzymes by carboxypeptidase results in a greatly reduced rate of fructose diphosphate (FDP) cleavage but not fructose 1-phosphate (F-1-P) cleavage; however, the binding of FDP or F-1-P is not affected by carboxypeptidase treatment.

Dihydroxyacetone phosphate (DHAP) has been reduced onto the lysine residue of the enzyme by sodium borohydride, indicating a Schiff base intermediate in the catalysis (Horecker et al., 1963). The pH profiles of FDP cleavage and hydrogen exchange upon DHAP binding are quite broad for class I enzymes (Richards and Rutter, 1961a).

Class II aldolases have been isolated from bacteria, blue-green algae, yeast and fungi. Aldolases in this class have a molecular weight of 70,000 to 80,000 in contrast to 150,000 to 160,000 for the class I enzyme, and probably consists of two identical subunits (Richards and Rutter, 1961 1961b). The possible role of lysine in catalysis of class II aldolases has not been clearly defined, since there is no inactivation of the enzyme in the presence of FDP with addition of sodium borohydride (Rutter, 1964). A high sulfhydryl lability of this enzyme is demonstrated by the requirement of sulfhydryl reagents in the isolation procedure (Rutter et al., 1966). The pH profiles of the hydrogen exchange with DHAP and of FDP cleavage are very sharp for class II enzymes in contrast to class I enzymes (Richards and Rutter, 1961b).

Both class I and class II aldolases have been discovered in Euglena and Chlamydomonas. In some cases mutants have been found in which one of the aldolases is absent (Rutter, 1964). Both aldolases must be controlled by the nuclear DNA because their formation is not correlated with chloroplast formation (Rutter, 1967).

Since this research was concerned with rabbit muscle

aldolase, a class I enzyme, the rest of the literature review will discuss the past work on the class I enzymes. Except for the recent isozyme work and the experiment by Tanford and Kawahara (1966), most of the remaining properties of aldolase to be discussed are consistent with the previously accepted three subunit model for rabbit muscle aldolase.

II. Physical Properties of Rabbit Muscle Aldolase

A. Native Enzyme

The physical properties of native rabbit muscle aldolase have been measured in several laboratories. Gralen (1939) demonstrated that rabbit muscle myogen A had a sedimentation coefficient of 8.0S. Taylor and Lowry (1956) first measured the weight-average molecular weight of the native enzyme using the Svedberg equation (see Methods). They obtained a molecular weight of 149,000 using a sedimentation coefficient of $s^{o}_{20,w} = 7.35S$ and a diffusion coefficient of $D^{o}_{20,w} = 4.63 \times 10^{-7}$ cm^2/sec. However, adiabatic cooling of the rotor was not taken into consideration; with this correction, the sedimentation coefficient is considerably increased (Kawahara and Tanford, 1966). Higher values for the sedimentation coefficient of the native enzyme have been obtained by Stellwagen and Schachman (1962) and by Deal *et al.* (1963) who found values of $s^{o}_{20,w} = 7.9S$ and 7.8S, respectively. Tanford and Kawahara (1966) estimated a value of $s^{o}_{20,w} = 8.0S$, using a sedimentation coefficient at a single concentration together with the concentration dependence calculated from

the regression formula of Stellwagen and Schachman (1962). Using an average value of 7.9S for the sedimentation coefficient and the above diffusion coefficient, a value of M(S/D) = 160,000 was obtained. Unfortunately, the value of the diffusion coefficient has not been confirmed.

Stellwagen and Schachman (1962) measured the molecular weight of the native enzyme by the sedimentation equilibrium technique at 5 mg/ml and found an apparent weight-average molecular weight of 142,000. Using the short column sedimentation equilibrium technique, Lewis and Hass (1963) obtained a weight-average molecular weight at three concentrations of 140,000, in good agreement with the previous value. Finally, Kawahara and Tanford (1966), using the high speed equilibrium technique of Yphantis (1964), found an apparent weight-average molecular weight of 158,000 at 2 mg/ml.

The intrinsic viscosity of native aldolase has been determined by two laboratories (Stellwagen and Schachman, 1962; Hass, 1964) to be 4.0 cc/g. The intrinsic viscosity of aldolase indicates that it has a very compact structure, and thus is a fairly globular protein, not an elongated rod or random coil. Using the formula of Scheraga and Manderkern (1953) relating sedimentation coefficient, intrinsic viscosity, and molecular weight, a molecular weight of 161,000 was obtained for the native enzyme by Kawahara and Tanford (1966). The β parameter used in the above formula had a value of 2.12×10^6; this value is commonly used for ellipsoidal molecules with moderate axial ratios and for spherical

molecules. The sedimentation coefficient used was the same as that used in the previous M(S/D) calculation, i.e. 7.9S.

Values of 0.742 cc/g (Taylor and Lowry, 1956) or 0.745 cc/g (Hass, 1964), both at 20°C, for the partial specific volume have been reported for native aldolase.

B. Subunits

The enzyme has been dissociated by urea (Stellwagen and Schachman, 1962), by guanidine HCl (Schachman and Edelstein, 1966a,b), by sodium dodecyl sulfate (Schachman, 1960), by acid (Deal et al., 1963; Stellwagen and Schachman, 1962; Westhead and Boyer, 1963), and by alkaline pH (Sine and Hass, 1966) into subunits approximately one-third the molecular weight of the native enzyme. Although the original subunit molecular weight values of Schachman and Edelstein were too low for the three subunit model, they corrected these values upward where they did agree with the three subunit model using the assumption that there was preferential interaction of water with protein. This correction for the 3, 5, and 7 M guanidine HCl solutions changed the respective apparent molecular weights from 46,600, 43,000, and 38,300 to 50,000, 49,500, and 49,900.

Hade and Tanford (1967) have disputed their correction and provided evidence that, instead of preferential of water with protein, actually there was preferential interaction of guanidine with protein. Thus, in contrast to the previous correction where the molecular weight was increased, Hade and

Tanford's correction would decrease the molecular weight. Thus the molecular weight of 46,600-38,300 should be corrected downward to about 44,000-36,000.

Kawahara and Tanford (1966) also measured the molecular weight of the subunits in guanidine HCl, but included β-mercaptoethanol as well. By the sedimentation equilibrium technique they obtained an average molecular weight for two experiments of 41,000 using a partial specific volume of $\bar{v}_{25} = 0.747$ cc/g. Since this experiment was done at essentially zero concentration, extrapolation to zero concentration was unnecessary.

Hass and Lewis (1963) reported the rapid formation of six subunits when the native enzyme was made alkaline to pH 12.5. However, they later retracted this with their conclusion that peptide bonds were being hydrolyzed at this pH (Hass, 1967). This later report also contained evidence for four subunits in aldolase. The results of Kawahara and Tanford (1966) and Hass (1967) are in agreement with those results reported here (see Chapter I-A in Results) which were completed in early 1965.

The value reported for the intrinsic viscosity of the subunits formed in acid is about 24.5 cc/g (Stellwagen and Schachman, 1962) which is in agreement with the results to be presented here (see Chapter II-B in Results). Kawahara and Tanford (1966) found an intrinsic viscosity of 35.3 cc/g for subunits produced in 6M guanidine HCl with 0.1M β-mercaptoethanol. This suggests that the acid dissociated

subunits were not completely unfolded, whereas the enzyme dissociated in guanidine were probably random coils. Tanford and Kawahara also reported an extrapolated sedimentation coefficient value for the subunits in this medium: $s^{o}_{20,w}$ = 1.8S.

They used their viscosity and sedimentation data to give an additional independent calculation of the molecular weight. Again, to use the formula of Scheraga and Manderkern (1953) to obtain the molecular weight, a β value characteristic of that for random coils must be used. A plot of the intrinsic viscosity vs. the molecular weight for various enzymes dissociated into random coils with guanidine HCl and β-mercaptoethanol has shown that the intrinsic viscosity (η) varies linearly with the molecular weight (M) according to the formula: $(\eta) = M^{0.68}$ (Tanford, 1966). The β value for random coils used for these molecular weight determinations was 2.5×10^{6}. Using this β value the previously mentioned values for intrinsic viscosity and sedimentation coefficients, and a partial specific volume of 0.747 cc/g at 25°C, Kawahara and Tanford (1966) calculated a molecular weight for aldolase subunits to be 42,000.

III. Chemical and Catalytic Properties of Rabbit Muscle Aldolase

Aldolase is a relatively unique enzyme because one of its substrates can be reduced stereospecifically onto its active site by sodium borohydride (Grazi et al., 1962). This

method has been used to quantitatively estimate the number of binding sites per mole of native enzyme. Using this technique, the number of estimated binding sites has risen from one to three (Horecker et al., 1963; Lai et al., 1964; Ginsburg and Mehler, 1966) during the last several years. Investigations using other techniques led to values from one to three binding sites: (1) Westhead et al. (1963) found one binding site using ultracentrifugal or equilibrium dialysis methods; (2) Castellino and Barker (1966) found three binding sites for D-arabinitol-1,5-diphosphate-1-^{14}C when the diphosphate was equilibrated with enzyme and run through a Bio-Gel P-6 column or analyzed by the partition-cell ultracentrifugation technique; and (3) Ginsburg (1966) found three binding sites when a mixture of radioactively labeled DHAP and native aldolase was rapidly percolated through a G-50 (coarse) Sephadex column.

Winstead et al. (1963) found a dissociation constant for the FDP-aldolase complex of $k_d = 4 \times 10^{-6}M$. An association constant for DHAP of $ka = 1.6 \times 10^{-3}M$ was also reported; thus FDP is bound much more strongly than DHAP. The K_m value of $1.0\text{-}1.5 \times 10^{-5}$ (Richards and Rutter, 1961) is somewhat higher than the dissociation constant for FDP; this is expected since the K_m also includes the catalytic rate constant. Carboxypeptidase treatment of aldolase lowers the K_m, but does not lower the k_d for DHAP or FDP (Dreschlser et al., 1959). These data taken together suggest that the catalytic rate constant is decreased by the loss of the c-terminal

tyrosine; this data provides a mechanism whereby carboxypeptidase treatment of aldolase decreases the V_{max} to 7% of that of the native enzyme (Dreschlser et al., 1959).

A. C-Terminal Analysis

Dreschlser et al. (1959) reported that 93% of the activity of native aldolase was lost upon treatment with carboxypeptidase. When the digestion was conducted in H_2O^{18} and the liberated C-terminal amino acids were isolated and characterized (Kowalsky and Boyer, 1960), approximately three moles of C-terminal tyrosine were found per 149,000 g of protein. Tyrosine was measured by the Folin-Ciocalteu method or the Goodwin and Morton U-V absorption method. Winstead and Wold (1964) also calculated three C-terminal tyrosines from experiments using carboxypeptidase A and B. In addition, by the hydrazinolysis method they found 2.47 and 2.68 moles of tyrosine per 149,000 g of enzyme.

Carboxypeptidase treatment does not alter the spectrophotometrically demonstrated DHAP-enzyme or FDP-enzyme complex; this fact is in agreement with the observation that C-terminal tyrosine is not involved in the binding, but is necessary for the catalytic process of FDP cleavage.

B. N-Terminal Analysis

Udenfriend and Velick (1951) first reported 1.9 and 2.3 moles of proline per 149,000 g of aldolase using I^{131}-p-iodophenylsulfonyl chloride. Hass (1964) reported 3.98

moles of proline per 142,000 g of aldolase by the Edman procedure.

Winstead and Wold, in their attempt to explain the discrepancy in the literature for the number of subunits (3 or 6 subunits) of aldolase, hypothesized that the N-terminal amino acid might have been "masked" by other groups such as an acetyl group. This would then explain why only 3 or 4 moles of proline (instead of 6) were detected per mole of enzyme. Since at least one other glycolytic enzyme, enolase, was known to possess an N-acetylated amino-terminal amino acid (Winstead and Wold, 1964a), they decided to investigate this possibility in aldolase. In their attempt, they found no evidence for an N-acetylated amino-terminal amino acid upon treatment with hog kidney acylase; they therefore concluded that the previous data which indicated that aldolase possessed three or four N-terminal prolines was correct.

C. Sulfhydryl Content

The number of sulfhydryl groups in aldolase has been reported to be between 28 (Swenson and Boyer, 1957; Westhead et al., 1963) and 29 (Benesch et al., 1955), depending on the method used. Swenson and Boyer used a spectrophotometric assay of sulfhydryl groups with p-mercuribenzoate, whereas Benesch et al. (1955) measured -SH groups by amperometric Ag titration. Approximately 10 -SH groups can be titrated without loss of catalytic activity (Swenson and Boyer, 1957). It appears then that at least 10 -SH groups are on or near the

surface of the enzyme. If 8M urea is added to unfold the enzyme, more and more -SH groups are exposed until finally, all 28 -SH groups are available for titration. Stellwagen and Schachman (1962) found 27.1 -SH groups in 8M urea; this value agrees with the previous two results.

IV. Isozymes of Aldolase

The isozyme studies yield information which is very pertinent to subunit structure; in fact, the results from the isozyme work strongly support a model in which rabbit muscle aldolase contains four subunits, and it is hard to reconcile this work with a three subunit model.

Penhoet et al. (1966) have reported three different aldolases in mammalian tissues, each with its own distinctive catalytic, electrophoretic, chromatographic, and immunochemical properties. Acid dissociation and reassociation of any two of the parental aldolases yield five membered sets by electrophoresis, with the two parental type aldolases enclosing this set. Any purified hybrid can also reproduce the five membered set upon reversible dissociation. In addition to producing hydrids artifically, in vitro, hybrids are also found in tissues containing more than one parental type aldolase. For example, adult brain and testes contain all the A-C hybrids; on the other hand, the adult liver and kidney contain all the A-B hybrids.

The sequential appearance of isozymes of aldolase during embryogenesis will now be considered. In the early stages,

isozyme A is the only form synthesized. Then as organogenesis takes place, synthesis of isozyme C results in the formation of A-C hybrids, for example, when differentiation of the ectoderm into brain tissue begins. Correspondingly, the synthesis of isozyme B results in the formation of A-B hybrids when differentiation of the endoderm into kidney and liver occurs (Rutter et al., 1967).

The isozymes have also been characterized by the ratios of their catalytic activity using fructose diphosphate (FDP) and fructose-1-phosphate (F-1-P) respectively, as substrates. After treatment with carboxypeptidase, residual activities and FDP/F-1-P ratios are almost equivalent. This suggests that the function of the C-terminal tyrosine in the catalytic process is different for each of the different isozymes. However they do seem to have certain similarities with respect to their catalytic sites; all three isozymes are reduced and inactivated by $NaBH_4$ in the presence of FDP, indicating a common Schiff base intermediate.

The production of five isozyme hybrids from the parental types suggests that aldolase is composed of four subunits (Rutter et al., 1967). In addition to this evidence, Rutter and coworkers have found that when they dissociated radioactive aldolase A (isolated from mice grown on ^3H leucine) together with non-radioactive aldolase C (isolated from rabbits), reassociated again, and separated the resultant 5 hybrids by electrophoresis, they discovered the following percentages of specific radioactivities (0% representing the

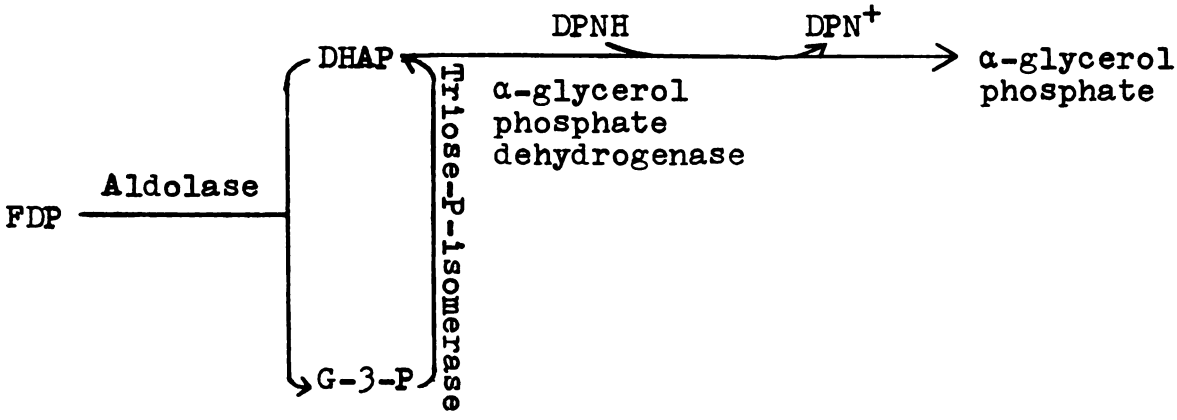
C_4 tetramer): 0%, 25%, 50%, 75% and 100%. These results, of course, support, if not prove, the four subunit model for aldolase.

METHODS

I. Preparation and Assay of Rabbit Muscle Aldolase

Aldolase was prepared by the method of Taylor *et al.* (1948) as modified by Kowalsky and Boyer (1960). After at least three recrystallizations the crystalline suspension was stored in 50% saturated ammonium sulfate at 4°C. Enzyme concentrations were determined at 280 mμ, using the extinction coefficient, $E_1^{0.1\%}_{cm} = 0.91$ (Baranowski and Niederland, 1949). All preparations were homogeneous in the analytical ultracentrifuge and those that were analyzed on polyacrylamide gel electrophoresis gave a single band.

Aldolase was assayed by the procedure of Richards and Rutter (1961) as represented in the following coupled reaction sequence:



The reaction was followed, spectrophotometrically, by the decrease in DPNH absorbance at 340 mμ. The concentrations

of the various components in the 0.4 ml assay were the following: 0.012M FDP (Sigma), 0.003M DPNH (P and L Laboratories), 0.02 mg/ml triose-p-isomerase-α-glycerol phosphate dehydrogenase mixture (Sigma), and 0.2M tris HCl (Sigma), pH 7.5. The FDP used for most of the work was 75% pure, and where it was thought that the impurity might interfere with the results, such as in the inhibition studies, FDP (Sigma) of 98% purity was used. All aldolase preparations had a specific activity of 13.2-13.5 μmoles FDP cleaved/min/mg of enzyme at 25°C and pH 7.5.

II. Ultracentrifugal Analysis

All experiments were performed in a Spinco Model E ultracentrifuge equipped with a phase plate as a schlieren diaphragm. Photographic plates were measured with a Bausch and Lomb microcomparator.

A. Sedimentation Velocity and Diffusion Experiments

Sedimentation velocity experiments were run either with 30 mm or 12 mm single sector cells, at 50,740 rpm in an An E rotor for the former cells and at 59,780 rpm in an An D rotor for the latter cells. Sample volumes for the 30 mm cells and 12 mm cells were 1.25 ml and 0.55 ml, respectively. The longer columns had the advantage of greater sensitivity and could be used for sample concentrations as low as 0.3 mg/ml. Sedimentation coefficients were calculated from the following equation:

$$s = \frac{1}{(t-t_o)\omega^2} \ln \frac{r_p\ (t)}{r_p\ (t_o)}$$

where r_p is the peak maximum in the refractive index gradient curve of picture taken at time t_o (first picture to be measured) or at time t (later pictures), ω is the rotor velocity in radians/sec and s is the sedimentation coefficient in Svedberg units (S).

The diffusion coefficient experiments were determined with a double sector synthetic boundary cell (12 mm) at 4,059 rpm using an An D rotor. A solvent volume of 0.41 ml was used in one sector, and a sample solution volume of 0.14 ml was used in the other sector. The diffusion coefficient was calculated from the following equation (Schachman, 1957):

$$D = \frac{m}{4\pi 60}$$

where m is the slope of a line from a graph which plots $Area^2/Height^2$ vs. time (minutes). The diffusion coefficients were calculated manually using the above graph.

Sedimentation and diffusion coefficients were corrected to 20°C and water (Schachman, 1957). Densities of the solvent were measured at the same temperature as the centrifuge studies with the aid of a hydrometer. Since sedimentation and diffusion coefficient studies were usually performed in parallel, the same temperature was used in both series of experiments. The precise temperature was obtained from the RTIC meter on the ultracentrifuge.

To calculate molecular weights from sedimentation coefficients and diffusion coefficients, the M(S/D) formula

of Svedberg (1940) is used:

$$M = \frac{s\ RT}{D\ (1-\bar{v}\rho)}$$

where R is the gas constant, 8.31×10^7 ergs/mole/degree, T is the absolute temperature, $\bar{v}$ is the partial specific volume, and ρ is the density.

B. Sedimentation Equilibrium and Archibald Molecular Weight Analysis

Standard 12 mm double sector cells were used for the sedimentation equilibrium experiments, whereas both 12 mm and 30 mm double sector cells (the latter for greater sensitivity) were used in the Archibald molecular weight analysis. A volume of 0.06 ml of protein sample was used in one sector, while a solvent volume of 0.02 ml more than the combined height of sample volume and flurocarbon oil (FC-43) was used in the other sector. The flurocarbon oil (0.05 ml, ordinarily) was used to produce column height separation when more than one cell was run at a time.

For the subunit analysis, short column sedimentation equilibrium techniques (Van Holde and Baldwin, 1958) were used along with a three cell arrangement to shorten the experimentation time. The time to reach equilibrium in the subunit analysis was usually 36 hours. The weight-average molecular weight was calculated from the following equation:

$$\frac{1}{M_2^{app}} = \frac{\omega^2\ (1-v\rho)}{RT}\ \frac{C^o}{C_b - C_m}\ \frac{r_b^2 - r_m^2}{2}\ ;$$

$$\text{but} \quad \frac{1}{M_2^{app}} = \frac{1}{M_2} + B_1 \frac{(C_m + C_b)}{2} ,$$

To obtain true weight-average molecular weight Mw, the quantity, $1/M_2^{app}$ (the "apparent" signifies that the molecular weight was measured at a finite concentration), must be extrapolated to zero protein concentration (expressed as $(C_m + C_b)/2$), and the reciprocal be taken. In the above equations, M_2 is the true molecular weight, B_1 is the first virial coefficient, r_m and r_b are the distances from the center of rotation to the meniscus and the bottom of the cell, respectively, and C_m and C_b are the concentrations at the meniscus and the bottom of the cell, respectively. C^o, the original protein concentration, was determined from area measurements of synthetic boundary experiments, similar to those described for the diffusion coefficient experiments.

The Z-average molecular weights, M_z, were also calculated and these "apparent" values were obtained from the following equation:

$$\frac{1}{r_b}\left(\frac{dC}{dr}\right)_b - \frac{1}{r_m}\left(\frac{dC}{dr}\right)_m = \frac{M_z^{app}\,(1-\bar{v}\rho)\,\omega^2\,(C_b - C_m)}{RT}$$

where $(dC/dr)_b$ and $(dC/dr)_m$ are the concentration increments at the bottom and the meniscus of the cell, respectively. The apparent M_z values were also extrapolated to zero protein concentration to obtain the true Z-average molecular weight.

Although it usually takes at least 24 hours to reach equilibrium (and longer when using guanidine HCl solvents) in the sedimentation equilibrium technique, the Archibald tech-

nique can be used to calculate molecular weight very early in the run (within 2 hours). Since the meniscus or the bottom of the cell does not allow any solute pass through these points, conditions for equilibrium are satisfied at all times at these two points. The same equation, which applies to the rest of the points of the cell at equilibrium, then applies at all times for the meniscus and bottom of the cell; the calculation of the molecular weight for the meniscus of the cell is given below:

$$M = \frac{RT}{(1-\bar{v}\rho)\ \omega^2} \frac{(dC/dr)_m}{r_m\ C_m}$$

These experimental parameters have been previously defined. If the subscript (m) is changed to the subscript (b), this equation could then be used to calculate the molecular weight from data obtained from the bottom of the cell.

Archibald and sedimentation equilibrium calculations and the plotting of the data were carried out by a Control Data Corporation 3600 computer with a fully tested program which included a statistical analysis.

III. Viscosity

Viscosity was measured by means of a Cannon-Ubbelohde capillary dilution viscometer. The temperature was maintained at 7.85°C and regulated to within ± 0.002°C. The densities of the solvents were determined with a hydrometer. The densities of the protein solutions were calculated from the partial specific volume, protein concentrations, and solvent

densities as outlined by Schachman (1957). All protein and solvent solutions were filtered through polyvinyl chloride Metricel Type VM-6 Millipore filters (pore size of 0.45 μ) under air pressure.

The equation used to calculate the specific viscosity η_{sp} was the following:

$$\eta_{sp} = \frac{\rho t}{\rho' t'} - 1$$

where ρ' and ρ are the densities (cc/g) of the protein solution and the solvent solution, respectively, and t' and t are the flow times (seconds) for the protein solution and solvent solution, respectively. The reduced viscosity and the intrinsic viscosity are defined in the text.

A portion of the crystalline aldolase suspension was centrifuged and the pellet was dissolved in 0.001M EDTA, 0.05M NaCl, and 0.01M citrate buffer, pH 5.45. The stock solution was then dialyzed against this buffer for 48 hours at 4°C. Solvents containing 0.001M EDTA, 0.05M NaCl, and 0.5M citrate buffer at the appropriate pH were used to produce the desired species: native, intermediate, or subunits. To prepare the final solution for viscosity measurement, 4.8 ml of enzyme stock solution were diluted with 0.6 ml of the appropriate solvent. To make the final dilution solvent, 4.8 ml of the dialysate were diluted with 0.6 ml of the same 0.5M citrate (Na) buffer. This dilution solvent was then used to dilute the enzyme solution in the viscometer. Three concentrations of native enzyme and two concentrations of acid dissociated

subunits were examined to check the experimental technique and verify the published values. The viscosity of the intermediate was determined as a function of time after acid addition at two concentrations at pH 3.40.

IV. Bio-Gel P-150 Column Preparation

Bio-Gel P-150 (50-150) was allowed to swell in 0.001M EDTA, 0.05M NaCl, and 0.1M citrate (Na), pH 3.35, and poured into a column (1.5 cm x 75 cm) equipped with a large funnel to facilitate a single addition of gel. After about 10 cm of gel had settled in the column, the elution buffer was allowed to percolate through the system.

To determine the void volume, 0.1% dextran (Sephadex) was layered on the column and fractions were taken until the dextran peak appeared. The volume obtained in this interval was taken as the void volume.

V. Sucrose Density Gradient Centrifugation

The procedure used for the sucrose density gradient sedimentation velocity (SDSV) experiments was that of Martin and Ames (1961). The experiments were performed with the aid of a Beckman Model L Analytical Ultracentrifuge at a speed of 40,000 rpm (except where noted) and -6°C. The linear gradient of 4.6 ml was 5% to 20% sucrose. This, together with an applied sample volume of 0.1 ml, made a total volume of 4.7 ml. Ten drop fractions were collected per tube and the total number of tubes were noted to the first decimal point. The

following procedure was used to convert fraction number (tube number) to the distance traveled from the meniscus (cm): (1) cm/tube = 3.62 cm/total number of tubes, (2) "corrected number of tubes" = total number of tubes - $\frac{(0.1)(\text{total number of tubes})}{4.7 \text{ ml}}$ (3) (corrected tube number from meniscus) = "corrected number of tubes" - (fraction-tube number of each tube collected), (4) (corrected tube number from meniscus) x (cm/tube) = (distance traveled from meniscus in cm).

Activities (units/ml) were then plotted against the (distance traveled from meniscus in cm). Peak positions (in cm traveled from meniscus) were then used to calculate the sedimentation coefficient of the dimer species using the following relationship:

$$\frac{s^{\text{dimer}}}{s^{\text{tetramer}}} = \frac{\text{Distance traveled}^{\text{dimer}}}{\text{Distance traveled}^{\text{tetramer}}}$$

where s^{tetramer} was taken as 7.9S, which is an average sedimentation coefficient ($s^{o}_{20,w}$) determined by the conventional moving boundary technique using the Model E ultracentrifuge.

RESULTS

I. The Approach to the Problem and Initial Studies

As outlined in the introduction, the primary objective of this research was to achieve an understanding of the mechanism of subunit association of rabbit muscle aldolase: of special interest was the role played by the 3.5S obligate intermediate in both the dissociation and association processes of this enzyme. But before a meaningful study on the mechanism could be undertaken, the subunit structure of the native enzyme had to be defined as well as possible. Although essentially all the available evidence at that time was consistent with the three subunit models for native aldolase, a report by Hass and Lewis (1963) had provided evidence that aldolase dissociated rapidly into 6 subunits when a solution of native enzyme was made alkaline to pH 12.5.

The first objective then of this investigator was to repeat the subunit molecular weight analysis to solve this problem. To effect complete dissociation, the best dissociation solvent system known, guanidine HCl and β-mercaptoethanol, was used.

The second objective was to characterize the 3.5S intermediate. However, before the physical characterization

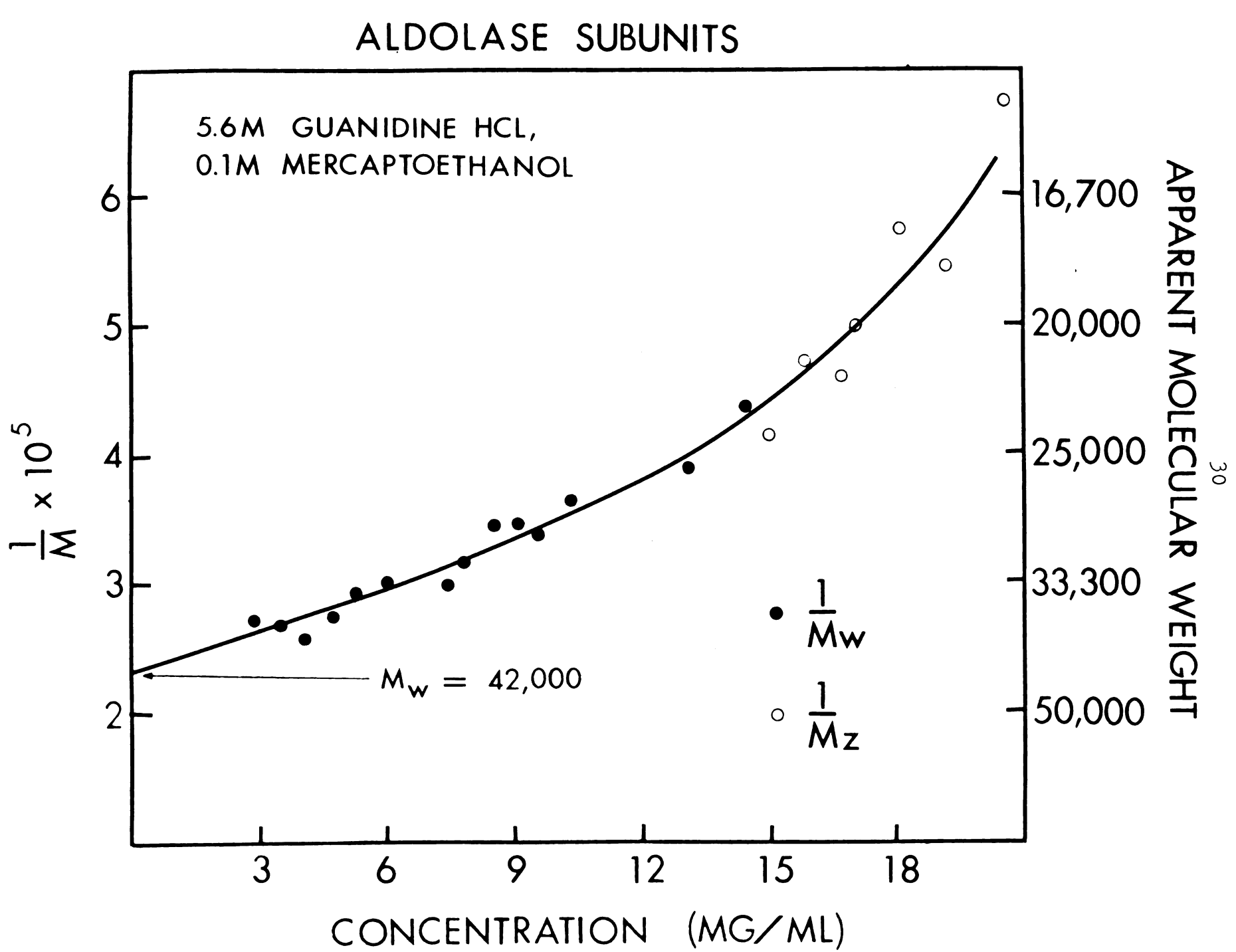
of the intermediate could be conducted, the effect of pH had to be investigated to find the optimum pH for 3.5S intermediate formation. Such a study constitutes the second major section of this introductory chapter (I) and involves an analysis of the sedimentation velocity patterns for native, intermediate, and subunits in the narrow pH range of 3.0 to 4.0.

A. Determination of Rabbit Muscle Aldolase Subunit Molecular Weight in Guanidine HCl and β-Mercaptoethanol

Preparation of the aldolase subunits for molecular weight analysis by the sedimentation equilibrium technique was as follows: a portion of the crystalline aldolase was centrifuged, and the resulting pellet was dissolved in a solution containing 5.6M guanidine HCl, 0.15M NaCl, 0.001M EDTA, and 0.04M tris buffer at pH 7.5. This enzyme solution (approximately 16 mg/ml) was then dialyzed for 48 hours at 4°C against the above solution. Following dialysis the enzyme solution was diluted with dialysate to yield several protein concentrations. Synthetic boundary and short column sedimentation equilibrium experiments (Van Holde and Baldwin, 1958) were performed with the use of the Spinco Model E analytical ultracentrifuge.

Figure 1 shows the weight-average molecular weight of aldolase subunits as a function of protein concentration. There is a strong concentration dependence and the data is somewhat scattered. Using the extrapolated value of 42,000

Figure 1. Extrapolation of the apparent weight-average (M_w^{app}) and apparent Z-average (M_z^{app}) molecular weights to zero concentration. The concentrations were expressed as $(C_m + C_b)/2$ for M_w^{app} and $(C_m + C_b)$ for M_z^{app} (Van Holde and Baldwin, 1958). For details, see text and methods.


ALDOLASE SUBUNITS
5.6M GUANIDINE HCL,
0.1M MERCAPTOETHANOL
$\frac{1}{M} \times 10^5$
6
5
4
3
2
M_w = 42,000
$\frac{1}{Mw}$
$\frac{1}{Mz}$
16,700
20,000
25,000
33,300
50,000
APPARENT MOLECULAR WEIGHT
3
6
9
12
15
18
CONCENTRATION (MG/ML)

(Figure 1) and assuming a native enzyme molecular weight between 142,000 (Schachman and Stellwagen, 1962) and 150,000 (Taylor and Lowry, 1956), the number of subunits was calculated to be between 3.4 and 3.6. Thus these results were not consistent with the six subunit model of aldolase, nor were they consistent with the trimer model. Therefore, in addition to the trimer model, the possibility of a tetramer model had to be considered. It should be mentioned, however, that the tetramer was not consistent with any of the extensive chemical or physical data on aldolase in the literature and was not seriously considered by this investigator until the analysis of the 3.5S intermediate showed an inconsistency with the trimer model.

After this work was complete, two other laboratories (Schachman and Edelstein, 1966; Kawahara and Tanford, 1966) published results of the molecular weight of aldolase subunits in guanidine HCl. Although their experiments were of a limited nature (3 molecular weight determinations by the former investigators and 2 determinations by the latter), both of their results were in good agreement with ours. (See Literature Review for more information on their work.)

B. Determination of the pH Range for Formation of the 3.5S Aldolase Intermediate

For reasons given in the introductory paragraphs of this chapter, sedimentation velocity experiments were performed to determine the most favorable conditions of formation

and stability for the 3.5S intermediate.

Approximately 90 mg of enzyme crystals were centrifuged, and the resulting pellet was dissolved in 18 mls of a solution containing 0.001M EDTA, 0.05M NaCl, and 0.01M citrate (Na) buffer at pH 5.45. This sample was then dialyzed for 36 hours. Nine parts of this dialyzed solution were then acidified with one part of solutions at the appropriate pH containing 0.001M EDTA, 0.05M NaCl, and 0.5M citrate (Na) buffer to yield solutions at the indicated pH values (between pH 3.0 and pH 4.0). After a one hour incubation at $4^{o}C$ and 4 mg/ml, sedimentation velocity experiments were performed for the different samples at 59,780 rpm, using the Spinco Model E analytical ultracentrifuge.

The results in Figure 2 show the effect of pH on the dissociation of aldolase. The top line represents unfolded native enzyme with an apparent sedimentation coefficient of 6.5S. If the pH is lowered to pH 3.6, a transition from unfolded native enzyme to the intermediate occurs, as shown by the upper dashed line. At lower pH values, below pH 3.2, a second transition from the intermediate to monomers occurs, as shown by the second dashed line. Since these experiments show that the intermediate is formed between pH 3.1 and pH 3.6, let us examine the sedimentation velocity patterns in this pH range.

Sedimentation velocity profiles are depicted in Figure 3 at the indicated pH values. In Figure 3A the upper peak, at pH 3.1, is skewed towards monomers with some intermediate

Figure 2. The effect of pH on the structure of aldolase. The protein concentration was 4.0 mg/ml. Each of the final solutions contained the following components: 0.001M EDTA, 0.05M NaCl, and 0.059M citrate (Na) buffer. The amount of each species present at each pH is proportional to the degree of shading, filled circles representing 100% of that species. See text for further details.

