PART I

STUDIES ON THE MOTILITY AND BIOCHEMISTRY OF CILIA

PART II

CHITINASE ACTIVITY DURING *DROSOPHILA* DEVELOPMENT

Thesis by

Sandra Winicur

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1971

(Submitted August 17, 1970)

this thesis is dedicated

with love and gratitude

to my mother

my first and finest teacher

ACKNOWLEDGMENTS

" 'Beyond the Wild Wood comes the Wide World,' said the Rat."

The Wind in the Willows
Kenneth Grahame

When I first came here, Cal Tech seemed a strange and awesome place. Despite its friendly welcome, it impressed me as a Wild Wood and I often found it hard to see the forest for the trees. After seven years now, the Biology Department has begun to feel like home — which, as everyone knows, means it's time to say thank you and leave for the Wide Wide World.

The most wonderful part of the Biology Department has always been, to me, the people. I suppose there are congenial folk everywhere, but I believe the blend of warmth, wit and wisdom in this department to be unique. To all of you who were never too busy to help, to all who had time to talk, to laugh and to listen, my sincerest thanks. May your memories of Cal Tech be as rich and pleasant as mine.

More specifically, there are those who stand out in bold relief. I acknowledge sincerely and gratefully the following people; some I certainly couldn't have done without, and as for the rest, I wouldn't have wanted to try:

Professor H.K. Mitchell - for support and detachment, guidance and freedom, advice and information, and the wisdom with which he tempers them all - and especially for providing a true environment

of science in which to work.

Professor C.J. Brokaw - for introducing me to a totally captivating experimental system and for providing me the liberty to discover and come to terms with my own abilities and limitations - I am most truly thankful.

Dr. Stuart Goldstein - always a friend and colleague, for unfailing, matter of course acceptance of some gross eccentricities, for constant and ready support, and most of all, for coming back.

Alan Blumenthal - for help and advice given honestly, competently and wisely, and yet so naturally and generously that it will probably surprise him to be singled out here.

Zoltán Tökes - for never letting me quit.

Peter Cross - for clean cilia.

Robbi Hunt - for excellent typing on short notice and for her expert knowledge of the anatomy and physiology of a thesis.

the rest of the guys in the Mitchell group - for stimulating and enlightening discussion.

USPH Training Grant GM 00086 - for the support.

Zev - who said "cafeteria" at 20 months and "MacDonald's" at 22, for being and brightening.

and most of all

Dan - for loving me just the way I am and letting me know it.

ABSTRACT

Part I

Cilia isolated from Tetrahymena after inducing ciliary shedding by addition of calcium chloride to cells suspended in an ethanol solution have been shown capable of motility in the presence of ATP. The potential for motility was restored by treating the isolated cilia with glycerol, ethylene glycol or digitonin-sucrose solutions. Up to 80% of these cilia were motile upon addition of ATP or ADP. These cilia showed an optimum reactivation temperature of 16° C.

The cilia are most motile in concentrated solution. Addition of xylose and dextrose, both of which are present in high concentration in native cilia, cause a sharp increase in percent motility.

The ATPase activity was also studied under different conditions of motility.

Regeneration of cilia by Tetrahymena deciliated by pH 5 treatment was observed. This regeneration took about 7 hours.

Part II

Before both larval molts in Drosophila melanogaster, the chitin in the cuticle is digested to a significant degree by the molting fluid. A spurt of chitinase activity appears just before each molt, drops sharply after the first molt and begins to rise again just about the time that chitin degradation becomes visible in electron micrographs. The level of enzyme activity per mg of soluble protein or per mg of wet weight reached just before the second molt is about twice that before the first, and this declines gradually after the molt until puparium formation.

The activity measured per individual larva however starts a slow rise after hatching with a slight peak at the second molt and continues to rise to a point mid way between the second molt and puparium formation. This indicates that some of the measured chitinase activity may be due to the activity of another enzyme, either a different chitinase, a lysozyme, or possibly a chitin synthetase, which would be most active during the third instar.

Data exist supporting the presence of more than one enzyme with chitinase activity. Two fractions can be separated by ammonium sulfate precipitation and three peaks can be separated on a DEAE-Sephadex column.

The enzyme activity is stable, with no loosely bound cofactor and a single isoelectric point about 3.8. Chitinase activity was measured by a viscometric assay on a substrate of chitosan, a partially deacetylated solubilized product of chitin.

TABLE OF CONTENTS

PART I

STUDIES ON THE MOTILITY AND BIOCHEMISTRY OF CILIA

CHAPTER I

General Introduction

Early observations

Although the existence of cilia was first reported in 1675 by A. van Leeuwenhoek (1), and the name "cilia" was originated in 1786 by O.E. Müller (2), the serious physiological study of these organelles did not begin until they were observed in mammals. This occurred, according to Sir James Gray, in 1834, when Purkinje and Valentin discovered ciliated epithelium on the oviduct walls of vertebrates (3). Cilia or flagella in some form have since been identified in practically all the major animal and most plant groups (4).

The mechanism for motility in these structures was originally thought to be situated within the cells. Older theories considered cilia and flagella as inert rods moved by mechanical action at the basal body or by the passage of water or cytoplasm through fixed internal channels (5). A contractile theory was proposed by Sharpey in 1835 (6). He noticed that cilia bent throughout their length rather than just at the base, and suggested that they contained "muscular substances" by which they could be bent or extended. The significance of this idea was not realized at the time, but comparisons between cilia and muscle have been a constant source of experiment and speculation ever since.

Differences between cilia and flagella

The difference between cilia and flagella is a functional one. Typically, cilia move fluid at right angles to the long axis of the beating cilium, therefore parallel to the surface bearing cilia, and

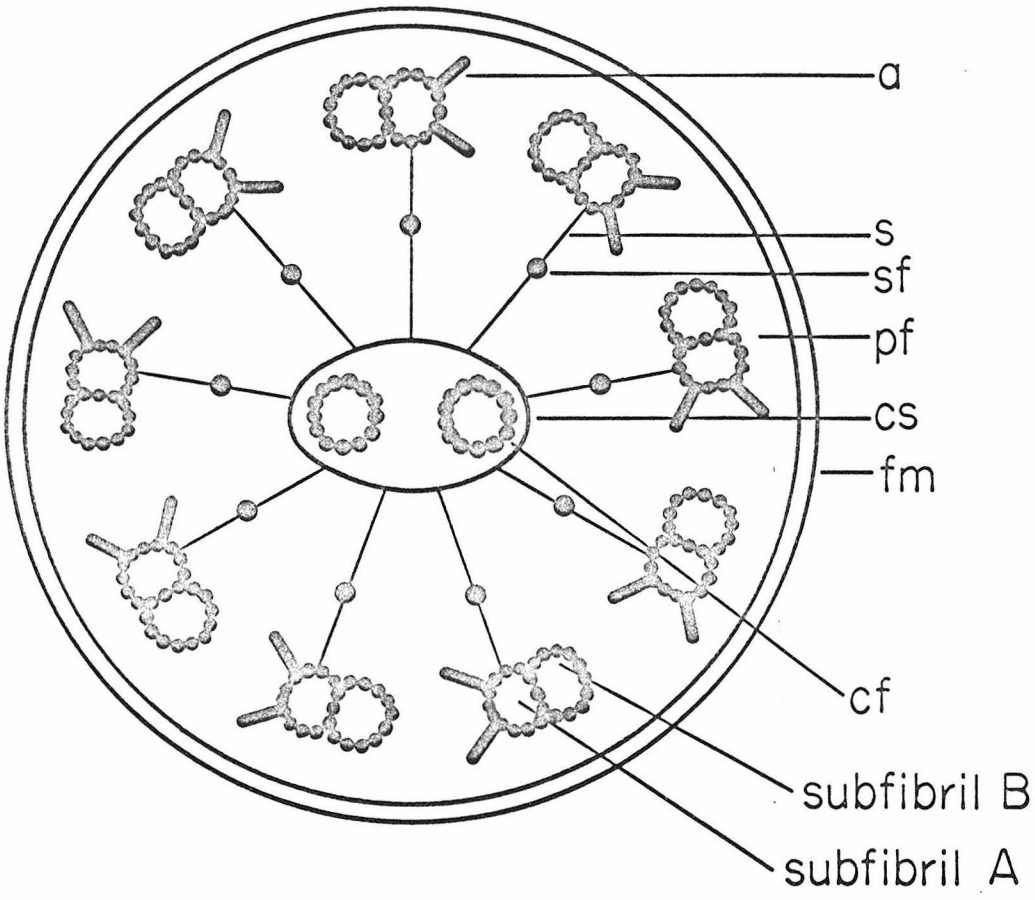
flagella move fluid along the length of the flagellar axis, normal to the surface bearing flagella (4). Flagella are generally longer, but the ranges of size overlap and the cross-sections of both in the electron microscope are identical.

The diameter of cilia and flagella is generally about 0.2 μ, and the length of cilia varies between 5 and 20 μ, the length of flagella, 5 and 200 μ (4). However, cilia can exist combined into compound structures which are much longer, up to 2000 μ (4).

Structure of cilia and flagella

The first electron microscope pictures of the familiar pattern of nine outer and two inner fibrils were published by Manton and Clarke in 1952 (7). Many additional features have since been elucidated, and a representative cross-sectional diagram is shown in Figure 1. Each outer fibril is composed of two microtubular subfibrils, each of about 250 Å in diameter, referred to as subfibrils A and B. A pair of arms projects from subfibril A. A set of radial spokes and secondary fibrils are generally seen between the central and outer fibrils. The central fibrils are also about 250 Å, and Ringo has found that each central and outer microtubule has about 13 subunits in its circumference (10). The central fibrils are surrounded by a sheath which has a spiral composition with a 120 Å pitch (11). This set of nine outer and two inner fibrils with their auxiliary attached structures is known as the axoneme. The ciliary membrane is continuous with the plasma membrane of the cell and appears to be a typical three-layered cell membrane (4).

Figure 1. Diagram of cross-section of a flagellum viewed from basal end. a = arm; cf = central fibril; cs = central sheath; fm = flagellar membrane; pf = peripheral fibril; s = spoke; sf = secondary fibril (8, 9).


a
s
sf
pf
cs
fm
cf
subfibril B
subfibril A

Chemical composition of cilia and flagella

The chemical analysis of ciliary content performed by Culbertson (12) showed the cilium to be about 75% protein, 20% lipid, 3-5% carbohydrate and 0.4% RNA. Some nucleotides were found, which were primarily adenine nucleotides. Similar analyses of other flagellar systems (13, 14, 15) gave comparable results, with the exception of the carbohydrate data, which ranged from less than 1% to 8% in different reports. The main carbohydrates identified by Culbertson were xylose and glucose, with lesser amounts of three oligosaccharides. The lipids present seemed to be predominantly membrane lipids (12).

Motility in isolated cilia and flagella

Mechanically isolated flagella were first found to be capable of movement by Engelmann, who observed beating in tails of frog spermatozoa severed between the head and midpiece (16). Hoffmann-Berling, in 1954, prepared flagellar models by extraction of grasshopper sperm with aqueous glycerol (17), as has been done with muscle models (18, 19). As with muscle, the flagellar models showed motility upon addition of ATP. The flagella moved rhythmically, even if separated from the head and midpiece (20). The glycerol apparently altered the membrane barrier to ATP. ATP is now generally accepted as the energy source for flagellar motility. The similarity of flagellar ATPase and muscle myosin in this regard was suggested by Engelhardt (21, 22).

Brokaw (23), in 1961, obtained wave propagation and forward progression of glycerinated Polytoma flagella isolated from the cells by reacti-

vation with ATP. Cilia from the ciliate protozoan Tetrahymena, isolated in 60% glycerol, are also motile in ATP (24), and the phenomenon appears in most similar systems. These systems specifically require Mg^{++} for motility (17, 22, 23) and show little or no reactivation in nucleoside triphosphates other than ATP (23).

Isolated cilia and flagella seem to require an intact base for motility, and procedures that rupture the flagellum part way along its shaft also inactivate it (5, 16, 25, 26). The reason for this is not known, but it is not due to a requirement for the basal body, which is left behind in the cell when the cilium is released (13, 27), and which has never been observed in electron micrographs taken of the base of free flagella (28).

ATPase of cilia

Davies' proof in the early 1960's that ATP is the primary energy source for muscle contraction (29) implicates ATP in every system that displays ATP-generated motility. This is well substantiated in flagellar systems. A number of different methods have been used for the isolation of flagella and the study of their enzymatic activity (some of which are described in references 14, 17, 21, 23, 27, 30, 31, 32, 33, 34). Generally, flagellar ATPases in situ preferentially split ATP, are Mg^{++} and/or Ca^{++} activated, have alkaline pH optima and show Michaelis-Menton constants comparable to the lower extreme of the range reported for myosin (about 10^{-5} M) (35). ADPase activity, which is high in protozoan cilia, but not sperm tails, is probably due to an ATP-AMP

phosphotransferase (23, 34, 36, 37), and other nucleoside triphosphates may be dephosphorylated in varying degrees up to 25% of the activity with ATP (23, 36).

The best characterized ATPase is that of Tetrahymena cilia, isolated and studied by Gibbons (38). This enzyme, called "dynein", is extractable at low ionic strength in two active fractions, a 14 S monomer (600,000 molecular weight) and a 30 S polymer which appears in the electron microscope to be short filaments of globules looking like the 14 S protein (39). The enzyme activity requires Mg^{++}, Ca^{++} or a similar divalent cation, and has a K_m with Mg^{++} of 3.5×10^{-5} (14 S) or 1.1×10^{-5} (30 S) (36).

A dynein with similar characteristics has been isolated from sea urchin sperm by extraction in low ionic strength, but only in the monomeric (11 S) form (40).

Relation between ATPase and motility

Brokaw has calculated that the dephosphorylation of one ATP molecule/dynein molecule/beat would provide adequate energy for flagellar motility (41). However, the existence of ATPase activity in flagella in which motility has been destroyed, with a specific activity of the same order of magnitude as in motile ones (24, 25, 27, 38), indicates the requirement for other parameters, such as a high degree of structural integrity, to transform chemical energy to mechanical energy.

The relationship between structural integrity, motility and ATPase activity is a complicated one. Brokaw found that glycerinated sea urchin

sperm lose up to half their ATPase activity and become completely immotile if they are broken by Vortex mixing (25). He called this movement-coupled dephosphorylation of ATP the "ATPase activity difference". He also observed an increase in ATPase activity from extended pretreatment of the sperm in salt solutions (42), which he interpreted as an uncoupling of ATPase activity.

Solubilization and fine structure of cilia

The largest block to the characterization of the proteins of cilia has been the apparent insolubility of the structure, requiring drastic methods of analysis (12, 14, 27). This was shown to be entirely attributable to the insolubility of the ciliary membrane (38), and the dissolution of this membrane by digitonin or low ionic strength allowed fractionation and characterization of the protein components.

Gibbons, in an extremely elegant series of experiments on Tetrahymena (38), selectively extracted a fraction with ATPase activity from the axonemal protein at low ionic strength. Recombination of these fractions in the presence of Mg^{++} restored the arms to their positions on subfibril A of the nine outer fibrils, as seen in the electron microscope, and also restored ATPase activity to the axonemal fraction. Some characteristics of the enzyme which forms these arms, dynein, have already been discussed.

Axonemal proteins of the central and outer fibers can be prepared by methods of isolation similar to those used for purification of actin (43, 44, 45). The outer fibers of Tetrahymena cilia separate

into a protein with a molecular weight of 104,000, which breaks down in guanidine hydrochloride to fragments of 55,000 molecular weight (43). These can be further fractionated thermally into the tubulin of the A and B subfibrils, which differ only in small regions of the polypeptide chain (46).

Similarities between cilia and muscle

The similarities between cilia and muscle are obvious and tempting sources for speculation (47). The preparation of glycerinated flagellar models in the manner of muscle models has already been mentioned. Many theories exist describing flagellar motility in terms of contraction-relaxation phenomena (47), and flagellar proteins have often been isolated and studied using techniques devised for muscle.

Enzymology Dynein, the ATPase of flagellar systems, resembles myosin enzymatically in many ways. It is fairly specific for ATP and has a specific activity similar to that of myosin. Activation requires either Mg^{++} or Ca^{++}, which resembles actomyosin, myosin being specific for Mg^{++} (48). Gibbons and Rowe point out that dynein seems closely related to myxomyosin, the ATPase of slime molds (39).

Burnasheva (31) found an ATPase extract from bull sperm flagella she called "spermosin". This extract interacted with muscle actin to form an actomyosin-like compound which, like actomyosin, decreased its viscosity upon addition of ATP.

ADPase activity is due to a myokinase-like molecule (an ATP-AMP phosphotransferase) which is strongly bound to the axonemal protein and is much larger than muscle myokinase (36).

Structure Dynein has about the same molecular weight and subunit composition as myosin (48). Axonemal proteins of the central and outer microtubular fibrils can be broken down into proteins which resemble actin in their amino acid composition and physico-chemical properties (43, 44, 45), but are extremely dissimilar in their tryptic and chymotryptic peptides (49). Outer fibers of sea urchin sperm flagella have been found to contain one mole of tightly bound guanine nucleotide/mole (55,000) of protein, comparable to the bound adenine nucleotide on actin (50).

Motility In myofibrils and actomyosin gels, ATP causes a decrease in hydration, a phenomenon known as superprecipitation. An increase in hydration results under similar conditions in cilia. Both the 30 S dynein and the outer fibers are required for this phenomenon (51, 52). A relaxing factor effect similar to that in muscle has also been reported in glycerinated invertebrate sperm (53).

The Huxley-Hanson model for muscle contraction by a sliding filament system (54) is paralleled by the data of Satir indicating that a sliding filament mechanism is responsible for ciliary movement (55). His electron micrographs show that when a cilium bends, the fibers on the inside of the bend extend at the tip, suggesting that the fibers slide relative to each other without changing their length.

Research objectives

The object of the experiments reported in Chapter II was to study motility and ATPase activity of Tetrahymena cilia under the same conditions,

i.e. conditions under which the cilia have good motility. Most of the data on ciliary motility have been published previously (56).

The work on ciliary proteins and regeneration reported here in Chapters III and IV was originated in an attempt to identify the precursors of cilia within the cell, hopefully in a system that was induced by deciliation to actively regenerate cilia. The immunologic and electrophoretic results did not turn out well enough to pursue this line of study and the attempt was discontinued in favor of other areas of research. Other researchers have found similar systems unamenable to immunological approach (57).

Tetrahymena cilia were used in all these experiments since the organism is easily grown axenically and the cilia can be collected readily.

CHAPTER II

Motility and ATPase of *Tetrahymena* Cilia

INTRODUCTION

Cilia isolated from Tetrahymena by the addition of Ca^{++} salts, or salts of other cations, to cells suspended in an ethanol solution have been studied chemically and enzymatically (13, 27, 38, 58, 59), but until now have never been shown capable of motility in the presence of ATP despite an active ATPase. These methods, however, provide very clean preparations of cilia in a relatively short time.

Glycerination procedures for isolation of cilia, on the other hand, take much longer, and the cilia are generally not as clean. Gibbons, however, has demonstrated motility in cilia isolated by glycerination techniques (24) similar to those developed for grasshopper sperm tails (20) and Polytoma flagella (23). These cilia in the presence of ATP showed motility of short duration (the majority for less than five minutes) and low frequency (2-3 waves/second) (24).

This chapter describes similar results obtained with ethanol-calcium isolated cilia that were treated with glycerol before ATP was added. Since the energy for motility in the in situ cilium is probably derived from ATP breakdown, the ATPase activity and motility were studied under conditions appropriate for motility.

Permeability of ciliary membranes to ATP has been accomplished by means of glycerol, detergents and EDTA (24, 38). These and other methods were tested in this system.

MATERIALS AND METHODS

Growth of Tetrahymena

Stocks of the ciliate protozoan Tetrahymena pyriformis W (obtained from Dr. Jay S. Roth, University of Connecticut) were maintained at 25° C in DeLong culture flasks (500 ml capacity) containing 200 ml of a medium composed of 1% Difco Proteose-Peptone, 0.25% Difco Yeast Extract and 0.1% dextrose.

Cilia were collected from late log phase Tetrahymena grown for 2½ to 3 days at 25° C in 8 liters of the above medium (less dextrose), with Dow Corning Antifoam A Spray added to control foaming. The carboys were aerated at the rate of 2.5 to 3 liters/minute.

Isolation of Cilia

Most of the cilia studied were isolated by a method similar to Gibbons' modification of the procedure of Watson and Hopkins (13, 38).

