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of Aryl-β-Glucosidase in Neurospora.   (1975)
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The effect of guanidine hydrochloride (GuHCl) on the stability and
the state of aggregation of Neurospora aryl-β-glucosidase isozymes "Y"
and "W" was studied in one wild type and several exotic strains by two
major methods. Purified conidial "Y" enzyme was treated with GuHCl and
separated by electrophoresis on cellulose polyacetate and thin-layer gel
(TLG) filtration on Biogel P-150. The inactivation characteristics in
time of crude conidial washes and purified "Y" and "W" preparations were
determined using p-nitrophenyl-β-D-glucopyranoside as the substrate.

The generation of the smaller isozyme "W" from purified conidial
"Y" was observed in the exotic strain P-212 with various concentrations
(2 to 5M) of GuHCl, but not in the wild type strain STA-4 with 3M GuHCl.
When P-212 "Y" was incubated with GuHCl at 4 C or at room temperature in
the presence of 0.005M 2-mercaptoethanol, a new enzymatically active
intermediate with a mobility on TLG between that of "Y" and "W" was
observed.

The inactivation curves of STA-4 aryl-β-glucosidases showed both
"Y" and "W" enzymes to be rapidly inactivated by even low GuHCl concentra-
tions. In contrast, the inactivation curves of P-212 aryl-β-glucosidases
indicated that these enzymes, following a sharp initial decline, were
reactivated in the presence of low GuHCl concentrations. At higher GuHCl
concentrations, the initial decline was followed by a more gradual one.
Crude conidial washes and purified "W" from exotic strain P-113 exhibited
a pattern of inactivation in 4M GuHCl similar to that observed with P-212
at higher concentrations of GuHCl, as did the aryl-β-glucosidases of exotic

strain P-278. P-113 conidial "Y" was completely deactivated by even brief exposure to 3-4M GuHCl. When the half-times of Neurospora aryl-β-glucosidase isozymes "Y" and "W" in GuHCl were determined using linear regression analysis, the calculated values showed that, for all strains and all concentrations of GuHCl used, the smaller isozyme, "W", was more stable than the larger isozyme.

From these studies it appeared that "W" generated from "Y" (called "nascent W") is less stable than is the "native W" from conidial washes. A possible explanation for this effect is presented. The GuHCl inactivation characteristics of these enzymes are discussed in light of current theories of protein denaturation and a theory as to the exact isozyme relationship of "W" and "Y" is proposed on the basis of the research described in this thesis.

CHEMICAL DENATURATION STUDIES OF TWO ISOZYMES

OF ARYL-β-GLUCOSIDASE IN NEUROSPORA

by

Susan Rawles Morton

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APPROVAL PAGE

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INTRODUCTION

Isozymes

With the development of the high-resolution "zymogram" method of electrophoresis by Hunter and Markert in 1957, a large number of enzymes have been demonstrated to exist in more than one molecular form. Such multiple forms of an enzyme, derived from the same organism and having similar or identical catalytic activities, were designated isozymes by the 1959 definition of Markert and Møller (Scandalios, 1969). Although there is much general disagreement on a single, simple definition of the term isozyme, the word (with its alternative spelling isoenzyme) has been accepted by the Committee on Biochemical Nomenclature of the International Union of Biochemistry and enjoys a wide acceptance (Shaw, 1969).

Markert has suggested that his "operational" definition can be modified or broadened to include such terms as allelic, nonallelic, homopolymeric, heteropolymeric, comformational, and isokinetic. George Brewer (1970) has included a complete discussion of these terms in his recent book, An Introduction to Isozyme Techniques. Shaw (1969) has classified isozymes into two basic categories in his review on the subject: (1) those which are distinctly different polypeptides and are presumably produced from different genetic sites (designated "primary" isozymes) and (2) those which result from secondary alterations in the structure of a single polypeptide species (designated "secondary" isozymes) which may be in vitro artifacts in many cases. Several nongenetic urease isozymes have been generated from jack bean α-urease by Fishbein, Nagarajan, and Scurzi

(1973). These isozymes presumably represent examples of this last cate-
gory. Shaw states, however, that primary isozymes are the only isozymes
that can be considered biologically significant, and, for that reason,
the following discussion will be confined mainly to this category.

When isozymes were first discovered, it was hypothesized by Kimura
and others that the frequency of isozymes and of enzyme polymorphism in
general would be low in natural populations due to the high evolutionary
cost, or "genetic load", involved (Johnson, 1974). The occurrence of
isozymes among plants and animals has been shown to be so widespread,
however, that isozymes appear to be the rule rather than the exception as
previously believed (Scandalios, 1974). While a survey of the literature
tends to bear this out, negative data are for the most part not published,
so that one cannot determine from such a survey which enzymes do not
occur as isozymes; thus, an absolutely accurate estimate of the relative
frequency of isozymes is not possible (Shaw, 1969). Shaw, however,
estimates that something on the order of half of all enzymes occur as
isozymes in a wide variety of organisms.

Some examples from the literature will serve to demonstrate the
number and variety of enzymes which have been shown to exist as isozymes
in microorganisms, "higher" plants, and animals. Erickson and Steers
(1970a,b), Melchers and Messer (1971), and Hartl and Hall (1974) have
demonstrated isozymes of β-galactosidase in Escherichia coli and several
related bacterial strains and Pollard and Steers (1973) have elucidated
the isozyme structure of Bacillus megaterium β-galactosidase. The chem-
istry and subunit structure of native yeast hexokinase isozymes have been
determined by Schmidt and Colowick (1973a,b). Fernandez-Moran, Reed,

Koike, and Willms (1964) have shown that the pyruvate dehydrogenase complex of _Escherichia coli_ consists of three isozymes associated in a polyhedral structure. Flury, Heer, and Fiechter (1974) have demonstrated two isozymes of malate dehydrogenase in _Schizosaccharomyces pombe_, one of which is formed in the mitochondria of glucose-repressed cells and the other in the cytoplasm of fully derepressed cells. The multiple molecular forms of enzymes in higher plants have been extensively reviewed by Scandalios (1969) who describes the existence and significance of the isozymes of amylase, catalase, alcohol dehydrogenase, esterase, leucine aminopeptidase, peptidase, and peroxidase in a variety of plants. A large number of enzymes which exist as isozymes have been found in man, including lactate dehydrogenase (Nance, Claflin, and Smithies, 1963; Zonday, 1963), salivary amylase (Karn, Shulkin, Merritt, and Newell, 1973), acid phosphatase of skin fibroblasts (Kaye and Nadler, 1974), and creatine phosphokinase of muscle and brain (Witteveen, Sobel, and DeLuca, 1974), and variations in human isozyme patterns are utilized for a variety of clinical tests.

A fundamental question that is often raised concerning the existence of isozymes is: Why has natural selection permitted the existence of two forms of an enzyme in the same organism? In other words, what is the significance of isozymes? Although a complete discussion of this question is not possible with our present inadequate knowledge, a brief summary of some of the pertinent information which has so far become available would be beneficial. First, as Shaw (1969) points out, it is important to understand that, although two isozymic forms may exhibit identical catalytic activity, there are often significant differences between them.

These differences are often functionally related to the significance of the enzyme to the organism. According to Shaw (1969), the three major types of differences between isozymes are variations in their: (1) role in development and differentiation of tissues, (2) regulation, and (3) enzyme activity.

Many isozymes have been demonstrated to differ in terms of the development and differentiation of the organism in which they are found (Masters and Holmes, 1972; Scandalios, 1974). John Scandalios (1974) in his excellent review on the subject of isozymes in the development and differentiation of plants suggests three common isozyme "fluctuations" which have been encountered by a number of investigators in a variety of organisms. They are: (a) distinct isozymes in different tissues of a given organism, referred to as "tissue specificity" by Shaw (1969); (b) some isozymes may be present in a tissue at a given developmental stage but absent in another; and (c) genetically identical isozymes may be present in different tissues but in varying quantities. Perhaps the most famous example of a tissue specific isozyme system is lactate dehydrogenase (LDH). For example, the LDH of breast muscle and heart muscle in the chicken are clearly separate entities, as determined from physiological, enzymatic, and immunological criteria (Cahn, Kaplan, Levine, and Zwilling, 1962). These two lactate dehydrogenases form molecular hybrids which change in composition during development. Shaw (1969) cites malate dehydrogenase of rat kidney, which is readily separated into mitochondrial and cytoplasmic fractions, as an example of tissue-specific isozymes.

Other examples of developmental differences in isozyme patterns are common. Coston and Loomis (1969) have demonstrated that the two

varieties of β-glucosidase which occur at two different stages in the
morphogenesis of Dictyostelium discoideum are electrophoretically distinct
from each other and thus represent an example of changes in isozyme com-
position associated with development. Atherton (1973) has shown ontogenic
differences between the acetyl and butrylcholinesterase isozymes present
in the cerebellum of chick embryos at 10 days and those present at 14
days. This suggested to him that the "switch" from the 10-day isozyme
complex to the 14-day complex represents a differentiating step requiring
genetic control.

Another aspect of the significance of isozymes concerns regulatory
isozymes - those which respond differently to changes in the cellular
environment. The example cited above of the two isozymes of malate de-
hydrogenase in Schizosaccharomyces pombe which differ in their response
to glucose would fall into this category. Chancellor-Maddison and Noll
(1963) have demonstrated electrophoretically that Euglena grown on auto-
trophic medium has two malate dehydrogenases whereas Euglena grown on
heterotrophic medium has only one MDH, thus showing that the molecular
forms of malate dehydrogenase in Euglena depend in part upon the nutri-
tional environment of the organism. Goodfriend, Sokol, and Kaplan (1966),
working with LDH from monkey cell cultures, reported that the rate of
synthesis of LDH was regulated by the concentration of oxygen in some
cells, particularly muscle cells. Tsao and Madley (1969) observed a
definite modification of phosphofructokinase isozymes in Neurospora crassa
as a result of depletion of nutrients in the culture medium. The gradual
shift in the electrophoretic pattern of phosphofructokinase isozymes with
increasing age of the mycelium demonstrates a possible role of environmental

factors in the occurrence of isozymes. The mechanisms of such regulatory phenomena are not yet completely understood, but it is certain that they are under complex genetic regulatory control (Shaw, 1969).

Although the zymogram technique has been responsible for the discovery of numerous isozyme systems, it tends to emphasize catalytic similarities between isozymes and thus is inadequate for demonstrating possible significant kinetic differences between them. Two general approaches to the study of functional differences between isozymic forms are described by Shaw (1969) which have successfully demonstrated kinetic differences among a great number of isozymes, including alcohol dehydrogenase in Rhesus monkey, glucose-6-phosphate dehydrogenase of mammals, and lactate dehydrogenase in a variety of organisms.

It is tempting to hypothesize, on the basis of these kinetic differences between isozymes, that one form of the enzyme provides an advantage over the other in a particular situation and that the two forms have thus been evolved through selection (Shaw, 1969). An opposing view is held by Kimura and Ohta who claim that the majority of enzyme polymorphisms have no significance to the survival or reproduction of the organism and thus are "adaptively neutral" (Johnson, 1974). Yamazaki and Maruyama (1973) offer mathematical evidence that supports their hypothesis. On the other hand, Bryant (1974), in investigating the adaptive significance of enzyme polymorphisms in five species of arthropods and three species of rodents, obtained results which indicate that enzyme polymorphisms are adaptively important in their response to environmental heterogenity and, thus, are not selectively neutral. Johnson (1974) supports the idea that polymorphism among enzyme loci (including isozymes)

is related to metabolic regulatory function and presents some evidence

that indicates that enzyme polymorphisms and isozymes are not selectively

neutral. Although such supporting evidence does exist, Shaw (1969) states

that in no single instance has the selective advantage of an isozyme

system been adequately demonstrated and to accomplish such a demonstration

would likely be a frustrating and difficult task. It is hoped that future

investigations will be designed to provide the necessary proof or disproof

of the selective advantage of isozymes.

Perhaps the most important implication of isozyme systems is the

fact that isozymes may differ in primary structure because they are encoded

in different genes, which may be allelic or nonallelic. These multiple

gene products can be observed directly through the use of electrophoresis

or other isozyme techniques and the effect of mutation on these products

can be tested. Many fascinating studies on the genetic control of iso-

zymes in a wide variety of organisms have been reported, but it is

possible to mention only a few. In some cases, isozymes have been dis-

covered because the genetics of a particular system was already known.

Smith and his coworkers (1963) found at least two isozymes of the enzyme

xanthine dehydrogenase in Drosophila. It was already known that the

synthesis of XDH was controlled by at least two genes, rosy (ry) and

maroon-like (ma-1), and the results of those experiments indicated that

these genes controlled the isozymes by producing two different polypeptides

that assemble in groups of three or four. Yau and Lindegren (1967) have

demonstrated that the melezitose locus (mz) in Saccharomyces is responsible

for the production of three different α-glucosides having different

electrophoretic mobilities and substrate specificities. The review by

John Scandalios (1969) referred to previously describes in great detail

the genetic control of the isozymes of the various plant enzymes mentioned

in the reference. The genetic control of alcohol dehydrogenase (ADH) in

a tetraploid species of wheat was investigated by Gary Hart (1969). The

wild type strain showed three bands of ADH activity corresponding to one

or the other of two electrophoretic patterns, phenotype I and phenotype II.

Results of reciprocal backcrosses indicated that the phenotypic difference

observed is controlled at a single locus, Adh, by two codominant alleles,

Adh^1 and Adh^2. Halsall and Catcheside (1971) have studied the 3-deoxy-D-

arabino-heptulosonate 7-phosphate synthase (DAHP synthase) system in

Neurospora crassa and have found that there are three DAHP synthase iso-

zymes, each subject to feedback inhibition by one of the three aromatic

amino acids, phenylalanine (Phe), tyrosine (Tyr), and tryptophan (Trp).

Mutations inactivating each isozyme separately have been isolated: arom-6

(DAHP synthase Tyr), arom-7 (DAHP synthase Phe), and arom-8 (DAHP synthase

Trp). A second class of mutations has been found which renders each iso-

zyme insensitive to allosteric inhibition. Since these two classes of

mutation fail to complement each other, they were considered to be alleles

of a single structural gene for each isozyme. The hypothesis that arom-6,

arom-7, and arom-8 can be considered to be the structural genes for DAHP

synthase isozymes was strengthened by the authors' finding of a new class

of pleiotrophic mutants which affect both the activity of the isozymes and

their sensitivity to feedback inhibition.

Even with this inadequate survey of the literature concerning iso-

zymes, it should be clear that isozymes provide what John Scandalios (1969)

has called a "natural 'built-in' marker system" for investigations into

the biochemistry, genetics, and developmental biology of organisms. With the use of isozyme systems whose genetic control has been resolved, it is now possible to study interallelic complementation and the mechanism and molecular basis for heterosis and differential gene action in the development of higher organisms (Scandalios, 1969). Another aspect of gene function that can be effectively examined by using isozyme markers is gene dosage effects. Organelle-specific isozymes could also be used to answer questions relating to the coding of specific enzymes (Scandalios, 1969). Shannon, Ballal, and Harris (1973), on the basis of their results from starch gel electrophoresis of six enzymes from nine species of Polyphorus, have suggested that isozyme banding patterns might prove valuable in determining taxonomic relationships among the wood-rotting fungi.

Fungi have proven to be extremely valuable research tools in the development of the fields of molecular biology and biochemical genetics. They can be studied as microorganisms (with the concomitant advantages of short life cycle, convenient growth and mating characteristics, and ease of obtaining sufficient numbers of progeny for dependable genetic analysis), yet, as stated in a recent review by Metzenberg (1972), their protein regulatory mechanisms may be similar to those of higher organisms due to their possession of eukaryotic chromosomes. Neurospora crassa is a particularly useful organism in this connection due to its well-defined genetic background and its convenient growth characteristics. For the same reasons, Neurospora is an especially suitable organism to use for the investigation of isozymes and their functional and physiological roles.

Many enzymes in Neurospora have been found to exist as isozymes.
Bates and Woodward (1964), Johnson (1969), Johnson and DeBusk (1970a,b),
and Lester and Byers (1964) have reported the existence of at least three
isozymes of Neurospora crassa β-galactosidase. A large enzyme with a pH
optimum of 7.5 is strongly bound to the cell and has a transglycosidation
activity which appears to be involved in the induction of the enzymes,
since no induction occurs in a mutant which has lost this activity
(Johnson, 1969). Two smaller enzymes, with pH optima at 4.2 and 4.5,
are released to the medium when the mold is grown on lactose, with the
pH 4.5 enzyme being the major extracellular form during the early stages
of induction. The dehydrogenase enzymes of Neurospora crassa have been
studied by Tsao (1962). When malate, isocitrate, glucose-6-phosphate,
and 6-phosphogluconate dehydrogenases from the mycelia of several wild
strains of Neurospora were subjected to zone electrophoresis in starch
gel, four electrophoretically distinct malate dehydrogenases, a single
isocitrate dehydrogenase, three glucose-6-phosphate dehydrogenases, and
two 6-phosphogluconate dehydrogenases were obtained regardless of the
strain or the growth conditions. The only strain differences noted were
the relative intensity of the various isozyme bands. Sundaram and Fincham
(1964) have investigated the glutamate dehydrogenase complex of Neurospora
crassa and have found a mutant enzyme that is interconvertible between
electrophoretically distinct active and inactive forms. Fincham and
Garner (1967) later reported that the transition from the inactive to the
active form in both the wild type and the mutant is dependent upon a very
slight shift in pH. Benveniste and Munkres (1973) have explored the
effects of ionic concentration and pH on the isozymes of mitochondrial

malate dehydrogenase from Neurospora crassa. Five active isozymes of 105,000, 91,000, 78,000, 65,000, and 39,000 daltons were observed when the enzyme was extracted from mycelia and centrifuged in a sucrose gradient with 5mM tris-Cl at pH 9.0. The number of isozymes observed was reduced in alkaline solutions of monovalent cations at 100mM or divalent cations at 10mM. Multiple forms of esterases in Neurospora have been described by Reddy (1971) and Sagarra (1973) and the results of crosses between different strains led these authors to conclude that at least four independent esterase systems controlled by two or three separate alleles exist in Neurospora.

