CHEMICAL AND PHYSICAL CHARACTERIZATION OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM CALF LIVER

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
STEVEN LEVINE
1969

THESIS

LIBRARY
Michigan State
University

This is to certify that the

thesis entitled

CHEMICAL AND PHYSICAL CHARACTERIZATION OF
UDP-GLUCOSE PYROPHOSPHORYLASE
FROM CALF LIVER

presented by

STEVEN LEVINE

has been accepted towards fulfillment
of the requirements for

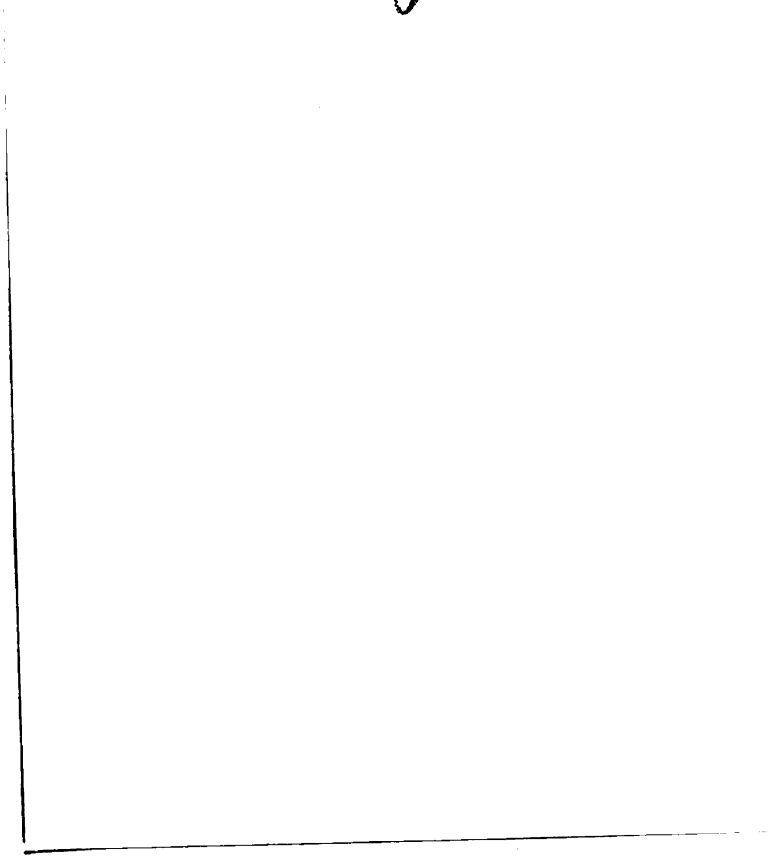
Ph.D. degree in BIOCHEMISTRY

Date SEPTEM

O-169

L-184

Brag 7/15/91 W



ABSTRACT

CHEMICAL AND PHYSICAL CHARACTERIZATION OF
UDP-GLUCOSE PYROPHOSPHORYLASE
FROM CALF LIVER

By

Steven Levine

Uridine diphosphate glucose, which is formed from glucose-1-phosphate and uridine triphosphate with the concomitant liberation of inorganic pyrophosphate, is an extremely important intermediate in carbohydrate metabolism. The crystallization of uridine diphosphate glucose pyrophosphorylase, an enzyme that catalyzes the above transformation, has afforded an opportunity to study the relationship of its structure to its function. The physical and chemical characterization of this protein is the purpose of this research.

The pyrophosphorylase was found to be a large polydisperse enzyme composed of large discrete subunits. The optical properties, renaturation tendency, chemical composition, and subunit structure of the enzyme were also determined.

CHEMICAL AND PHYSICAL CHARACTERIZATION OF UDP-GLUCOSE PYROPHOSPHORYLASE FROM CALF LIVER

By

Steven Levine

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1969

562809
7-1-70

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. R. G. Hansen without whose guidance and inspiration this work could not have been completed.

Thanks are also extended to Dr. Pat Oriel of Dow Chemical Company for performing the optical rotatory dispersion experiments and for his assistance in the interpretation of the data. Acknowledgement is made to Dr. Bill Rutter for allowing us the use of his laboratory to perform the electrofocusing experiments. Acknowledgement is also made to Miss Doris Bauer for her aid in the amino acid analysis. The author also wishes to thank Dr. W. C. Deal for his help with the analytical ultracentrifuge experiments. Special thanks are extended to Dr. W. A. Wood whose interest in the author and his work is greatly appreciated.

TABLE OF CONTENTS

TABLE OF CONTENTS - Continued

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES - Continued

INTRODUCTION

The enzyme uridine diphosphate glucose pyrophosphorylase (UTP:α-D-glucose-1-phosphate uridylyltransferase, E. C. 2.7.7.9) catalyzes the biosynthesis of the important coenzyme uridine diphosphate glucose (UDP-glucose) from glucose-1-phosphate (Glc-1-P) and uridine triphosphate (UTP) with the concomitant formation of inorganic pyrophosphate (PP_i).

$$UTP + Glc\text{-}1\text{-}P \underset{}{\overset{Mg^{2+}}{\rightleftharpoons}} UDP\text{-glucose} + PP_i \qquad (1)$$

The enzyme appears to be ubiquitous in nature (4-8, 13-17, 19-27) and has been crystallized from calf (28), lamb, goat, sheep, rabbit (32), and human (30) livers. The enzyme also has been obtained in a highly purified state, if not crystalline, from rabbit muscle.

The calf liver protein is the subject of this research. In the original work of Albrecht et al. (28), the pyrophosphorylase was estimated to account for over 0.3% of the extractable protein of calf liver and to have a molecular weight of approximately 400,000. Polydispersity was noted, but contamination could not be eliminated as its cause.

This thesis describes: (1) a more complete study on the molecular weight of the native pyrophosphorylase and its subunits

and some information concerning the nature of the minor molecular components; (2) the optical properties, renaturation tendency, chemical composition, and subunit structure of the crystalline enzyme.

LITERATURE REVIEW

Uridine diphosphate glucose pyrophosphorylase belongs to a general class of enzymes which catalyze the formation of various nucleoside diphosphate sugars according to the following reaction:

$$XTP + S\text{-}1\text{-}P \rightleftharpoons XDPS + PP_i \qquad (2)$$

The notation, X, can be various purine or pyrimidine nucleosides and the notation, S, can be various sugars. The important nucleoside diphosphate sugar, UDP-glucose, is biosynthesized by this route. UDP-glucose, or as some have called it, "activated glucose," is an exceedingly important compound because of its glycosyl transferring ability and its role in the interconversion of sugars and formation of hexoses, pentoses and uronic acids (1-4).

While studying the metabolic route for the conversion of galactose-1-phosphate to glucose-1-P in bakers' yeast, Leloir et al. in 1949-50 determined that a necessary thermostable cofactor in the conversion was uridine diphosphate glucose (5, 6). Shortly thereafter, in 1952-53, an enzyme was discovered in yeast by Kalckar and Munch-Petersen et al. (7, 8) that would catalyze the pyrophosphorolysis of UDP-glucose to form glucose-1-phosphate and uridine triphosphate. This type of pyrophosphorolytic cleavage, although extremely important to the eventual understanding of the

C-4 interconversion of hexoses, was not new. In 1948-50, Kornberg et al. had demonstrated the pyrophosphorolytic cleavage of some nucleoside diphosphate compounds (9-11).

Several years after its discovery, Munch-Petersen purified the UDP-glucose pyrophosphorylase from yeast approximately 260-fold with an overall yield of 15% (12). She noted that the enzyme was unstable in dilute solutions, even in the presence of a 3 mg/ml solution of bovine serum albumin, and that 60-75% of the activity remained after three month's storage. The enzyme exhibited a broad pH profile (pH 6.5-8.0) and the reaction was stimulated by magnesium at an optimal concentration of 2 mM. In addition to the fact that NaF at a concentration of 0.05 M and ethylenediaminetetraacetate (EDTA) at a concentration of 0.01 M had no inhibitory effect, the presence of cysteine also had no affect on the reaction. Following some radioactive exchange experiments, she proposed the possible existence of a uridylated intermediate in the reaction sequence.

The decade or so after 1953 was a time of identification of the pyrophosphorylase for UDP-glucose from many sources. After the 1953 demonstration by Kalckar et al. (13) of the existence of the pyrophosphorylase in galactose adapted Saccharomyces fragilis, Smith et al. (14, 15) described its presence in nuclei of guina pig liver and mammary glands, and Burma et al. (16) in 1956 noted its activity in sugar beet leaves.

In 1957 Neufeld et al. (17) found the pyrophosphorylase in mung bean seedlings and a host of other plant sources. After

partially purifying the enzyme, they noted the need for a divalent metal and found that Mg^{2+}, Mn^{2+}, and Co^{2+} would serve equally as well. Further work on the characterization of the mung bean enzyme followed the next year from Ginsburg's laboratory (18). After purifying the enzyme 800-fold, he noted that the pH optimum was about 8.0 and the Michaelis constants (Km) for UDP-glucose and PP_i were 1.1×10^{-4}M and 2.3×10^{-4}M respectively. His purified protein was not very stable and lost 90% of its activity even in the presence of mercaptoethanol after storage for 2 weeks at $-7^{\circ}C$.

In that same year the pyrophosphorylase was found in the plant Impatiens holstii (19) and in pea seeds (20). The purified enzyme from pea seeds exhibited a broad pH range (pH 7.0-9.0) and its activity was not affected by fluoride or orthophosphate anions, arsenate at a concentration of 10 mm, p-chloromercuribenzoate (PMB) at a concentration of 1 mm, or Hg^{2+} ions at a concentration of 0.1 mM. Mn^{2+} (1mM) was as effective a divalent metal as was Mg^{2+} (5mM) and Co^{2+} and Ni^{2+} stimulated at a concentration of 2.5 mM. For this enzyme EDTA at a concentration of 0.01 M inhibited the reaction by 90%.

Using rabbit muscle as their source, Villar-Palasi et al. (21) purified the pyrophosphorylase 1300-fold and noted that the stability point (pH 9.8) was different from the point at which the enzyme was most active (pH 7.5).

Shortly afterwards, in 1961, Basu et al. (22) purified 30-fold a human brain pyrophosphorylase that showed an absolute requirement for Mg^{2+}. Although the enzyme was not affected by

reducing agents such as cysteine and glutathione, 0.0001 M PMB caused the enzyme to lose 50% of its activity. It should be noted that the pyrophosphorylase has been reported in other human tissues (23-26).

More recently, in 1965, Kamogawa et al. (27) purified the E. coli K-12 pyrophosphorylase 280-fold. They noted that as with other pyrophosphorylases, the pH profile was broad (pH 7.5-9.0). With Mg^{2+} optimal for activity, they determined the apparent equilibrium constant to be 5.0 in the direction of UTP formation. NaF, PMB, and UMP at 2 mM concentrations were not inhibitory.

In 1966 Albrecht et al. (28), using calf liver as a source of the enzyme, finally obtained a 300-fold purified and crystalline UDP-glucose pyrophosphorylase. When the base and sugar portion of the nucleoside diphosphate sugar was varied from uracil or glucose, the rate of the reaction was only a small percentage of that found for UDP-glucose. The protein was apparently present in large quantities in calf liver, as it represented more than 0.3% of the total extractable protein, and the specific activity and turnover number were found to be 240 and 83,000 respectively. The molecular weight of the protein was estimated to be 400,000 and all the substrates had Michaelis constants of about 10^{-5}M except UTP which was 2×10^{-4}M. Although Mg^{2+} was optimal at a concentration of 2 mM, Co^{2+} and Mn^{2+} were 25% as effective at a comparable concentration. The enzyme apparently did not require reducing agents for stabilization.

It is important to note the many factors that indicate that UDP-glucose pyrophosphorylase is the major catalyst for UDP-

glucose biosynthesis; (1) the large amount of the enzyme in calf liver, (2) the high rate specificity for UDP-glucose, (3) the low Km for UTP and glucose-1-phosphate, (4) the high turnover number, and probably of less importance under physiological conditions, (5) the favorable equilibrium constant (approximately 0.3 in the direction of UDP-glucose biosynthesis).

More recently, DeFazio (29), employing the crystalline calf liver enzyme and using new spectrophotometric and chromatographic techniques, determined very accurately that the apparent equilibrium constant was 0.2 in the direction of UDP-glucose synthesis.

Because the pyrophosphorylase has recently been crystallized from sources other than calf liver, it is now possible to compare the proteins on the bases of their physical and chemical characteristics, along with the comparison of their reaction mechanisms. Extremely important is the recent purification and crystallization by Knop (30) of the pyrophosphorylase from human liver. The 500-fold purified and crystalline human liver pyrophosphorylase was almost homogeneous as judged by sucrose density gradient ultracentrifugation, polyacrylamine gel electrophoresis, and sedimentation in the analytical ultracentrifuge. The pyrophosphorylase exhibited the usual broad alkaline pH profile. Mg^{2+} was most active at a concentration of 3 mM, but Co^{2+} could serve as the divalent metal at only 14% of the rate with Mg^{2+}. Also, the human liver enzyme required the presence of a reducing agent to protect against inactivation.

Since the original study of Villar-Palasi *et al.*, Bass *et al.* (31) have highly purified, if not crystallized, the pyrophosphorylase

from rabbit muscle. The enzyme exhibited one band on polyacrylamide gel columns, but two bands on sucrose density gradient columns. The protein also required reducing agents to prevent against inactivation.

Still in progress are the investigations of Gillett et al. (32). They were able to crystallize the pyrophosphorylase from lamb, goat, and rabbit liver. Preliminary experiments indicate that the enzymes from lamb and goat are extremely similar, if not identical, to the calf liver pyrophosphorylase. These conclusions have come from similarities in gross crystal structure, electrophoretic results on polyacrylamide gel columns, and the apparent lack of necessity for protection by reducing agents. On the other hand, the rabbit liver pyrophosphorylase is a sulfhydryl requiring pyrophosphorylases and its gross crystal structure and electrophoretic behavior are different from the calf, lamb, goat, and human liver preparations.

Many patients suffering from the hereditary disorder known as galactosemia are able to metabolize a small amount of galactose. Due to the reports of Isselbacher et al. (33, 34) and other authors (17, 35, 36) relating to a separate UDP-galactose pyrophosphorylase pathway in these galactosemics, Ting et al. (37) and Knop (30) have studied the question in relationship to the crystalline UDP-glucose pyrophosphorylase. The results of both studies support the conclusion that there is not a separate UDP-galactose pyrophosphorylase. Their conclusions were based on the fact that, throughout the purification of the calf and the human liver

pyrophosphorylases, the UDP-galactose/UDP-glucose activity ratio remained unchanged.

EXPERIMENTAL PROCEDURE

Materials and Methods

Reagents

All chemicals were purchased from commercial sources and where critical, the sources are indicated. Crystalline UDP-glucose pyrophosphorylase was prepared according to the procedure of Albrecht et al. (28) as modified by Gillett et al. (38).

Spectrophotometry and Measurement of Enzyme Activity

Spectrophotometry and the estimation of pyrophosphorylase activity were determined as described by Albrecht et al. (28). Assay system 1 was routinely used.

Absorption Spectroscopy

A solution of approximately 1 mg/ml of pyrophosphorylase in 0.01 M tricine buffer (pH 8.36) was dialyzed against 1000 volumes of the above buffer for 18-24 hours. Values for the actual protein content were obtained using the molar extinction coefficient, and were checked with the Lowry procedure (39). The spectra were taken at 24°C in a Cary 15 Spectrometer in 1.5 ml quartz cells with a 1 cm light path.

Determination of the Extinction Coefficient

Approximately 50 mg of a crystalline suspension of the pyrophosphorylase, that was stored in 0.01 M tricine buffer (pH 8.5),

which was 20% in ammonium sulfate, was diluted to about 6 ml with 0.01 M sodium phosphate buffer (pH 7.0). The material was then dialyzed against 1 liter of the 0.01 M phosphate buffer for 24 hours. The slight amount of precipitate was removed by centrifugation and the solution was redialyzed for a total of 3 days against changes amounting to 10 liters of fresh phosphate buffer. Dry weight was determined after high-speed centrifugation which resulted in a clear solution.

Metal stainless steel planchets were used as tares. The planchets were heated in a drying oven at 105°C for 24 hours, and then allowed to equilibrate to room temperature in a dessicator over $CaCl_2$ for 1 1/2 hours. After the equilibration period, the planchets were removed with forceps and weighed with a semi-microanalytical balance. To insure a constant weight, further heating, equilibration, and weighing were performed at 5 and 10 hours after the initial weighing.

Five samples of the pyrophosphorylase and two of the outside dialysis solution were pipetted into the planchets. All planchets were then dried at 85°C for 6 hours and then at 105°C until a constant weight was obtained.

Extinction readings were made on the sample at 280 mμ, 278.5 mμ and 260 mμ. Dilutions of this solution were measured on spectrophotometers which were independently standardized.

Cross-standardization with the Lowry method involved Bovine Serum Albumin (BSA) as the comparison standard. The dry weight value was also cross-standardized with the 280/260 method of Warburg and Christian (40).

Carbohydrate Content

The phenol-sulfuric acid method of Dubois et al. (41) was used to determine the carbohydrate content of the pyrophosphorylase. Galactose was used as the internal carbohydrate standard and ovalbumin was used to check the sensitivity of the method to protein-bound carbohydrates.

Ultracentrifugation

All ultracentrifugation experiments were performed near 5°C in a Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics. Schlieren patterns were read with the aid of a Bausch and Lomb or Gaertner microcomparator. Sedimentation velocity analyses were performed at 59,780 rpm and diffusion coefficient analyses were performed at 4,059 rpm. Diffusion coefficients (D) were calculated by the height-to-area analysis (42) and were converted as were the sedimnetation coefficients (S) to standard conditions of water at 20°C. Double sector synthetic boundary cells were used for the diffusion coefficient analyses.

Sedimentation equilibrium analysis was done by the short column technique of Van Holde and Baldwin (43). The solution column depth was routinely 1.7 mm (0.06 ml of protein solution). The rotor velocities were 3,000 for the native enzyme and 20,400 for the enzyme dissociated in guanidine hydrochloride. Sedimentation equilibrium experiments were allowed to proceed for 24 hours for the native and from 24-36 hours for the dissociated enzyme.

Molecular weights, M(s/D), that were evaluated from sedimentation and diffusion data were analysed by the formula of

Svedberg (44):

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

where R is the gas content, T is the absolute temperature, ρ is the solvent density, and $\bar{v}$ is the partial specific volume.

The apparent weight-average molecular weights determined from sedimentation equilibrium experiments were calculated from the equation:

$$\frac{1}{M_2^{App}} = \frac{(1-v\rho)\ \omega^2}{RT} \qquad \frac{C_o}{C_b-C_m} \qquad \frac{r_b^2 - r_m^2}{2}$$

where ω is the angular velocity, C_o (determined from a synthetic boundary experiment) is the initial protein concentration, C_b and C_m, and r_b and r_m are the concentrations and radii at the bottom and meniscus of the solution column, respectively.

The apparent z-average molecular weights were calculated from the equation:

$$\frac{1}{r_b}\left(\frac{dc}{dr}\right)_b - \frac{1}{r_m}\left(\frac{dc}{dr}\right)_m = \frac{M_z(1-vp)\ \omega^2}{RT}\ (C_b-C_m)$$

where $\left(\frac{dc}{dr}\right)_b$ and $\left(\frac{dc}{dr}\right)_m$ are the concentration gradients at the bottom and the meniscus of the solution column.

The intrinsic weight-average and z-average molecular weights were obtained by extrapolation of the apparent molecular weights to infinite dilution. Concentrations for extrapolation of the

apparent molecular weights were evaluated as $(c_m + c_b) / 2$ for the weight-average molecular weights and $c_m + c_b$ for the z-average molecular weights (43).