On the third day of growth, an 8 liter culture of Tetrahymena, about 3×10^5 cells/ml, was harvested in a Lourdes continuous flow centrifuge at 300 x g. This took about 45 minutes. The centrifuge was then cooled down to 4° C and most of the remaining operations were carried out at that temperature.

The cells were washed twice in a solution of 30 mM NaCl and 200 mM sucrose, collected each time at 300 x g for 3 minutes, and brought to 64 ml in the NaCl - sucrose solution. They were then added to 250 ml of cilia removal solution (11% ethanol, 2.5 mM EDTA, 15 mM Tris, 150 mM

sucrose, 30 mM KCl, brought to pH 8.3 at 1° C with thioglycollic acid). To this, 3 ml of 1 M $CaCl_2$ was rapidly added with mixing. This step removed the cilia from the cells, and the cell bodies were collected at 100 x g for 8 minutes. The supernatant was removed by aspiration down to the top of the pellet.

The centrifuge was cooled down to 0° C and the cilia spun out at 15,000 x g for 20 minutes. The pellet weight was generally about 0.1 g, seldom less, and sometimes ranged as high as 0.18 g.

Occasionally cilia were isolated by a glycerination technique similar to those of Brokaw and Gibbons (23, 24).

Protein Determinations

Since the cilia tended to clump and distribute unevenly when being pipetted for ATPase assays, a method for protein determination was required that could be performed on each individual sample in order to obtain meaningful values for specific activity. This method had to be performable directly on the TCA-precipitated protein remaining in the test tubes after the supernatants were removed to measure ATPase activity.

At first, both the Lowry (60) and the Biuret (61) methods were used. However, many reagents often present in the ATPase assays, such as thioglycollate, sugars and glycerol, interfered either by retarding color formation or by causing false positive results.

After preliminary trials of several qualitative tests for protein, the Xanthoproteic reaction was found to be amenable to quantitative

expression. Ninhydrin, Millon-Nasse and OD 280/260 determinations were rejected for various reasons, but the Xanthoproteic reaction showed good, reproducible results which could be standardized (62). The yellow-gold color produced in this reaction is due to nitration of aromatic groups in the protein by nitric acid. Tyrosine and tryptophan are reported to be the most reactive of the amino acids.

The assay as finally developed was as follows:

1. Up to 0.2 ml protein solution (0.5 to 5.5 mg total) was brought to 0.5 ml with distilled water in a small test tube. The protein was precipitated with 0.1 ml 57% TCA, centrifuged in an International Clinical Centrifuge at 3000 rpm for 10 minutes, and the supernatant poured off and discarded.
2. The pellet was dissolved in 0.1 ml of 1 N NaOH and left standing for an hour.
3. To this, 0.2 ml of 70% HNO_3 was added carefully and slowly, and the test tubes were covered with refluxing spheres and put in a boiling water bath for 10 minutes. The tubes were then cooled in a room temperature water bath for about 20 minutes.
4. The samples were neutralized with 0.4 ml of 10 N NaOH and the color was left to develop from one hour to overnight.
5. Each sample was diluted into 3 ml of distilled water and the optical density determined at 400 mμ.

The absorption spectra in Figure 2 show how the boiling time and wave length for absorption were determined. At 400 mμ, the optical density was independent of the duration of boiling. A heating time

Figure 2. Xanthoproteic Assay: absorption spectrum between 390 and 450 mμ. Assays were done on 2.2 mg protein. Boiling times in HNO_3 were varied for each curve.

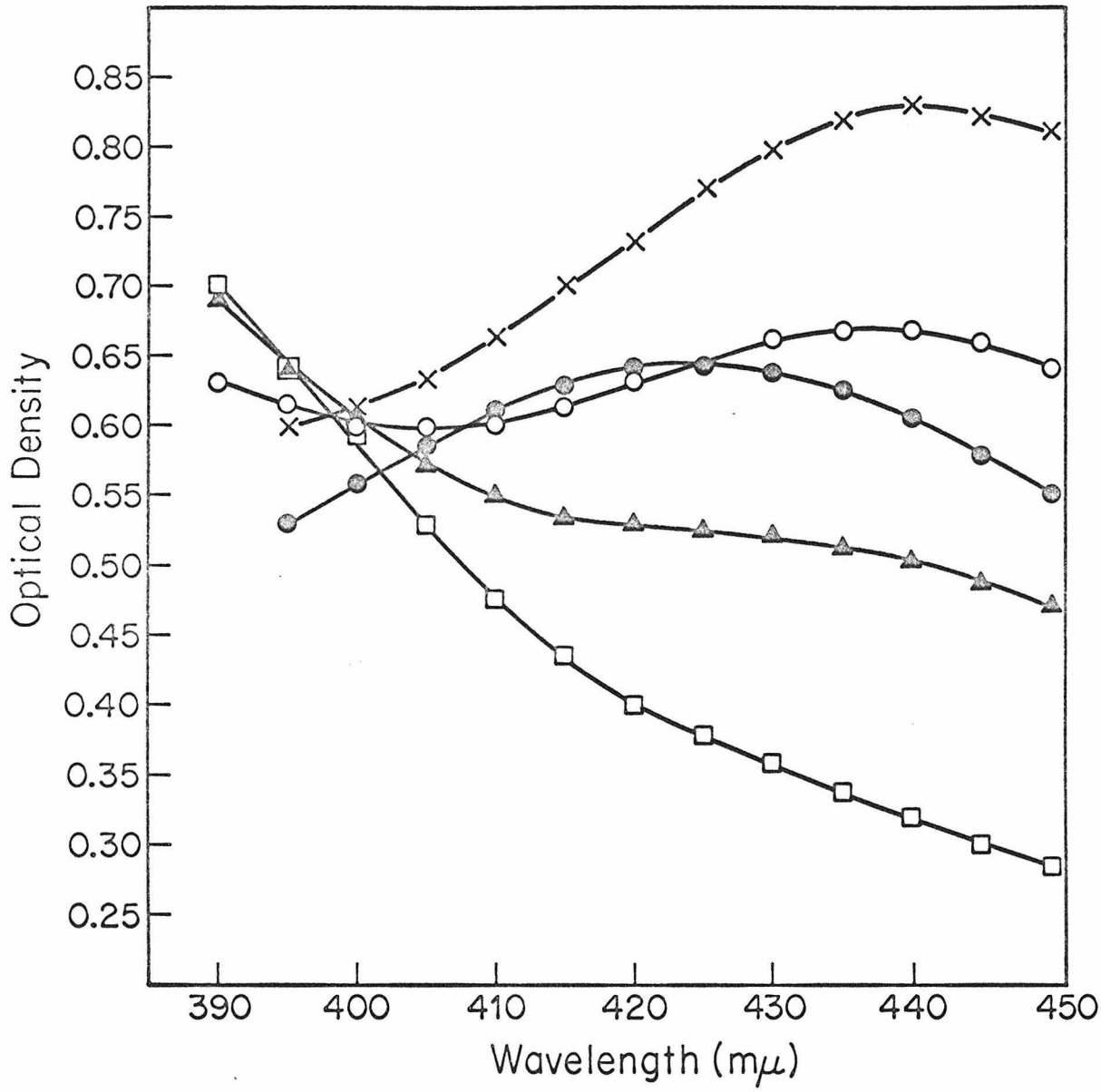
● = 0 minutes

X = 5 minutes

o = 10 minutes

▲ = 22 minutes

□ = 55 minutes


0.85
0.80
0.75
0.70
0.65
0.60
0.55
0.50
0.45
0.40
0.35
0.30
0.25
Optical Density
390
400
410
420
430
440
450
Wavelength (mμ)

of 10 minutes was chosen as the time at which a slight error in wave length would cause the least error in optical density.

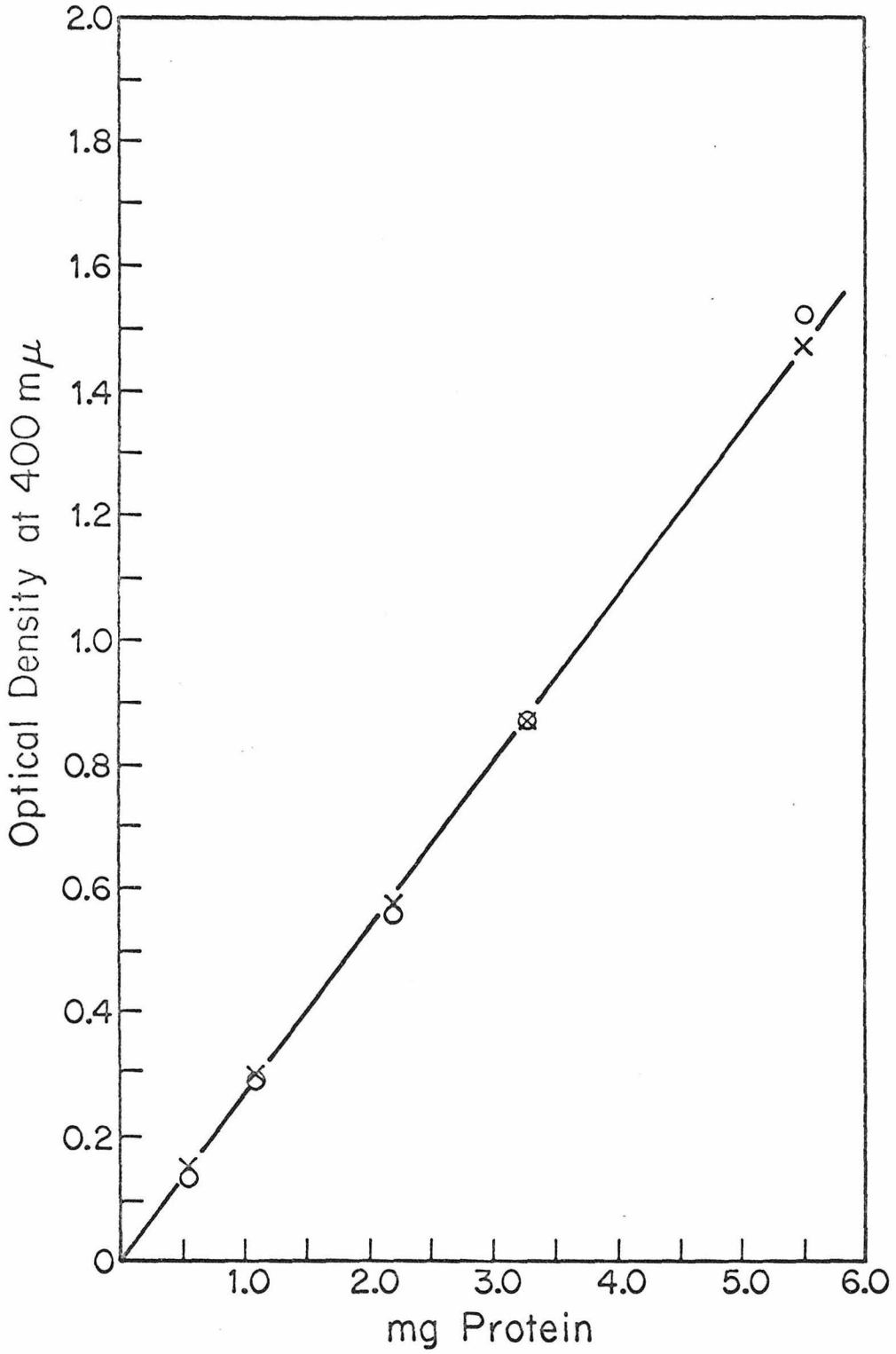
The stability of the color with time is shown in Figure 3, as is the linearity of the assay over the range studied. This range was chosen to conform to the amount of protein present in the ATPase assays, but since the color required extensive dilution to be readable (Step 5), this assay could probably be easily scaled down to much smaller amounts of protein. No interfering substances were encountered.

All the methods of protein determination studied were standardized against bovine serum albumin.

Figure 3. Xanthoproteic Assay: protein concentration vs. optical density at 400 mμ.

O = after color developed for one hour

X = after color developed overnight


Optical Density at 400 mμ
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
1.0
2.0
3.0
4.0
5.0
6.0
mg Protein

Motility assays on cilia

To study the motility of the ethanol-calcium isolated cilia, the 15,000 x g pellet of cilia was cooled to about 0° C and combined with five volumes (unless otherwise stated) of an extraction solution at 0°C. The cilia were resuspended gently with a 5 inch Pasteur pipette. Care was taken to prevent the extraction solution from getting too cold, as this caused the cilia to freeze before extraction was accomplished and subsequently to fragment. The total elapsed time from the addition of the ethanol solution to the addition of the final extraction solution was usually 1.5 hours. The motility of the cilia could be reactivated after 30 minutes by diluting the suspension of cilia with two or three parts of ATP solution at 19° C. Motility was studied from 30 minutes to 3 hours after addition of extraction solution, during which time reactivation of ciliary motility was essentially unchanged. Movement was measured microscopically by counting the beat frequency with the aid of a stop watch.

With the exception of the experiments on the effect of temperature, cilia were observed in a room maintained at 19° C. One drop of cilia suspension was added to two or three drops of reactivation solution and mixed rapidly with a glass rod. A small drop of the mixture was immediately placed on a glass slide, covered gently with a cover slip and observed by phase-contrast microscopy at 1000 x magnification. The size of the drop was found to be important in producing repeatable results, and the best results were obtained from a drop of 8 to 9 µl.

A 40 ml container of water placed over the 6 volt light source in the Zeiss microscope base served as a heat filter. By using a glass slide with a built-in thermocouple, it was determined that this filtered illumination did not raise the temperature of the slide more than a fraction of a degree over that produced by dark-field illumination at a lower magnification.

The apparatus used to regulate and determine temperature in the studies on the effects of temperature is described by Holwill and Silvester (63).

All the extraction solutions used with the exception of Tris-EDTA and TMSK contained in addition to the extracting reagent, 50 mM KCl, 2.5 mM $MgSO_4$ and 20 mM Tris-thioglycollate buffer, pH 8.3 at 1° C. The Tris-EDTA solution is identical to Gibbons' Tris-EDTA solution for membrane disruption of ethanol-calcium isolated cilia, containing 0.1 mM EDTA and 1 mM Tris-thioglycollate buffer, pH 8.3 at 1° C. TMSK solution, designed by Gibbons for maintaining ciliary shape, contained 25 mM KCl, 2.5 mM $MgSO_4$, 200 mM sucrose and 30 mM Tris-thioglycollate buffer, pH 8.3 at 1° C (38).

The reactivation solutions contained, in addition to ATP or ADP (unless otherwise stated), 50 mM KCl, 2.5 mM $MgSO_4$ and 20 mM imidazole-thioglycollate buffer, pH 6.8 at room temperature. The ATP concentration in the reactivation solution was generally 0.5 mM. Although visual estimations of the percent of motile cilia give only approximate values, the results were checked on a number of occasions with investigators working on similar systems (Dr. Stuart Goldstein, Dr. Michael

Holwill, Dr. Charles Brokaw) who agreed in substance with the reported data.

ATPase determinations

At first, a modification of the Fiske-SubbaRow method for determination of inorganic phosphate was used to measure the ATPase activity of cilia (64), but this was discarded due to interference by the rapid ATP breakdown during the time of color development.

A modification of the Taussky-Shorr method for phosphate determination was substituted (65). This assay is slightly less sensitive than the above, but full color development occurs in less than 2 minutes, and less than 3% of the ATP is destroyed in the course of the assay.

In this method, 0.3 ml of ATPase assay medium (identical to the reactivation solutions for motility) and 0.1 ml of cilia in glycerol extraction solution (generally about 50 mg/ml) were combined for the desired amount of time. The temperature was controlled around 16° C by means of a refrigerated water bath. The reaction was stopped and the protein precipitated with 0.1 ml of 57% TCA. Samples were filtered on Whatman #1 filter paper and 0.3 ml was pipetted into a test tube. To this was added 1 ml of $FeSO_4$-NH_4 molybdate Reagent as described by Taussky (65). Test tubes were Vortex mixed and the color was determined spectrophotometrically at 700 mμ in 2 minutes.

Since the thioglycollate in the ATPase assay solution affected both the amount and the duration of color development, all blanks and standards were run in the presence of thioglycollate.

RESULTS AND DISCUSSION

When ethanol-calcium isolated cilia were diluted directly into a reactivation solution containing 0.5 mM or 1.0 mM ATP, the membrane appeared to swell and the cilia curl up, or alternatively, fragment. However, if these cilia were observed within 15 seconds of dilution into ATP, an occasional cilium could be seen to beat for a while before it was inactivated.

When cilia were diluted with 5 volumes of an extraction solution containing 70% glycerol (as used directly to isolate cilia in the glycerination procedures) and subsequently diluted into a reactivation solution with 0.5 or 1.0 mM ATP at 16° C, they retained their shape and did not appear to swell. Initially about 50% and occasionally as high as 80% of the cilia were motile, but this fraction decreased rapidly, about half of the motile cilia stopping within the first minute. These cilia therefore retained the potential for motility but were more fragile than their glycerol isolated counterparts (24), which were motile at room temperature.

These data also indicate that the ciliary membrane after ethanol-calcium isolation was not severely damaged, and was permeable to water.

Effect of dilution on ciliary motility

The concentration of the cilia turned out to be an extremely critical factor in determining their motility. If dilution with glycerol extraction solution was increased from 1:5 to 1:20, only about one half as

many motile cilia were subsequently observed, and higher dilutions caused an even greater diminution of motility. However, washing the cilia in TMSK solution and reprecipitating them before extraction did not noticeably alter the results. Some factor which is required for motility appears to be extracted from the cilia into the glycerol medium. Similar dilution results have been observed by other investigators (24).

Diluting the cilia (which were already in glycerol extraction solution) 1:10 with ATP reactivation solution rather than the usual 1:3 produced one third to one half the normal amount of motile cilia. In a 1:20 dilution there was very little motility.

Addition of 3% bovine serum albumin to the reactivation solution caused an increase of greater than 20% in the motility observed after a routine (1:5) glycerol extraction, but did not affect the reduced motility after extraction at 1:20, nor did it improve motility when directly included in the extraction solution. The beneficial effect of albumin, and of the polyvinylpyrrolidone used in other reactivation systems (66, 67), may be an osmotic effect. A rough calculation suggests that the soluble matrix protein within the cilium, about 25% of the total protein (38), represents approximately a 2% protein solution. (The parameters for ciliary size here are considered to be the same as those for Paramecium cilia (68) and the protein/cilium is derived from the data of Watson and Hopkins (13)). Alternatively, the main effect of albumin and polyvinylpyrrolidone may be simply to reduce sticking of the cilia to the slide and cover glass.

ATPase assay

Since the ATPase activity was being studied as a function of motility, it had to be followed under conditions in which the cilia were motile. The requirement displayed by the cilia for being highly concentrated imposed severe restrictions on the ATPase assay, and made kinetic studies extremely difficult. The cilia in each assay were in such high concentration that the ATP concentration (between 0.5 and 1.0 mM required for motility) was constantly diminishing at a rapid rate. For this reason, most of the assays described in this thesis were terminated at 2 minutes, the earliest point at which total inorganic phosphate production was consistently high enough to be measured reproducibly.

Preparations of cilia with good initial ATPase activity ranged from those with the rate of enzyme activity remaining fairly constant over a period of 10 minutes to those with a steadily diminishing ATPase activity. In general, preparations that had been in glycerol extraction solution for the longest times before being assayed showed the highest initial activity. In one experiment, after 6 hours in glycerol extraction solution, the cilia retained less than 20% of the motility they had shown after 1 hour, but the ATPase activity had increased more than 25%. Comparable time effects have been observed by Brokaw with glycerinated sea urchin sperm tails (42). One possible explanation is that a progressive deterioration of the ciliary structure with time uncouples the ATPase from some rate-limiting conformation. This is especially interesting in view of the fact that 14 S dynein has a higher specific activity than 30 S dynein under some conditions (36). Alternatively, prolonged glycerination may expose more sites to the substrate.

The ATPase rate variations among different preparations could not be correlated to motility differences, appearance of the cilia preparation or cell age. ATPase determinations were generally done after the motility observations on a given preparation were concluded, so the time the cilia remained in glycerol before the enzyme activity was assayed varied. Because these variations made comparisons from experiment to experiment difficult, most analyses of ATPase activity discussed here represent a single typical experiment.