Metzenberg (1964) and Trevethick and Metzenberg (1964) found that Neurospora invertase can exist in two forms, "light" and "heavy". The "light" form, the subunit, is formed from "heavy" invertase under a variety of conditions that promote dissociation. Both forms of the enzyme are active and are present in crude mycelial extracts and crude conidial washes. Results from experiments with Neurospora protoplasts, which secrete predominately "heavy" invertase, indicate that the aggregation of subunits into the "heavy" form must occur at some site interior to the cell membrane during, or soon after, their synthesis. Meachum, Colvin, and Braymer (1971) found that the "heavy" invertase has an approximate molecular weight of 210,000 and the "light" invertase has an approximate molecular weight of 51,000, thus indicating a tetrameric structure for the enzyme.

In investigating the effect of carbon source on isocitrate lyase formation in Neurospora crassa, Sjogren and Romano (1967) found that the enzyme formed in the presence of acetate and that formed in the presence

of glucose differed in a number of physical properties, including pH optima, Michaelis constants, and sensitivity to inhibition by phosphoenol pyruvate. Heat inactivation studies of the two enzymatically active components eluted from a diethylaminoethyl cellulose column confirmed the presence of multiple forms of isocitrate lyase in Neurospora. More recently, Rougemont and Kobr (1973) have found that isocitrate lyase-2 is the isozyme formed in cultures that have been derepressed by acetate.

Hill and Sussman (1963) have found that the isozymes of trehalase are similar in substrate specificity, response to inhibitors, pH optima, and Michaelis constants; however, small differences in the rate of inactivation of these enzymes at 50 C were detected. Shih-an Yu, Garrett, and Sussman (1971) have elucidated the genetics of these two electrophoretically distinct trehalases and have found that they are controlled by two alleles of the same gene. The activity and heat stability of trehalase from both Neurospora mycelia and ascospores has recently been investigated by Hecker and Sussman (1973) who found that high temperatures (37 C) and low ionic strengths tended to favor the dissociation of trehalase into two species, whereas low temperatures and high ionic strengths tended to promote the formation of high molecular weight aggregates.

The β-glucosidase enzyme system is of particular interest and importance due to its participation in the degradation of cellulose to CO_2 and water and its consequent role in the carbon cycle. Metzenberg (1972) recently stated that the control of the β-glucosidases, particularly in fungi, is complicated by the existence of multiple enzymes with overlapping substrate specificities. The multiple forms of β-glucosidases have been separated, purified, and characterized in a variety of organisms,

including Myrothecium verrucaria (Hash and King, 1958a,b), Aspergillus niger (Murti and Stone, 1961), Stachybotrys atra (Jermyn, 1962), Saccharomyces fragilis and Saccharomyces dobzhanskii (Fleming and Duerksen, 1967a,b), Saccharomyces lactis (Marchin and Duerksen, 1968a,b), Dictyostelium discoideum (Coston and Loomis, 1969), Botrydiploidia theobromae Pat. (Umezurike, 1971 and 1975), Chaetomium thermophile var. coprophile N. var. (Lusis and Becker, 1973), and Lycopersicon esculentum L. (tomato) (Sobtka and Stelzig, 1974).

For nearly fifteen years, research has been done on the β-glucosidase system of Neurospora crassa and multiple forms of β-glucosidase have been observed in this organism as well. Eberhart (1961) and Berger and Eberhart (1961) reported finding high aryl-β-glucosidase and cellobiase activity in conidial washes of several Neurospora strains. Mahadevan and Eberhart (1962) later isolated a mutant, designated gluc-1, which exhibits less than 10% of the normal aryl-β-glucosidase activity, but whose other physical properties did not appear to differ significantly from the enzymes of standard wild type strains. The results of heterokaryon experiments (Mahadevan and Eberhart, 1962) and further genetic analysis (Eberhart, Cross, and Chase, 1964) indicated that the gluc-1 mutation was a dominant regulatory gene which reduced aryl-β-glucosidase activity but had no effect on cellobiase. The aryl-β-glucosidase of both the wild type (Mahadevan and Eberhart, 1964a) and the mutant (Mahadevan and Eberhart, 1964b) have been purified by ammonium sulfate precipitation and anion exchange chromatography and the physical properties of the purified enzymes were also found to be quite similar, providing further evidence that the gluc-1 mutation is regulatory, rather than structural, in function.

A second mutation, designated cell-1, was isolated by Myers and Eberhart (1966) and was found to exhibit constituitive production of cellobiase and an exogenous cellulase. Although cell-1 is also regulatory in character, aryl-β-glucosidase levels are not affected which is consistent with the idea that gluc-1 is the sole regulatory gene for aryl-β-glucosidases in Neurospora. Myers and Eberhart (1966) demonstrated that cell-1 was unlinked to gluc-1 and also performed heterokaryon tests which revealed that cell-1 is recessive to cell-1$^+$.

Eberhart and Beck (1970) isolated an apparent allele of gluc-1, designated gluc-2, which results in less than 1% of the normal aryl-β-glucosidase activity. These authors also found that aryl-β-glucosidase is primarily a mural (associated with the cell wall) enzyme, while cellobiase is cryptic (endocellular) in intact-cell preparations, and they were able to further determine the physical properties of both aryl-β-glucosidase and cellobiase. Eberhart and Beck (1973) have recently reported differences in the induction patterns of these two enzymes which suggest that they represent two fundamentally different classes of disaccharidases: (1) enzymes which are broadly inducible, as in the case of aryl-β-glucosidase; and (2) enzymes with highly specific induction requirements, of which cellobiase is an example.

Madden (1971), in a survey of several exotic strains of Neurospora, discovered a second aryl-β-glucosidase, designated "W", in addition to the previously reported aryl-β-glucosidase ("Y") and there is some evidence for the existence of an additional aryl-β-glucosidase isozyme, "V" (Hartis, unpublished data). Madden (1971) hypothesized that the enzyme activity of "W" could be related to "Y" in one of several possible ways:

1. It could be a structurally distinct protein with substrate specificity overlapping that of "Y", such as acetolacetate synthetase (Halpern and Umbarger, 1959).

2. It could differ from "Y" in the proportion of invariant kinds of subunits, as with lactate dehydrogenase (Cahn, Kaplan, Levine, and Zwilling, 1962).

3. It could be a subunit or polymer of "Y", as exists with invertase (Metzenberg, 1964).

4. It could be due to the association of a carbohydrate or other moiety with the enzyme, similar to that described for the β-glucosidases of Stachybotrys atra (Jermyn, 1962).

Many new and exciting things are now being done with isozyme systems, including in vitro synthesis of enzyme subunits, as with messenger-RNA directed synthesis of alkaline phosphotase monomers (Dohan, Rubman, and Torriani, 1971), and in vitro synthesis of hybrids of different tissue-specific isozymes, as with pig and chicken heart lactate dehydrogenase (Saito, 1972) and bovine liver and skeletal muscle pyruvate kinases (Dyson and Cardenas, 1973). Perhaps the most fascinating studies which have been done are dissociation and denaturation studies which aid in the determination of the subunit structure of isozymes. Dissociation by ultracentrifugation has been used to elucidate the subunit relationship of aspariginase (Scholtan and Lie, 1971) and urea has been used in a similar way to investigate the subunit interactions of β-galactosidase from Escherichia coli K12 (Shifrin and Steers, 1967). Dissociation studies with guanidine hydrochloride (GuHCl) by Apella and Markert (1961) were the primary source of the evidence which led them to propose the tetrameric structure of lactate dehydrogenase.

Although the current study was originally designed to investigate the occurrence and physical properties of the new aryl-β-glucosidase isozyme "W" in standard laboratory strains of Neurospora crassa, the major emphasis was later shifted to the determination of the exact relationship between "W" and the larger aryl-β-glucosidase isozyme "Y". Dissociation and denaturation studies with guanidine hydrochloride were performed on the aryl-β-glucosidases of several strains of Neurospora crassa in order to determine which, if any, of the possibilities suggested by Madden (1971) represents the actual case. As the study progressed, the chemical in-activation characteristics in time of one wild type and several exotic strains were determined using the synthetic β-glucoside, p-nitrophenyl-β-D-glucopyranoside, as the substrate. The results of these inactivations were analyzed and interpreted in light of the current theories of protein denaturation. Due to the extreme complexity of the denaturation process, a preliminary discussion of protein denaturation is included in the following section.

Protein Denaturation

Prior to the 1960's, protein chemists and enzymologists were concerned primarily with the native protein, its structure and the reasons for it. Charles Tanford, in his excellent review on the subject (Tanford, 1968 and 1970), designated the determination of the structure of myoglobin by Kendrew and his coworkers in 1961 as the best starting point for his literature survey. Since that time, entire volumes have been written on the exceedingly complex subject of protein denaturation (Joly, 1965) and current literature abounds with new and different approaches to the

problem. It is apparent that a complete discourse on the subject of
protein denaturation is well beyond the scope of this discussion, and even
to limit one's consideration specifically to enzyme denaturation would
still present one with an overwhelming amount of information. A somewhat
condensed version of some of the paramenters involved appears in the final
chapter of Laidler and Bunting's recent book, The Chemical Kinetics of
Enzyme Action, and, since it is also a review which emphasizes the present
subject of enzyme denaturation, it will be used as a basis for the follow-
ing discussion.

Protein denaturation occurs when a protein is heated or treated in
various other ways so that its three dimensional structure is altered.
The denaturation of enzymes, when it leads to the loss of their catalytic
activity, is specifically referred to as inactivation. It is difficult
to define denaturation precisely since different types of treatment may
bring about different changes in a protein. Laidler and Bunting quote an
early definition of Wu which states that denaturation is a change in the
native protein whereby it becomes insoluble in one or more of its former
solvents. Another definition, one by Neurath and his coworkers, states
that denaturation must be a non-proteolytic modification of the native
protein which results in some perceptible change in the properties of the
protein. This definition excludes the mere hydrolysis of the protein,
but does not exclude dissociation into smaller subunits or aggregation
into larger molecules, two changes which do commonly occur due to the
action of denaturation agents.

Denaturing agents can be roughly divided into two groups, physical
agents and chemical agents. The first group includes such "mechanical"

factors as heat, very high hydrostatic pressure (5000-10,000atm.), ir-
radiation by ultraviolet light or ionizing radiation, and ultrasonic
waves. Some of the chemical agents suggested by Tanford (1968) and
Laidler and Bunting (1973) include guanidine hydrochloride (GuHCl), urea,
salts other than GuHCl (i.e., LiBr, $CaCl_2$, KSCN, NaBr, NaCl, KCl, and
other guanidinium salts, such as guanidinium thiocyanate or GuHSCN),
acids and bases, organic acids (such as dichloroacetic acid and tri-
fluoroacetic acid), alcohols (including simple aliphatic alcohols, 2-
chloroethanol, dioxan, ethylene glycol, and other polyhedric alcohols),
other simple organic reagents (such as foramide), and detergents (such as
dodecyl sulfate). Laidler and Bunting suggest, as does Joly (1965), that
enzymes are actually chemical denaturing agents since the initial effect
of proteolytic enzymes on protein substrates is to bring about denatura-
tion, after which the proteins are more sensitive to the hydrolytic action
of the enzyme. Cassman and Schachman (1971) found that the denaturation
of beef liver glutamate dehydrogenase in 2.5M GuHCl rendered this enzyme
more susceptible to proteolytic degradation. It is important to emphasize
that these various agents act in collaboration with each other and with
other factors which influence the denaturation process. For example,
denaturation by acid often proceeds faster if it is performed along with
the addition of heat.

Tanford's 1968 definition of protein denaturation states that pro-
tein denaturation is a "major change" from the original native structure
of the protein without alteration of the amino acid sequence and without
breaking any of the primary chemical bonds which join one amino acid to
another. This more specific definition gives one an inkling of what

actually happens when a protein is denatured. There are changes in the secondary and tertiary bonding of the protein, resulting in a change in the general shape of the molecule. There is some disagreement as to what actually constitutes a "major" change. Tanford points out that a major conformational change must be "cooperative" since ordered structures are cooperative in nature, involving many amino acid residues which must be removed as a unit rather than one at a time. Anfinsen (1973) cites as an example of such "cooperativity" a nuclease fragment which represents 85% of the total amino acid sequence, yet exhibits only 0.12% of the activity of the native enzyme. The further addition of the 23 remaining residues restores the stability required for activity. The transition from native to denatured state is a "steep" transition, which generally occurs within a narrow range of temperature, pH, or concentration of denaturing agent. The "quasi-native" states, illustrated by the ability of some globular proteins to exist in more than one compact, globular configuration, do not constitute evidence of a "major conformational change" and, hence, do not represent denaturation. On the other hand, enzyme inactivation, though it may result from what is actually a minor conformational change, is certainly a major change in the physical properties of the enzyme. A somewhat simpler approach to the explanation of what happens when a protein is denatured is given by Laidler and Bunting. They list six changes which they term "property changes" which often occur when a protein is denatured, even though not all of them will necessarily occur with a given protein. These "property changes" include: 1) decrease in solubility, 2) loss of crystallizability, 3) change in overall molecular shape, 4) increase in chemical reactivity, 5) increase in susceptibility

to attack by proteolytic enzymes, and 6) loss of biological activity (for enzymes, catalytic activity; for proteins like hormones, the ability to regulate biological functions).

The process of denaturation may be easily reversible or it may be irreversible. The denaturation of a protein is considered irreversible if the simple return to the native environment will not restore the native configuration. If the process is irreversible, it is sometimes, but not always, the result of a secondary reaction which follows the original "major conformational change" (Tanford, 1968). In general, denaturation under gentle conditions is more likely to produce reversible denaturation, while more vigorous conditions produce an irreversible effect. Most studies have been done with reversible systems because they are easier to study and to interpret. Examples from the literature will be included in a later section.

Since protein denaturations involve the alteration of a single reactant species, protein denaturation reactions have traditionally been considered to be unimolecular and thus to follow first order kinetics. In some situations, this is indeed the case, as has been found with the denaturation of haemoglobin by alkali (Perutz, 1974). Laidler and Bunting (1973) have examined a large volume of experimental data and have found many examples of orders higher than unity. Indeed, many of the earlier investigators made no "proper" determination of order but instead assumed that the reaction followed first-order kinetics. By "proper" determination is meant the determination of both the order with respect to concentration (or the true order) and the order with respect to time. Laidler and Bunting designate four main classes of behavior: 1) both

orders are unity, 2) the order with respect to concentration is unity, but the order with respect to time is greater than unity, 3) the order with respect to time is unity, but the order with respect to concentration is less than unity, and 4) both orders of reaction are greater than unity. A variety of mechanisms is possible with these four types of behavior, but a discussion of all of them is not possible here. Denaturations corresponding to type 1 and type 4 reactions would be considered first-order reactions, however. Joly (1965) also agrees that denaturations can rarely be described as one-step monomolecular processes and he suggests that, on the basis of chemical models, it is often necessary to implicate several reactions following several pathways in several steps.

The reagent chosen for the chemical denaturation studies reported in this thesis was guanidine hydrochloride (GuHCl). Most proteins with an ordered native structure undergo a notable transition upon the addition of GuHCl (Tanford, 1968). The transition is usually complete at concentrations of from 6M to 8M at room temperature, although higher concentrations may be needed to effect a conformational change for very stable proteins. All proteins that have undergone a complete transition in the presence of GuHCl have been found to be random coils, a fact which may help explain the finding of Maddy and Kelly (1971) that GuHCl is an inadequate dispersive agent for membrane proteins. GuHCl is generally considered to be a very effective denaturant and has been used to elucidate the subunit structure of various proteins, including the 7S protein from soybean globulin (Koshiyama, 1971), porcine pituitary lutenizing hormones (Courte and Willemont, 1972), lactate dehydrogenase (Apella and Markert, 1961), glyceraldehyde-3-phosphate dehydrogenase (Amelunxen, Noelken, and

Singleton, 1970), and glutamic dehydrogenase (Cassman and Schachman, 1971). Since a reagent that leads to the loss of all noncovalent structure will disrupt all noncovalent bonds between polypeptide chains, GuHCl has also been frequently used in determinations of the molecular weights of the constituent polypeptide chains of proteins (Erickson, 1970; Fish, Mann, and Tanford, 1969; Fish, Reynolds, and Tanford, 1970; Heinz and Prosch, 1971; Klaus, Nitecki, and Goodman, 1972); Mann and Fish, 1972; Reisler and Eisenberg, 1969; and Rydén, 1972).

GuHCl is a strong electrolyte and electrostatic interactions have little or no importance in concentrated solutions of the reagent (Tanford, 1968). The pH of the reaction is therefore not critical in terms of the effectiveness of the reagent, although it may be of extreme importance when the protein to be denatured is considered. For example, at alkaline pH, proteins with both cysteine and cystine residues experience an effect known as "scrambling" of the disulfide bonds by the disulfide interchange reaction described by Anfinsen (1973). This scrambling can lead to aggregation, gelation, or precipitation of the protein.