Sedimentation, diffusion, and equilibrium calculations were performed with statistical analysis on a CDC 3600 computer with programs kindly supplied by Dr. W. C. Deal. The densities and viscosities of solutions not containing guanidine hydrochloride were calculated using reference tables (44, 45). The relative viscosity of solutions containing guanidine hydrochloride were measured at temperatures near those of the centrifuge experiments with a Cannon-Ostwald viscosimeter. Densities were measured pycnometrically using water to calibrate the pycnometer at identical temperatures to those used for the guanidine hydrochloride solutions.

To prevent interference from products of mercaptoethanol and guanidine hydrochloride (46), freshly distilled mercaptoethanol was used and synthetic boundary experiments were performed immediately after dialysis. Native samples were dialyzed for 24-48 hours and the denatured enzyme was dialyzed for 36 hours with rapid stirring.

The partial specific volume utilized in these studies was calculated from the amino acid analysis (see amino acid analysis section) and found to be 0.74 ml/gm.

Optical Rotatory Dispersion (ORD)

ORD measurements were performed on a Cary Model 60 Spectropolarimeter and critical wavelength readings were checked on a Durrum-Jasco ORD/uv-5 Optical Rotatory Dispersion Recorder. All readings were made under a nitrogen purge at room temperature (Ca. 24°C) for the Durrum-Jasco machine and in a thermostatically controlled environment at 27°C in the Cary 60.

For the Cary 60 studies, 45 mg of the pyrophosphorylase in crystalline suspension was added to a pH 8.5 solution of 0.01 M tris-HCl (Mann Ultra Pure) that was 0.10 M in KCl to make a solution of about 14 mg/ml. The solution was then dialyzed for several days against the above buffer system. Before rotatory dispersion analysis, the solution was diluted and the protein content was determined by the Lowry method and then converted to a dry weight basis. To obtain readings on the native protein in the 195-500 mμ spectral range, the protein solutions were varied from 0.26 - 1.30% in layers that were 0.1 mm to 1 cm.

The denatured pyrophosphorylase was prepared by diluting the native enzyme solution with an 8M solution of guanidine hydrochloride that was 0.01 M in tris-HCl (pH 8.50) and 0.01 M in KCl to obtain a 6 M solution. The pyrophosphorylase was allowed to remain in the denaturing solvent at room temperature for about 2 hours prior to the rotatory dispersion measurements. Readings were made on a 0.16% solution in cells that varied from 1 mm to 1 cm in layer thickness throughout the 220-500 mμ spectral range.

All observed rotations were corrected for the buffer blank and converted to specific rotations ($[\alpha]_\lambda^T$) by use of the formula:

$$[\alpha]_\lambda^T = \frac{(\alpha_{obs})\ (100)}{(l)\ \ (c)}$$

where α_{obs} is the corrected observed reading of the protein solution, l is the path length in decimeters, c is the concentration in gm/100 ml, and T is the temperature in degrees centigrade. Reduced mean residue rotations, ($[m']_\lambda^T$), were computed from the specific rotations according to the formula:

$$[m']_\lambda^T = \left(\frac{MRW}{100}\right)\left(\frac{3}{n^2 + 2}\right)\left([\alpha]_\lambda^T\right)$$

where MRW is the mean residue weight and is taken as 111 for the pyrophosphorylase and n is the refractive index of the solvent at wavelength λ.

The solvent for the native enzyme was assumed to be water at 20°C and the refractive index at various wavelengths was evaluated according to the following Duclaux-Jeantet formula (47):

$$n^2 = 1.762530 - 0.0133998\lambda + \frac{0.00630957}{(\lambda^2 - 0.0158800)}$$

where the wavelength, λ, is in microns.

The refractive index for the 6 M guanidine hydrochloride solution was evaluated from the following basic Sellmeier equation (48, 49):

$$n^2 = 1 + 0.9934\lambda^2/(\lambda^2 - 15067)$$

where λ is measured in mμ.

Visible rotatory dispersion was determined according to the following one term Drude relationship:

$$[\alpha]_{\lambda}^{T} = \frac{A}{\lambda^2 - \lambda_c^2}$$

where λ_c is the dispersion constant and is obtained from the slope of a plot of $[\alpha]_{\lambda}^{T}\lambda^2$ vs. $[\alpha]_{\lambda}^{T}$.

The Moffitt a_o and b_o parameters were evaluated according to the phenomenological equation of Moffitt and Yang (50):

$$[\alpha]_{\lambda}^{T} = \frac{a_o\lambda_o^2}{(\lambda^2-\lambda_o^2)} + \frac{b_o\lambda_o^4}{(\lambda^2-\lambda_o^2)^2}$$

or

$$[\alpha]_{\lambda}^{T}(\lambda^2-\lambda_o^2) = a_o\lambda_o^2 + \frac{b_o\lambda_o^4}{(\lambda^2-\lambda_o^2)}$$

Using a λ_o of 212 mμ, 216 mμ, and 220 mμ for various spectral ranges, a_o and b_o values were determined from the intercept and slope, respectively, of a plot of $[\alpha]_{\lambda}^{T}(\lambda^2-\lambda_o^2)$ vs. $\lambda_o^4/(\lambda^2-\lambda_o^2)$.

Titration of Cysteine Residues with Ellman's Reagent (DTNB)

All titrations with DTNB [5,5'-dithiobis (2-nitrobenzoic acid)] were performed at 25°C in a total reaction volume of 0.5 ml. The liberated thionitrobenzoate anion was quantitated at 412 mμ by the extinction coefficient of 13.6 reported by Ellman (51). For experiments involving pH dependent titrations, the extinction coefficient of the thionitrobenzoate anion was taken to be constant over the pH 6.0 to 10.0 range, since it had been shown to vary little in this region (52). The enzyme

concentration was determined by its extinction coefficient or by the method of Lowry. EDTA was included in all titrations at a concentration of 20 mM. When guanidine hydrochloride or urea was used as the denaturant, a freshly recrystallized sample or a Mann Ultra Pure product was used. All titrations were initiated by the addition of 10 µl of 10 M DTNB (Aldrich) in 0.1 M phosphate buffer (pH 7.0). Extraneous reaction of DTNB with the solvent system was corrected for by using a blank solution that contained all the components except the protein.

Titration of Cysteine Residues with p-Mercuribenzoate (PMB)

The procedure used to determine the sulfhydryl content of the pyrophosphorylase with PMB was based on the spectrophotometric method of Boyer (53) and Benesch et al. (54). The PMB (Sigma) was purified according to Boyer's directions and standardized immediately before use. The reported extinction coefficient of 1.69×10^4 (pH 7.0) at 233 mµ was used.

All titrations and PMB standardizations were performed in 5.0 M urea because the native enzyme became turbid upon titration. The pyrophosphorylase samples were dialyzed overnight against 1000 volumes of 0.05 M phosphate or pyrophosphate buffers. The initial solution volume was 1.0 ml, and a reference cell that contained all the components except the protein was used to correct for the reaction of PMB with the buffer system. Ten µl aliquots of the standardized PMB solutions were added to both the reference and protein cells, and optical density increments were recorded at 250 mµ immediately after the addition. The

reaction was considered complete when further aliquots of PMB failed to produce an absorbance increment. The optical density increments for the protein solution were corrected for dilution by the PMB additions, and for the reference readings.

Amino Acid Analysis

The sample was prepared according to the procedure of Moore and Stein (55). Five mg samples of salt-free and lyophilized pyrophosphorylase were placed in constricted heavy-walled pyrex tubes (16 x 125 mm). The protein was then suspended in 1.0 ml of 6N HCl and the tubes were frozen, degassed, and sealed with the aid of a vacuum pump which reduced the pressure to below 50 microns of mercury. Hydrolysis was carried out at $110^{\circ} \pm 1^{\circ}C$ for the indicated times and the contents were evaporated twice to dryness at $40^{\circ}C$ in 10 ml flasks on a rotary evaporator. The residues were readily taken up in 1.0 ml of water, and the amino acid content of the hydrolysates were determined with a Technicon Amino Acid Analyzer using a procedure based on the Piez and Morris (56) modification of the Spackman, Stein, and Moore (57) procedure. Each analysis was performed in about 24 hours with a 150 cm column loaded with type A chromobeads.

Tryptophan was estimated separately by two of the more common methods: (1) the chemical p-dimethylaminobenzaldehyde (PDAB) technique of Spies and Chambers (58, 59); (2) the spectrophotometric determination of Goodwin and Morton (60). Tyrosine was also estimated using the latter technique.

In the PDAB method, the L-tryptophan (General Biochemicals) and PDAB (Sigma) were purified according to the directions of Spies and Chambers. Standards and pyrophosphorylase samples, estimated to contain 10-150γ of tryptophan were analyzed. Quantities of solution A (13.92 mg of L-tryptophan and 348 mg PDAB dissolved in 116.0 ml of 19 N sulfuric acid) and solution B (300 mg PDAB dissolved in 100 ml of 19 N sulfuric acid) were mixed in 25 ml glass-stoppered erlenmeyer flasks to give a final volume of 10.0 ml and the desired tryptophan content. In the case of the pyrophosphorylase, 10.0 ml of solution B were added to the solid salt-free, lyophilized protein. All flasks were mixed and allowed to remain in darkness at room temperature for 12 hours. One tenth ml of 0.04% sodium nitrite was added to each flask, and after mixing and incubation for 30 minutes, the optical densities of the solutions were read at 600 mμ in a Coleman Jr. Spectrophotometer. The tryptophan content of the pyrophosphorylase was determined from the standard curve.

In the spectrophotometric determination of tryptophan, approximately 2.5 mg of the lyophilized, salt-free pyrophosphorylase was dissolved in 0.1 ml of 0.1 N NaOH and the exact concentration of one-tenth and one-twentieth dilutions of the stock enzyme were measured every 10 mμ from 280 mμ to 360 mμ. Readings were also taken at 294 mμ. The 280 mμ and 294 mμ readings were corrected for haze by extrapolation of the optical densities in the 320-360 mμ region. The moles of tryptophan and tyrosine were determined by the equations of Goodwin and Morton.

In addition to the DTNB and PMB titrations (see respective sections), the cysteine content of the pyrophosphorylase was evaluated by chromatographic analysis of the acid-hydrolyzed, reduced and S-carboxymethylated (RSCM) enzyme. RSCM pyrophosphorylase were prepared according to the procedure of Crestfield, Stein, and Moore (61). Fifteen to twenty-five mg of the salt-free, lyophilized pyrophosphorylase were placed in a 15 ml screw-cap vial. Urea (3.61 g of a Mann Ultra Pure product) and the following solutions made up in O_2 free water were added: 0.30 ml of a 50 mg/ml solution of disodium EDTA, 3.0 ml tris buffer (5.23 g plus 9.0 ml 1.0 N HCl diluted to 30 ml with water), and 0.1 ml 2-mercaptoethanol. The solution was made up to the 7.5 ml mark with water and 8 M urea that was 0.2% in EDTA was used to fill the vial to the 12.0 ml mark. A layer of nitrogen gas was then placed over the top of the solution. After incubation for 4 hours at room temperature, 0.268 g of iodoacetic acid[1] (Eastman) in 1.0 ml of 1.0 N NaOH (adjusted to pH 8.5 with concentrated NaOH) was added. Further manipulation was performed in the dark. After 15 minutes, the solution was dialyzed against either 0.01 M NH_4HCO_3 or 50% acetic acid to eliminate the components of the reaction. Both the solution in the case of the acetic acid dialysate and the suspension in the case of the NH_4HCO_3 dialysate were shell frozen and lyophilized. The acetic acid dialyzed sample tended to form a glass.

---

[1]The iodoacetic acid was recrystallized from a diethylether-hexane solution and dried <u>en vacuo</u> over silica gel.

Electrophoresis on Cellulose Acetate Strips

A 7 mg/ml solution of the crystalline pyrophosphorylase that was 0.05 M in sodium bicarbonate buffer (pH 11.0) and 6M in urea was dialyzed for 24 hours against the same buffer system prior to electrophoresis. Cellulose acetate strips (2.5 x 18 cm) were soaked for 1 hour in the outside dialysis solution used above. Excess solution was blotted from the strips with the aid of a piece of filter paper. Five µl were streaked across the strip, without scratching the cellulose acetate surface or spotting the edges of the strip. The still moist, but thoroughly impregnated strip was subjected to electrophoresis at a constant voltage (65-70 volts per strip) in a Shandon Electrophoresis Apparatus for 3 hours at 4°C. The strips were then gently blotted with filter paper and fixed by submersion for 10 minutes in 3% trichloroacetic acid (TCA). The excess solution was removed by blotting and the strips were stained for 10-15 minutes by submerging them in 0.2% Ponseau S in 3% TCA. The excess dye was removed by repeated washings of the strips in 5% acetic acid prior to drying.

Isoelectric Focusing

Isoelectric focusing was performed on an LKB 8101 electrofocusing column with a 110 ml capacity. The technique used was described by Svensson (62) and Vesterberg and Svensson (63). A step-wise 0-50% sucrose density gradient was used with an equal mixture of pH 3-10 and pH 5-8 LKB carrier ampholytes which were used at a final concentration of 1% (w/v). All solutions including

those of the cathode and anode were made 6 M in urea. To prevent oxidation and reduction, sulfuric acid and ethylenediamine were added to the anode and cathode solutions, respectively. Six mg of the pyrophosphorylase in 0.5 ml were added to an intermediate fraction of the less dense solution, and solid urea was added to a concentration of 6 M. The analysis was performed for 48 hours at 4°C and final focusing was accomplished at 900 volts. The column was then drained and 9 drop fractions were collected and were analyzed at 280 mμ for protein.

Trypsin Digestion and Peptide Mapping

Fingerprinting experiments on the pyrophosphorylase were carried out by standard procedures (64, 65). Trypsin digestion was found to proceed best when the enzyme was heat denatured. A 10 mg/ml solution of the pyrophosphorylase in 0.2 M NH_4HCO_3 (pH 8.6) was dialyzed for 24 hours against the same buffer. The solution was then transferred to a small glass vial, and the protein was coagulated by heating the vial at 90°C for 6 minutes. The tube was then cooled by emersion in an ice bath. The protein was dispersed by use of a small magnetic stirring bar. A 2% protein ratio of TPCK-trypsin (Worthington Biochemicals Corporation) was added over a period of 20 hours. Trypsin was a freshly prepared 5 mg/ml solution in 0.001 N HCl. The clear and colorless solution was centrifuged to eliminate a trace amount of suspended material and the digest was then shell-frozen and lyophilized.

The white fluffy digest proved to be insoluble in water, but soluble in dilute ammonia water (1:15). Two to three mg of the

digest was routinely used for each map and was applied in enough ammonia water to make about a 50 mg/ml solution.

Chromatography was performed in the first direction on full sheets of Whatman 3 MM chromatography paper (18.5 x 22.25 cm). Discrete spots were observed when the solvent system was n-butanol: pyridine: acetic acid: water (90:60:18:72). The chromatograms were routinely developed in a chromatocab for 27 hours at room temperature and then air-dried overnight. Electrophoresis was performed in the second direction in a Gilson Model D Electrophorator. The chromatograms were exposed to 2100 volts for 2 hours in the pH 3.5 buffer system containing pyridine: acetic acid: water (1:10:289), and the papers were again air-dried overnight.

The peptides were visualized by dipping the papers in 0.25% ninhydrin (General Biochemicals) in acetone; the spots were allowed to develop at room temperature for 3 hours, and then in the dark overnight. Tryptophan-containing peptides were visualized by dipping the ninhydrin-developed chromatograms in Ehrlich's stain (66) which was prepared by freshly mixing 900 ml acetone, 100 ml concentrated HCl, and 10 g p-dimethylaminobenzaldehyde. The HCl bleached all the peptides and after several minutes, the tryptophan-containing spots developed a blue color. The modified Sukaguchi reagent (67) was used to detect the presence of arginine on untreated maps. The chromatograms were dipped in 0.0125% α-napthol in absolute ethanol. After air drying, they were sprayed lightly with a solution of 1.5 ml of

4-5% sodium hypochlorite and 23.5 ml of 10% NaOH. Arginine peptides turned a light red color.

Cyanogen Bromide Cleavage

Cleavage of the pyrophosphorylase with cyanogen bromide was performed by the procedure of Gross et al. (68) as modified by Steers et al. (69). Three to four mg of RSCM-pyrophosphorylase in a glass-stoppered tube were dissolved in 2.0 ml of 70% formic acid. A 50-fold excess of cyanogen bromide (Eastman) over methionine (taken to be about 70 residues per mole) was added. The reaction was allowed to proceed at room temperature for 16-20 hours and then the digest was dried and the volatile components were removed by lyophilization. A white, fluffy digest was obtained.

The components of the digest were monitored by electrophoresis on 7.5% polyacrylamide gel columns with the pH 8.7 system described by Davis et al. (70). All gels were 5 M in urea and the digest (200-500 μg) was added to the sample gel in a 10 M urea solution, so that the final gel concentration was 5 M. Solubilization was also possible in a 1:15 solution of NH_4OH, but better acrylamide patterns were obtained with the urea system.

The subunits and the RSCM-enzyme were compared with the cyanogen bromide digests using 5% polyacrylamide gel columns that were 5 M in urea.

Subunit Reassociation

Dissociation and reconstitution experiments were performed according to the procedure of Deal (71). The dissociation medium contained 2.43 gm of urea (8 M), 0.1 ml of mercaptoethanol, 0.50 ml of 2 M ammonium sulfate, 2.40 ml of 0.47 M glycine buffer (pH 9.5), and water to make 5.0 ml. A crystalline suspension of the pyrophosphorylase (1.5 mg) was diluted to 1.0 ml with the dissociation mixture and a control system was prepared by dilution of the enzyme into a mixture containing everything except the urea. The control tube was warmed at 35°C to dissolve the crystals and both samples were allowed to remain for 2 hours at 3-4°C.

Reassociation experiments were attempted by two different procedures. The first method was the direct dilution of the control and dissociated enzyme into the system wherein activity was measured. A second procedure was the 1:100 dilution at 0°C of the dissociated enzyme into a reassociation mixture which contained 0.25 M imidazole buffer (pH 6.3), 0.2 M KCl, 0.03 M glutathione, and either 0.04 M DPN, 0.01 M UDPG or no additional component.

In the method that utilized the reassociation mixture, care was taken to keep all micropipettes at 0°C, and all dilutions were made slowly with stirring at that temperature to prevent precipitation. After the dilution at 0°C, the tubes were transferred to a water bath at 15-16°C, and aliquots were removed for activity measurements for times up to 3 hours.

RESULTS

Absorption Spectra

Figure 1 shows the typical aromatic amino acid spectra of the pyrophosphorylase. A maximum was observed at 278.5 mμ and a minimum at 255.0 mμ. Peaks were also noted in the spectra at 258.5 mμ, 265.0 mμ, 268.0 mμ, 283.5 mμ, and 292.0 mμ. The purified enzyme exhibited a 280/260 ratio of 1.6.

Extinction Coefficient

The protein samples were weighed with a precision of $\pm$0.025 mg, and the optical density readings were obtained with a precision of about 1%. It was found that in 0.01 M phosphate buffer (pH 7.0) a 0.1% solution of the pyrophosphorylase had an optical density of 0.719 in a 1 cm light path at 280 mμ. This corresponds to a molar extinction coefficient of 3.40×10^5 at this wavelength.