ATP concentration

As the ATP concentration was increased, the frequency of wave initiation in motile cilia increased from about 2 beats/second at 0.1 mM ATP to a maximum of 5-6 beats/second at 0.5 to 1.0 mM. The number of motile cilia was also highest where beat frequency was at a maximum. Further increase in ATP concentration caused a decrease in beat frequency and a corresponding decrease in the number of motile cilia, so that considerably less movement was seen at 2 mM ATP. By 10 mM ATP, the cilia began to deteriorate and vesiculate. Approximately the same results were obtained when the experiment was repeated with glycerol isolated cilia.

ATP is known to cause dissolution of ciliary structure at high concentrations (51), and the lack of motility at high ATP concentrations may be due to that effect. Alternatively, high concentrations of ATP may act by binding some ion necessary for motility.

The ATPase activity showed no trend that could explain these results (Figure 4). An order of magnitude estimate of the K_m of the ATPase can be extrapolated from the data. This value, about 6×10^{-5}, compares reasonably to other K_m's derived for similar systems (23, 35, 36).

Beat frequency at low ATP concentrations could be increased by 1 or 2 beats/second by increasing the Mg^{++} concentration in the reactivation solution to 5 mM. The adverse effect of high ATP concentration on motile cilia was not significantly affected by increasing the Mg^{++} concentration. At concentrations of 10 mM Ca^{++} or Mg^{++} all movement stopped. The inhibitory effect of high divalent cations has been observed routinely in other systems (20, 24).

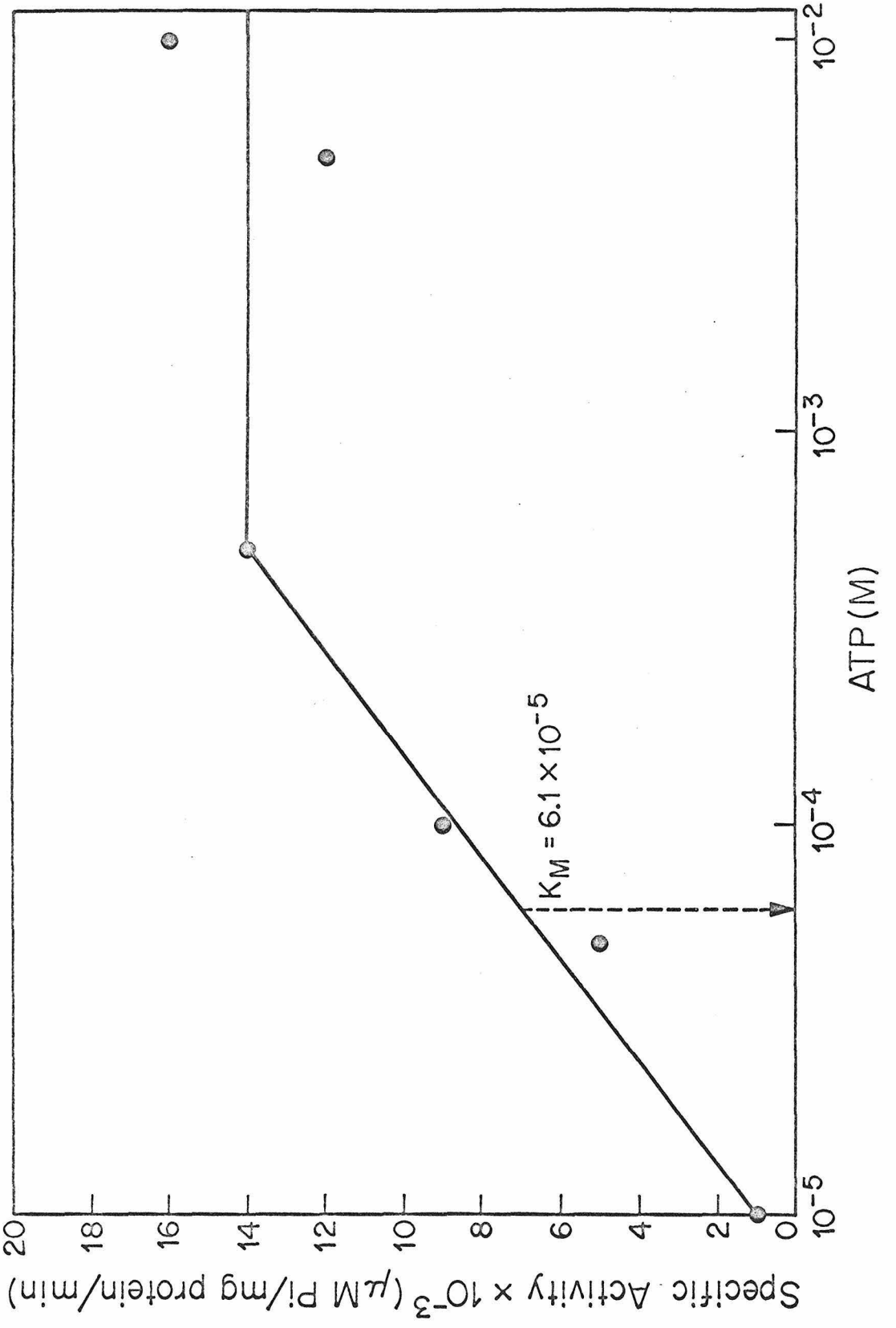
Effect of thioglycollate

The percent of motility of all preparations was improved to some degree by the use of imidazole-thioglycollate buffer rather than imidazole-HCl, so thioglycollate was included in all of the assays. In some preparations which showed extremely poor motility, thioglycollate increased the motility up to 400%, and even the most motile cilia preparations showed about a 15% improvement.

Effect of pH

The ATPase activity showed no strong pH optimum between pH 6.0 and 8.4, but dropped off slightly below pH 6.8.

Figure 4. Specific Activity of ATPase vs. ATP Concentration. Assays were run for 5' at 15.5° C with cilia diluted to a concentration of 15 mg/ml in glycerol extraction solution. In addition to ATP, the assay medium contained 50 mM KCl, 2.5 mM $MgSO_4$ and 20 mM imidazole-thioglycollate buffer, pH 6.8 at room temperature.


Specific Activity x 10^{-3} (μM Pi/mg protein/min)
20
18
16
14
12
10
8
6
4
2
0
10^{-5}
10^{-4}
10^{-3}
10^{-2}
ATP (M)
$K_M = 6.1 \times 10^{-5}$

Effect of temperature

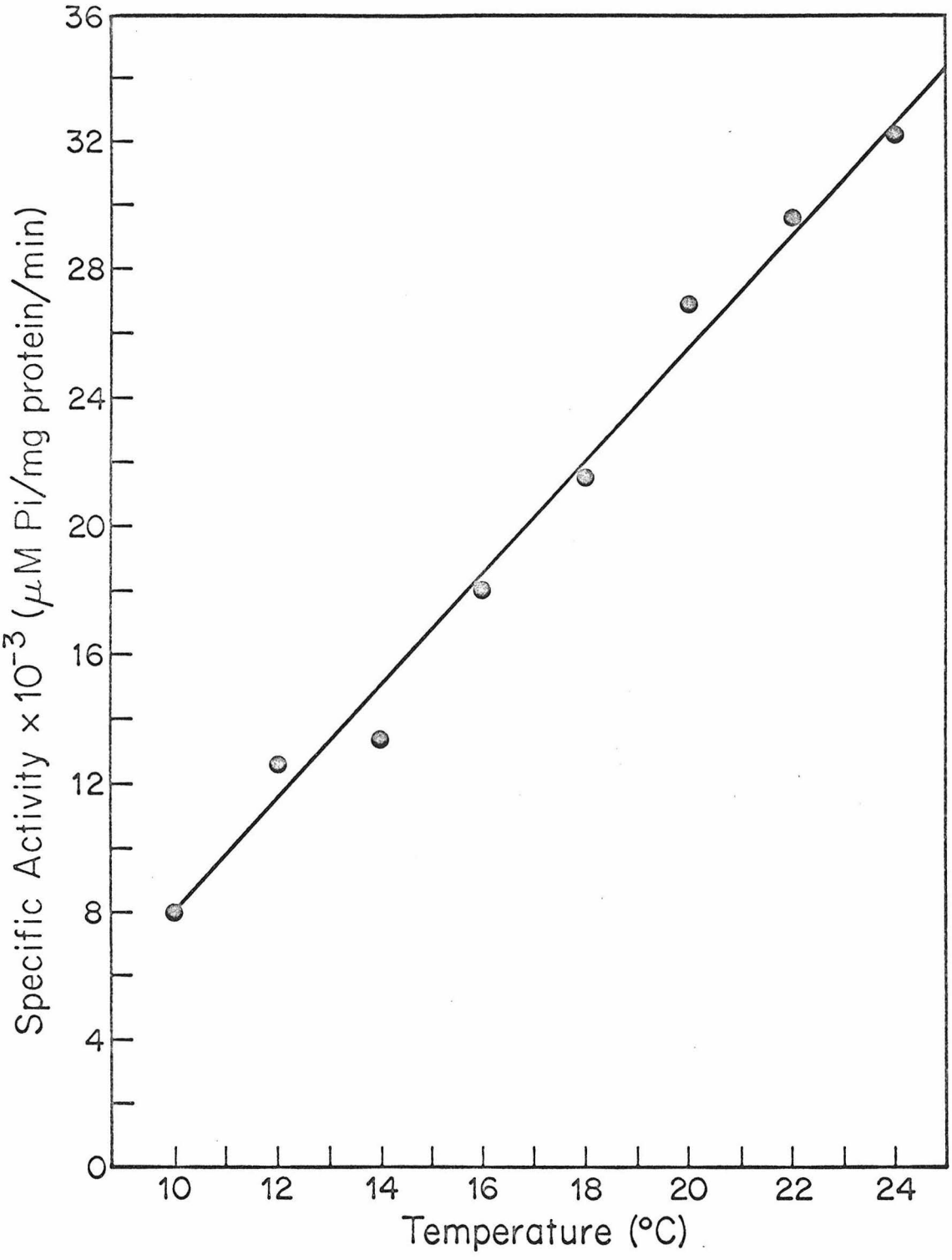
The motility of the ethanol-calcium isolated cilia was more sensitive to temperature variation than the glycerol isolated cilia. Motility was maximal at 16° C, and slightly less at temperatures up to 20° C, but fell off sharply outside this range, with only occasional movement at 24° or 12°. In contrast, the glycerol isolated cilia studied by Gibbons were observed at room temperature (24).

The ATPase activity increased linearly over the temperature range studied, with an increment in specific activity of 1.8×10^{-3}/ ° C (Figure 5). The increase in enzyme activity with temperature is generally expected to be steeper at higher temperatures, but in this highly structured multi-enzyme system, simple enzyme kinetics were not followed.

Activity in ADP

Ciliary motility was also reactivatable in ADP. Although some motility occurred at a concentration of 0.1 mM ATP, no movement occurred with ADP at this concentration. At 0.3 mM, about half as many cilia beat in ADP as in an equivalent ATP concentration, and then only after a 20 to 30 second lag period. The percent motility increased with increasing ADP concentrations, and at 2 and 3 mM, was as great as at optimal ATP. The results with ADP are comparable to the effect of ADP on Polytoma flagella (23) and probably indicate the conversion of ADP to ATP by an ATP-AMP phosphotransferase, the presence of which has been well substantiated in Tetrahymena cilia (37) and similar systems.

Figure 5. ATPase Activity vs. Temperature. Assays were run for 2' at 0.5 mM ATP. In addition to ATP, the assay medium contained 50 mM KCl, 2.5 mM $MgSO_4$ amd 20 mM imidazole-thioglycollate buffer, pH 6.8 at room temperature.


Specific Activity x 10^{-3} (μM Pi/mg protein/min)
0
4
8
12
16
20
24
28
32
36
10
12
14
16
18
20
22
24
Temperature (°C)

At 1 mM ADP, ADPase activity was measured at about 80% of the rate of ATPase activity (2', 16°C). In addition, the amount of inorganic phosphate measured after 30' of incubation with ATP as substrate was often higher than the total amount of ATP added. This indicates that the ATPase activity is actually a measure of dephosphorylation of the initial substrate ATP plus the ATP formed by activity of ATP-AMP phosphotransferase. The motility at a given ADPase concentration, then, is actually due to ATP at a concentration of less than 80% of that of the added ADP.

Effect of other reagents

Perhaps the greatest problem encountered in the course of these studies was the inability to dilute the cilia and still retain motility. In a search for conditions which would allow more dilution, and also in order to better characterize the biochemistry of motility, the motility of cilia was studied with various reagents added to the reactivation solutions. Colchicine had no effect on motility or ATPase activity at a concentration of 1 mM. Since colchicine is known to bind to some ciliary proteins (69), it may have been stopped by the vestiges of the ciliary membrane or by the polymerized state of the cilia from exerting any observable effect at that concentration.

Ouabain, which inhibits Na^+ and K^+ activated membrane ATPase activity, also showed no effect on ciliary ATPase at 1 mM, but caused a slight but reproducible improvement in the per cent and duration of motility.

A number of possible energy sources and related compounds were studied. There was no motility in the presence of GTP, and GTPase activity was less than 5% of the ATPase. A similar result was obtained by Brokaw with Polytoma flagella (23). Up to 1 mM AMP showed no effect on ciliary motility, alone or in combination with GTP.

Phosphocreatine, at 0.5 mM, 1.0 mM and 6.5 mM, caused no motility of cilia alone or with 1.0 mM AMP, and did not improve motility in the presence of ADP or ATP. Arginine kinase activity, however, has been found in cilia (70), and phosphoarginine should probably also be tried. Acetylcholine (1 mM) did not initiate motility, but when added at concentrations between 0.1 mM and 10.0 mM to ATP or ADP, caused a slight increase in beat frequency. Ribose-5-phosphate at 0.1 mM, also ineffective alone, caused a slight improvement in beat frequency and per cent motility in ADP and ATP.

Four sugars, ribose, xylose, dextrose and sucrose were studied for their effect on ciliary activity. Xylose and dextrose, at 1 mM, caused a notable improvement in ciliary motility in the presence of ATP. The beat frequency was extremely rapid, about 10 beats/second in both cases, and the per cent reactivation was also a bit improved. Ribose and sucrose showed no such effect, and all the sugars were inhibitory at 6.5 mM. Culbertson (12) has found extremely high amounts of xylose and dextrose present in cilia, and he also suggested the possibility that carbohydrate may be leached out of cilia during the preparative procedures. The added xylose and dextrose, then, may be replacing sugar naturally present in the cilium.

None of the sugars showed an effect on ciliary motility when added at 1 mM or 6.5 mM without ADP or ATP.

ATPase activity was measured in the presence of 1 mM concentrations of the above-mentioned sugars, and in no case did it differ significantly from normal. The effect of the sugars seems to be on some other structural parameter for motility, and not on the provision of energy.

Extraction solutions

The swelling and inactivation of ethanol-calcium isolated cilia when diluted directly into reactivation solution suggest that an intact semi-permeable membrane is still present, preventing the entry of externally applied ATP. The effect of a number of reagents on membrane permeability to ATP has been studied by means of motility measurements under the same conditions as were used for glycerol extraction.

Substitution of 70% ethylene glycol for glycerol in the extraction solution led to about 50% motility when the cilia were subsequently diluted into ATP reactivation solution. There was a noticeable lag period, up to half a minute, before full motility was observed. This differs from glycerol extracted cilia, which showed maximal motility immediately. The duration of movement was about three times as long as after glycerol extraction, but few cilia could still be reactivated after 3 hours in the ethylene glycol, in contrast to glycerol isolated cilia, which can be maintained for more than 3 hours.

Digitonin has been used for making reactivatable models of mammalian spermatozoa (66). Treatment of ethanol-calcium isolated cilia

with extraction solutions containing 0.5% digitonin and 60% sucrose led to about 40% motility; with 0.05% digitonin in 60% sucrose, about 20% motility. In both these cases there was a lag period similar to that with ethylene glycol. Less than 1% reactivation occurred following extraction with 0.05% digitonin in TMSK solution or with 60% sucrose alone. Cilia treated with digitonin alone were never reactivatable.

After both the ethylene glycol and digitonin-sucrose treatments, movement in ADP was sparse and slow at all concentrations studied. These solvents may have the effect of either inhibiting or dissolving the ciliary ATP-AMP phosphotransferase.

In Tris-EDTA solution, which Gibbons used to break down the membrane and extract proteins from ethanol-calcium isolated cilia (38), most of the cilia became swollen and curled. Less than 1% of the cilia subsequently showed motility in ATP reactivation solutions, but those that did moved rapidly, at 5 to 10 beats/second for 5 minutes or more. Addition of 0.2 M sucrose and 25 mM KCl to the Tris-EDTA had no effect.

Although the cilia maintained their shape after extraction with solutions containing 65% ethanol and with TMSK, no motility was observed. The maintenance of ciliary shape was probably due to isotonicity of the solutions.

CONCLUSIONS

The major difference observed between the ethanol-calcium isolated cilia and the glycerol isolated cilia is that the former are not permeable to ATP, but can be rendered permeable by treatment with glycerol. After this treatment, both cilia preparations are about equally motile, and have comparable ATPase activities. The ethanol-calcium isolated cilia are also a bit more fragile, as demonstrated especially by their temperature sensitivity.

The cilia are motile in concentrated solution, but seem to lose some component vital for motility upon dilution. Motility is evident roughly between 0.1 and 2.0 mM ATP, higher concentrations being adverse. Motility in ADP begins at 0.3 mM, and continues through 3 mM. Inorganic phosphate production with ADP as a substrate is about 80% of that with ATP.

Xylose and dextrose, both of which are naturally present in high concentrations in cilia (12), cause a sharp increase in per cent motility but have no effect at all on the ATPase activity.

Other methods than glycerination exist for making the ethanol-calcium isolated cilia motile, such as digitonin in sucrose, and ethylene glycol. All of these methods seem to dissolve the membrane but have the proper osmotonicity to retain functional ciliary structure.

These experiments were terminated because motility could no longer be demonstrated in the isolated cilia. All procedures were carefully checked and all solutions prepared many times, but the cause for this disruption of motility remains obscure.

CHAPTER III

Protein Components of *Tetrahymena* Cilia

INTRODUCTION

When good methods became available for isolating large quantities of clean cilia, and the problem of membrane solubility was overcome, it was then much easier to separate and analyse the proteins of the axoneme.

Gibbons developed two methods for solubilizing and fractionating Tetrahymena cilia (38). The first was by extracting the cilia with 0.5% digitonin, which solubilized the membrane, leaving bare axonemes containing most of the ATPase activity. These were further fractionated by dialysis against Tris-EDTA, which separated the central fibers and the arms of the outer fibers (Fraction I) from the nine paired outer fibers (Fraction II). Fraction I sedimented at 4, 14 and 30 S, with the first two peaks containing ATPase activity, as described in the General Introduction. Fraction II consisted of heterogeneous aggregates sedimenting between 4 and 50 S.

The second fractionation method was by dialysis of whole cilia against Tris-EDTA, which disrupted the membrane and dissolved Fraction I.

Watson and Hynes (58) ran starch gel electrophoresis on Fractions I and II by breaking disulfide bonds, then dissolving the fractions in 8 M urea. They saw up to 5 subunits in each fraction, with the same banding pattern (with quantitative variations) in each case.

Cilia have long been known as a good antigenic source. The strain-specific agglutinizing antigens for Paramecium (71) and Tetrahymena (72) are produced in high quantity by isolated cilia (71, 72). This antigen,

however, is also found in a purified deciliate body wall fraction, so is probably a component of the cell membrane.

Watson et al. (59), using gel diffusion techniques with antibodies made against untreated ciliary segments, found 6 precipitin lines against ciliary extracts made in water or 0.9% NaCl. Extracts of cilia prepared in 3 N acetic acid, which solubilized 90% of the protein, showed 3 precipitin lines, some identical to those against the saline extract. This drastic solubilization technique was probably required because of the insolubility of the ciliary membrane, and these precipitin bands may partly represent membrane protein.

The work described in this chapter involves a brief study of the protein components of cilia, similar to or based on those studies mentioned here.

MATERIALS AND METHODS

In addition to the Materials and Methods reported in Chapter II, the following procedures were used.

Solubilization of cilia

A variety of solutions were employed in the attempt to solubilize and characterize ciliary proteins. The following are from Gibbons' work on ciliary solubilization (38):

1. Tris-Mg Solution - 2.5 mM $MgSO_4$, 30 mM Tris-thioglycollate buffer, pH 8.3 at 1° C.
2. Tris-EDTA Solution - 0.1 mM EDTA, 1 mM Tris-thioglycollate buffer, pH 8.3 at 1° C.
3. digitonin in Tris-Mg Solution - 0.25 g digitonin was warmed in 30 ml water, cooled in ice. 0.03 g $MgSO_4 \cdot 7H_2O$ and 0.18 g Tris were added. The pH was brought to 8.3 with thioglycollic acid, and the volume was adjusted to 50 ml.