The action of GuHCl on proteins produces an effect which is very similar to that of urea. The effects of these two reagents can be explained on the basis of localized free energy changes (generally, lowering of the free energy) at hydrophobic side chains and peptide groups of the protein molecule (Tanford, 1970). Solvent perturbation studies by Solli and Herskovits (1973) have revealed that in "random-coil forming" solvents like urea and GuHCl, just as in helix-promoting alcohols, the hydrophobic interior folds of globular proteins and enzymes are gradually destroyed, which renders previously buried aromatic side chains accessible to the

solvent. Sugai, Yashiro, and Nitta (1973) found this to be the case in the reversible unfolding of α-lactalbumin by GuHCl. At about pH 5.50, two tryptophanyl residues buried in the interior of the native protein were considered to be exposed on its surface in the denatured state. Katz and his coworkers (1973) found that the influence of urea and GuHCl on volume effects of proteins was similar to that of water in two ways: the denaturing medium had an effect on the volume of the acid-base reaction they were studying and the denaturing agents altered the proteins and frequently "normalized" buried prototrophic groups. Ahmad and Salahudin (1974) made intrinsic viscosity measurements of proteins consisting of one polypeptide chain in 6M GuHCl and 9M urea in the presence of 2-mer-captoethanol at various temperatures in the range 25 to 55 C. They emphasized the importance of strictly controlled temperature conditions since their results suggested that some proteins which are well-behaved, linear random coils in denaturing solvents at 25 C show conformational anomalies at higher temperatures that are independent of amino acid com-position, chain length, and the nature of the denaturing solvent. They attributed these anomalies to some type of hydrophobic intramolecular interactions operating in these systems.

The mechanism of action of GuHCl is complicated and, since GuHCl is an electrolyte, is somewhat different from that of urea, although according to Gabel (1973), it has been proposed that the two reagents were similar in their denaturing mechanisms. Gabel studied the denatura-tion by urea and GuHCl of trypsin and N-acetylated trypsin derivitives bound to Sephadex and agarose gels. His finding that immobilization on Sephadex protected trypsin from denaturation by urea, but not by GuHCl,

led him to hypothesize that that the unfolding by GuHCl proceeds by a different activated state, since denaturation is reached in the same way by the isolated molecule or the molecule bound to the carrier. According to Tanford (1970), the neutral GuHCl molecule or the GuH$^+$ ion can be the ligand which actually binds to the protein molecule, although the exact site of interaction is not known. Green and Toms (1972) found that avidin molecules in which a fraction of the four binding sites were occupied by biotin did not dissociate completely in 6.4M GuHCl. Their results can be explained by assuming that the unfolding of unoccupied subunits followed by dissociation from the tetramer is initiated by penetration of GuHCl ions into the ligand binding site and, thus, disorganization of this region of the subunit. These authors propose a tentative generalization that when the intersubunit bonds are strong, ligand-binding sites are likely to be weak points in the protein structure and will be the main sites for attack by dissociation agents. Ligand-binding sites will therefore tend to stabilize the structure by direct competition. When the intersubunit bonds are relatively weak, ligands have little or no effect on the dissociation of subunits by denaturants.

Efforts to determine the kinetics of denaturation of GuHCl and urea have not always produced identical results. Wasserman and Burgner (1972) studied the kinetics of unfolding of dogfish muscle lactate dehydrogenase in GuHCl and found that their results were compatible with a one-step denaturation process involving a transition between native and completely unfolded molecules without the accumulation of stable intermediates. On the other hand, in the study mentioned above by Sugai et al. (1973) with α-lactalbumin, kinetic measurements revealed that the unfolding

of this protein by GuHCl was an apparent two state transition. The reversible dissociation of <u>Neurospora</u> <u>crassa</u> glutamine synthetase also appeared to be a two-step process (Ward and Kapoor, 1971). Joly (1965) cites Cristensen's study of the denaturation of β-lactoglobulin by urea in which he found it to be a four state transition process.

GuHCl is a hydrogen-bonding reagent which can also rupture the hydrogen bonds responsible for the secondary structure of many proteins, and, with the destruction of the secondary structure, the polypeptide chain unfolds and the aggregations of the chains dissociate. As mentioned previously, GuHCl played a large part in the elucidation of the structure of the isozymes of lactate dehydrogenase because of this ability. Dissociation of proteins by GuHCl and urea is usually reversible, especially at low concentrations of the denaturant. Renaturation is accomplished by the "removal" of the denaturant in one of two ways: by dialysis or by dilution into a large volume of a suitable buffer.

Since the final step in protein biosynthesis is the folding of the polypeptide chain into its native three-dimensional structure, an ideal approach to the study of this process is to examine the kinetics of refolding of proteins which have been transformed to random coils by GuHCl. Wong and Tanford (1973) have found that bovine carbonic anhydrase B is ideally suited for such studies because it contains no disulfide bonds and the GuHCl denaturation of this enzyme is a two-state process and distinct successive stages can be observed in both equilibrium and kinetic measurements. Ward and Kapoor (1971) studied the reversible inactivation and dissociation of glutamic synthetase of <u>Neurospora</u> <u>crassa</u> by urea. On partial inactivation by urea, the enzyme appears to consist of a dimeric

species and an intermediate partially unfolded form. The presence of substrates and effectors was found to be necessary to bring about a reversal of inactivation and a return of the enzyme to the native state. Peterman and Pavlovec (1971) were able to isolate, dissociate, and reassociate active subunits of rat liver ribosomes in 2 to 2.7M urea plus 1mM dithiothreitol. Sakamato, Hatfield, and Moyea (1972) found that GuHCl and urea had characteristic effects on the denaturation and renaturation of xanthosine 5'-phosphate aminase, depending on the concentration of the denaturant. At low concentrations (up to 1.2M GuHCl and 4.0M urea), the inactivation is caused by conformational changes and dissociation of the enzyme into subunits and is completely reversible. In 1.2M to 3.0M GuHCl and 4.0M and 8.0M urea, the reaction is irreversible due to the aggregation of the partially unfolded polypeptide chains. In 3.0M to 6.0M GuHCl, the molecule is extensively unfolded and is partially (15%) reassociable to the active form by removal of the denaturant.

The successful renaturation of some proteins appears to be a very complicated process. Tobes, Kuczenski, and Suelter (1972) found that the kinetics of renaturation of GuHCl-dissociated allosteric yeast pyruvate kinase were dependent on temperature. At temperatures from 9 to 30 C, the kinetics of the reaction were first order with a slightly greater yield of renatured enzyme at 20 C. At 0 C, the kinetics were not first order but were autocatalytic. Ullman and Monod (1969) studied the effect of divalent cations and protein concentration on renaturation of β-galactosidases from Escherichia coli. Their results indicate that two entirely different pathways may be followed by a solution of denatured protein during removal of the denaturant. At relatively high protein concentrations

and in the presence of certain divalent cations (like Ca^{++}, Mg^{++}, Mn^{++}, and Zn^{++}), multiple interactions between chains can occur, leading to the formation of an inactive precipitate. At lower concentrations of protein and in the absence of these ions, refolding of individual peptide chains onto themselves is favored, leading to the restoration of the native state. They also found that renaturation with GuHCl is less efficient than when urea is used as the denaturant. During the dialysis of GuHCl, chain to chain interactions may occur which would prevent the correct renaturation of individual chains, while, during renaturation from urea, this effect appears to be minimized. Kohn (1970) has identified nine variables which affect the renaturation of spinach leaf glyoxylic reductase from 6 and 8M GuHCl plus 0.1M 2-mercaptoethanol, including the pH of the diluting buffer, the temperature at dilution and during renaturation, the protein concentration at renaturation, and the presence of other substances, such as DPNH, glyoxylate, hydroxypyruvate, and bovine serum albumin. Yassan and Henkens (1972) found in their work with bovine carbonic anhydrase B that denaturation by GuHCl is thermodynamically reversible with or without Zn (II), even though this enzyme contains a specific zinc binding site. Refolding occurs at an extremely low rate, however, if Zn (II) is not present during the initial stages of the reaction, implying that Zn (II) is bound during the early steps of the folding of the polypeptide chain and, although it does not affect the final conformational state, thus influences the pathway of the reaction.

The reversible transition between native and denatured states of proteins is often a two-state process in the sense that states other than the native and denatured protein are never present in experimentally

significant amounts during the reaction. Kinetic studies by Ikai and Tanford (1971) of the denaturation and renaturation of proteins indicate metastable intermediates which are not on the direct pathway between native and denatured states. These discoveries suggested to them that the initial steps in the folding of a polypeptide chain may often be rapidly reversed and without influence on the ultimate result. Several subsequent investigations have served to support their findings. Carlsson, Henderson, and Lindskog (1973), studying the denaturation of human carbonic anhydrases in intermediate concentrations of GuHCl, found that the kinetics of this process is complex and the final products are not readily reactivated. These observations indicate that incorrectly folded molecules, rather than intermediates between the native and randomly coiled states, are formed under these conditions. The kinetics of the reactivation of enzymes which had been fully denatured by both GuHCl and urea also indicates that "incorrectly" folded molecules are formed. Waley (1973) found that the refolding of triose phosphate isomerase in low concentrations of GuHCl is a slow process. He attributed the slow rate of renaturation to the possibility that the incorrectly folded oligomers that might be formed may have to dissociate again before they can be transformed to the native protein, a view which is supported by Ikai and Tanford's work. Gibbons and Perham (1974) have described the reversible denaturation of citraconyl-aldolase by GuHCl and have found that suboptimal denaturing conditions produce an inactive species which is composed of an aggregation of sub-units. Since this inactive species can be at least partially reactivated in optimal conditions of denaturation-renaturation, the authors feel that it may represent an incorrectly folded intermediate similar to that suggested by Ikai and Tanford (1971).

The above general discussion of the process of protein denaturation is a brief, and perhaps incomplete, one, but it will serve as a background for the discussion of this author's own research. The effect of GuHCl on Neurospora aryl-β-glucosidase isozymes "Y" and "W" was studied in one wild type and several exotic strains of the bread mold with two general approaches. In the first, purified conidial "Y" was treated with GuHCl and separated by electrophoresis on cellulose polyacetate and by thin layer gel filtration on Biogel P-150. In the second, the inactivation characteristics in time of crude conidial washes and purified "Y" and "W" preparations were determined using the synthetic β-glucoside, p-nitro-phenyl-β-D-glucopyranoside, as the substrate.

MATERIALS AND METHODS

Chemicals

Biogel P-100, Biogel P-150, and Biogel P-200 were obtained from Bio-Rad Laboratories. Chitinase, Cleland's Reagent, 2-mercaptoethanol, p-nitrophenyl-β-D-glucopyranoside (PNPG), and sodium thioglycolate were purchased from Calbiochem. Bacto-agar was obtained from Difco, and Biocert yeast extract and glycerol was from Fisher Scientific Company. Sucrose was a product of Dixie Crystal. Lyphogel and all electrophoresis materials were purchased from Gelman. Urea was a product of Mallinckrodt Chemical Works. Guanidine hydrochloride (Ultrapure) was procured from Mann Research Lab. 4-methyl-umbelliferyl-β-D-glucopyranoside was obtained from Nutritional Biochemicals Company. Sephadex G-100 and Sephadex G-150 were products of Pharmacia, Inc. N-z-casein was purchased from Sheffield Chemical. Reagent grade guanidine hydrochloride and Tris-hydroxymethyl-aminomethane (Tris) were obtained from Sigma Chemical Company. Carbowax-Polyethylene Glycol Compound 20-M and Carbowax-Polyethylene Glycol Compound 6000 were purchased from Union Carbide.

Selection and Maintenance of Strains

This study was originally designed to investigate the occurrence and physical properties of the "new" aryl-β-glucosidase "W" in standard laboratory wild type strains of Neurospora crassa. The following wild type strains were obtained from the silica gel cultures available in our laboratory: 74-OR23-1A, Em692a, NC-OR-66, and STA-4. Although traces of

"W" can be found in conidial washes from all four of these strains, none were suitable for use in obtaining large quantities of "W" for purification and characterization experiments. When the emphasis of the study was shifted to the treatment of purified "Y" with guanidine hydrochloride, STA-4 was selected as the best wild type strain to use on the basis of its growth characteristics and its production of "W". When the necessity arose for a strain which produces more native "W", P-212, an exotic strain which was intensively studied by Madden (1971) and which contains "Y" and "W" in approximately a 50:50 ratio, was chosen for this purpose. As the study progressed, two more exotic strains, P-113 and P-278, were selected for guanidine hydrochloride experiments. Although these exotic strains are not proper N. crassa but are probably N. intermedia, they are genetically compatible with N. crassa in crosses. The strains used and their origins appear in Table 1.

TABLE 1

Neurospora Strains Used in the Study

Strain	FGSC No.	Source	Origin	Mating Type	β-glucosidases
74-OR23-1A	987	F.J. deSerres	St. Lawrence Wild Type	A	"Y" + "W" (trace)
Em692a	692	D.G. Catcheside	Emerson Wild Type	a	"Y" + "W" (trace)
NC-OR-66	none	W.K. Bates J.F. Wilson	Isolate of cross of Oak Ridge Wild Types	a	"Y" + "W" (trace)
STA-4	262	David D. Perkins	St. Lawrence Wild Type	A	"Y" + "W" (trace)
P-113	113	David D. Perkins	Australia	A	"Y" + "W"
P-212	212	David D. Perkins	Indonesia	A	"Y" + "W"
P-278	278	David D. Perkins	Singapore	A	"Y" + "W" (trace)

Stock cultures of 74-OR23-1A, Em692a, NC-OR-66, STA-4, and P-212 were maintained for transfer at room temperature on 8 ml of modified glycerol complete medium (Eberhart et al., 1964) containing 0.1% n-z-casein, 0.25% yeast extract, 1.5% agar, 2% Vogel's minimal salts medium (Vogel, 1956), 1% vitamin stock solution, 0.8% glycerol, and 1% sucrose. P-113 and P-278 were maintained by Eileen Hartis. Wild type strains and P-212 were transferred from silica gel approximately every six months to insure cultural continuity.

Growth and Harvest of Conidia for Crude Conidial Wash Preparations

To obtain large quantities of crude enzyme prepartions, it was necessary to use Erlenmeyer flasks for the growth of cultures. At the beginning of the study, large widemouth flasks (500 ml flasks containing 100 ml glycerol sucrose complete medium (GSCP) or 1000 ml flasks containing 300 ml of GSCP) were inoculated with a conidial suspension of the strain to be grown. The inoculated flasks were left at room temperature until the time of harvest (usually after seven days growth). Later, however, a method modified from Dr. William K. Bates employing a larger number of smaller flasks was used routinely. 250 ml widemouth Erlenmeyer flasks containing 50 ml of GSCP were inoculated with 1 ml of a dilute conidial suspension. In the case of strains which are often difficult to grow, such as 74-OR23-1A and Em692a, cultures were sometimes inoculated directly with conidia by "stabbing" the agar surface in the center and on each of four growing points directed at right angles to the central stab. The inoculated flasks were placed in the dark for 3 days at room temperature or in a 30 C incubator for 2 days. At this time, the mycelia had already

grown slightly less than half-way up the sides of the flasks and conidia
had already begun to appear on the aerial mycelia. The flasks were then
exposed to constant light until the time of harvest, which was usually
after a total of 7 days growth.

Crude enzyme preparations were obtained by a harvesting method
modified from Eberhart (1961) which is based on the solubility of extra-
cellular aryl-β-glucosidases in water. A suspension was made of the
conidia in each flask by adding a volume of sterile glass distilled water
approximately equal to the volume of media in the flask. Each flask was
shaken vigorously (with the cotton plug in place) to suspend the conidia.
The resulting suspension was filtered through two thicknesses of clean
cotton gauze pads to remove the mycelia. The filtrate was centrifuged
for 20 minutes in a desk model Sorvall centrifuge at a relative centri-
fugal force of 3,440 x g for small volumes of conidia or at 4 C in an
automatic refrigerated centrifuge (Model No. PR-J, International Equipment
Co.) at a relative centrifugal force of 3,000 x g. The conidial pellet was
resuspended in a small volume of glass distilled water and frozen for
future study. The supernatant fraction was placed in pre-boiled washed
dialysis tubing (Fisher Scientific Company) and concentrated with Carbowax
in the refrigerator for several hours until the desired concentration was
reached. This concentrate was frozen until needed.

Electrophoresis

During this study, two methods of electrophoresis were used. The
first employed the Gelman Electrophoresis Chamber (Model No. 51170-1)
using a cellulose polyacetate medium. Prior to the run, the chamber was

filled with 450 ml of chilled 0.1M potassium phosphate buffer at pH 6.0
which is close to the pH optima of cellobiase, another Neurospora β-gluco-
sidase. Although the pH optima of the aryl-β-glucosidases is 5.0,
electrophoretic separations done at this pH did not give clearly defined
bands (Madden, 1971 and unpublished data). Enzyme samples of ten micro-
liters were pipetted onto the Gelman electrophoresis applicator (Model
No. 51220) and were applied to the approximate center of Sepraphore 111
(1" x 6.75" or 1" x 6") cellulose polyacetate strips which had been pre-
soaked in 0.1M potassium phosphate buffer, pH 6.0. The origins were
carefully marked and the strips were tensioned on each side with small
magnets. Approximately seven samples could be run at the same time when
the strips were positioned in this manner. After the samples were in
place, the chamber was placed in the refrigerator and attached to a volt-
age regulated power supply. The voltage was increased to 250 volts and
the enzymes were allowed to separate for 20 minutes. At the end of the
separation, the strips were removed from the electrophoresis chamber and
were placed on Whatman No. 1 filter paper strips pre-soaked in a saturated
solution of the substrate, 4-methyl-umbelliferyl-β-D-glucopyranoside
(umbelliferone) and covered with plastic wrap to prevent evaporation.
As the β-glucosidic linkages were broken, a fluorescent moiety was
released which could be observed when the strips were exposed to a ultra-
violet light in a darkened room. The bands were marked on the plastic
wrap as they developed and were later transferred to a standard record
form.