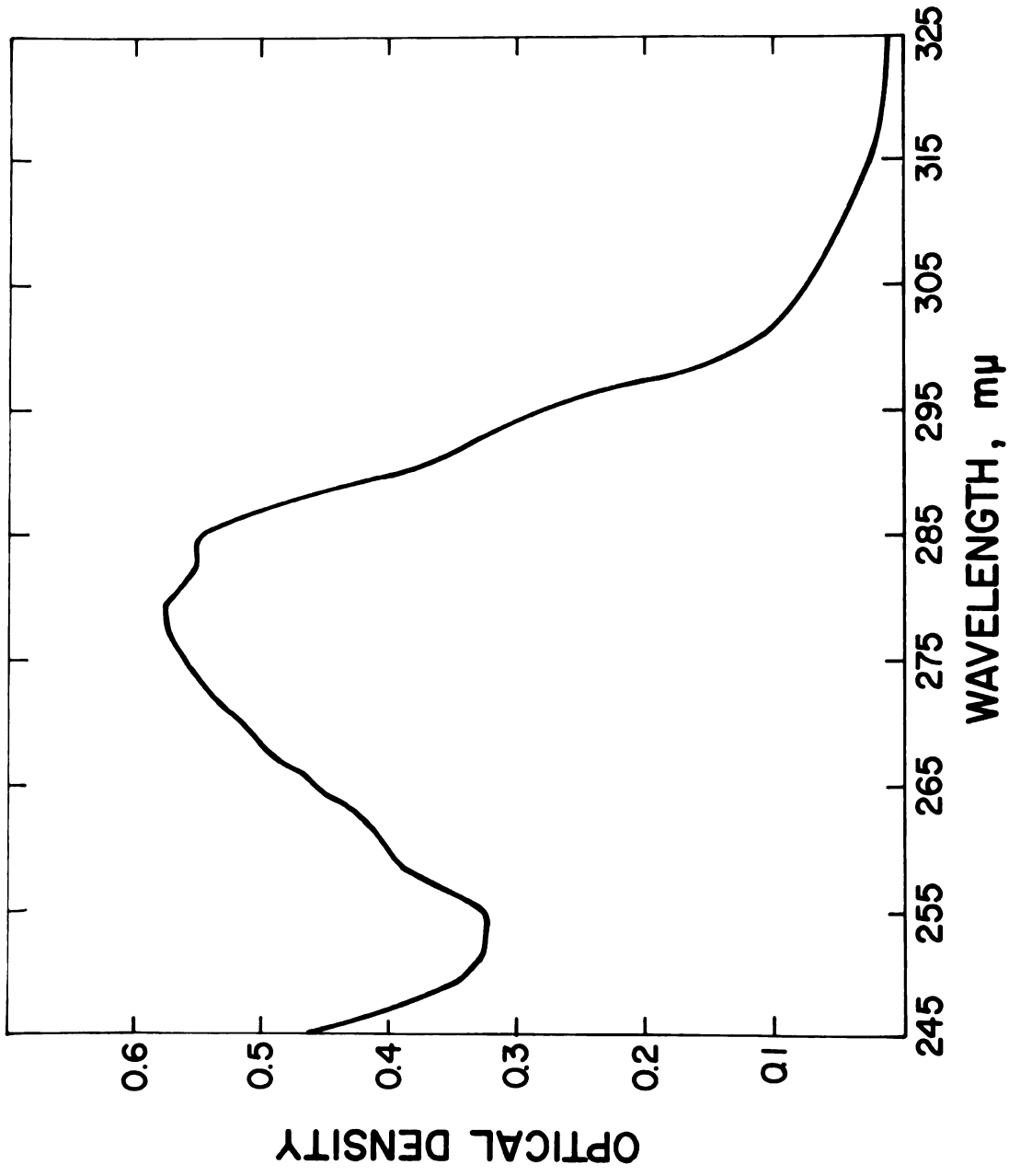
Protein estimated by dry weight was 93% of the values determined by the Lowry method with BSA as a comparison standard. Also, protein estimated by the 280/260 method of Warburg and Christian was 21% lower than the actual dry weight.

Carbohydrate Content

The carbohydrate content, as determined by the phenol-sulfuric acid method of Dubois *et al.*, was no more than 0.02%.

Figure 1. Ultraviolet absorption spectra of the pyrophosphorylase

The protein concentration was 0.80 mg/ml in 0.01 M tricine (pH 8.36). A Cary 15 Spectrometer was used with a 1 cm light path at a temperature of 24°C.


OPTICAL DENSITY
0.6
0.5
0.4
0.3
0.2
0.1
245
255
265
275
285
295
305
315
325
WAVELENGTH, mμ

Homogeneity and Molecular Weight of the Enzyme by Sedimentation and Diffusion Techniques

When two and three times recrystallized pyrophosphorylase was subjected to ultracentrifugal analysis, it became obvious that the pyrophosphorylase was polydisperse. With the major sedimenting component, there was at least one more rapidly sedimenting component (Figure 2). At concentrations (0.2-1%) of protein and conditions used in these experiments, the minor components amounted to about 4% or less of the total protein. Recrystallization of the enzyme up to six times has not altered the relative amount or mobility of these minor components. At times, two rapidly sedimenting components may be visualized. In an experiment that yielded three components, the sedimentation coefficients, $S^{0.4\%}_{20,w}$, were 13.22 for the principal component, 19.66 S and 25.08 S for the two minor components. Using the sedimentation coefficients and assuming spherical entities with a partial specific volume of 0.74 ml/gm, approximate molecular weight ratios of 1.0, 1.8 and 2.6 were obtained for the major component and the two minor components, respectively. It thus appears that multimers of the pyrophosphorylase molecule may exist. The results were essentially identical regardless of whether mercaptoethanol at a concentration of 0.1 M was included in the buffer system. The comparison of the mobilities of the native enzyme in the presence and absence of mercaptoethanol is shown in Figure 3. It thus appears that a reducing agent itself would not cause dissociation of the enzyme.

Figure 2. Schlieren sedimentation velocity pattern of the pyrophosphorylase

The temperature was 4°C and the enzyme was 3.2 mg/ml in 0.01 M tricine buffer (pH 8.5), 0.1 M in mercaptoethanol, and 0.1 M in NaCl. The diaphragm angle was 65° and sedimentation is from left to right.

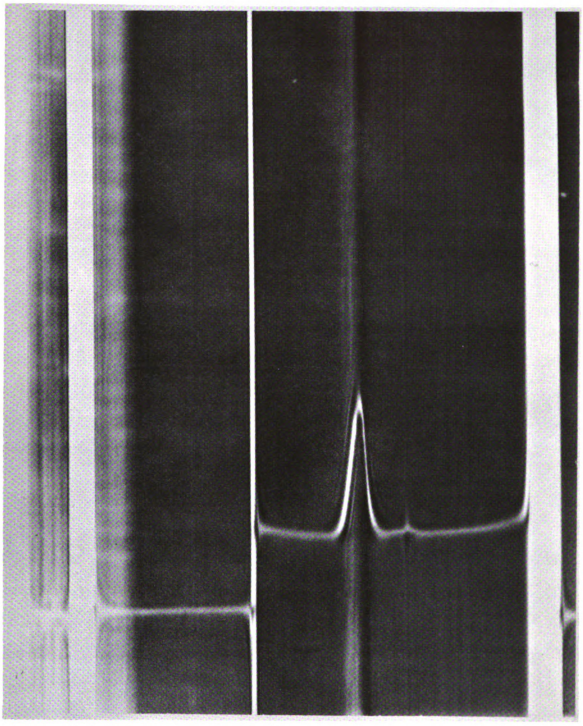


Figure 3. Relative sedimentation rates for the enzyme in the presence and absence of reducing agent

The upper pattern shows the enzyme in the presence of 0.1 M mercaptoethanol, and the lower pattern shows the enzyme in the absence of 0.1 M mercaptoethanol. Both samples were 0.01 M in tris-HCl (pH 8.5) and 0.1 M in NaCl.

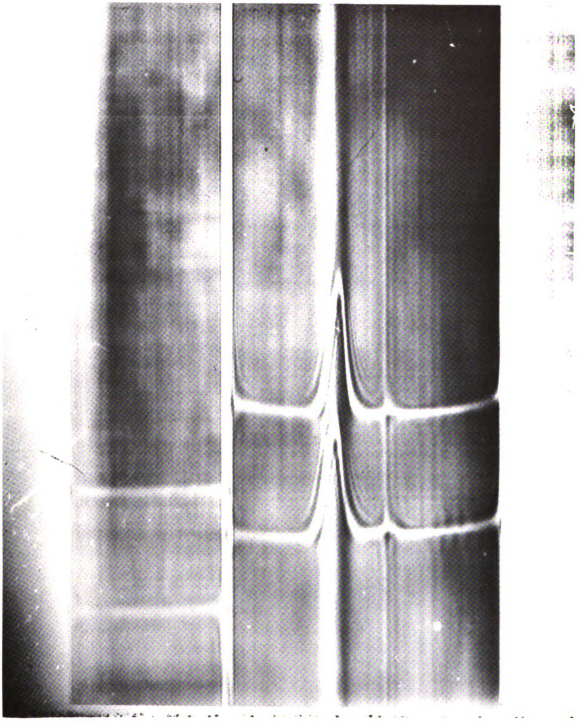
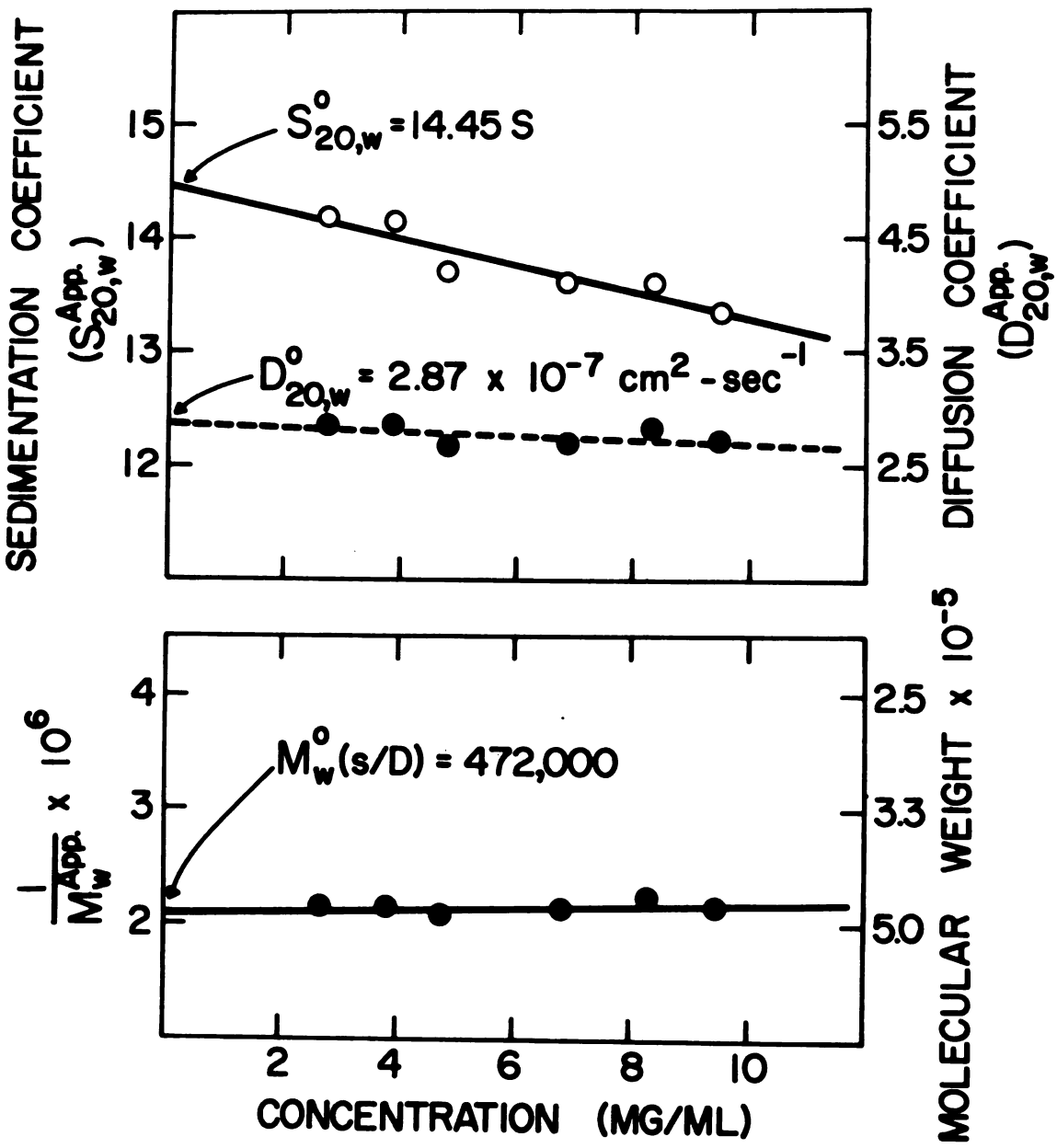


Figure 4. Apparent sedimentation coefficients, diffusion coefficients, and weight-average molecular weights for the principal component of the pyrophosphorylase

The enzyme was analyzed in 0.02 M triethanolamine buffer (pH 7.3), 0.1 M in NaCl, and 0.1 M in mercaptoethanol.


SEDIMENTATION COEFFICIENT ($S^{App.}_{20,w}$)
DIFFUSION COEFFICIENT ($D^{App.}_{20,w}$)
$S^{o}_{20,w}$ = 14.45 S
$D^{o}_{20,w}$ = 2.87 x 10^{-7} cm^2 - sec^{-1}
15
14
13
12
5.5
4.5
3.5
2.5
$\frac{1}{M^{App.}_{w}}$ x 10^{6}
M^{o}_{w}(s/D) = 472,000
4
3
2
2.5
3.3
5.0
MOLECULAR WEIGHT x 10^{-5}
2
4
6
8
10
CONCENTRATION (MG/ML)

Since the minor components represented a small fraction of the total protein, molecular weight determinations were made using the sedimentation and diffusion techniques. Sedimentation coefficients have varied under all conditions by about 6% and combination of sedimentation and diffusion data were therefore determined on the same sample. Table 1 compares the sedimentation behavior of the pyrophosphorylase under various conditions of buffer and pH, and different protein preparations. In some of the earlier work, the sedimentation coefficients varied by more than 6%, but it is believed that this simply represented an inaccurately known protein concentration. Figure 4 shows the extrapolation to infinite dilution of the apparent sedimentation and diffusion coefficients. The principal boundary was used for this analysis. Extrapolation to zero protein concentration yielded a value for $S^0_{20,w}$ of 14.45 and a value for $D^0_{20,w}$ of 2.87 x 10^{-7} cm^2/sec. Combination of the apparent sedimentation and diffusion coefficients have yielded a molecular weight for the principal component ($M^0_w(s/D)$) of 472 x 10^3 gm/mole. Although the sedimentation and diffusion coefficients varied in some cases, extrapolation to infinite dilution under various conditions of buffer, pH, and different protein preparation yielded values in close agreement. For example, an experiment in 0.01 M tricine (pH 8.5) that was 0.1 M in NaCl and 0.1 M in mercaptoethanol yielded a $S^0_{20,w}$ of 14.10 S, a $D^0_{20,w}$ of 2.72 x 10^{-7} cm^2/sec and a $M^0_w(s/D)$ of 485,000 gm/mole.

TABLE I

Sedimentation Coefficients for UDP-glucose Pyrophosphorylase Under Varying Conditions

Conditions	Prep-aration	Protein (mg/ml)	$S_{20,w}$
0.01 M tris-HCl; 0.1 M NaCl; pH 7.9	1	3.20	13.09
0.01 M tris-HCl; 0.1 M NaCl; 0.1 M MSH[a]; pH 7.9	1	3.20	13.22
0.01 M tricine; 0.1 M NaCl; 0.1 M MSH; pH 8.5	2	3.78	12.81
0.01 M tricine; 0.1 M NaCl; 0.1 M MSH; pH 8.5	3	3.98	12.94
0.01 M tricine; 0.1 M NaCl; 0.1 M MSH; pH 8.5	4	3.96	13.18
0.01 M tricine; 0.1 M NaCl; 0.1 M MSH; pH 8.5	5	4.00	13.03
0.01 M tricine; 0.1 M NaCl; 0.1 M MSH; pH 8.5	6	3.73	13.22
0.02 M triethanolamine; 0.1 M NaCl; 0.1 M MSH; pH 7.3	7	3.50	13.27
0.01 M bicine; 0.1 M NaCl; pH 8.5	7	4.00	13.49
0.01 M glycylglycine; 0.1 M NaCl; pH 8.5	7	4.00	13.08
0.02 M triethanolamine; 0.1 M NaCl; pH 8.5	7	4.00	13.22

[a]MSH = 2-mercaptoethanol

Molecular Weight of the Native Enzyme by the Sedimentation Equilibrium Technique

Sedimentation equilibrium experiments yielded molecular weight estimates that were less equivocal than the sedimentation and diffusion techniques. These experiments are shown in figure 5. Discrepancies are noted not only between the weight- and z-average molecular weights, but also between the limiting z-average and the intrinsic z-average molecular weights. Due to some scatter in the weight-average molecular weights, a value between 476,000 and 570,000 was found. The limiting value of 476,000 is in good agreement with that obtained from the sedimentation and diffusion techniques. Similarly, values of 606,000 and 1,052,000 were obtained for the limiting z-average and the intrinsic z-average molecular weights.

Homogeneity and Molecular Weight of the Subunits by Sedimentation, Diffusion, and Sedimentation Equilibrium Techniques

Figure 6 shows the relative rates of migration of the native enzyme in the lower pattern and the enzyme denatured in 5 M urea in the upper pattern. It appeared from this experiment that subunits of the pyrophosphorylase exist. Figure 7 shows the similarity in mobility of the denatured states of the pyrophosphorlase in urea and guanidine hydrochloride.

Sedimentation, diffusion and equilibrium studies were performed in 6 M guanidine hydrochloride and 0.1 M mercaptoethanol to characterize the pyrophosphorylase subunits. In this solvent

Figure 5. Apparent weight-average, z-average and limiting z-average molecular weights for the pyrophosphorylase

Concentrations were evaluated as $(C_m + C_b)/2$ for the weight-average and $C_m + C_b$ for the limiting and intrinsic z-average molecular weights. The analysis was performed at 5°C at a speed of 3,000 rpm. The buffer system contained 0.01 M tris-HCl (pH 8.3) that was 0.1 M in NaCl and 0.1 M in MSH.