Gel electrophoresis was performed on 7.5% acrylamide gels in a basic system prepared in 8 M urea after the manner of Jovin et al. (74). The proteins were added in 8 M urea or 20% sucrose for better layering. Gels were stained in 1% Amido Schwartz in a solvent of 5 methanol : 4 water : 1 acetic acid.

Immunotechniques were also used to characterize the proteins. Lyophilized whole cilia and cilia extract in 3.1 N HOAc were taken up in 0.9% saline and mixed with Fruend's Complete Adjuvant. Antibodies were produced by repeated injections into the toepads of rabbits.

Immunodiffusion and immunoelectrophoresis of the samples were performed on agar-coated slides (75). Immunodiffusion assays were also done against electrophoresed acrylamide gels of ciliary protein set in a well cut on an agar slide.

Treatment of dialysis tubing

This method for washing dialysis tubing was obtained from Dr. Anil Sadgopal.

One roll of dialysis tubing was soaked overnight in distilled water, washed thoroughly, boiled in water 30 minutes and washed again. It was then soaked at least 1 hour in 1% EDTA, washed and soaked overnight in 95% ethanol. After washing out the ethanol, it was stored refrigerated in 50% glycerol and washed thoroughly with distilled water before use.

RESULTS AND DISCUSSION

The ciliary proteins of Tetrahymena were fractionated by various methods and the fractions observed electrophoretically and immunologically. The cilia, isolated by Watson's ethanol extraction procedure, were frozen at -20° C until ready for use.

Between 20 and 30% of the total protein was extracted from these cilia by homogenization in water or dilute buffer. Other solubilization methods used included 3 N acetic acid and 10 M urea, each yielding about 90 to 95% of the total protein, and Gibbons' digitonin isolation, which, when preceded by a water extraction, put about 20% more of the protein into solution.

Electrophoresis of the fractions obtained by the above methods was done on acrylamide gel in 8 M urea in Tris buffer at pH 8.9. At this pH, most proteins migrated to the anode. Acetic acid extracts of the cilia showed seven distinct bands on the gels, indicating the presence of seven major protein species in large quantity in the cilia. The rf's in one typical gel were 0.12, 0.19, 0.31, 0.38, 0.45, 0.53, 0.83. The water extract, which did not contain any of the ATPase activity of the cilia, showed multiple diffuse banding and smearing on columns run in dilute salt without 8 M urea, but 2 or 3 distinct bands on urea columns, the two main bands being at 0.10 and 0.20. This seems to indicate that this fraction contains at least in part a polymerizable protein. The 10 M urea extract showed a pattern similar to that of 3 N acetic acid, with 10 bands visible. No bands were produced by extracts of cilia done with 0.6 M KCl or 0.6 M KI.

When 3 N acetic acid extracts and 8 M urea extracts were run on an acid acrylamide gel system (β-alanine system of Dr. Douglas Fambrough), a band appeared at 0.16 with each. No band was obtained from a 0.6 M KCl extract.

In double diffusion tests with antibody made against whole cilia, water extract of cilia formed 3 distinct bands. If left to develop 72 hours, 5 bands appeared against the digitonin extract of cilia (this would presumably include the bands in water extract), and one faint band appeared against a 0.6 M KCl extract of the precipitate from the digitonin extraction.

These results indicate that cilia contain a complex spectrum of proteins. The techniques used, however, did not lead to the development of a simple procedure to identify a major structural component of the cilia.

CHAPTER IV

Regeneration of *Tetrahymena* cilia

INTRODUCTION

Kinetosomes or basal bodies, the centriolar structures at the base of all cilia and flagella, remain in the cell after deciliation (13, 27, 28). The basal body is established as a necessary prerequisite for the development of the cilium (76), and this centriolar apparatus, with its ninefold symmetry, seems to be the morphological basis for the organization of the axoneme. Evidence exists that centrioles in many kinds of cells can become kinetosomes and originate cilia if properly stimulated (77). The basal bodies of Tetrahymena contain DNA ($\backsim 2 \times 10^{-16}$ g/basal body), whose function is yet unknown (78).

Regeneration of cilia from the site above the basal body in deciliated cells has been studied experimentally by a number of different methods. Hagen-Seyfferth induced flagellar shedding by the various methods of low pH, high pH, high temperatures or ethanol (78). Dubnau (79) and Rosenbaum and Child (80) studied regeneration of flagella removed by mechanical agitation. They found that the flagella elongated at a constantly decreasing rate in a process that depended on de novo protein synthesis. The site of assembly of the protein subunits was found autoradiographically to be at the tip of the growing flagellum.

Child studied regeneration of Tetrahymena cilia removed by sequential treatment of the cells with buffer at pH 6, Ca^{++} and shearing forces (81). He found that regeneration required oxidative phosphorylation, protein synthesis and DNA-dependent RNA synthesis. Colchicine, which inhibits the formation of the mitotic apparatus, the polymerization of actin (82)

and the elongation of ciliary buds in cultured fibroblasts (83), also inhibited reversibly the regeneration of Tetrahymena cilia but had no effect on the rates of RNA and protein synthesis. Since colchicine is known to bind to some protein subunits of cilia (69), its mechanism of action is probably through inhibition of polymerization.

Regeneration in Tetrahymena was observed here with the twofold purposes of (1) determining the physical state of the cells after deciliation, and (2) finding a system in which ciliary precursors could be studied, as was mentioned in the General Introduction.

MATERIALS AND METHODS

In addition to the Materials and Methods reported in Chapter II, the following procedures were used.

Deciliation

Cells from a 200 ml 2 day old culture of Tetrahymena were collected and resuspended in 25 ml of 0.05 N sodium acetate buffer at pH 5 in 0.1% EDTA. After gentle Vortex mixing, the cells were completely stripped of their cilia. At this point, the contractile vacuole was functioning in most of the cells. Cells were collected at 300 x g in a Lourdes centrifuge for 1 minute, resuspended in culture medium and observed at room temperature for ciliary regeneration up to 24 hours.

An earlier technique, obtained from F. Renaud, involved bringing the Tetrahymena to 0.01 M $CaCl_2$ after the addition of pH 5 buffer, but this step was found to be superfluous.

RESULTS AND DISCUSSION

Although contractile vacuole activity could be seen in most of the cells after they were deciliated by the ethanol-calcium procedure, washing them into fresh or used filtered proteose-peptone medium did not stimulate them to regenerate cilia within a few hours. These cells were generally two to three days old.

Washing a young (2 day) cell culture with Tris-EDTA and bringing it to 0.01 M $CaCl_2$ caused the cells to swim backwards and forwards with equal probability, but the addition of the Ca^{++} alone did not cause them to shed their cilia. After 15 minutes in the Ca^{++}, they swam much slower and became very uncoordinated.

Deciliating 2 day old cells by Renaud's pH 5 plus Ca^{++} technique, and resuspending them in fresh medium with 0.01 M Na phosphate buffer, pH 6.6, as has been included by Child for regeneration studies (81), resulted in ciliary regeneration on all cells that were still alive after four hours, but most of the cells were dead. A ten-fold increase in the buffer concentration resulted in an even higher mortality rate. The proteose-peptone medium itself buffers at pH 6.7, and phosphate at all pHs from 6.1 to 7.8 exhibited some toxicity.

The cilia were observed to come off the cells even before the Ca^{++} was added, so pH 5 shock was sufficient to deciliate Tetrahymena.

When the regeneration experiments were done as described in Materials and Methods, the cells were at pH 5 for less than 5 minutes total time. In all the preparations, at least half the cells maintained their shape

and contractile vacuole activity immediately after treatment, but the number of living deciliate cells decreased progressively. When the cells were replaced in used filtered (Whatman #1) medium rather than fresh medium some improvement was noted.

The best ciliary regeneration was obtained from cells about 36 hours old. Most of these survived the treatment, as indicated by contractile vacuole activity. These cells were completely stripped of their cilia, so that not even any oral cilia were visible. The cells were then divided into two lots and replaced in either new or used medium. The results of one such experiment are described in Table I.

These results confirm the report that Tetrahymena are not destroyed by deciliation, but are able to regenerate cilia again within a relatively short period of time (80). Since the normal generation time of Tetrahymena is about 6 to 8 hours, and no dividing cells were seen during ciliary regeneration, the deciliation process may not turn on protein synthesis but may merely delay cell division and avail itself of the normal protein synthetic patterns for generation of cilia.

This work was begun with the main goal of studying the synthesis of specific proteins during regeneration. Since the techniques tried for identifying specific proteins did not appear satisfactory (Chapter III), the regeneration studies were not pursued further.

TABLE I

Regeneration of Tetrahymena Cilia

TIME	NEW MEDIUM	USED MEDIUM
40 min.	No movement, no cilia visible.	No movement, no cilia visible.
2 hours	About 30% had oral cilia. Occasional cells had cilia scattered on surface, were slowly moving.	About 70% had oral cilia. Many had scattered cilia and were slowly moving.
4 hours	About 30% moved faster than at 2 hours, but not as fast as normal.	Most cells showed some motility.
6½ hours	Regeneration almost total. Cells moved almost as fast as normal.	Regeneration almost total. Cells like normal.
9 hours	Complete regeneration.	Complete regeneration.
24 hours	Cells had multiplied.	Cells had multiplied.

REFERENCES

1. Leeuwenhoek, A. van. Philos. Trans. London 12, 821 (1675).

2. Müller, O.E. Animalcula Infusoria, Fluviatilia et Marina Havniae. 367 pp (1786).

3. Gray, J. Ciliary Movement. Cambridge U. Press, London. 162 pp. (1928).

4. Sleigh, M.A. The Biology of Cilia and Flagella. Pergamon Press, New York, 242 pp. (1962).

5. Bishop, D.W. Physiol. Revs. 42, 1 (1962).

6. Sharpey, W. Cyclopedia of Anatomy and Physiology, ed. R.B. Todd, Longman, Brown, Green, Longman and Roberts, London 1, 606 (1835).

7. Manton, I., and B. Clarke. J. Exp. Bot. 3, 265 (1952).

8. Goldstein, S. Ph.D. Thesis, Calif. Inst. of Tech. (1968).

9. Sleigh, M.A. Handbook of Molecular Cytology, ed. A. Lima-de-Faria North Holland Publ. Co., Amsterdam , p. 1243 (1969).

10. Ringo, D.L. J. Ultrastruc. Res. 17, 266 (1967).

11. André, J. J. Ultrastruc. Res. 5, 86 (1961).

12. Culbertson, J.R. J. Protozool. 13, 397 (1966).

13. Watson, M.R. and J.M. Hopkins. Exp. Cell Res. 28, 280 (1962).

14. Jones, R.F. and R.A. Lewin. Exp. Cell Res. 19, 408 (1960).

15. Tibbs, J. Biochim. Biophys. Acta 28, 636 (1958).

16. Engelmann, T.W. Dictionnaire de Physiologie, ed. C. Richet, Paris 3, 783 (1898).

17. Hoffmann-Berling, H. Biochim. Biophys. Acta 14, 182 (1954).

18. Szent-Györgyi, A. Biol. Bull. 96, 140 (1949).

19. Weber, H. and H. Portzehl. Prog. Biophys. 4, 60 (1954).

20. Hoffmann-Berling, H. Biochim. Biophys. Acta 16, 146 (1955).

21. Engelhardt, V.A. Adv. in Enzymol. 6, 147 (1946).

22. Engelhardt, V.A. Bull. Acad. Sci. USSR Ser. Biol. p. 182 (1954).

23. Brokaw, C.J. Exp. Cell Res. 22, 151 (1961).

24. Gibbons, I.R. J. Cell Biol. 25, 400 (1965).

25. Brokaw, C.J. and B. Benedict. Arch. Biochem. Biophys. 125, 770 (1968).

26. Goldstein, S. J. Exp. Biol. 51, 431 (1969).

27. Child, F.M. Exp. Cell Res. 18, 258 (1959).

28. Rosenbaum, J. personal communication.

29. Davies, R.E., D.F. Cain, A.A. Infante, D. Klaupiks, and W.A. Eaton. Biochemistry of Muscle Contraction, ed. J. Gergeley. Little, Brown and Co., Boston. p. 463 (1964).

30. Tibbs, J. Biochim. Biophys. Acta 23, 275 (1957).

31. Burnasheva, S.A., M.V. Efremenko and M.N. Lyubimova. Biokhimiya 28, 442 (1963).

32. Watson, M.R., J.M. Hopkins and J.T. Randall. Exp. Cell Res. 23, 629 (1961).

33. Nelson, L. Biol. Bull. 109, 295 (1955).

34. Raff, E.C. and J.J. Blum. J. Cell Biol. 31, 445 (1966).

35. Bailey, J. Biochem. J. 36, 121 (1942).

36. Gibbons, I.R. J. Biol. Chem. 241, 5590 (1966).

37. Culbertson, J.R. Science 153, 1390 (1966).

38. Gibbons, I.R. Arch Biol. (Liège) 76, 317 (1965).

39. Gibbons, I.R. and A.J. Rowe. Science 149, 424 (1965).

40. Mohri, H., S. Hasegawa, M. Yamamoto and S. Murakami. Sci. Pap. Coll. Gen. Ed. U. Tokyo 19, 195 (1969).

41. Brokaw, C.J. Science 156, 76 (1967).

42. Brokaw, C.J. and B. Benedict. Submitted for publication.

43. Renaud, F.L., A.J. Rowe and I.R. Gibbons. J. Cell Biol. 36, 79 (1968).

44. Shelansky, M.L. and E.W. Taylor. Biophys. J. 8, A-187 (1968).

45. Shelansky, M.L. and E.W. Taylor. J. Cell Biol. 34, 549 (1967).

46. Stephens, R.E. and R.W. Linck. J. Mol. Biol. 47, 353 (1970).

47. Hayashi, T. Spermatozoan Motility, ed. D.W. Bishop. AAAS Publ. #72, Washington, D.C. p. 279 (1962).

48. Gibbons, I.R. Ann. Rev. Biochem. 37, 521 (1968).

49. Stephens, R.E. Science 168, 845 (1970).

50. Stephens, R.E., F.L. Renaud and I.R. Gibbons. Science 156, 1606 (1967).

51. Raff, E.C. and J.J. Blum. J. Biol. Chem. 244, 366 (1969).

52. Gibbons, I.R. J. Cell Biol. 26, 707 (1965).

53. Kinoshita, S. J. Fac. Sci. U. Tokyo, Sec. IV. 8, 427 (1959).

54. Hanson, J. and H.E. Huxley. Symp. Soc. Exptl. Biol. 9, 228 (1955).

55. Satir, P. J. Cell. Biol. 26, 805 (1965).

56. Winicur, S. J. Cell Biol. 35, C7 (1967).

57. Finck, H. and F.J. Wilson. Biophys. Soc. Abstr., 14th Ann. Mtng. p. 81a (1970).

58. Watson, M.R. and R.D Hynes. Exp. Cell Res. 42, 348 (1966).

59. Watson, M.R., J.B. Alexander and N.R. Silvester. Exp. Cell Res. 33, 112 (1964).

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

61. Colowick, S.P. and N.O. Kaplan. Methods in Enzymology, V. III, p. 450, Acad. Press, Inc., New York (1967).

62. Oser, B.L. Hawk's Physiological Chemistry 14th ed. V. IV, V, VI. McGraw Hill, New York (1965).

63. Holwill, M.E.J. and N.R. Silvester. J. Exptl. Biol. 47, 249 (1967).

64. Fiske, C.H. and Y. SubbaRow. J. Biol. Chem. 66, 375 (1925).

65. Taussky, H.H. and E. Shorr. J. Biol. Chem. 202, 675 (1953).

66. Bishop, D.W. Fed. Proc. 17, 15 (1958).

67. Brokaw, C.J. J. Exp. Biol. 40, 149 (1963).

68. Sedar, A.W. and K.R. Porter. J. Biophys. Biochem. Cytol. 1, 583 (1955).

69. Borissy, G.G. and E.W. Taylor. J. Cell Biol. 34, 533 (1967).

70. Watts, D.C. and L.H. Bannister. Nature 226, 450 (1970).

71. Preer, J.R. Jr. and L.B. Preer. J. Protozool. 6, 88 (1959).

72. Margolin, P., J.B. Loefer and R.D. Owen. J. Protozool. 6, 207 (1959).

73. Winicur, S. Unpublished results.

74. Jovin, T., A. Chrambach and M.A. Naughton. Analyt. Biochem. 9, 351 (1964).

75. Sanders, B. Personal communication.

76. Randall, J. and C. Disbrey. Proc. Roy. Soc., Ser. B. 162, 473 (1965).

77. Scherft, J.P. and W.T. Daems. J. Ultrastruct. Res. 19, 546 (1967).

78. Hagen-Seyfferth, M. Planta 53, 376 (1959).

79. Dubnau, D.A. Ph.D. Thesis, Columbia U., N.Y. University Microfilms, Ann Arbor, Mich. (#61-3427).

80. Rosenbaum, J.L. and F.M. Child. J. Cell Biol. 34, 345 (1967).

81. Child, F.M. J. Cell Biol. 27, 18A (1965).

82. Forsheit, A.B. and T. Hayashi. Biochim. Biophys. Acta 147, 546 (1967).

83. Stubblefield, E. and B.R. Brinkley. J. Cell Biol. 30, 645 (1966).

LIST OF ABBREVIATIONS

ADP adenosine diphosphate

AMP adenosine monophosphate

ATP adenosine triphosphate

DNA deoxyribonucleic acid

EDTA ethylenediamine tetraacetic acid, disodium salt

GTP guanosine triphosphate

P_i inorganic phosphate

RNA ribonucleic acid

TCA trichloroacetic acid

Tris Tris(hydroxymethyl)aminomethane

PART II

CHITINASE ACTIVITY DURING *DROSOPHILA* DEVELOPMENT

INTRODUCTION

Arthropod cuticle

One of the most distinctive characteristics in the evolution of arthropods is the development of an external skeleton. This exoskeleton, the cuticle, is a complex structure secreted by the hypodermis, a layer of epidermal cells resting on a basement membrane.

This integument is distinguishable into several separate regions. On the outside is the epicuticle, a thin layer composed of protein and lipid. Between this and the hypodermis lies the procuticle, which is subdivided into an outer exocuticle, brittle and often pigmented, and an inner endocuticle, lamellated and flexible. One of the principal constituents of the procuticle is the structural polysaccharide chitin, a β-1,4-linked linear polymer of N-acetylglucosamine units; this chitin is predominantly responsible for the lamellated format of the procuticle. The procuticle is composed of about half chitin and half protein, with small amounts of pigments, polyhydric phenols and inorganic materials.

Molting in arthropods

The problem of animal growth within a rigid, inextensible exoskeleton is solved by the process of molting, or ecdysis. During each intermolt period the hypodermis secretes a molting fluid or molting gel and underlays that with a new epicuticle, beneath which

chitin synthesis for the new cuticle occurs. The molting fluid solubilizes the old endocuticle, and the animal sheds its outgrown integument.

Plotnikov (1) and Tower (2), at the turn of the century, first suggested that the actual function of molting fluid was the digestion and solution of the inner layers of old cuticle. Wigglesworth demonstrated protease activity in molting fluid and suggested the existence of a chitinase (3), and Hamamura and Kanehara, in 1940, provided the first experimental proof of chitinolytic activity during molting in the silkworm Bombyx mori (4). In Crustacea, chitinase is elaborated permanently throughout the life cycle, and a burst of chitobiase activity (to be defined later) characterizes the molt (5). In insects, however, chitinase secretion is cyclic. In the silkworm, in which molting has been comprehensively studied, no chitinase activity is demonstrable during intermolt; it is secreted for the molting period and its activity drops sharply with the shedding of the old integument (5).

Molting in the silkworm

The role of chitinase in molting has been studied most completely in the silkworm. In studies on the pupal to adult molt in the Cecropia silkworm, Passonneau and Williams (6) observed that early molting fluid, during the first 2/3 of the intermolt stage, is a gel low in protease and chitinase activity. Liquefaction is accompanied by solubilization of the endocuticle and a 135x increase in chitinase activity. This is

probably due to the secretion of new enzyme rather than the activation of a prochitinase, because the addition of proteolytic enzymes to early molting fluid of a silkworm has no effect (7). Chitinolytic activity in the silkworm is very low during intermolt, and peaks sharply just before the larval and pupal molts (5).