The second method of electrophoresis employed the Gelman Sepratek
System. Prior to the run, the Sepratek buffer chamber (Model No. 51156,

Gelman Instrument Company) was filled with 200 ml of chilled 0.1M potassium phosphate buffer, pH 6.0. A 12.5 cm x 5.5 cm strip of Sepraphore Sepratek Medium pre-soaked in chilled 0.1M buffer was carefully blotted, tensioned on the support bridge, and placed in the buffer chamber. From one to eight enzyme samples were pipetted into the sample wells of the Sepratek applicator block with a clean Pasteur pipet. The "push-button" applicator was placed on the applicator block and "loaded" with the enzyme samples. The loaded applicator was placed over the closed buffer chamber and the samples were applied through the applicator slot. For concentrated enzyme samples, only one application was necessary, but for more dilute samples (especially guanidine hydrochloride-treated preparations) several applications were required. After the samples were applied, the electrodes were attached to the chamber. The entire apparatus was then placed in the refrigerator and the electrodes were attached to the voltage regulator. The voltage was increased to 200 volts and the enzymes were allowed to separate for 20 minutes. At the end of the run, the electrodes were removed and the origin was carefully marked. The cellulose strip was removed from the buffer chamber and placed on a piece of Whatman No. 1 filter paper pre-soaked in a saturated solution of umbelliferone. The strip was covered with a sheet of plastic wrap on which the bands were marked as the fluorescence appeared. The Sepratek method of electrophoresis was especially useful for separation of enzymes treated with guanidine hydrochloride. Since the samples were applied at the same time, in equal amounts, and at the same origin, any changes observed in enzyme activity or in electrophoretic mobility could thus be safely attributed to the effect of guanidine hydrochloride on the enzymes and not to any experimental error in application.

Thin-layer Gel Filtration

Thin-layer gel filtration (TLG) was used routinely throughout the study as a method of separating "Y" from "W" and of observing the effect of guanidine hydrochloride on these enzymes. In TLG, polymeric molecules (such as enzymes and other proteins) are separated on the basis of size by movement (usually _descending_ movement) through a shallow layer of swollen gel. Throughout this study, TLG was performed in the Pharmacia TLG-apparatus (obtained from Pharmacia Fine Chemicals AB) utilizing a synthetic maximum resolution polyacrylamide gel matrix. At the beginning of the study, Biogel P-200 (-400 Mesh), spread on 20 x 40 cm glass plates, was used. This gel has an exclusion limit of 200,000 daltons and a fractionation range of 30,000-200,000 daltons; thus, it is suitable for use with globular proteins the size of "Y" (168,000 daltons) and "W" (40,000 daltons). It has the disadvantage, however, of requiring 48 hours at room temperature to achieve maximum separation. Substitution of Biogel P-150 for Biogel P-200 and 20 x 20 cm plates for 20 x 40 cm plates solved both of these problems. Biogel P-150, which has an exclusion limit of 150,000 daltons and a fractionation range of 15,000-150,000 daltons, is also suitable for use with molecules the size of "Y" and "W", requires only 24 hours at room temperature to hydrate, and requires only 2.5-3 hours at room temperature to achieve a good separation.

The gel was prepared by _slowly_ and _carefully_ suspending the required amount of gel (5.0 g for P-200 and 7.5 g for P-150) in 200 ml of the standard eluting buffer, 0.05M potassium phosphate, pH 6.0. Hydration was routinely carried out at room temperature. In order to normalize the ratio between the stationary (gel) and mobile (eluting buffer) phase

volumes, it was necessary to set up the plate the night before the run in order to allow at least 12 hours for equilibration to occur. 60-65 ml of the eluting buffer was placed in the upper buffer well and 40-45 ml in the lower buffer well if one 20 x 40 cm plate was to be used. If two 20 x 20 cm plates were to be used, 40-45 ml of buffer was placed in the central well and 30-35 ml in the lower. The swollen gel was carefully spread on a thoroughly cleaned and dried plate to a thickness of 0.6mm with the TLG spreader. The spread plate was placed in the TLG chamber and the gel layer was connected to the buffer wells by 17.5 x 5.0 cm Whatman No. 3 filter paper "bridges". After the lid of the chamber was firmly set in place, the system was elevated to 10° and was allowed to equilibrate over-night.

For most experiments, a maximum of six or seven samples were run per plate. With the TLG apparatus horizontal, samples were applied through the sample slits with a clean micropipet. Although a variety (5-20 micro-liters) of sample volumes were employed, samples of 5 microliters were used most frequently as suitable spot sizes were obtained and more samples could be run on one plate with this volume. The concentration of the sample is also an important factor to be considered. Viscosity, caused by too high a sample concentration, cause zone distortion (streaking) and reduces the rate of migration. This was not a problem in preparations treated with guanidine hydrochloride as the enzyme concentration of such samples was very low, but control samples of high activity were diluted with distilled water to reduce the viscosity effect and thus give these samples a mobility more like that of the more dilute guanidine-treated samples.

When all samples had been applied, the plate was elevated to start the buffer flow. Plates spread with Biogel P-200 were generally run at an angle of 10° for 8-9 hours or at 15° for 5-6 hours. Plates of Biogel P-150 were inclined to an angle of 15° and were allowed to run for 2.5 hours. TLG runs were always carried out in the dark and the lid of the apparatus was covered with aluminum foil to help minimize leakage of air into the system.

At the end of the run, the system was lowered and the plate was removed from the chamber. A sheet of Whatman No. 1 filter paper corresponding to the size of the plate and pre-soaked in a saturated solution of the substrate umbelliferone was carefully pressed on the gel and the entire plate was covered with plastic wrap to prevent evaporation. The presence of aryl-β-glucosidase activity was observed as fluorescence when the plate was viewed under ultra-violet light in a darkened room. Enzyme spots were marked on the plastic wrap as they occurred and were later transferred to a permanent record.

Column Gel Filtration

When large quantities of purified enzyme were needed, column gel filtration was used routinely as a convenient method of separating the extracellular aryl-β-glucosidases in a crude conidial wash. Like TLG, column gel filtration accomplishes the separation of enzymes and other proteins on the basis of size by movement through swollen gel. The gel in this case is loosely packed in a cylindrical column. Column fractionation was performed utilizing a jacketed Pharmacia K50 column and a synthetic polyacrylimide gel matrix. At the beginning of the study,

Biogel P-100, 100-200 Mesh, was used for column separations, but later the column was repacked with Biogel P-150, 100-200 Mesh.

The conidial wash to be separated on the column was concentrated with Carbowax after harvest until at least a 5-10 fold concentration was achieved. Since the enzyme activity is diluted with the eluting buffer, it was necessary to have a very concentrated preparation at the outset. This concentrated preparation was spun at least twice at 3,000 x g. Prior to the column run, the concentrate was assayed with PNPG to check on its activity. If the column run was to take place at a later date, the concentrated wash could be frozen without loss of activity until needed.

When a freshly packed column was to be used, a small volume (1-2 ml) of Blue Dextran 2000 was applied to the column the day before the column run in order to check the evenness of the flow and the uniformity of the packed bed. The column was always washed during the day and overnight with 1-2 liters of the eluting buffer, 0.05M potassium phosphate, pH 6.0.

Immediately prior to the column run, the concentrated wash was thawed and assayed with PNPG. At least ten milliliters of the concentrate was slowly and carefully applied through the 4-way valve with a 10 cc syringe. About 10 minutes was required to apply the enzyme and great care was taken that air bubbles were not allowed to enter the system. Between 20 and 30 cm of hydrostatic pressure was obtained with a Mariotte flask, giving a flow rate of 1-2 ml/min. The packed bed of the column was approximately 42.0-43.0 cm x 5.0 cm, the variation in the length of the column being due to slight differences in packing. After the application of the enzyme, the column was allowed to run until 200 ml of bed buffer had passed at which time the collection of fractions was begun.

At the beginning of the study, fractions were collected utilizing an unrefrigerated automatic fraction collector (Model No. A, ISCO). The fraction collector was set to collect at 3.5 minute intervals, which, at a flow rate of 1-2 ml/min., gave individual fraction volumes of ± 4 ml. 72 such fractions were normally collected in this manner. This method of collecting fractions had the advantage of giving uniform fraction volumes. It had the disadvantage, however, of giving small fraction volumes, which, with the elution pattern of our enzyme system, is not necessary. Another disadvantage was that the fractions must be removed from the collector and refrigerated as soon as they are collected to prevent loss of activity.

Later in the study, a more satisfactory method of fraction collection was developed. Fractions were collected by a water driven automatic fraction collector immersed in a water bath set at 4 C. (Both the fraction collector and the water bath were obtained from Buchler Instruments.) The water clock was set to collect approximately every 15 minutes, giving a per fraction volume of 15-20 ml. From 30-32 fractions were collected in this manner, corresponding to about 500 ml of bed buffer. (Since the fractions began to be collected as soon as the column was connected to the buffer reservoir via the 4-way valve, the first few fractions (approximately 200 ml) contained no aryl-β-glucosidase activity.) This method of collection not only permitted larger fraction volumes, but also kept the fractions cold until they could be assayed and frozen.

The fractions collected after 200 ml of bed buffer had passed were assayed with PNPG to determine the net aryl-β-glucosidase activity. The data from the assays was then plotted to obtain an elution profile. The

exact aryl-β-glucosidase character of the fractions was determined by electrophoresis of every fifth fraction collected by the "old" method or by TLG of the fractions to be used in further experiments. In the case of fractions collected by the "old" method (in small volumes), fractions of similar activity were pooled and frozen for future use. Fractions collected by the "new" method did not have to be pooled and were merely frozen until needed.

PNPG Assay Methods

The substrate used in routine quantitative assays of aryl-β-glucosidase activity was p-nitrophenyl-β-D-glucopyranoside (PNPG). When the β-glucosidic linkage of this compound is hydrolyzed, p-nitrophenol is released. At an alkaline pH, this compound is converted to a chromogenic form which can be analyzed colorimetrically. The discontinuous colorimetric method described by Eberhart (1961) was modified in two ways for use in this study.

For quick assays, a short method utilizing a PNPG stock solution with a concentration of 2 mg/ml was employed. The stock solution was usually freshly prepared for the experiment in question, but it could be refrigerated for short periods or frozen for longer periods. For the short assay, 0.1 ml of the PNPG stock solution was mixed with 0.1 ml of the enzyme preparation to be assayed, giving a final substrate concentration of 1 mg/ml. Larger sample volumes could be used as long as the final concentration of PNPG was 1 mg/ml. At the end of the desired incubation time (usually 10-20 minutes at room temperature), the reaction was stopped by the addition of 0.1 ml 1M Tris. The alkaline pH of 9.2 which resulted

converted all the released nitrophenol to its yellow-colored form. Color controls in which the enzyme was mixed with Tris prior to the addition of the substrate were run on each enzyme sample. The optical density of each sample was read at 410 mµ on the Beckman Spectrophotometer. The net aryl-β-glucosidase activity of each sample was determined by subtracting the optical density of the control from the total optical density of the corresponding sample. The readings were usually recorded as net optical density, but the data of some experiments was converted to optical density per minute.

For assays of guanidine hydrochloride treated preparations, a longer method which corresponds more closely to the discontinuous method referred to above was used. A stock solution of PNPG (10 mg/ml distilled water) was prepared when needed. 0.1 ml of this stock solution was diluted with 0.7 ml 0.1M potassium phosphate buffer, pH 6.0, and was mixed with 0.2 ml of the enzyme sample to be assayed, giving a final PNPG concentration of 1 mg/ml. (Each 0.2 ml enzyme sample contained enzyme and guanidine in a 1:1 ratio for treated samples or enzyme and distilled water in a 1:1 ratio for control samples.) After the required incubation time (usually 20-60 minutes at room temperature, depending on the activity of the original untreated enzyme sample), the reaction was stopped by the addition of 0.5 ml 1M Tris, thus producing the colored form of the nitrophenol. Color controls for each enzyme sample were prepared by combining 0.1 ml of the enzyme solution to be assayed with 0.1 ml glass distilled water. To this mixture was added 0.5 ml 1M Tris and, after mixing, 0.7 ml dilute PNPG. The optical density of each sample was read at 410 mµ on the Beckman Spectrophotometer. The net aryl-β-glucosidase activity of each sample was

determined by subtracting the optical density of the control from the optical density of its corresponding sample. The activity was recorded as net optical density and as activity per minute. The activity was plotted against time on a semi-log scale as net optical density values.

Chemical Denaturation and Inactivation Methods

Although a small number of denaturation experiments were performed using urea as the denaturing agent, the chemical agent used for the large majority of the denaturation and inactivation studies to be described in this thesis was guanidine hydrochloride (GuHCl). The early experiments with GuHCl were performed using Sigma reagent grade GuHCl, but most of the inactivation studies to be reported below were done utilizing Mann Ultrapure GuHCl as the denaturant. During this study, two major methods using GuHCl were developed.

The first method developed involved the treatment of purified P-212 conidial "Y" with GuHCl in an attempt to cause the conversion of "Y" to "W" or some other enzymatically active component. Immediately prior to the start of the experiment, a fresh solution of the desired concentration (usually 4-8M) was prepared by suspending the correct amount of GuHCl in 1 ml of 0.1M acetate buffer, pH 4.0. (A pH of 4.0 was used in the early experiments with GuHCl in the interest of repeating exactly some very preliminary experiments in this area which were done by Brenda Madden in the summer of 1971. This pH was used throughout the study for purposes of consistency.) If a reducing environment was to be used, Cleland's Reagent or 2-mercaptoethanol to a concentration of 0.01M was added to the solution. Sigma GuHCl solutions of high concentration (6M) had to be

heated gently and stirred with a magnetic stirrer to solubilize the guanidine and then filtered to remove insoluble residues. Although these residues probably consisted mostly of impurities, there was a possibility that the desired molarity had not been reached. By contrast, Mann Ultra-pure GuHCl solutions of concentrations as high as 10-12M could be obtained with no difficulty at all. Once prepared, the GuHCl solution was left at room temperature until needed.

After the guanidine solution was prepared, the enzyme preparation (partially purified concentrated P-212 "Y") to be used was thawed in a water bath at room temperature and then assayed with PNPG. A 1:1 mixture of enzyme to guanidine was prepared by combining 20 microliters of each reactant in a small spot test plate, giving final concentrations of 0.005M for the reducing agent, 0.05M for the acetate buffer, and of one-half the original concentration for the guanidine solution. Mixtures were made and allowed to incubate at room temperature for a specified time (usually 120, 90, 60, 45, 30, and 15 minutes). At the end of the incubation time, samples were removed from the reaction mixtures and separated by electro-phoresis, TLC, or both. For electrophoretic separations, 10 microliter samples were removed from each of the reaction mixtures with a micro-pipet and applied to the applicator block. After application to a 12.5 x 5.5 strip of Sepraphore cellulose polyacetate with the Sepratek applicator (3-4 applications), the samples, along with a P-212 crude conidial wash control and a P-212 "Y" control, were separated by electrophoresis using standard Sepratek procedure and "assayed" with umbelliferone as described in the methods. For thin layer separations, 5 microliter samples were removed (generally from only the 30, 60, 90, and 120 minute reaction

mixtures) and were applied (along with the appropriate controls) to a plate of Biogel P-150 (-400 Mesh), spread to a thickness of .6 mm. The system was elevated to 15° and was allowed to run in the dark at room temperature for 2.5 hours at the end of which time the enzymes were scored with umbelliferone.

Later in the study, variations in this method were developed using TLG as the separating technique. In parallel to molecular weight determinations in which GuHCl was placed in the separating medium (Heinz et al., 1971), Biogel P-150 (-400 Mesh), was prepared in 0.05M potassium phosphate, pH 6.0, containing GuHCl to a concentration of 0.1M. After swelling, the "guanidine gel" was spread on a 20 x 20 cm glass plate to a thickness of 0.6mm. The buffer wells were filled with 0.05M potassium phosphate-buffered 0.01M GuHCl. The remaining steps in preparation, equilibration, and running of the plate were as described in the methods. The plate was used to separate samples from a GuHCl experiment identical to the one described above. After the run, the enzymes were scored with umbelliferone in the usual manner.

Another variation utilizing TLG aided in exploring the effect of temperature on the GuHCl reaction. 0.1 ml of a P-212 "Y" enzyme concentrate was incubated with 0.1 ml of a freshly prepared 8M GuHCl solution (in 0.1M acetate, pH 4.0) at 4 C, giving final concentrations of 4M GuHCl and 0.05M acetate. Mixtures were made and were allowed to incubate for 120, 90, 60, and 30 minutes. At the end of 2 hours, 5 microliter samples were removed and applied to a 20 x 20 cm plate of Biogel P-150 (-400 Mesh), which had been equilibrated overnight in the cold. The plate was elevated to 15° and allowed to run for 2.5 hours. At the end of the run, the plate

was removed from the refrigerator and the enzymes were scored with umbelliferone. Experiments were also done in which the reaction took place at 4 C, but separation on Biogel P-150 was carried out at room temperature in the usual manner.