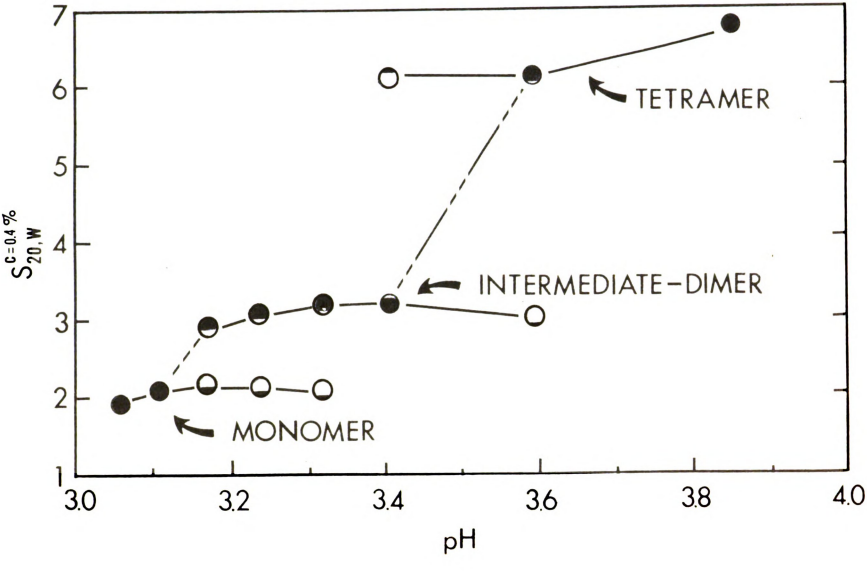
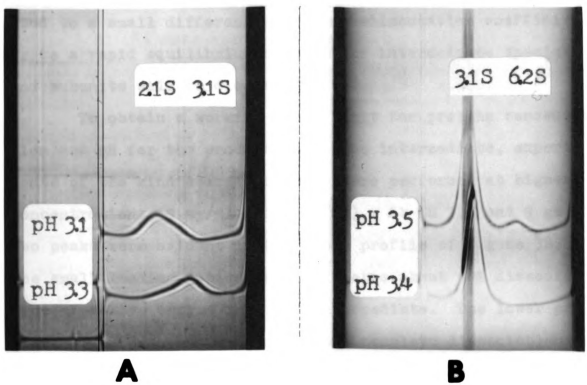

7
6
5
4
3
2
1
$S^{c=0.4\%}_{20,w}$
TETRAMER
INTERMEDIATE-DIMER
MONOMER
3.0
3.2
3.4
3.6
3.8
4.0
pH

Figure 3. Sedimentation velocity patterns of aldolase for the pH range between pH 3.1 and pH 3.5. Sedimentation is from left to right. The times at which pictures were taken after reaching speed (59,780 rpm) were 140 minutes for photograph 3A and 80 minutes for photograph 3B.

A. Upper curve: 4 mg/ml at pH 3.1
   Lower curve: 4 mg/ml at pH 3.3

B. Upper curve: 9 mg/ml at pH 3.5
   Lower curve: 6 mg/ml at pH 3.4


21S 31S
pH 3.1
pH 3.3
A
31S 62S
pH 3.5
pH 3.4
B

present on the leading edge. At pH 3.3, in the lower pattern, the peak with a sedimentation rate of 3.1S is skewed towards intermediate, with some monomers present on the trailing edge. Thus the intermediate and subunit peaks are not resolved completely; whether this result can be attributed to a small difference between sedimentation coefficients, or to a rapid equilibrium between the intermediate species and subunits will be discussed later.

To obtain a working upper limit for protein concentration and pH for the production of the intermediate, experiments of the kind described above were performed at higher concentrations (9 mg/ml and 6 mg/ml). At pH 3.5 and 9 gm/ml, two peaks were evident in the upper profile of Figure 3B; the small leading native peak indicates about 90% dissociation of native enzyme into the intermediate. The lower profile, at pH 3.4, and 6 mg/ml, shows complete dissociation of native enzyme into the intermediate. Thus complete resolution of the native and intermediate peaks is clearly visible. This complete resolution ruled out a rapid equilibrium between native enzyme and the intermediate species or native enzyme and monomers.

Thus pH 3.5 is too high at 9 mg/ml to give complete dissociation into the intermediate species, whereas pH 3.3 at 4 mg/ml is too low and causes further dissociation into monomers. Since the intermediate is essentially the only species present between pH 3.3 and pH 3.45, further physical characterization of this intermediate was examined in this pH range.

II. The Nature of the 3.5S Aldolase Intermediate

Before the quantitative results on the intermediate analysis are presented, it is desirable to consider the different dissociation mechanisms by which a tetramer or a trimer molecule could give rise to a 3.5S intermediate (Figure 4). A measure of the asymmetry of each of the different intermediates considered was determined by calculating f/f_o, the frictional ratio. This ratio can be calculated from the following relationship:

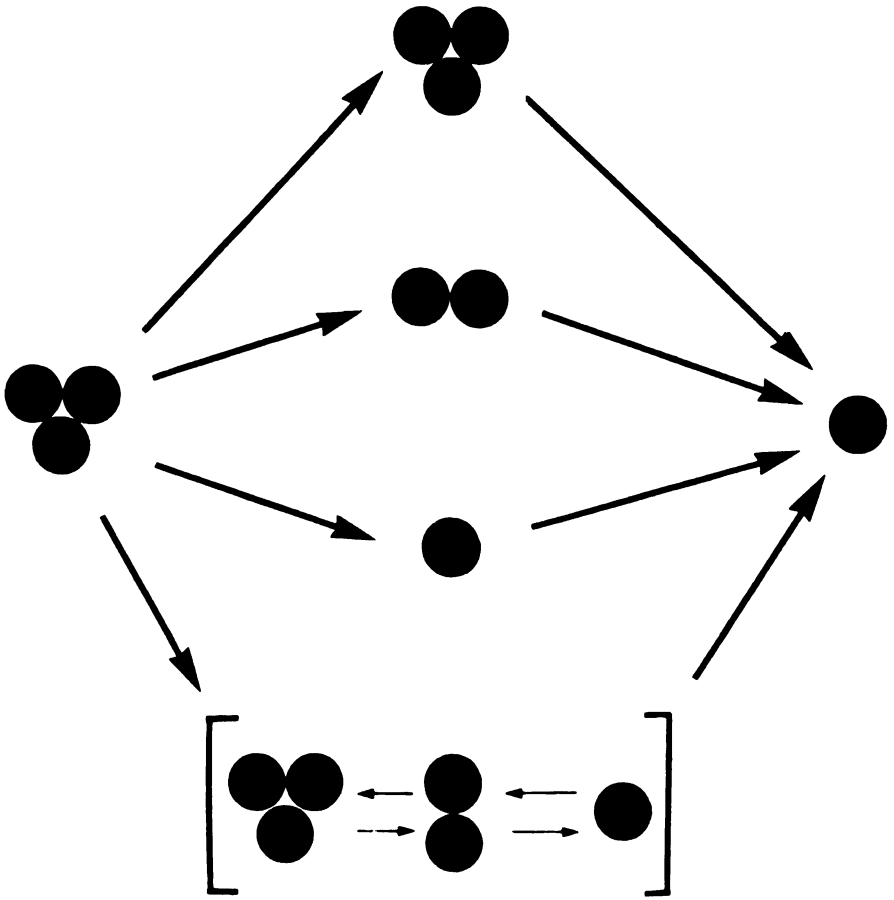
$$\frac{f}{f_o} = \frac{S^o_{max}}{S^o_{exp}}$$

where S^o_{max} is the theoretically determined sedimentation coefficient for perfect spheres and S^o_{exp} is the experimentally determined sedimentation coefficient for the aldolase intermediate, i.e. 3.5S. The only factor which changes in the above relationship is S^o_{max}, and this changes according to the molecular weight assumed for each of the different intermediates considered. The reader is referred to Figure 5 from which the theoretically determined S^o_{max} values were obtained, since it shows the dependence of S^o_{max} on changes in molecular weight. Thus, for example, to accomodate a trimer or a tetramer intermediate, the intermediate would have to be extremely unfolded ($f/f_o = 3.0$); on the other hand, in order for the intermediate to be a monomer, it would have to be very tightly folded ($f/f_o = 1.2$). Similarly, a dimer intermediate would have a conformation with

Figure 4. The reactions depicted here are the most likely mechanism by which a tetramer or a trimer molecule could give rise to a 3.5S intermediate. The frictional ratios (f/f_o), a measure of asymmetry, for each of the different intermediates were calculated to be (1) extremely unfolded trimers or tetramers (f/f_o = 3.0), (2) moderately unfolded dimers (f/f_o = 1.9), and (3) tightly folded monomers (f/f_o = 1.2). See text for the formula used to obtain these ratios and for other details.

TRIMER MODEL

INTERMEDIATE-3.5S



TETRAMER MODEL

INTERMEDIATE-3.5S

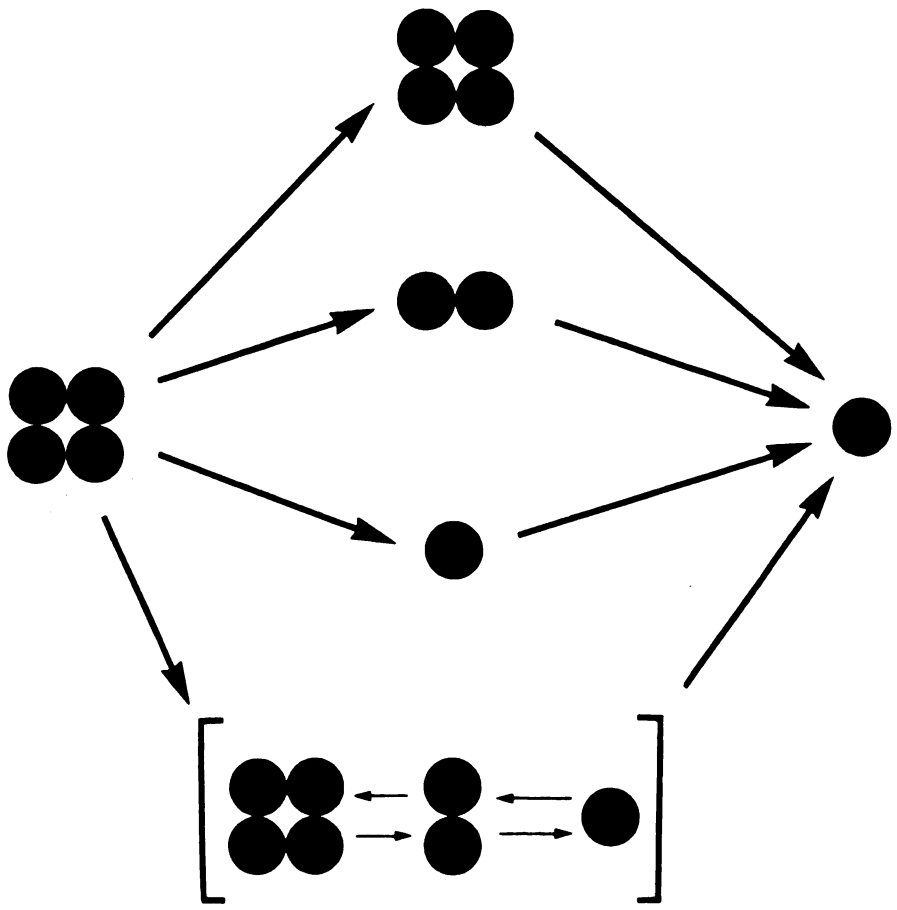
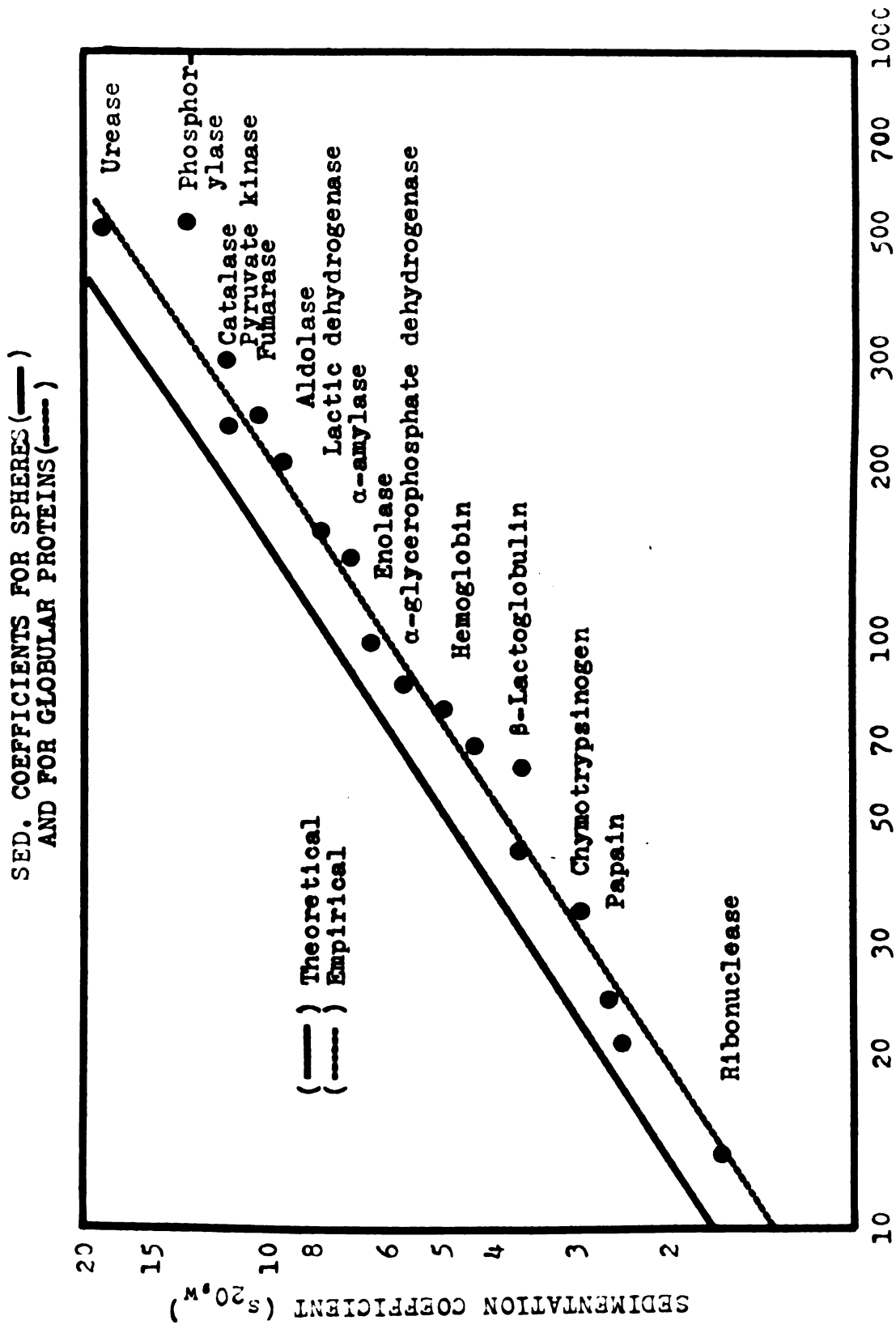


Figure 5. Sedimentation coefficients vs. molecular weights for perfect spheres and globular proteins. The solid line represents the theoretically calculated values, and the dashed line represents the empirical results calculated from experimental data published in the literature (taken from W.H. Holleman's Ph.D. thesis).


SED. COEFFICIENTS FOR SPHERES (——)
AND FOR GLOBULAR PROTEINS (– – –)
(——) Theoretical
(– – –) Empirical
Urease
Phosphor-
ylase
Catalase
Pyruvate kinase
Fumarase
Aldolase
Lactic dehydrogenase
α-amylase
Enolase
α-glycerophosphate dehydrogenase
Hemoglobin
β-Lactoglobulin
Chymotrypsinogen
Papain
Ribonuclease
SEDIMENTATION COEFFICIENT ($s_{20,w}$)
20
15
10
8
6
5
4
3
2
10
20
30
50
70
100
200
300
500
700
1000
MOLECULAR WEIGHT x 10^{-3}

a f/f_o between the two extremes ($f/f_o = 1.9$). The last possibility, a rapid equilibrium, would be too complex to analyze in f/f_o terms.

As mentioned previously, a rapid equilibrium between native enzyme and dimers or native enzyme and monomers has been already excluded (see Section B of Chapter I); however, a rapid equilibrium between dimers and monomers must still be considered as one of the possibilities for the 3.5S intermediate.

The physical characterization of the 3.5S intermediate was approached as follows: First, an analysis for the possible existence of a rapid equilibrium was conducted (Section A). Next, sedimentation and diffusion coefficients were measured and molecular weight values calculated by the M(S/D) technique, under different conditions of dimer preparation, pH, and temperature (Sections B and C). Then, viscosity analysis of the 3.5S intermediate was conducted (Section D); the results will be shown to support the molecular weight analysis in the previous two sections and exclude other possibilities which were described in the introductory paragraphs (see also Figure 4).

A. Analysis for a Rapid Equilibrium by an Investigation of the Concentration Dependence of Sedimentation Coefficient and Molecular Weight by the Archibald Technique

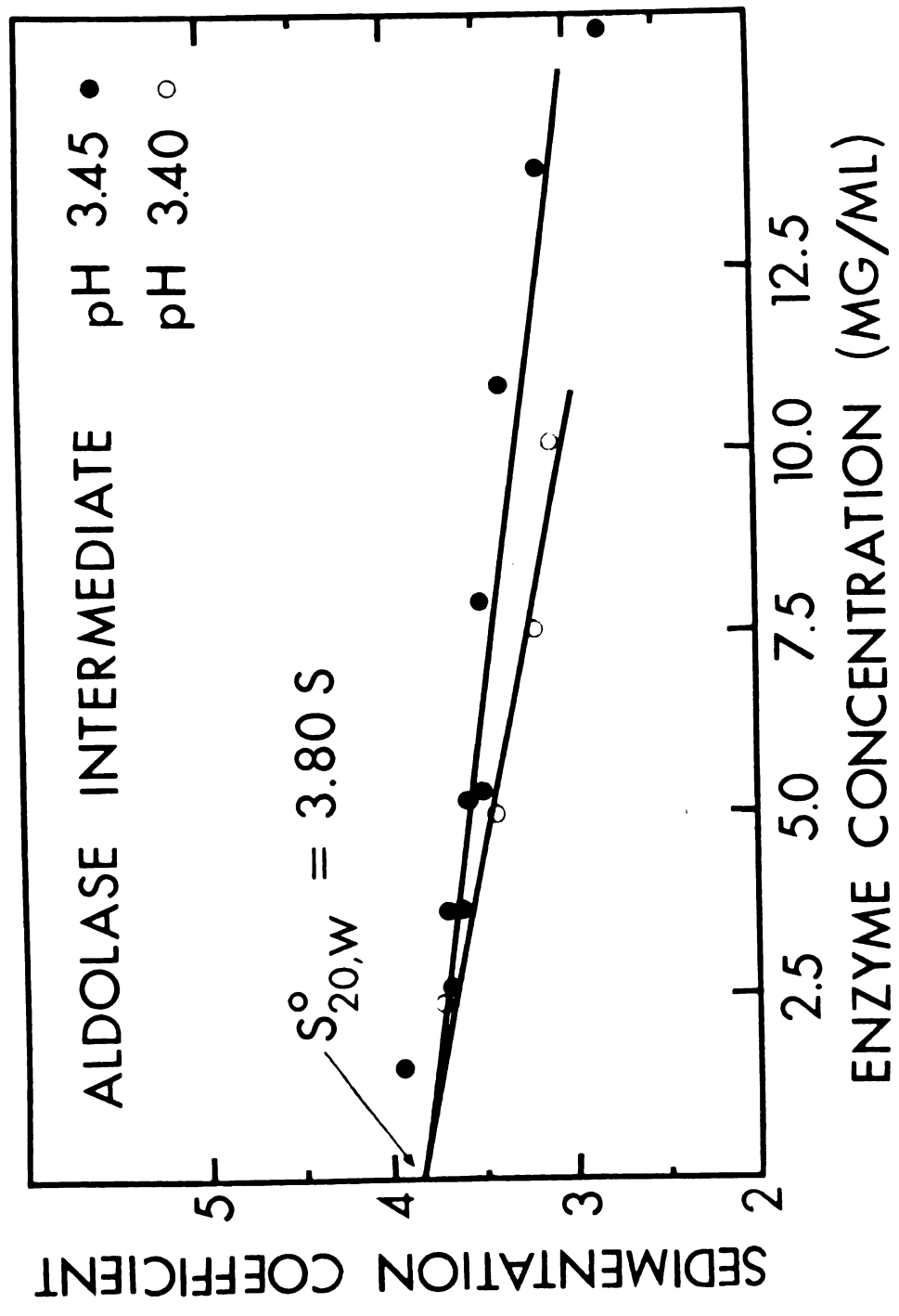
To test the 3.5S intermediate species for the possible

existence of a rapid equilibrium, sedimentation coefficient analysis (for a theoretical treatment of this method, see Fugita (1962)) and molecular weight analysis using the Archibald technique were chosen. The study by Rao and Kegeles (1958) of the rapid equilibrium of α-chymotrypsin using the Archibald method has shown the potential usefulness of such an analysis.

An enzyme solution of approximately 12 mg/ml, previously dialyzed against a solution of 0.01M citrate (Na), 0.05M NaCl, and 0.001M EDTA, was acidified with acid solution, 0.5M citrate (Na), 0.05M NaCl, and 0.001M EDTA (nine parts of the former enzyme solution to one part of latter 0.5M citrate solution) to yield a final pH of 3.45. This solution was then dialyzed for 24 hours. The dialyzed solution was then diluted to 10.4, 9.0, 8.0, 6.0, 4.0, 3.0, 2.0, and 1.2 mg/ml. Sedimentation and diffusion coefficients were then performed simultaneously with the molecular weight analysis by the Archibald technique. For the sedimentation coefficient and Archibald molecular weight determinations, the four lowest concentrations were run in 30 mm cells for better sensitivity (2.5x); the remaining concentrations for these determinations and all concentrations for the diffusion coefficient determinations, however, were run in the more commonly used 12 mm cells.

The sedimentation coefficient data is shown in Figure 6 (closed circles). For a rapid equilibrium, a line with a negative slope at high concentrations and a positive slope

Figure 6. Extrapolation of sedimentation coefficients to zero protein concentration at pH 3.45 (closed circles) and pH 3.40 (open circles).


ALDOLASE INTERMEDIATE
pH 3.45 ●
pH 3.40 ○
$S^{\circ}_{20,w}$ = 3.80 S
SEDIMENTATION COEFFICIENT
5
4
3
2
2.5
5.0
7.5
10.0
12.5
ENZYME CONCENTRATION (MG/ML)

or decreased negative slope at low concentrations is expected; but this was not found. Instead, the graph shows a linear line with a negative slope which, upon extrapolation to zero concentration, yields a sedimentation coefficient of $s^{o}_{20,w}$ = 3.80S. This result then contradicts what one might expect for a rapid equilibrium between different molecular weight species such as between monomers and dimers; on the other hand, it is indicative of a single molecular entity such as monomers, dimers, trimers, or tetramers.

The results from the Archibald molecular weight analysis could not be interpreted by the above analysis because the values were so badly scattered. This scattering might have been expected, however, since the sedimentation velocity patterns exhibited substantial amounts of aggregate or undissociated native enzyme. It was later discovered that the enzyme is not completely dissociated at pH 3.45 and thus, that pH 3.45 was too high.

Diffusion coefficients were also measured from experiments run simultaneously with the Archibald analysis; the results are presented in Figure 7 (closed circles). The details of the diffusion coefficients are presented in a later section (Section B), so a discussion of those results will be deferred until then.

Following the Archibald molecular weight analysis, conventional short column sedimentation equilibrium experiments were performed on the intermediate using different solution column lengths, produced by using different volumes

of 0.02 mg, 0.06 ml, and 0.09 ml, respectively. A qualitatively analysis of the curves indicated the presence of higher molecular weight material. That aggregate was present was attested by the fact that increased slopes were observed near the bottom of the cell. Furthermore, the slopes increased with time over a four day period and thus never attained final equilibrium; these results indicated that aggreagte was continuously being formed over this time period.

In an attempt to reduce aggregation, thought to be caused by non-specific interaction of the intermediate, and to try to obtain complete dissociation of native enzyme into the intermediate, molecular weight analysis M(S/D) was conducted with what was thought to be two improvements: (1) the pH was lowered from pH 3.45 to pH 3.40 to effect complete dissociation, and (2) the total experimentation time was decreased from 5 days to 12 hours to eliminate to the fullest extent possible, the effects of aggregation with time. Also, a new method for intermediate formation was used as described in the following section.

B. Sedimentation Coefficient, Diffusion Coefficient, and Molecular Weight M(S/D) of the 3.5S Intermediate at pH 3.40

Aldolase crystals were spun down, and the pellet was dissolved in 2 ml of a solution containing 0.001M EDTA, 0.05M NaCl, and 0.01M citrate (Na), pH 6.9. The enzyme

solution was then dialyzed against this pH 6.9 solution for six hours to remove any residual ammonium sulfate present (if this dialysis was not done, the enzyme precipitated out of solution in the following acidification procedure). After dialysis a 1 ml aliquot of this solution was acidified by direct addition of 1 ml of a solution containing 0.001M EDTA, 0.05M NaCl, and 0.1M citrate (Na), pH 3.20 (this solution was first diluted five-fold to further reduce the salt concentration during the dissociation procedure). After acidification, the protein solution was dialyzed for three hours against the following buffer: 0.001M EDTA, 0.05M NaCl, and 0.1M citrate (Na), pH 3.40. The solution containing the intermediate was then diluted with the dialyzate to yield four concentrations: 10, 8, 5, and 2.5 mg/ml. Sedimentation and diffusion coefficients were immediately determined at 7°C.

Sedimentation velocity patterns revealed only a small amount of aggregate or undissociated native in the first two samples (10 and 2.5 mg/ml); but more aggregate was observed in the second set of samples (8 and 5 mg/ml), indicating aggregate formation with time.

The extrapolated value for the sedimentation coefficient is shown to be $s^{o}_{20,w} = 3.80S$ (Figure 6, open circles) and agrees closely with previous results at pH 3.45--both were determined at 7°C. Presumably, the reason the slope was more negative in this experiment than before was the lower ionic strength ($\mu = 0.035$ vs. 0.11).

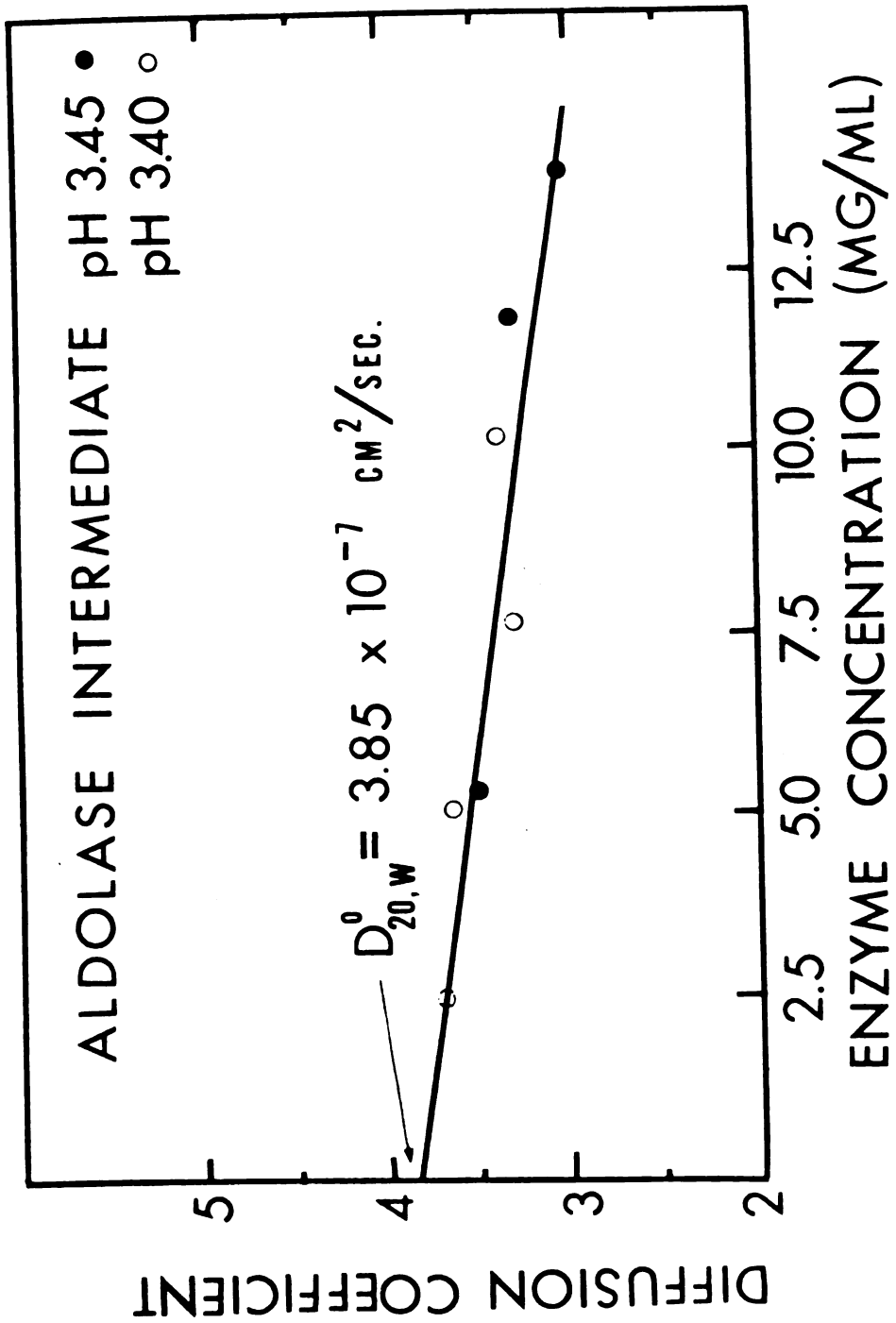
The diffusion coefficient, extrapolated to zero protein concentration, is $D^{o}_{20,w} = 3.85 \times 10^{-7}$ cm^2/sec., (Figure 7, open circles). This data also includes three points from the previously mentioned experiments at pH 3.45 (Section A). Again, the temperature was maintained at 7°C for both experiments.

The molecular weight obtained from the above data is M(S/D) = 91,000 ± 4,000. This value is too high for dimers of subunits of molecular weight, 40,000; a value between 80,000 and 86,000 was expected. Since the molecular weight may have been raised by the presence of a small amount of aggregate, this experiment was repeated at pH 3.35 and 2°C in an attempt to see whether such a molecular weight was obtained under conditions where the 3.5S intermediate might be more stable.

C. Sedimentation Coefficient, Diffusion Coefficient, and Molecular Weight M(S/D) of the 3.5S Intermediate at pH 3.35

To reduce the amount of aggregation to a minimum, sedimentation coefficients and diffusion coefficients were determined as soon as possible (1 hour) after acidification, omitting the dialysis in these experiments. The buffering capacity of the protein was calculated from the amino acid composition. This effect was then compensated for in the acidification procedure--the resultant enzyme stock was pH 3.34, only 0.03 pH units less than the solvent.

Figure 7. Extrapolation of the diffusion coefficients to zero protein concentration at pH 3.45 (closed circles) and pH 3.40 (open circles).


ALDOLASE INTERMEDIATE pH 3.45 •
pH 3.40 ○
$D^0_{20,w}$ = 3.85 x 10^{-7} cm^2/sec.
DIFFUSION COEFFICIENT
5
4
3
2
2.5
5.0
7.5
10.0
12.5
ENZYME CONCENTRATION (MG/ML)

The enzyme was prepared somewhat differently than before, but the main features remained the same. Freshly prepared enzyme was dialyzed for 36 hours. In the first 12-hour period, the enzyme was dialyzed against 0.001M EDTA, 0.05M NaCl, 0.01M citrate (Na), pH 7.00, to remove any residual ammonium sulfate. In the next 24-hour period the enzyme was dialyzed against 0.001M EDTA, 0.05M NaCl, and 0.003M citrate (Na), pH 7.00, with one solvent change after 12 hours; this was to lower the citrate concentration and buffering capacity of the enzyme solution. A test dilution was then run to check for precipitation; none was formed and so the entire sample was acidified 1.25-fold (eight volumes of the former to two volumes of the following pH 2.90 buffer: 0.001M EDTA, 0.05M NaCl, and 0.5M citrate (Na), pH 2.90) to a final pH of 3.35. Sedimentation coefficients and diffusion coefficients were determined immediately after a one hour incubation at 0°C. Simultaneously, the molecular weight analysis by the Archibald technique was begun.

Unfortunately, the results from the Archibald molecular weight experiments were inconclusive because of the uncertainty found in computing the area under the curve of dC/dr vs. r in the schlieren patterns. Since the vertical lines at the meniscii were very curved at high speeds and not coincident, the direct subtraction of the solution line from the solvent line did not properly subtract out the sedimentation of the solvent itself. This had to be

corrected by shifting the curves until they coincided. The resulting uncertainty was as large as 16%. The uncorrected values ranged from 87,000 to 130,000, whereas the corrected values (see Table I) ranged from 96,000 to 116,000. Because of this error, these molecular weight values should be interpreted with caution.

Extrapolation of the sedimentation coefficient to zero concentration gave a value of $s^{o}_{20,w} = 3.45S$ (Figure 8); this value (at 2°C and pH 3.35) is 0.35S units below the previous values (at 7°C and pH values of 3.45 and 3.40). This difference in the sedimentation coefficient is probably indicative of a more unfolded dimer at lower pH values and lower temperatures. A lower pH is expected to unfold the molecule to a greater extent and lower temperature is expected to unfold the molecule by lowering the intramolecular hydrophobic interactions. Also, the lower temperature would be expected to reduce the collision frequency and thus, prevent aggregation. In fact, no aggregation was observed in the sedimentation velocity patterns in contrast to the previous experiments.

The extrapolated value for the diffusion coefficient was $D^{o}_{20,w} = 3.73 \times 10^{-7}$ cm^2/sec (Figure 9), a value 0.12 units less than the previous value. This decrease is practically within experimental error, but it may reflect a more unfolded dimer, in agreement with the sedimentation coefficient data.

Since no apparent aggregation was observed in the

Figure 8. Extrapolation of the sedimentation coefficients to zero protein concentration for the rabbit muscle aldolase intermediate at pH 3.35. See text for details.

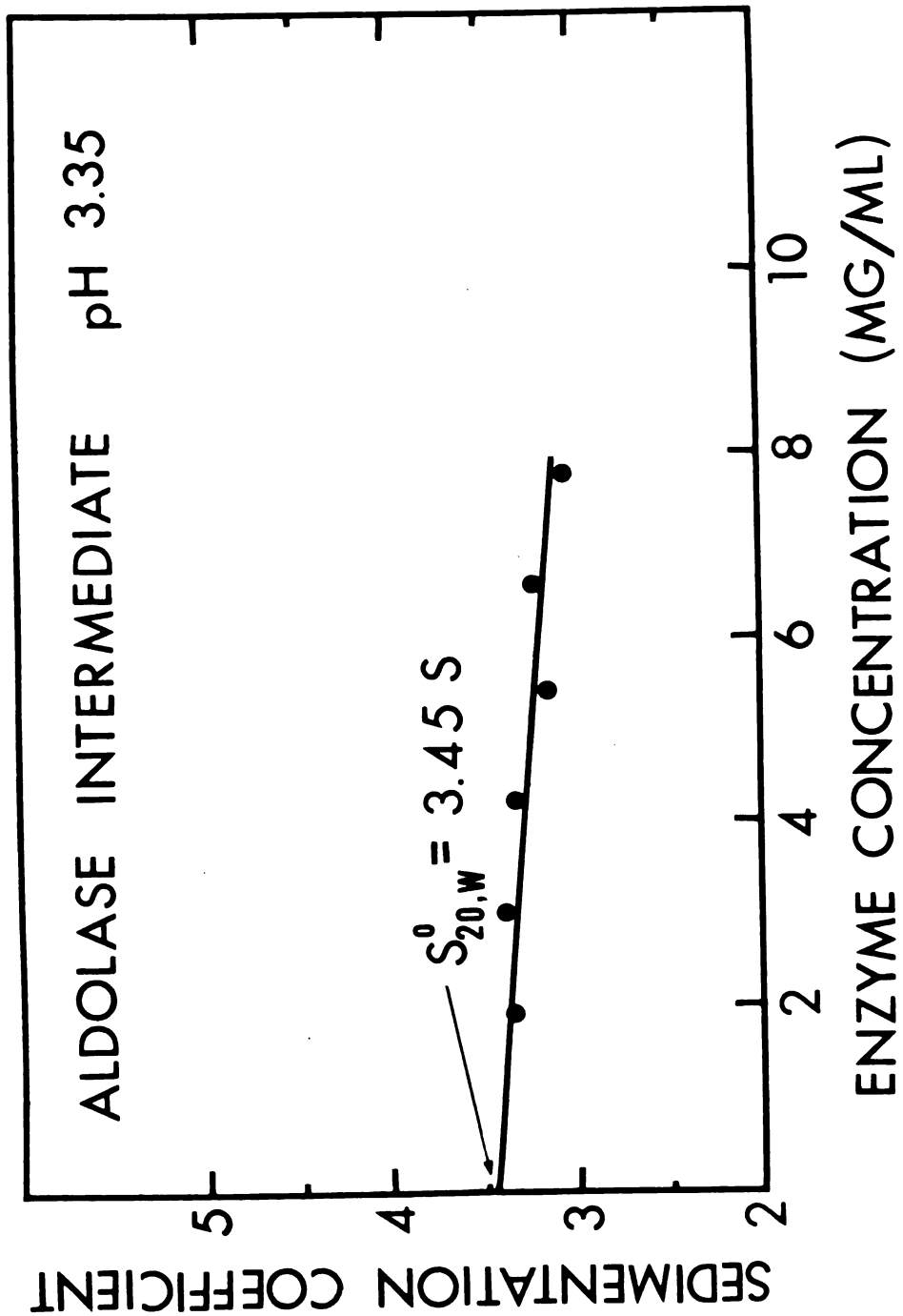

ALDOLASE INTERMEDIATE pH 3.35
$S^0_{20,w}$ = 3.45 S
SEDIMENTATION COEFFICIENT
5
4
3
2
2
4
6
8
10
ENZYME CONCENTRATION (MG/ML)

Figure 8. Extrapolation of the sedimentation coefficients to zero protein concentration for the rabbit muscle aldolase intermediate at pH 3.35. See text for details.