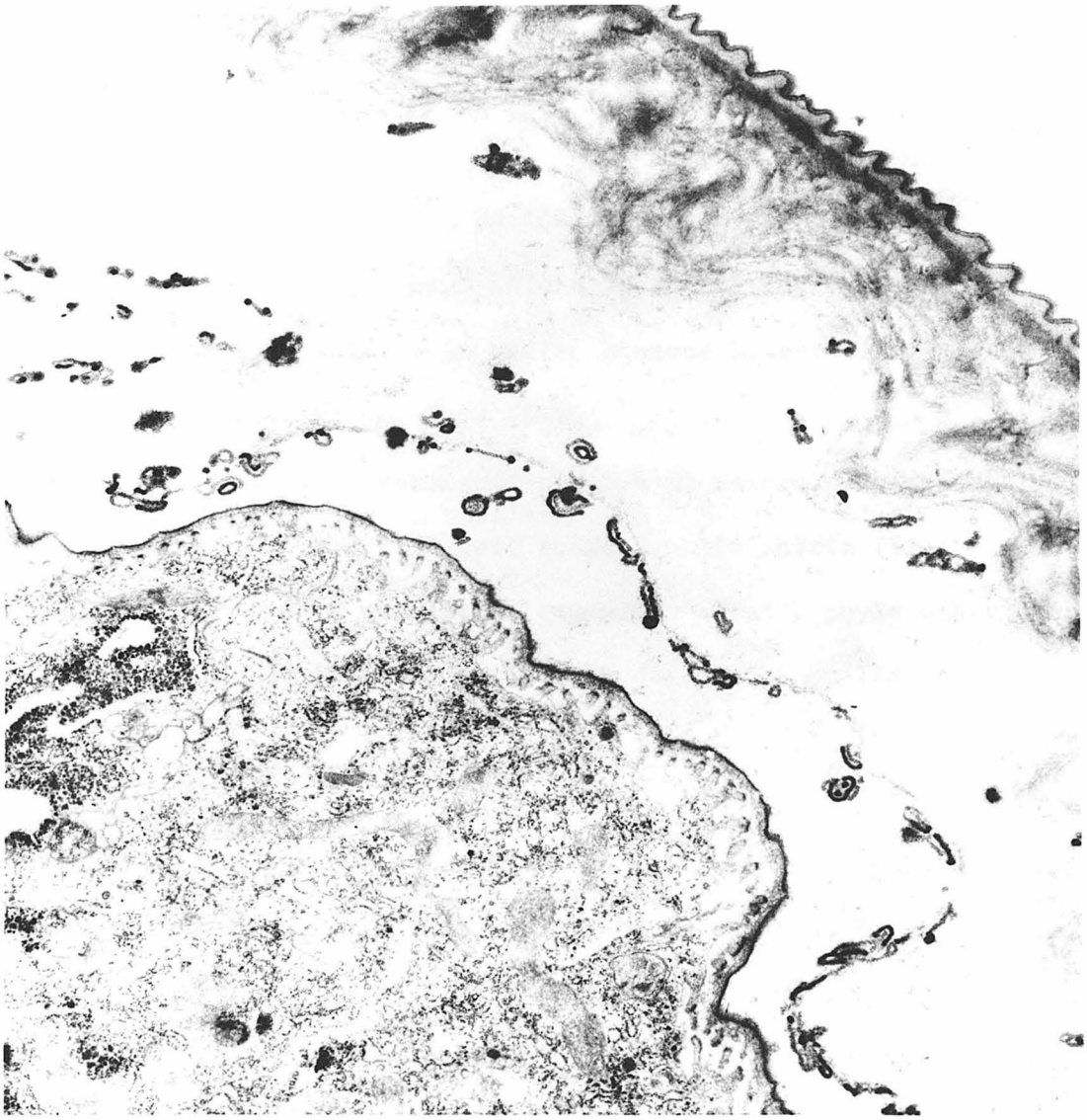
Although the digestion products of the molting fluid are reabsorbed directly into the animal, as shown by absorption of such compounds as neutral red (3), fluorescein (8), C^{14}-glycine (6) and C^{14}-glucose (9) in normal and ligated silkworm prenymphs, the enzymes are probably filtered out by the epidermis, and at least some of the chitinase is recoverable from air-dried cast larval skins (5, 10, 11).

Molting in Drosophila

The molting pattern in the fruit fly Drosophila melanogaster varies a bit from the silkworm. Drosophila has two larval molts, at which extensive chitin degradation occurs. Unlike the silkworm, the molting fluid is not secreted far in advance of endocuticle degradation and there is no obvious chitin breakdown during the pupal to adult transition.

In recent electron micrographs taken by Mitchell et al (12) of the second larval instar, covering 48 to 72 hours, one can see the synthesis and subsequent degradation of the chitinous endocuticle occurring directly above the epidermal cells (Figure 1). The new epicuticle forms and endocuticle begins to be deposited only 4 hours before the molt. A similar situation prevails during the first instar. A diagram of the

Figure 1. Degradation of the endocuticle in late second instar Drosophila melanogaster larvae. The chitin lamellae in the undigested remainder of the endocuticle are apparent under the old epicuticle; the endocuticle has been digested away from the space below it by molting fluid. The new epicuticle can be seen forming along the border of the epithelial cell. (1 μ = 1.97 cm) (12).



life cycle of Drosophila with the pattern of chitin synthesis and development is shown in Figure 2 (12, 13).

Chitin

The development of chitin as the major component of the arthropod exoskeleton is not surprising. Chitin is an amazingly sturdy and resistant polymer, insoluble in water, aqueous bases, dilute acids, alcohol and all organic solvents (14).

Chitin appears in several other roles in nature. Most fungal cell walls and most groups of soil fauna contain chitin (15). As would be expected with such a ubiquitous compound, several phyla other than Arthropoda have developed the ability to digest and utilize chitin.

Chitinases in nature

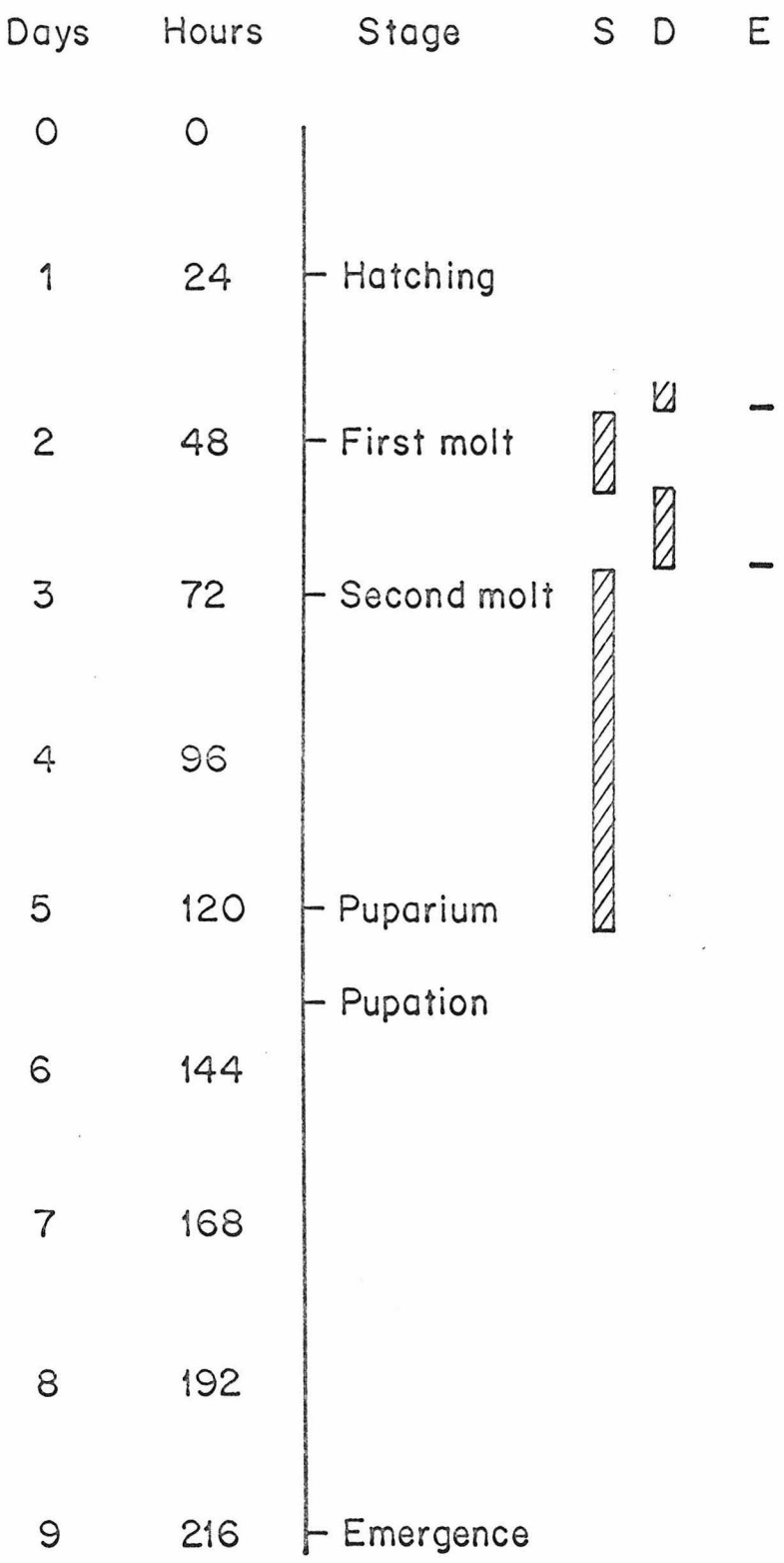
The existence of chitinolytic bacteria has been known since 1905 (16) and chitinase has since been observed in numerous bacterial species, especially Streptococci, where it has been well studied (17, 18). It is also prevalent in several varieties of fungi (15, 19). Chitinase has been found in higher plants such as almonds (20) and bean seeds (21), and its role here is speculated as being fungicidal. The gut of some species of oligochaetes (22, 23) and several gastropod mollusks (24) also contain chitinolytic enzymes, but there is good evidence that in at least one specie of snail the chitinase activity is produced by strains of enteric bacteria (25). Insects manifest chitinase activity elsewhere than just in the molting fluid; small amounts of activity have been found in the gut, hemolymph and saliva of the cockroach (11).

Figure 2. Drosophila melanogaster life cycle. Stages of chitin synthesis and degradation from 40 hours are shown (12, 13).

S = chitin synthesis phases

D = chitin degradation phases

E = epicuticle synthesis phases


Days
Hours
Stage
S
D
E
0
0
1
24
Hatching
2
48
First molt
3
72
Second molt
4
96
5
120
Puparium
Pupation
6
144
7
168
8
192
9
216
Emergence

Characteristics of chitinases

Hydrolysis of chitin to N-acetylglucosamine in all well-characterized systems turns out to be due to a chitinolytic series of two consecutively acting hydrolases (7). The first is chitinase, an endoenzyme hydrolyzing the chitin chain into shorter chain lengths and generally ineffective on polymers shorter than a tetrose. The second enzyme in the series is a chitobiase, which hydrolyses the bioses and trioses to N-acetylglucosamine. This sequence was first demonstrated in 1939 by Zechmeister and Toth (20) and has been consistently reported in bacteria (17), fungi (26), higher plants (21) and arthropods (5, 27).

All the chitinases studied have a pH optimum in the acid range, generally between 3.5 and 5.5. The molecular weight is usually below 50,000, and no co-factor requirement has ever been identified. These enzymes demonstrate a remarkable stability; the streptococcal chitinase is stable to freezing for over 2 years and has a half life of 40 days at 37° C and 230 days at 5° C (11, 15, 17, 28, 29, 30).

Often 2 or 3 fractions with chitinolytic activity can be separated in the extract from a given organism (17, 29), but whether or not these are all true chitinases remains to be seen, since chitin synthetase has been shown to be reversible in at least one case (31), and lysozyme (from egg white) is known to digest colloidal chitin (32). The 3 chitinases isolated from Streptococcus by Jeuniaux show some synergistic effect on recombination (17), but no synergism was found by Powning and Irzykiewicz between 2 purified cockroach chitinases (29).

Research objectives

The object of the work described here was to elucidate the pattern of chitinolytic activity during the life cycle of the fruit fly, *Drosophila melanogaster*. Chitinase as a molting enzyme is closely tied to the control of development in *Drosophila*, and characterization of the role of this enzyme would provide a good system in which to study some of the factors affecting development.

Efforts were also made to determine the number of chitinases present in the organism, and preliminary purifications and characterization were attempted.

MATERIALS AND METHODS

Culture and collection of Drosophila

Drosophila melanogaster of the Oregon R wild type were raised and collected essentially as described by Mitchell and Mitchell (33). To increase egg yields, the flies were kept in a larger lucite box (28 x 24 x 24 in.), in which twice as many flies, about 50,000, could be accommodated. Eggs were collected in the bottom tray of lucite collecting boxes (12 x 4 x 4 in.) for 1½ to 2 hours as described (33). Enough water to keep the environment humid was added on the second day, and a yeast slurry on the third if the larvae were to be maintained past the second instar.

Homogenization of larvae

Larvae collected for study were washed onto nylon net, cleaned from food fragments by floating them in 10% NaCl, washed thoroughly and air dried on filter paper. They were either frozen in liquid nitrogen and stored at -70° C for later use or homogenized immediately (generally at a concentration of 200 mg larvae/ml in water or 10^{-3} M Cleland's Reagent) in a ground glass homogenizer of 30 ml capacity with a motor-driven pestle. Thorough homogenization of the larvae was accomplished by lifting the homogenizer against the rotating pestle 300 to 400 times.

The homogenate was centrifuged in a Servall SM-24 head at 28,000 x g for 15 minutes, and the supernatant fraction was pipetted off and

retained. This was either used immediately or frozen at -16° C. No significant differences in enzyme activity were observed as a result of freezing either the larvae or the larval supernatant.

Life cycle studies

For the study of chitinase activity during the life cycle of Drosophila melanogaster, the eggs were collected and maintained as previously described. Eggs, larvae or pupae of the desired age were gathered and homogenized in water at a concentration of 200 mg/ml. The homogenates were centrifuged, and the supernatants retained and frozen until the isolation of animals at various stages of the life cycle was completed; then they were thawed and assayed together for enzyme activity. A graph of the life cycle of D. melanogaster is shown in Figure 2.

For the study of some of the later stages an effort was made to synchronize the cultures at the second molt. Larvae just at and following the second molt float in 70% NaCl (34), so the floaters were separated and discarded at one or two points during the late second instar. However, the continued immersion in NaCl seemed to delay molting in some cases, and may account for some scatter in the data.

Chitosan preparation

Chitosan, a soluble product of the partial deacetylation of chitin, was commercially available from K & K Labs.

About 35 g of chitosan were ground to powder with porcelain balls in a ball mill of 1 liter capacity for 3 days. The powder was thoroughly washed with water over a Buchner funnel. To this, 900 ml of 10% acetic acid was added slowly and the mixture stirred to dissolve overnight. Chitosan is acid soluble, and precipitates out of solution around pH 7. The chitosan was dialysed at first briefly against water, then against 0.4 M NaCl in 0.1 M sodium acetate buffer, pH 5.0, to equilibrium.

To 500 ml of the above, about 900 ml of the NaCl + acetate buffer was added, bringing the η_{sp} of the chitosan to 12.3. The chitosan concentration of the solution was 15.0 mg/ml.

Viscometric assay

Of the three most commonly used techniques for measuring chitinase activity (viscometric assay of chitosan breakdown, measurement of turbidometric decrease in a chitin suspension, and colorimetric determination of N-acetylglucosamine production) (28), the viscometric assay was chosen as the most practical for following chitinase activity during the life cycle of the fly. Chitosan is well documented as a substrate for chitinase activity (15, 28), and the assay is simple and rapidly performed.

The turbidometric assay was not favored because of the probable interference due to varying turbidity of the supernatant fraction of crude larval homogenates, and the exact colorimetric determination of N-acetylglucosamine seemed an arduous procedure easily interfered with by other sugars and more suited for measuring the activity of an

exoenzyme such as chitobiase, splitting off terminal sugar moieties, than an endoenzyme like chitinase.

The assay procedure used was modelled after that of Tracey (15). To start the assay, 0.6 ml of the sample to be assayed for enzyme activity was added to 3.0 ml of the dialysed chitosan solution in a 28 ml test tube in a water bath maintained at 34° C. After 30 minutes, 3.0 ml of this mixture were transferred to a U-type capillary viscometer in the same water bath and the flow time through the viscometer measured to 0.1 second with a stopwatch. Mixing of the reaction during the 30 minute assay procedure was found to be superfluous, and Reynolds has found that mixing did not assume significance in a similar system until the temperature was brought below 28° C (35).

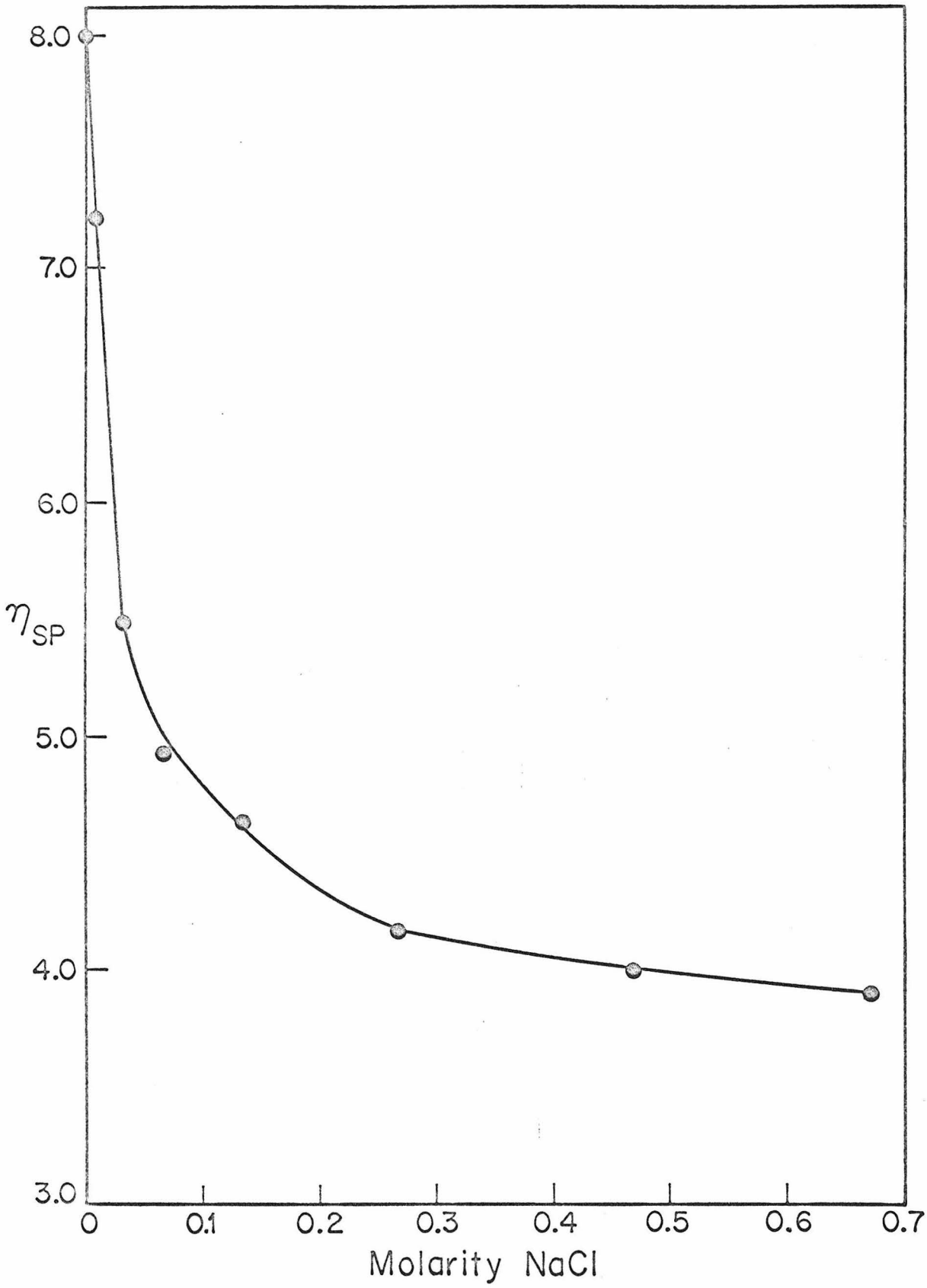
The viscometers, made by H.K. Mitchell, had a 5 ml capacity in their reserve bulb, a 1 ml capacity in the measuring bulb, and a 1 mm bore in the capillary tubing. Smaller bore led to coating and clogging of the tube by the chitosan.