The second major method developed in the study explored the overall response in time of crude conidial washes, and purified conidial "Y" and "W" to inactivation by GuHCl. A method using the standard PNPG assay was devised in the summer of 1971 by Brenda Madden and was utilized for her early experiments. I later modified this method to provide more information about the quantitative effect of GuHCl on our enzyme system and its standard assay. This was the method used throughout most of the study and the one used with Mann Ultrapure GuHCl. Immediately prior to the start of the experiment, a GuHCl solution of the desired concentration was prepared in 0.1M acetate buffer, pH 4.0. In the early experiments, Cleland's Reagent was added to a concentration of 0.01M, but later experiments were performed in the absence of any reducing agent. The enzyme preparations (usually a crude conidial wash and a purified "Y" and "W" prep for each strain to be tested) were thawed in a water bath at room temperature. 0.1 ml of each enzyme preparation was pipetted into each of 7 7.5 cm x 1.0 cm appropriately marked test tubes with an Oxford Laboratory Sampler. At time 0, 0.1 ml of the GuHCl solution was added to each of the 7 enzyme samples. The tubes were gently shaken to mix the contents. After one minute incubation at room temperature, 0.8 ml of dilute PNPG (0.1 ml stock PNPG (10 mg/ml) diluted with 0.7 ml 0.1M potassium phosphate, pH 6.0) was added with a 1 ml syringe to the tube which had been designated as "late addition of GuHCl". The PNPG effectively diluted the GuHCl so that the

activity assayed essentially represents the activity remaining after 1 minute's incubation in GuHCl. These samples were allowed to incubate with PNPG at room temperature for a suitable time period (usually 20 minutes or longer for less active preps) when the reaction was stopped by the addition of 0.5 ml Molar Tris. The other 6 samples were assayed with PNPG in the same way at the end of their appropriate incubation times (15, 30, 45, 60, 90, and 120 minutes). During the course of the experiment, controls were prepared for each strain. To control for the inhibitory effect of GuHCl on the PNPG assay itself, "simultaneous addition of GuHCl" samples were prepared by adding 0.1 ml GuHCl and 0.8 ml PNPG at precisely the same time to a test tube containing 0.1 ml of the enzyme preparation. The "simultaneous addition" samples were allowed to incubate at room temperature for an appropriate time. Untreated controls were prepared by diluting 0.1 ml of the enzyme prep with 0.1 ml glass distilled water and were assayed with PNPG as above. Color controls were prepared by combining 0.1 ml of the enzyme preparation, 0.1 ml glass distilled water, 0.5 ml Tris, and 0.8 ml PNPG. The activity of all samples was read as the O.D. at 410 mμ on the Beckman Spectrophotometer. The net activity of each sample was determined by subtracting the O.D. of the appropriate control from the O.D. of the sample. The results were recorded as net optical density and were plotted against time on a semi-log scale.

For routine experiments with most strains at most concentrations of GuHCl, the "standard method" described above was satisfactory. With P-212 aryl-β-glucosidases and 4M GuHCl, however, the results obtained were not strictly repeatable. Late in the study, the "standard method" was improved to eliminate variations which might arise from slight differences in mixing

or in the temperature of the tubes. The entire experiment was carried out in a 20 C water bath. Enzyme concentrates were thawed in the water bath and the freshly prepared GuHCl and dilute PNPG solutions were placed in the water bath until they were needed. At time 0, a 1:1 mixture of enzyme to GuHCl was made in a 10.0 x 1.5 cm test tube which was immersed in the 20 C water bath. 0.2 ml samples were removed from this mixture after 1, 15, 30, 45, 60, 90, and 120 minutes incubation and were placed in 7.5 cm x 1.0 cm test tubes containing 0.8 ml dilute PNPG for assay. Simultaneous addition samples and untreated and color control samples for each enzyme concentrate were prepared in separate tubes as described in the "standard method". These improvements were tested by employing duplicate and even triplicate samples in the early runs and were found to effectively eliminate the two sources of error mentioned above and to produce much more reliable results.

The data obtained from the inactivation experiments were submitted to statistical analysis in order to determine quantitative half-time values for crude conidial washes, conidial "Y", and conidial "W" in the presence of guanidine hydrochloride. Half-time values were calculated using multiple regression analysis based on the $\log_{10}$ of the enzymatic activity. A least squares program based on the equation $\log_{10} Y = A_0 + A_1 t$ was used with a programmable calculator. The incubation times (0, 1 min., 15 min., etc.) were entered as the t values and the corresponding optical density data were entered as the Y values. The $t_{1/2}$ value for that particular data set was calculated directly from the data by the program. Each set of data was entered at least twice in order to eliminate possible mechanical errors in entering the numbers.

For curves with a relatively slight amount of "scatter", this method proved satisfactory; however, in cases where activation was observed, all data points could not be used in the calculation of the half-time. Instead, only the point of highest activation and those following it were entered. For purposes of these calculations, an <u>apparent</u> activation was not considered a "true" activation unless at least two previous data points were obtained below the point of activation. A minimum of three data points were used for each calculation, with from 6 to 8 being the average number. If three points could not be obtained before all activity had disappeared (as, for example, in the case of P-113 "Y"), a low value of .0001 was entered as the "Y" value of the third point so that a half-time could be calculated for that particular data set. After the half-time was recorded, the y-intercept (A_0) and the slope (A_1) of the regressions line described by the equation were also recorded. In addition, the program was designed so that, once the half time has been calculated from a set of experimental data, the same or different t values could be entered and the corresponding Y values obtained. A straight line based on the calculated slope and y-intercept could then be drawn for comparison with the data points. T values of 0, 1, 5, 10, 15, 30, 45, 60, 75, 90, 105, and 120 were entered for each set of experimental data and the corresponding Y values of the straight line given by the calculated A_0 and A_1 were recorded for reference.

RESULTS

At the outset, this study involved an intensive survey of several available prototrophic wild type strains of Neurospora crassa (specifically, 74-OR23-1A, Em692a, NC-OR-66, and STA-4) in order to determine the presence of the "new" aryl-β-glucosidase "W" discovered by Eberhart and Madden (Madden, 1971, and unpublished data). The strain having the "best" or most abundant "W" present was to be used to obtain large quantities of "W" for purification and characterization of its physical properties. Development of a quantitative method for determining the precise amount of "W" present in a mixture of the two aryl-β-glucosidases was also a primary objective of this study as it was originally designed. Although the emphasis of the study was later shifted to chemical denaturation of Neurospora aryl-β-glucosidase isozymes, the subject of the majority of the research to be described in this thesis, it is of interest to note here some of the findings which were brought to light by this early work.

Determination of Growth Conditions for Optimal Production of "Y" and "W"

In order to determine the best growth conditions for optimal production of "Y" and "W" in all wild types, the strain 74-OR23-1A was selected as a representative strain and was grown at various temperatures (20 C, 25 C, and 28 C) and on two different media (Vogel's minimal plus 1% sucrose and glycerol sucrose complete media). Four 500 ml Erlenmeyer flasks containing 100 ml each of Vogel's minimal media (VS2) and four flasks containing 100 ml each of glycerol sucrose complete media (GSCP)

were inoculated with a few drops of a conidial suspension of 74-OR23-1A and were placed in an incubator set at 20 C. Eight flasks were inoculated in the same way for growth at 25 C and at 28 C. To investigate the optimum time for harvest of the conidia, one flask of each experimental condition was harvested after 5, 7, 9, and 11 days growth (with the exception of one set of 20 C flasks which were not ready for harvest at 5 days which consequently left two 20 C flasks to be harvested at 13 days). After harvest, the crude washes for each experimental condition were assayed with PNPG to determine their total aryl-β-glucosidase activity. The results of this assay appear in Table 2 and Figure 1. The optimum conditions of growth and harvest suggested by these results were growth on GSCP media at 25 C and harvest at 7 days, although the 0.040 units difference observed with minimal media is probably not an extremely significant difference. This conclusion is based not only on total activity recovered, as the same optical density value was obtained with MM at 25 C for 9 days, but also on the shortest incubation time that still yields high aryl-β-glucosidase activity.

The results also show that cultures grown at 20 C on GSCP contained more aryl-β-glucosidase activity than those grown at that temperature on VS2, with a peak in activity at 9 days for GSCP and at 11 days for VS2. In contrast, cultures grown at 28 C on VS2 contained more extracellular aryl-β-glucosidase activity than those grown at 28 C on GSCP (with one exception at 7 days growth), with a peak in activity at 5 days for VS2 and a slight peak at 11 days for GSCP. Activity at 28 C on VS2 was highly irregular, however, and was also generally poor on GSCP at this temperature. Although 25 C was considered the optimum temperature for growth, 20 C gave

TABLE 2

Production of Aryl-β-Glucosidase Isozymes
"Y" and "W" Under Various Growth Conditions

Prep #	Media	Temp.	Day Harvested	Optical Density at 410mμ	Y activity	W activity
1	VS2	25°	5 da	.035	+	−
2	GSCP	25°	5 da	.140	+++	−
3	VS2	28°	5 da	.320	+++	±
4	GSCP	28°	5 da	.010	±	−
5	VS2	20°	7 da	.142	++	−
6	GSCP	20°	7 da	.230	++++	−
7	VS2	25°	7 da	.320	++++	+
8	GSCP	25°	7 da	.360	++++	++
9	VS2	28°	7 da	.040	+	+
10	GSCP	28°	7 da	.095	+++	+
11	VS2	20°	9 da	.190	+++	+
12	GSCP	20°	9 da	.290	+++	+
13	VS2	25°	9 da	.360	+++	+
14	GSCP	25°	9 da	.240	++++	++
15	VS2	28°	9 da	.110	+++	+++
16	GSCP	28°	9 da	.085	+++	++
17	VS2	20°	11 da	.240	+++	−
18	GSCP	20°	11 da	.250	++++	±
19	VS2	25°	11 da	.260	++++	+
20	GSCP	25°	11 da	.320	++++	++
21	VS2	28°	11 da	.250	+++	+
22	GSCP	28°	11 da	.105	++++	−
23	VS2	20°	13 da	.115	++++	++
24	GSCP	20°	13 da	.185	+++	+

Figure 1. Aryl-β-Glucosidase Activity Produced by Neurospora crassa Wild-type Strain 74-OR23-1A Under Various Conditions of Growth.
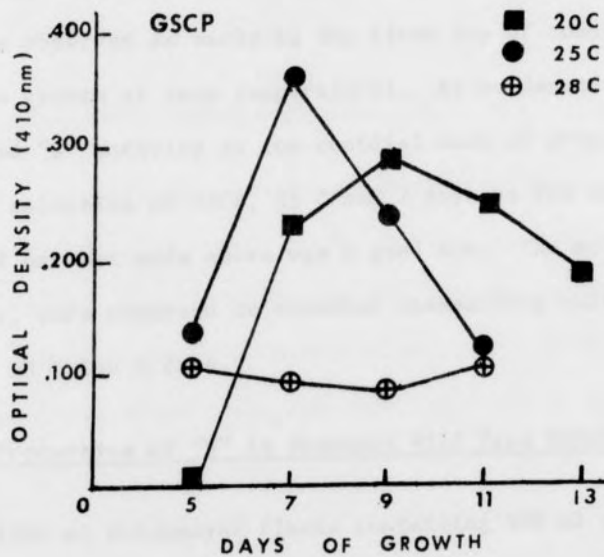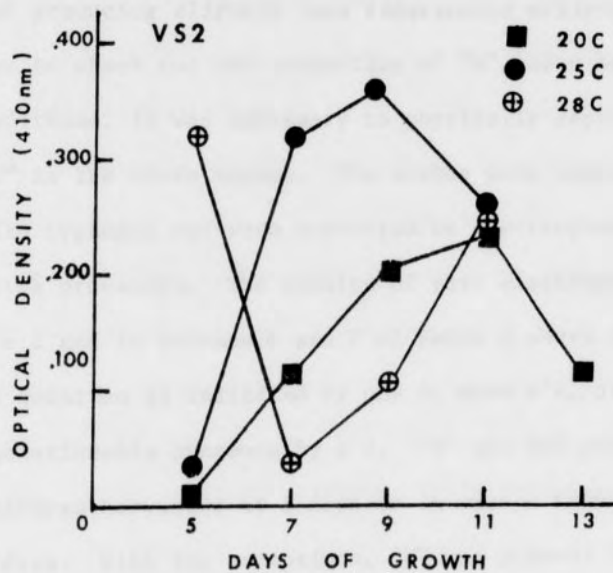
an even more regular pattern of growth on both types of media. This temperature has the disadvantage, however, of taking longer to give good activity and of producing slightly less recoverable activity.

In order to check for the production of "W" under these 24 experimental conditions, it was necessary to physically separate it from its isozyme "Y" in the crude washes. The washes were concentrated approximately 25X with Lyphogel and were separated by electrophoresis using standard Sepratek procedure. The results of this electrophoresis are shown in Figure 1 and in columns 6 and 7 of Table 2 where the presence of the isozyme in question is indicated by one or more +'s, its absence by a -, and its questionable presence by a ±. "W" was not present in conidial washes from cultures harvested at 5 days or in washes from cultures grown at 20 C for 7 days. With two exceptions, "W" was present to some extent under all other growth conditions. This result differs from those obtained by Madden (1971) with the exotic strain P-212 in which evidence of "W" production was observed as early as the first day of conidiation (approximately 3 days growth at room temperature). As evidenced by the presence of good "Y" and "W" activity in the conidial wash of prep number 8 (see Table 2), the selection of GSCP, 25 C and 7 days as the optimum conditions for growth and harvest made above was a good one. The most active "W" bands, however, were observed in conidial washes from cultures grown on both media at 28 C for 9 days.

Evidence for Production of "W" in Standard Wild Type Strains

Three 1000 ml Erlenmeyer flasks containing 300 ml of GSCP media each were inoculated with a conidial suspension of each of the four

standard <u>Neurospora</u> wild type strains mentioned above. The flasks were grown at 25 C and were to be harvested at 7 days, but were not sufficiently conidiated for harvest until 9 days. The yield of this harvest was too low to obtain a sufficiently concentrated preparation, so it was decided to try a different method of growth. The method modified from Dr. Bates was used successfully to obtain a substantial quantity of enzyme from each wild type strain. After concentration (approximately 10-fold) with Carbowax, the conidial washes were separated by electrophoresis with Sepratek and by thin-layer gel filtration on Biogel P-200. The results of the electrophoretic separation experiments appear in Figure 2. The electrophoretic mobility (mm/20 min., 200 V) of "Y" was 12 and that of "W" was 7 for all strains tested. Although small amounts of "W" were found in all four wild types with both separation techniques used, it appeared that <u>Neurospora crassa</u> wild type stocks in common laboratory usage do not contain sufficient "W" for purification and characterization experiments.

Investigation of a Quantitative Assay for "W"

Some preliminary work was done toward development of a quantitative method for determining the amount of "W" present in a mixture of the two aryl-β-glucosidases. A method based on the two assays used in our laboratory - the quantitative PNPG assay and the qualitative umbelliferone assay - was developed whereby the two isozymes could be crudely quantified. The enzymes in a crude conidial wash were separated electrophoretically in the Gelman Model No. 51170-1 electrophoresis chamber and the isozyme bands were marked using umbelliferone as the substrate. The

Figure 2.   Sepratek Electrophoresis of Crude Conidial Washes of Some
            Neurospora Wild-type Strains.

STRAIN   origin

NC-OR-66

STA-4

EM692a

74-OR23-1A

P-212

strips were cut on either side of each band to separate each individual "Y" and "W" band from the Sepraphore strip. Each separate band was then placed in a 5 ml disposable beaker containing 1 ml dilute PNPG (1 mg/ml). After one hour's incubation, the reaction was stopped by the addition of 0.5 ml Molar Tris. The optical density values obtained when samples of the solutions were read in the Beckman Spectrophotometer did provide a crude estimate of the relative activity of each isozyme. Experiments conducted using this method, however, were not repeatable and the results were so unusual that they will not be reported in this thesis.

The unusual results and the lack of repeatability, however, raised the question that some uncontrolled factor (or factors) was interfering with the ability of the system to produce reliable results. Among the possibilities were the pH of the reaction, the attraction of the Sepraphore electrophoresis medium for the enzyme, and the time necessary for elution of the enzyme from the Sepraphore paper. These three factors were investigated in an interesting experiment the results of which appear in Table 3. The PNPG reaction was run at both pH 5.0 (the pH optima of both "Y" and "W") and pH 6.0 (the standard pH for electrophoresis, PNPG, and umbelliferone reactions). A concentrated crude conidial wash preparation of 74-OR23-1A was assayed under the following conditions for 10 min and for 1 hour at both pH 5.0 and 6.0:

1. The enzyme was applied directly to the Sepraphore electrophoresis paper (presoaked in 0.1 M potassium phosphate buffer of the appropriate pH) and was placed in the substrate (diluted with buffer of the appropriate pH).

TABLE 3

Investigation of the Quantitative Assay Method for "W"

	pH 5.0 10 min reaction		pH 6.0 10 min reaction		pH 5.0 60 min reaction		pH 6.0 60 min reaction	
	O.D. @ 410mμ	O.D.- control	O.D. @ 410mμ	O.D.- control	O.D. @ 410mμ	O.D.- control	O.D. @ 410mμ	O.D.- control
1. Enzyme applied directly to Sepraphore paper	.130	.105	.085	.070	.520	.480	.400	.365
2. Enzyme added to the substrate, but Sepraphore paper placed in mixture	.155	.130	.105	.090	.680	.640	.420	.395
3. Enzyme directly mixed with substrate	.220	.195	.150	.135	.900	.860	.510	.475
4. Color control (enzyme mixed with Tris prior to addition of substrate)	.025	–	.015	–	.040	–	.035	–

2.   The enzyme was mixed directly with the substrate, but a piece of Sepraphore paper was placed in the mixture to test for possible interference of the paper itself.