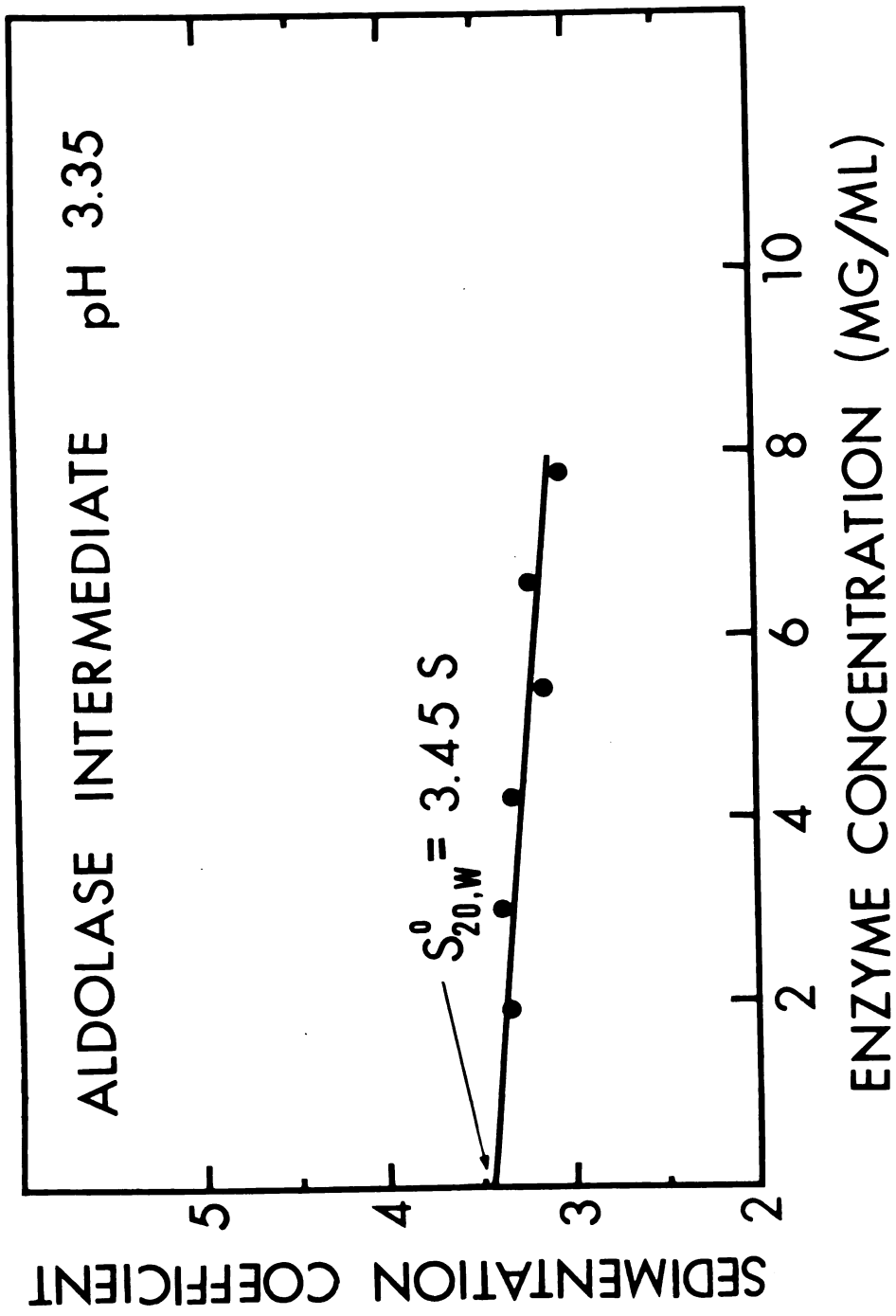
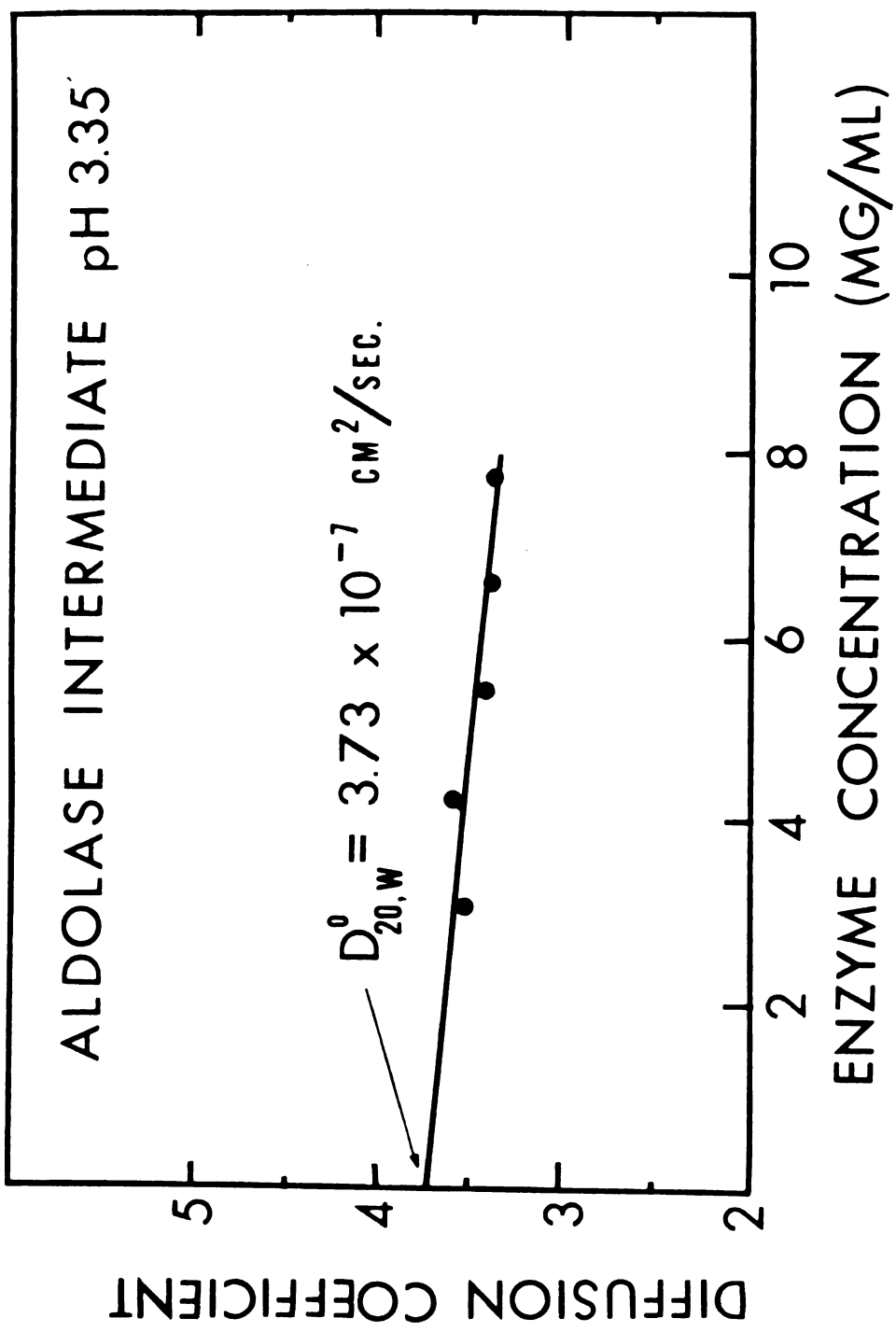

ALDOLASE INTERMEDIATE pH 3.35
$S^0_{20,w}$ = 3.45 S
SEDIMENTATION COEFFICIENT
5
4
3
2
2
4
6
8
10
ENZYME CONCENTRATION (MG/ML)

Figure 9. Extrapolation of the diffusion coefficients to zero protein concentration for the rabbit muscle aldolase intermediate at pH 3.35. See text for details.


ALDOLASE INTERMEDIATE pH 3.35
$D^0_{20,W}$ = 3.73 x 10^{-7} cm^2/sec.
DIFFUSION COEFFICIENT
5
4
3
2
2
4
6
8
10
ENZYME CONCENTRATION (MG/ML)

sedimentation velocity patterns, these values for sedimentation coefficient and diffusion coefficient were considered the most reliable. For this reason, these values will be used in all discussions of the intermediate.

The molecular weight can be calculated from the sedimentation coefficient and diffusion coefficient data, using the published values for the partial specific volume. The published values for partial specific volume were determined at 20° and 5°, and there is some uncertainty as to the interpolation procedure to use to correct for temperature dependence. Thus, the amount of correction upon temperature change on the partial specific volume is from 0.000365/degree (Hunter, 1966) to 0.001/degree (Taylor and Lowry, 1956). If the former is correct, a partial specific volume of 0.735 cc/g should be used to compute the sedimentation coefficient; this yields a value of $s^{o}_{20,w}$ = 3.45S. But if the latter is correct, a corrected partial specific volume of 0.722 cc/g is obtained; from this, a sedimentation coefficient of $s^{o}_{20,w}$ = 3.26S is computed. Then by using these values, 3.26S and 3.45S, and a partial specific volume of 0.742 cc/g at 20°C (Taylor and Lowry, 1956) in the Svedberg equation, the respective values for the molecular weight are 83,000 and 86,000. A summary of the molecular weight results for the intermediate at pH 3.35, and pH values at pH 3.40 and pH 3.45, are presented in Table I.

The molecular weight of the 3.5S intermediate of 83,000 to 86,000 is most consistent with a dimer molecule,

TABLE I

PHYSICAL PROPERTIES OF 3.5S ALDOLASE INTERMEDIATE

pH	$s^{o}_{20,w}$	$D^{o}_{20,w}$	% PURITY OF INTERMEDIATE	M(S/D)	MOLECULAR WEIGHT (ARCHIBALD)
3.40-3.45	3.80S	3.85	70-85	91,000	130,000-200,000
3.35	3.45	3.73	85-95	83,000-86,000	96,000-116,000

since these molecular weights are slightly more than twice the molecular weight of aldolase subunits, assuming a value of 40,000 (see Results in Chapter I, Section A).

To provide supporting evidence for the identification of the intermediate as a dimer of aldolase subunits, further physical information on the intermediate structure was obtained from intrinsic viscosity measurements. It will be also shown that other possibilities, which were described in the introduction of this chapter, such as unfolded native enzyme or folded subunits are excluded.

D. Determination of the Intrinsic Viscosity of the 3.5S Intermediate of Aldolase and Comparison with that for the Native and Subunits

Let us first give a few definitions to make clear the nomenclature for different types of viscosities. Specific viscosity η_{sp} is defined by the following identity, $\eta_{sp} = (\eta' - \eta)/\eta$, where η' is the viscosity measured for the macromolecular solution and η is the viscosity for the pure solvent. But as such, specific viscosity is not too useful for comparisons between different macromolecular solutions since it is proportional to concentration. On the other hand, reduced viscosity η_{red} is independent of concentration (for ideal solutions) and is given by the relationship:

$$\frac{\eta_{sp}}{c} = \eta_{red}$$

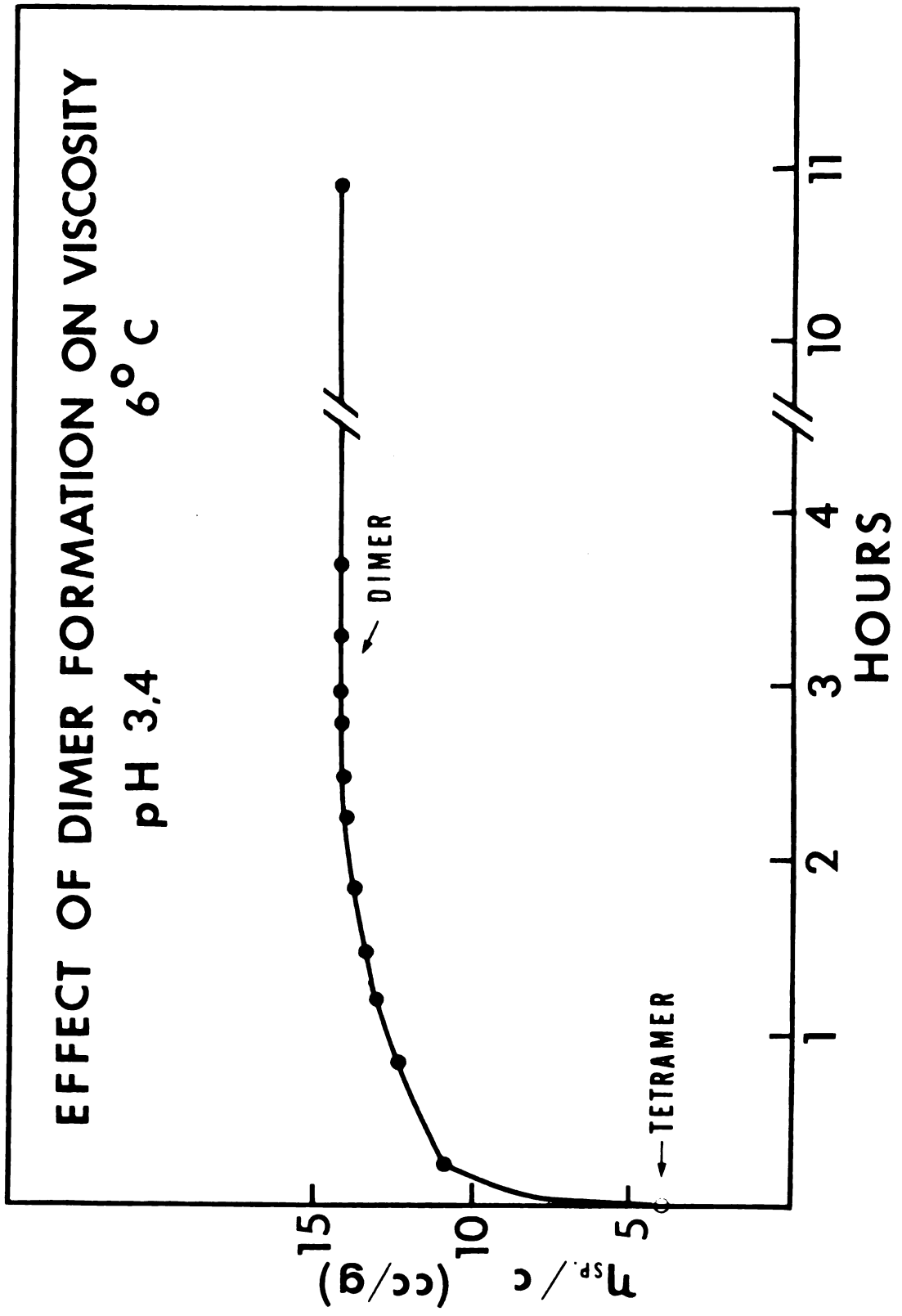
However, since most macromolecular solutions are not ideal,

the reduced viscosity is usually extrapolated to zero concentration, and this extrapolated value, the most useful quantity, is commonly called the intrinsic viscosity $[\eta]$.

Before presenting the experimental details and the results, it is of interest to first predict what viscosity one might expect for each of the different intermediates. If, in fact, the intermediate is a distinct species, such as extremely unfolded tetramers or trimers, unfolded dimers, or folded subunits, intrinsic viscosity should be a very sensitive tool to help distinguish between these possibilities--independent of the previous molecular weight analysis. For example, if the intermediate is folded monomers, the intrinsic viscosity should be close to those values found for globular proteins, i.e. about 4 cc/g. Alternatively, unfolded tetramers or timers would have to have a much higher intrinsic viscosity than subunits, i.e. higher than 24 cc/g. The intrinsic viscosity for an moderately unfolded dimer, however, would be mid-way between 4 cc/g and 24 cc/g.

In the first experiment, kinetics of intermediate formation were followed beginning immediately after addition of acid. The final conditions were pH 3.4, 8.3 mg/ml, and 6°C. (See Methods for preparation of dimers). After 3 hours the reduced viscosity for the dimer intermediate reached a limiting value of 14.5 cc/g, which was constant for at least 10 hours (Figure 10). In a similar experiment at a slightly lower concentration of 6.2 mg/ml, a reduced viscosity of 14.0 cc/g was obtained. These data, together with the viscosities

Figure 10. Effect of dimer formation on reduced viscosity. See text for details.


EFFECT OF DIMER FORMATION ON VISCOSITY
pH 3,4
6° C
DIMER
TETRAMER
HOURS
1
2
3
4
10
11
5
10
15
$\eta_{sp.}/c$ (cc/g)

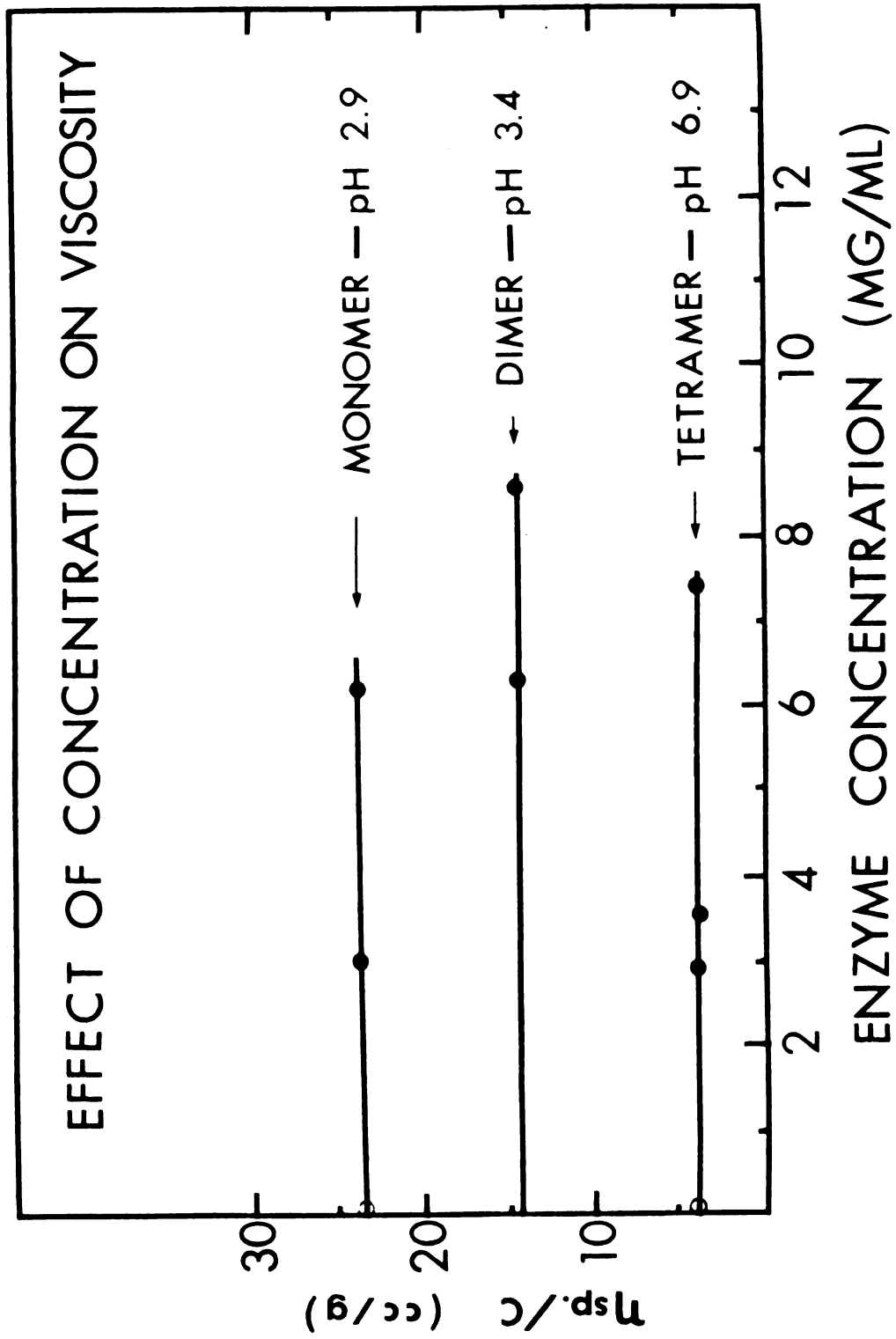
determined for the native enzyme and subunits, are reported in Figure 11 (closed circles); here it is seen that our values for reduced viscosities for the native enzyme and subunits extrapolate to the same intercepts and yield the same intrinsic viscosities as those (open circles) reported by Stellwagen and Schachman (1962). The intrinsic viscosity of 14.0 cc/g for the intermediate is approximately half-way between the value of 4 cc/g for the native enzyme and 24 cc/g for the subunits (Figure 11). As discussed in the introduction of this section, the fact that the viscosity for the intermediate is mid-way between 4 cc/g (folded native) and 24 cc/g (unfolded subunits) suggests, independently of the molecular weight analysis, that the intermediate is a moderately unfolded dimer.

An independent method for the measurement of asymmetry of the dimer molecule involves calculating the β parameter from the following equation (Scheraga, 1961):

$$\beta = \frac{s\,[\eta]^{1/3}}{M^{2/3}\,(1-\bar{v}\rho)}$$

Thus, by using the experimentally determined values for the intrinsic viscosity (14.0 cc/g), sedimentation coefficient (3.45S), and molecular weight (84,000) for the dimer molecule, the β value from the above formula is calculated to be 2.28 to 2.45 x 10^6. This value results in an axial ratio of about 8 to 11 (see Table II on page 6 of Scheraga's book (1961)) assuming a prolate ellipsoid. The value of β for an oblate ellipsoid is essentially independent of axial ratio in this range.

Figure 11. Extrapolation of the reduced viscosity to zero protein concentration for native enzyme, dimers, and monomers at the indicated pH values. See text for details.


EFFECT OF CONCENTRATION ON VISCOSITY
MONOMER — pH 2.9
DIMER — pH 3.4
TETRAMER — pH 6.9
30
20
10
2
4
6
8
10
12
ENZYME CONCENTRATION (MG/ML)
ηsp./C (cc/g)

The values for the axial ratios, together with the pertinent physical data from this and other sections are summarized in Table II.

In conclusion the viscosity of the 3.5S intermediate is most consistent with a dimer. The following discussion shows how the physical data in Table II excludes other possibilities for the intermediate, such as tightly folded subunits, or extremely unfolded native enzyme.

If we assume the intermediate is subunits with a molecular weight of 40,000, these subunits must be tightly folded--a molecule of this molecular weight must be very compact ($f/f_o = 1.3$) to have a sedimentation coefficient of 3.5S (Figure 5). But for reasons given earlier the observed value of 14.0 cc/g is not consistent with a folded globular protein; furthermore, using the β value calculated from the previous formula, an axial ratio of 400:1 is estimated. Thus a contradiction results: it is obvious that the subunits cannot be both very compact and extremely unfolded. Therefore, the intermediate cannot be subunits, since the symmetry data cannot be reconciled using a molecular weight of about 40,000.

We next analyze the possibility that the intermediate might be an unfolded polymeric (trimer or tetramer) enzyme. The same data is used, with the molecular weight being changed to that of trimers or tetramers, i.e. 142,000 to 160,000. For a molecule of this molecular weight to possess a sedimentation coefficient as low as 3.5S, it must be

TABLE II

PHYSICAL PROPERTIES OF ALDOLASE MONOMERS, DIMERS, AND TETRAMERS

SPECIES	pH	INTRINSIC VISCOSITY	MOLECULAR WEIGHT	AXIAL[f] RATIO
MONOMER	2.9	23.5 cc/g	38,300-46,600[a] 41,000-42,000[b] [c]	13
DIMER	3.4	14.0 cc/g	83,000-86,000[d]	8-11
TETRAMER	6.9	4.0 cc/g	142,000[e] 160,000[b]	6

a. Edelstein and Schachman, 1966.

b. Kawahara and Tanford, 1966; and Yphantis, 1968. This appears to be the most probable value.

c. See Results, Chapter I-A.

d. See Results, Chapter II-B, C.

e. Stellwagen and Schachman, 1962.

f. Axial ratios are not to be taken literally--includes a factor of hydration.

extremely asymmetric (f/f_o = 3.0, see Figure 5). When the intrinsic viscosity of 14.0 cc/g, a molecular weight of 150,000, and a sedimentation coefficient of 3.5S are combined in the β formula, a value for β of 1.5×10^6 is calculated. But theoretically the lowest possible β value is 2.12×10^6 which applies for perfect spheres; this value can only increase with increasing asymmetry. Therefore, this β value (1.5×10^6) takes a value not allowed and results in a contradiction: that this molecule of 150,000, assumed to be very asymmetric, is required to be more symmetric than a perfect sphere. Hence the results from the viscosity experiments demonstrate that the intermediate is not folded monomers, or unfolded trimers, or unfolded tetramers. But, in fact, is most consistent with a dimer of aldolase subunits. These results from the viscosity analysis, then, do indeed support the conclusions from the molecular weight analysis: that the 3.5S aldolase intermediate is a moderately unfolded dimer.

E. Analysis for Incomplete Dissociation and Non-specific Aggregation of Dimers: Attempts to Separate Dimers from Higher Molecular Weight Material and Estimate Dimer Purity

1. Effect of Salt and Temperature on Homogeneity of Dimer Preparations

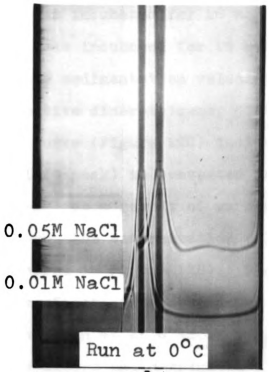
Aldolase crystals were centrifuged and dissolved in the following solution: 0.001M EDTA, 0.05M NaCl, and 0.003M

citrate (Na), pH 7.00. The enzyme solution was then dialyzed against this buffer for 15 hours; the final enzyme concentration was 13.9 mg/ml. After dialysis the enzyme solution was divided into equal fractions. Fraction one was not dialyzed further; but fraction two was dialyzed for 5 more hours against the above buffer solution, diluted five-fold with water. Both solutions were then dissociated by acidification to form dimers with a 1.25-fold dilution with 0.5M citrate (Na), 0.001M EDTA, and 0.05M NaCl, pH 2.90. After one hour, the solutions were then analyzed by sedimentation velocity experiments.

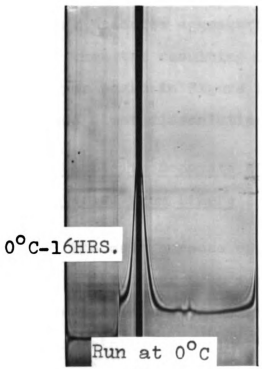
The results (Figure 12A) show that the sample, which was dialyzed the second time (fraction two) against the 0.01M NaCl, buffer solution to remove most of the salts, was essentially free of higher molecular weight material; while the other sample, dialyzed against the 0.05M NaCl, buffer solution, had a substantial amount of material sedimenting at a faster rate. Thus it seemed best for subsequent experiments to remove almost all the salts before acid dissociation.

For the second part of this study, to test the effect of higher temperatures on the stability of dimers, a sample of dimers at 11.0 mg/ml from the above preparation was divided into two parts and treated as follows: (1) Sample I was incubated for 16 hours at 0°C and the relative distribution of tetramers (and aggregate) and dimers was analyzed by conventional moving boundary sedimentation velocity (Figure 12B). (2) Sample II was subdivided into two additional parts. The

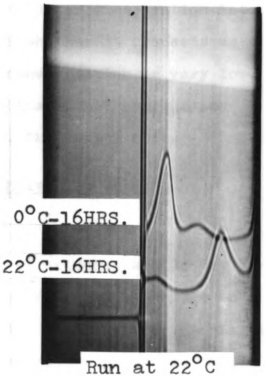
Figure 12. Sedimentation velocity patterns of different dimer preparations exposed to various conditions. Sedimentation is from left to right. The times at which pictures were taken after reaching speed (59,780 rpm) were 60 minutes for photograph 12A and 12B and 25 minutes for photograph 3C. The main peak in A, B, and C (upper curve only) was shown in previous experiments to be the dimer intermediate with a sedimentation coefficient of approximately 3.1 (4 mg/ml). See text for details.


0.05M NaCl
0.01M NaCl
Run at 0°C

A


0°C-16HRS.
Run at 0°C

B


0°C-16HRS.
22°C-16HRS.
Run at 22°C

C

first part (II-0°) was incubated for 16 hours at 0°C and the other part (II-22°) was incubated for 16 hours at 22°C. Then both were analyzed by sedimentation velocity experiments to determine the respective dimer-tetramer distributions. The peaks in the upper curve (Figure 12C) indicate that some of the dimer sample (left peak) is converted into tetramer or aggregate (right peak) as a result of warming the sample (II-0°) to 22°C to run the sedimentation velocity experiment (compare to the control in Figure 12B). The lower curve in Figure 12C show the effect of incubation at 22°C instead of 0°C for 16 hours, and run at 22°C as in sample (II-0°): essentially all the dimers had aggregated within this period of incubation, and some of the dimers had dissociated into monomers. It is not known whether dimer dissociation into monomers precedes aggregation, or whether it succeeds aggregation when the resulting dimer concentration is very low. The upper peaks in Figure 12C indicate that aggregation precedes dimer dissociation, i.e. the latter case.

2. Attempts to Separate Higher Molecular Weight Material from Dimers

The main purpose of these experiments was to try to separate the dimer from aggregate in order to be able to measure the molecular weight of the 3.5S intermediate sample by sedimentation equilibrium. Separation by sedimentation velocity separation cells, sucrose density centrifugation, Bio-Gel filtration, and Sephadex filtration was tried. It

should be emphasized that sedimentation velocity experiments showed no evidence of any higher molecular weight material in the dimer preparations. However, sedimentation equilibrium, a technique much more sensitive to small amounts of heterogeneity, indicated some higher molecular weight material to be present.

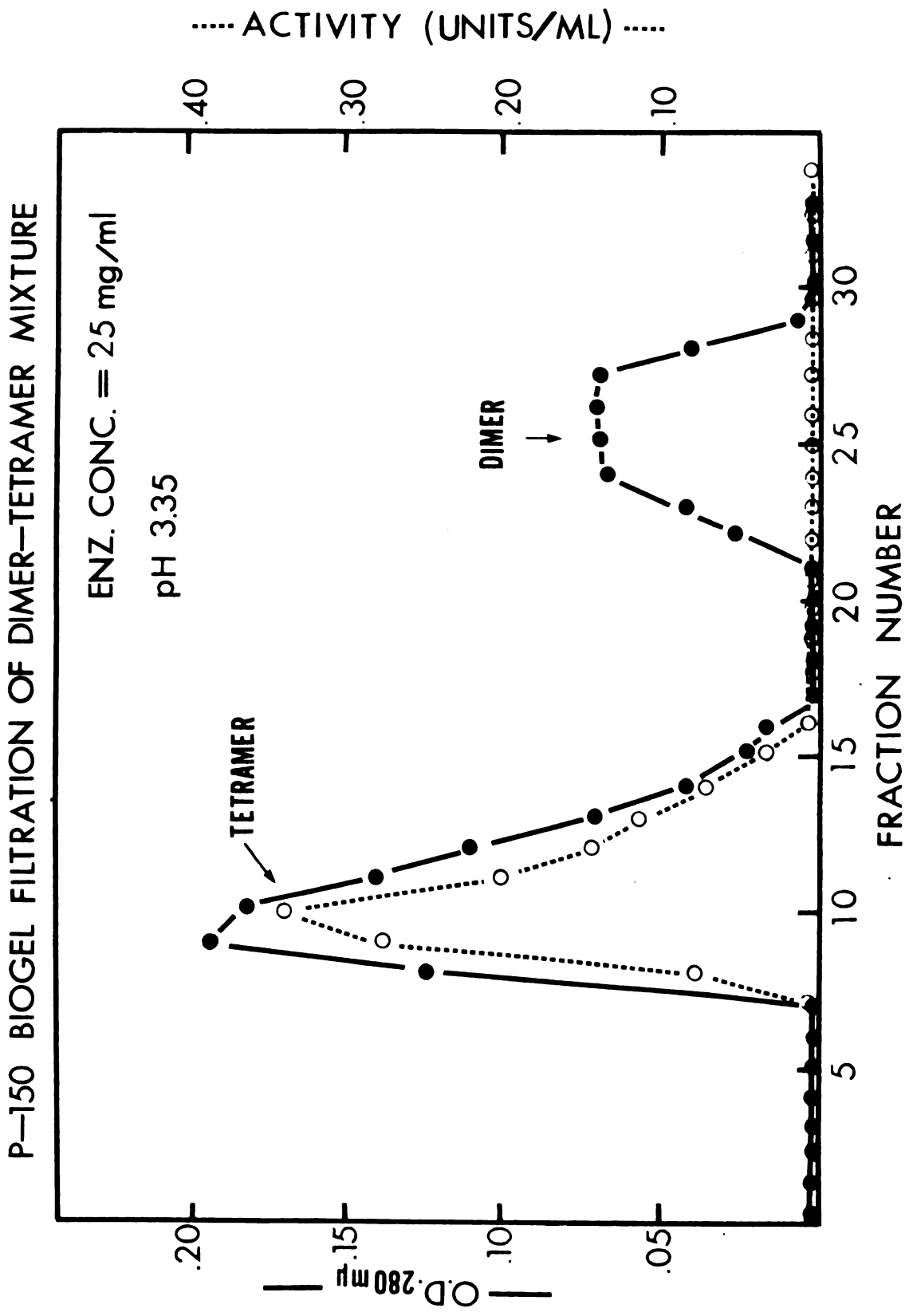
Both of the first methods, separation by sedimentation velocity separation cells and separation by sucrose density sedimentation velocity experiments, were unsuccessful.

The Bio-Gel and Sephadex filtration experiments, however, were successful in the attempt to separate the dimers from higher molecular weight material.

The procedure for preparation of the protein sample was as follows: Aldolase crystals were spun down, dissolved in 0.001M EDTA, 0.05M NaCl, and 0.003M citrate (Na) buffer at pH 7.0, and dialyzed against this solvent for 24 hours. The dialysate was diluted five-fold with water, and dialysis continued for another 12 hours. Four parts of this enzyme solution were then acidified with one part of a solution containing 0.001M EDTA, 0.05M NaCl, and 0.5M citrate (Na), pH 3.35. A 0.5 ml sample of dimers at 25 mg/ml was applied to a column (80 x 1.5 cm) containing Bio-Gel P-150, equilibrated in the pH 3.35 citrate buffer at 4°C.

Five ml fractions were collected and the optical density was determined at 280 mμ. The results are shown (closed circles) in Figure 13. The void volume, determined with a 1% dextran marker, was 55 ml and is equivalent to the first 11

Figure 13. P-150 Bio-Gel filtration of an aldolase sample at pH 3.35 and 25 mg/ml. Protein concentration was determined by absorbance at 280 mμ (shown in graph as solid lines) and activity was determined after reactivation of each fraction at pH 7.5 (shown in graph as broken lines). See text for details.