The NaCl was required in the dialysed chitosan solution because of the extreme sensitivity of chitosan viscosity to changes in ionic strength at low ionic strengths, as shown in Figure 3. At a concentration of 0.4 M NaCl, small changes in salt concentration have virtually no effect on chitosan viscosity.

The experimental data are expressed in terms of specific viscosity, η_{sp}, which is calculated as follows:

$$\eta_{sp} = \frac{\text{flow time of unknown liquid}}{\text{flow time of water}} - 1$$

Figure 3. Effect of salt on the viscosity of chitosan. Assays were run on a chitosan solution of 5.7 mg/ml at 35° C.


8.0
7.0
6.0
5.0
4.0
3.0
η_{SP}
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Molarity NaCl

The assay was standardized for measuring enzyme activity by using known concentrations of CalBioChem Chitinase Lot#73217. A 1 mg/ml solution of CalBioChem Chitinase was prepared in water and centrifuged for 15 minutes at 28,000 x g in a Servall SM-24 head to eliminate debris. The η_{sp} of various dilutions of the supernatant fraction were determined and the results plotted against enzyme concentration for a standard curve. Since this curve covered the range of enzyme activity in the larval extracts, arbitrary units were designated along the abscissa, 1 unit being equivalent to the η_{sp} of the supernatant of 0.1 mg/ml of CalBioChem Chitinase under the conditions used in this assay (Figure 4). Experimental results were read off this standard curve as units/ml of sample assayed.

Protein determinations

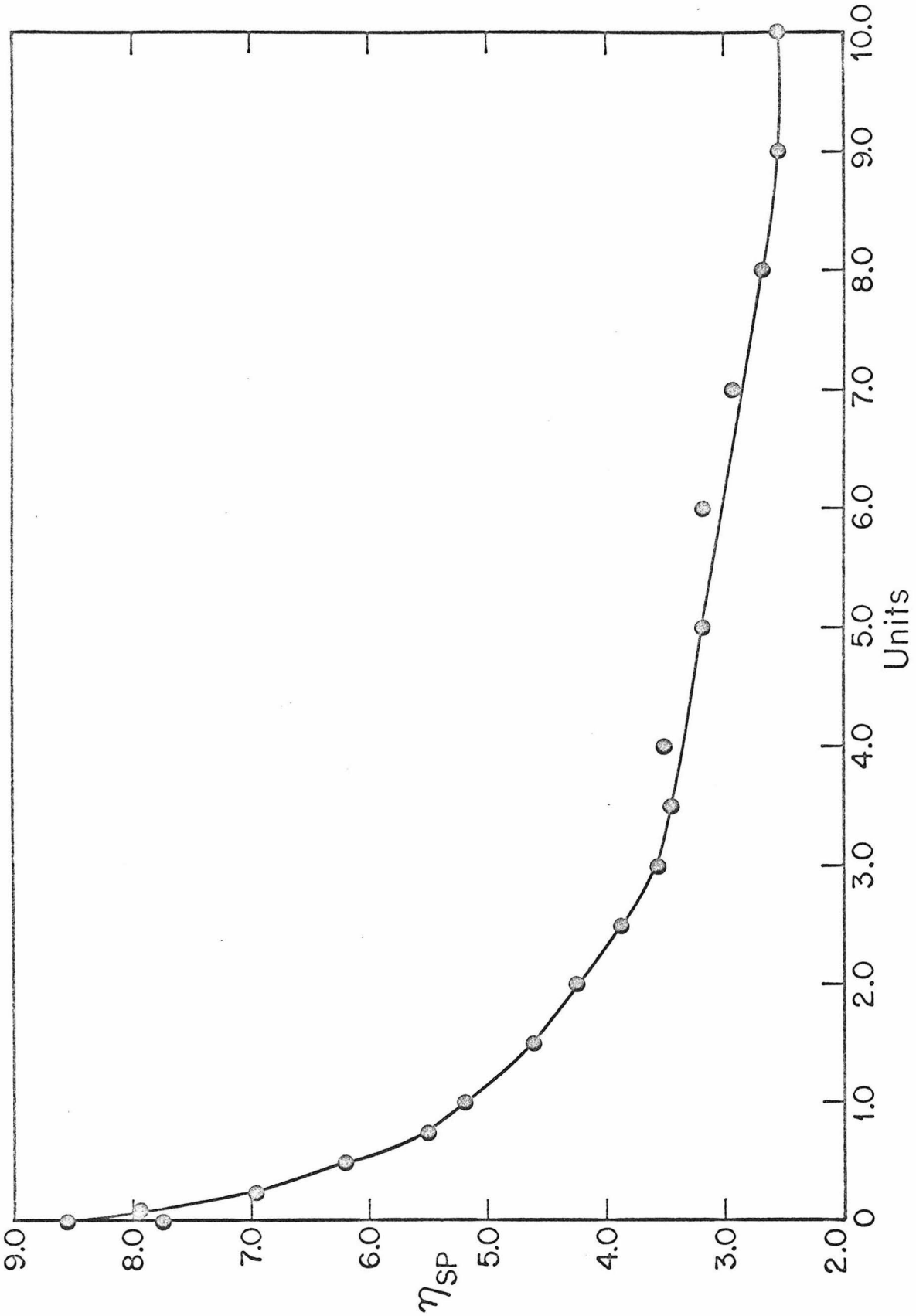
Up to 1 mg of protein was precipitated from solution with 10% TCA and heated at 80° C for 15 minutes. The samples were cooled and centrifuged for 20 minutes at full speed in a Servall Superspeed Desk Centrifuge. The supernatant TCA was removed with a bent tip capillary pipette and the procedure was repeated.

The precipitate was dissolved in 0.2 ml of 0.2 M NaOH, followed by 0.5 ml Biuret Reagent (36), and the OD at 550 mμ read after 30 minutes. The assay was standardized against bovine serum albumin.

Ammonium sulfate precipitation

The $(NH_4)_2SO_4$ precipitation of protein was always done on the pH 5.4 soluble fraction of the larval supernatant. After the acid

Figure 4. Standard curve with CalBioChem Chitinase. Assays were done by mixing 2.5 ml of a 15.0 mg/ml chitosan solution at 0.4 M NaCl in 0.1 M sodium acetate buffer, pH 5.0, with 0.5 ml of chitinase solution and measuring the viscosity after 30 minutes at 34° C. Each unit represents the activity of the 28,000 x g supernatant of 0.1 mg/ml CalBioChem Chitinase.


9.0
8.0
7.0
6.0
η_{SP}
5.0
4.0
3.0
2.0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
Units

precipitation with 0.1 M sodium acetate buffer, all the activity was retained in the soluble fraction. The protein samples were then brought to the desired salt concentration by adding either saturated or solid $(NH_4)_2SO_4$ and leaving them to stand in the cold room for 14 hours. The precipitated proteins were centrifuged out, resuspended in the acetate buffer and dialysed against the buffer for about 24 hours.

Ultrafiltration

Enzyme concentrating and desalting were often performed on Diaflo membranes UM-10, PM-30 or XM-50 in Amicon ultrafiltration cells Model #12 (10 ml) or Model #52 (65 ml). Nitrogen pressure was used between 15 and 30 psi.

Isoelectric focusing

Isoelectric focusing was done in an LKB 8101 Electrofocusing Column in a sucrose density gradient set up according to the instruction manual, with the protein sample incorporated into a few of the central test tubes. Ampholyte ranges of pH 3-10 and pH 3-6 were studied, and fractions were collected by hand in graduated test tubes, using a peristaltic pump to collect regions with a large protein precipitate.

Column chromatography

Bio-Gel P-10 Bio-Gel P-10, 50 to 150 mesh, was swelled in distilled water overnight and washed repeatedly in distilled water. A 60 ml column, 18 cm x 2 cm, was poured. The void volume was determined with Blue Dextran 2000 and the included volume by running 2 M KCl over the column and checking the efflux with a conductivity meter. The sample was layered on the top of the column, and 5 ml fractions were collected during elution on a Gilson fraction collector. The OD_{280} of the tubes was determined to check the location of the protein fraction.

Bio-Gel CM-2 Bio-Gel CM-2, 100 to 200 mesh, was swelled in distilled water, washed in 2 M KCl in 0.05 N sodium acetate buffer, pH 5.5, and equlibrated in 0.05 N acetate buffer. An 18 ml column, 1.5 cm x 16 cm, was poured and the larval supernatant was layered on the top. Fractions of 5 ml were collected during elution and the protein peaks located as described for Bio-Gel P-10.

DEAE-Sephadex A-25 DEAE-Sephadex A-25 was swelled and washed in 0.05 M Tris pH 8.2 in 10^{-3} M Cleland's Reagent. A 60 ml column, 18 cm x 2 cm, was poured. The larval supernatant was layered on the top and the flow rate was adjusted to 4 drops/minute. A linear gradient of 125 ml 0.05 M Tris pH 8.2 and 125 ml 2 M KCl in 0.05 M Tris pH 8.2, both in 10^{-3} M Cleland's Reagent, was set up. The column was run at $4^{o}C$ under a pressure head of 30 cm, and 5 ml fractions were collected.

RESULTS

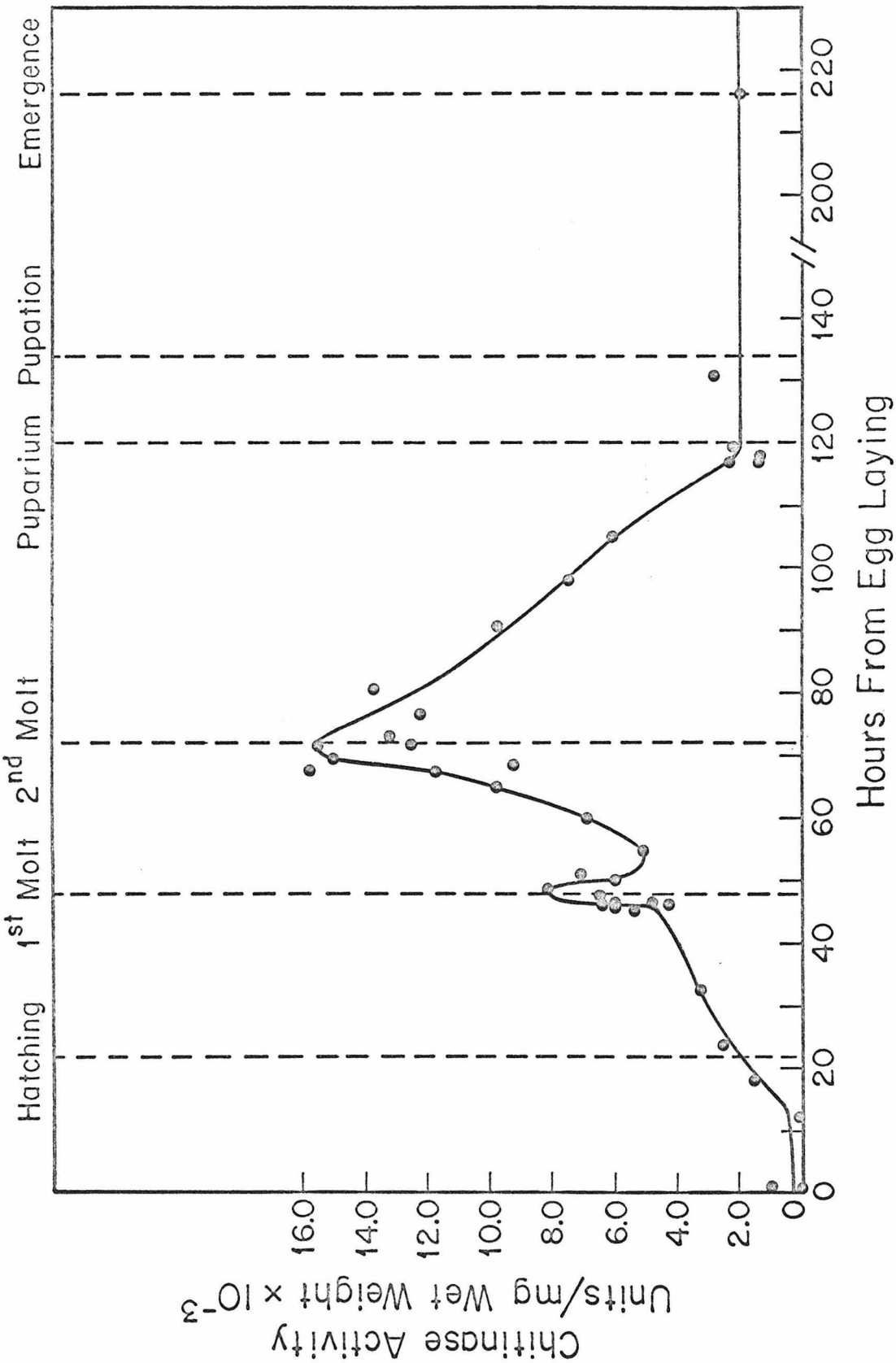
Life cycle

Chitinase activity in Drosophila has been followed from egg to emergence by the viscometric assay described in Materials and Methods. Each determination, at each stage in the life cycle, was performed on the supernatant fraction of 200 mg wet weight Drosophila/ml extract. Chitinase activity of the homogenate is about equal to that of the supernatant, so all the activity is in the soluble fraction.

The pattern of chitinase activity/mg wet weight is shown in Figure 5. The activity, which is lowest in the egg, rises gradually from hatching to 46 hours, at which point it starts to rise abruptly until the first molt. It drops off rapidly after the molt about to the level of activity at 46 hours and starts to climb again at 55 hours, peaking at the second molt (72 hours) at double the value obtained at the first. The decline of chitinase activity between second molt and puparium is gradual and only at puparium formation, 120 hours, does the activity become steady at a constant low level which is maintained through emergence. The enzyme activities represented here were measured in one complete series of experiments, but the results of other experiments done at various stages of the life cycle always fell in the vicinity of these curves.

Although both Drosophila larval wet weight and total soluble protein rise steadily through the life cycle the ratio of total soluble protein (i.e. protein in the supernatant extract) to wet weight shows

Figure 5. Chitinase Activity: units/mg wet weight through the life cycle. Activity of the supernatant fraction of an extract of 200 mg/ml Drosophila was assayed viscometrically with a chitosan substrate at intervals through the life cycle as described in Materials and Methods. Each time point represents the approximate midpoint of an egg laying period of about 1½ hours.


Chitinase Activity
Units/mg Wet Weight × 10^{-3}
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
Hatching
1st Molt
2nd Molt
Puparium
Pupation
Emergence
0
20
40
60
80
100
120
140
200
220
Hours From Egg Laying

variations due to cyclic fluctuations in wet weight from water storage, build up and degradation of chitin, etc. Figure 6 shows the graph of mg soluble protein/mg wet weight. A drop in soluble protein can be observed before each molt and continuing through it, and a maximum of soluble protein/wet weight appears during late third instar, at about 105 hours. The scatter around each molt probably indicates the difficulty in precisely synchronizing the animals at a period of rapid change in the parameter being measured.

In Figure 7, the chitinase activity/mg soluble protein has been calculated. The peaks of chitinase activity at each molt are intensified and the activity is focused a bit more around each molting period, but the pattern of activity is not significantly changed from that in Figure 5.

Pre-imago Drosophila were weighed at intervals from zero to 140 hours. The average weight of an animal varies from 0.008 mg in the egg to a high of 2.030 mg during the third instar (see Figure 8). This represents a change of greater than 2 orders of magnitude in the weight and therefore in the number of larvae represented in each determination. In order to follow what was happening to an individual larva during its growth, the amount of chitinase activity/wet weight of a single larva was calculated and is depicted in Figure 8.

This presents a totally different picture than the activity/mg wet weight or soluble protein. Chitinase activity in a single larva rises steadily from hatching and peaks during third instar in the vicinity of 100 hours. Activity falls from that point until puparium formation, where it levels off.

Figure 6. Variation in soluble protein/wet weight in pre-imago *Drosophila* from egg laying to emergence. The boxes enclose the scatter in these values around each molt and the curve is drawn through the mean.

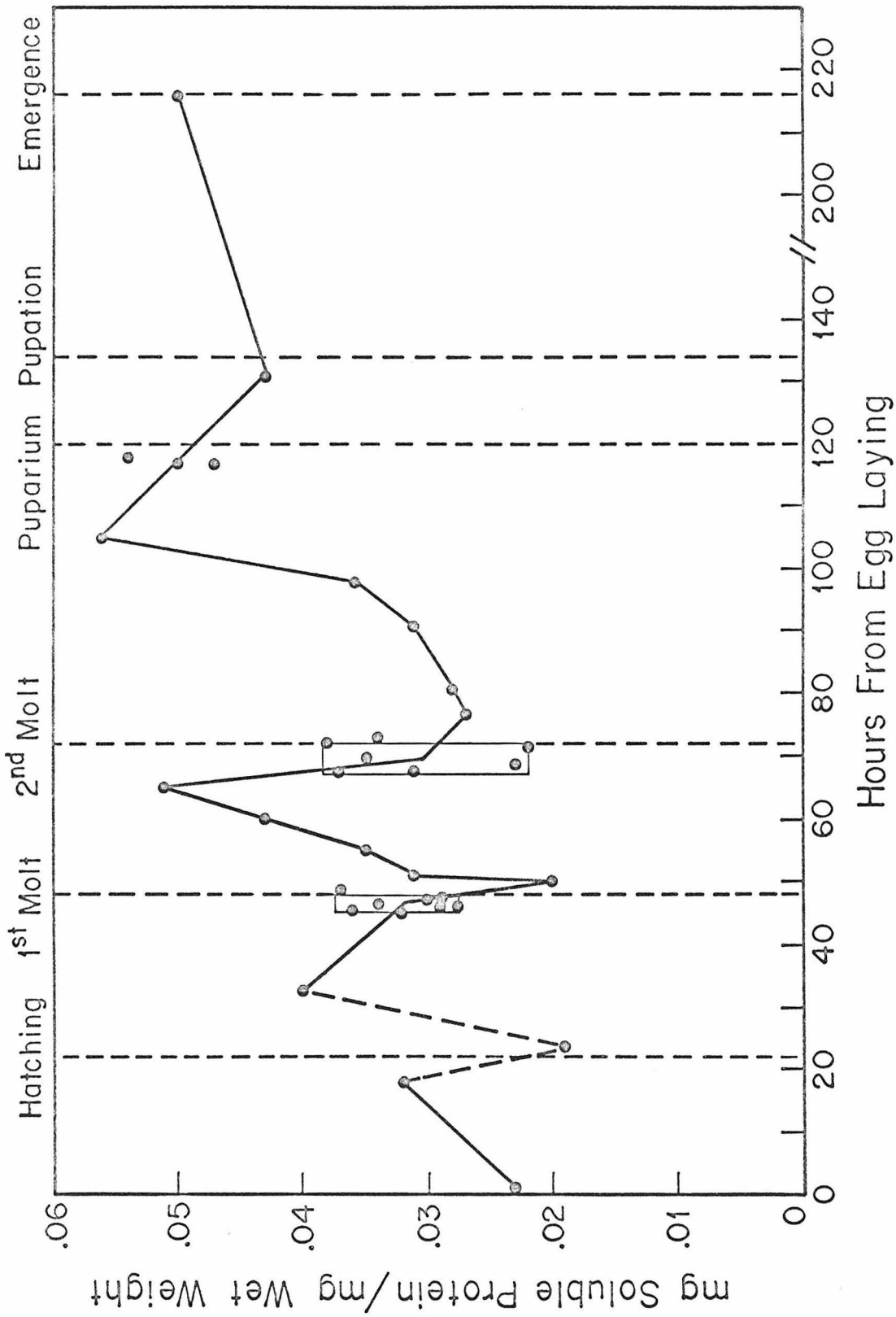

mg Soluble Protein/mg Wet Weight
.06
.05
.04
.03
.02
.01
0
Hatching
1st Molt
2nd Molt
Puparium
Pupation
Emergence
0
20
40
60
80
100
120
140
200
220
Hours From Egg Laying

Figure 7. Chitinase Activity: units/mg soluble protein through the life cycle. Activity was determined as described for Figure 5 and calculated/mg soluble protein according to the data in Figure 6.