3.   The enzyme was directly mixed with the substrate.

4.   The enzyme was mixed with 1M Tris prior to the addition of the substrate, thus providing a color control.

The results indicate that running the PNPG reaction at pH 5.0 was more likely to produce readable results than pH 6.0. Not only was the activity significantly greater at pH 5.0 than pH 6.0, but the effect was increased with time as evidenced by a comparison of the values obtained at 10 min incubation with those obtained after one hour's incubation. This result was not surprising since pH 5.0 is known to be the pH optima of both "Y" and "W". The results of this experiment also indicated that the Sepraphore paper, in some as yet undetermined way, interfered with the reaction by absorbing or "holding back" part of the enzyme. Even when the paper was placed in the mixture after the enzyme had been added to the substrate, the paper still absorbed some of the enzyme, resulting in a decrease in the amount of activity recovered.

Although a good quantitative assay for "W" was not developed, the assay method described above would perhaps still be feasible if relatively concentrated preparations were used. Then the loss of as much as 50% of the activity would not prevent one from obtaining readable results. The amount of activity would have to be reduced proportionally so that the relative activities of the enzymes (i.e., the ratio of "Y" to "W") would not be altered by the failure to elute all of the enzyme. Much more work needs to be done in this area as a good assay which would distinguish

between "Y" and "W" in a mixture of the two would be an invaluable, time-saving research tool.

Preliminary Experiments with Guanidine Hydrochloride

Since we were unsuccessful in finding a wild type strain with sufficient "W" for purification and characterization experiments and since more information about the exact relationship of "W" and "Y" would be useful in future investigation of an assay for "W", we decided to modify our original goals. Although the aryl-β-glucosidase system is considered to be an isozyme system in several of the exotic strains tested by Madden and, to a lesser degree, in standard wild type strains, the exact isozyme relationship of "Y" and its isozyme "W" is not known. The aryl-β-glucosidase designated "Y" is known to be the larger enzyme by its thin layer and column gel filtration characteristics and from molecular weight determinations by Madden (1971). Whether the aryl-β-glucosidase system is homopolymeric or heteropolymeric, "W", the smaller enzyme, would be at least in part a subunit of "Y". It should therefore be possible to generate or produce "W" from a purified preparation of "Y" with guanidine hydrochloride (GuHCl), urea, or any of the other chemical denaturants commonly used for this purpose. Using guanidine hydrochloride (Sigma Chemical Company) as the denaturant, several preliminary experiments were conducted.

When an old mycelial"Y" preparation from Reta Beck was thawed, incubated with 3M GuHCl, and separated by TLG on Biogel P-200, changes in the mobility of the "Y" enzyme which might indicate the production of one or more smaller enzymes were observed due to the action of GuHCl over time.

A crude STA-4 conidial wash which had been shown by TLG to contain no detectable "W" was also treated with 3M GuHCl and a less mobile slur of activity was produced in time. When samples of the wash were incubated with various concentrations of GuHCl for one hour and were separated by TLG on Biogel P-200, final GuHCl concentrations as high as 6-10M completely destroyed all aryl-β-glucosidase activity. Final concentrations in the range of 2-4M appeared to be most suitable for GuHCl denaturation experiments.

Purification of Wild Type Conidial "Y" for Denaturation Studies

Experiments such as those briefly described above, however, can not actually show that the "W" (or any other smaller enzymatically active molecule) observed has been produced from "Y" by the action of GuHCl in time - unless the enzyme preparation to be treated is initially free of "W". A simple and efficient way to accomplish this is gel column fractionation. Ten milliliters of a concentrated STA-4 conidial wash was placed on a Pharmacia K50 column of Biogel P-100, 100-200 Mesh, and was separated according to the procedure described in the Methods. A bimodal elution profile was obtained (Figure 3) and both electrophoresis and TLG showed only "Y" to be present in the earlier fractions and only "W" to be present in the later fractions, with intermediate fractions containing a mixture of the two molecular species. The "purest" (i.e., earliest) "Y" fractions of good activity were used for GuHCl treatment experiments. Although the "Y" obtained from a single column run was not in any sense chemically "pure", such a partially purified preparation was quite suitable for the denaturation studies under consideration here.

Figure 3. Elution Profile of Extracellular β-Glucosidases of STA-4
from a Biogel P-150 Column.
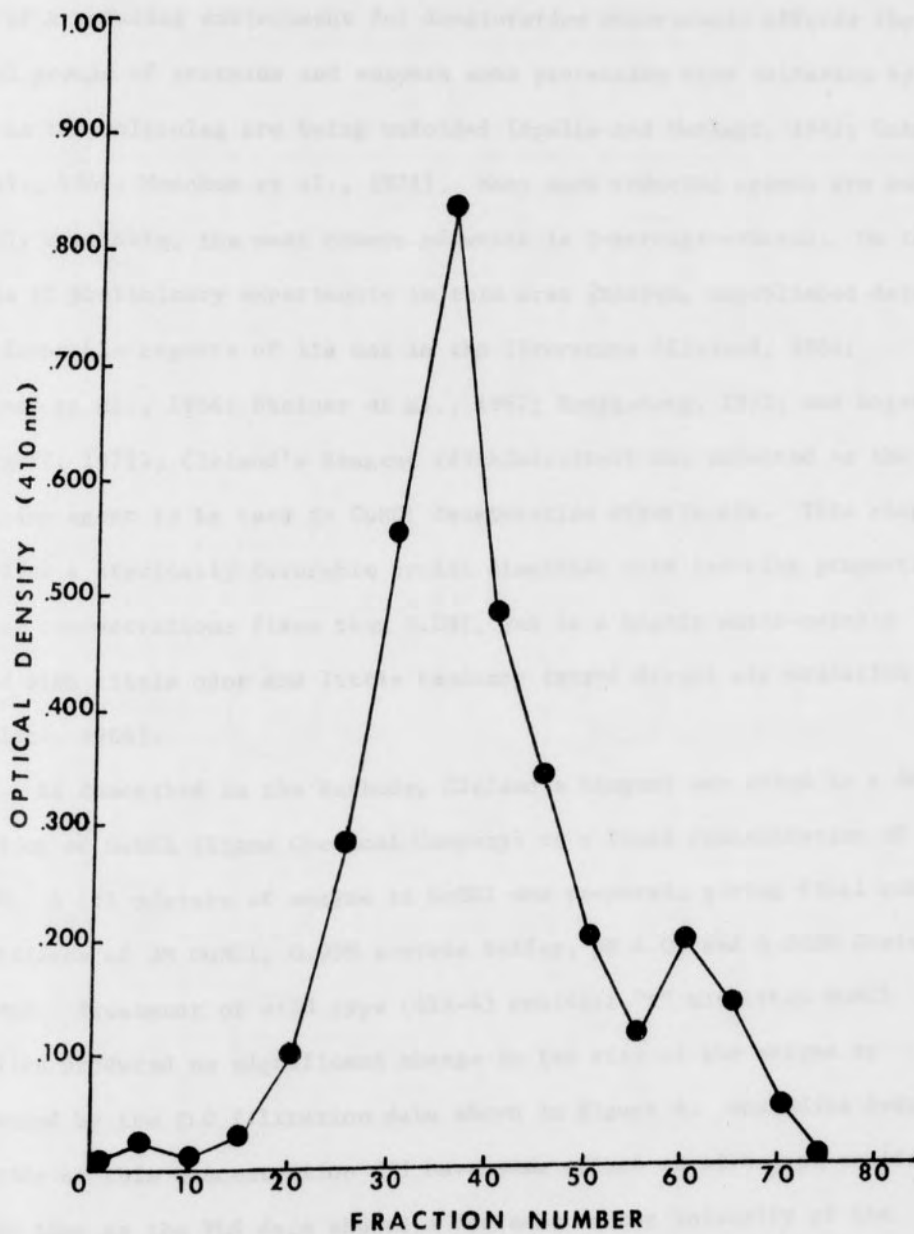
Treatment of Purified Wild Type (STA-4) Conidial "Y" with 3M GuHCl and 0.005M Cleland's Reagent

The literature reports the finding of some investigators that the use of a reducing environment for denaturation experiments affords the thiol groups of proteins and enzymes some protection from oxidation by air as the molecules are being unfolded (Apella and Markert, 1961; Cahn et al., 1962; Meachum et al., 1971). Many such reducing agents are currently available, the most common of which is 2-mercaptoethanol. On the basis of preliminary experiments in this area (Madden, unpublished data) and favorable reports of its use in the literature (Cleland, 1964; Mourand et al., 1966; Steiner et al., 1967; Konigsberg, 1972; and Rogers and Chargaff, 1972), Cleland's Reagent (dithiotreitol) was selected as the reducing agent to be used in GuHCl denaturation experiments. This reagent provides a sterically favorable cyclic disulfide with reducing properties at low concentrations (less than 0.1M), yet is a highly water-soluble solid with little odor and little tendency toward direct air oxidation (Cleland, 1964).

As described in the Methods, Cleland's Reagent was added to a 6M solution of GuHCl (Sigma Chemical Company) to a final concentration of 0.01M. A 1:1 mixture of enzyme to GuHCl was prepared, giving final concentrations of 3M GuHCl, 0.05M acetate buffer, pH 4.0, and 0.005M Cleland's Reagent. Treatment of wild type (STA-4) conidial "Y" with this GuHCl solution produced no significant change in the size of the enzyme as evidenced by the TLG filtration data shown in Figure 4. Guanidine hydrochloride at this concentration did have some effect on wild type conidial "Y" in time as the TLG data showed a decrease in the intensity of the

Figure 4.   Thin-layer Gel Filtration of Purified STA-4 "Y" Incubated
with 3M GuHCl and 0.005M Cleland's Reagent.
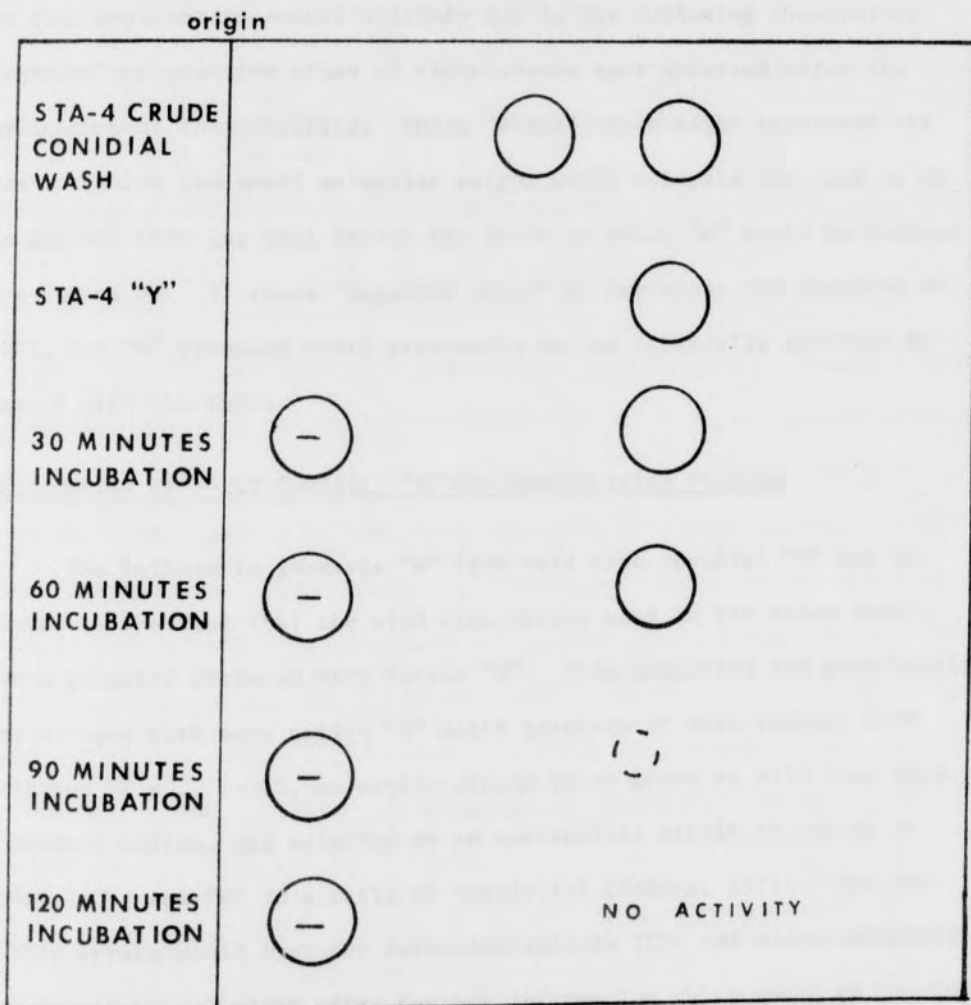
enzyme spots in time with umbelliferone as the substrate. There was some question that "W" might have been produced, but was so labile that prolonged contact with GuHCl in the gel prior to the actual separation might have destroyed it. It is also possible that destruction of any generated "W" occurred due to prolonged contact with GuHCl after the separation, but this explanation seemed unlikely due to the following observation. "Negative" or quenched areas of fluorescence were observed after the application of the substrate. These "blank" spots might represent the point at which the small molecular weight GuHCl molecule was held up in the gel and they lag well behind the point at which "W" would be located (see Figure 4). If these "negative areas" do represent the location of GuHCl, any "W" produced would presumably not be critically affected by contact with the GuHCl.

Purification of P-212 Conidial "Y" for Denaturation Studies

The failure to generate "W" from wild type conidial "Y" may be related to the fact that the wild type strain used in the above experiments normally produces very little "W". This suggested the possibility that strains with more native "W" might generate it more readily from their own "Y's". P-212, an exotic strain which grows at wild type rate on minimal medium, was selected as an appropriate strain to use as it produces "Y" and "W" in a ratio of nearly 1:1 (Madden, 1971). The use of this strain would test our detection methods (TLG and electrophoresis with Sepratek) and might offer further information which would be helpful in future investigations with a standard wild type.

A concentrated and dialyzed P-212 conidial wash prepared by Madden and frozen in June 1970 was separated on a Pharmacia K50 column of Biogel P-150 (100-200 Mesh). Fractions of similar activity with PNPG were pooled and a bimodal elution profile was obtained (see Figure 5). TLG and electrophoresis of the fraction pools obtained indicated the presence of "Y" alone in the early fraction pools and the presence of "W" alone in the later pools. The purest "Y" pools of good activity from this separation were used in a number of GuHCl denaturation experiments.

Treatment of P-212 Conidial "Y" with 3M Sigma GuHCl and 0.005M Cleland's Reagent

When P-212 conidial "Y" was incubated with 3M GuHCl and 0.005M Cleland's Reagent (DTT) and was separated by TLG filtration, changes in the mobility of this enzyme in time were observed. These results indicated that the molecular size of P-212 conidial "Y" was altered by treatment with 3M GuHCl. The activity of the P-212 conidial "Y" enzyme was lowered by 3M GuHCl in time as evidenced by the reduction in intensity of the enzyme spots in time. When P-212 conidial "Y" treated with 3M GuHCl and 0.005M Cleland's was separated by electrophoresis, "slurs" of activity which may represent active enzyme pieces were observed. With increased incubation in 3M GuHCl, these "streaks" of activity may be said to "approach" "W", as shown in Figure 6.