P—150 BIOGEL FILTRATION OF DIMER—TETRAMER MIXTURE
ENZ. CONC. = 25 mg/ml
pH 3.35
TETRAMER
DIMER
..... ACTIVITY (UNITS/ML) .....
— O.D. 280 mμ —
FRACTION NUMBER
.20
.15
.10
.05
40
30
20
10
5
10
15
20
25
30

fractions. After the protein determination was completed, the fractions were diluted five-fold with 0.2M imidazole (Na) pH 7.5, incubated for 2 hours at 0°C and then for an additional hour at 22°C to reactivate the inactive dimers and native enzyme (open circles, Figure 13). The higher specific activity, 9.3, was obtained with fraction 11; therefore, this peak was essentially all native enzyme which was only unfolded at pH 3.35 and not dissociated. Thus even at pH 3.35, high protein concentration (25 mg/ml) prevented complete dissociation. The fractions were too low in protein concentration to be analyzed further by ultracentrifugal experiments. However, an attempt was made in the Sephadex filtration experiments to concentrate the protein for further analysis. The method of protein concentration and the results will be described below.

Following the separation by Sephadex filtration, tubes from the dimer peak and from the aggregate or tetramer peak were separately pooled and concentrated for subsequent sedimentation velocity analysis. The concentration of protein was accomplished by absorbing the water from the dialysis bag containing the protein solution with G-25 Sephadex. Subsequent sedimentation velocity patterns of the concentrated dimer fraction revealed a molecule sedimenting at the same rate as the dimer, whereas the tetramer-aggregate peak which was also concentrated by G-25 Sephadex showed the presence of native enzyme and aggregate. Presumably, the aggregate was formed after separation by gel filtration in the concen-

tration step and because of this, further physical analysis of the dimer was not pursued.

Summarizing this chapter, the molecular weight of the intermediate is most consistent with a dimer of aldolase subunits. The best conditions for the production of the dimer are (1) very low salt (0.01M NaCl), (2) 0°-2°C, (3) pH 3.35, and (4) final enzyme concentration between 1.0 and 10.0 mg/ml. Also, the dimer is more reactive at higher temperatures and consequently aggregates. And if the native enzyme is not essentially free of salt before dissociation, the production of the intermediate is hampered; the product aggregates and in fact, if too high a salt concentration is present, the enzyme precipitates out of solution.

The sedimentation coefficient is probably not affected very much by any aggregation present because of the "kinetic purification" during the run. What is meant by this is that the aggregate, which has a much higher sedimentation rate will move down the cell faster and be separated completely from the intermediate boundary. Consequently, it will not interfere with the slower moving boundary of the intermediate. On the other hand, the diffusion coefficient is a weight-average of the diffusion coefficients of all the components in the solution. Thus the aggregate indicated by the sedimentation equilibrium experiments would tend to yield a diffusion coefficient too small compared to what it should be; therefore, proper correction of the diffusion coefficient would increase it and consequently would decrease the

molecular weight, according to the Svedberg equation. An estimation of this error would be helpful in defining the accuracy of the molecular weight data. The following section describes attempts to estimate the error by an empirical determination of the purity of the dimer preparation.

3. An Estimation of the Purity of the Dimer Preparation by Comparing the Areas of the Dimer and Native Enzyme Peaks in the Sedimentation Velocity Patterns

A stock solution of native enzyme at 7.4 mg/ml was prepared in the usual manner (see Section C-1 above) and divided into two parts. Part I was diluted 1.25-fold with the second stock solution dialysate, while part II was acidified 1.25-fold with the pH 2.90 citric acid buffer used to prepare dimers. After one hour both were analyzed by a sedimentation velocity experiment, and the areas of each peak were measured and compared. The ratio of dimers to tetramers was about 0.85; thus a maximum value of 15% is the estimate for undissociated tetramer-aggregate present. To calculate a corrected diffusion coefficient for the dimer, and thus the percentage error in this value, (and the molecular weight value), the following weight-average relationship for diffusion coefficients was used:

$$D_{Mixture} = D_{Aggregate}\ (15\%) + D_{Dimer}\ (85\%) = 3.73 \times 10^{-7}\ cm^2/sec$$

where 3.73×10^{-7} cm^2/sec is the experimental value for the mixture. If one now assumes a diffusion coefficient, 3.0×10^{-7} cm^2/sec (a minimal value for a molecule with a molecular

weight of 300,000, unfolded to the same extent as dimers at this pH), the corrected value for the diffusion coefficient for the dimer species is calculated to be 3.90×10^{-7} cm^2/sec. This increase in the diffusion coefficient for the dimer intermediate is approximately 4% and would decrease the molecular weight of the dimer from the 83,000-86,000 range to 80,000-83,000 range. A value in the range of 80,000 to 84,000 is exactly what is predicted for a dimer of aldolase subunits with a molecular weight in the 40,000-42,000 range (see Results in Chapter I).

III. Characterization of the Reactivation Reaction From pH 3.3 Dimers

Since tetrameric aldolase can be selectively dissociated into dimers (pH 3.3) or into monomers (pH 2.8) (see Results in Chapter II), two distinct intersubunit binding sites must exist, one for monomer-monomer interaction and a different one for dimer-dimer interaction. Selective dissociation was expected from an isologous tetramer[1] (pseudo-tetrahedral) in which two isologous dimers, each with an axis of symmetry, are associated into the tetramer with a new axis of symmetry formed (Changeus, 1964). But what does the effect of breaking the native enzyme into dimers have on the catalytic properties of the newly formed dimer, presumably each with half of the four catalytic sites. Presumably, when dimers are formed, new previously unexposed hydrophobic areas then become exposed to the aqueous medium and may cause a conformational change in the dimer to minimize the hydrophobic exposure to water; this could alter the topology at the active site and render the dimer inactive. Alternatively, there may be little conformational change upon dissociation into dimers. Thus activity analysis of the dimer would be a

---

[1] Isologous Association: The domain of bonding involves two identical binding sets.

Even though rabbit muscle aldolase is now thought to have two different subunits, α and α', the domain of bonding must still be equivalent for both subunits since they show no apparent preferential interaction for each other in association-dissociation studies (see isozyme section in Literature Review).

sensitive technique to test the effect of dissociation on the conformation of the active site. An analysis for possible catalytically active dimers, therefore, was the subject of research to be described in the rest of this thesis: (1) In this chapter (III), the immediate aim was to find conditions which would produce complete reactivation of activity from pH 3.3 dimers, free of any aggregation, and an analysis for pH 3.3 dimer catalytic activity. (2) Chapter IV describes an investigation of the factors which inhibit dimer association at higher pH's under conditions in which the native enzyme remains as a tetramer and still possesses catalytic activity. (3) Chapter V will be concerned with the final analysis for active dimers at higher pH values (pH 5.5 and pH 7.5).

It was possible to define the conditions for total recovery without the necessity of a complete systematic study. The key experiments which showed the effects of variables upon recovery are described below.

Since the method for producing dimers was the same throughout the rest of this chapter--but is unique for this chapter--this procedure will be described first. Native enzyme was dialyzed against 0.01M citrate (Na), pH 7.0, for 24 hours at 0°C. This stock solution was diluted two-fold with 0.025M citrate (Na), pH 2.35, to final pH of 3.3 and incubated for one hour at 0°C. Usually the final dimer concentration was between 1.0 and 6.5 mg/ml. Kinetics of inactivation demonstrated that the reaction is essentially com-

plete in 35 minutes.

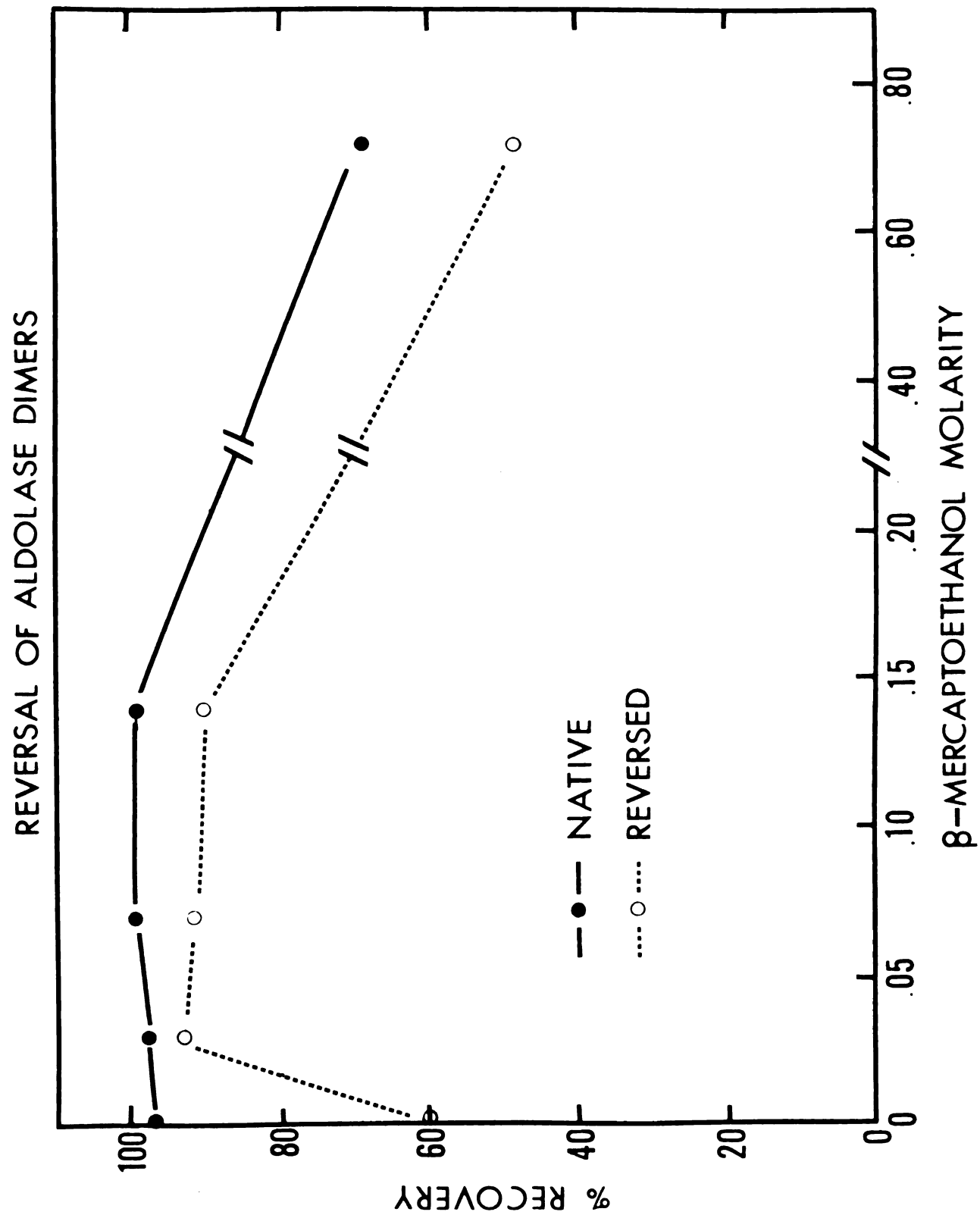
Since the experimental conditions for obtaining optimum recovery of activity were developed in successive stages from experiment to experiment, comparison between different experiments will be limited. However, any comparisons that can be made will be emphasized in the text.

A. Effect of β-Mercaptoethanol on Native Enzyme and Reversal of Catalytic Activity from pH 3.3 Dimers

The first variable to be tested for its effect upon reversal recovery was β-mercaptoethanol. After dimers were formed at a concentration of 6.50 mg/ml, they were diluted 500-fold at 0°C to a final enzyme concentration of 0.013 mg/ml with 0.3M Imidazole buffer, pH 7.9, containing the appropriate concentration of β-mercaptoethanol. After incubating 5 minutes at 0°C the solutions were brought to 20°C for 15 minutes before assaying. For the control, native protein solutions were diluted in the same manner except the pH was never changed. The results (Figure 14) show that low concentrations of β-mercaptoethanol (0-0.15M) stimulate recovery of activity but have relatively little effect on the native enzyme. However, at higher concentrations of β-mercaptoethanol (0.7M) both the native control and reversal samples lost activity; presumably, this is an unfolding effect of the solvent on the enzyme.

Another critical factor in obtaining total recovery of activity upon reversal was pH. The effect of pH at

Figure 14. Effect of β-mercaptoethanol on native enzyme and reversal of catalytic activity from pH 3.3 dimers. Reversal conditions were pH 7.9, 5 minute incubation at 0°C followed by a 15 minute incubation at 20°C, and a final enzyme concentration of 0.013 mg/ml. See text for details.


REVERSAL OF ALDOLASE DIMERS
% RECOVERY
100
80
60
40
20
0
.05
.10
.15
.20
.40
.60
.80
β-MERCAPTOETHANOL MOLARITY
NATIVE
REVERSED

various times of incubation is described in the following section.

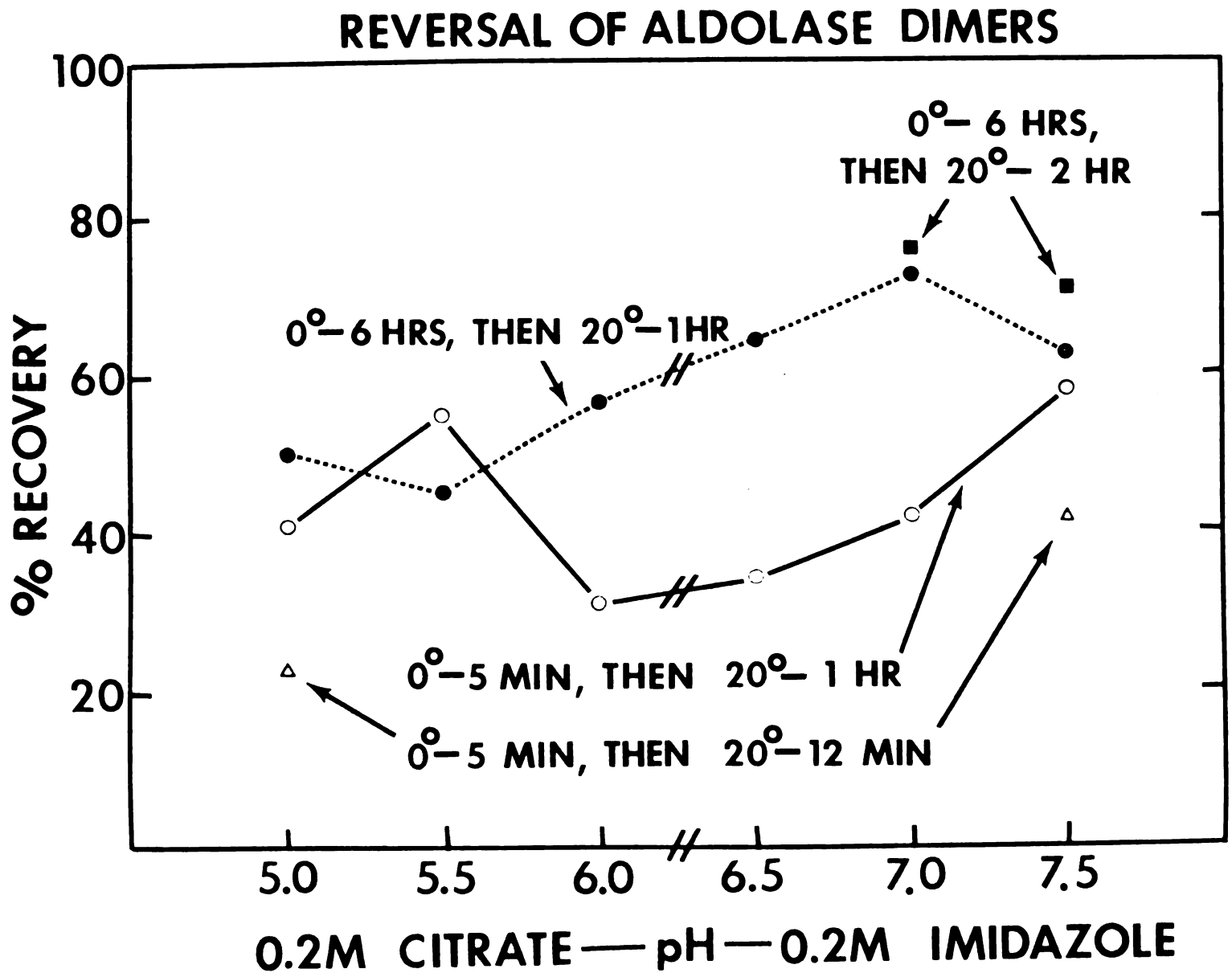
B. Effect of pH on Reassociation from pH 3.3 Dimers at Various Times of Incubation at 0°C and 20°C

The procedure for testing the effect of pH was quite similar to that in the β-mercaptoethanol experiments. After preparing the dimer (6.5 mg/ml), aliquots of 10 µl were diluted into 5.0 ml of 0.2M Imidazole buffer or 0.2M citrate buffer at the appropriate pH and incubated in this reactivation medium for various times--the final concentration was again 0.013 mg/ml.

Almost 80% recovery was obtained after incubation in the pH 7.0 reassociation medium for 6 hours at 0°C, followed by a 2 hour incubation at 20°C (Figure 15). The overall pH profile was quite broad both for the 5 minute incubation and the 6 hour incubation at 0°C. The one point at pH 5.5 may be in error, but any downward correction would just increase the broadness of the curve.

As judged from the last two experiments, recoveries are best in 0.1M β-mercaptoethanol, pH near neutrality, and incubation in the reassociation medium for 6 hours at 0°C followed by 2 hours at 20°C. These conditions--except the 6 hour incubation at 0°C which was changed to 1 hour at 0°C--were used in the reversal procedure in the next experiment.

Figure 15. Effect of pH on reversal of pH 3.3 dimers at various times of incubation at 0°C and 20°C. See text for details.

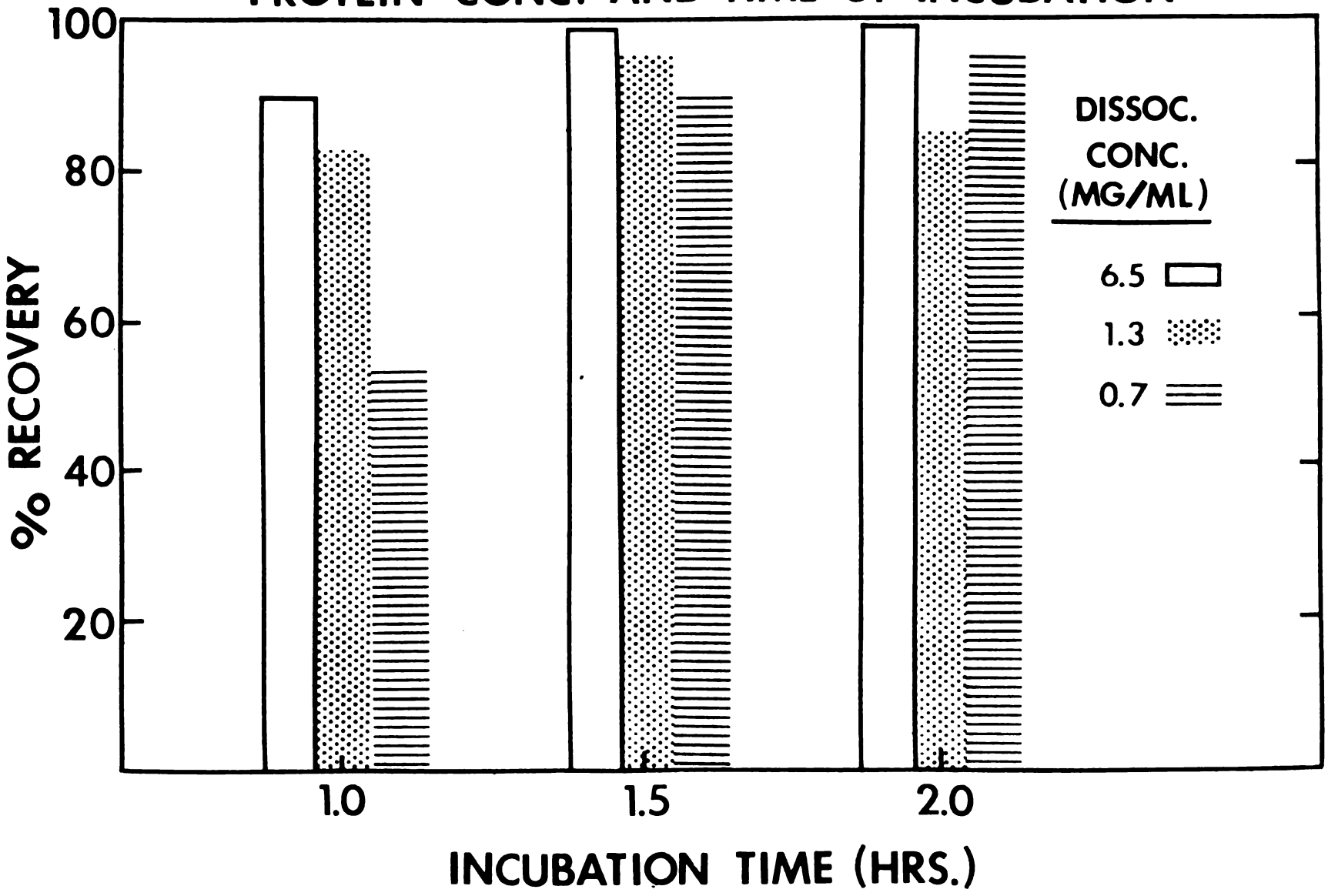

REVERSAL OF ALDOLASE DIMERS
100
80
60
40
20
% RECOVERY
0°– 6 HRS,
THEN 20°– 2 HR
0°–6 HRS, THEN 20°–1HR
0°–5 MIN, THEN 20°– 1 HR
0°–5 MIN, THEN 20°–12 MIN
5.0
5.5
6.0
6.5
7.0
7.5
0.2M CITRATE — pH — 0.2M IMIDAZOLE

C. Effect of Dimer Concentration and Time of Incubation in the Dissociation Medium: Attainment of Conditions for 90%-100% Recovery of Activity

In contrast to the previously described experiments, in which reversal conditions were varied, the purpose of this experiment was to investigate various conditions in the dissociation medium. It was thought that dimers might be aggregating with time and thus cause a decreased activity recovery in the reversal studies; moreover, high enzyme concentrations would tend to increase this aggregation. Thus an experiment was designed to test two effects: (1) dimer concentration and (2) incubation time in the dissociation medium.

Enzyme solutions were acidified as before to form dimers at a concentration of 6.5 mg/ml. This solution was immediately diluted with dimer solvent to 1.3 mg/ml and 0.70 mg/ml; these three solutions were then incubated at $0^{\circ}C$ for 1.0, 1.5, and 2.0 hours. After the incubation period the three dimer solutions were diluted into the reassociation medium of 0.3M Imidazole buffer, pH 7.9, and 0.1M β-mercaptoethanol at $0^{\circ}C$, to a final enzyme concentration of 0.013 mg/ml. After one hour at $0^{\circ}C$, the solutions were warmed to $20^{\circ}C$ for two hours and assayed. The results reported in Figure 16 show that at least six dimer samples had been reactivated to 90-100% of the control. Furthermore, the results show that the recovery of enzymatic activity was not significantly altered by dimer concentration at incubation

Figure 16. Effect of dimer concentration and time of incubation in the dissociation medium: attainment of conditions for 90%-100% recovery of activity. The reversal conditions were pH 7.9, 0.1M β-mercaptoethanol, one hour incubation at 0°C followed by a two hour incubation at 20°C, and a final enzyme concentration of 0.013 mg/ml. For other details, refer to the text.


EFFECTS OF DISSOCIATION CONDITIONS ON REVERSAL:
PROTEIN CONC. AND TIME OF INCUBATION
100
80
60
40
20
% RECOVERY
DISSOC.
CONC.
(MG/ML)
6.5
1.3
0.7
1.0
1.5
2.0
INCUBATION TIME (HRS.)

times at 1.5 and 2.0 hours.

Although not shown in the graph, other studies have shown that the % recovery of the sample which was incubated for one hour at 6.5 mg/ml in the dissociation medium can be increased from 55% to 98% by simply increasing the reversal concentration from 0.013 mg/ml to 0.065 mg/ml. The activity remained constant at 98% for at least 5 hours.

The % recoveries of activity are all based on duplicate controls (arbitrarily placed at 100%) which were put through the same incubations and dilutions but not the change in pH. Any change in activity due to instability in the experimental samples should also occur in the control. The controls had a specific activity (μmoles/min/mg) of 12.2 and 12.3, close to the reported value of 12.5.

In conclusion, the main purpose of these experiments was to find conditions for complete recovery of enzyme activity upon reversal of dissociation into dimers. This experiment accomplished this purpose by identifying conditions where at least 6 samples were reversed 90-100% and 2 samples were reversed 98%. Also, at higher concentrations in the reversal mixture (0.065 mg/ml vs. 0.013 mg/ml) three more samples were reversed 98% (the 0.065 mg/ml was not graphically represented).

Now that the conditions for complete reversal of catalytic activity had been found, the rest of the work described in this chapter was concerned with the following two problems: (1) the question of whether pH 3.3 dimers are

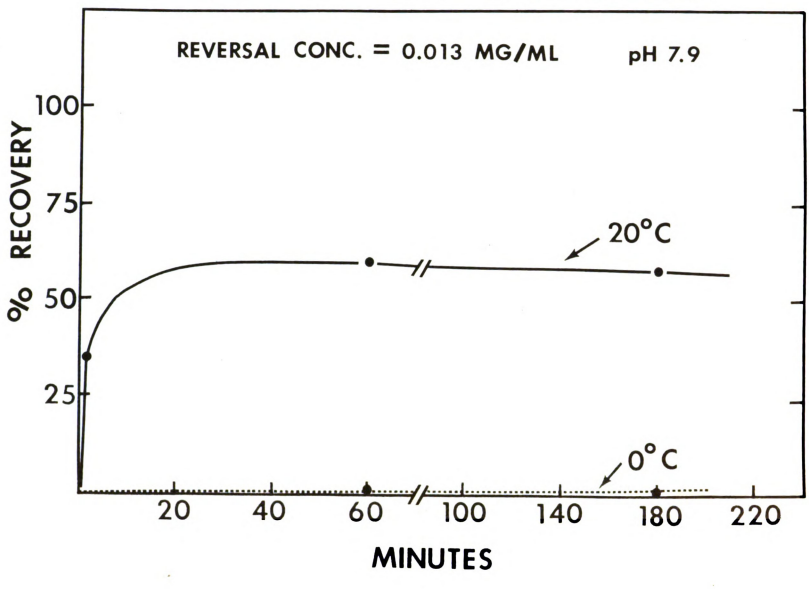
active; the next three sections describe studies on the effects of temperature and dimer concentration on the kinetics of reactivation and half-live of reversal, and (2) the question of whether the substrate, FDP, could inhibit reactivation and/or dimer association.

D. Recovery as a Function of Time at Two Temperatures

Dimers (pH 3.3) were prepared as before and incubated for 2 hours in the dissociation medium at an enzyme concentration of 0.75 mg/ml. These dimers were then diluted 50-fold to a final concentration of 0.013 mg/ml with 0.3M Imidazole buffer, pH 7.9, and incubated at 0°C or 20°C. Aliquots were taken at various times and tested for activity. Activity recoveries at these two temperatures are shown in Figure 17. Kinetics show that the initial rate of recovery was very fast (40% in 2 minutes) at 20°C, and the reaction was essentially over in 20 minutes. When compared to the native control, the recovery was approximately 60% after one hour at 20°C. At 0°C, however, only a small amount of activity was found. Later, it was shown that a 24 hour incubation at 0°C produced about 10% reactivation. Thus reactivation was occurring at 0°C, although it was very slow.

The increased reversal at the higher temperature was probably not only due to increased collision frequency of the diffusion-limited process of association but also due to increased dimer interaction strength caused by stronger

Figure 17. Recovery as a function of time at two temperatures. See text for additional information.


EFFECT OF TEMP. ON DIMER REVERSAL
REVERSAL CONC. = 0.013 MG/ML
pH 7.9
% RECOVERY
100
75
50
25
20°C
0°C
20
40
60
100
140
180
220
MINUTES

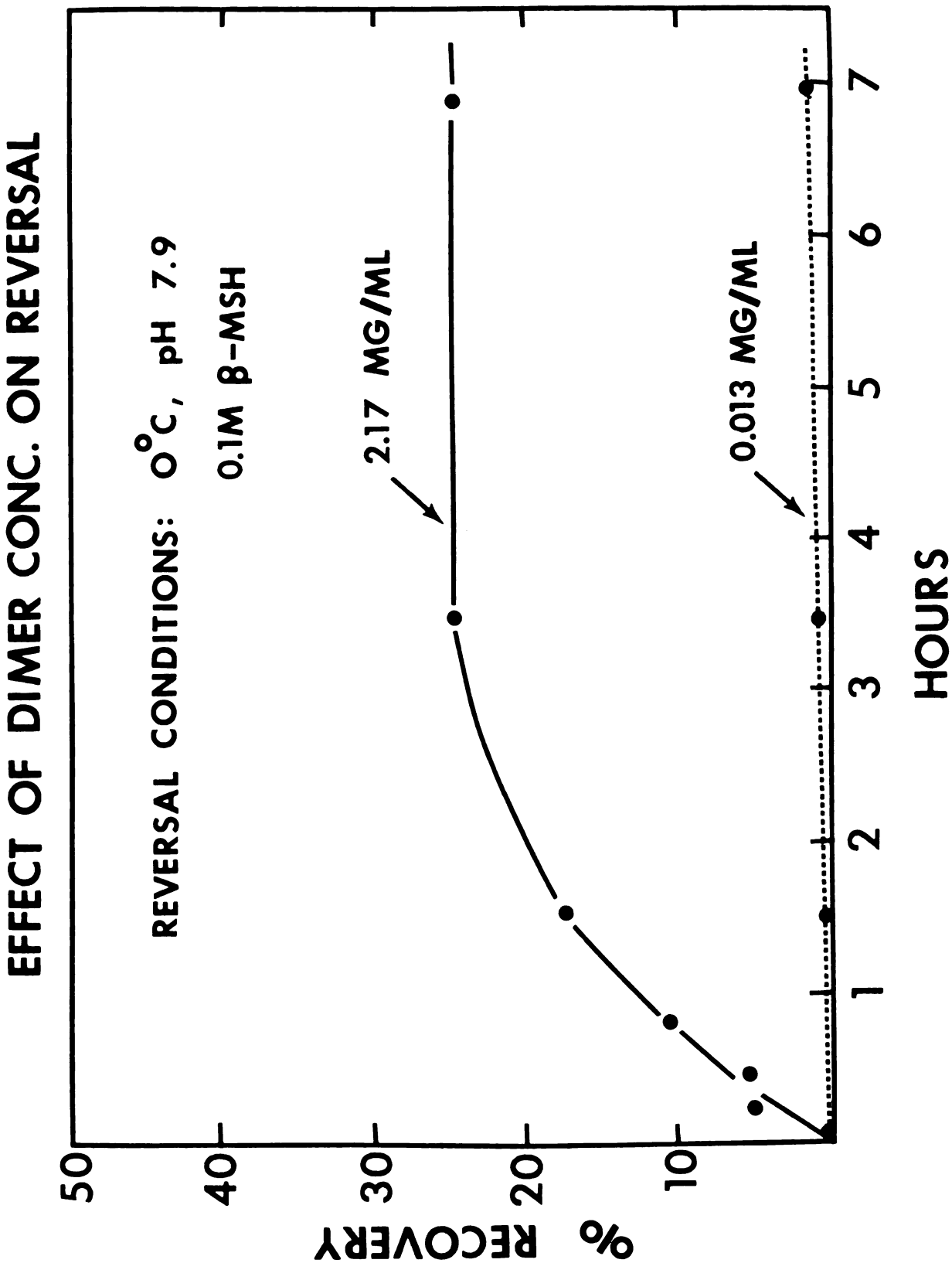
hydrophobic bonding at 20°C over that at 0°C. If the reversal process involves an association reaction, it should be concentration dependent; in particular, higher concentrations should also increase the rate of reversal. Experiments to test for concentration dependence of the reassociation process are described in the following section.

E. Kinetics of Reversal Process at Two Concentrations

After dimers were prepared, they were diluted into the reversal mixture, 0.3M Imidazole buffer, pH 7.9 and 0.1M β-mercaptoethanol at 0°C to a final concentration of 2.17 mg/ml and 0.013 mg/ml. Aliquots were removed at various times and assayed.

As can be seen in Figure 18 where kinetics of reversal are plotted for the two concentrations, the higher concentration indeed increases the rate of reversal, but only 25% recovery of activity was obtained at this concentration. This low recovery was probably due to non-specific aggregation, since a white precipitate had formed after two or three hours. At the lower concentration much less reversal was evident; by the time the higher concentration (2.17 mg/ml) plateau of reversal was reached, only about 1% reversal had occurred at the lower concentration (0.013 mg/ml). However, after longer times (24 hours) the lower concentration sample had reversed about 10%. The concentration dependence of reactivation at 0°C is consistent with a model in which an inactive dimer must associate before activity is obtained.

Figure 18. The reversal of pH 3.3 dimers as a function of time at two enzyme concentrations. See text for details.


EFFECT OF DIMER CONC. ON REVERSAL
REVERSAL CONDITIONS: 0°C, pH 7.9
0.1M β-MSH
2.17 MG/ML
0.013 MG/ML
% RECOVERY
50
40
30
20
10
1
2
3
4
5
6
7
HOURS

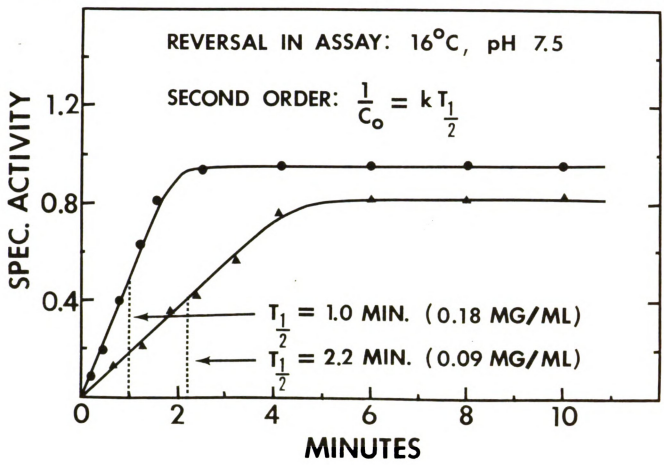
Thus the pH 3.3 dimer is probably inactive at 0°C.

But the activity assays show significant lag times; perhaps the pH 3.3 dimer which is inactive at 0°C folds in the assay at higher temperatures and becomes active. If the pH 3.3 dimers must associate before reactivation occurs, the effect of concentration on the half-time for this reaction should reflect a second order reaction. The following experiment examines the effect of concentration on half-time of reactivation in the assay.

F. Effect of Concentration on Half-Time of Reactivation in the Assay (16°C)

Dimers were produced at 3.25 mg/ml and incubated for at least 1.5 hours at pH 3.3. Then 10 µl and 20 µl aliquots were diluted into an assay to a final concentration of 0.09 mg/ml and 0.18 mg/ml at 16°C (16°C instead of the normal assay temperature of 25°C to prevent precipitation which occurs at the higher temperature) and assayed at 16°C. Relative specific activities from typical assays are reproduced in Figure 19. The half-time for reversal for 0.18 mg/ml sample was 1.0 minutes; whereas the half-time for 0.09 mg/ml dimer sample was 2.2 minutes. If pH 3.3 dimers were active upon folding in the assay, the half-time for this unimolecular reaction at various dimer concentrations should be constant; however, the results show that the half-time was reduced by approximately two when the dimer concentration was increased by a factor of two. This inverse

Figure 19. Effect of dimer concentration of half-life of reversal in the assay at 16°C and pH 7.5. See text for other details.


EFFECT OF DIMER CONC.ON HALF-LIFE OF REVERSAL
REVERSAL IN ASSAY: 16°C, pH 7.5
SECOND ORDER: $\frac{1}{C_o} = k T_{\frac{1}{2}}$
SPEC. ACTIVITY
1.2
0.8
0.4
$T_{\frac{1}{2}}$ = 1.0 MIN. (0.18 MG/ML)
$T_{\frac{1}{2}}$ = 2.2 MIN. (0.09 MG/ML)
0
2
4
6
8
10
MINUTES

relationship is consistent with a second order reaction which follows the half-time equation, $1/C_o = kt_{\frac{1}{2}}$. From this relationship a second-order rate constant was calculated to be $k = 1.35 \times 10^4$ M liters/mole-sec at 16°C. These results then are consistent with a model in which dimers must associate before catalytic activity is obtained.

G. Effect of FDP on Reactivation of pH 3.3 Dimers in the Assay

In the course of previous studies it was observed that when FDP was present in the assay before pH 3.3 dimers were diluted into the assay, much lower activities were found than if FDP was added last. Subsequent studies showed that if a pH 3.3 dimer sample at 0.0125 mg/ml was added to the assay before FDP was added, substantial recovery of activity was obtained after a lag period of one to three minutes; but if FDP was present before dimers were added, no recovery of activity was obtained even after ten minutes. Moreover, if higher concentrations (0.025 mg/ml) of dimers were used, the FDP inhibition of reactivation was partially overcome. The fact that the FDP inhibition of reactivation is enzyme concentration dependent suggests that FDP somehow binds to the dimer and prevents a conformational change required for dimer association.