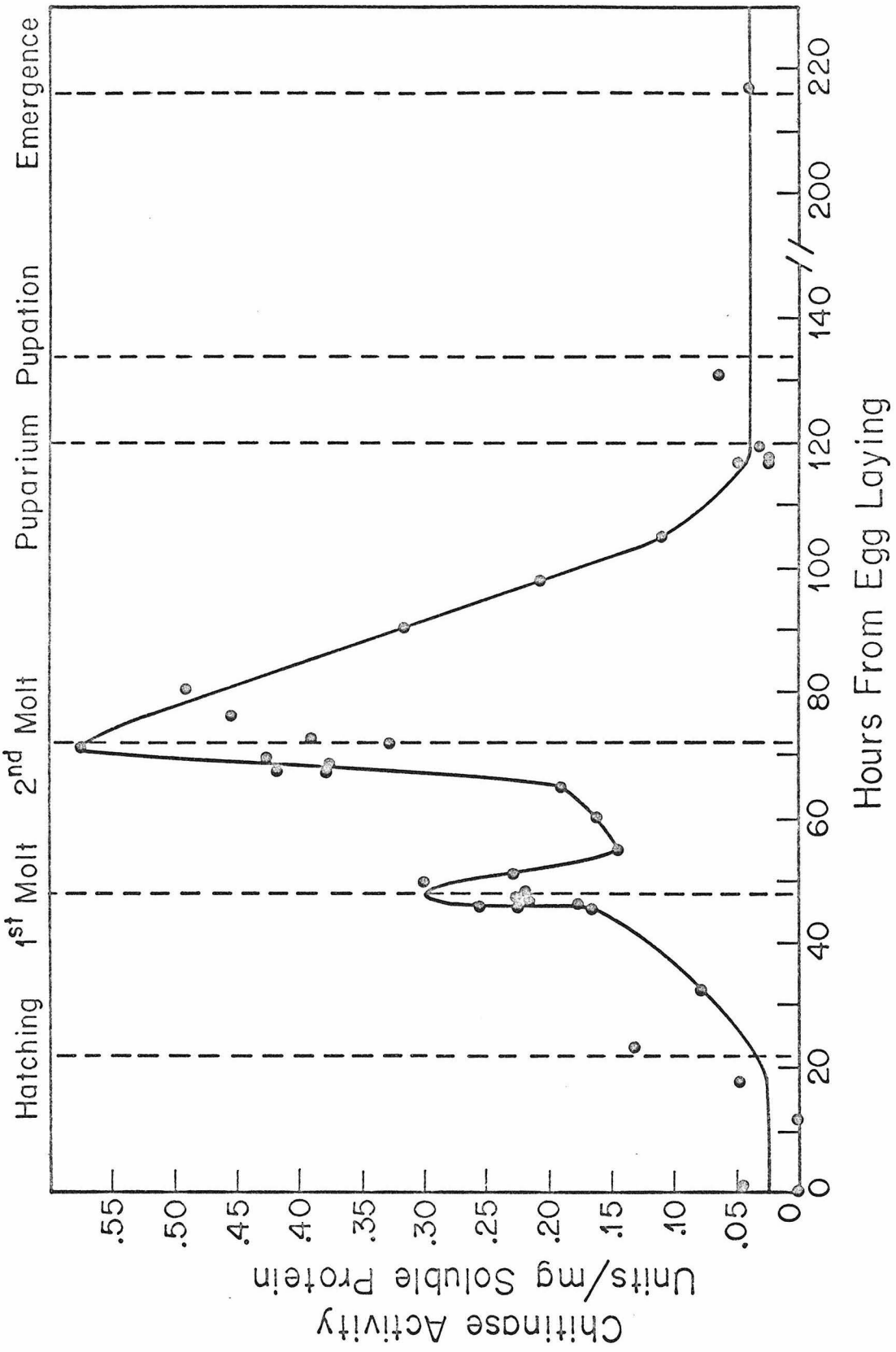
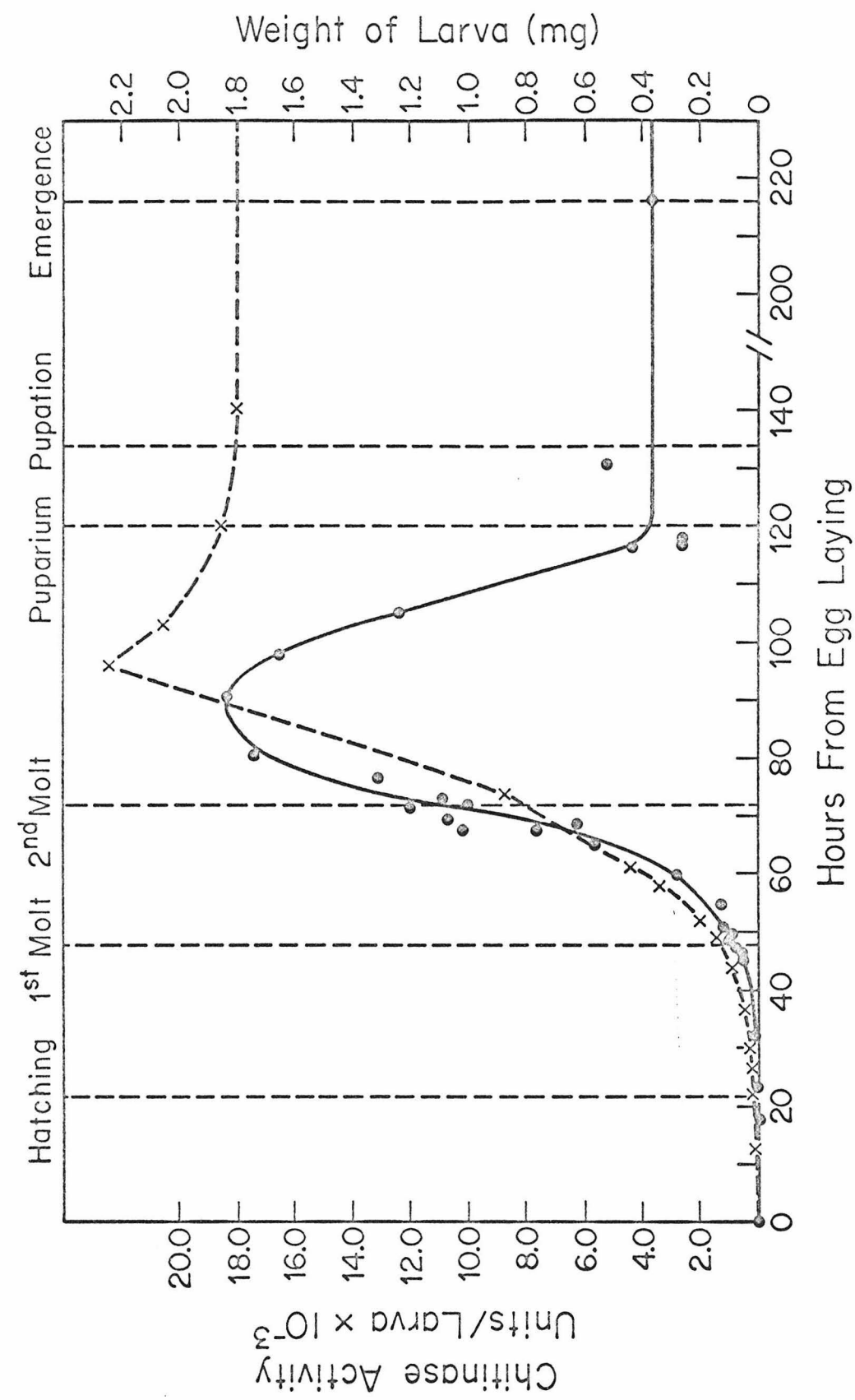

Chitinase Activity
Units/mg Soluble Protein
.55
.50
.45
.40
.35
.30
.25
.20
.15
.10
.05
0
Hatching
1st Molt
2nd Molt
Puparium
Pupation
Emergence
0
20
40
60
80
100
120
140
200
220
Hours From Egg Laying

Figure 8. ● = Chitinase Activity: units/individual larva through the life cycle. Activity was determined as described for Figure 5.

x = Variation in weight of an individual larva through the life cycle.


Weight of Larva (mg)
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Emergence
Pupation
Puparium
2nd Molt
1st Molt
Hatching
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
0
20
40
60
80
100
120
140
200
220
Hours From Egg Laying
Chitinase Activity
Units/Larva x 10⁻³

Since the mg soluble protein/mg wet weight is not constant, but varies depending on the stage of the life cycle (Figure 6), the values for units/larva were adjusted to reflect this variation. Figure 9 represents the chitinase activity as units/larva x mg soluble protein/mg wet weight. The activity rises slowly with a slight peak at the second molt and reaches a maximum at 105 hours, which is also the maximum for mg soluble protein/mg wet weight. The activity generally follows the units/larva curve of Figure 8.

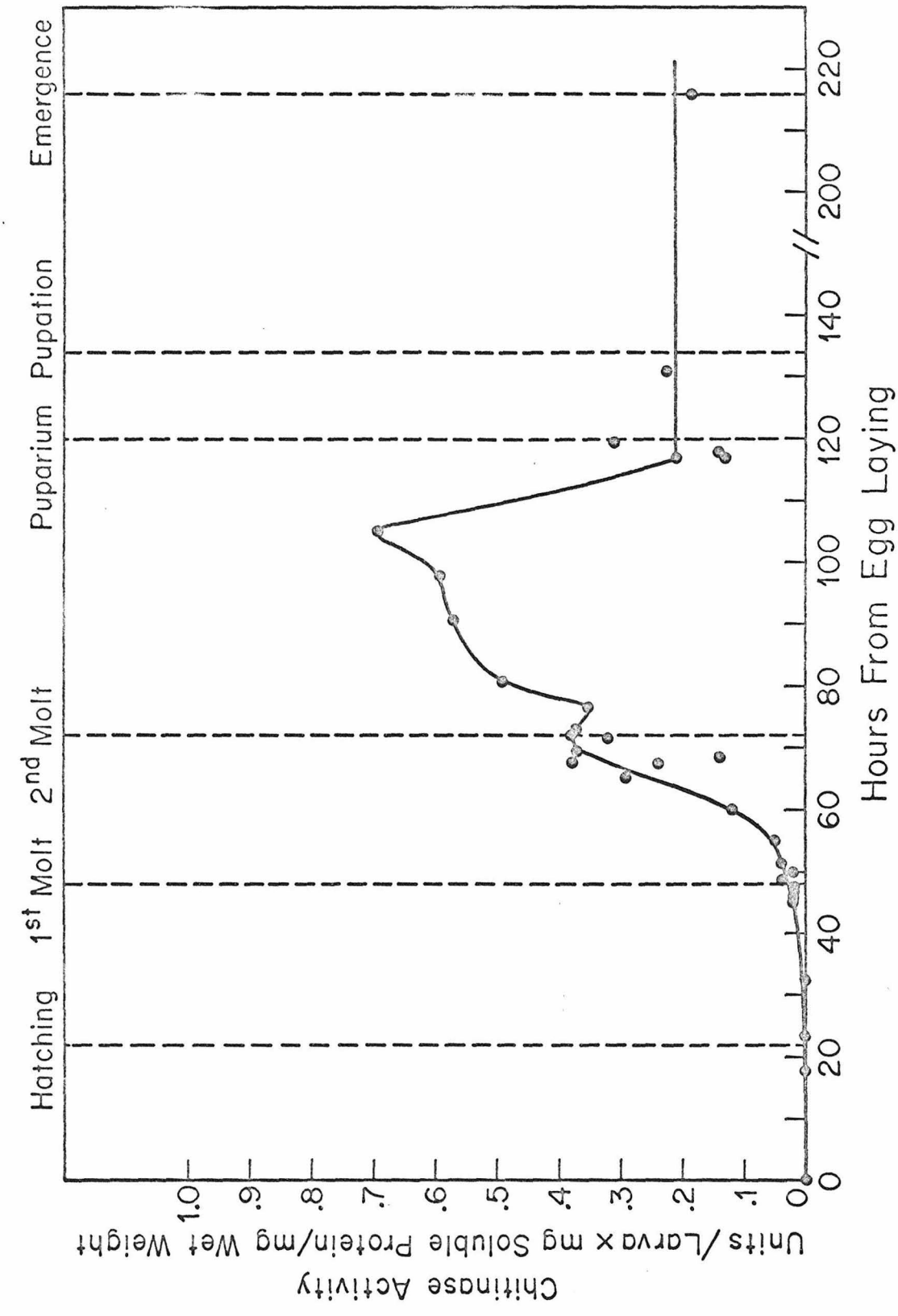
Properties of the assay

In order to study the properties and attempt a purification of chitinase, late second instar larvae were collected in bulk, frozen in liquid nitrogen and stored at -70° C. They were thawed as needed and homogenized in water or 10^{-3} M Cleland's Reagent. These demonstrated no loss in activity over fresh homogenized larvae. In most of the following studies, the larvae were homogenized in 10^{-3} M Cleland's Reagent because the black color that develops from phenol oxidase activity interfered with protein determinations. Neither Cleland's Reagent nor phenol oxidase activity, however, had any effect on the chitinase activity.

Since the standard curve was set up with CalBioChem Chitinase, a dilution series was run with larval supernatant to check the accuracy of the curve with the larval system.

The viscometric assay was also tested for the effect of protein on the assay. There was no change in the viscosity of the chitosan solution

Figure 9. Chitinase Activity: units/individual larva calculated for mg soluble protein/mg wet weight through the life cycle. Activity was determined as described for Figure 5.


Chitinase Activity
Units /Larva x mg Soluble Protein/mg Wet Weight
1.0
.9
.8
.7
.6
.5
.4
.3
.2
.1
0
Hatching
1st Molt
2nd Molt
Puparium
Pupation
Emergence
0
20
40
60
80
100
120
140
200
220
Hours From Egg Laying

when boiled larval supernatant was assayed (~4 mg/ml protein), nor was there an effect from a 2 mg/ml solution of bovine serum albumin.

Properties of the enzyme

Cofactor requirement *Drosophila* chitinase has no loosely bound cofactor that can be separated by dialysis, ultrafiltration or by running the larval extract over a Bio-Gel P-10 column.

On dialysis of the enzyme against water or 0.05 M sodium acetate buffer pH 5.5, 80 to 100% of the activity was maintained.

Ultrafiltration of the larval supernatant was done in a Model 12 Amicon ultrafiltration cell (10 ml capacity) on a UM-10 Diaflo membrane, which has an exclusion limit of 10,000 molecular weight. At about 5° C, under a pressure of 30 psi, 10 ml of larval supernatant were concentrated to 1 ml, reconstituted to 10 ml in 0.05 M Tris buffer (made to pH 7.7 at room temperature) in 10^{-3} M Cleland's Reagent. Over a period of 4 days the larval supernatant was concentrated to 1 ml and rediluted 4 times. Samples from each dilution were diluted 1:1 with water or with the ultrafiltrate from the first concentration and assayed for chitinase activity. The results are shown in Table I. The activation that occurs after ultrafiltration is a somewhat ubiquitous phenomenon, appearing often after freezing, treatment at pH 5.5 and on some columns. The reason for this effect is unknown.

A Bio-Gel P-10 column was run in distilled water as described in Materials and Methods. This gel has a molecular weight exclusion limit

TABLE I

Ultrafiltration of Larval Supernatant on a UM-10 Diaflo Membrane

Sample	Larval Supernatant + Water 1:1 (units)	Larval Supernatant + Ultrafiltrate 1:1 (units)
Original extract	0.40	-
First dilution	0.55	0.50
Second dilution	0.65	0.65
Third dilution	0.60	0.60
Fourth dilution	0.60	0.60
Water alone	0	-
Ultrafiltrate alone	-	0

of 10,000. An aliquot of 3 ml of larval supernatant containing 4.2 units of activity were layered on the top. About 4.0 units, 95% of the activity, were recovered in the void volume. Recombining the enzyme fraction with an aliquot from the included volume did not alter the activity.

Ca^{++} and Mg^{++} had no effect on enzyme activity when added in concentrations of 5×10^{-3} M, 5×10^{-2} M or 10^{-1} M.

Stability Chitinase activity in Drosophila extracts is very stable, as shown in Table II.

Isoelectric focusing When an isoelectric focusing column was run over the pH range 3 to 10, the chitinase activity focused in one peak between pH 2.80 and 3.95. Running the column over a narrower range, pH 3 to 6, and collecting 5 ml fractions, focused the activity in a single peak between pH 3.7 and 3.9 with the isoelectric point probably about pH 3.8 (see Figure 10). Roughly 60% of the enzyme activity could be accounted for, and about a five-fold purification with respect to protein concentration was accomplished.

Preliminary purification of chitinase

Acid precipitation Samples of larval supernatant were diluted 1:1 with 0.1 M sodium acetate buffer at pHs 5.5, 4.9, 4.5 and 4.0. The precipitates were centrifuged out at 28,000 x g and rediluted in 0.1 M acetate buffer pH 5.5. Supernatants and precipitates were assayed for chitinase activity, and the results are given in Table III. Treatment at both pH 4.9 and 5.5 precipitates about 60% of the protein, yielding

TABLE II

Stability of Chitinase Activity in the Larval Supernatant

#	Sample	Units
1	Larval supernatant from fresh larvae (late second instar) immediately after preparation	2.0
2	Larval supernatant of #1 frozen 24 hours	1.9
3	Larval supernatant of #1 frozen 4 days	1.9
4	Larval supernatant of #1 refrigerated 24 hours	1.9
5	Larval supernatant of #1 refrigerated 23 days (very black)	2.0
6	Larval supernatant of #1 at 55° C for 1 minute	1.3
7	Larval supernatant of #1 boiled for 1 minute	0
8	Larval supernatant of #1 with 1 mg of $\sum$ pronase added to the viscometric assay	1.9
9	Larval supernatant of larvae frozen in liquid nitrogen and stored at -70° C for a few weeks, immediately after preparation	2.0

Figure 10. Isoelectric focusing column over the pH range 3 to 6. Fractions of 5 ml were collected and assayed for chitinase activity viscometrically with a chitosan substrate as described in Materials and Methods. The high background viscosity at low pH, diminishing toward higher pH, is due to the viscosity effect of the sucrose in the gradient in which the column is run.

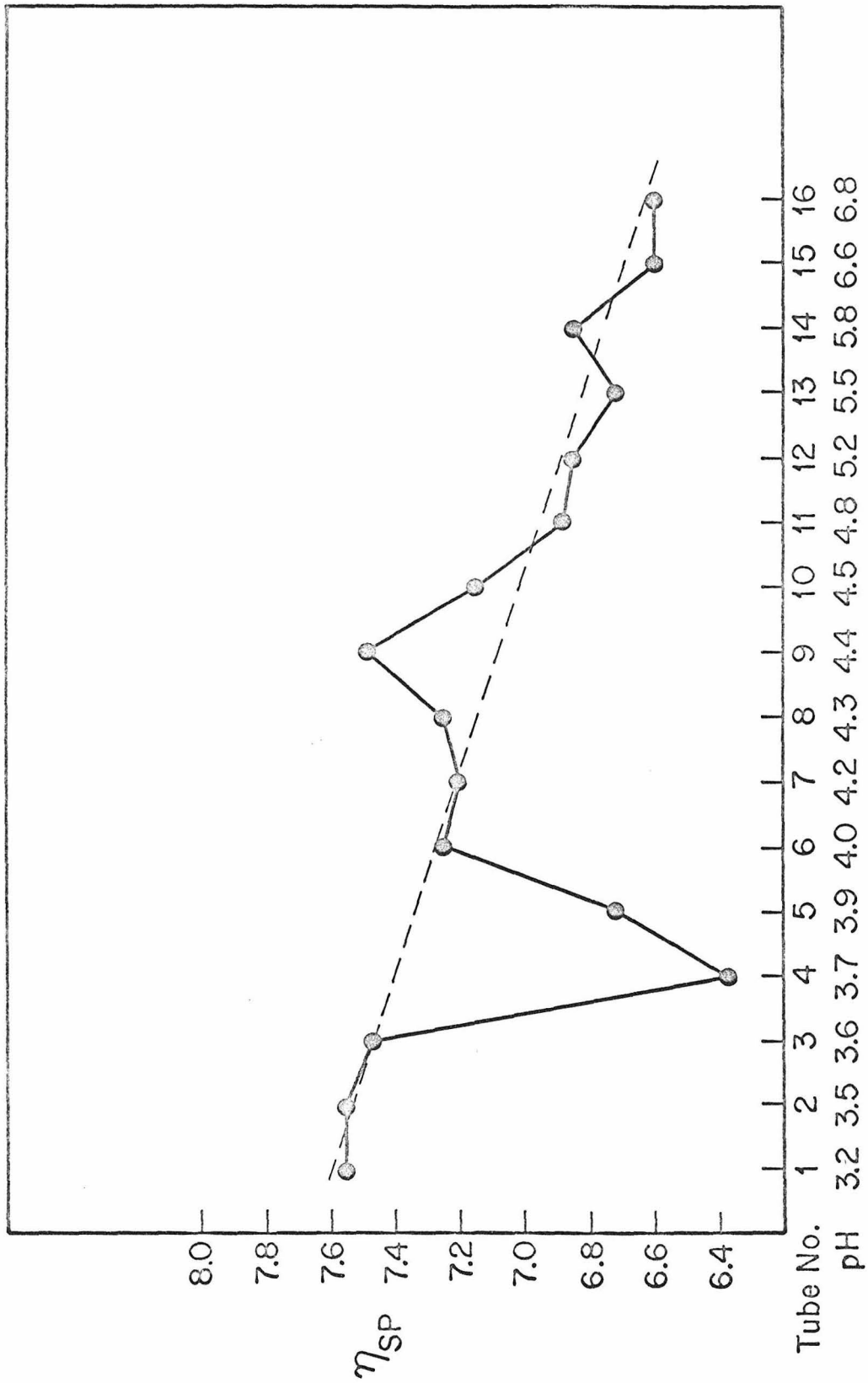

8.0
7.8
7.6
7.4
7.2
7.0
6.8
6.6
6.4
η_{SP}
Tube No. 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
pH 3.2 3.5 3.6 3.7 3.9 4.0 4.2 4.3 4.4 4.5 4.8 5.2 5.5 5.8 6.6 6.8

TABLE III

Acid Precipitation of Larval Supernatant

pH Treatment of Larval Supernatant	Units in Supernatant	Units in Precipitate	Total Units	% Recovery
Untreated larval supernatant	10.9	-	10.9	
pH 5.5	14.0	0.4	14.4	132
pH 4.9	5.9	1.6	7.5	69
pH 4.5	1.7	1.9	3.6	33
pH 4.0	0.3	1.6	1.9	17

a two- to three-fold purification at pH 5.5, and essentially no purification at pH 4.9.