Freshness and Purity of GuHCl Solutions

It was observed during the early work with Sigma GuHCl that results of experiments using GuHCl solutions prepared prior to the day of the experiment were not strictly comparable to results obtained with freshly

Figure 5. Elution Profile of Extracellular β-Glucosidases of P-212
from a Biogel P-150 Column.
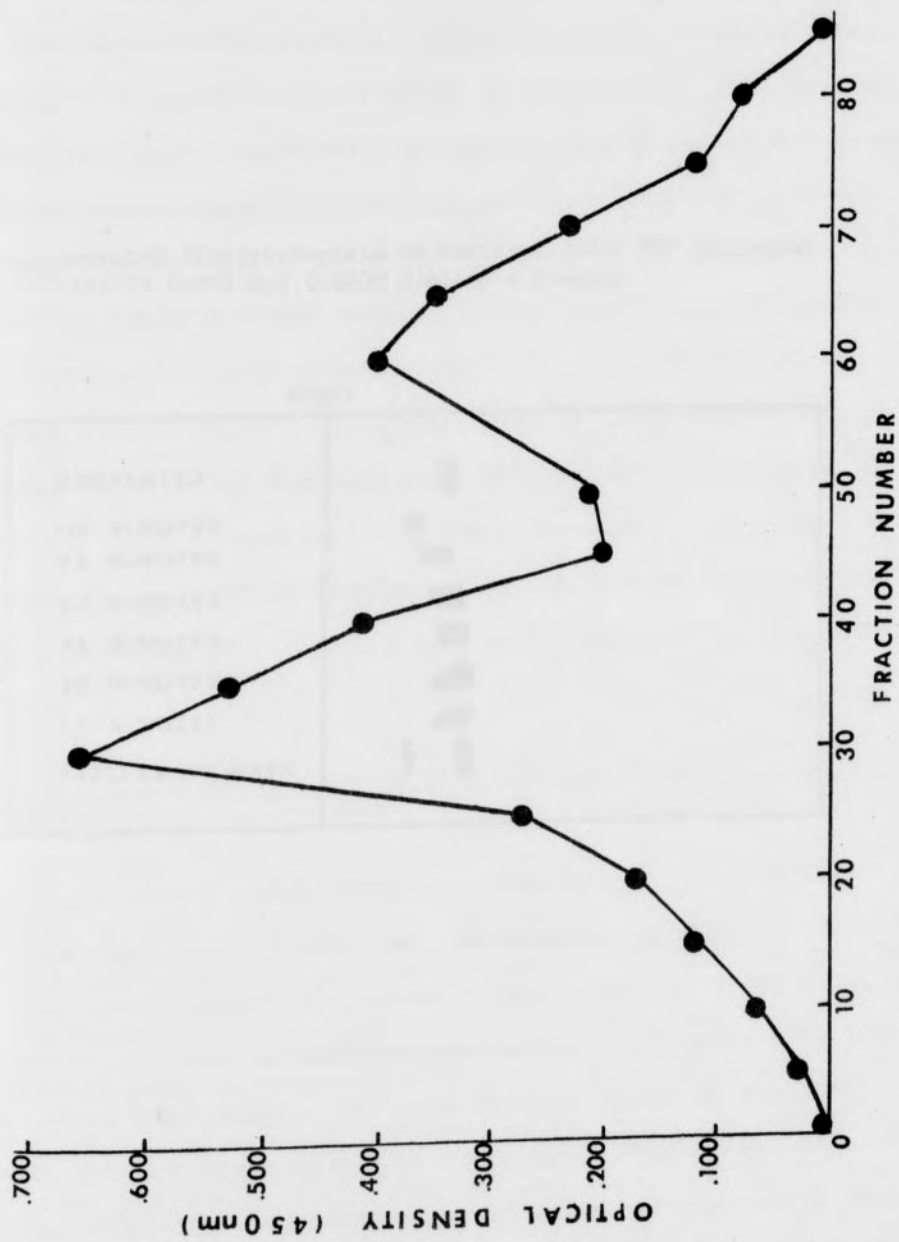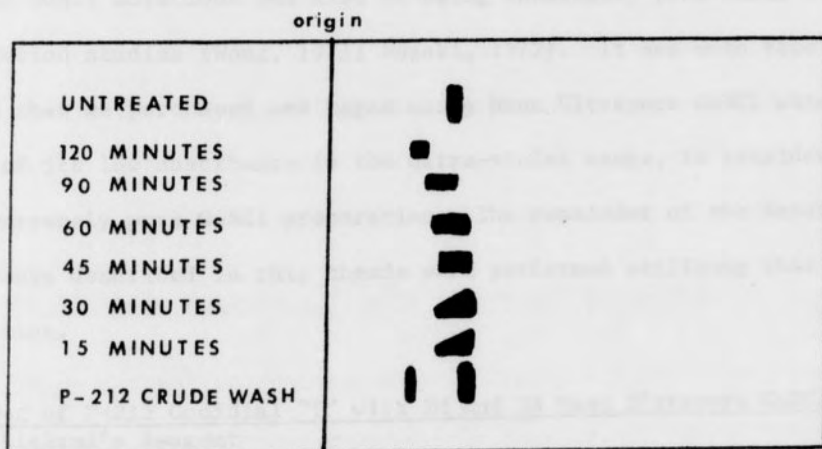
Figure 6. Sepratek Electrophoresis of Purified P-212 "Y" Incubated
with 3M GuHCl and 0.005M Cleland's Reagent.

origin

| UNTREATED |
| 120 MINUTES |
| 90 MINUTES |
| 60 MINUTES |
| 45 MINUTES |
| 30 MINUTES |
| 15 MINUTES |
| P-212 CRUDE WASH |

prepared solutions of GuHCl. Solutions of GuHCl kept at room temperature for more than a few hours appeared to lose part of their previous ability to denature our enzymes. References to GuHCl in the literature generally mention that the solutions used were freshly prepared. In order to keep the results as comparable as possible and the experimental procedures as uniform as possible, it was decided to make up GuHCl solutions immediately prior to the start of the experiment in small quantities sufficient for the experiment in question.

The literature not only stresses the importance of using freshly prepared GuHCl solutions but also of using chemically pure GuHCl for denaturation studies (Wong, 1971; Nozaki, 1972). It was with this fact. in mind that we purchased and began using Mann Ultrapure GuHCl which, by virtue of its low absorbance in the ultra-violet range, is considered to be an extremely pure GuHCl preparation. The remainder of the denaturation experiments described in this thesis were performed utilizing this GuHCl preparation.

Treatment of P-212 Conidial "Y" with 2M and 3M Mann Ultrapure GuHCl and 0.005M Cleland's Reagent

Although Mann Ultrapure GuHCl is a much purer preparation than reagent grade Sigma GuHCl, preliminary experiments with Mann GuHCl indicated that freshly prepared solutions of concentrations in the range of 2-4M would still be most suitable for denaturation experiments with this compound. When P-212 conidial "Y" incubated with 2M and 3M Mann GuHCl and 0.005M Cleland's was separated by TLG on Biogel P-150, generation of new enzymatically active molecules with mobilities corresponding to that of "W" was observed. When similarly treated preparations were separated

by electrophoresis on Sepraphore III, definite spots of "W" activity were also observed. The results of both TLG and electrophoresis experiments for 3M GuHCl are shown in Figures 7 and 8. The TLG data indicated that the generation of "W" from "Y" was not always accompanied by the complete disappearance of P-212 "Y" activity. On the contrary, an even more interesting phenomenon occurred in these experiments. The activity of the P-212 "Y" did not merely decline in time as in earlier experiments with Sigma GuHCl. Instead, an initial decline was followed by an activation of the P-212 "Y" sample, which, depending on the time sequence of activation, may or may not be followed by a second decline. The time sequence of this activation did vary considerably and the results of many experiments did not suggest any single logical pattern. The activation was observed in both thin-layer and electrophoresis experiments to occur as early as 30 minutes incubation or as late as 120 minutes incubation.

Treatment of P-212 Conidial "Y" with 4M and 5M Mann GuHCl

In an attempt to discover whether higher concentrations of GuHCl would alter the denaturation pattern heretofore observed, several experiments were performed with P-212 "Y" and 4M and 5M GuHCl. 4M GuHCl caused the conversion of P-212 "Y" to "W" in the first 30 minutes of incubation as evidenced by TLG filtration of treated P-212 "Y" preparations. Unless complete destruction of all enzyme activity was achieved, P-212 "Y" activity did not disappear as "W" was produced; however, no true activation effect was observed at this concentration with the time sequence used (30, 60, 90, and 120 minutes incubation).

Figure 7.   Thin-layer Gel Filtration of Purified P-212 "Y" Incubated
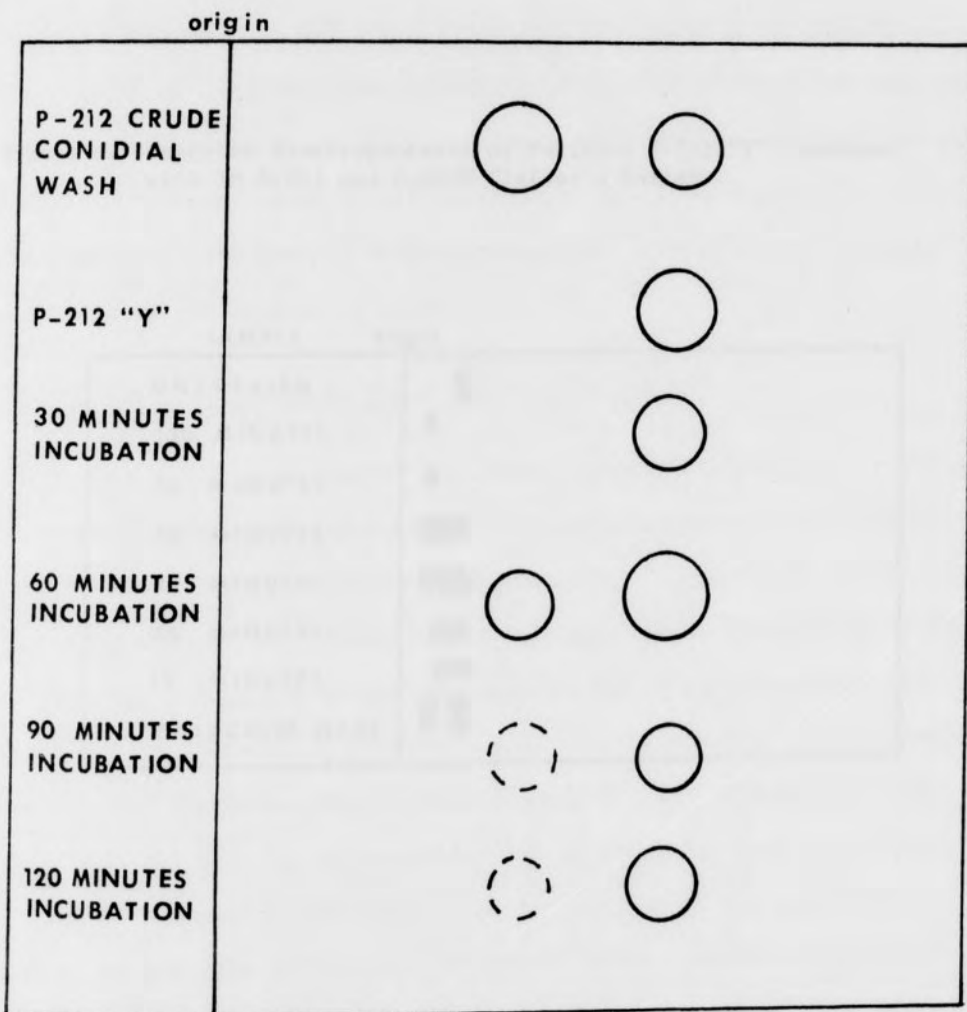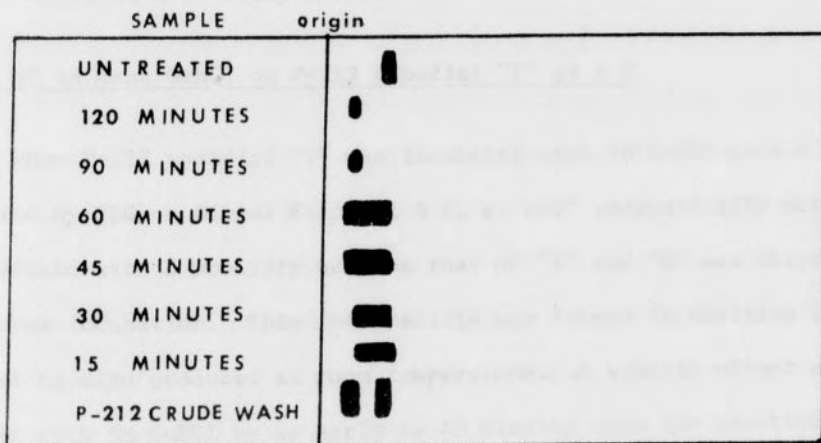   with 3M GuHCl and 0.005M Cleland's Reagent.

Figure 8. Sepratek Electrophoresis of Purified P-212 "Y" Incubated with 3M GuHCl and 0.005M Cleland's Reagent.

5M Mann GuHCl caused the conversion of "Y" to "W" in the first 5 minutes of incubation. As with lower concentrations, P-212 "Y" activity did not disappear completely as "W" was produced - unless all enzyme activity was destroyed. A slight increase in activity due to the action of 5M GuHCl over time was noted by as early as 15 minutes incubation. The results of other experiments indicated that this activation can occur as late as 30 minutes at this concentration of GuHCl. The loss in enzyme activity at these higher concentrations was quite severe and TLG plates sometimes required several hours contact with the substrate before the results could be accurately marked.

Effect of 4M Mann GuHCl on P-212 Conidial "Y" at 4 C

When P-212 conidial "Y" was incubated with 4M GuHCl at 4 C and was separated by TLG on Biogel P-150 at 4 C, a "new" enzymatically active intermediate with a mobility between that of "Y" and "W" was observed by 90 minutes incubation. This intermediate was formed in addition to the "W" that is also produced at room temperature. A similar effect was observed with 5M GuHCl by as early as 45 minutes when the reaction was run at 4 C. The difference in mobility was due to the action of GuHCl on the enzyme and not due to irregular flow of or position of the samples on the plate as changing the position of the samples on the plate did not affect the mobility difference. The most critical factor in producing this effect was the incubation of the GuHCl and the enzyme at 4 C as evidenced by the fact that a definite "new" intermediate was observed even when the separation took place at room temperature. The results of a typical experiment in which the separation took place at 4 C is shown in

Figure 9. These results indicate that the conditions of separation may be responsible in some mechanical way for part of the effect as 90 minute samples on plates run at room temperature fail to show a significant mobility difference as they did when plates were run at 4 C. Instead, the new intermediate appeared somewhere between 105 and 120 minutes incubation. The possible reasons why this intermediate had never been observed in experiments performed at room temperature will be discussed in a later section.

Effect of 5M GuHCl and 0.005M 2-Mercaptoethanol on P-212 Conidial "Y"

The possible formation of a new intermediate was also observed at room temperature when P-212 "Y" was incubated with 5M GuHCl and 0.005M 2-mercaptoethanol. This intermediate was not observed under these conditions in the absence of 0.005M 2-mercaptoethanol. In addition to the formation of the intermediate, a definite activation effect was observed under these conditions of separation. Both of these effects can be seen in the TLG data shown in Figure 10. Since these phenomena were observed in several similar experiments, they are probably not experimental artifacts.

Comparison of the "Stabilizing Ability" of Cleland's Reagent and 2-Mercaptoethanol in 2M GuHCl

As mentioned earlier, Cleland's Reagent was selected as the reducing agent of choice not only because of its slight odor and other convenient properties, but also in the interest of repeating some of Brenda Madden's unpublished experiments. Although 2-mercaptoethanol is an extremely volatile liquid with an unpleasant thiol odor, it has historically

Figure 9.   Thin-layer Gel Filtration at 4 C of Purified P-212 "Y"
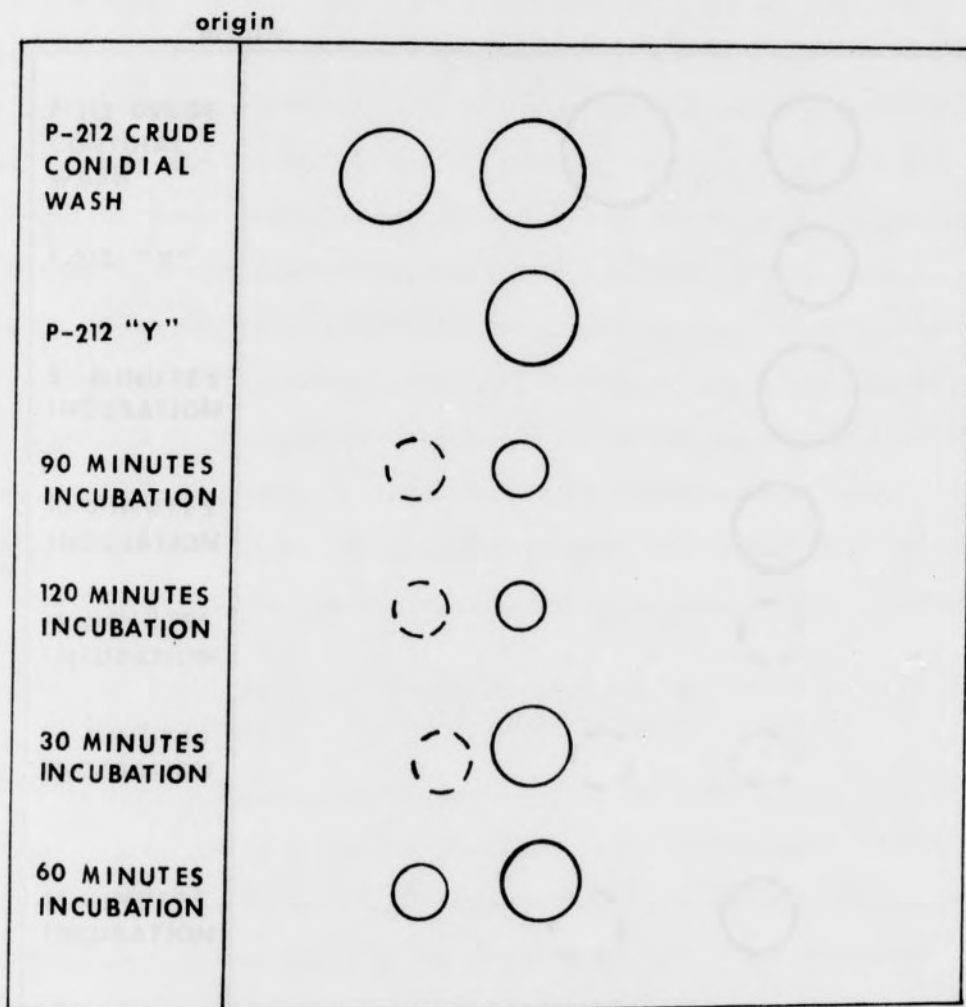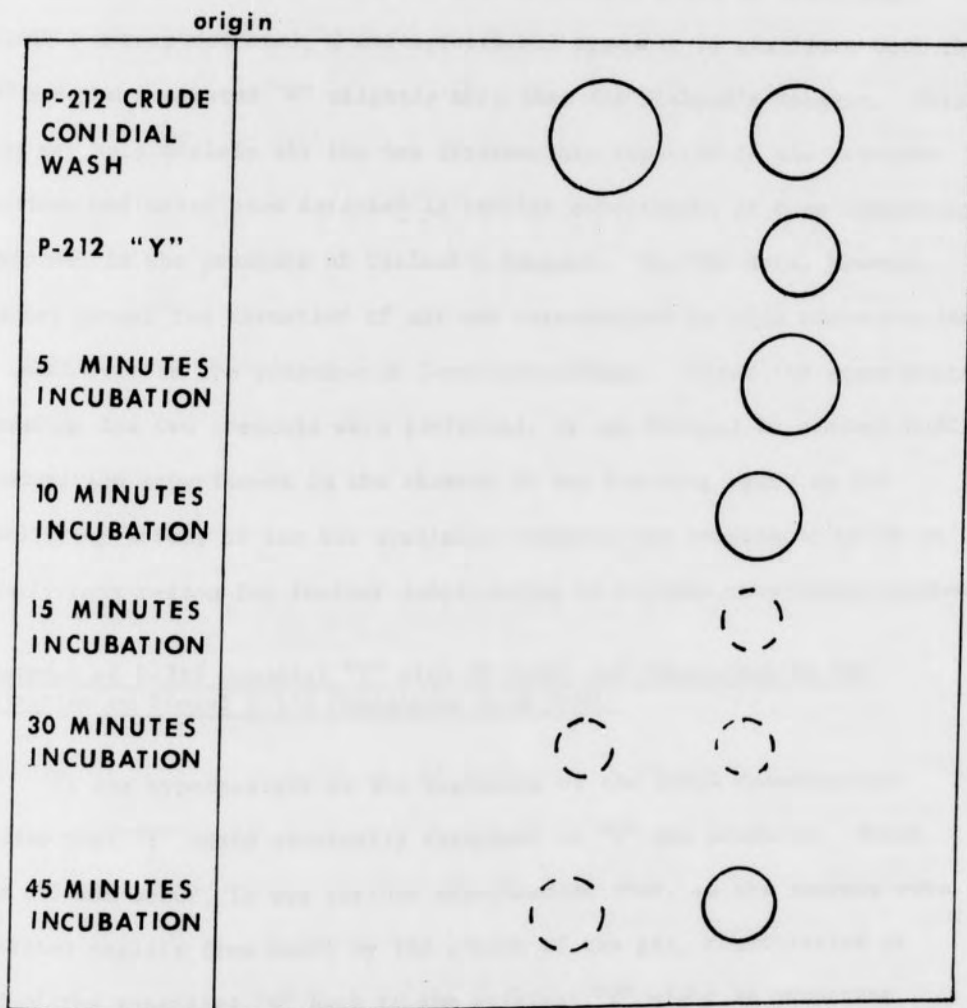   Incubated with 4M GuHCl at 4 C.