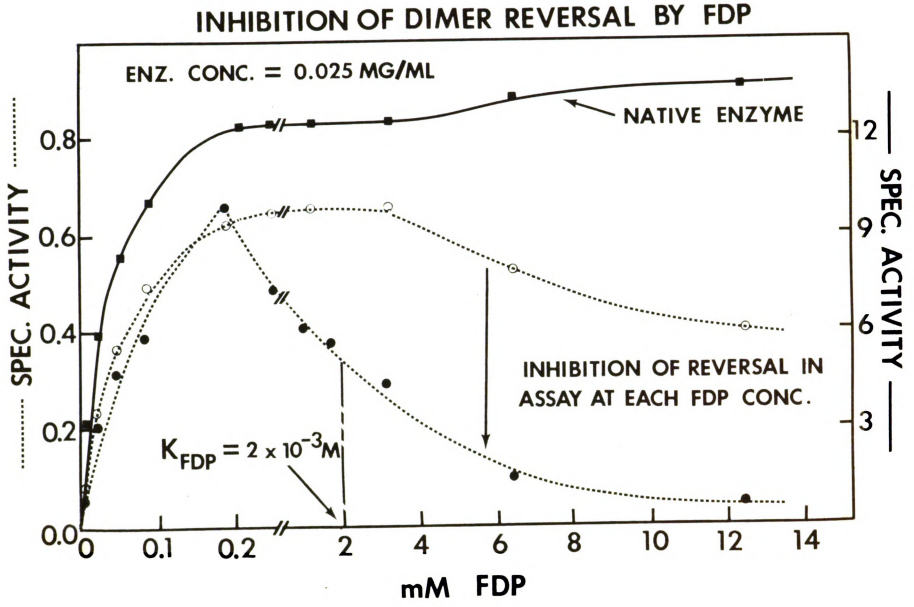
Thus experiments were designed to test the effect of various concentrations of FDP on initial activities of dimers at a dimer concentration of 0.025 mg/ml in the assay. The

values were then compared to dimers incubated in the assay without FDP under identical conditions. The effect of FDP on the native enzyme was also determined to make sure that the native enzyme was not inactivated by FDP. The concentration of FDP which caused a 50% inhibition of activity over the control was considered to be a measure of the binding constant for FDP inhibition (Figure 20):

$$K_{\text{FDP Binding}} = 2 \times 10^{-3} \text{ moles/liter}$$

This is approximately 500 times higher than the FDP binding constant ($k_d = 4 \times 10^{-6}$M) for the native enzyme. Evidence which will be presented later (Chapter IV, Section B-7) supports this data by the demonstration that FDP does indeed inhibit association of the pH 3.3 dimer. Furthermore, the binding of FDP to the pH 3.3 dimer is probably through the phosphate groups of FDP since pyrophosphate also inhibits dimer association. It has been shown that two phosphate binding sites exist at each FDP binding site on the native enzyme (see Literature Review); perhaps these binding sites still persist in the pH 3.3 dimer but in a slightly different conformation. When FDP binds to the pH 3.3 dimer, it presumably freezes the molecule in the altered conformation and prevents association.

Figure 20. Inhibition of dimer reversal under assay conditions by FDP. The solid squares represent the normal substrate saturation for native aldolase. The circles represent activities obtained 10 seconds after pH 3.3 dimers were diluted into the assay in the presence of FDP (closed circles) or in the absence of FDP (open circles). In the latter case FDP was added immediately after enzyme was added.


INHIBITION OF DIMER REVERSAL BY FDP
ENZ. CONC. = 0.025 MG/ML
NATIVE ENZYME
SPEC. ACTIVITY
SPEC. ACTIVITY
INHIBITION OF REVERSAL IN ASSAY AT EACH FDP CONC.
$K_{FDP} = 2 \times 10^{-3} M$
mM FDP
0.0
0.2
0.4
0.6
0.8
3
6
9
12
0
0.1
0.2
2
4
6
8
10
12
14

IV. A Study Preliminary to the Analysis for Active Dimers of Aldolase: Attainment of Aldolase Dimers Under Conditions Where Native Tetramers are Active

This study is a general investigation of the physical parameters which affect dimer reactivation and association at pH values between pH 3.35, where the inactive, unfolded dimer is formed (see Results in Chapter III), and pH 7.5, where the native tetramer is stable. It was thought that the 3.5S dimer was inactive at pH 3.35 because the low pH used caused it to be unfolded; thus it seemed that at a higher pH the dimer might remain or become folded and have activity. There are two obvious approaches for trying to produce dimers with activity at pH values between pH 3.35 and pH 7.5: (1) try to produce active dimers directly from tetramers by dissociation without unfolding, by varying factors which aid dissociation, such as low protein concentrations, temperature changes, and small changes in pH; or (2) produce unfolded dimers at pH 3.35 and then find conditions where the unfolded dimer folds, but does not associate, as the pH in increased towards neutrality (Figure 22). The former approach seemed more reasonable since an inactive species, the pH 3.35 dimer, would not have to be a transient intermediate from active tetramers to active dimers, so it was attempted first.

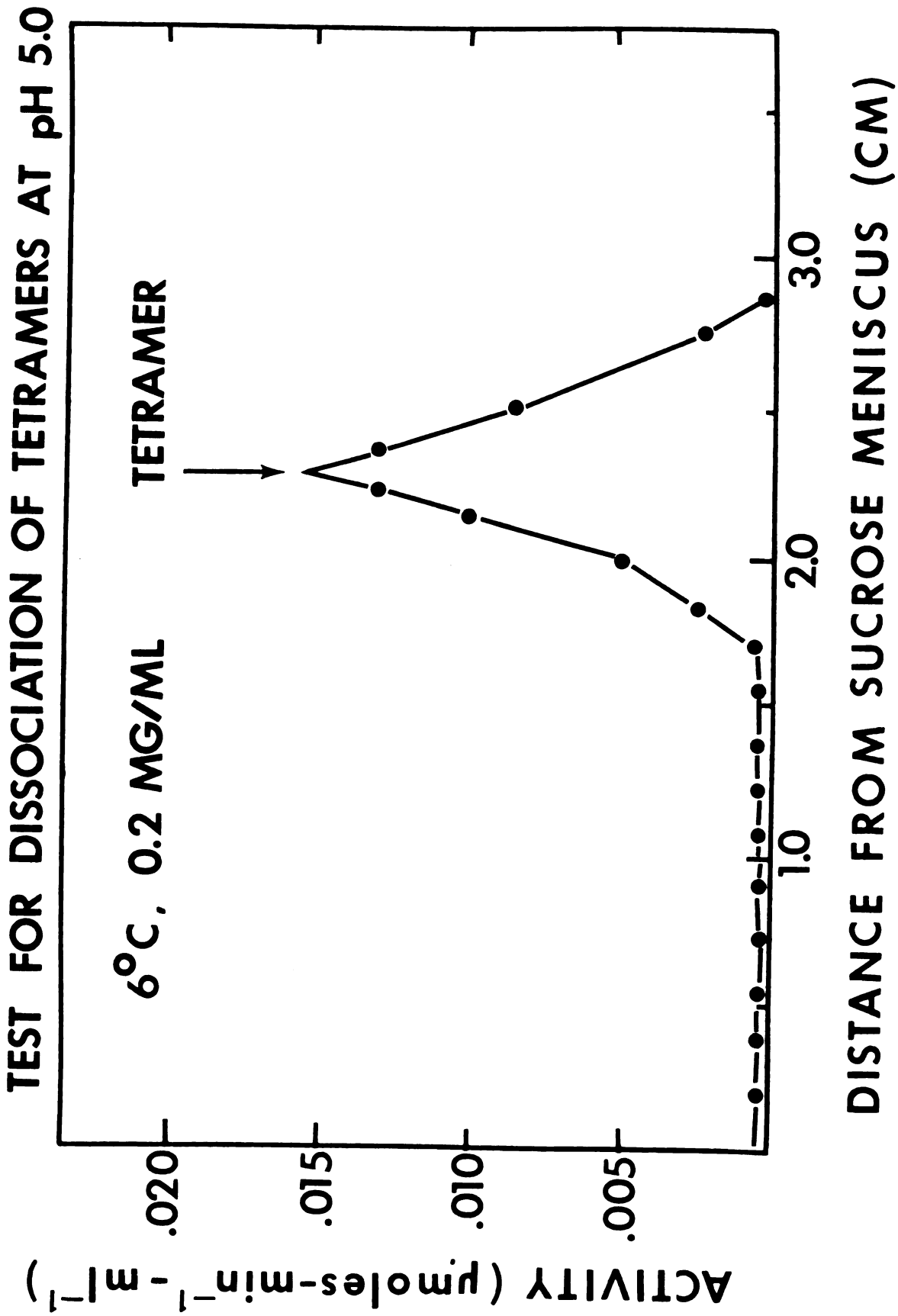
A. Test for Active Dimer Production by Dissociation of Tetramers

The aim of this experiment was to investigate the first possibility above: dissociating tetramers into dimers with catalytic activity at higher pH values than in previous methods. It seemed reasonable that this could be accomplished by lower enzyme concentrations and higher temperatures. Sucrose density centrifugation was the method of choice to analyze this problem, activity measurements being the only method sensitive enough to detect protein at these low concentrations.

Aldolase crystals were centrifuged, dissolved in 0.2M citrate (Na) buffer at pH 5.0, and diluted with this solution to the following protein concentrations: 2.0 mg/ml, 0.2 mg/ml and 0.04 mg/ml. Then 10 µl aliquots were taken from each of these solutions and applied to the top of separate sucrose gradient solutions in 5 ml plastic centrifuge tubes. (For the procedure for sucrose density gradient preparation, refer to Methods.) After centrifuging for 20 hours (up to 40 hours in other experiments to be described later), the gradient tubes were removed, holes were punched in the bottom of the tubes, and 10 drop fractions were collected and assayed for aldolase activity.

The results from a typical sucrose density sedimentation velocity (SDSV) run are shown in Figure 21. The concentration chosen was the intermediate concentration, 0.2 mg/ml. The finding of a single, fairly symmetrical peak for

Figure 21. Test for dissociation of tetramers at pH 5.0 into active dimers. This is a SDSV pattern of native aldolase at 6°C and 0.2 mg/ml. The identification of the peak as tetramers was determined by comparison to other experiments under similar conditions in which glyceraldehyde-3-phosphate dehydrogenase was used as a marker. For further experimental details, see text.


TEST FOR DISSOCIATION OF TETRAMERS AT pH 5.0
6°C, 0.2 MG/ML
TETRAMER
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
.020
.015
.010
.005
1.0
2.0
3.0
DISTANCE FROM SUCROSE MENISCUS (CM)

all concentrations demonstrated that tetrameric aldolase was the only active species present. Since the presence of protein was analyzed by activity assay, only the active species would have been detected; inactive dimers or subunits would have gone undetected. However, we have an indirect check for inactive enzyme; the percentage recovery of active tetramers can be calculated for each concentration. The percentage recovery for the highest concentration, 2.0 mg/ml, was 97%, to be compared with 65% for 0.2 mg/ml and 60% for 0.04 mg/ml. It is not known whether the loss of activity recovery is a result of inactive tetramers or inactive dimers; the greater loss at lower protein concentrations is consistent with the latter.

If dimers had been formed at these low concentrations, they could have been unfolded, and thus inactive because of the low temperatures used. Reasoning that perhaps a higher temperature might avoid any such unfolding of a dimer, this experiment was therefore repeated as before, with the exception of increasing the temperature to about 20°C. Again, the results showed only a single symmetrical peak present at the tetramer position. In agreement with the previous results, lower percentage recoveries were obtained at lower concentrations: 61% at 2.0 mg/ml compared to 22% at 0.04 mg/ml. Since the recoveries were lower in this experiment at comparable enzyme concentrations, higher temperatures must have caused this inactivation. As before, it was not known if dimers were formed; if they were formed, they were inactive.

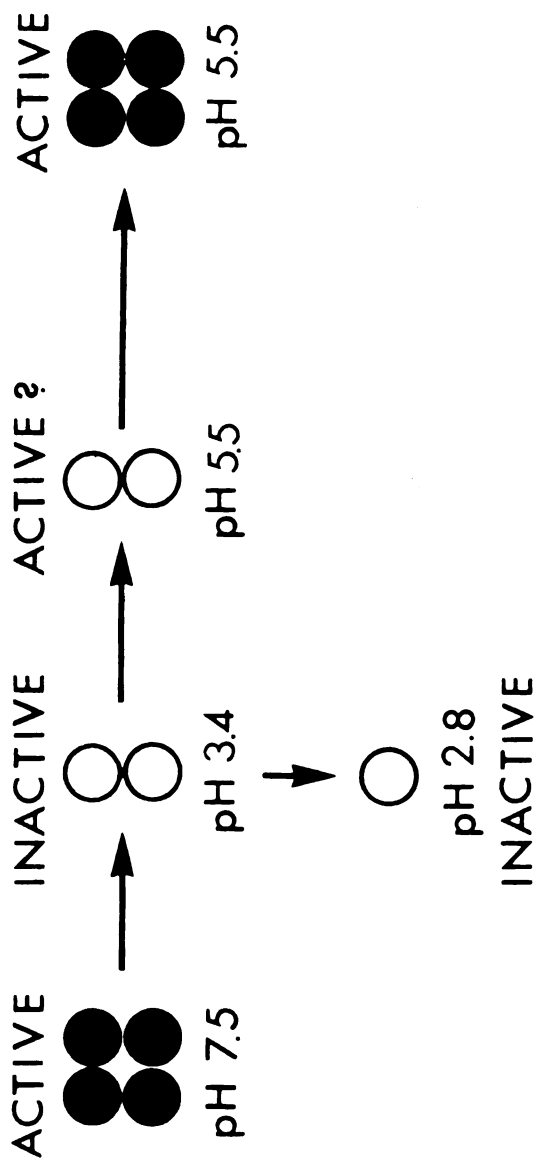
Therefore, the second approach was investigated (Figure 22). First dimers were produced at pH 3.35; these inactive dimers were then placed under various conditions which inhibited association but which presumably might allow selective folding of the inactive dimer to an active dimer--if such was possible.

B. Test for an Active Dimer by Studying the Effect of Various Conditions on Selectively Folding (Without Association) the pH 3.35 Unfolded Dimer at Higher pH Values

Since in this section the procedures for many of these studies are similar in outline, differing only in some details at the various steps, a general flow-sheet will be used in the legend of each figure with the specific differences emphasized in the text.

In the following series of experiments dimers were produced under previously tested conditions (see Chapter II) at pH 3.35. When the dimer samples had to be diluted further at pH 3.35, the following buffer was used for the dilution step: 0.001M EDTA, 0.05M NaCl, and 0.1M citrate (Na), pH 3.35. Most experiments entailed, in addition to the above dilution step, diluting the dimer solution five-fold to a higher pH with 0.2M citrate (Na) and subsequently testing the effect of variables such as dimer concentration, temperature, sucrose concentration, substrate concentration, and incubation time on the dimer-tetramer distribution. The relative dimer-tetramer

Figure 22. This model shows the approach of producing a dimer at pH 3.4 and then raising the pH to a higher value and testing for dimer association or dimer catalytic activity.


ACTIVE
pH 7.5
INACTIVE
pH 3.4
pH 2.8
INACTIVE
ACTIVE ?
pH 5.5
ACTIVE
pH 5.5

distribution was analyzed by sucrose density gradient sedimentation velocity (SDSV) experiments (followed by activity analysis of the collected fractions). It was necessary, however, to positively first identify the dimer as a dimer, in order to be confident of the results.

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was used as a marker. In the aldolase test sample two peaks were usually present: the leading peak was identified as the tetramer species (7.9S), (sedimenting the same distance as native enzyme), whereas the slower moving peak was identified as the dimer species with a sedimentation coefficient between 3.5S and 4.3S which is equal to or higher than the experimentally determined values of 3.45S to 3.80S. The sedimentation coefficients for the dimer were determined previously (see Results in Chapter II) by conventional moving boundary sedimentation velocity experiments in aqueous solutions using the analytical model E ultracentrifuge.

Since pH seemed to be the most influential factor in reactivation and association, the first experiments were designed to determine the minimum pH necessary to prevent dimer association but still exhibit activity reversal. The first pH range to be tested was pH 4.0 to pH 5.5, since the tetramer has been shown to be stable and remain as a tetramer at pH 5.0 (see Results in previous section at 2.0 mg/ml).

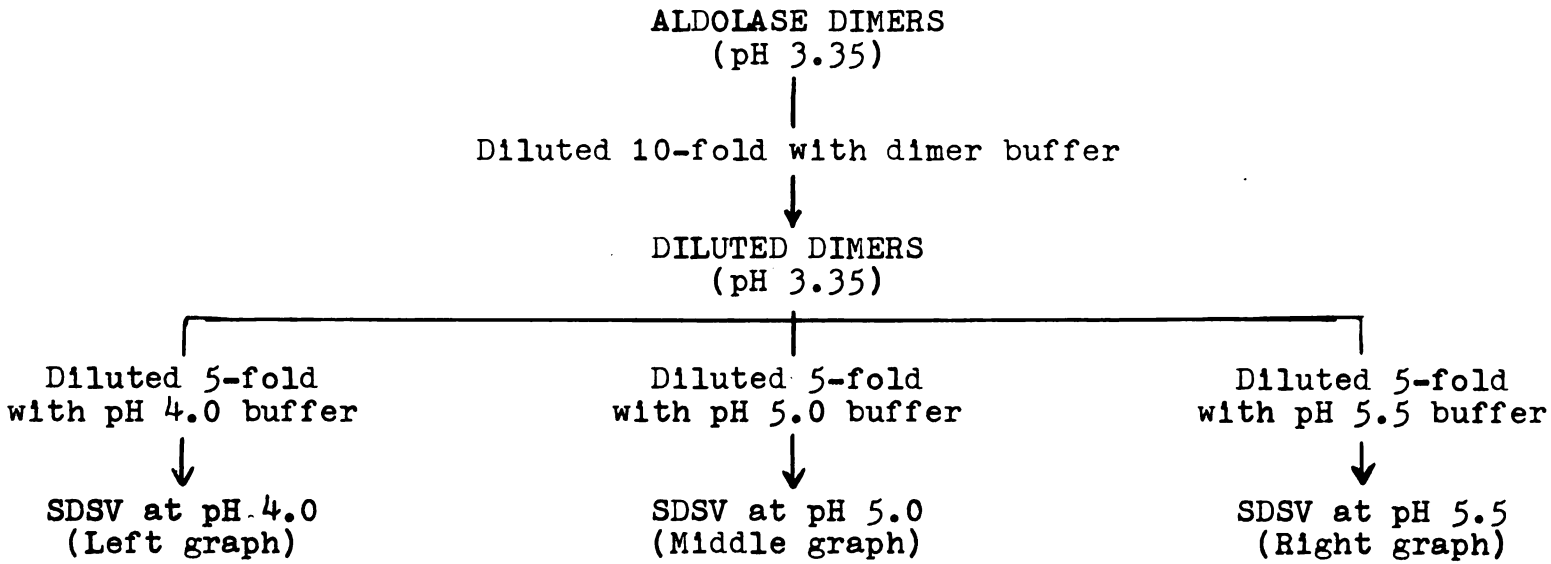
1. Effect of pH on the Reassociation of Dimers to Tetramers After a 15 Minute Incubation

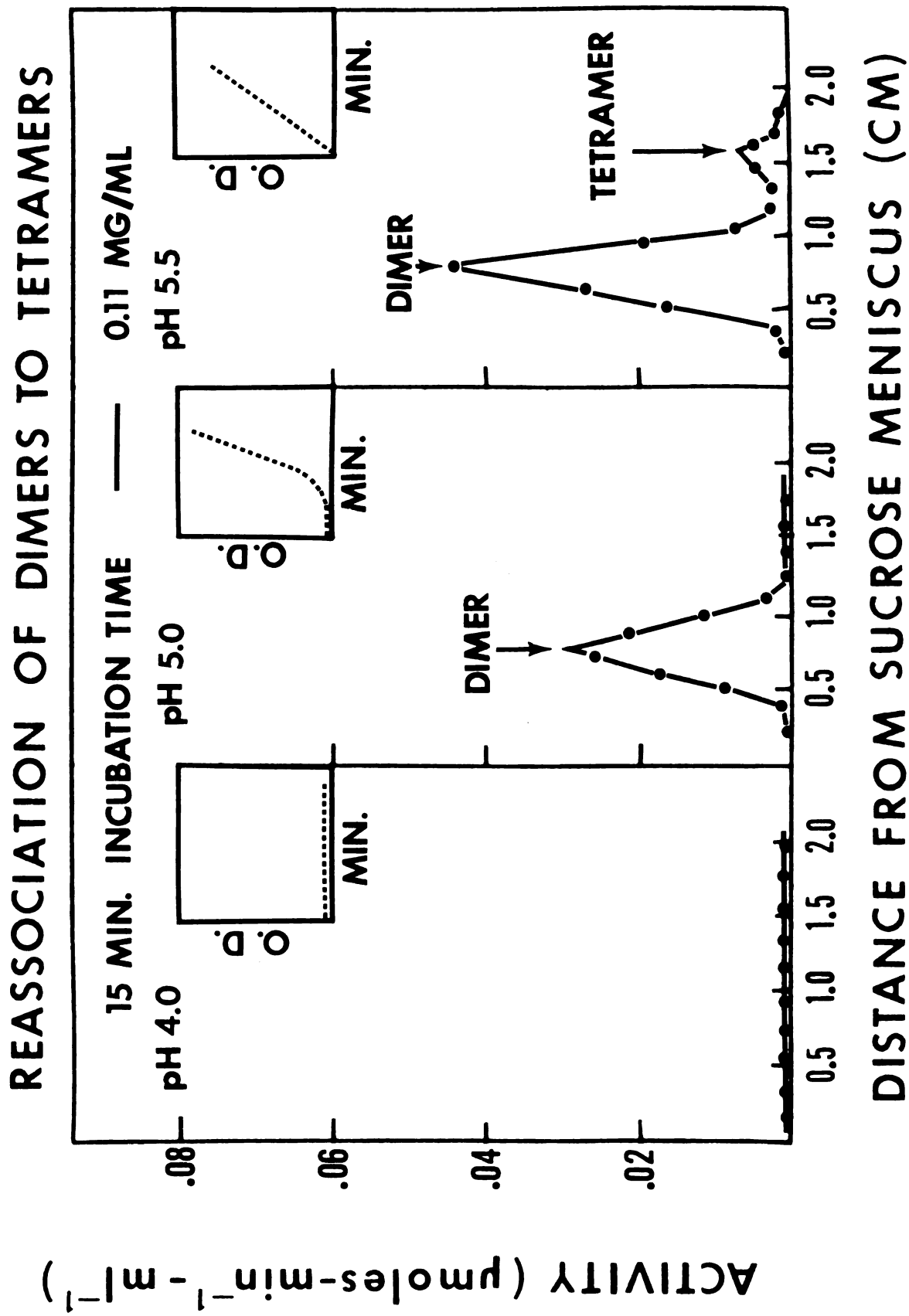
The design of this experiment was as follows: dimers were diluted five-fold to a final concentration of 0.11 mg/ml with pH 4.0, 5.0 and pH 5.5 citrate (Na) buffers, incubated for 15 minutes, and analyzed by SDSV in sucrose gradients also at the respective pH values. Patterns from the activity analysis for pH 4.0, 5.0 and pH 5.5 samples are shown in the left, middle, and right graphs, respectively, of Figure 23. There was no activity in the pH 4.0 sample (see sample assay in left insert); in fact, at this pH, only at very much higher concentrations was reactivation in the assay possible.

The initial activities of the pH 5.0 sample were almost zero but showed a gradually increasing activity with time in the assay. Typical activity assay profiles are given by the inserts. Because of the pronounced lag times at pH 5.0 which probably represent association (see insert in middle graph of Figure 23 for a typical assay), final activities were plotted for the pH 5.0 sample.

In contrast to the pH 4.0 and pH 5.0 samples, the pH 5.5 fractions immediately gave activities and these were constant (little or no lag times were observed; see insert in right graph, Figure 23). This observation was very exciting at the time, since it seemed to provide overwhelming evidence for the capability of dimers to be active; however, later discoveries tempered this observation (see Results in Chapter V). At any rate, this fact suggested that the pH 5.5 system

Figure 23. The effect of pH on dimer association after a 15 minute incubation at each pH. The results from the SDSV analysis for pH 4.0, pH 5.0, and pH 5.5 are shown in left, middle, and right graphs, respectively. Sample assays at each pH are represented schematically in the inserts of each graph; they provide tests for reassociation in the assay. Final activities are plotted for pH 5.0 and pH 4.0; however, initial activities are plotted for pH 5.5 fractions. The sedimentation coefficients for the dimer at pH 5.0 and pH 5.5 were calculated to be 3.8S and 3.9S, respectively (see Methods for method of calculation). See text for other details.




REASSOCIATION OF DIMERS TO TETRAMERS
15 MIN. INCUBATION TIME
0.11 MG/ML
pH 4.0
pH 5.0
pH 5.5
MIN.
DIMER
TETRAMER
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
DISTANCE FROM SUCROSE MENISCUS (CM)
.08
.06
.04
.02
0.5
1.0
1.5
2.0

would probably be the best to analyze, even though a small amount of association did take place, as shown by the small tetramer peak present. Thus in answer to our earlier question: dimer association could be completely inhibited by lower pH values, but reactivation without significant lag times was not possible at pH values of 5.0 or lower at this concentration--although apparently possible at pH 5.5.

But only one concentration (0.11 mg/ml) was investigated in this experiment. Therefore, the effect of concentration on the reactivation process was next investigated. To ascertain then if reactivation could be accomplished at pH 4.0, higher concentrations were investigated. These experiments along with the pH 5.5 concentration dependence experiments--which were more important since the pH 5.5 dimer gave immediate activity in the assay--were designed to test the effect of dimer concentration on reactivation and dimer association.

2. Concentration Dependence of Reassociation at pH 4.0 and pH 5.5

a. Effect of Dimer Concentration on Reassociation at pH 4.0 After a 15 Minute Incubation

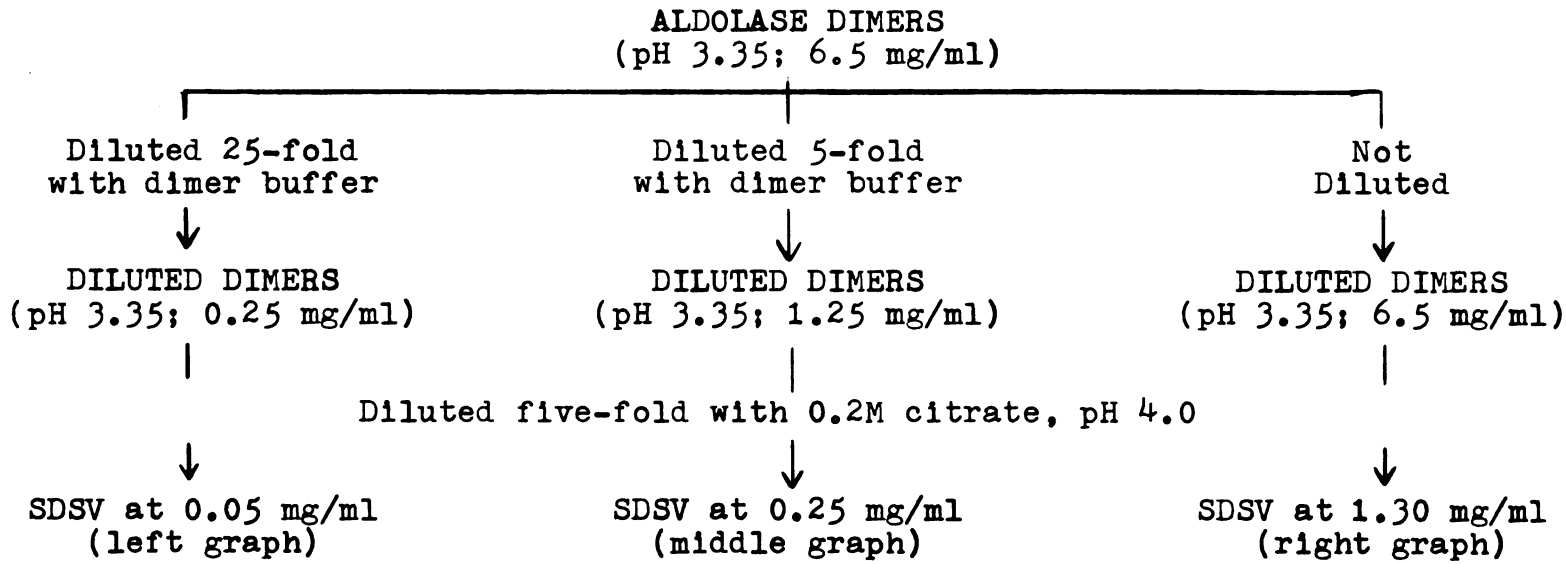
Dimers were diluted five-fold into a pH 4.0 citrate (Na) buffer solution to a final concentration of 0.05, 0.25, and 1.30 mg/ml, incubated for 15 minutes at 0°C, and analyzed by SDSV. The two lower concentration samples were completely inactive and showed no reassociation in the assay,

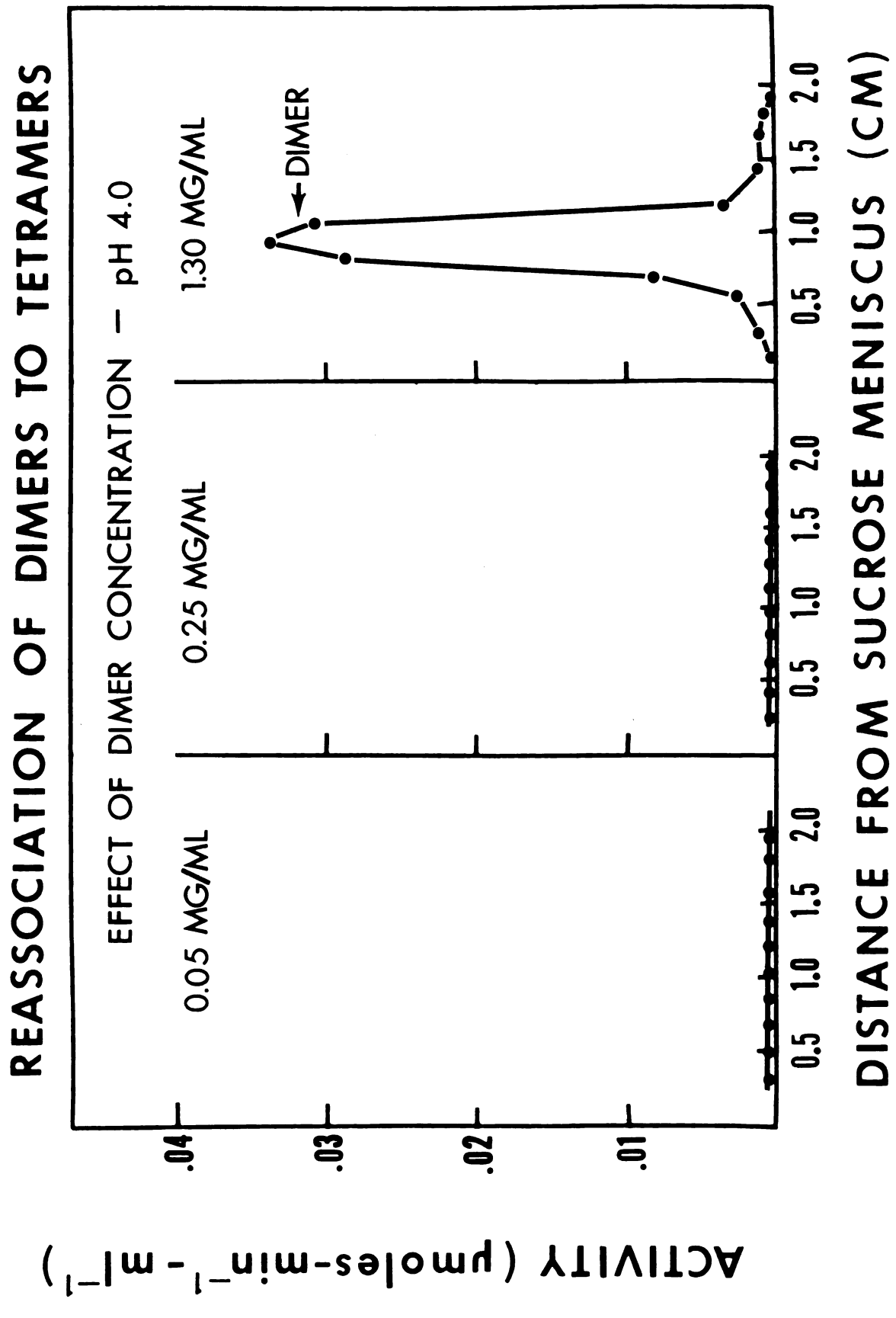
even after 15 minute incubation (see Figure 24). Samples at the highest concentration, 1.30 mg/ml, were also inactive initially; but in contrast to those at the lower concentrations, some reactivation occurred during the incubation period in the assay. Since reactivation was concentration dependent, this indicated that reassociation had also taken place. For obvious reasons, final activities were plotted in Figure 24. Two conclusions are suggested from this data: (1) association into tetramers had to occur before catalytic activity was obtained at pH 4.0, and therefore dimers were inactive at pH 4.0, and (2) pH 4.0 dimers which had been incubated for several minutes in the assay at pH 7.5 and 25°C at 0.03 mg/ml were inactive. The possibility that the dimer concentration had some influence on a folding reaction is not excluded, although it seems unlikely. Thus, these results apparently contradict the evidence for active dimers in the previous section.

b. Effect of Dimer Concentration on Reassociation at pH 5.5 After 3.5 Hours

In contrast to the previous experiments where dimer concentrations had to be increased to very high levels (1.30 mg/ml) to reactivate the dimers and activation took place only in the pH 7.5 assay, for experiments at pH 5.5, dimer concentration had to be decreased to avoid association. This decrease in dimer concentration (0.12 mg/ml to 0.03 mg/ml) was used to accomodate a study of the following problems:

Figure 24. The effect of dimer concentration on dimer association at pH 4.0 after a 15 minute incubation. Analysis by SDSV at pH 4.0 of the respective dimer concentrations (0.05, 0.25, and 1.30 mg/ml) is shown in left, middle, and right graphs, respectively. The identification of the peak in the right graph as a dimer was by the peak position after a 20 hour run at 40,000. There was no activity in the two lower concentration samples even after a 10 minute incubation in the assay; however, since there was some reactivation from the dimer sample at 1.30 mg/ml with time, final activities were plotted in the right graph. See text for details.




REASSOCIATION OF DIMERS TO TETRAMERS
EFFECT OF DIMER CONCENTRATION — pH 4.0
0.05 MG/ML
0.25 MG/ML
1.30 MG/ML
DIMER
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
.04
.03
.02
.01
0.5 1.0 1.5 2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

(1) demonstrate that dimer association into tetramers, a bimolecular reaction, was indeed concentration dependent, and (2) try to find at lower protein concentration conditions which exhibit complete inhibition of dimer association.

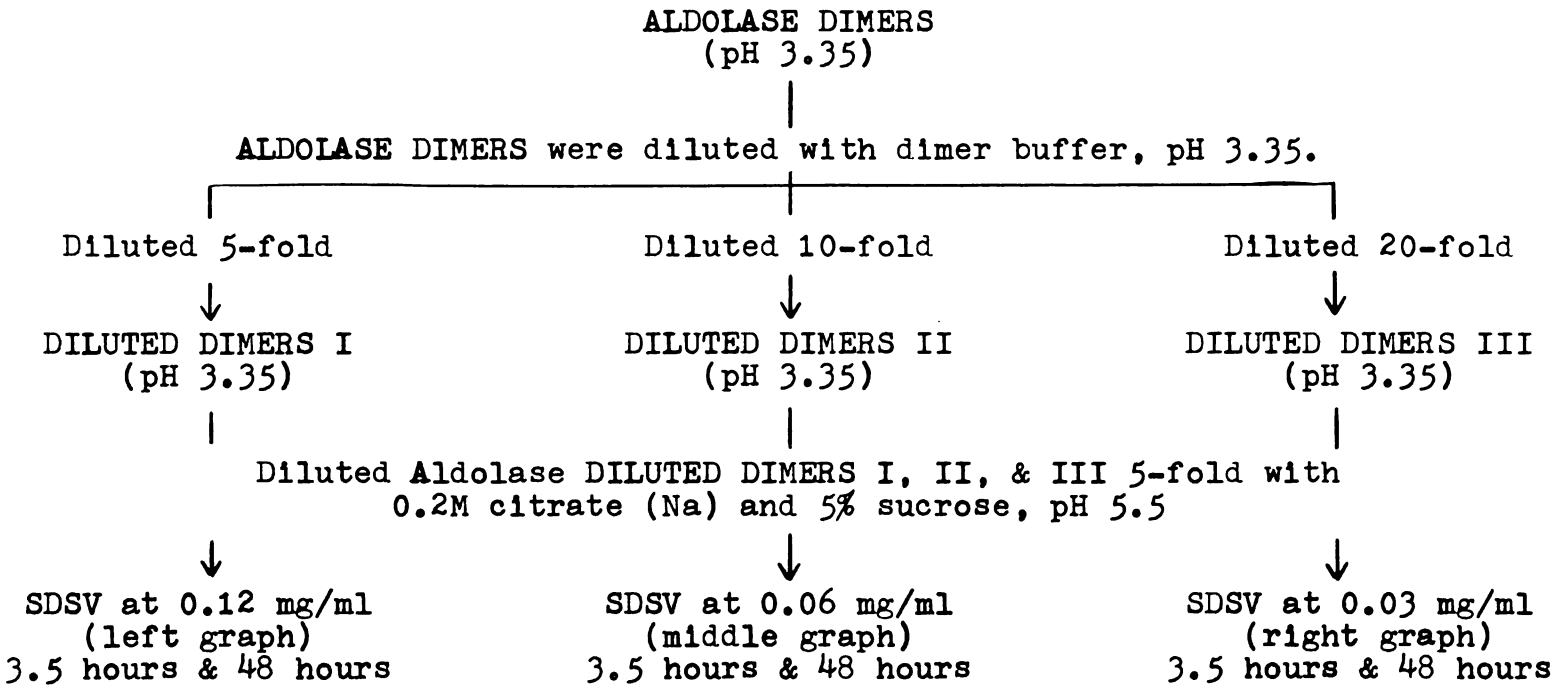
Dimers were produced at pH 5.5 at three different protein concentrations, 0.12, 0.06, and 0.03 mg/ml, incubated at pH 5.5 at 4°C for 3.5 hours, and analyzed by SDSV experiments (see Figure 25) at -6°C.