Ammonium sulfate precipitation Results from $(NH_4)_2SO_4$ precipitation first showed that more than one enzyme might be operant. Ammonium sulfate precipitation was always preceded by pH 5.5 precipitation of the larval supernatant. Chitinase activity was precipitable in 2 peaks, the first at 30 to 50% ammonium sulfate, which contained 56% of the activity and 35% of the protein, and a later peak in the 50 to 70% fraction containing 16% of the activity and 30% of the protein.

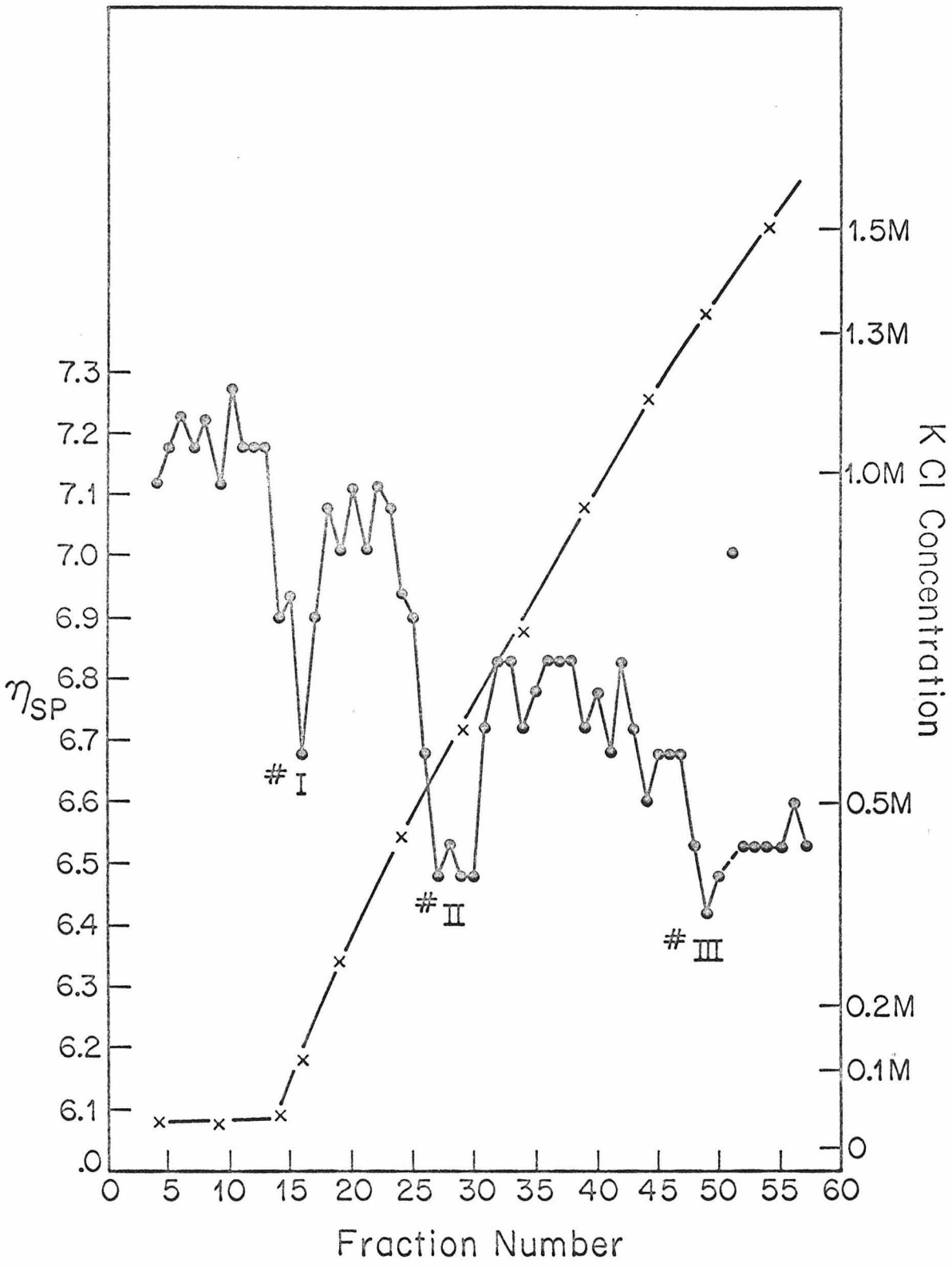
Column chromatography on Bio-Gel CM-2 A column of Bio-Gel CM-2, a cation exchanger, was set up and 6 ml of larval supernatant containing 8.4 units of activity were added to the top. A stepwise elution was performed in 5 steps with 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M KCL in 0.05 M acetate buffer pH 5.5. All the activity, 9.8 units, and more than 90% of the protein were recovered in the void volume, so a slight activation of chitinase was observed by this treatment also.

Column chromatography on DEAE-Sephadex A-25 A column of DEAE-Sephadex A-25, an anion exchanger, was set up as described in Materials and Methods. The larval supernatant, 6 ml containing 13.8 units, was layered on top and a linear gradient was run from 0 to 2 M KCl. About 95% of the OD_{280} was recovered but only 42% of the activity, in 3 separate peaks (Figure 11). Peak #1 contained 2.15 units, 15.6% of the activity, peak #2 had 3.50 units, 25.4% of the activity and peak #3 had 0.2 units, 1.5%.

Figure 11. Chromatographic Separation of Chitinases on DEAE-Sephadex. 13.8 units in 6 ml were applied to a column of 18 cm x 2 cm and the column was eluted by a linear gradient from 0 to 2 M KCl in 0.05 M Tris pH 8.2 in 10^{-3} M Cleland's Reagent. The viscosity decrease with Fraction Number is due to the effect of the increasing salt concentration on the viscosity of the chitosan.

● = chitinase activity

x = KCl concentration


7.3
7.2
7.1
7.0
6.9
6.8
6.7
6.6
6.5
6.4
6.3
6.2
6.1
.0
η_{SP}
#I
#II
#III
1.5M
1.3M
1.0M
0.5M
0.2M
0.1M
0
K Cl Concentration
0 5 10 15 20 25 30 35 40 45 50 55 60
Fraction Number

DISCUSSION

Life cycle

Events preceding the sloughing off of the larval case during molting in Drosophila are separable into a number of parallel sequences which can be followed biochemically and electron microscopically. The event of most concern to this thesis is the production of the enzyme chitinase for the digestion of endocuticular chitin before the emergence of the larva. The production of molting fluid by the epithelial cells and the thinning of the cuticle before each molt lead to the supposition that there is a cyclic production of chitinase as a component of the molting fluid, and that this chitinase is lost, along with the rest of the molting fluid, with the epicuticle at molting. The testing of this hypothesis is the primary purpose of these experiments.

As would be expected, there are 2 definite peaks of chitinase activity in the pre-imago Drosophila, one appearing at each molt. The chitinase activity/mg wet weight, shown in Figure 5, was considered to be a rough measurement due to the dependence of the wet weight on such phenomena as the cyclic nature of the thickness of the endocuticle and the rapid intake of water and food by the larvae directly after each molt. Chitinase activity/mg protein was considered a better determination of actual developmental changes in Drosophila chitinase, so the ratio of mg protein to mg wet weight was determined (Figure 6).

As expected from the afore-mentioned hypothesis, the ratio of soluble protein to wet weight starts to diminish before each molt, continuing for a short time beyond it. The peak of soluble protein in late third instar has also been observed in D. melanogaster by other researchers (37).

The chitinase activity/mg soluble protein (Figure 7) shows the 2 peaks of activity much more sharply defined at the molts. The expected production of chitinase activity before molting is well substantiated from these graphs.

It is interesting to compare the data for chitinase activity with the physical picture of the cuticle at these stages. Mitchell et al (12) have taken a series of electron micrographs of the cuticle which elucidate the pattern of synthesis and degradation of chitin (e.g. Figure 1). The time sequence of these events is depicted in Figure 2.

The visible degradation of the chitinous lamellae of the endocuticle begins at about 56 hours in the second instar and reaches a maximum at about 68 hours (digestion probably proceeding submicroscopically until molting). At 56 hours, the chitinase activity also begins to rise, so this enzyme is becoming active just as degradation begins. This would seem to indicate that the activity of the peaks is definitely due to the molting enzyme (at least during the second instar). There is also a change in the appearance of the cells at this time as they change over from their role in chitin synthesis to their role in chitin degradation. They change from sturdy cells with microvilli and rough endoplasmic reticulum to cells with a faint cell membrane, vacuolated cytoplasm and generally secretory appearance (12).

If all the chitinase activity were in the molting fluid, one would expect the activity to be completely lost with the loss of cuticle and molting fluid directly following the molt, as seems to be the case in the silkworm (5). There are 2 points however which stand out in Figure 7. The first is the substantial background of chitinase activity apparent throughout the life cycle, and the second is the slow decline of activity after the second molt. These data are probably not due to larval retention of molting fluid chitinase, because although some components of the molting fluid are resorbed into the animal (3, 6, 8, 9), the enzymes generally seem to be excluded (5, 10, 11).

In order to see if this gradual decline of activity was just due to enzyme being retained by the growing larvae or actually due to new enzyme synthesis, the weight/larva was measured and the total chitinase activity in an individual larva was determined (Figure 8). For the reasons mentioned before, this activity was adjusted for the ratio of soluble protein to wet weight (Figure 9). These results indicate quite clearly that chitinase activity increases fairly steadily in the individual larva up until 90 hours (Figure 8) or 105 hours (Figure 9), with a small peak of activity at the second molt. The apparent gradual decline in activity from 72 hours noticed in the measurement of activity/mg protein (Figure 7) is simply due to the rate of larval growth being so much more rapid than the rate of enzyme production.

The most probable explanation for these results is the presence of an enzyme or enzymes in addition to molting fluid chitinase. There are 3 possible candidates for this role:

1) Some of the enzyme activity could be due to another chitinase. In the cockroach, although the highest activity was in the integument, substantial chitinase activity was located in the gut, hemolymph and saliva (38). In Streptococcus, 4 separate chitinase fractions have been purified (17, 18). It is possible that molting fluid chitinase is not the only chitinase in Drosophila.

2) Some activity could be due to a lysozyme-like enzyme. Egg white lysozyme is known to degrade chitin and related compounds (32), and Drosophila larvae may possess a similar enzyme.

3) Chitin synthetase has been shown in at least one instance to be a very reversible enzyme (31). Chitin synthesis occurs during a good part of the second instar and all of the third (Figure 2), and it is not inconceivable for the synthetase, in the presence of a large excess of substrate, to be responsible for some of the measured activity. In support of this hypothesis, the peak of chitinase activity in Figure 9 is about at the visible maximum of chitin formation, a period of rapid chitin synthesis. Chitin deposition becomes slight after 105 hours (12).

Although the Neurospora chitin synthetase requires UDP to hydrolyse chitin, the requirement for hydrolysis in Drosophila may not be as stringent. Alternatively, the measured activity due to chitin synthetase may represent the availability of UDP in the soluble fraction of larval extract.

Preliminary data exist for assuming that more than one enzyme is active in this system. Results with ammonium sulfate precipitation

suggest the presence of 2 separate enzyme fractions, and 3 peaks of activity were obtained from gradient elution of the DEAE-Sephadex column.

Properties of the assay

Since the substrate in the viscometric assay is chitosan, a partially deacetylated, solubilized derivative of chitin, rather than chitin itself, the question must necessarily be asked what indications exist that hydrolysis of chitosan is a true reflection of the presence of chitinase. First, there is support in the literature for this point of view. Researchers on other systems have found that viscometric assays of chitin derivatives adequately characterize chitinase activity (15, 28, 30). Then, the conformity of these data to the electron micrographs of the second instar (12), to the expected physiological results and to the results for chitinase distribution in other insects studied by other means (5) are a strong indication that true chitinase activity is being revealed. The enzyme under study also displays properties like those of other observed chitinases, as will be described later.

It is not known whether the site of chitinase action in chitosan is between 2 glucosamine residues or if the enzyme requires at least one acetylglucosamine moiety of the partially deacetylated polymer for hydrolysis, but the latter possibility is more probable.

Although the viscometric assay is a simple and rapid one, and good for analysis of crude extracts because it is not interfered with by color or turbidity, it suffers from the disadvantage of being extremely

susceptible to pH variations. Since the assay was standardized against a commercial streptococcal chitinase which has its own pH optimum, pH optimum curves could not be run. A different soluble chitin derivative described in the literature as a substrate for a viscometric chitinase assay, glycolchitin, is reported to lack the pH and salt sensitivity of chitosan (30), and may be worth considering as a substrate for future assays.

The results from the acid precipitation experiment indicate that pH 5 may not be the ideal pH for assaying this enzyme, since some 30% of the activity is lost by treatment at this pH. The fact that this can be determined by a pH 5 assay procedure probably testifies to the protective power of the chitosan substrate for the enzyme under adverse conditions. Increased chitinase stability in the presence of chitin has been demonstrated by Jeuniaux (17). The recovery of 60% of the enzyme activity around pH 3.7 in the isoelectric focusing experiments is probably due to the protective effect of the sucrose in the gradient in which the column is run, since precipitation of the enzyme at pH 4.0 without protection eliminates more than 80% of the activity.

In analysing the efficacy of this assay, we must also consider the possibility that all the chitinase may not be reaching the supernatant fraction of the larval extract. Chitinase is known to bind to cast larval skins (10, 11) and affinity of this enzyme for its substrate has been used as a method of purification (17). A significant fraction of the chitinase may be discarded with the particulate matter of the larval homogenate. The fact that homogenate and supernatant have the

same activity may be due to chitin-bound chitinase not being active in the assay. Since chitinase is quite stable, this hypothesis might be tested by letting the larval extract sit long enough for the chitinase to hydrolyse and free itself from the chitin in the homogenate and then by centrifuging the homogenate and testing the supernatant for an increase in chitinase activity.

Properties of the enzyme

As has been mentioned, the properties of the chitinase studied in this thesis resemble those of other chitinases described in the literature, with regard to enzyme stability and to lack of a cofactor requirement. Table II describes the gratifying stability of the enzyme to the usual storage conditions, which simplifies long term experiments and comparisons. Not only is there an absence of any requirement for loosely bound cofactor, but some procedures which would separate out a small molecule often tend to produce some enhancement of enzyme activity, as if something slightly inhibitory were being removed.

The isoelectric point of chitinase was found to be about pH 3.8. It is interesting, but may or may not be relevant, that the isoelectric point of endocuticle in the blowfly has been determined as 3.4 (39).

REFERENCES

1. Plotnikov, W. Z. Wiss. Zool. 76, 333 (1904).

2. Tower, W.L. Biol. Bull Woods Hole 10, 176 (1906).

3. Wigglesworth, V.B. Quart. J. Mic. Sci. 76, 269 (1933).

4. Hamamura, Y. and Y. Kanehara. J. Agr. Chem. Soc. 16 , 907 (1940).

5. Jeuniaux, C. Bull. Soc. Zool. France 86, 590 (1961).

6. Passonneau, J.V., and C.M. Williams. J. Exp. Biol. 30, 545 (1953).

7. Florkin, M. Proc. Int. Symp. Enz. Chem. Ser 2, 390 (1957).

8. Jeuniaux, C. Arch. Int. Physiol. Biochim. 66, 121 (1958).

9. Bade, M.L. and G.R. Wyatt. Biochem. J. 83, 470 (1962).

10. Jeuniaux, C. Actes. Soc. Linneene Bord. 97, 1 (1957).

11. Waterhouse, D.F., R.H. Hackman and J.W. McKellar. J. Insect Physiol. 6, 96 (1961).

12. Mitchell, H.K., U.M. Weber-Tracy and G. Schaar. J. Exp. Zool., submitted for publication.

13. Strickberger, M.W. Experiments in Genetics with Drosophila. John Wiley & Sons, Inc., N.Y. (1962).

14. Hackman, R.H. The Physiology of Insects. ed. M. Rockstein, Vol. III. Acad. Press, N.Y. p. 471 (1964).

15. Tracey, M.V. Biochem. J. 61, 579 (1955).

16. Beneke, W. Botan. Zeit. 63, 227 (1905).

17. Jeuniaux, C. Arch. Int. Physiol. Biochim. 67, 597 (1959).

18. Berger, L.R. and D.M. Reynolds. Biochim. Biophys. Acta 29, 522 (1958).

19. Ōtakara, A. Agr. Biol. Chem. 25, 54 (1961).

20. Zechmeister, L., G. Toth and E. Vajda. Enzymologia 7, 170 (1939).

21. Powning, R.F. and H. Irzykiewicz. Comp. Biochem. Physiol. 14, 127 (1965).

22. Tracey, M.V. 2nd Int. Congr. Biochem. Paris, 242 (1952).

23. Tracey, M.V. Nature 167, 776 (1961).

24. Jeuniaux, C. Mem. Acad. Roy. Belg. Classe Sci. 28, 7 (1954).

25. Jeuniaux, C. Arch. Int. Physiol. 58, 350, 352, 354 (1950).

26. Ōtakara, A. Agr. Biol. Chem. 25, 494 (1961).

27. Wyatt, G.R. Adv. in Insect Physiol. 4, 287 (1967).

28. Jeuniaux, C. Methods in Enzymology Vol. III. Acad. Press, N.Y., p. 644 (1967).

29. Powning, R.F. and H. Irzykiewicz. J. Insect. Physiol. 13, 1293 (1967).

30. Ōtakara, A. Agr. Biol. Chem. 25, 50 (1961).

31. Glaser, L. and D.H. Brown. J. Biol. Chem. 228, 729 (1957).

32. Berger, L.R. and R.S. Weiser. Biochim. Biophys. Acta 26, 517 (1957).

33. Mitchell, H.K. and A. Mitchell. Drosophila Information Service 39, 135 (1964).

34. Mitchell, H.K. personal communication.

35. Reynolds, D.M. J. Gen. Microbiol. 11, 150 (1954).

36. Colowick, S.P. and N.O. Kaplan. Methods in Enzymology V. III, p. 450. Acad. Press, Inc., N.Y. (1967).

37. Church, R.B. and F.W. Robertson. J. Exptl. Zool. 162, 337 (1966).

38. Waterhouse, D.F. and J.W. McKellar. J. Insect Physiol. 6, 185 (1961).

39. Dennell, R. Proc. Roy. Soc. Ser. B. 133, 348 (1946).

LIST OF ABBREVIATIONS

η_{sp}	specific viscosity
TCA	trichloroacetic acid
Tris	Tris(hydroxymethyl)aminomethane
UDP	uridine diphosphate

"Every woman needs a career of some sort to fall back on."

— my mother