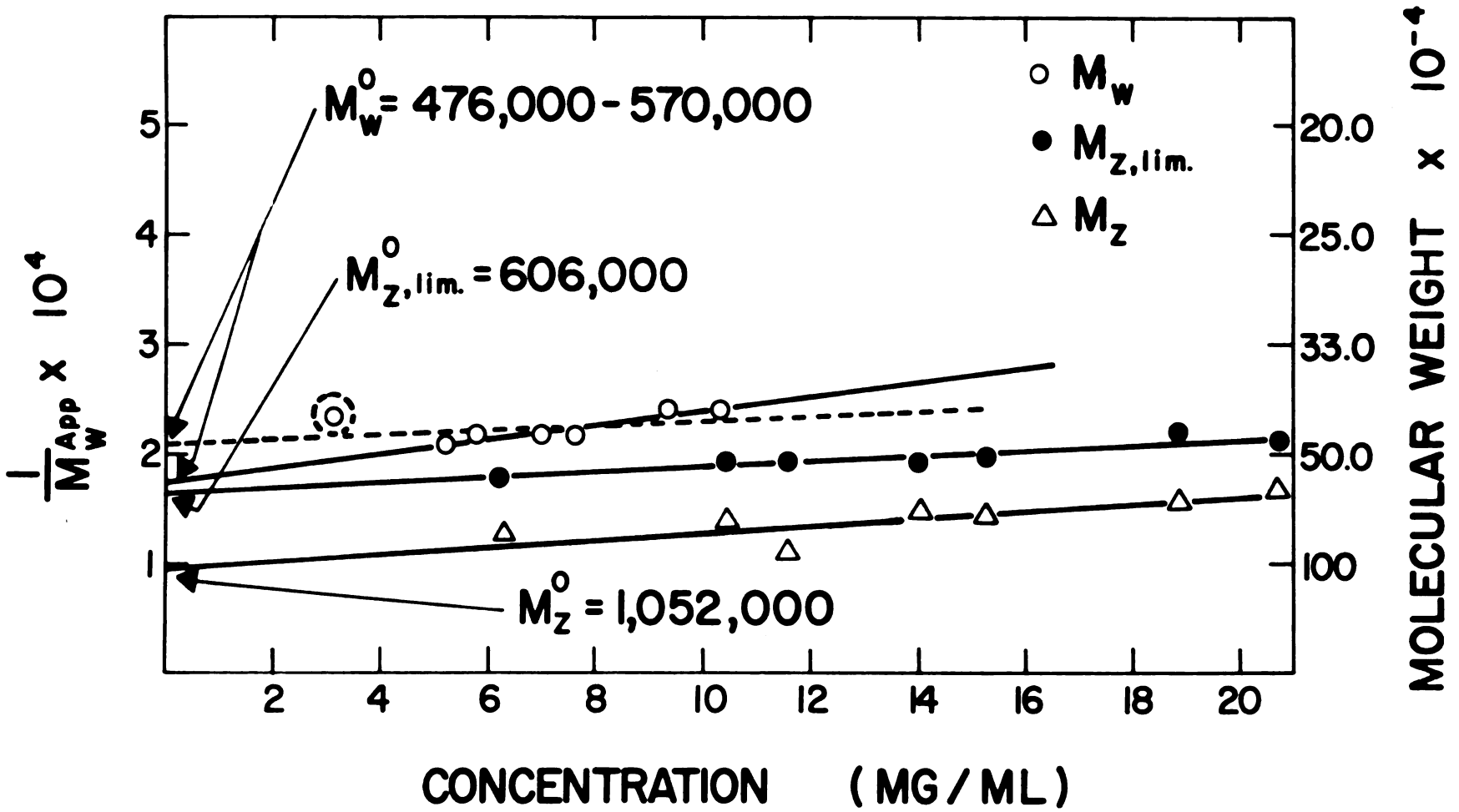
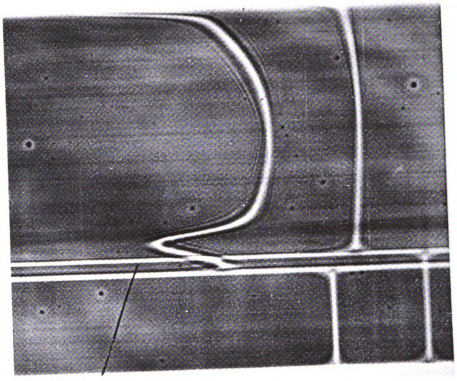

M_w^0= 476,000 - 570,000
$M_{z,lim.}^0$ = 606,000
M_z^0 = 1,052,000
M_w
$M_{z,lim.}$
M_z
$\frac{1}{M_w^{App}} \times 10^4$
MOLECULAR WEIGHT x 10^{-4}
CONCENTRATION (MG/ML)
1
2
3
4
5
2
4
6
8
10
12
14
16
18
20
20.0
25.0
33.0
50.0
100

Figure 6. The relative sedimentation rates for the native protein and the subunits

The pictures were taken 1 1/2 hours after attaining 59,780 rpm and were spaced by 32-minute intervals. The lower pattern shows the native enzyme at 4 mg/ml in 0.02 M triethanolamine buffer (pH 8.5) that was 0.1 M in NaCl. The upper pattern shows the subunits at 4 mg/ml in 0.02 M triethanolamine (pH 7.8) that was 0.01 M in NaCl and 5 M in urea. The temperature was 6.6°C.



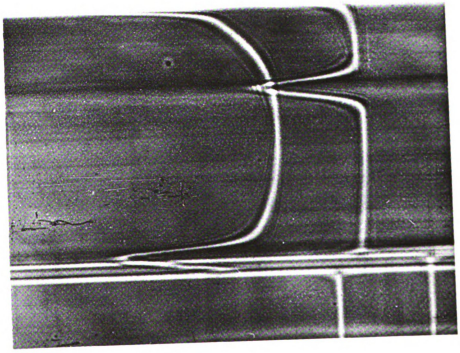
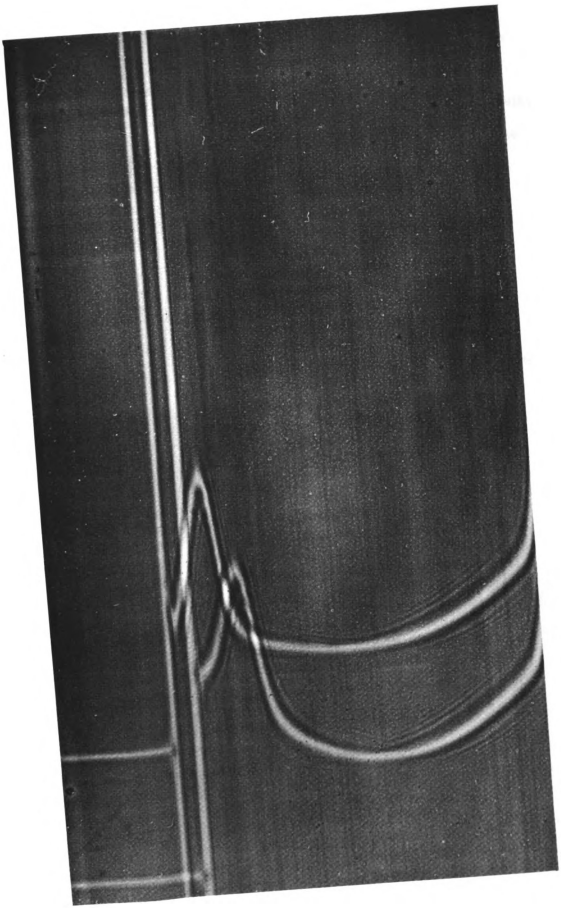


Figure 7. The relative rates of sedimentation of the subunits in guanidine hydrochloride and urea

In both cases the enzyme was 3.5 mg/ml in 0.01 M NaCl. The upper pattern shows the enzyme in 7 M urea (pH 8.1) while the lower pattern shows the enzyme in 5 M guanidine hydrochloride (pH 7.6). The rotor speed was 59,775 rpm and the temperature was 4.2°C.



system, the sedimentation coefficient decreased markedly, but the diffusion coefficient remained essentially the same as the native enzyme. Figure 8 shows the extrapolation to infinite dilution of the apparent sedimentation and diffusion coefficients of the subunits. Using a partial specific volume which was the same as the native enzyme and uncorrected for preferential solvent effects, a $S^{0}_{20,w}$ of 2.15 S and a $D^{0}_{20,w}$ of 2.90×10^{-7} $cm^2/sec.$ was obtained. Combination of $S^{0}_{20,w}$ and $D^{0}_{20,w}$ gave a $M^{0}_{w}(s/D)$ of 69,100.

Low speed sedimentation equilibrium experiments yielded a value for the molecular weight of the subunits in good agreement with that obtained from the Svedberg relationship. Figure 9 shows the extrapolation to infinite dilution of the apparent weight- and z-average molecular weights. The fact that both extrapolated to 67,500 indicated that the subunits were similar, if not identical, in mass.

Production of these subunits is accompanied by a major change in shape of the protein, as judged by molecular weight and frictional coefficient analysis. The sedimentation and diffusion coefficients are lower than expected for a globular protein of molecular weight 68,000. Furthermore, a frictional ratio of 2.8 was found for the subunits as compared with 1.5 for the native enzyme. The subunits are, therefore, highly unfolded.

Figure 8. Apparent sedimentation and diffusion coefficients and weight-average molecular weights of the subunits

The dissociating solvent system was 0.01 M tris-HCl (pH 8.5) that was 6 M in guanidine hydrochloride, 0.1 M in MSH, and 0.1 M in NaCl.

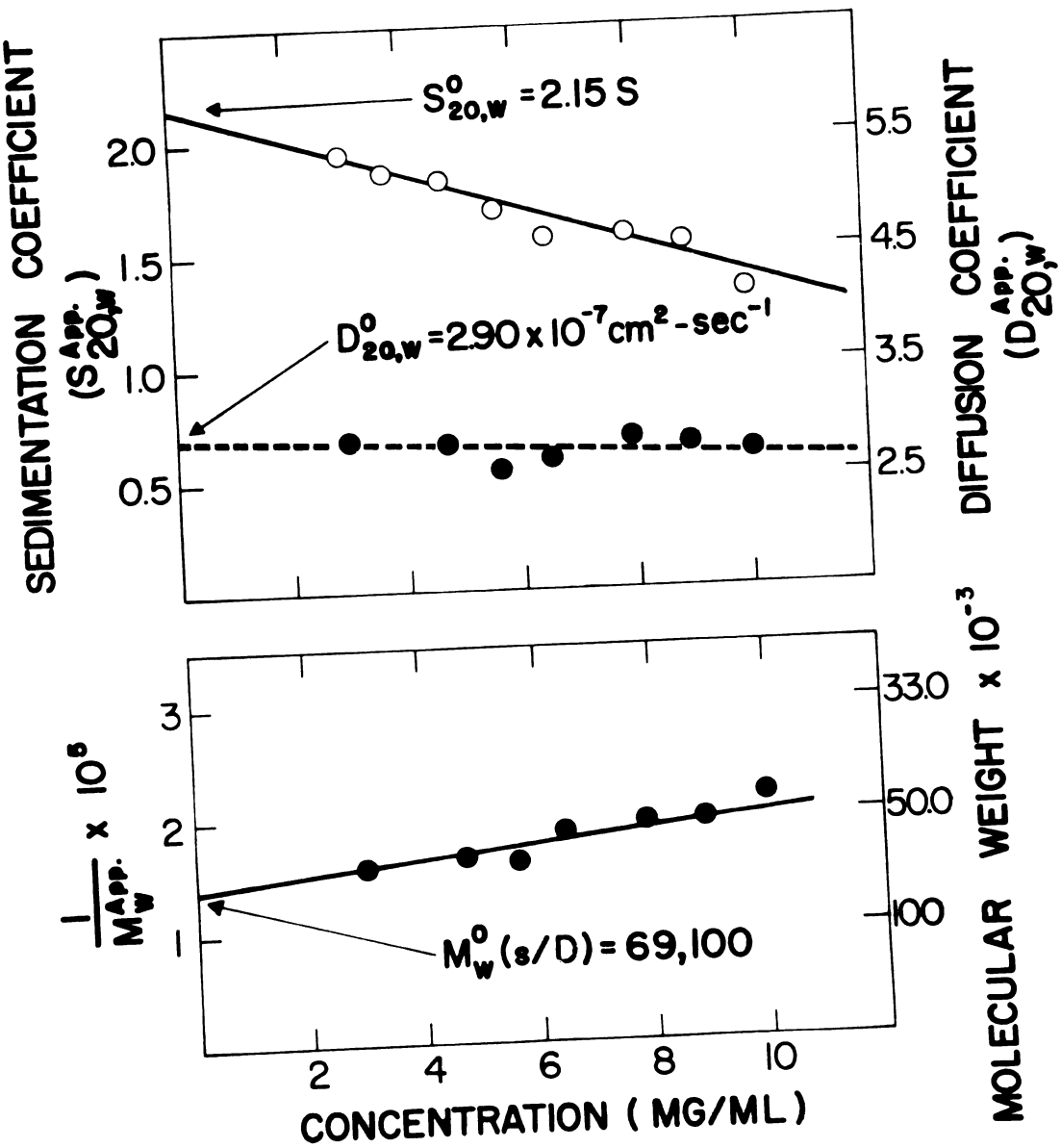
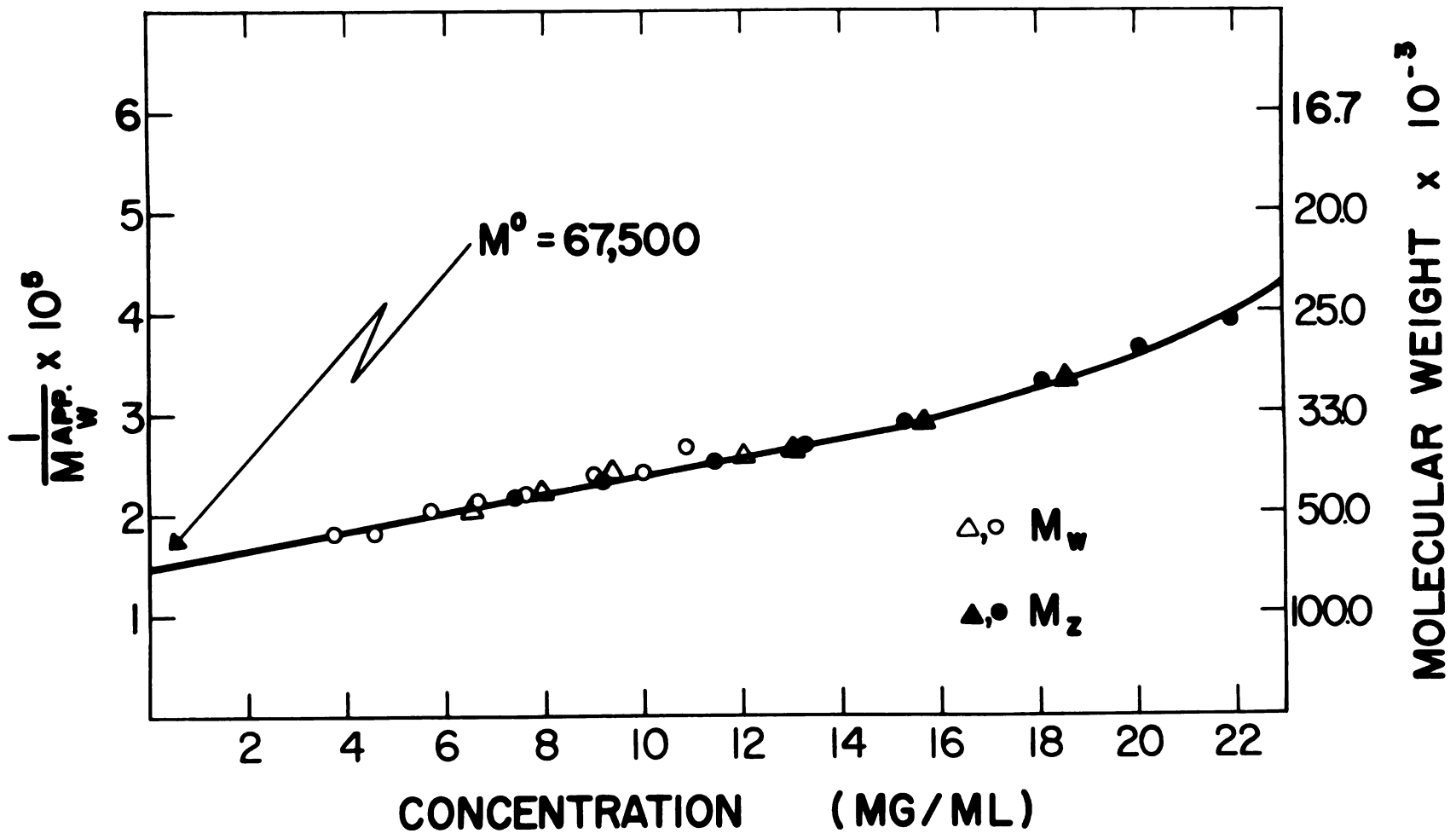

SEDIMENTATION COEFFICIENT ($S^{App.}_{20,w}$)
DIFFUSION COEFFICIENT ($D^{App.}_{20,w}$)
$S^0_{20,w}$ = 2.15 S
$D^0_{20,w}$ = 2.90 x 10^{-7} cm^2 - sec^{-1}
2.0
1.5
1.0
0.5
5.5
4.5
3.5
2.5
$\frac{1}{M^{App.}_w}$ x 10^5
MOLECULAR WEIGHT x 10^{-3}
M^0_w (s/D) = 69,100
3
2
1
33.0
50.0
100
2
4
6
8
10
CONCENTRATION (MG/ML)

Figure 9. The apparent weight-average and z-average molecular weights of the subunits

The weight- and z-average molecular weights were analyzed as described in figure 5 and the dissociating system was the same as described in figure 8. The analyses were carried out at 5°C for 24-36 hours at 20,400 rpm. Various preparations of the pyrophosphorylase are indicated by the symbols -Δ- and -O-.


$\frac{1}{M_w^{APP.}} \times 10^5$
6
5
4
3
2
1
$M^0 = 67{,}500$
△,○ M_w
▲,● M_z
16.7
20.0
25.0
33.0
50.0
100.0
MOLECULAR WEIGHT x 10^{-3}
2
4
6
8
10
12
14
16
18
20
22
CONCENTRATION (MG/ML)

Optical Rotatory Dispersion

When native and denatured pyrophosphorylase were subjected to optical rotatory dispersion analysis in the 190-330 mμ spectral region, the results shown in figure 10 were obtained. Figure 11 shows an expanded plot of the specific rotation versus wavelength in part of the visible and near ultraviolet regions. In these regions, the specific rotation decreased in a regular fashion for both the native and denatured states. Re-evaluation of these data according to the graphic procedure of Yang and Doty (72) is shown in figure 12. This procedure involved plotting $[\alpha]_\lambda \lambda^2$ versus $[\alpha]_\lambda$. The fact that these plots are linear for both the native and denatured states indicates that the one term equation of Drude (73) is adequate to account for the visible and near ultraviolet dispersion. The dispersion constant, λ_c, which was obtained from the slope of the Yang and Doty plot was found to be 246 mμ and 216 mμ for the native and denatured enzyme, respectively.

Analysis of the rotatory dispersion of the native enzyme in the 280-500 mμ spectral range according to Moffitt and Yang is shown in figure 13. A value of 212 mμ for λ_o was adequate to obtain linear Moffitt plots in the 350-600 mμ spectral range (74, 75). Choosing a λ_o of 212, 216, and 220 mμ, the linearity of the Moffitt plots was extended to 320, 310, and 285 mμ, respectively. Values for a_o of -271, -204, and -200 deg-cm^2/decimole and values for b_o of -123, -87, and -76 deg-cm^2/decimole were obtained when λ_o was 212, 216, and 220 mμ, respectively.

Figure 10. Ultraviolet optical rotatory dispersion of the native and denatured enzyme

The symbol -●- represents the native enzyme and -O- represents the enzyme denatured in 6 M guanidine hydrochloride. Both solutions were 0.01 M in tris-HCl (pH 8.50) and 0.10 M in KCl. The native enzyme varied in protein concentration from 0.521% to 0.260% in layer thicknesses of 0.02 to 0.001 decimeters. The denatured enzyme was 0.163% in layer thicknesses that varied from 0.1 to 0.001 decimeters. The analysis was performed at 27°C.