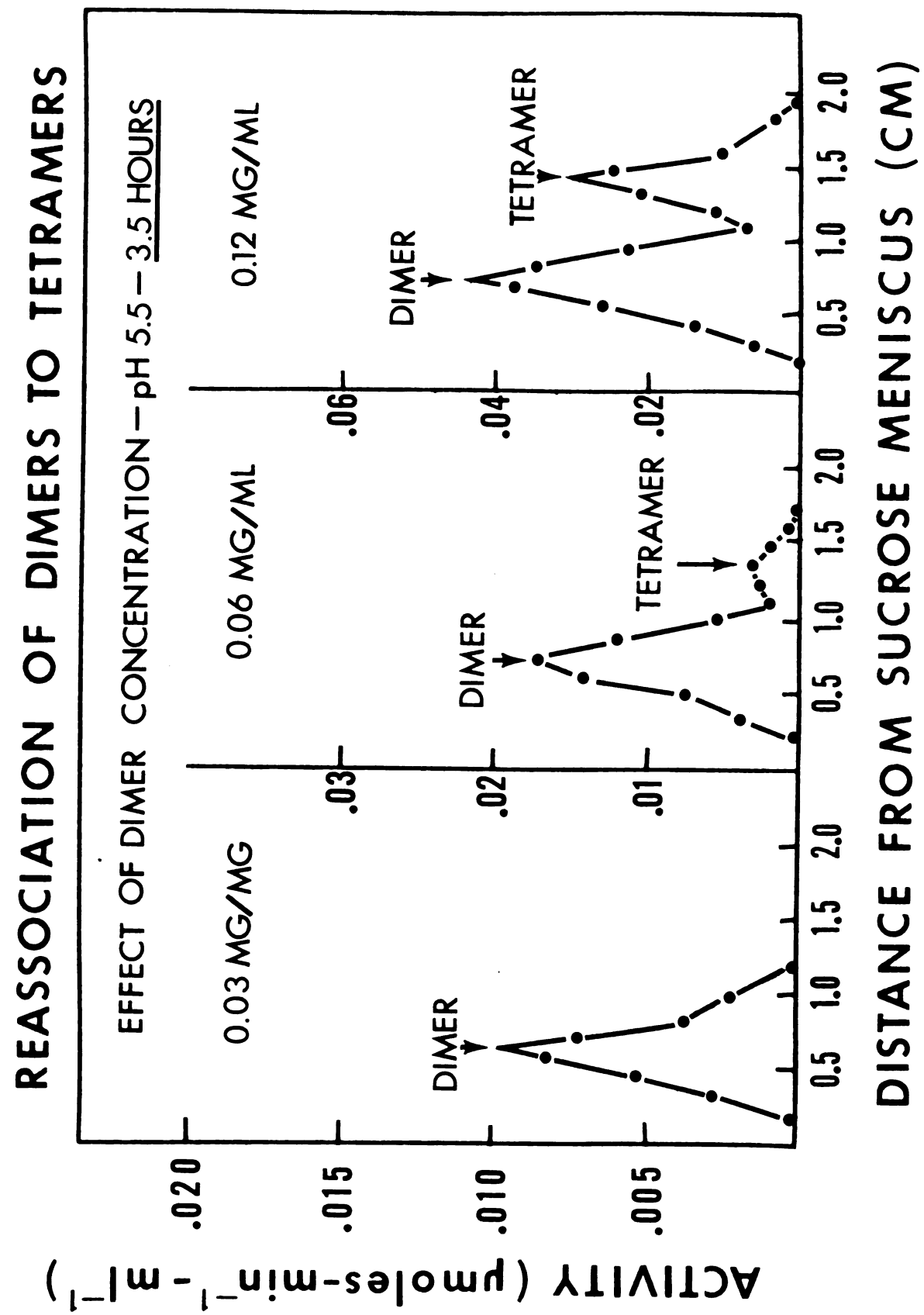
The results clearly demonstrated that dimer association was concentration dependent and that 0.03 mg/ml was probably the lowest concentration which could be used in later experiments and still retain good sensitivity. Moreover, since essentially no association took place in 3.5 hours at this concentration, and since immediate activity was present in the dimer peak, this strongly suggested that pH 5.5 dimers were active. Nevertheless, the possibility that the activity might be due to tetramer formation from dimers during the incubation had to be considered.

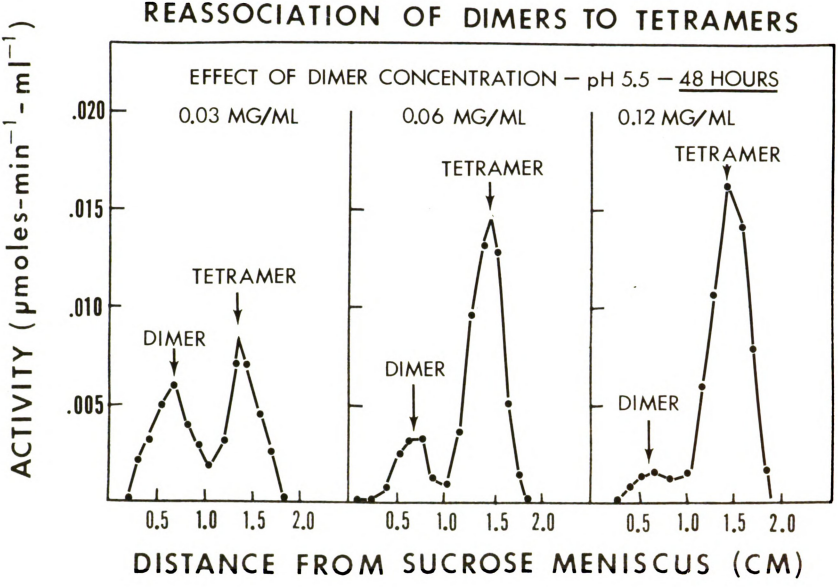
3. Effect of Incubation Time on Association at Various Concentrations at pH 5.5

This experiment was a part of 2-b above. In this part of the experiment, after the 3.5 hour incubation reported in 2-b, the pH 5.5 dimers were incubated for an additional 48 hours. The effect of incubation for 3.5 hours or for 48 hours at the three dimer concentrations

Figures 25 and 26. Effect of dimer concentration on the reassociation of dimers to tetramers after 3.5 hours and 48.0 hours incubation at pH 5.5. The sedimentation coefficients for the dimer in the left, middle, and right graphs of Figures 25 are 3.8S, 3.9S, and 4.1S, respectively; and those values for the dimer in the left, middle, and right graphs of Figure 26 are 3.8S, 4.1S, and 3.4S, respectively.




REASSOCIATION OF DIMERS TO TETRAMERS
EFFECT OF DIMER CONCENTRATION — pH 5.5 — 3.5 HOURS
0.03 MG/MG
0.06 MG/ML
0.12 MG/ML
DIMER
TETRAMER
ACTIVITY (μmoles-min⁻¹ - ml⁻¹)
.020
.015
.010
.005
.03
.02
.01
.06
.04
.02
0.5 1.0 1.5 2.0
DISTANCE FROM SUCROSE MENISCUS (CM)


REASSOCIATION OF DIMERS TO TETRAMERS
EFFECT OF DIMER CONCENTRATION – pH 5.5 – 48 HOURS
0.03 MG/ML
0.06 MG/ML
0.12 MG/ML
TETRAMER
DIMER
ACTIVITY (μmoles-min⁻¹ - ml⁻¹)
.020
.015
.010
.005
0.5
1.0
1.5
2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

(0.12, 0.06, and 0.03 mg/ml) is depicted in Figures 25 and 26. As expected, longer incubation times promoted increased association--even at 0.03 mg/ml (Figure 26) some association took place, in contrast to the results for the 3.5 hour incubation where, at 0.03 mg/ml (Figure 25), no association took place.

But if the association is occurring continuously with time, then why doesn't association take place during the 20 hour centrifugation period, and cause one broad peak? The following analysis gives one possible explanation. If the amount of tetramerization at 0.03 mg/ml, for a 20 hour incubation period, is estimated by interpolation from the 48 hour incubation data, about 15% of the total dimers would have associated. If this activity were then distributed in the gradient position between the dimer and tetramer peaks--such a distribution process would be expected to occur during the 20 hour centrifugation period--the activity due to this association would be too low to be detectable at any one point in the gradient. Thus two sharp peaks would still emerge and it would appear as if association had not occurred during the centrifugation period. Thus, whether this explanation is the correct one is not known; however, it is consistent with the data.

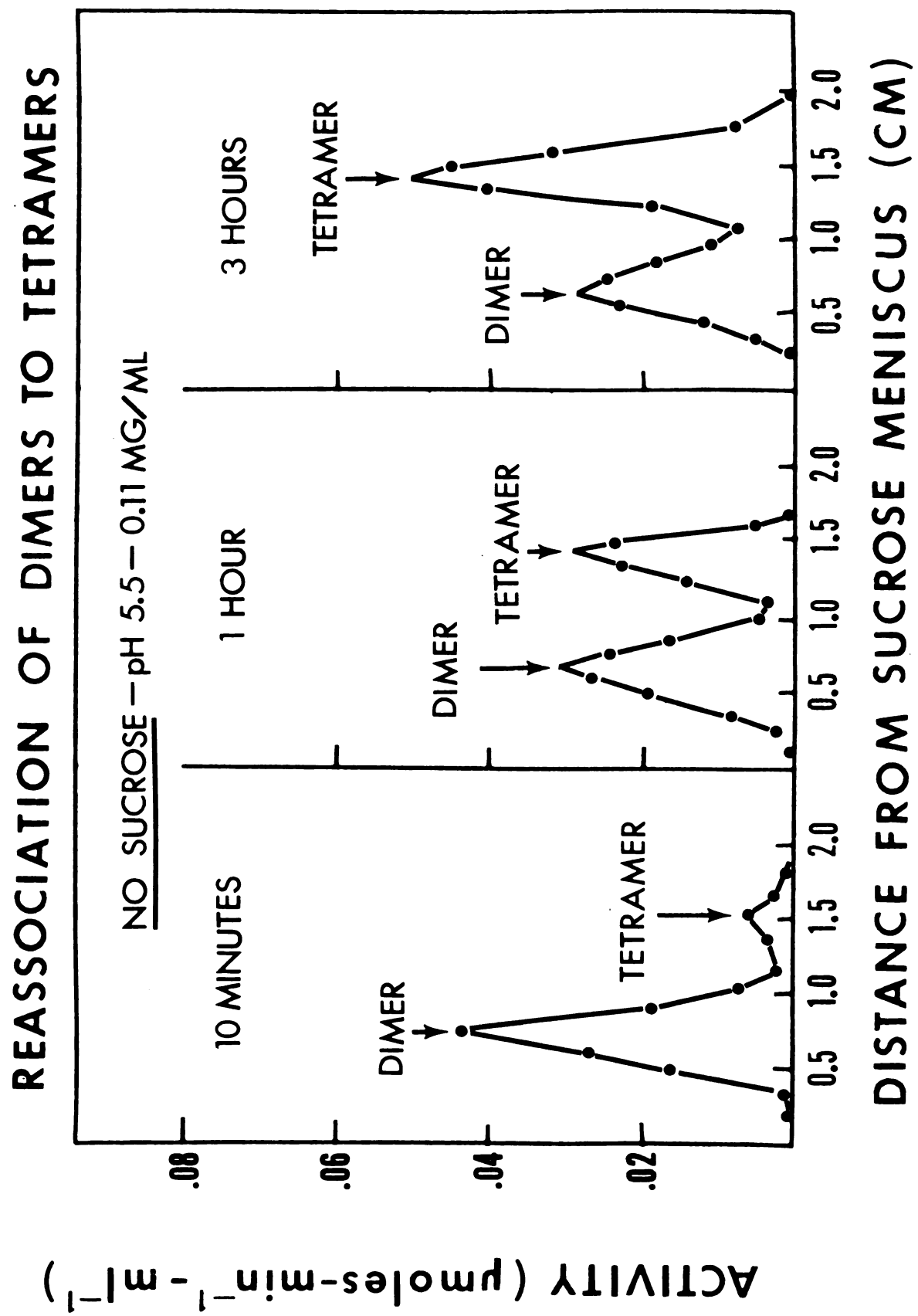
Finally, the major conclusions from these experiments as to the best combination of factors are the following: (1) pH 5.5, (2) initial dimer concentrations of 0.03 mg/ml, and (3) short incubation times (less than four hours),

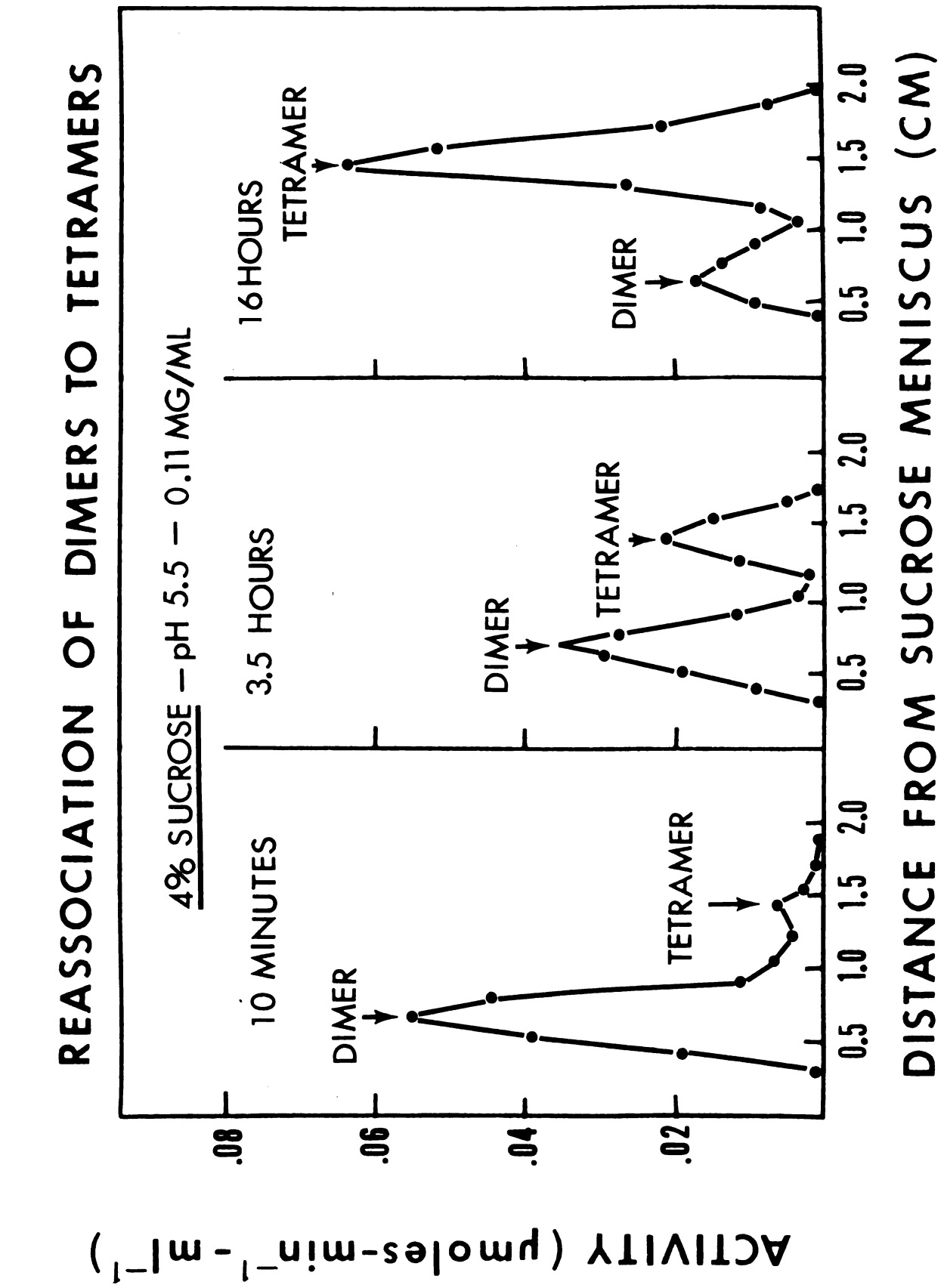
especially since enzyme from the dimer peak was active immediately upon assaying. There was another variable, however, which might have been expected to influence the rate of dimer association; that variable was sucrose. Sucrose solutions are very viscous, especially at low temperatures, and could effectively inhibit the diffusion-limited bimolecular reaction.

4. Effect of Sucrose Concentration on Dimer Association at pH 5.5

To test the effect of sucrose, dimers were prepared in the usual manner (see legend of Figures 27 and 28 for details) at pH 3.35 and diluted to pH 5.5. Dimers at pH 5.5 were then incubated with or without 4% sucrose for various times at 4°C and 0.11 mg/ml and analyzed by SDSV. The samples without sucrose were incubated for 10 minutes, 1 hour, and 3 hours (depicted in left, middle, and right graphs, respectively, of Figure 27); whereas the samples with 4% sucrose were incubated for 10 minutes, 3.5 hours, and 16 hours (depicted in the left, middle, and right graphs, respectively, of Figure 28). The conclusion from this experiment was that sucrose does indeed inhibit dimer association into tetramers. The results show that samples containing 4% sucrose had to be left for longer times to allow the same amount of association found for the control samples without sucrose (Figures 27 and 28). For example, the dimer-tetramer distribution favored tetramers (40%-60%) in

Figures 27 and 28. Effect of sucrose concentration on dimer association at pH 5.5 after various incubation times. SDSV analysis of dimer-tetramer distributions without sucrose after incubation for 10 minutes, 1 hour, and 3 hours is shown in left, middle, and right graphs, respectively, of Figure 27. SDSV analysis of dimer-tetramer distributions with 4% sucrose after incubation for 10 minute, 3.5 hours, and 16 hours is shown in the left, middle, and right graphs, respectively, of Figure 28. The sedimentation coefficients for the dimer in the left, middle, and right graphs of Figure 27 and 4.5S, 3.8S, and 3.5S, respectively. The sedimentation coefficients for the dimer in the left, middle, and right graphs of Figure 28 are 4.2S, 4.0S, and 3.5S, respectively. (See Methods for the equation used for the dimer sedimentation coefficient determination.) Procedure for pH 5.5 dimer preparation was the same as that used for Figures 25 and 26 (left graph) with the exception that either no or 5% sucrose was present in final pH 5.5 dilution step. See text for details.


REASSOCIATION OF DIMERS TO TETRAMERS
NO SUCROSE—pH 5.5—0.11 MG/ML
10 MINUTES
1 HOUR
3 HOURS
DIMER
TETRAMER
ACTIVITY (pmoles-min⁻¹-ml⁻¹)
.08
.06
.04
.02
0.5 1.0 1.5 2.0
DISTANCE FROM SUCROSE MENISCUS (CM)


REASSOCIATION OF DIMERS TO TETRAMERS
4% SUCROSE – pH 5.5 – 0.11 MG/ML
10 MINUTES
3.5 HOURS
16 HOURS
DIMER
TETRAMER
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
.08
.06
.04
.02
0.5 1.0 1.5 2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

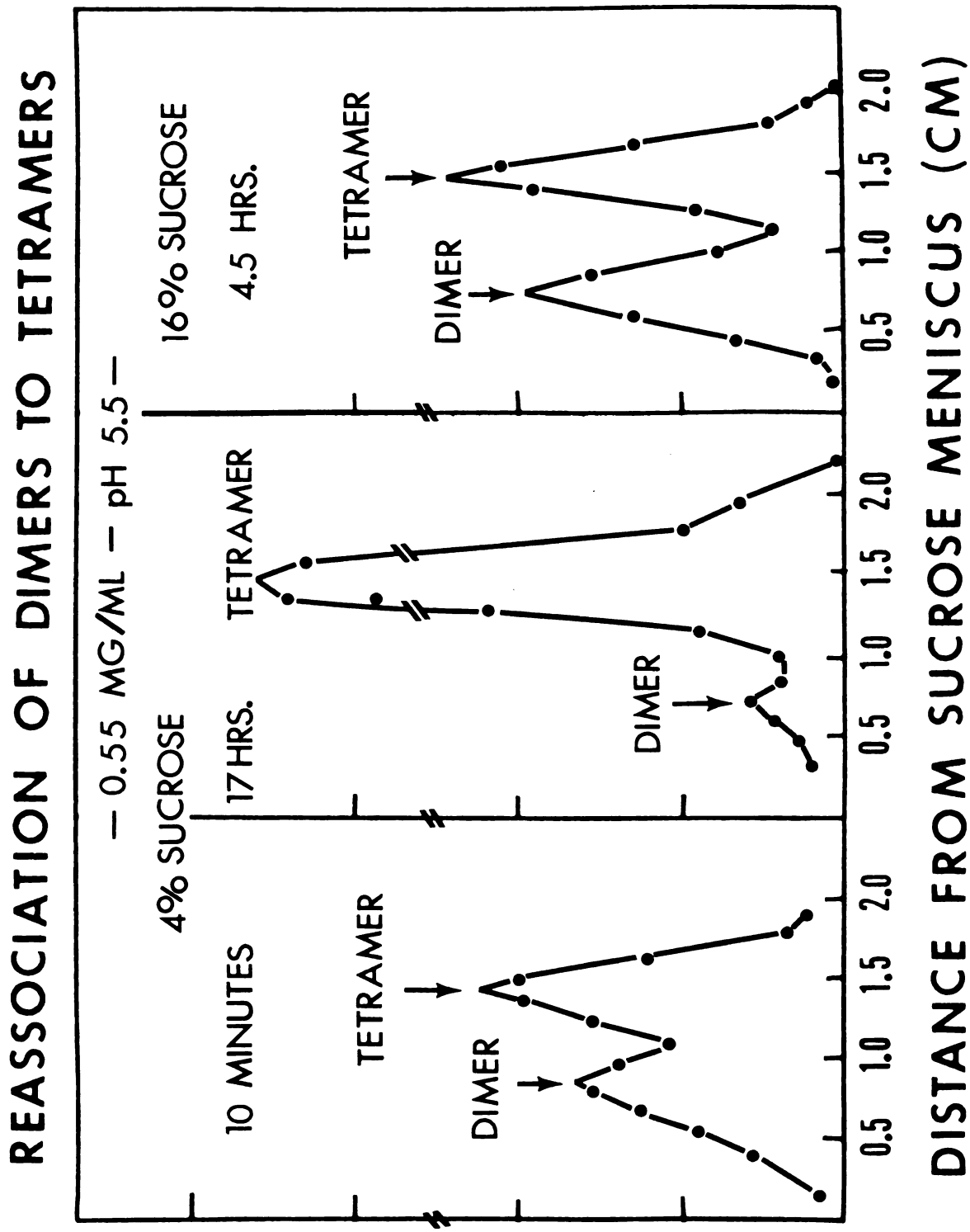
the sample without sucrose after a 3 hour incubation; whereas in the sample with sucrose after a slightly longer time of incubation (3.5 hours), the dimer-tetramer distribution still favored dimers (60%-40%).

Although association was inhibited by sucrose, it was expected that higher protein concentrations would overcome the sucrose inhibition and yield rapid association. In addition to testing this possibility, it was also of interest to test the ability of sucrose concentrations higher than 4% to inhibit dimer association. Anticipating the effects to be obtained, the time of incubation was changed, in the experiments to follow (respectively), to obtain a similar dimer-tetramer distribution in the higher dimer concentration and in the higher sucrose concentration samples.

When pH 5.5 dimers were incubated for only 10 minutes at 0.55 mg/ml (instead of 0.11 mg/ml), approximately 60% of the area was found in the tetramer peak (Figure 29, left graph); but in the previous experiment (Figure 28) conducted at 0.11 mg/ml, it had required a much longer time (4.5 hours) to obtain the same amount of tetramer. Both samples contained 4% sucrose. When the 0.55 mg/ml sample (for details of procedure, see legend of Figure 29) was allowed to react for 4.5 hours in 4% sucrose, about 85% reversal was obtained by activity analysis; almost complete association had taken place by 17 hours (Figure 29, middle graph).

The third experiment in this series was to gain further information concerning the strength of sucrose inhibi-

Figure 29. Effect of higher protein concentration and higher sucrose concentration (16%) on dimer association at pH 5.5. The procedure was the same for samples depicted in the left and middle graphs as that used in the previous experiment at 4% sucrose except that the dimer concentration was 0.55 mg/ml instead of 0.11 mg/ml. And the only difference between the sample depicted in the right graph and those in the middle and left graphs is that 16% sucrose was present. The sedimentation coefficients for the dimer in the left, middle, and right graphs are 4.9S, 4.1S, and 4.1S, respectively. See Methods for calculation of sedimentation coefficients. Also, see text for other details.


REASSOCIATION OF DIMERS TO TETRAMERS
— 0.55 MG/ML — pH 5.5 —
4% SUCROSE
10 MINUTES
17 HRS.
16% SUCROSE
4.5 HRS.
TETRAMER
DIMER
.8
.4
.2
.1
0.5 1.0 1.5 2.0
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
DISTANCE FROM SUCROSE MENISCUS (CM)

tion of association. Dimer samples (0.55 mg/ml) at pH 5.5 were incubated for 4.5 hours in the presence of 16% sucrose and analyzed by SDSV. The conclusion was that 16% sucrose inhibited dimer association more than 4% sucrose.

The results (Figure 29, right graph) show that the dimer-tetramer distribution favored dimers (40%-60%) after the 4.5 hour incubation at 16% sucrose. In comparison, in the previous experiment (Figure 29, left graph) at the same dimer concentration but at 4% sucrose, the dimer association rate was much faster: only 5 minutes were required to obtain a similar distribution; after 4.5 hours, the reaction was 85% complete. Since the enzyme concentration was diluted four-fold in this experiment, the activity scale for Figure 29, left graph, was consequently increased four-fold so that all three graphs in Figure 29 could be compared. The reason for the four-fold dilution of the enzyme was to accomodate a dilution of the 16% sucrose solution to 4%, so that the enzyme sample incubated in 16% sucrose could be layered on the 5%-20% sucrose gradient without disturbing it. There are two reasons why this method should not affect the results: (1) the dilution was accomplished immediately before the SDSV analysis, and (2) previous studies at 0.14 mg/ml had demonstrated that a 5 minute incubation did not produce any association in 4% sucrose (see Figure 27, left graph).

Sucrose was also found to be able to inhibit non-specific aggregation as well as the specific association into tetramers. Thus, when a sample of pH 3.35 dimers was diluted

into 0.2M imidazole buffer at pH 7.9 to a final enzyme concentration of 1.3 mg/ml with no sucrose, immediate aggregation occurred, as indicated by a white precipitate formed; but when 20% sucrose was present, no precipitation was evident. Also the percent recoveries of activity increased from 45% with no sucrose, to 70% with 20% sucrose.

In conclusion, these experiments show that sucrose did indeed strongly inhibit association, as expected, but the inhibition was still not complete. As stated in the introduction of this chapter (IV), for a study of active dimers, it was desirable to be able to use experimental conditions (1) where a sample of dimers exhibited activity upon assaying--which has been achieved with the pH 5.5 dimer system--but (2) where dimer association was completely inhibited. Any additional inhibition of association would aid in achieving the second objective; for this reason, all reversal solutions were made 4% in sucrose for all subsequent experiments.

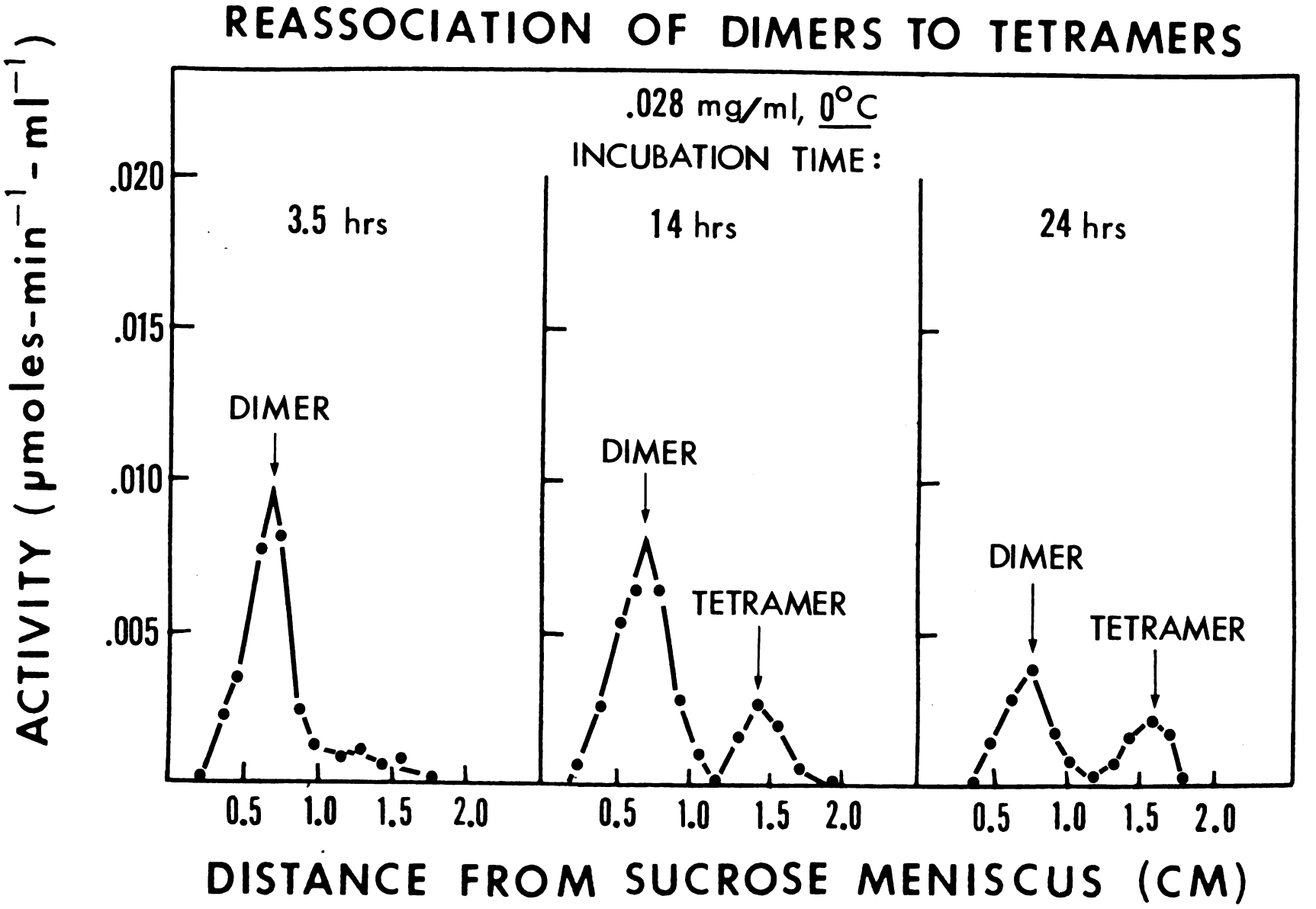
One final variable which had not been investigated was temperature. Since in previous experiments the reaction mixtures were incubated at 4°C and analyzed by SDSV at -6°C, it was of interest to test incubation temperatures of 0°C and -6°C. The results from these experiments could then be compared with the 4°C experiments to see if the lower temperatures would more effectively inhibit dimer association.

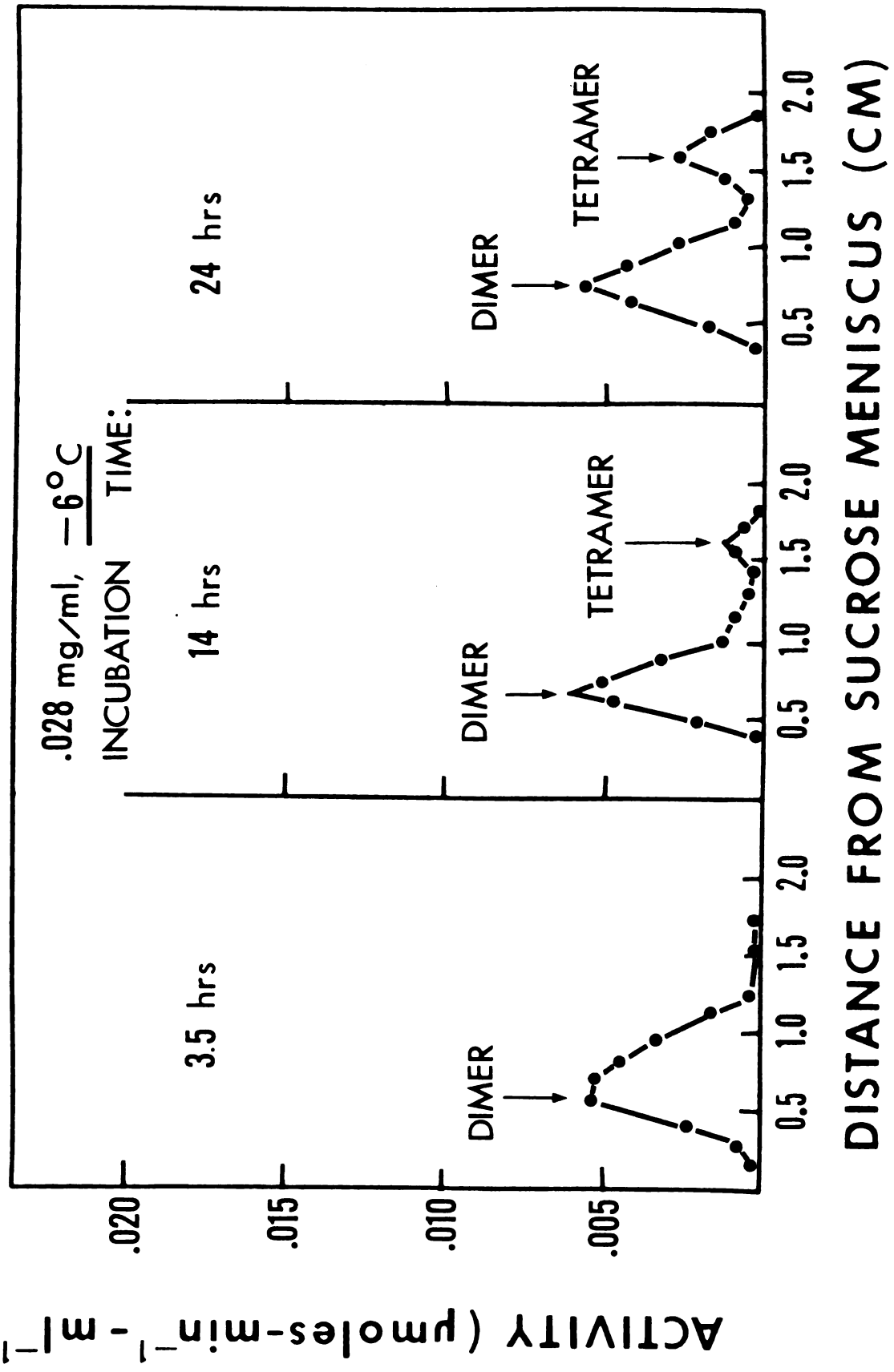
5. Effect of Incubation at 0°C and -6°C for Various Times at 0.03 mg/ml and pH 5.5

Since the inhibitory effect of lower temperatures (0°C and -6°C vs. 4°C) on dimer association was of primary interest in this experiment, and since association was measured by rate of sedimentation in SDSV experiments, no special precautions for temperature regulation were necessary once the dimer and tetramer peaks were separated, i.e. after SDSV analysis. However, the following special precautions were needed and taken for that part of the experiment preceding the SDSV separation, to make sure that the temperature of the rotor, gradients, and enzyme solutions did not rise above 0°C or -6°C: (1) Rotors were cooled to 0°C and -6°C by incubation at -20°C for 12 minutes and 20 minutes, respectively. (2) All gradients were cooled at 4°C for 3 hours, followed by (a) incubation of three gradients at -20°C for 7 minutes to lower the temperature to 0°C, and by (b) incubation of the other three gradients at -20°C for 12 minutes to lower the temperature to -6°C. (3) The pH 5.5 dimer samples were maintained at 0°C and -6°C in salt-ice baths. That these procedures accomplished their objective was verified by using a thermocouple probe to measure the temperature in an earlier trial run.

A sample of pH 3.35 dimers was prepared (see legend of Figures 30 and 31 for details), diluted five-fold to pH 5.5 at 0°C and -6°C to a final dimer concentration of 0.03 mg/ml, incubated for 3.5, 14, and 24 hours, and analyzed by

Figures 30 and 31. Effect of incubation at 0°C and -6°C on dimer association into tetramers at the indicated incubation times. The effect of the incubations at 0°C are depicted in Figure 30, whereas the effect of incubation at -6°C are shown in Figure 31. The sedimentation coefficients of the dimer in the left, middle, and right graphs of Figure 30 are 3.7S, 3.6S, and 3.7S, respectively, and those values in Figures 31 are 3.3S, 3.5S, and 3.7S, respectively. (See Methods for the calculation of the dimer sedimentation coefficients.) Refer to the text for further details.


REASSOCIATION OF DIMERS TO TETRAMERS
.028 mg/ml, 0°C
INCUBATION TIME:
3.5 hrs
14 hrs
24 hrs
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
.020
.015
.010
.005
DIMER
DIMER
TETRAMER
DIMER
TETRAMER
0.5
1.0
1.5
2.0
DISTANCE FROM SUCROSE MENISCUS (CM)


REASSOCIATION OF DIMERS TO TETRAMERS
.028 mg/ml, —6°C
INCUBATION TIME:
3.5 hrs
14 hrs
24 hrs
DIMER
TETRAMER
ACTIVITY (μmoles-min⁻¹- ml⁻¹)
.020
.015
.010
.005
0.5 1.0 1.5 2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

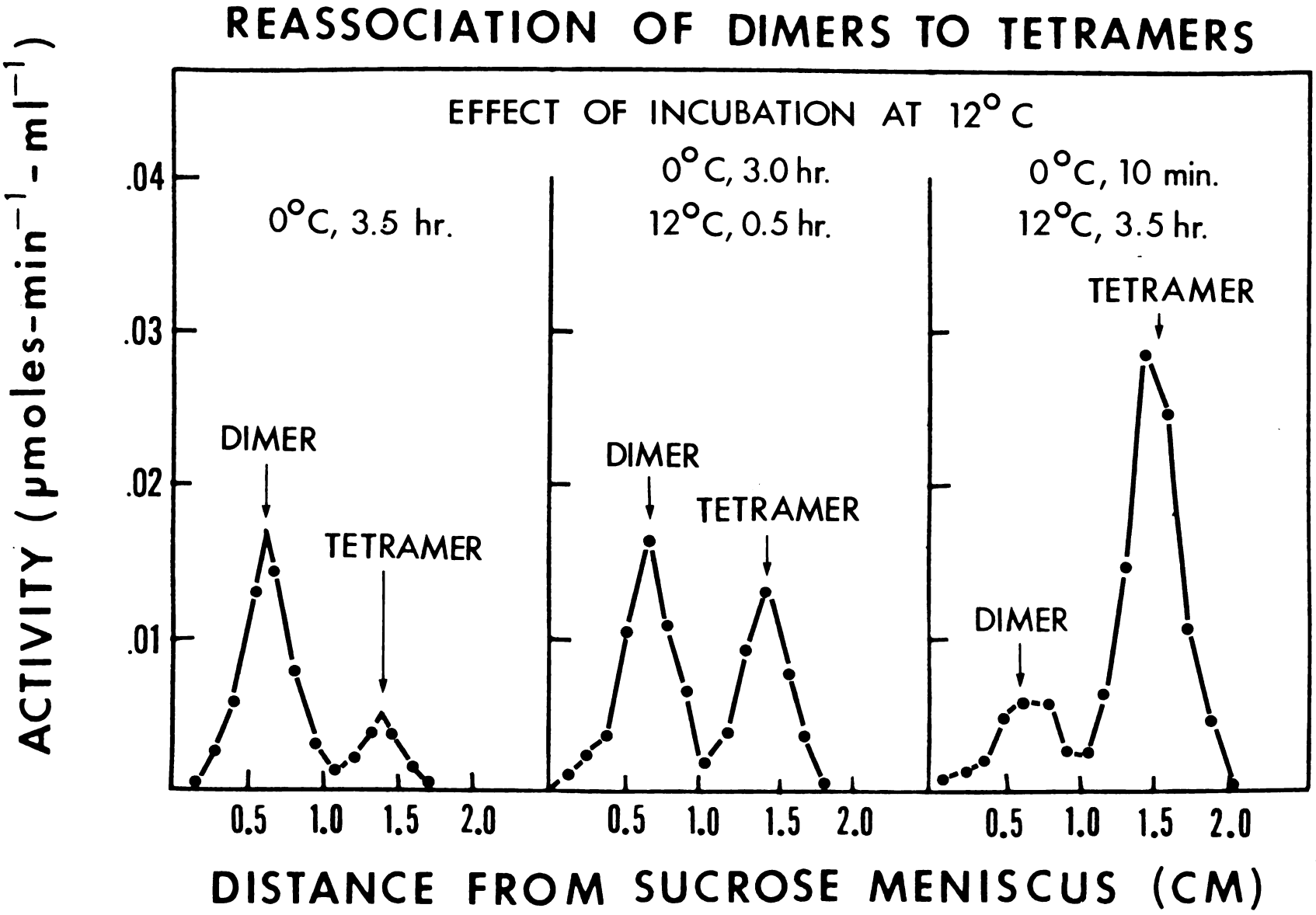
SDSV. The results (Figures 30 and 31) show that only 3-6% of the dimers had associated by 20 hours; thus almost complete inhibition of association was found at both 0°C and -6°C, using a protein concentration of 0.03 mg/ml.

Since lower temperatures strongly inhibited association, an experiment was designed to test the effect of incubation at higher temperatures (12°C) on dimer association into tetramers. Although somewhat unlikely, it was hoped that increased temperatures might aid dimer folding and thus produce active dimers, without significant association.

6. Effect of Incubation at a Higher Temperature (12°C) on Dimer Association

Dimers were diluted five-fold into the 4% sucrose solutions at pH 5.5 and incubated for various times at 0°C and 12°C at a final concentration of 0.05 mg/ml. The control was incubated for 3.5 hours at 0°C while the two experimental samples were incubated for (a) 3.0 hours at 0°C, followed by 0.5 hours at 12°C (sample #1), or for (b) 10 minutes at 0°C, followed by 3.5 hours at 12°C (sample #2). The control and experimental samples, 1 and 2, were then analyzed by SDSV. The activity patterns obtained for the control and experimental samples, 1 and 2, are depicted in the left, middle, and right graphs, respectively, of Figure 32. The results demonstrate that increased temperature (12°C vs. 0°C) stimulates the rate of dimer association into tetramers and significantly, the stimulation is time-dependent.

Figure 32. Effect of a higher temperature (12°C) on dimer association into tetramers. Dimers were incubated at pH 5.5 for a total time of 3.5 hours at the indicated temperatures (0°C or 12°C). Sedimentation coefficients of the dimer in the left, middle, and right graphs are 3.5S, 3.7S, and 3.8S, respectively. (See Methods for the calculation of the S values.) For more details, refer to the text.


REASSOCIATION OF DIMERS TO TETRAMERS
EFFECT OF INCUBATION AT 12° C
0°C, 3.5 hr.
0°C, 3.0 hr.
12°C, 0.5 hr.
0°C, 10 min.
12°C, 3.5 hr.
DIMER
TETRAMER
DIMER
TETRAMER
TETRAMER
DIMER
.04
.03
.02
.01
0.5
1.0
1.5
2.0
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
DISTANCE FROM SUCROSE MENISCUS (CM)

Presumably, the dimers are already somewhat folded and the increased temperature increases the collision frequency of the diffusion-limited association reaction. "Hydrophobic bonding" is also stronger at 12°C than at 0°C and thus may help stimulate dimer association.

7. Effect of Substrate on Dimer Association at pH 5.0 After a 4 Hour Incubation

Even though dimers at pH 5.0 were not catalytically active (see experimental results in Section B-1 in this chapter), the possibility existed that they might still bind the substrate, i.e. FDP. If the dimers did bind substrate, the substrate might affect dimers in two ways: (1) The substrate might stabilize a folded dimer structure; this would allow dimer activity to be tested. (2) The substrate might change the conformation of the dimers in such a way as to stimulate dimer association.

In general, substrates, when bound to their native enzymes frequently tighten their structures and stabilize them against denaturation and dissociation. If the substrate could bind to its (postulated) subpolymer binding site and induce a conformational change to a more folded state, it conceivably could shift the subpolymer-polymer equilibrium towards association. The other possibility is that FDP could induce an active dimer formation. The substrate could stabilize the dimer structure in a conformation which was catalytically active while the physical

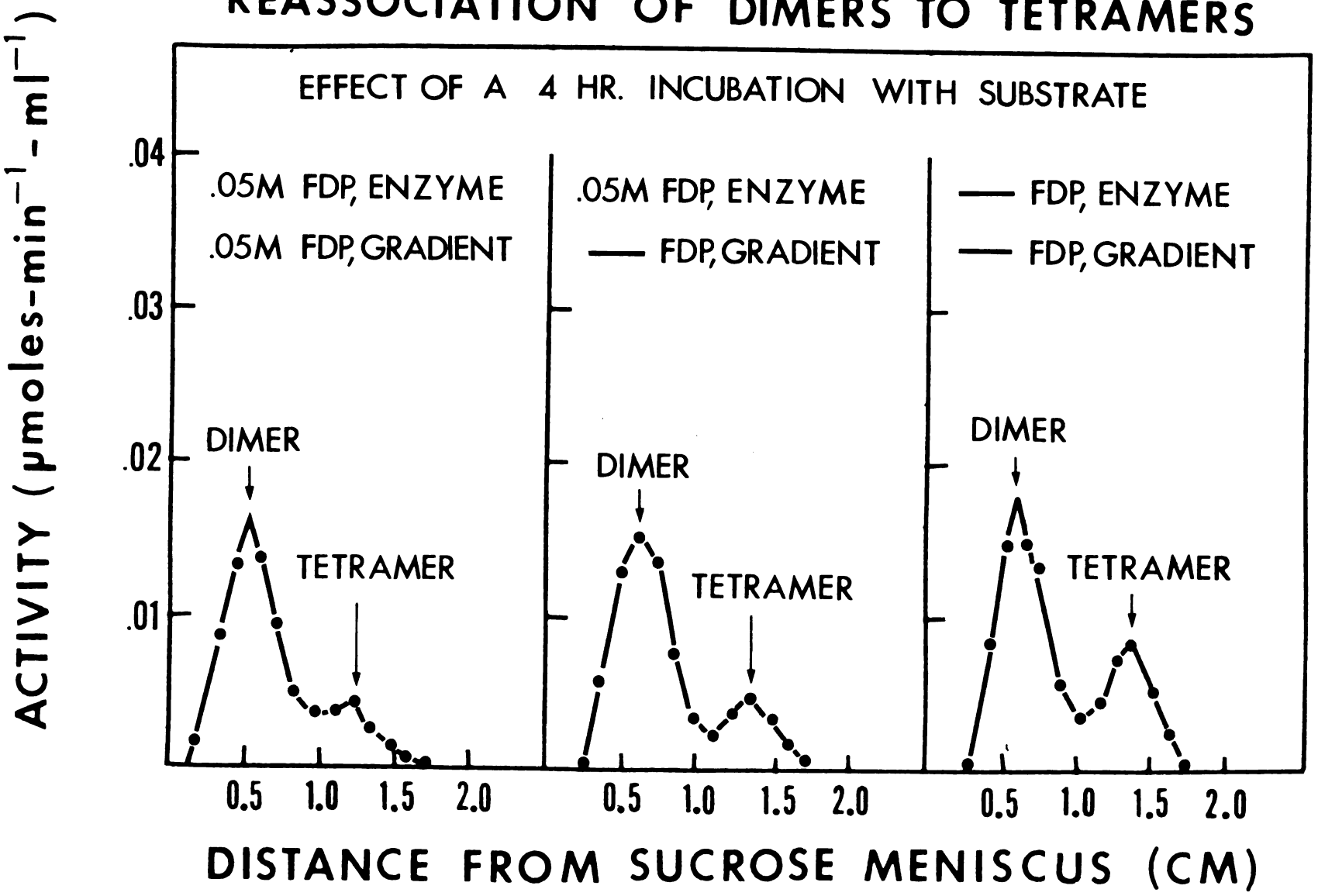
forces such as pH, low temperatures, low protein concentration could be used to inhibit dimer association. Examples of enzymes whose substrates stabilize them against unfolding and denaturation are phosphofructokinase, pyruvate kinase, and carboxypeptidase.

The purpose of the following experiment was to test whether such an effect of substrate might occur with rabbit muscle aldolase dimers at pH 5.0. It was also of interest to see whether the substrate might have effect on the aldolase dimers when FDP was present in the sucrose gradient solutions during centrifugation.

Dimers were prepared, diluted 5-fold into pH 5.0 citrate buffer to a final concentration of 0.08 mg/ml, incubated for 4 hours in the presence and absence of 0.05M FDP, and analyzed by SDSV. The activity pattern for the test sample which was both incubated in FDP and run in a gradient containing FDP is shown in the left graph of Figure 33. The activity pattern for this test sample should be compared to that for the control, which was incubated without FDP and run in an FDP-free gradient (see right graph of Figure 33 for the control pattern). For the effect of incubating with FDP and running without FDP in the gradient, refer to the middle graph in this figure.

A comparison of the test sample and control sample shows that FDP did not stimulate association or dimer reactivation as originally postulated; in fact if anything, FDP inhibited association. Other SDSV experiments showed that

Figure 33. Effect of substrate on dimer association into tetramers after a 4 hour incubation at pH 5.0. In the sample depicted in the left and middle graphs, FDP was present with enzyme sample during the 4 hour incubation, whereas no FDP was present in the sample in the right graph. FDP was present only in the gradient of the experiment depicted in the left graph. Sedimentation coefficients of the dimer in the left, middle, and right graphs are 3.5S, 3.7S, and 3.6S. (See Methods for the calculation of the S values.) Refer to text for details.


REASSOCIATION OF DIMERS TO TETRAMERS
EFFECT OF A 4 HR. INCUBATION WITH SUBSTRATE
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
.04
.03
.02
.01
.05M FDP, ENZYME
.05M FDP, GRADIENT
.05M FDP, ENZYME
FDP, GRADIENT
FDP, ENZYME
FDP, GRADIENT
DIMER
TETRAMER
DIMER
TETRAMER
DIMER
TETRAMER
0.5
1.0
1.5
2.0
0.5
1.0
1.5
2.0
0.5
1.0
1.5
2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

pyrophosphate also inhibited dimer association (these experiments will not be reported) and suggested that the FDP inhibition was probably through the phosphate binding site on the dimer which remains partially intact after dissociation. These results are consistent with the FDP inhibition of dimer reactivation and dimer association found in Chapter III-G.

V. Analysis for Active Dimers

In the previous chapter it was shown that aldolase dimers could be produced at pH 5.5 and, after SDSV analysis, exhibited immediate activity upon assaying the dimer peak. Significantly, the activity assays from the SDSV fractions were linear with time, showing no lag times and thus, there apparently was no reactivation in the assay. In this chapter we explore further the question of whether the dimers are catalytically active. This involves detailed analysis of the kinetics of the reversal process of pH 5.5 dimers, not subjected to SDSV analysis, (Section B--first part) and further experiments related to the possibility that dimers might be inactive--with the "apparent" dimer activity being due to formation of heretofore undetected active tetramers at some point in the analysis. In particular, we analyzed the two possibilities that the apparent dimer activity might be due to tetramer formation either (1) during SDSV analysis or immediately after SDSV analysis and before the assay procedure (Section A), or (2) in the assay (Section B--second part). The two possibilities were both tested directly by SDSV analysis for tetramers in the products in the two cases--in the first case, by SDSV "rerun" of the dimer fraction from the initial SDSV, and in the second case, by SDSV analysis of samples taken directly from the assay.

In Section B (first part), kinetics of activity reversal from pH 5.5 dimers (0°C), not subjected to SDSV analysis, were analyzed to see if this technique would answer the

question of whether dimers were active or not. The pH 5.5 dimers, not subjected to SDSV analysis, were not active initially in contrast to the pH 5.5 dimers which were subjected to SDSV separation. Therefore, the immediate question was whether the reactivation of the inactive (initially) pH 5.5 dimer was first-order or second-order with respect to dimer concentration. A first-order reversal process from pH 5.5 dimers would be consistent with active dimers (although other possibilities exist, see Section B of this chapter); whereas a second-order process would require a dimer association step before activity reversal and would therefore, be inconsistent with active dimers.

But as will be shown, the results from these two sections were quite perplexingly contradictory. These descriptive words understate the dilemma of this complicated research problem. Fortunately, Section C resolved this "apparent" dilemma and provides a unifying touch to this chapter--and for that matter, to chapter III as well. However, as will be shown, there still remains a discrepancy between the results with pH 5.5 dimers which have been subjected to SDSV analysis and those which have not; the former seem to be active but the latter do not.

A. Test for Dimer Association After the SDSV Analysis but Before the Assay Procedure

1. Effect of Post-SDSV Incubation at -6°C, 4°C, and 12°C On Activity: An Indirect Test

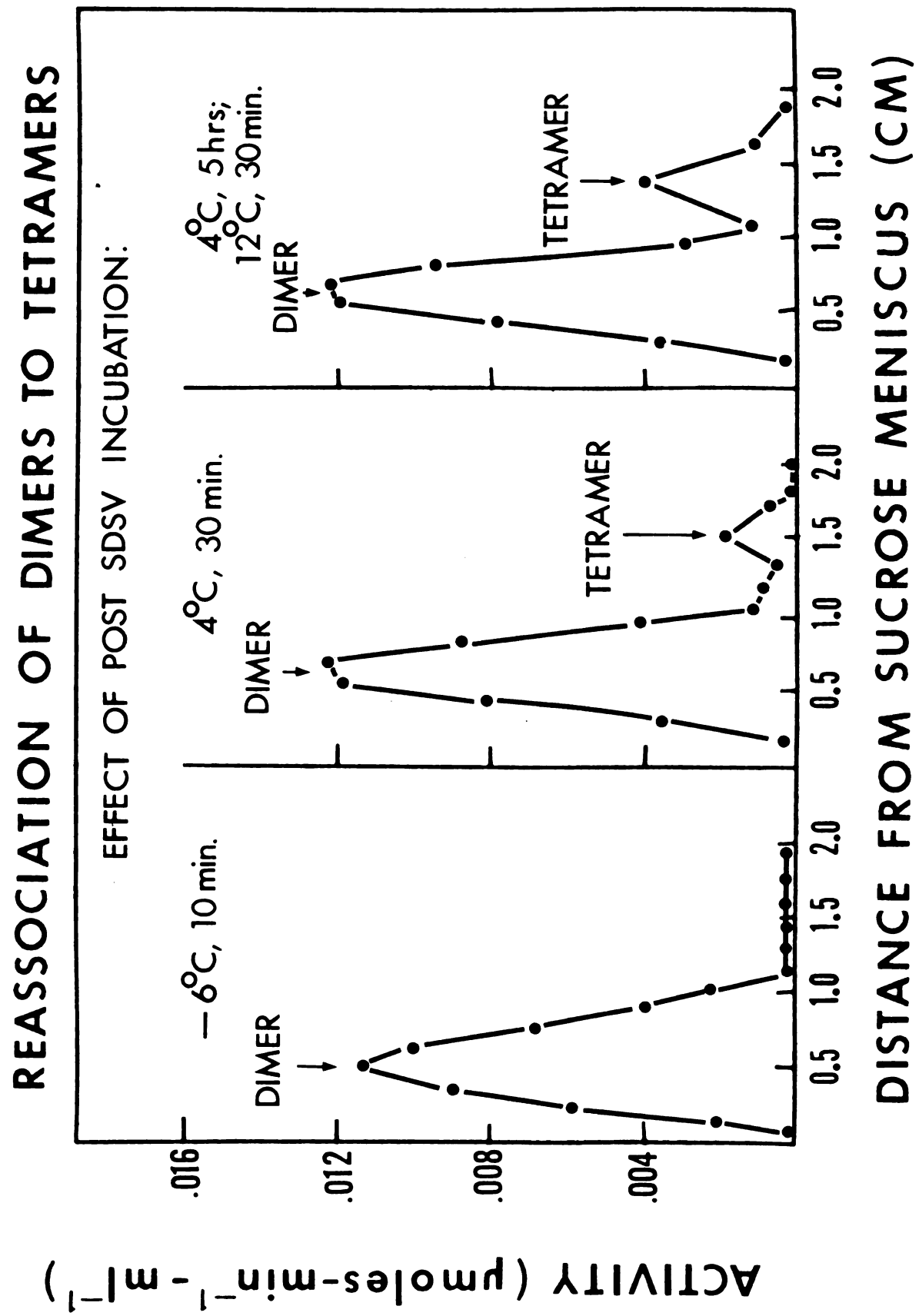
This experiment was designed to test for activation--and thus association (assuming that this correlation can be drawn)--after the SDSV run. It was thought that the elution temperature of 4°C might allow dimer association after the run and might be responsible for the immediate activity found in the dimer peak; thus it seemed that going to lower temperatures would avoid this problem if it were occurring. We thus decided to attempt to conduct an experiment at -6°C, taking special care to insure that at no time did the temperature of the solutions rise above this. Thus special precautions were used in this procedure to insure that at all stages of the experiment, the solutions were kept at -6°C. Procedures outlined before for the previous -6°C experiment (see Section B-5 of Chapter IV) were used again here for the first part of the experiment through the SDSV run. However, an additional feature in this experiment was the maintenance of a temperature of -6°C for the gradient tubes and fractions collected following the SDSV run. Cooling coils with circulating ethylene glycol-water solvent were used to maintain the plastic centrifuge gradient tubes at -6°C during the drop collection procedure, and fractions were collected in glass tubes equilibrated at -6°C using a

salt-ice bath.

A sample of pH 3.35 dimers was diluted five-fold to pH 5.5 to a final concentration of 0.04 mg/ml, incubated for 3.5 hours at -6°C, and aliquots applied to three gradients for subsequent SDSV analysis.

After SDSV runs, the control gradient was eluted at -6°C and assayed; its activity pattern is represented in the left graph of Figure 34. Another gradient was incubated at 4°C for 30 minutes, eluted at 4°C and assayed immediately. The activity pattern obtained is represented in the middle graph of Figure 34. This sample was then assayed again after two incubations, an additional 4.5 hour incubation at 4°C, followed by a 0.5 hour incubation at 12°C; the resulting activity pattern is represented in the right graph of Figure 34. The results show that little or no reactivation occurred upon incubation at higher temperatures (cf. 4°C and 12°C to -6°C); but there was, however, some preferential reactivation (two-fold) of the tetramer peak with incubation at the higher temperature. This data suggested that since there was no increase in activity in the dimer peak with time of incubation, no association of dimers had occurred after the SDSV analysis; however, the possible complication of simultaneous activation and dimer inactivation at the different incubation conditions due to instability cannot be excluded. Moreover, the validity of our analysis depends upon the assumption that the material in the dimer peak is dimers alone (not active tetramers) in the -6°C control.

Figure 34. Effect of post-SDSV incubation at the indicated temperatures on dimer reactivation. See text for details.


REASSOCIATION OF DIMERS TO TETRAMERS
EFFECT OF POST SDSV INCUBATION:
— 6°C, 10 min.
4°C, 30 min.
4°C, 5 hrs;
12°C, 30 min.
DIMER
TETRAMER
ACTIVITY (μmoles-min⁻¹ - ml⁻¹)
.016
.012
.008
.004
0.5 1.0 1.5 2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

Unless unequivocal proof of the correctness of this assumption can be provided, a shadow of doubt will be cast on this conclusion. This direct proof will be presented in the next section.

2. Direct Test for Possible Tetramers in the Dimer Peak

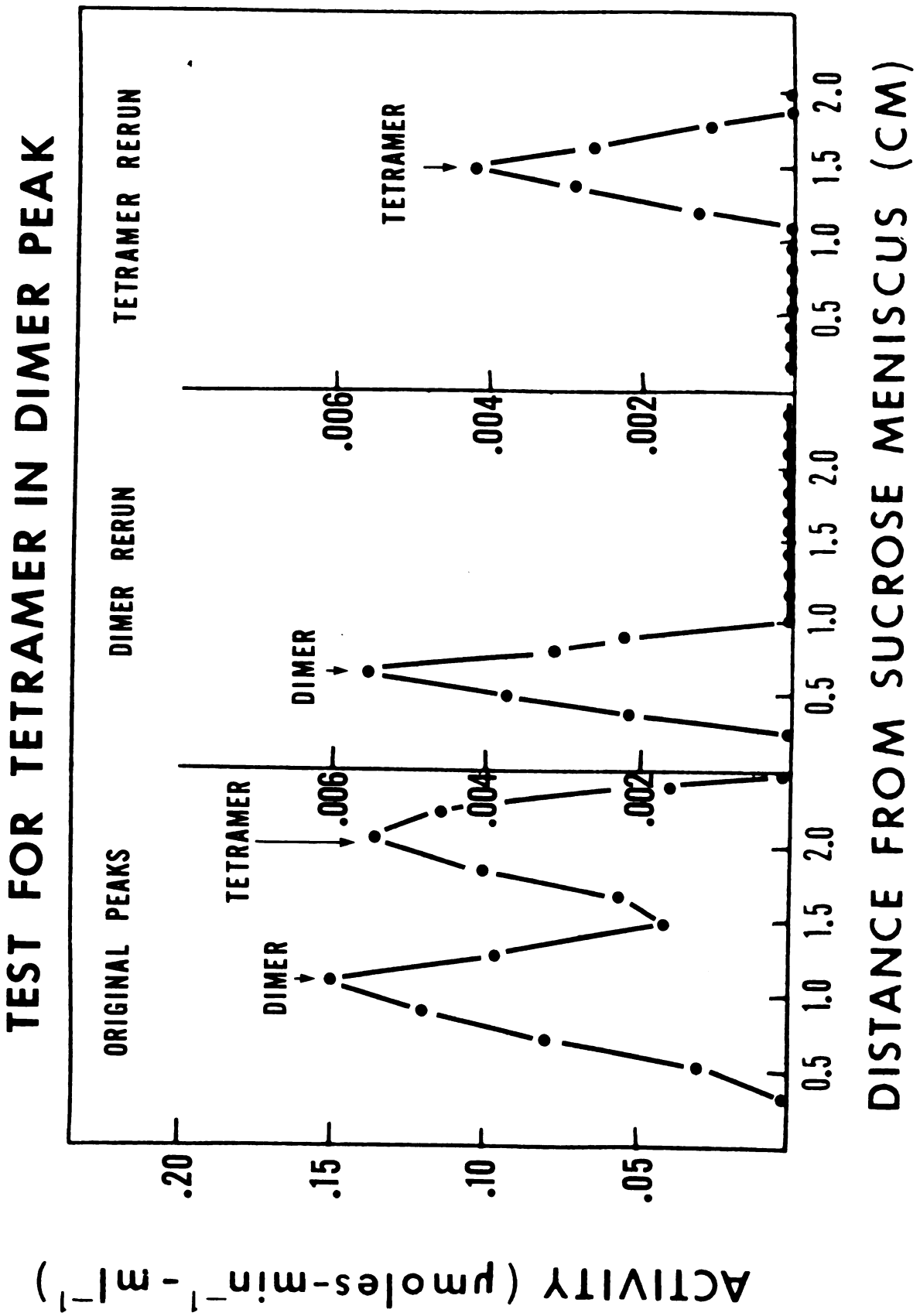
To test for the presence of tetramers in the dimer peak from SDSV analysis, an aliquot from the dimer peak fraction was "rerun" in another SDSV experiment. The special precautions used in the previous section were again used here to insure temperature regulation at -6°C.

To accomplish this "double" SDSV experiment, the initial enzyme concentrations in the first SDSV experiment had to be higher, 0.6 mg/ml, so that the second SDSV experiment would yield enzyme concentration high enough for assay detection. In addition, the first SDSV centrifugation time was extended to 40 hours to insure complete separation of the dimer and tetramer peak; however, the "rerun" experiments were centrifuged for the normal 24 hour period.

The "original" peaks from the first sucrose density experiment are shown in the left graph of Figure 35. Aliquots from these "original" dimer and tetramer peak fractions were applied to two new sucrose gradients and run again. The results from the "dimer rerun" and "tetramer rerun" experiments are shown in the middle and right graphs, respectively, of Figure 35.

As shown in the middle graph, there is no tetramer

Figure 35. Test for tetramer in dimer peak by "dimer rerun" and "tetramer rerun" SDSV experiments after the "original" SDSV separation. The sedimentation coefficients of the dimer peaks in the left and middle graphs are 4.1S and 3.6S, respectively. (See Methods for calculation of S values.) See text for details.


TEST FOR TETRAMER IN DIMER PEAK
ORIGINAL PEAKS
DIMER RERUN
TETRAMER RERUN
DIMER
TETRAMER
DIMER
TETRAMER
ACTIVITY (pmoles-min⁻¹-ml⁻¹)
.20
.15
.10
.05
.006
.004
.002
0.5
1.0
1.5
2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

contamination in the dimer peak; thus dimers remain as dimers after SDSV analysis and do not associate before the assay procedure. If the activity in the dimer peak had been due to tetramer contamination, a tetramer peak similar to the activity pattern of the "tetramer rerun" experiment would have been found in the "dimer rerun" experiment. This then shows that the sensitivity of the assay would have been sufficient to detect tetramers, had they been present.

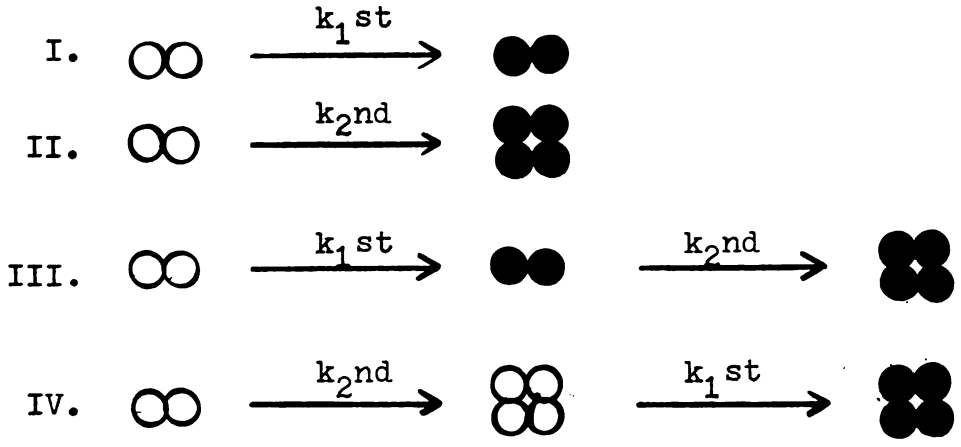
Other experiment, identical in procedure with the exception that the dimer concentration was reduced to $\frac{1}{2}$ and $\frac{1}{4}$ the concentration used in this experiment, produced results identical to those presented above.

The conclusion then from this section is that the activity found in the dimer peak is not due to tetramer contamination formed before the assay procedure. Thus the linear activity rates from the dimer peak after SDSV must be due to either (1) active dimers or (2) association in the assay. The following section deals with the experiments which were designed to test for association in the assay.

B. Test for Dimer Association in the Assay

Since most of the following experiments involve a kinetic analysis, it is best to discuss the various mechanisms before presenting the experimental details. Listed below are the four most likely reaction mechanisms followed by the appropriate explanations. The open symbols represent subunits of an inactive enzyme and the closed symbols repre-

sent subunits of an active enzyme. The k's are the reaction rate constants, a k_1st symbol being used for a 1^{st} order reaction and k_2nd for a second-order reaction.



The first mechanism postulates that dimers are active and that a folding process is necessary to regain activity; it predicts that the reaction rate for producing active enzyme (filled circles) from inactive enzyme (open circles) depends upon the dimer concentration to the first power. In addition, the half-time should be independent of concentration, as expected for a first-order process, such as a folding reaction.

The second mechanism postulates that dimers are inactive, so that association is necessary to regain activity. The rate of association in such a process should be concentration-dependent and therefore, the rate of activity recovery should be concentration-dependent, and in particular, it should be proportional to C^2. Thus the kinetics of activity recovery would be expected to be second-order in this mechanism. In addition, the half-time should depend

concentration to the first power.

The third mechanism is identical to mechanism I, with the additional feature that active dimers associate into active tetramers. A kinetic analysis would not distinguish between these two mechanisms (I and III); however, a physical analysis of the dimer-tetramer distribution in the assay would make this distinction (see Section B-2 below).

The fourth mechanism postulates that dimers are inactive and must associate into inactive tetramers before a final folding reaction produces active tetramers. The rate of association in such a process should be concentration-dependent, but the rate of activity reversal should be independent of concentration. Since the overall rate of activity recovery will be measured, the kinetics will reflect the one step in the reaction which is rate-limiting. Thus at higher concentration, the kinetics of activity recovery might be expected to be first-order; but at very low concentrations, where the association reaction must become rate-limiting, the kinetics of activity recovery would be expected to be second-order. In particular, the initial rate of recovery should be proportional to C_o^2. Likewise, the half-times should be independent of concentration at high concentrations; whereas at low concentrations, they should depend upon concentration to the first power.

Thus we have three tests which should help us to distinguish between the different possibilities. If the unfolded pH 5.5 dimers can be active with only a folding process

required, then the kinetics of activity recovery should be first-order and the half-times should be independent of initial concentration of unfolded dimers (mechanisms I and III). On the other hand, if the unfolded pH 5.5 dimers must associate to be active, then the kinetics of activity recovery should be second-order and the half-times should depend on the concentration to the first power (mechanism II, and over a wider concentration range, mechanism IV). As stated previously, a distinction between I and III cannot be made by a kinetic analysis; only a physical analysis of the dimer-tetramer composition in the assay will distinguish between these two possibilities.

The first experiment in this section was a kinetic analysis of activity recovery; this experiment should distinguish between mechanisms I (III) and mechanism II. The second experiment was designed to distinguish between mechanism I and mechanism III by a physical analysis.

1. Kinetics of Activity Reversal from pH 5.5 Dimers (0°C) Not Subjected to SDSV Analysis: Determination of the Rate Constant and the Order of Activity Recovery from pH 5.5 Dimers at 25°C and pH 7.5

In contrast to all the previous experiments in Chapters IV and V, pH 5.5 dimers were not separated by SDSV before the activity analysis. Instead, the following procedure was used: Various concentrations of pH 3.35 dimers were diluted five-fold in the usual manner to pH 5.5 at 0°C.

Using chilled micropipettes, aliquots were immediately removed from the pH 5.5 stock solutions (before any association could take place, i.e. 0-10 seconds) and diluted into the pH 7.5 assay at 25°C (mixing immediately); the cuvettes were then incubated for various times before adding substrate to determine catalytic activity. The concentration range used in the assay in this study was between 0.0032 mg/ml and 0.032 mg/ml.

Before turning to the results, the problem of inactivation should be discussed. A significant amount of inactivation was found after the initial reactivation period of 5 to 10 minutes. Because of this inactivation, final activities (after 30-60 minutes) were unreliable and therefore, half-times could not be determined at each concentration. It was evident that the problem was more acute at lower concentrations. However, since the inactivation did not affect the initial reactivation rates, the other method of determining reactivation order could be studied. This method, to be described below, is the kinetic analysis of the dependence of the initial rates of reactivation upon initial dimer concentrations.

Specific activities were determined from the initial optical densities changes. Reactivation curves were then determined by plotting specific activity changes as a function of time for each concentration studied. Initial rates of reactivation $(dC/dT)_{t=0}$ were then plotted against initial dimer concentrations (C_0) raised to the first or second

power for first-order and second-order kinetic reactions, respectively. These data are shown in Figures 36 and 37, respectively.

When $(dC/dT)_{t=0}$ was plotted against C_0^2 for a second-order reaction, a curved line was obtained[2] (Figure 37). On the other hand, when $(dC/dT)_{t=0}$ was plotted against C_0 for a first-order reaction, a straight line was obtained (Figure 36). From the slope of this line, a first-order rate constant was calculated to be $k_1 st = 2.4 \times 10^{-3}$/sec. From these results, it follows that in the concentration range studied the rate-limiting step is a first-order reaction. Therefore, the production of an active enzyme from pH 5.5 dimers is a folding reaction, and not an association reaction. Mechanism II (see introduction to this section) must be excluded since the rate-limiting step in this mechanism is a second-order reaction. The other three mechanisms (I, III, and IV) are consistent with these results. Mechanisms I and IV are included in Figure 36 (as both mechanisms, I and II, preclude an active dimer, only one of them was included in the graph).