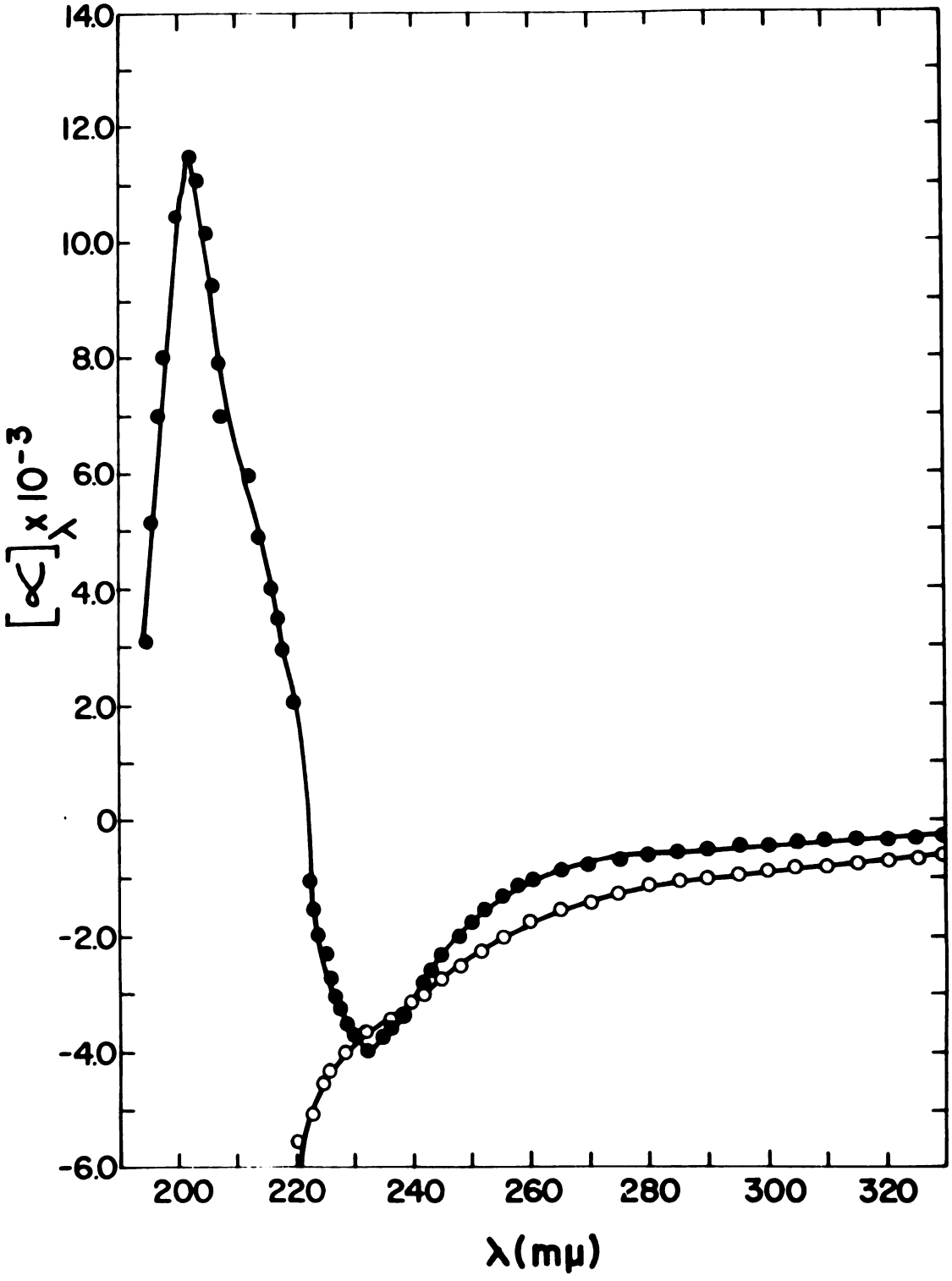

14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
-2.0
-4.0
-6.0
$[\gamma]_\lambda \times 10^{-3}$
200
220
240
260
280
300
320
λ(mμ)

Figure 11. Visible and near ultraviolet optical rotatory dispersion of the native and denatured enzyme

The symbol -●- indicates the native enzyme solution and -O- indicates the enzyme denatured in 6 M guanidine hydrochloride. The conditions of the analysis are given in figure 10. The native enzyme was 1.303% in a layer thickness of 0.1 decimeter, and the denatured enzyme was 0.163% in the same layer thickness.

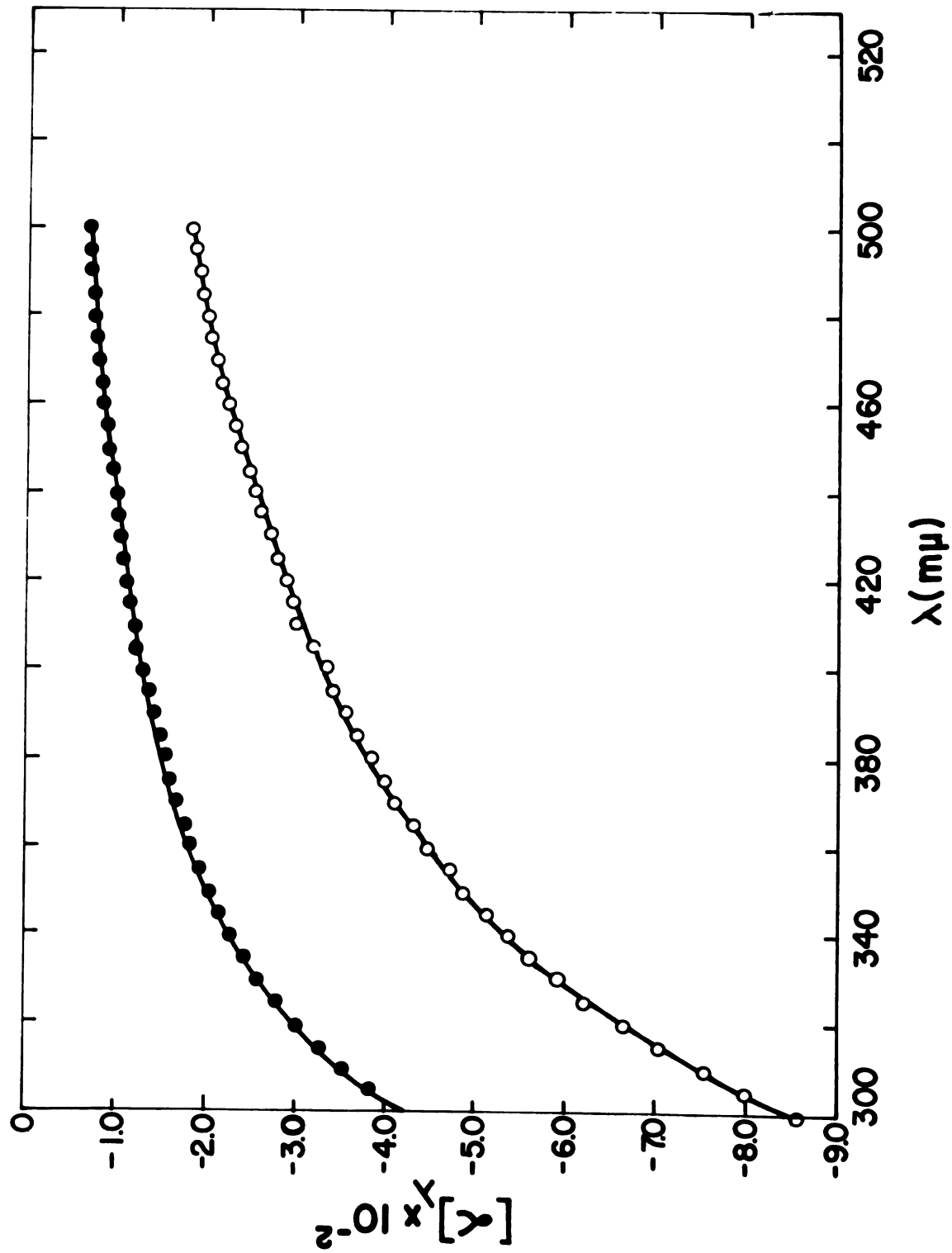

0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
-7.0
-8.0
-9.0
300
340
380
420
460
500
520
λ(mμ)
[α]λ x 10⁻²

Figure 12. Drude plots in the visible and near ultraviolet regions for the native and denatured enzyme

The symbol -●- depicts the native enzyme and -O- the enzyme denatured in 6 M guanidine hydrochloride. The conditions are given in figure 10.

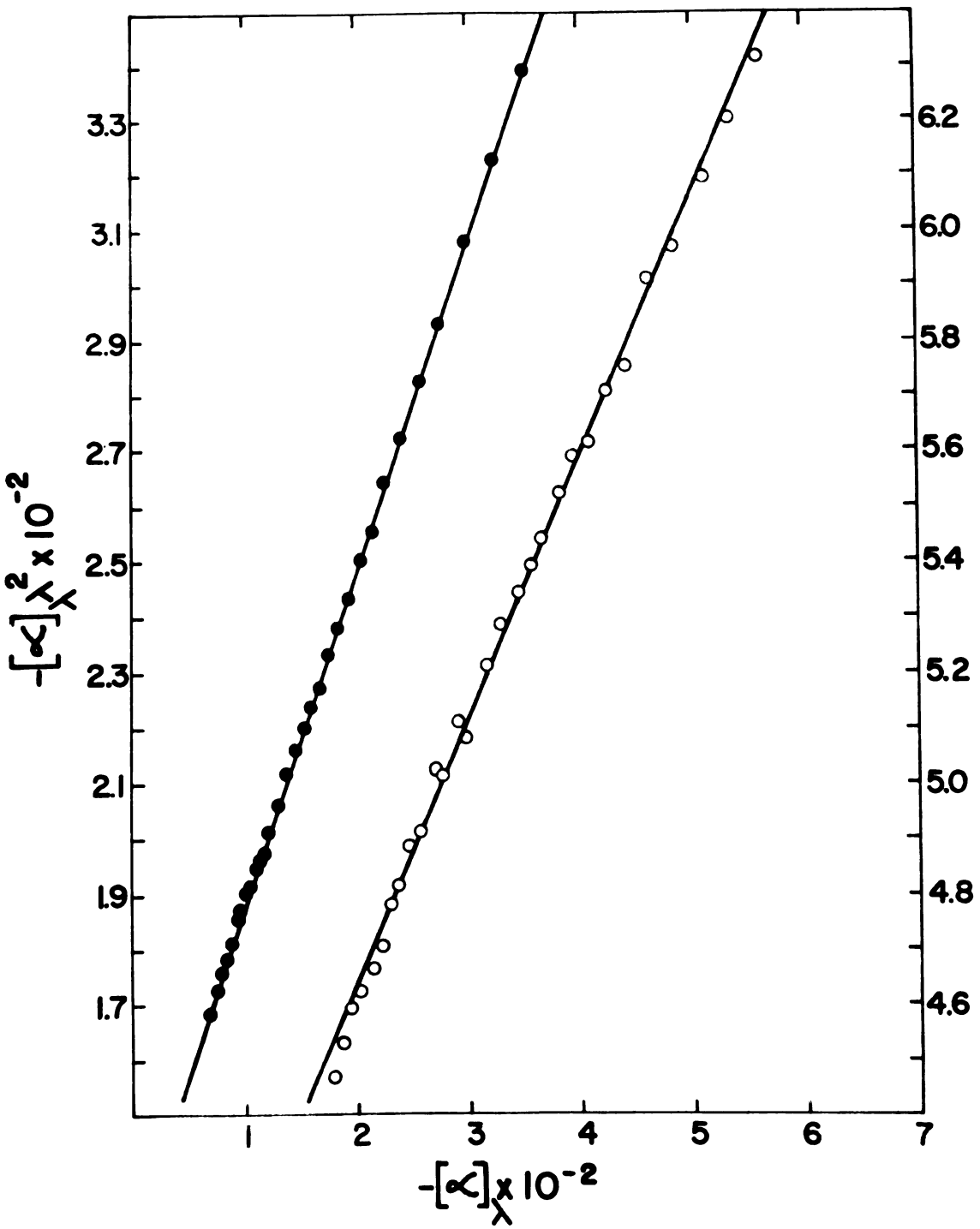
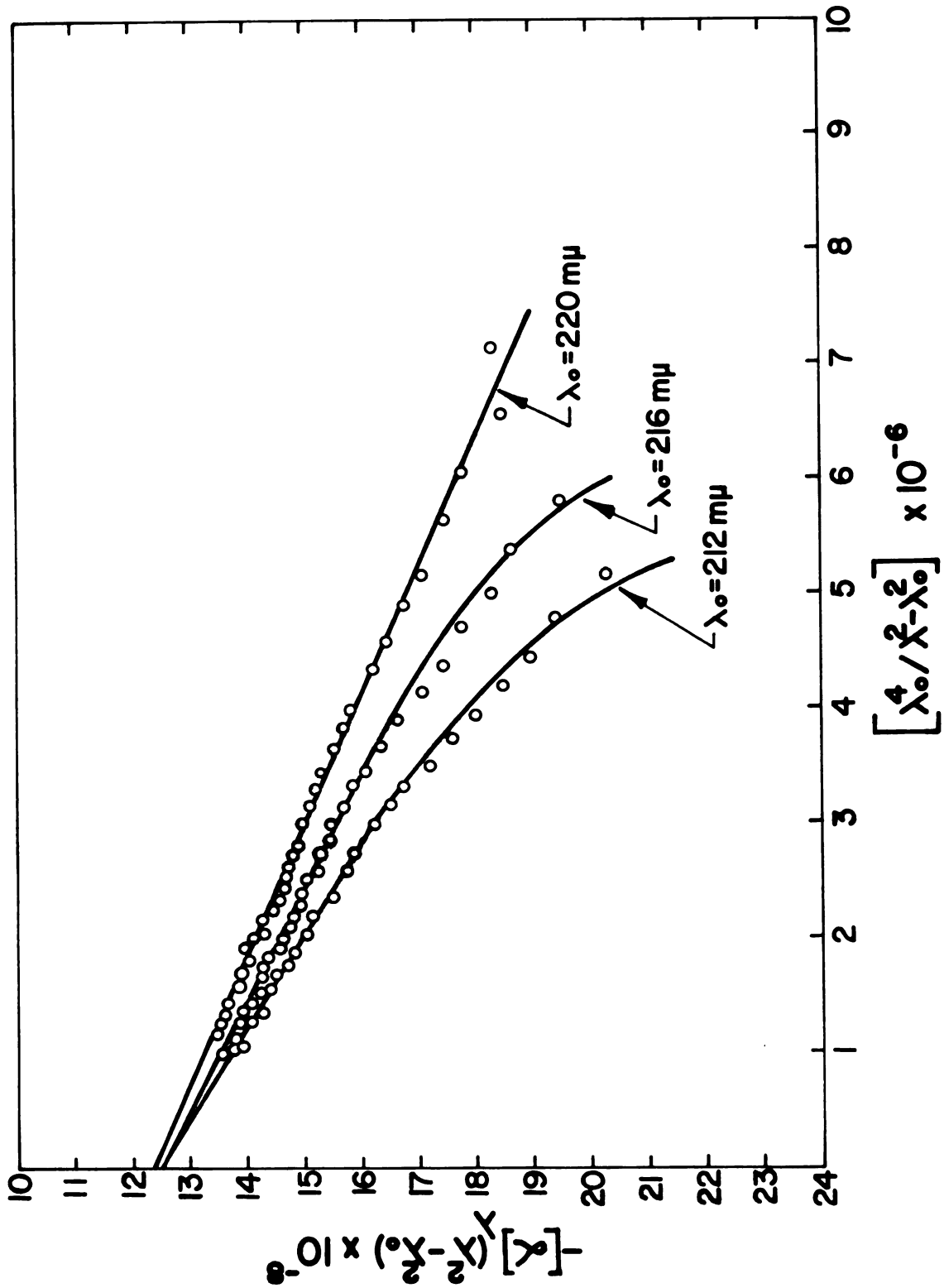

3.3
3.1
2.9
2.7
2.5
2.3
2.1
1.9
1.7
6.2
6.0
5.8
5.6
5.4
5.2
5.0
4.8
4.6
1
2
3
4
5
6
7
$-[\alpha]_\lambda^2 \times 10^{-2}$
$-[\alpha]_\lambda \times 10^{-2}$

Figure 13. Moffitt-Yang plots of the native enzyme in the 280-500 mμ spectral region

See figure 10 for the experimental conditions.


$-[\alpha]_\lambda(\lambda^2-\lambda_0^2) \times 10^{-8}$
$\left[\frac{4}{\lambda_0/\lambda^2-\lambda_0^2}\right] \times 10^{-6}$
λ_0=220 mμ
λ_0=216 mμ
λ_0=212 mμ

The Moffitt plot of the data for the enzyme denatured in 6 M guanidine hydrochloride is shown in figure 14. A λ_o of 212 mμ was utilized and the a_o and b_o values in this solvent system were -642 deg-cm^2/decimole and approximately 0 deg-cm^2/decimole, respectively.

A summary of the optical rotatory dispersion parameters of the native and denatured enzyme is given in table II. There was a blue shift of 1 mμ of the cotton effect trough and a red shift of 4 mμ of the cotton effect peak from the accepted values for helical proteins and synthetic polypeptides. Also, the cotton effect extrema showed relatively low amplitudes when compared with values for helical polyglutamic acid ($[m']_{233}$ of 13,000-16,000° (76,77) and $[m']_{198}$ of 70,000-80,000° (76,78)).

Estimates of helicity have varied depending upon which method of calculation was utilized. Using a value of 212 mμ and 246 mμ for the denatured and native dispersion constants and 212 mμ and 254 mμ for 0 and 30% helicity of reference polyglutamic acid (74), a helical content of 21.4% was calculated. Also, employing a b_o of -123 deg-cm^2/decimole (λ_o of 212 mμ) for the native pyrophosphorylase and -630 deg-cm^2/decimole for the b_o of helical polyglutamic acid, a helical content of 20% was calculated. The excellent agreement in the calculated helical content from the above methods is not substantiated analyzing the amplitudes of the cotton effect trough and peak (77). A value of 10% or less was obtained from the amplitude of the 233 mμ trough. Standard values for the peak regions are still uncertain, but a helical content of about 16% was calculated when a suggested amplitude was utilized.

Figure 14. Moffitt-Yang plot of the denatured enzyme in the 280-500 mμ spectral region

See figure 10 for the experimental conditions.

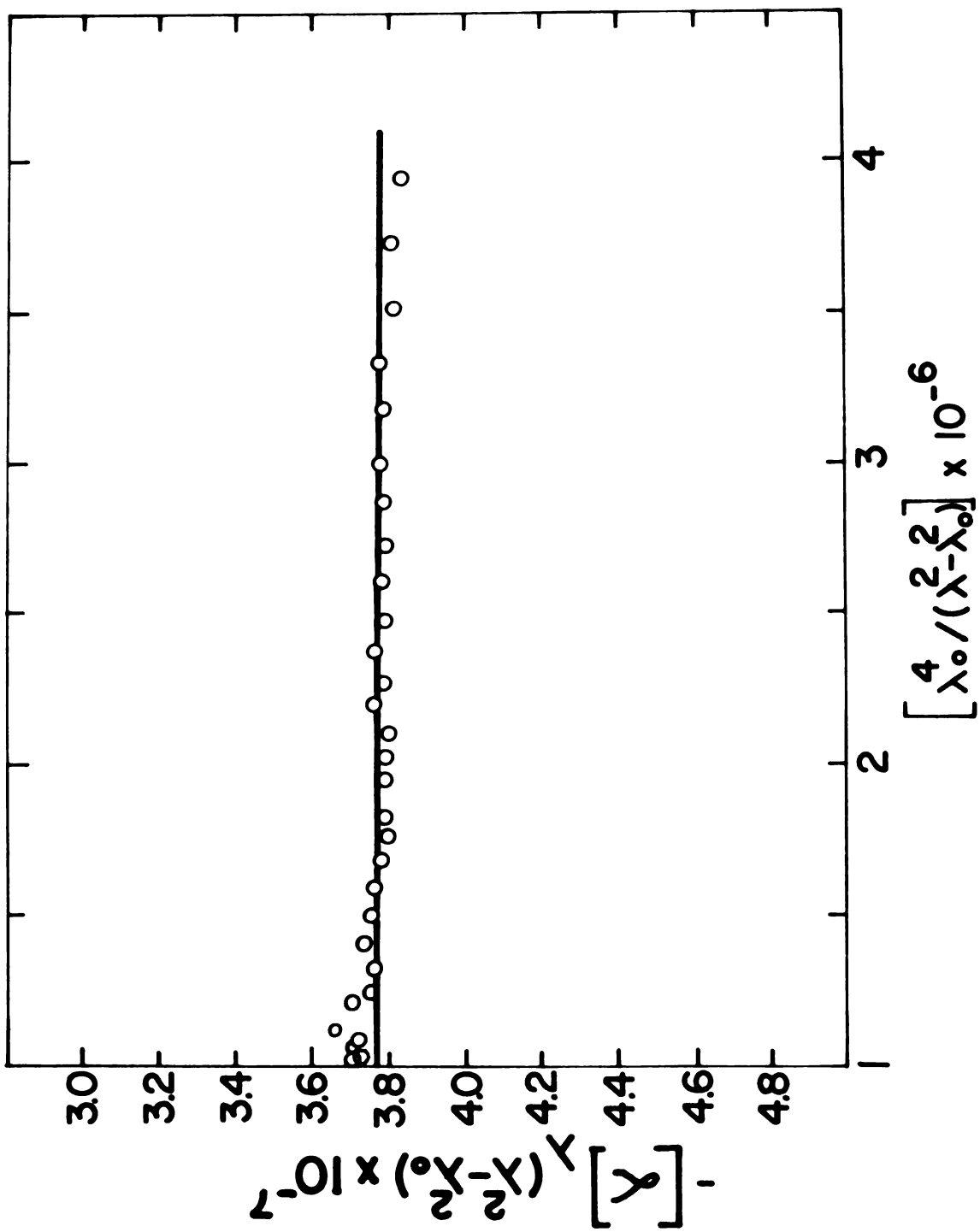

$-[\alpha]_\lambda(\lambda^2-\lambda_o^2)\times10^{-7}$
3.0
3.2
3.4
3.6
3.8
4.0
4.2
4.4
4.6
4.8
1
2
3
4
$[\lambda_o^4/(\lambda^2-\lambda_o^2)]\times10^{-6}$

TABLE II

Optical Rotatory Dispersion of UDP-glucose Pyrophosphorylase

General Parameter	Native	Denatured
$[\alpha]^{24^\circ}_{500\ m\mu}$	-67.5°	-179.0°
λ_c	246 mμ	216 mμ
λ_{trough}	232 mμ	—
$[m']_{233\ m\mu}$	-3312°	-2667°
$\lambda_{crossover}$	222 mμ	—
$[\alpha]^{24^\circ}_{maximum}$	11,494°	—
$\lambda_{maximum}$	202 mμ	—

Moffitt Parameter	Native	Denatured
λ_o	a) 212 mμ b) 216 mμ c) 220 mμ	212 mμ
a_o	a) -271[a] b) -204 c) -200	-642[a]
b_o	a) -123[a] b) - 87 c) - 76	0[a]
Spectral range	a) 320-500 mμ b) 310-500 mμ c) 285-500 mμ	310-500 mμ

[a]Units of deg-cm^2/decimole

Titration of Cysteine Residues with Ellman's Reagent

The rate of reaction of the sulfhydryl groups of a several month old preparation of the pyrophosphorylase at pH 7.5 is illustrated in figure 15. At this pH, 9-11 SH groups of the native enzyme reacted in less than 2 minutes. In 6 M guanidine hydrochloride, 14-16 SH groups immediately reacted.