Since the first-order rate constant found above does not distinguish between mechanism I in which only dimers

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[2]The "pseudo" second-order rate constant at low concentration, i.e. for the linear portion of this curvilinear line, was calculated to be $k_2 nd = 6.4 \times 10^4$ liters/mole-sec at 25°C and pH 7.5. This can be compared with the second-order rate constant calculated in Chapter III, Section F: $k_2 nd = 1.4 \times 10^4$ liters/mole-sec at 16°C and pH 7.5.

Figure 36. Test for first-order kinetics of pH 5.5 dimer reversal in the assay (pH 7.5 and 25°C). See text for details.

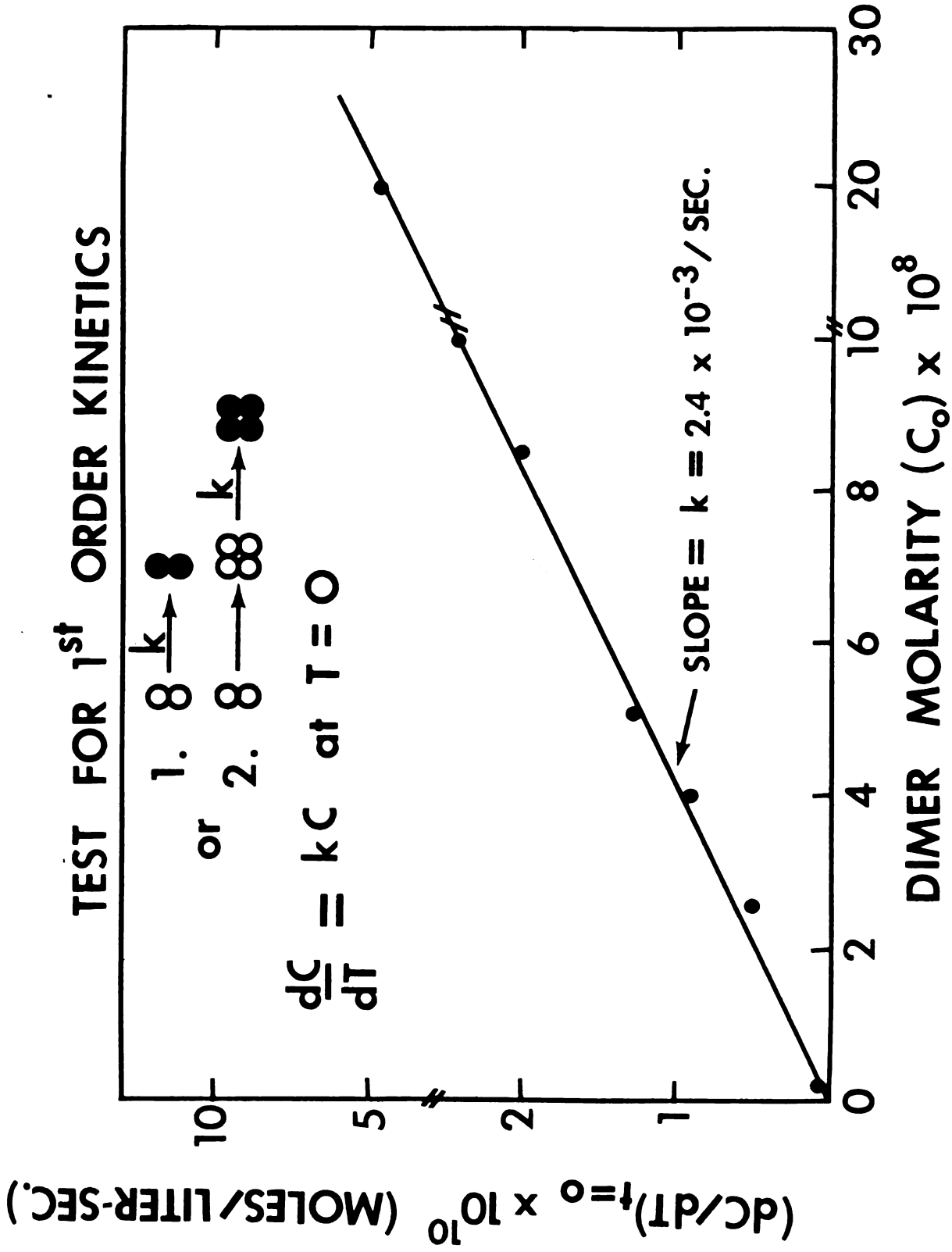
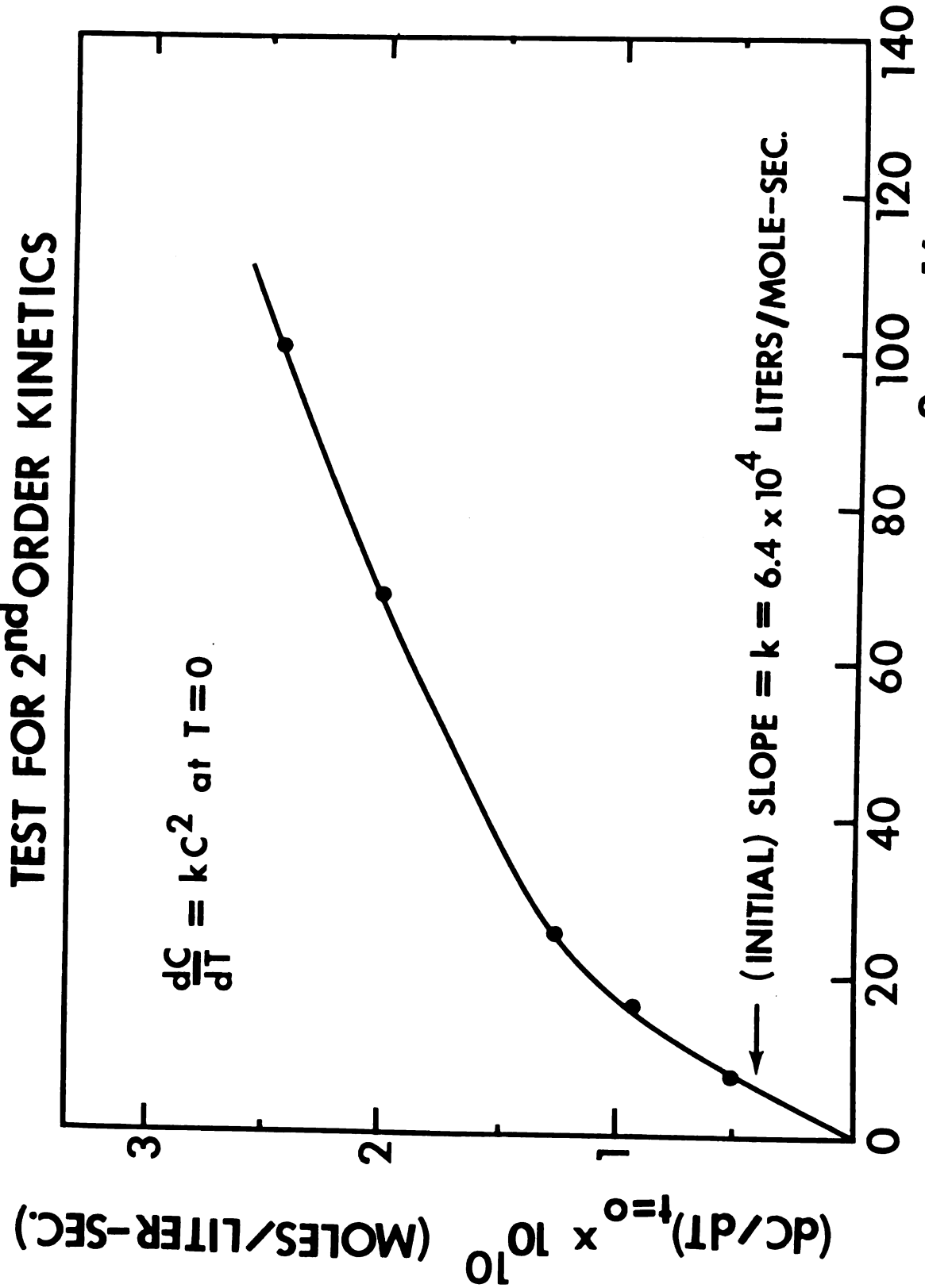

TEST FOR 1st ORDER KINETICS
1. ● —k→ ●
or
2. ● —k→ ●●
dC/dT = kC at T = O
SLOPE = k = 2.4 x 10⁻³/SEC.
(dC/dT)t=o x 10¹⁰ (MOLES/LITER-SEC.)
DIMER MOLARITY (Co) x 10⁸
10
5
2
1
0
2
4
6
8
10
20
30

Figure 37. Test for second-order kinetics of pH 5.5 dimer reversal in the assay (pH 7.5 and 25°C). See text for details.


TEST FOR 2nd ORDER KINETICS
$\frac{dC}{dT} = kC^2$ at T=0
(INITIAL) SLOPE = k = 6.4 x 10^4 LITERS/MOLE-SEC.
$(dC/dT)_{t=o} \times 10^{10}$ (MOLES/LITER-SEC.)
DIMER MOLARITY $(C_o)^2 \times 10^{16}$
0
20
40
60
80
100
120
140
1
2
3

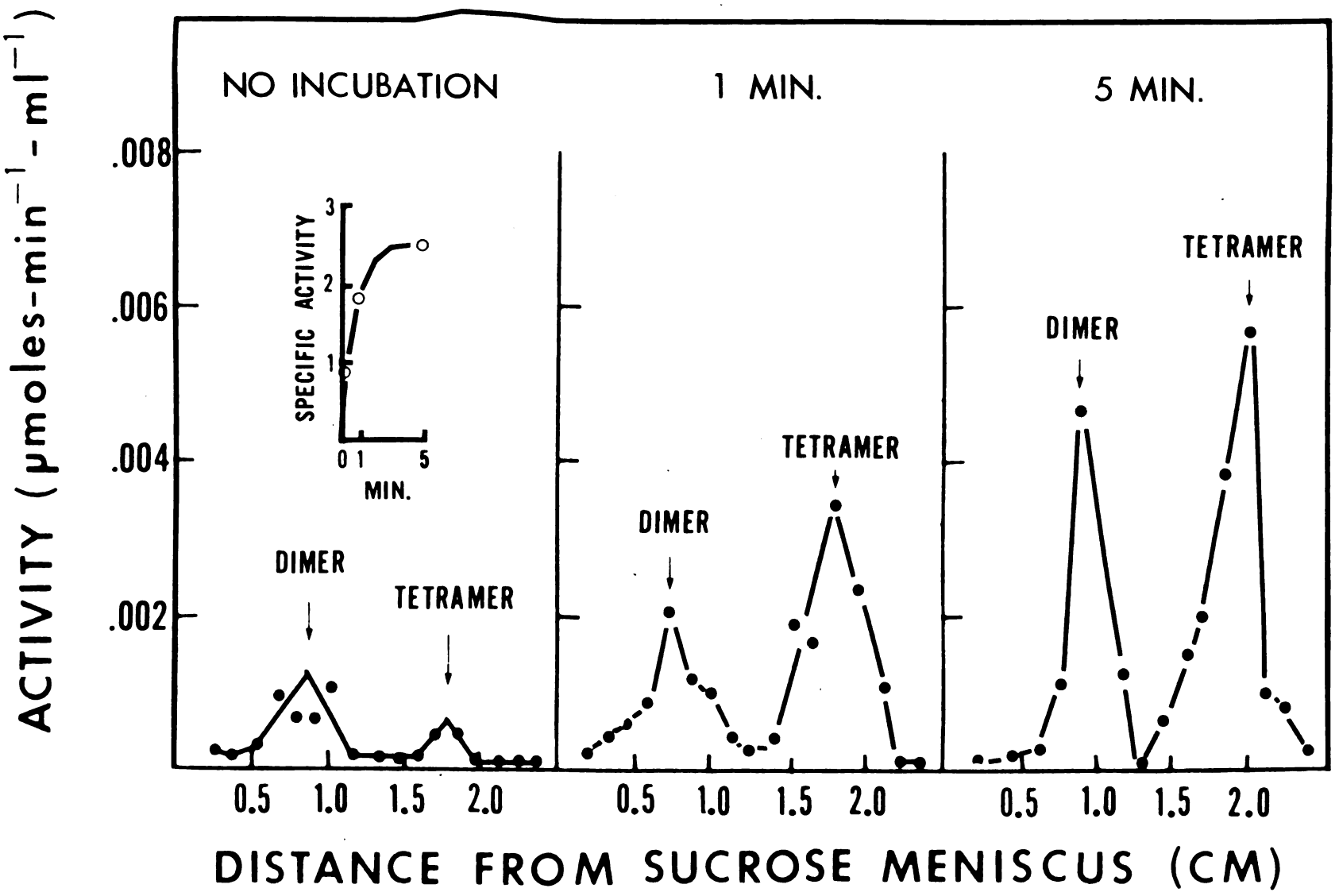
are present and mechanisms I and IV in which both dimers and tetramers are present, the experiments in the next section were designed to determine whether tetramer formation accompanied the reactivation by directly measuring the dimer-tetramer distribution in the assay after various times of incubation.

2. Direct Test for Possible Dimer Association in the Assay: SDSV Analysis After Incubation of Dimers in the Assay

The purpose of this experiment was to test for possible dimer association in the assay after various periods of incubation. To accomplish this, it was necessary to determine the relative dimer-tetramer distributions in the assay by the SDSV method of analysis.

Dimers at pH 5.5 were diluted into assay mixtures, complete except for FDP, at 0.016 mg/ml, 25°C and pH 7.5, and incubated for 0, 1, and 5 minutes, respectively, before beginning the assay by addition of FDP. The initial activities in the insert of Figure 38 show a reactivation due to incubation in the assay. To determine the dimer-tetramer distribution in the assay at the various incubation times, 0.1 ml aliquots were removed within about 15 seconds from the respective assay samples after activity analysis, cooled to 0°C to "freeze" the dimer-tetramer distribution, and layered on a pH 5.5 sucrose gradient. Subsequent SDSV analysis for the assay incubation times of 0, 1, and 5

Figure 38. SDSV analysis of composition in assay at various times after adding pH 5.5 dimers to the assay. The insert shows the amount of reactivation for each of the indicated times in the assay. See text for details.


SED. VEL. ANALYSIS AFTER INCUBATION OF DIMERS IN ASSAY
NO INCUBATION
1 MIN.
5 MIN.
ACTIVITY (μmoles-min⁻¹-ml⁻¹)
.008
.006
.004
.002
SPECIFIC ACTIVITY
3
2
1
0 1 5
MIN.
DIMER
TETRAMER
DIMER
TETRAMER
DIMER
TETRAMER
0.5 1.0 1.5 2.0
0.5 1.0 1.5 2.0
0.5 1.0 1.5 2.0
DISTANCE FROM SUCROSE MENISCUS (CM)

minutes are shown in the left, middle, and right graphs, respectively, of Figure 38. Since the amount of tetramer in the 5 minute sample is greater than that in the 1 minute sample, these results show that dimer association does take place in the assay, and that the amount of association is proportional to the incubation time.

The question which now arises is, "Why does the amount of activity in the dimer peak also increase with time in the assay?" This raised the possibility that the dimers might become catalytically active, as a result of folding of dimers in the assay, i.e. mechanism III. However, an alternate possibility was that the apparent activation associated with the dimer peak was an artifact caused by (1) using assay micropipettes which were not chilled or by (2) the method of analyzing the test samples. That is, the 5 minute sample was analyzed first, followed by the 2 and 1 minute samples, respectively. During the interval between assays, the material might have become inactivated. This would have caused a reduction of peak height in the left graph over that of the right graph. Further support for this "inactivation" explanation was furnished by two other experiments where special precautions were taken to insure that the assay micropipettes were chilled (-20°C) and where the order of assaying the samples was reversed, i.e. tubes one, two, and then three. Under these circumstances, there was no increase in the activity in the dimer peak, in direct contradiction to the previous results. However, the activity in the tetramer

peak did increase as before. Thus the apparent increase in the activity in the dimer peak as shown in Figure 38 appears not to be real, but rather due to inactivation and the order of assaying the samples.

These results are consistent with mechanism IV in which the only active species is tetramers, but the data do not unequivocally exclude mechanism III in which both active dimers and active tetramers are formed. That is, both association and folding could be occurring, each of which could yield activity--Figure 38 supports this contention but the inactivation explanation contradicts this. Therefore both mechanisms will be retained for further examination. On the other hand, since we have indeed shown that an active tetramer can form in the assay, mechanism I is excluded, as this mechanism does not allow the formation of an active tetramer in the assay. Mechanism II has already been excluded in previous section.

In conclusion then, only two mechanisms remain: (a) mechanism III, which postulates that the formation of an active dimer occurs first, followed by the formation of an active tetramer, and (b) mechanism IV, which postulates that the formation of an inactive tetramer occurs first, followed by the formation of an active tetramer (no active dimer intermediate). Therefore, final proof of mechanism III or IV rests on disproving one of these two mechanisms. This again will have to be done by a kinetic analysis, and this can most conveniently be done directly in the assay as in the

previous kinetic analysis.

To exclude the active dimer mechanism (III), it will have to be shown (a) that the concentration-dependence of the activity recovery changes from first-order (found in the previous section) to second-order (or at least, becomes greater than first-order) or (b) that the half-time of the activity recovery vary with dimer concentration. But since further quantitative analysis for a change in half-time at still lower concentrations would be clouded by the inactivation process under the present assay conditions, a study was undertaken with the express intent of eliminating the inactivation problem. The successful completion of this study then made it possible to conduct an analysis of half-times at various initial dimer concentrations.

C. Proof that Dimers are not Catalytically Active

1. Stimulation of Reactivation in the Assay with Bovine Serum Albumin and/or β-Mercaptoethanol to Aid Later Kinetic Analysis at Lower Concentrations

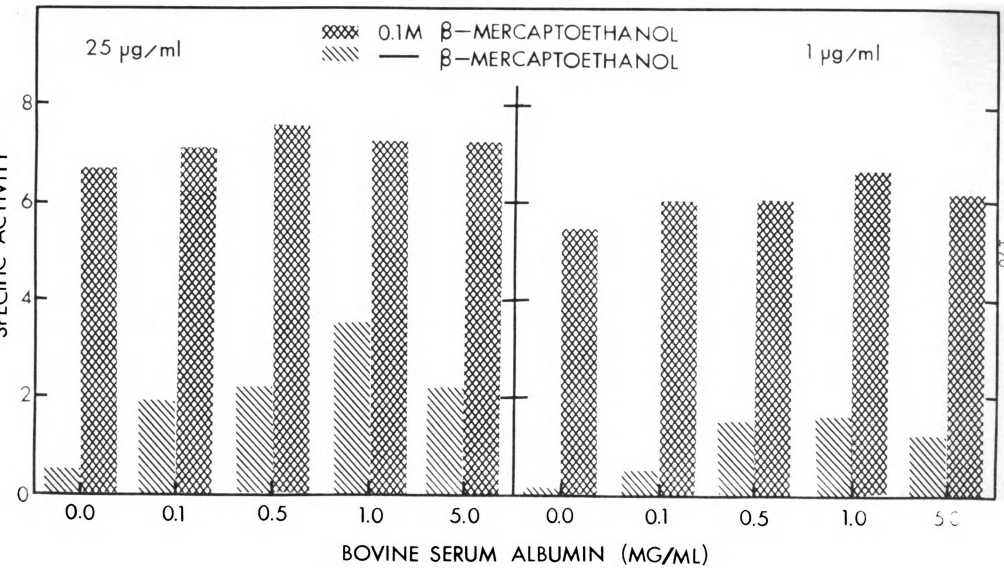
The minimum requirements for a meaningful kinetic analysis of reactivation from inactive dimers in the assay are (1) no association for at least 5 minutes at pH 5.5 and (2) no inactivation in the assay for at least 30 minutes. To eliminate any association at pH 5.5 before the kinetic analysis, the temperature was controlled to -6°C. In line with the second requirement above, the experiments in this section were designed to stabilize the dimers against the

inactivation.

A sample of pH 3.35 dimers was diluted five-fold to pH 5.5 and incubated in a -6°C salt-ice bath. Within 1 minute, 10 µl aliquots were removed with chilled micropipettes (-20°C) and quickly added to the assay cuvettes with mixing. After a 30 minute incubation at 25°C in the assay cuvette, which contained the appropriate concentrations of BSA and/or 0.1M β-mercaptoethanol, FDP was added to determine the activity. The final dimer concentration in the assay was either 0.025 mg/ml or 0.001 mg/ml. Four different concentrations of BSA were tested: 0.1, 0.5, 1.0, and 5.0 mg/ml.

The results shown in Figure 39 demonstrated that although BSA was effective alone in stimulating reversal at both enzyme concentrations, 0.1M β-mercaptoethanol was far superior in its ability to stabilize the dimers in the reversal process against inactivation. Moreover, when β-mercaptoethanol was present, the BSA effect was less pronounced. The combination of both β-mercaptoethanol and BSA, however, did produce greater stabilization than either one alone. Therefore, in the following kinetic analysis both were used in the assay.

Figure 39. Effect of bovine serum albumin (BSA) and 0.1M β-mercaptoethanol (β-MSH) on dimer reversal in the assay after a 30 minute incubation. The dimer concentrations (25 μg/ml and 1 μg/ml) shown in the upper corners of the graph were the final dimer concentrations in the assay. See text for details.


EFFECT OF BSA AND β-MSH ON DIMER REVERSAL IN ASSAY
25 µg/ml
0.1M β—MERCAPTOETHANOL
β—MERCAPTOETHANOL
1 µg/ml
8
6
4
2
0
0.0
0.1
0.5
1.0
5.0
0.0
0.1
0.5
1.0
5.0
BOVINE SERUM ALBUMIN (MG/ML)

2. Kinetic Analysis of Reversal in the Assay Over 150-Fold Dimer Concentration Range (0.00016 mg/ml to 0.025 mg/ml): Demonstration That Dimer Association into Tetramers, Followed by a Tetramer Conformational Change, Precedes Catalytic Activity

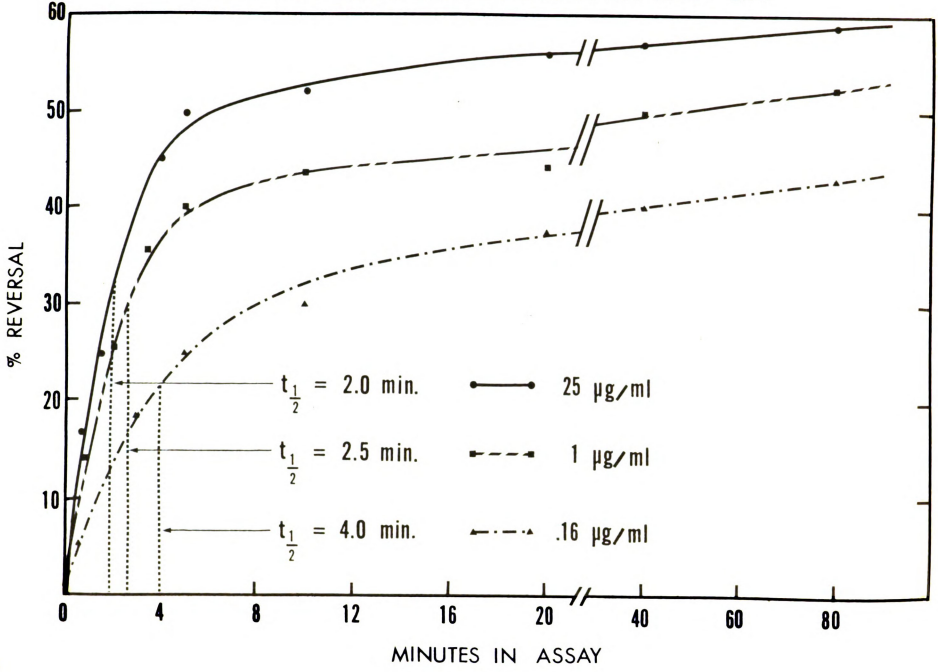
Now that we had obtained conditions in which the pH 5.5 dimer was stable in the assay, it was possible to undertake quantitative experiments on the kinetics of the activity recovery upon reversal.

The same procedure was used in this experiment as in the previous experiment except a third enzyme concentration (0.00016 mg/ml) was added, and dimers were incubated for various times in the assay instead of the constant 30 minute incubation period used before. Both 0.1M β-mercaptoethanol and BSA (1 mg/ml) were present in the assay.

The kinetics in Figure 40 show that the half-times increase from 2.5 minutes to 5 minutes when the dimer concentration is lowered from 0.001 mg/ml to 0.00016 mg/ml. Furthermore, when the experiment was repeated with only 0.1M β-mercaptoethanol in the assay, essentially the same results were obtained.

Therefore Mechanism III, which postulates an active dimer, is not consistent with this data. The correct mechanism (IV) and the only remaining mechanism for the conversion of inactive pH 5.5 aldolase dimers into catalytically active enzyme is that aldolase dimers must first associate into tetramers and then go through a conformational change

Figure 40. Effect of dimer concentration on half-lives of reactivation. See text for details.


EFFECT OF DIMER CONCENTRATION ON HALF—LIFE
% REVERSAL
60
50
40
30
20
10
0
4
8
12
16
20
40
60
80
MINUTES IN ASSAY
$t_{\frac{1}{2}}$ = 2.0 min.
$t_{\frac{1}{2}}$ = 2.5 min.
$t_{\frac{1}{2}}$ = 4.0 min.
25 µg/ml
1 µg/ml
.16 µg/ml

before catalytic activity appears. Thus under all conditions studied in this research, aldolase dimers have been shown to be catalytically inactive; only the tetramer possesses catalytic activity.

DISCUSSION

As stated in the introduction: "The 3.5S intermediate is of special interest for its significance to protein biosynthesis since the intermediate may also occur in the process of subunit association in living cells." But before a meaningful role for the intermediate in the subunit association could be presented, the subunit structure of native aldolase, a problem not unequivocally resolved, had to be reexamined.

Subunit Structure of Rabbit Muscle Aldolase

When aldolase was dissociated into subunits with a 0.1M β-mercaptoethanol, 5.6M guanidine HCl solution, the subunits were found to have a weight-average molecular weight of 42,000. This is in agreement with the results subsequently published in the literature (see Literature Review). These results, together with the recently published molecular weight of 160,000 for the native enzyme (Kawahara and Tanford, 1966), strongly suggested that aldolase was composed of four subunits (further confirming evidence is presented in the Isozyme section of the Literature Review). In addition, the characterization of the 3.5S intermediate as a dimer of aldolase subunits, which is described below, provides further support for the four subunit models of rabbit muscle aldolase.

Characterization of the 3.5S Aldolase Intermediate as a Dimer of Subunits

The extrapolated sedimentation coefficient for the dimer was found to be $s^{o}_{20,w}$ = 3.80S at pH 3.40 or pH 3.45 at 7°C and $s^{o}_{20,w}$ = 3.45S at pH 3.35 and 2°C. The f/f_o value which was taken as a measure of the asymmetry of a molecule was calculated to be f/f_o = 1.9 for the dimer; this can be compared to the more compact structure, f/f_o = 1.3, for the native enzyme, or to the more unfolded structure, f/f_o = 2.3, for acid dissociated subunits.

The extrapolated diffusion coefficient for the dimer was found to be $D^{o}_{20,w} = 3.85 \times 10^{-7}$ cm^2/sec at pH 3.40 or pH 3.45 at 7°C and $D^{o}_{20,w} = 3.73 \times 10^{-7}$ cm^2/sec at pH 3.35 and 2°C.

The molecular weight of the 3.5S intermediate of aldolase was calculated from the M(S/D) technique (using extrapolated S and D values) to be 83,000-86,000, approximating a dimer of aldolase subunits (42,000). The 3.5S intermediate was produced and was stable under the following conditions: pH 3.35, 0-2°C, and low salt (0.01M NaCl) during the dissociation. Some higher molecular weight aggregate was found as shown by sedimentation equilibrium in agreement with 108,000 limiting value (Deal et al., 1963).

An estimate of the inaccuracy of the diffusion coefficient due to aggregation was made by an empirical determination of the amount of aggregate present in the intermediate preparations. It was shown that the impurity would

lead to a diffusion coefficient too low and the maximum error was 4%. With this correction, the diffusion coefficient was increased from 3.73×10^{-7} cm^2/sec to 3.90×10^{-7} cm^2/sec. Using the Svedberg equation for M(S/D), this correction decreased, in turn, the molecular weight from 83,000-86,000 to 80,000-83,000. Significantly, these values are most consistent with a dimer of aldolase subunits (40,000 to 42,000).

That the intermediate is indeed a dimer is strongly supported by the measurements of another hydrodynamic parameter, the intrinsic viscosity. Upon formation of the intermediate, the apparent (6.2 or 8.3 mg/ml) intrinsic viscosity of the native aldolase molecule (4 cc/g), shows a rapid initial increase to 9 cc/g within 10 minutes; then it slowly attains a limiting value of 14.5 cc/g in 3 hours, and remains constant for at least 10 hours. These data suggest that the intermediate is: (1) formed rapidly, (2) stable, (3) somewhat unfolded, and (4) a dimer of aldolase subunits. In addition, the viscosity data was shown to exclude the only other possibilities for the intermediate: (1) tetramers, (2) trimers, and (3) monomers.

Thus, in contrast to the previously-held trimer model, the mechanism of subunit association in living cells for the tetramer model of aldolase is somewhat anticlimactic, especially with the physical characterization of the intermediate as a dimer. That is, the most likely mechanism of subunit association into tetrameric aldolase is a simply two consecutive bimolecular association reaction: (1) subunit

association into dimers, followed by another bimolecular reaction, (2) dimer association into tetramers. This then concludes the primary objective of this thesis: to characterize the 3.5S intermediate of rabbit muscle aldolase.

The discovery that the 3.5S intermediate was a dimer, together with the fact that aldolase contained at least 3 catalytic sites (see Literature Review) led to the second major aim of this thesis: "to answer the question of whether aldolase dimers could possess catalytic activity independent of their 'partner dimer' in the native tetramer."

But before the question of active dimers could be examined, a study was conducted to find the proper conditions for complete reversibility of activity from pH 3.3 dimers. The basis for such a study was to eliminate, as much as possible, any non-specific aggregation that might interfere with the analysis for active dimers.

Activity Reversal from the pH 3.3 Dimers: Attainment of Conditions for Complete Reversibility

Studies on the reversibility of dissociation to produce the pH 3.3 dimers have shown that 100% recovery of activity can be obtained under the following conditions: (1) pH 7.9, (2) 0.1M β-mercaptoethanol, (3) 1 hour incubation at 0°C followed by a 2 hour incubation at 20°C, and (4) reversal concentrations between 0.013 mg/ml and 0.065 mg/ml.

The rate of activity reversal from pH 3.3 dimers was

found to be temperature-dependent and concentration-dependent. The rate of activity recovery in the assay at 16°C was found to be second-order with respect to dimer concentration (0.09-0.18 mg/ml). The second-order rate constant was calculated to be $k = 1.35 \times 10^4$ liters/mole-sec. These results suggested that the pH 3.3 dimer was inactive and had to associate into tetramers before catalytic activity was regained.

FDP Inhibition of Reversal

In the course of these studies, it was discovered that when FDP was present in the assay before pH 3.3 dimers were diluted into the assay, much lower activities were found than if FDP was added last. Since it was previously shown that the pH 3.3 dimer was inactive, the observation that FDP inhibited activity reversal coupled with the observation that this inhibition was strongly dependent upon dimer concentration suggested that the FDP was preventing dimer association. Perhaps the binding site on the native enzyme for FDP had remained partially intact after dissociation into dimers. This hypothesis was supported by a later experiment (Chapter IV) where it was shown by SDSV analysis at pH 5.0 that FDP also inhibited dimer association. The subsequent observation that pyrophosphate could substitute for FDP in this inhibition supported the above contention that the inhibition by FDP was due to binding of the effector to the phosphate binding site on the dimer, perhaps

"freezing" the dimer in a conformation which prevented a necessary folding reaction which preceded association. On the other hand, it may have interfered with the association reaction directly. More detailed studies on the effect of various concentrations of FDP showed that, at 0.17 mM FDP concentration, FDP would no longer inhibit reversal. At 50% inhibition, a dissociation constant for FDP dissociation from the dimer was calculated to be 4×10^{-3}M FDP, and can be compared to published FDP dissociation constant for the native enzyme, $k_d = 4 \times 10^{-6}$M FDP. In conclusion to this section, conditions for complete reversibility were found as there were few problems with non-specific aggregation. The pH 3.3 dimer was inactive and therefore had to associate before catalytic activity was regained. And finally, FDP was found to inhibit dimer association into active tetramers. These results should be kept in mind, especially the inactive pH 3.3 dimer results, when discussing the studies in the next section which are preliminary to the analysis for active dimers at higher pH values.

The Study Preliminary to the Analysis for Active Dimers of Aldolase: Attainment of Aldolase Dimers Under Conditions Where Native Tetramers are Active

The approach to finding an active dimer was two-fold: (1) The first approach was by direct dissociation of the active tetramer into a possible active dimer without passing through an inactive dimer intermediate. (2) The second

approach was to form dimers at pH 3.35 and then raise the pH to find conditions which would allow the selective folding of the pH 3.35 dimer without association into tetramers. The method used to physically separate the dimer and tetramers was sucrose density gradient sedimentation velocity (SDSV) experiments.

In the first approach no evidence for an active dimer was found at pH 5.0 at 7°C or 20°C by direct dissociation of tetramers. Therefore, the second approach was investigated. The results showed that when the pH of pH 3.35 dimers was raised to pH 4.0 or pH 5.0, association was inhibited; but reactivation in the assay yielded essentially zero initial rates so that dimers at these pH values must have been inactive too. In contrast to these results, the pH 5.5 dimers which were incubated and separated by SDSV immediately gave activity and were constant with little or no log times. Subsequent studies on the concentration-dependence of dimer association at pH 5.5 showed that only about 3% dimer association had occurred in a 20 hour period at -6°C and 0.03 mg/ml. However, incubation at higher temperatures (0°C, 4°C, and 12°C) stimulated dimer association. In other studies, it was shown that increased concentrations of sucrose (4%, 16% and 20%) could more effectively inhibit dimer association, when compared to lower sucrose concentrations. In conclusion to this chapter (IV), pH 5.5 dimers subjected to SDSV analysis exhibited immediate activity upon assaying with essentially no lag times; therefore, it was

under these conditions (except the SDSV analysis was ommitted) that the final analysis for active dimers was investigated.

Analysis for Active Dimers (Not Subjected to SDSV Analysis)

The last chapter (V) of this thesis is devoted entirely to proving whether the pH 5.5 dimer at -6°C was active when assayed at pH 7.5 and 25°C. To examine this question, a kinetic analysis of activity reversal, together with the physical analysis of the dimer-tetramer distribution in the assay, from -6°C, pH 5.5 dimers (not subjected to SDSV analysis) was undertaken. The only way to "prove" anything by kinetics is to disprove all other likely reaction mechanisms--the remaining mechanism, of course, being consistent with all the known physical and kinetic data.

At high concentrations in the assay, data was obtained which was consistent with a first-order reaction, but not a second-order reaction. This excluded mechanism II which postulates that inactive dimers must associate directly into active tetramers with no intermediates.

A physical analysis of the dimer-tetramer distribution in the assay at various times during the reversal process showed increasing tetramer formation; this excluded the mechanism which postulates that inactive dimers must fold into active dimers with no subsequent association into active tetramers.

Thus only two mechanisms remained at this time in the

kinetic analysis: (1) inactive dimers folding to active dimers which subsequently associated into active tetramers (mechanism III), or (2) inactive dimers associated into inactive tetramers which subsequently folded to form active tetramers (mechanism IV). Both mechanisms were consistent with the previous two results that (a) a first-order reaction preceded a molecule with activity (active dimers in mechanism III above or active tetramers in mechanism IV above), and (b) tetramers were formed in the assay (tetramers were formed in both mechanisms.

To exclude mechanism III, the half-times for reversal should have increased at lower concentrations. On the other hand, it would be much more difficult to exclude mechanism IV. If it was found that the half-times did not change at lower concentrations, reversal would begin to show changes in half-times. Fortunately for the analysis of this problem (but unfortunate for the active dimer model) the half-times at lower concentration showed an increase. These results then excluded the active dimer model that postulated that inactive dimers folded in the assay to form active dimers which subsequently associated into active tetramers. The only remaining model was the following:

At high concentrations ---- 1st order; $k_1 = 2.4 \times 10^{-3}$/sec

At low concentrations ---- 2nd order; $k_2 = 6.4 \times 10^{4}$ l/m-sec

$$\frac{k_1}{C} = k_2$$

at 3 µl/ml or 0.003 mg/ml, the rates and half-lives for a first-order and for a second-order reaction are equal.

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