In order to detect if the DTNB reactable SH groups of the pyrophosphorylase were sensitive to pH, titrations were performed in the pH 6.0 to 10.0 range. The titrations proceeded for 15 minutes before the final readings were taken. Figure 16 shows the effect of pH on the number of SH groups that react readily with DTNB. There was a gradual increase in the number of SH groups with increasing pH, and the maximum value found was 16 moles of SH per mole of protein. Other preparations have shown the same increasing trend in reactable groups, but the absolute number has been lower than that indicated in figure 16. In fact, preparations that were a year old (stored in a crystalline state in $(NH_4)_2SO_4$) yielded from 1-13 readily reactable SH groups in the native state and from 3-16 readily reactable groups in the denatured state. The various preparations did not appear to have significantly different sedimentation coefficients. At present, 16 moles of SH per mole of protein represents the maximum number found. After these are titrated in the native state, the enzyme maintained 70% of the activity of the unreacted protein.

As expected, 0 SH groups per mole of protein was found when the reduced and unreduced S-carboxymethylated pyrophosphorylase was titrated with DTNB.

Figure 15. Rate of reaction of the sulfhydryl groups of the native and denatured enzyme with Ellman's reagent

Both reactions contained 0.1 M tris-HCl buffer (pH 7.5), 20 mM EDTA, 0.2 mM DTNB, and 1.230 mg/ml of pyrophosphorylase. The denatured enzyme solution was 6 M in guanidine hydrochloride.

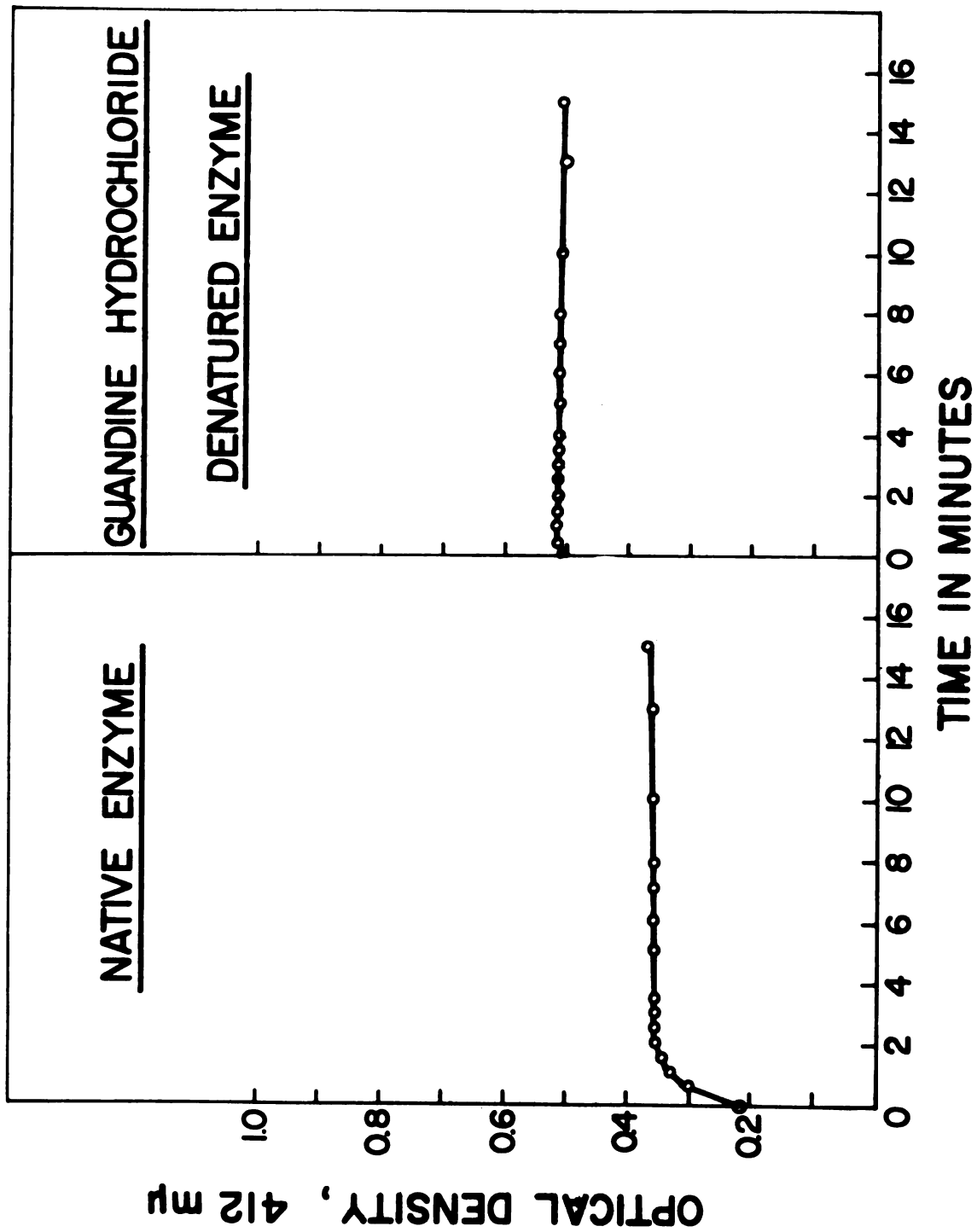
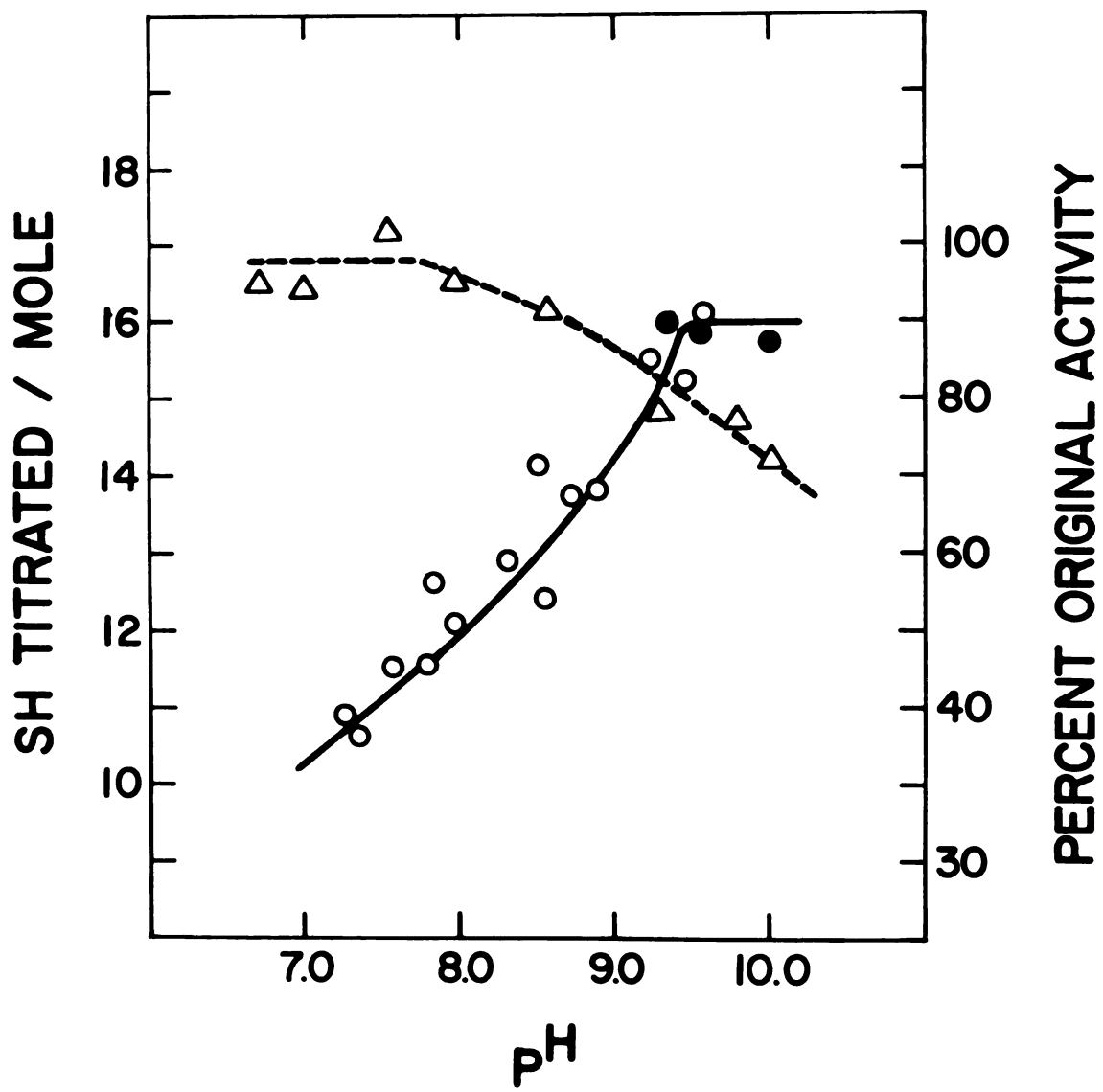

NATIVE ENZYME
GUANDINE HYDROCHLORIDE
DENATURED ENZYME
OPTICAL DENSITY, 412 mμ
1.0
0.8
0.6
0.4
0.2
0 2 4 6 8 10 12 14 16 0 2 4 6 8 10 12 14 16
TIME IN MINUTES

Figure 16. The pH dependence of the number of SH groups titrated per mole of protein

All titrations contained 0.05 M glycylglycine (-O-) or 0.05 M glycine (-●-) buffer that was 20 mM in EDTA and 0.2 mM in DTNB, and the protein concentrations were about 1.0 mg/ml in each titration. Samples were dialyzed overnight against the appropriate buffer prior to titration. The symbol -Δ- represents the percent of activity relative to an untitrated sample at that pH.


SH TITRATED / MOLE
18
16
14
12
10
PERCENT ORIGINAL ACTIVITY
100
80
60
40
30
7.0
8.0
9.0
10.0
pH

Titration of Cysteine Residues with p-Mercuribenzoate

The spectrophotometric titration of the sulfhydryl groups of a several month old preparation of the pyrophosphorylase with PMB is depicted in figure 17. The end point in this titration corresponded to 8 sulfhydryl groups that were available for mercaptide formation per mole of protein. Up to 12 sulfhydryl groups per mole of enzyme have been reacted when the buffer was 0.05 M pyrophosphate (pH 7.5) instead of 0.05 M phosphate buffer.

Amino Acid Composition and Partial Specific Volume

The amino acid composition of the pyrophosphorylase is given in table III. Cysteine was determined from a DTNB titration of the denatured enzyme. Conflicting values for the analysis of cysteine based on the S-carboxymethylation, PMB, and DTNB methods necessitated the choice of one of these techniques. The DTNB method, however, suffers from the fact that only cysteine and not total half-cystine is determined and therefore the value found must be taken to be a minimum. Moreover, chromatographic analysis of the hydrolyzed, reduced and S-carboxymethylated protein has indicated a higher value for the half-cystine content. However, the assessment of half-cystine has been complicated by the unexpected finding of 0 moles of S-carboxymethylcysteine for the hydrolyzed, unreduced protein.

Twenty-one residues of tryptophan per mole of pyrophosphorylase is presently the best estimate. This was determined by the Goodwin and Morton spectrophotometric method and was in relatively good

Figure 17. Titration of the sulfhydryl groups of the enzyme with PMB

The PMB concentration was 7.32×10^{-4}M and the pyrophosphorylase was 8.68×10^{-6}M in 0.05 M phosphate buffer (pH 7.0) that was 5 M in urea. Additions of the PMB were made to 1.0 ml of the protein solution.

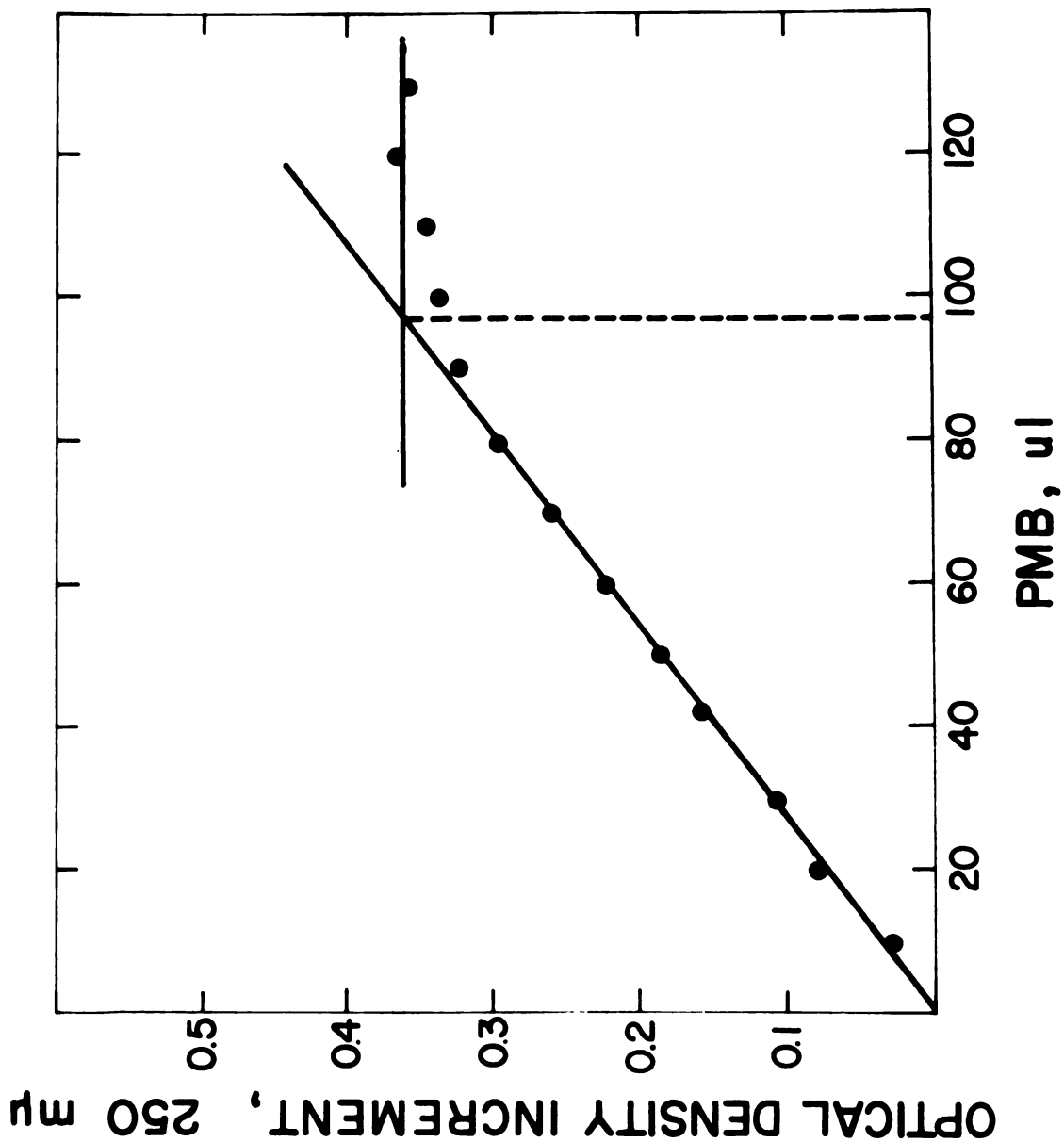

OPTICAL DENSITY INCREMENT, 250 mμ
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
120
PMB, μl

TABLE III

Amino Acid Composition of UDP-glucose Pyrophosphorylase

Amino Acid	Time of hydrolysis			Average or Extrapolated Values	Residues per Mole[d]
	20 hrs	42 hrs	72 hrs		
	Mole ratio[a]				
Asp . . .	1.060	1.080	1.084	1.076	502
Thr . . .	.473	.491	.445	.520	243
Ser . . .	.616	.591	.484	.680	318
Glu . . .	.972	1.008	.961	.980	458
Pro . . .	.452	.491	.433	.459	214
Gly . . .	.613	.633	.610	.619	289
Ala . . .	.364	.391	.363	.373	174
Val . . .	.668	.733	.687	.696	325
Met . . .	.156	.166	.145	.156	73
Iso . . .	.504	.533	.539	.525	245
Leu . . .	1.000	1.000	1.000	1.000	467
Tyr . . .	.257	.225	.239	.240	112
Phe . . .	.389	.408	.397	.398	186
Lys . . .	.720	.708	.714	.714	333
His . . .	.194	.216	.191	.200	93
Arg . . .	.375	.400	.372	.382	178
Cys . . .				.034[b]	16
Try . . .				.045[c]	21

[a]Relative to Leucine taken as 1.000

[b]Minimum value determined from a DTNB titration of the denatured enzyme.

[c]Determined by the Spectrophotometric technique (60).

[d]The molecular weight was taken to be 480,000.

agreement with the chemical method of Spies and Chambers which yielded 26 residues of tryptophan per mole of enzyme. The spectrophotometric method was used because it afforded an independent check on the amount of tyrosine obtained from the amino acid analyzer. One hundred and sixteen residues of tyrosine per mole of enzyme determined by the spectrophotometric method was in good agreement with 112 residues determined by the amino acid analyzer.

The partial specific volume of the pyrophosphorylase, calculated according to the method of McMeeken and Marshall (79) is shown in table IV. The partial specific volumes of the amino acids were obtained from Cohn and Edsall (80). Little difference was noted in $\bar{v}$ when estimates of glutamine and asparagine were used in the calculation. A value of 0.739 ml/gm was calculated for the partial specific volume of the pyrophosphorylase.

Subunit Electrophoresis on Cellulose Acetate Strips

Because the native enzyme did not yield a discrete band under all conditions, cellulose acetate electrophoresis was performed in the presence of a dissociating concentration of urea. Figure 18 shows the migration toward the anode of the dissociated enzyme at pH 11.0. In the denatured state the enzyme migrated as a discrete band.

Isoelectric Focusing of the Subunits

In contrast to other experiments in 6 M urea, isoelectric fractionation revealed that the pyrophosphorylase was heterogeneous. Figure 19 shows the electrofocusing optical density

TABLE IV

Calculation of the Partial Specific Volume
From the Amino Acid Analysis

Amino Acid Residue	Residues per Mole	% by Weight of Residue	Specific Volume of Residue[a]	% by Volume of Residue
Asp	502	12.15	0.59	7.17
Thr	243	5.16	0.70	3.61
Ser	318	[illegible].82	0.63	3.67
Glu	458	12.40	0.66	8.18
Pro	214	4.38	0.76	3.33
Gly	289	3.49	0.64	2.23
Ala	174	2.61	0.74	1.93
Val	325	6.73	0.86	5.83
Met	73	2.00	0.75	1.50
Iso	245	5.83	0.90	5.25
Leu	467	11.10	0.90	9.99
Tyr	112	3.83	0.71	2.72
Phe	186	5.73	0.77	4.41
Lys	333	8.97	0.82	7.36
His	93	2.69	0.67	1.80
Arg	178	5.84	0.70	4.09
Cys	15	0.38	0.63	0.24
Try	21	0.84	0.74	0.62
TOTAL	---	$\sum_i W_i = 99.99$	----	$\sum_i W_i V_i = 73.93$

$$\bar{v} = \sum_i W_i V_i / \sum_i W_i = .739_4 \text{ ml/gm}$$

[a]Taken from Cohn and Edsall (80).

Figure 18. Electrophoresis of the subunits on cellulose acetate strips

Electrophoresis of the subunits (35 μg) was performed for 3 hours at 70 volts in 0.05 M bicarbonate buffer (pH 11.0) that was 5 M in urea. The protein was stained with 0.2% Ponseau S in 3% TCA. The temperature was 4°C.

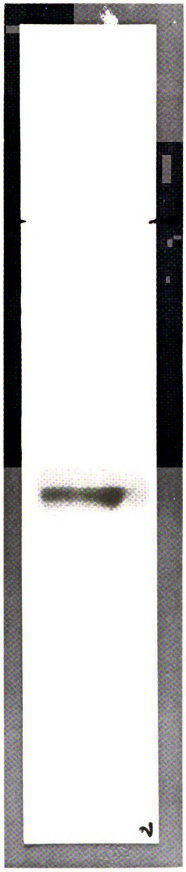
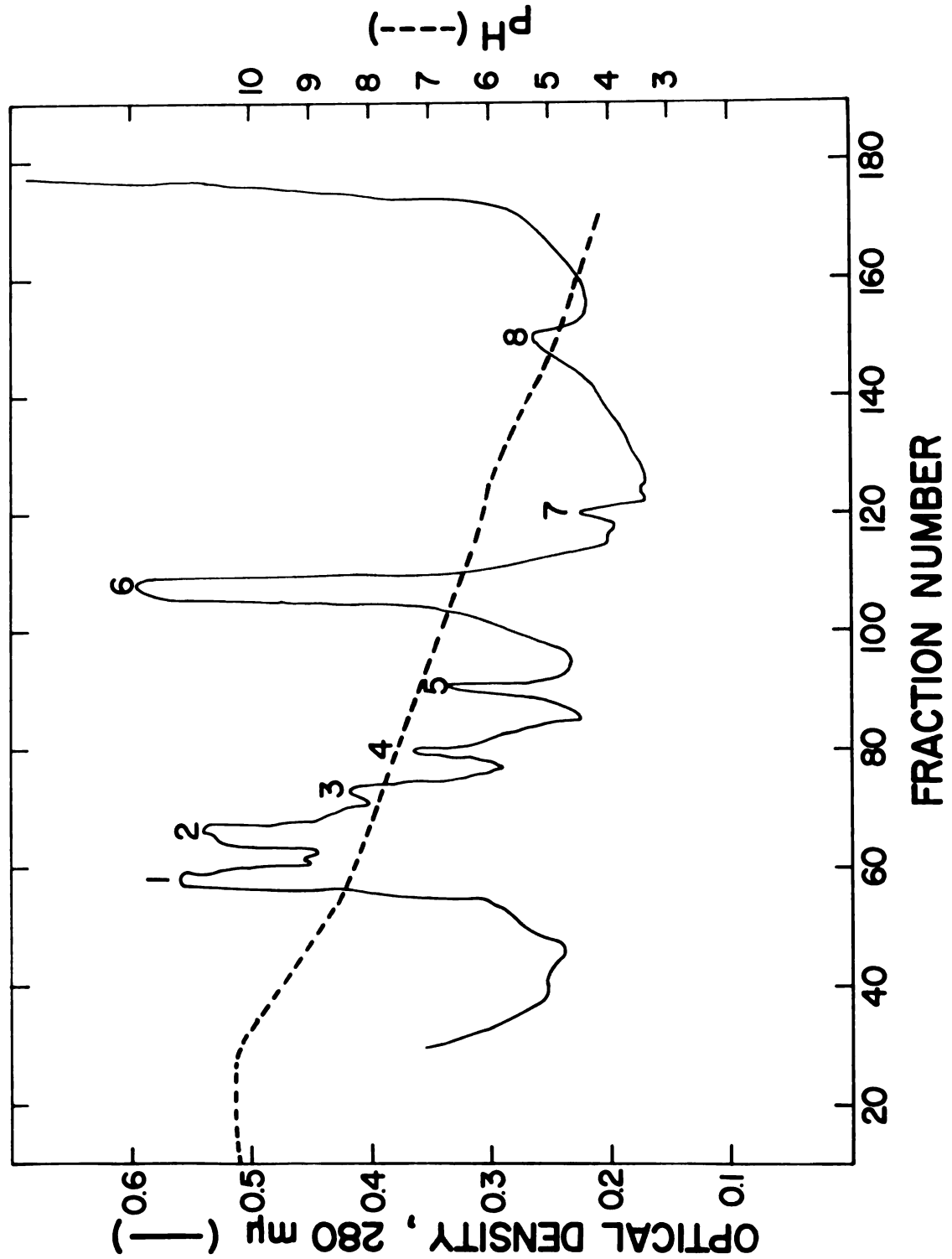

origin
2

Figure 19. Isoelectric focusing of the subunits

Isoelectric focusing of 6 mg of the subunits was performed in 6 M urea as described in the Materials and Methods section. The line (——) indicates absorbance at 280 mμ and the line (---) indicates the pH gradient.


OPTICAL DENSITY, 280 mμ (——)
0.6
0.5
0.4
0.3
0.2
0.1
pH (----)
10
9
8
7
6
5
4
3
20
40
60
80
100
120
140
160
180
FRACTION NUMBER
1
2
3
4
5
6
7
8

and pH profiles of the subunits. At least eight fractions could be detected.

An attempt was made to analyze each fraction on the basis of its tendency to reassociate to form active enzyme. The following reassociation procedure was used. Fifty µl of each fraction was diluted at 0°C to 1.0 ml with 0.01 glycylglycine buffer (pH 7.5) which was 0.1 M in ammonium sulfate and 0.1 M in mercaptoethanol. The solution was then incubated for 1 1/2 hours at 16°C and analyzed for the presence of pyrophosphorylase activity. Using this procedure, only peaks 1, 2, 3, and 4 showed activity, and the activity was approximately in the same ratio as the peak heights. Fraction 6 which was present in the largest amount was devoid of activity. When this fraction was mixed with other fractions in the reassociation experiments, no additional recovery was noted. Further characterization of this fraction indicated that protein was not present. At the present time, the nature of this fraction is unknown.

Trypsin Digestion and Peptide Mapping

Although the length of time for trypsin digestion of the heat denatured pyrophosphorylase apparently varied from preparation to preparation, the peptide maps were very similar. Best results were obtained with the procedure adopted, but no technique completely eliminated all "core" material. The likelihood of chymotryptic contamination was essentially ruled out since the results were identical whether trypsin (Worthington) was an L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone treated or untreated sample.

A fingerprint of 2.5 mg of the tryptic digest is shown in figure 20. A summary of the peptide staining pattern of several maps is given in table V. The total number of ninhydrin-positive peptides varied from 52-62 and arginine-containing peptides varied from 14-19, while only one tryptophan-containing peptide was seen.

Cyanogen Bromide Cleavage

The polyacrylamide pattern of a cyanogen bromide digest of the RSCM-pyrophosphorylase is shown in figure 21. Ten to eleven fragments could be detected from various preparations.

Gel electrophoresis of the subunits showed one diffuse band. However, upon total S-carboxymethylation of the protein, a very lightly staining second band was detected on polyacrylamide gel columns. The mobility and amount of this fraction has varied; but in all cases, it represented only a small percent of the principal component, and the cyanogen bromide fragmentation pattern appeared to be very similar. The origin of the minor band is as yet unknown, but may be due to the carboxymethylation procedure.

Reassociation of the Subunits

When the dissociated enzyme was diluted directly into the assay cuvette, the activity increased with time from zero. This fact was indicative of a time dependent formation of active from inactive enzyme. Due to the low recovery of active enzyme by this procedure, an alternate reassociation method was next used. When

Figure 20. Fingerprint of a trypsin digest of the enzyme

The sample (2.5 mg) was applied to the paper in dilute ammonia water and subjected to chromatography in the first direction and electrophoresis in the second as described in the methods section. Lightly staining ninhydrin-positive spots are indicated by dashed lines.

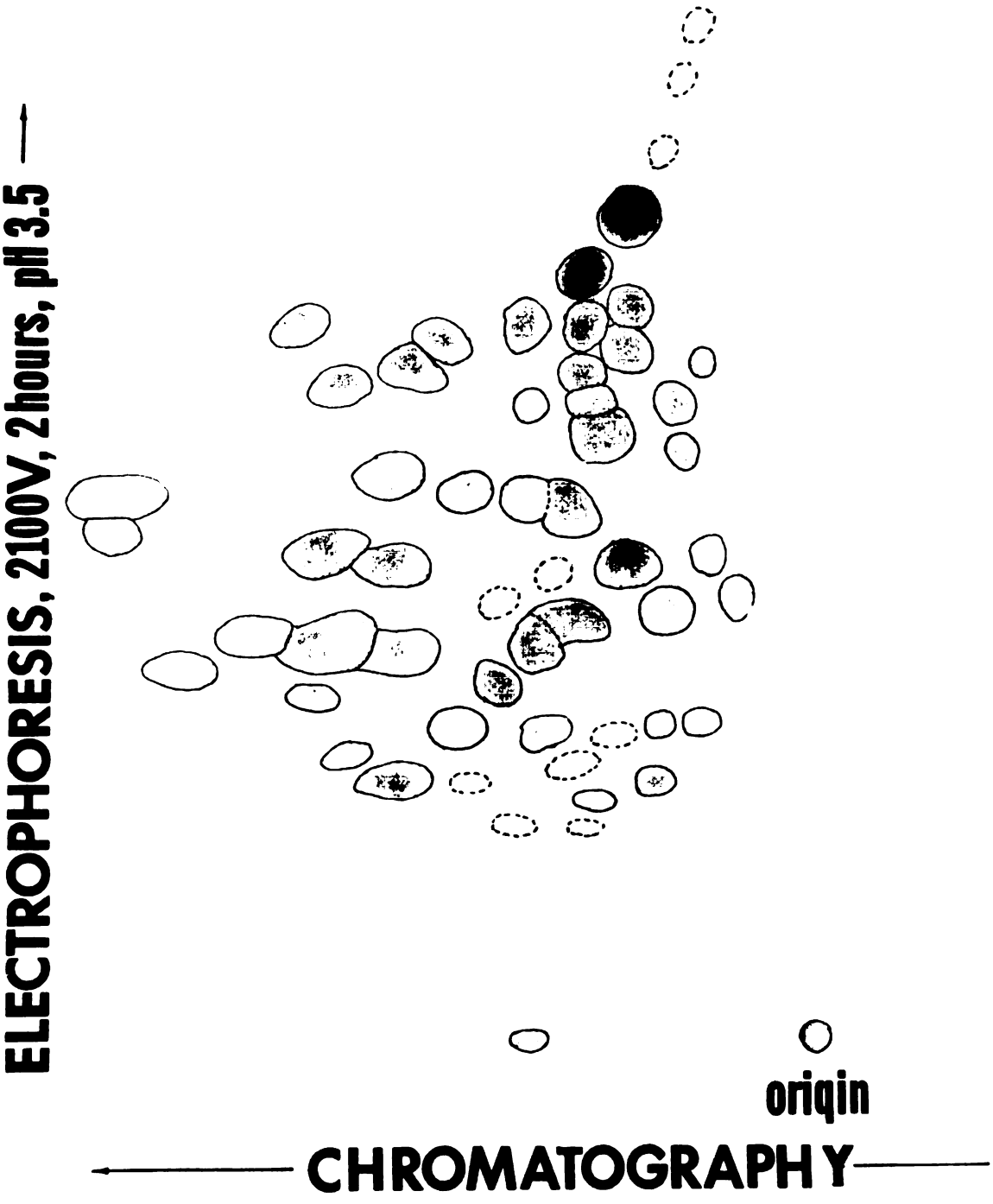

ELECTROPHORESIS, 2100V, 2hours, pH 3.5
oriqin
CHROMATOGRAPH Y

TABLE V

A Summary of the Tryptic Digests of

UDP-glucose Pyrophosphorylase

Amino Acids	Residues/mole[a]	Number of Peptides	
		Predicted[b]	Found
Lysine plus Arginine[c]	511	64	52-62
Arginine[d]	178	22	14-19
Tryptophan[e]	20-26[f]	2-3	1

[a]The molecular weight taken as 480,000.

[b]Based on 8-identical subunits.

[c]Ninhydrin-positive peptides.

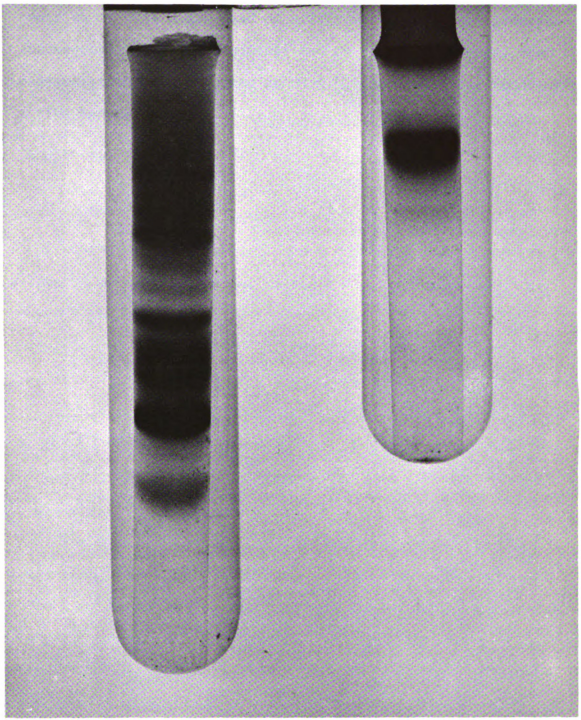
[d]Sakaguchi-positive peptides (67).

[e]Ehrlich-positive peptides (66).

[f]See amino acid analysis section.

Figure 21. Polyacrylamide patterns of a reduced and S-carboxymethylated sample of the enzyme before and after cleavage with cyanogen bromide

The left pattern represents 250 μg of the sample cleaved with cyanogen bromide and the right pattern represents 200 μg of an uncleaved sample. The cleaved sample was developed for 1 hour on a 7.5% gel column while the uncleaved sample was developed for 1 1/2 hours on a 5% gel column. Both samples were developed at a constant amperage of 5 ma/tube and after staining for 1 hour with napthol blue black (1% in 7% HoAc), they were destained electrophoretically.



compared with the undissociated control, 35-75% of the pyrophosphorylase activity was recovered after three hours of incubation in this reassociation mixture at 15°C and pH 6.4. As expected, addition of 0.04 M NAD^+ had no effect on the recovery process. Furthermore, 0.01 M UDP-glucose had no effect. However, when the reassociation was performed at pH 5.5, there was less recovery of active enzyme.

DISCUSSION

UDP-glucose pyrophosphorylase from calf liver is a relatively large enzyme. Unequivocal molecular weight determinations are complicated by this fact and also by the presence of small quantities of more rapidly sedimenting components. Preliminary analysis of these more rapidly sedimenting components in the ultracentrifuge indicated that the pyrophosphorylase might be a polymerizing system in which the rate of association is slow. This hypothesis has received substantiation by Levine et al. (81). They observed that the same distribution of material that was seen in the ultracentrifuge could also be seen on polyacrylamide gel columns and that each component contained pyrophosphorylase activity. Furthermore, the components which were separable by sucrose density gradient ultracentrifugation could be re-equilibrated to yield all the components of the original system.

Hydrodynamic studies of the principal component in the analytical ultracentrifuge yielded a molecular weight of about 480,000 as determined by sedimentation and diffusion techniques. The results were identical whether or not mercaptoethanol was included in the solvent system. This fact indicated that the mechanism of association is presumably not by disulfide formation.

The use of the low speed sedimentation equilibrium technique for the determination of the molecular weight of the pyrophosphorylase presented serious problems in obtaining unequivocal results.

One limitation of the technique was the low speed (3,000 rpm) at which the ultracentrifuge had to be used. In addition to this limitation, the low speed technique is abnormally sensitive to small amounts of high molecular weight material; and since molecular weight averages are determined, serious reservations must be placed in the analysis under this conditions.

Assuming that the higher molecular weight material is a dimer of the 480,000 molecular weight enzyme and that it represents about 4% by weight of the total material present, it may be calculated that the weight-average and the z-average molecular weights should be 495,600 and 507,900, respectively. The calculated z-average molecular weight is substantially lower than was observed and presumably indicated an exaggeration of the molecular weight due to a species outside the limitation of the low speed technique.

It must therefore be concluded that, due to the "purification" of material during the sedimentation experiments, the sedimentation and diffusion techniques are more representative of the true molecular weight of the pyrophosphorylase molecule. Similarly, the average molecular weights as determine from sedimentation equilibrium must be taken as a maximum, but presumably indicated a molecular weight range for the enzyme.

Subunits of the pyrophosphorylase were produced in 5 M urea or 6 M guanidine hydrochloride. The highly unfolded subunits which were produced in 6 M guanidine hydrochloride appeared to be homogeneous in size and to have a molecular weight of about

68,000. Confidence may be placed in the subunit analysis due to the good agreement between the sedimentation-diffusion and sedimentation equilibrium methods. Dissociation was obtained in the absence of reducing agents, again indicating no interchain disulfide bonds. Using 480,000 for the native molecular weight and 68,000 for the subunit molecular weight, seven subunits per mole were calculated.

Electron micrographs have revealed that the pyrophosphorylase is tetrameric (81). The subparticle molecular weight was calculated to be 53,000, a figure which, when combined with the native molecular weight would indicate an octomer model.

In addition to circumstances which have precluded the calculation of an unequivocal molecular weight of the native enzyme, there are also factors which contribute to the uncertainty in estimating absolutely the molecular weight of the subunits. Corrections for the partial specific volume of proteins ($\bar{v}$) in high concentrations of guanidine hydrochloride are still debatable (82-87). Some sources have indicated that $\bar{v}$ should be lowered by 0.01 - 0.02 ml/gm under these conditions. Furthermore, others have indicated that $\bar{v}$ should also be lowered somewhat for experiments conducted, as these were, at low temperatures (44, 88-90). Both of these corrections if applied would tend to make the bouyancy correction factor ($1-\bar{v}p$) higher and the molecular weight correspondingly lower. If the value of 0.72 ml/gm were used for the partial specific volume in the subunit analysis at low temperatures, a molecular weight of about 60,000 would be obtained and this would be in excellent agreement with an octomer model. In view of the

present hydrodynamic data and the subparticle structure that has been revealed from the electron micrographs, the conclusion is supported that the pyrophosphorylase must be octomeric in nature and that the subunits are similar, if not identical, in mass.

Optical rotatory dispersion measurements were obtained in the 190-500 mμ region for the native enzyme and in the 220-500 mμ region for the denatured enzyme. The more levorotatory behavior of the denatured state was expected on the basis of the helix to coil transitions of other systems (74). The dispersion constant of 246 mμ for the native enzyme is in the 230-280 mμ range which has been found for most globular proteins studied to date. In addition to this, the dispersion constant for the denatured protein of 216 mμ is in the 210-230 mμ range which has been found for many denatured proteins and is only slightly higher than 212 mμ, a value that has been accepted for randomly coiled synthetic polypeptides.

The optical rotatory dispersion data for the pyrophosphorylase closely resemble those of a group of proteins studied by Jirgensons (91), and the comparison is shown in table VI. All the proteins appeared to have low negative b_o values (λ_o of 220 mμ) and similar cotton effect characteristics. Beta structure was suspected in some cases.

Table VII summarizes some of the characteristics of various secondary structures (92). The possibility of β-structure is further supported by the blue shift of the cotton effect trough and the red shift of the cotton effect maximum. However, the

TABLE VI

Some Optical Rotatory Dispersion Parameters of UDP-glucose Pyrophosphorylase and a Group of Proteins

Protein	$-b_o$[a]	$-[\alpha]_{233}$	$-[\alpha]_{228}$	$[\alpha]_{maximum}$	Maximum at (mμ)
Deoxyribonuclease[b]	74[c]	4,000°	4,300°	17,500°	202
Glucose Oxidase[b]	57	3,600	3,900	22,000	202
α-Chymotrypsin[b]	23	3,400	3,300	13,500	201
α_1Acid Glycoprotein[b]	20	3,300	2,800	12,000	205
UDP-glucose Pyrophosphorylase	76	3,900	3,400	11,500	202

[a] λ_o of 220 mμ.

[b] Values taken from Jirgensons (91).

[c] Units of deg-cm^2/decimole.

TABLE VII

Cotton Effects of L-Polypeptides and
UDP-glucose Pyrophosphorylase

Confirmation or Protein	trough, mμ	crossover, mμ	peak, mμ
α Helix[a]	232-233	~224[b]	198-199
	182-184	~190	
β-form[a]	229-230	~220	205
	~190	~196	
Coil[a]	238 (small)	198	228 (small)
	204-205		189
Poly-L-proline[a]	210	203	194
UDP-glucose Pyrophosphorylase	232	222	202

[a]Values taken from Yang (92).

[b]With a shoulder near 215 mμ.

212 mμ shoulder presumably indicates the presence of some helical regions. Thus, the present hypothesis is that the pyrophosphorylase may contain α-helical, random coil, and β-regions. Substantiating evidence from infrared spectroscopy would be informative.

In view of the possible presence of structures other than the α-helix and random coil, semiquantitative estimates of helicity must be viewed with some reservation. However, all facts indicated that the enzyme is a protein of relatively low helical content. Discrepancies in the calculated helical content were noted using different methods of calculation and this fact might be a further indication of the presence of structures other than the α-helix and random coil.

The denatured pyrophosphorylase exhibited the typical optical rotatory dispersion spectrum of randomly coiled polypeptides and proteins. Furthermore, a b_o of 0 (λ_o of 212 mμ) is interpreted to indicated the lack of helical regions.

Titration of the sulfhydryl groups of the pyrophosphorylase with DTNB and PMB has resulted in some interesting findings. The rapidity of the reaction of the cysteine residues of the native protein at a given pH with DTNB indicated that some of these groups were quite available to solvent, and presumably they are on the surface of the protein or in a cleft. The gradual increase in the number of titratable SH groups with this reagent suggested a pH-dependent conformational change that resulted in an opening of the protein structure at high pH to make more SH groups available for titration. The magnitude of this conformational change must be small because little change was noted in the

sedimentation coefficient in this pH range. The observed difference in the absolute number of groups titrated with different preparations might indicate that some were more highly oxidized than others. There appeared to be a decrease in the number of titratable SH groups with increasing age of the preparation. However, the maximum number of 16 moles of SH per mole of protein was found for some preparations that were a year old. The fact that 70% of the control activity was maintained after 16 cysteine groups were titrated with DTNB indicates that the sulfhydryl groups are presumably not essential for activity.

The value for the cysteine content which was determined on a limited number of preparations was lower when PMB was used as the titrant than when DTNB was used. Two residues per subunit were found with DTNB while 1.0 to 1.5 residues per subunit was found with PMB. The reason for the lower value with PMB is as yet uncertain, but may reflect the possible oxidation problem that has been mentioned for the DTNB titrations or perhaps neighboring group influence on the reactability of the various sulfhydryl groups. It is clear that more preparations need to be titrated with PMB.

Nothing abnormal was noted in the amino acid composition of the pyrophosphorylase. As expected from other proteins, glutamic and aspartic acids were present in the largest amount and cysteine and tryptophan, followed by methionine, were present in the lowest amounts. There was no detectable carbohydrate material bound to the purified protein. The calculated partial specific volume of 0.739 ml/gm is also to be expected for an average globular protein.

Since the analytical ultracentrifuge data indicated that the subunits of the enzyme were similar in mass, if not identical, experiments were performed to assess the further likelihood that they are chemically identical. Electrophoresis on cellulose acetate strips indicated that the subunits were similar, if not identical, in charge. Furthermore, polyacrylamide gel electrophoresis, a method which is dependent on both charge and mass, indicated that the subunits were similar in these respects. The unequivocal interpretation of a single species suffers from the fact that polyacrylamide patterns of the intact protein which was in the native state, denatured, or derivatized always yielded material at the origin and in the sample gel.

Fingerprinting experiments were also performed to assess the likelihood of subunit homology. From the known specificity of trypsin for lysine and arginine residues and from the number of these residues present, one would expect 512 peptides if there were no repeating sequences in the primary structure. Fifty-two to sixty-two ninhydrin spots were observed. Thus, one-eighth to one-tenth the number of ninhydrin spots that would have been expected if there were no repeating sequence was observed. This result is consistent with eight to ten homologous subunits and is in good agreement with the prediction of eight subunits by other methods. The number of observable arginine-containing spots varied somewhat from preparation to preparation, but the higher values are consistent with nine to ten homologous subunits. A lower than expected number of tryptophan-containing peptides was observed. Several explanations may account for this. First, the

two methods used for tryptophan analysis have been shown to be unreliable in some instances (93). Other explanations might be that the two or three residues of tryptophan were located on the same peptide or free tryptophan, or a very small peptide containing tryptophan, occurred because of an unusual sequence and was not detected. Still a further possibility might be that not all the tryptophan-containing peptides gave a positive test with Ehrlich's reagent. Due to the fact that a lower than expected number of tryptophan spots was observed and the number of arginine-containing spots varied, the conclusion of eight to ten homologous subunits must be viewed cautiously. However, the results do not negate this possibility.

Cyanogen bromide cleavage yielded 10-11 bands which were observed on polyacrylamide gel columns. From the number of fragments, the specificity of cyanogen bromide for methionine peptides, and the number of these residues per mole, eight extremely homologous, if not identical, subunits were again postulated. For this conclusion, complete cleavage of the methionine bonds and complete reduction and S-carboxymethylation of all disulfide linkages was assumed. This fact has been demonstrated by Steers et al. for β-galactosidase (69) using the same cleavage system. When monitored on polyacrylamide gel columns, the presence of a second faint band for the RSCM-pyrophosphorylase was found. In light of the single diffuse band found for the underivatized subunits, this is not yet understood.

Electrofocusing experiments, on the other hand, indicated the enzyme was heterogeneous in 6 M urea. At least eight fractions could be detected. These results are compatible with: (1) the presence of differently charged subunits which were present in varying amounts, (2) nonequivalent binding of small ions to a single identical subunit, or (3) modification of the protein due possibly to proteolytic digestion during fractionation. It appeared from the electrofocusing experiments that at least four fractions have the competence to form active pyrophosphorylase molecules with approximately equal specific activities. These preliminary results do not in themselves rule out the possibility of chemically identical subunits.

Reassociation experiments demonstrated that active enzyme could be reconstituted from a sample denatured in 8 M urea. There was a strong pH dependence on the reassociation process, and presumably the higher recovery at more alkaline pH can be correlated with the alkaline pH stability range of the protein (28). The high recovery in these preliminary experiments was encouraging because of the prospects for further optimizing the yield of active enzyme. Furthermore, the high recovery of the pyrophosphorylase activity demonstrated an important biochemical principle, which is that the information necessary for the proper tertiary structure of some proteins resides in the linear sequence of their component amino acids.

SUMMARY

Uridine diphosphate glucose pyrophosphorylase which was recrystallized as many as six times exhibited molecular polydispersity. Analysis in the ultracentrifuge pointed to the presence of polymers. The molecular weight of the principal component was estimated from sedimentation and diffusion analysis and found to be about 480,000. This molecular weight was roughly substantiated by low speed sedimentation equilibrium analysis.

In the solvent system containing 6 M guanidine hydrochloride and 0.1 M mercaptoethanol, pyrophosphorylase subunits were produced that had a molecular weight of about 68,000 with a value of 0.74 ml/gm for $\bar{v}$ (60,000 with a $\bar{v}$ of 0.72 ml/gm).

The pyrophosphorylase exhibited a typical aromatic amino acid spectrum, and the extinction coefficient was determined with concomitant dry-weighing experiments. Optical rotatory dispersion studies indicated a low helical content with the possibility of some β-structure regions.

Chemical studies demonstrated a typical protein amino acid content without bound carbohydrate material. The total number of cysteine groups which would readily react with Ellman's reagent was pH dependent. Although the number of SH groups per mole appeared to depend on the preparation chosen and possibly its age, a maximum of about 2 per subunit was obtained. Other

methods have indicated the possibility of a larger number for the total half-cystine analysis.

Polyacrylamide and cellulose acetate electrophoresis, and cyanogen bromide digestions were consistent with subunit homology, if not identity. Certain aspects of the fingerprinting experiments also supported this hypothesis, but due to the variance in the arginine-containing spots and the observation of a lower than expected number of tryptophan-containing spots, the results are less conclusive. Preliminary electrofocusing experiments indicated the presence of different electrophoretic species under denaturing conditions.

The subunits produced in urea appeared to be catalytically inactive, but active pyrophosphorylase was able to be reconstituted with a high overall yield.

REFERENCES

1. Newfeld, E. F., and Hassid, W. Z., Advan. Carbohydrate Chem., 18, 309 (1963).

2. Cabib, E., Ann. Rev. Biochem., 32, 554 (1963).

3. Ginsburg, V., Advan. Enzymol., 26, 35 (1964).

4. Neufeld, E. F., and Ginsburg, V., Ann. Rev. Biochem., 34, 601 (1965).

5. Caputto, R., Leloir, L. F., Trucco, R. E., Cardini, C. E., and Paladini, A. C., J. Biol. Chem., 179, 497 (1949).

6. Caputto, R., Leloir, L. F., Cardini, C. E., and Paladini, A. C., J. Biol. Chem., 184, 333 (1950).

7. Kalckar, H. M., and Cutolo, E., II^{e} Congr. Intern. de Biochemie, Paris, 260 (1952).

8. Munch-Petersen, A., Kalckar, H. M., Cutolo E., and Smith, E. E. B., Nature, 172, 1036 (1953).

9. Kornberg, A., J. Biol. Chem., 176, 1475 (1948).

10. Kornberg, A., J. Biol. Chem., 182, 779 (1950).

11. Schrecker, A. W., and Kornberg, A., J. Biol. Chem., 182, 795 (1950).

12. Munch-Peterson, A., Acta Chem. Scand., 9, 1523 (1955).

13. Kalckar, H. M., Braganca, B., and Munch-Peterson, A., Nature, 172, 1038 (1953).

14. Smith, E. E. B., and Mills, G. T., Biochim. Biophys. Acta, 13, 386 (1954).

15. Smith, E. E. B., and Mills, G. T., Biochim. Biophys. Acta, 18, 152 (1955).

16. Burma, D. P., and Mortimer, D. C., Arch. Biochem. and Biophys., 62, 16 (1956).

17. Neufeld, E. F., Ginsburg, V., Putnam, E. W., Fanshier, D., and Hassid, W. Z., Arch. Biochem. Biophys., 69, 602 (1957).

18. Ginsburg, V., J. Biol. Chem., 232, 55 (1958).

19. Ganguli, N. C., J. Biol. Chem., 232, 337 (1958).

20. Turner, D. H., and Turner, J. F., Biochem. J., 69, 448 (1958).

21. Villar-Palasi, C., and Larner, J., Arch. Biochem. Biophys., 86, 61 (1960).

22. Basu, D. K., and Bachhawat, B. K., J. Neurochem., 7, 174 (1961).

23. Anderson, E. P., Kalckar, H. M., Kurahashi, K., and Isselbacher, K. J., J. Lab. Clin. Med., 50, 469 (1957).

24. Seits, I. F., Luganova, I. S., and Vladimivova, A. D., Biokhimiya, 28, 295 (1963).

25. Blinov, M. N., Vopr. Med. Khim., 10, 163 (1964).

26. Halprin, K. M., and Ohkawara, A., J. Invest. Dermatol., 46, 43 (1966).

27. Kamogawa, A., and Kurahashi, K., J. Biochem., 57, 758 (1965).

28. Albrecht, G. J., Bass, S. T., Seifert, L. L., and Hansen, R. G., J. Biol. Chem., 241, 2968 (1966)

29. DeFazio, K., Master of Science thesis, Michigan State University, 1968.

30. Knop, J., Master of Science thesis, Michigan State University, 1969.

31. Bass, S. T., and Hansen, R. G., in preparation.

32. Gillett, T. A., and Hansen, R. G., in preparation.

33. Isselbacher, K. J., J. Biol. Chem., 232, 429 (1958).

34. Isselbacher, K. J., Science, 127, 28 (1958).

35. Gitzelman, R., personal communication to R. G. Hansen, Sept. 1968.

36. Abraham, H. D., and Howell, R. R., J. Biol. Chem., 244, 545 (1969).

37. Ting, W. K., and Hansen, R. G., Proc. Soc. Exptl. Biol. Med., 127, 960 (1968).

38. Gillett, T. A., Levine, S. L., and Hansen, R. G., in preparation.

39. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem., 193, 265 (1951).

40. Warburg, O., and Christian, W., Biochem. Z., 310, 384 (1941).

41. Dubois, K. A., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F., Anal. Chem., 28, 350 (1956).

42. Schachman, H. K., Methods Enzymol., 4, 2 (1957).

43. Van Holde, K. E., and Baldwin, R. L., J. Phys. Chem., 62, 734 (1958).

44. Svedberg, T., and Pedersen, K. O., The Ultracentrifuge, London, Oxford University, Clarendon Press, 1940.

45. Kawahara, K., and Tanford, C., J. Biol. Chem., 241, 3288 (1966).

46. Steinmetz, M. A., and Deal, W. C., Jr., Biochemistry, 5, 1399 (1966).

47. Dorsey, N. E., Properties of Ordinary Water-Substance, Reinhold, New York, 280 (1940).

48. Hooker, T. M., Jr., Doctor of Philosophy thesis, Duke University, 1966.

49. Tanford, C., Kawahara, K., Lapanje, S., Hooker, T. M., Jr., Zarlengo, M. H., Salahuddin, A., Aune, K., and Takagi, T., J. Am. Chem. Soc., 89, 5023 (1967).

50. Moffitt, W., and Yang, J. T., Proc. Natl. Acad. Sci. U. S., 42, 596 (1956).

51. Ellman, G. L., Arch. Biochem. Biophys., 82, 70 (1959).

52. Kemp, R. G., and Forest, P. B., Biochemistry, 7, 2596 (1968).

53. Boyer, P. D., J. Am. Chem. Soc., 76, 4331 (1954).

54. Benesch, R., and Benesch, R. A., Methods Biochem. Anal., 10, 60 (1962).

55. Moore, S., and Stein, W. H., in Methods in Enzymology, Colowick, S. P., and Kaplan, N. O., editors, Academic Press Inc., New York, 819 (1963).

56. Piez, K. A., and Morris, L., Anal. Biochem., 1, 187 (1960).

57. Spackman, D. H., Stein, W. H., and Moore, S., Anal. Chem., 30, 1190 (1958).

58. Spies, J. R., and Chambers, D. C., Anal. Chem., 20, 30 (1948).

59. Spies, J. R., and Chambers, D. C., Anal. Chem., 10, 1249 (1949).

60. Goodwin, T. W., and Morton, R. A., Biochem. J., 40. 628 (1946).

61. Crestfield, A. M., Moore, S., and Stein, W. H., J. Biol. Chem., 238, 622 (1963).

62. Svensson, H., Arch. Biochem. Biophys., Suppl. 1, 132 (1962).

63. Vesterberg, O., and Svensson, H., Acta Chem. Scand., 20, 820 (1966).

64. Ingram, V. M., Biochim. Biophys. Acta, 28, 539 (1958).

65. Katz, A. M., Dreyer, W. J., and Anfinsen, C. B., J. Biol. Chem., 234, 2897 (1959).

66. Smith, I. Nature, 171, 43 (1953).

67. Bennett, J. C., in Methods of Enzymology, Colowick, S. P., and Kaplan, N. O., editors, Academic Press Inc., New York, 330 (1968).

68. Gross, E., and Witkop, B., J. Biol. Chem., 237, 1856 (1962).

69. Steers, E., Jr., Craven, G. R., Anfinsen, C. B., and Bethune, J. L., J. Biol. Chem., 240, 2478 (1965).

70. Davis, B. J., Ann. N. Y., Acad. Sci., 121, 404 (1964).

71. Deal, W. C., Jr., Biochemistry, in press.

72. Yang, J. T., and Doty, P., J. Am. Chem. Soc., 79, 761 (1957).

73. Drude, P., Lehrbuch der Optik, 2nd ed., S. Hirzel Verlag, Leipzig, 1906.

74. Urnes, P., and Doty, P., Advances in Protein Chem., 16, 401 (1961).

75. Blout, E. R., in Optical Rotatory Dispersion, Djerassi, C., editor, McGraw-Hill, New York, 238 (1960).

76. Sogami, M., Leonard, W. L., Jr., and Foster, J. F., Arch. Biochem. Biophys., 100, 206 (1963).

77. Yang, J. T., and McCabe, W. J., Biopolymers, 3, 209 (1965).

78. Blout, F. R., Schmier, I., and Simmons, N. S., J. Am. Chem. Soc., 84, 3193 (1962).

79. McMeekin, T. L., and Marshall, K., Science, 116, 142 (1952).

80. Cohn, E. J., and Edsall, J. T., eds., Proteins, Amino Acids and Peptides, Reinhold, New York, 370 (1943).

81. Levine, S., Gillett, T. A., Hageman, E., and Hansen, R. G., J. Biol. Chem., in press.

82. Kielley, W. W., and Harington, W. F., Biochim. Biophys. Acta., 41, 401 (1960).

83. Noelken, M. E., Federation Proc., 26, 777 (1967).

84. Marler, E., Nelson, C. A., and Tanford, C., Biochemistry, 3, 279 (1964).

85. Hade, E. P. K., and Tanford, C., J. Am. Chem. Soc., 89, 5034 (1967).

86. Castellino, F. J., and Barker, R., Biochemistry, 7, 2207 (1968).

87. Edelstein, S. J., and Schachman, H. K., Federation Proc., 23, 1248 (1966).

88. Hunter, M. J., J. Phys. Chem., 70, 3285 (1966).

89. Taylor, J. F., and Lowry, C., Biochim. Biophys. Acta., 20, 109 (1956).

90. Reithel, F. J., and Sakora, J. D., J. Phys. Chem., 67, 2497 (1963).

91. Jirgensons, B., J. Biol. Chem., 240, 1064 (1965).

92. Yang, J. T., in Poly-α-Amino Acids, Vol. I, Fasman, G. D., and Timasheff, S. N., editors, Marcel Dekker, Inc., New York, Chapter 6 (1967).

93. Light, A., and Smith, E. L., in The Proteins, Vol. 1, Ed. 2, Neurath, H., editor, Academic Press, New York, 1 (1963).