Figure 10.   Thin-layer Gel Filtration of Purified P-212 "Y" Incubated
with 5M GuHCl and 0.005M 2-Mercaptoethanol.

been the reagent of choice when a reducing environment was required (Apella and Markert, 1961; Cahn et al., 1962; Meachum et al., 1971). In experiments in which P-212 conidial "Y" enzyme was incubated for 30, 60, 90, and 120 minutes with both 4M GuHCl and 0.005M Cleland's and 2M GuHCl and 0.005M 2-mercaptoethanol, 2-mercaptoethanol appeared to stabilize both the "Y" and the generated "W" slightly more than did Cleland's Reagent. This fact may help explain why the new intermediate reported in the previous sections had never been detected in earlier experiments at room temperature performed in the presence of Cleland's Reagent. The TLG data, however, did not reveal the formation of any new intermediate at this concentration of GuHCl even in the presence of 2-mercaptoethanol. After the experiments comparing the two reagents were performed, it was decided to conduct GuHCl denaturation experiments in the absence of any reducing agent as the stabilizing effect of the two available reagents was considered to be an insufficient reason for further complicating an already complicated system.

Treatment of P-212 Conidial "Y" with 2M GuHCl and Separation by TLG Filtration on Biogel P-150 Containing 0.1M GuHCl

It was hypothesized at the beginning of the GuHCl denaturation studies that "Y" would eventually disappear as "W" was produced. Since this did not occur, it was further hypothesized that, as the enzymes were separated rapidly from GuHCl by the action of the gel, renaturation of part of the generated "W" back to the original "Y" might be occurring. The presence of very low levels of GuHCl in the separating medium might serve to prevent this from happening. When P-212 conidial "Y" treated with 2M GuHCl in the usual way was separated on Biogel P-150 containing

0.1M GuHCl, discrete "Y" and "W" spots were observed. The intensity of the "W" spots was approximately equal to that of the "Y" spots. This was in contrast to the result obtained in previous denaturations in which the generated "W" was always much less active than the "Y". This suggested the possibility that this method was at least partially effective in protecting the newly formed "W". At the same time, however, these low levels of GuHCl appeared to have some denaturing action on the "W" as evidenced by long "tails" or slurs of activity which appeared to lag behind the "W" spots as shown in Figure 11. The P-212 "Y" control, which had not been treated with GuHCl prior to application to the plate, was even acted upon in the same way by the GuHCl in the gel (Figure 11). Although some renaturation possibly was prevented by this method, it was not completely satisfactory due to the continued action of the GuHCl in the gel on the enzyme samples.

Overall Response in Time of P-212 and STA-4 Conidial Aryl-β-Glucosidases to Inactivation by 3M Mann GuHCl

Although relative amounts of inactivation and activation can be assessed with umbelliferone as the substrate, a more quantitative measure of the time course of inactivation was necessary. The method utilizing the standard PNPG assay described in the Methods section was used to obtain inactivation curves of crude conidial washes, purified conidial "Y", and purified conidial "W" from a number of Neurospora strains. Figure 12 shows a typical activation curve obtained with P-212 aryl-β-glucosidases in 3M GuHCl. Figure 13 shows a cruve obtained with STA-4 enzymes in 3M GuHCl. Later, the data obtained from the inactivation curves were used to calculate half-time values for these enzymes in GuHCl. The half-times of

Figure 11. Thin-layer Gel Filtration of Purified P-212 "Y" Incubated
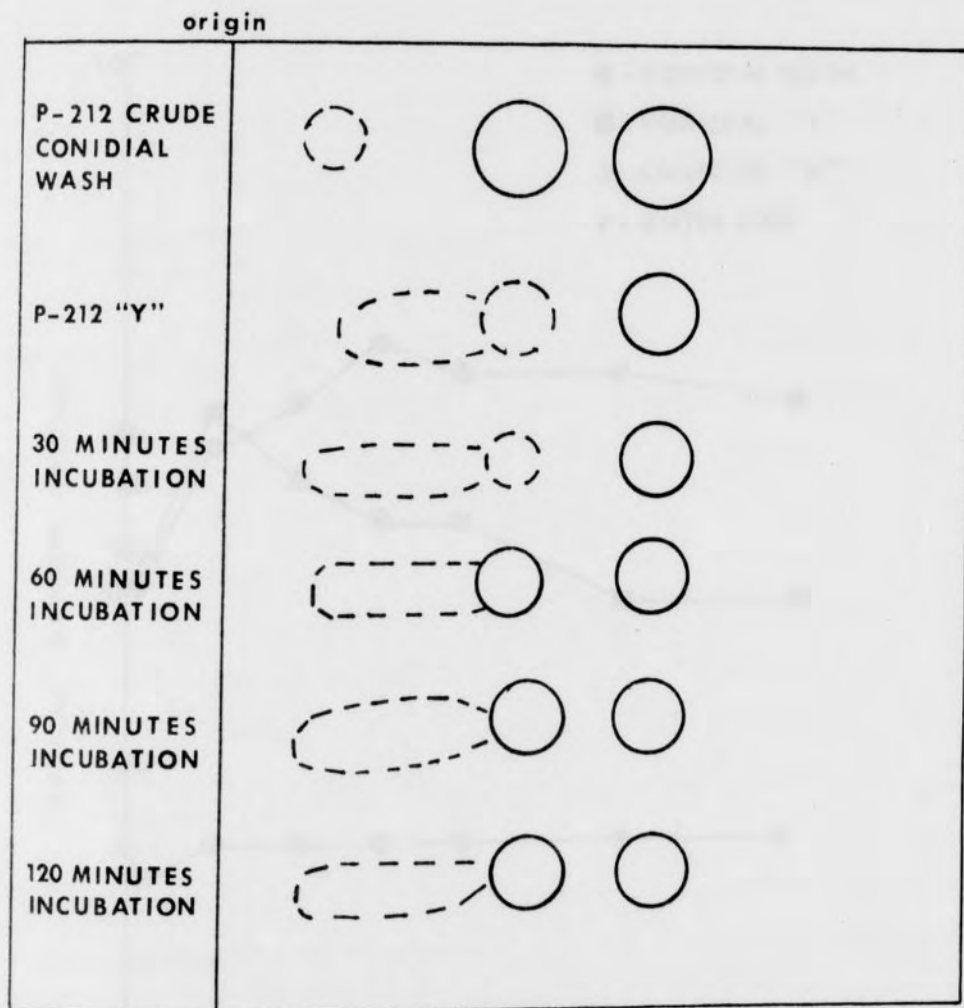with 2M GuHCl and Separated in Biogel P-150 Containing
0.1M GuHCl.

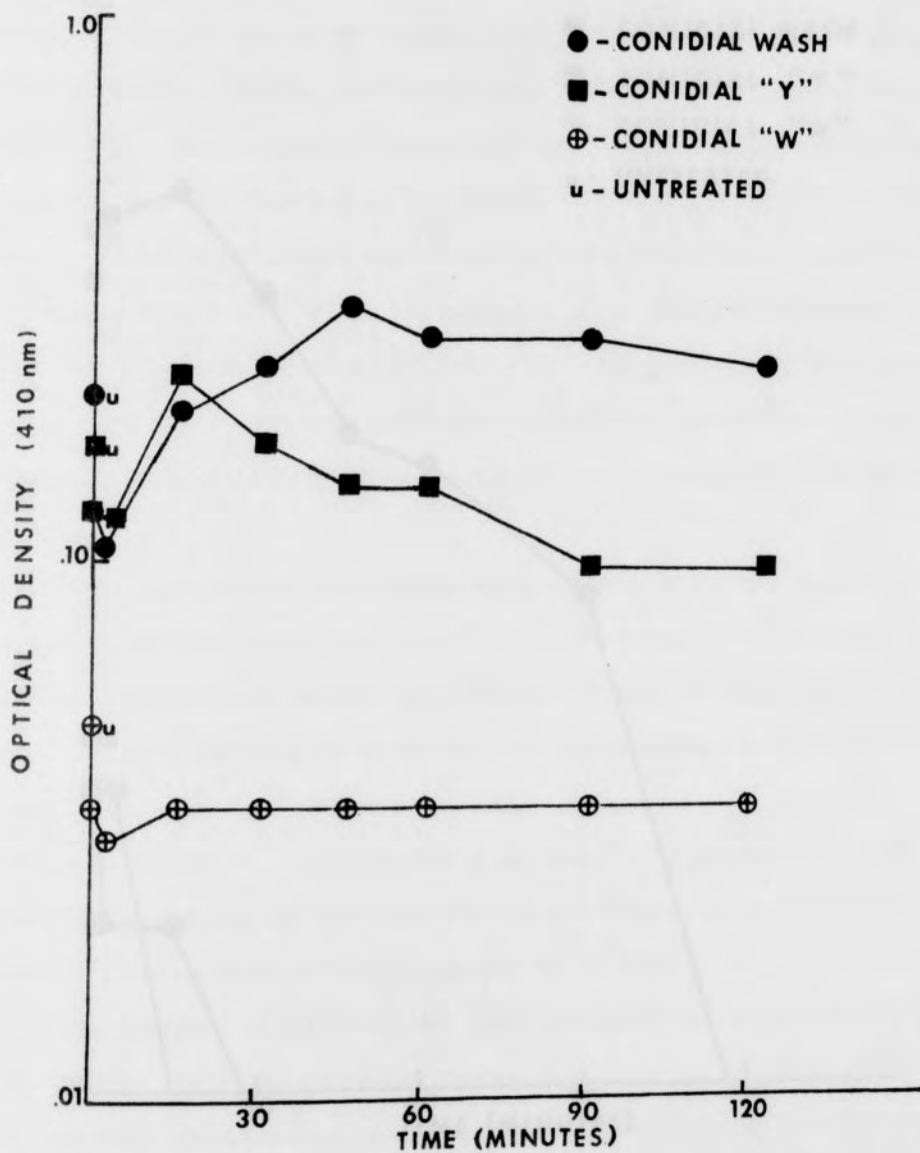Figure 12. Inactivation Curves of P-212 Conidial Aryl-β-Glucosidases in 3M GuHCl.

Figure 13. Inactivation Curves of STA-4 Conidial Aryl-β-Glucosidases in 3M GuHCl.
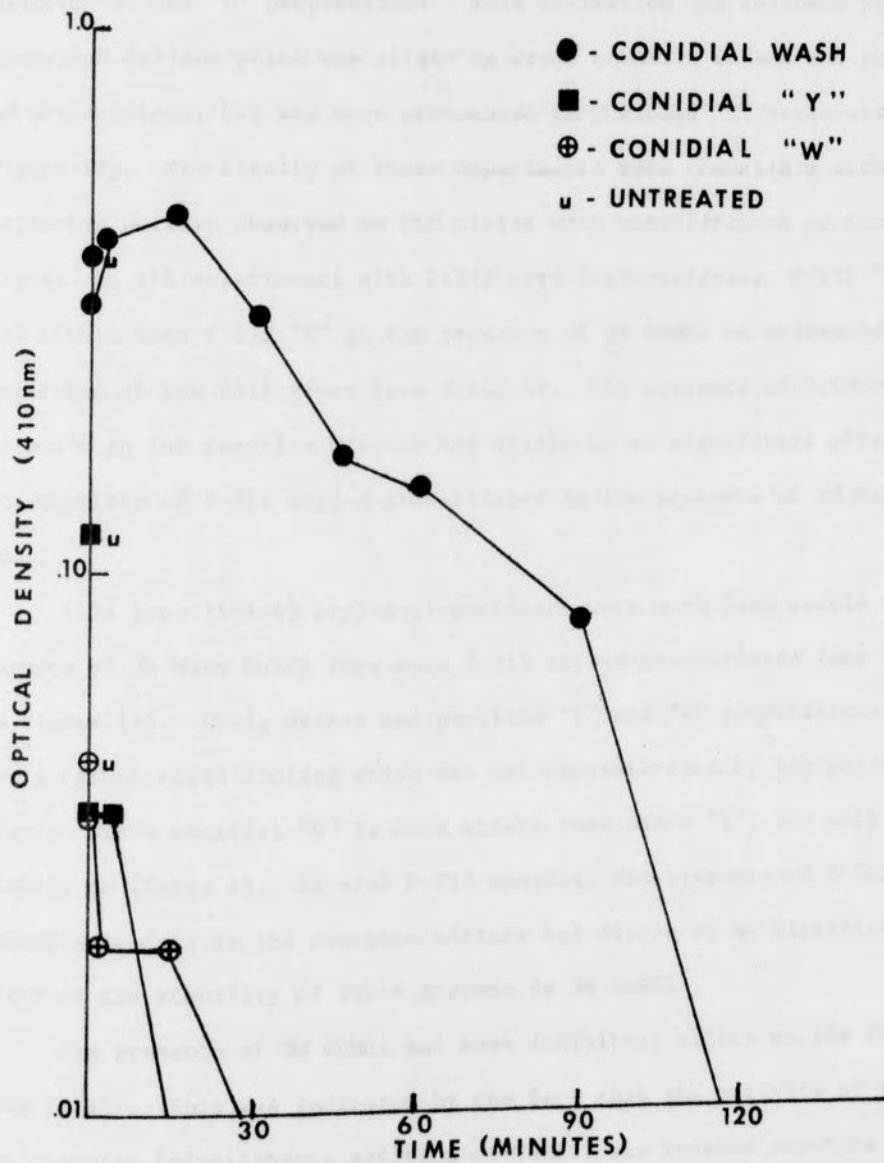
crude conidial washes, conidial "Y", and conidial "W", as calculated by the logarithmic least squares method for four strains, appear in Table 4.

3M Mann GuHC1 produced an initial decline in P-212 enzyme activity which was followed by an activation in both crude conidial washes and in purified "Y" and "W" preparations. This activation was followed by a subsequent decline which was slight in crude conidial washes and purified "W" preparations, but was more pronounced in purified "Y" preparations (Figure 12). The results of these experiments were compatible with the activation pattern observed on TLG plates with umbelliferone as the substrate. In all experiments with P-212 aryl-β-glucosidases, P-212 "W" was more stable than P-212 "Y" in the presence of 3M GuHC1 as evidenced by a comparison of the half-times (see Table 4). The presence of 0.005M Cleland's in the reaction mixture had little or no significant effect on the stability of P-212 aryl-β-glucosidases in the presence of 3M Mann GuHC1.

Wild type (STA-4) aryl-β-glucosidases were much less stable in the presence of 3M Mann GuHC1 than were P-212 aryl-β-glucosidases (see Table 4 and Figure 13). Crude washes and purified "Y" and "W" preparations exhibited a rather rapid decline which was not characterized by any activation effect. STA-4 conidial "W" is more stable than STA-4 "Y", but only slightly so (Table 4). As with P-212 enzymes, the presence of 0.005M Cleland's Reagent in the reaction mixture had little or no significant effect on the stability of STA-4 enzymes in 3M GuHC1.

The presence of 3M GuHC1 had some inhibitory effect on the PNPG assay itself. This was indicated by the fact that the activity of the "0 time" samples (simultaneous addition of GuHC1) was reduced anywhere from 50-80% as compared with untreated values.

TABLE 4

Half-Times of Neurospora Aryl-β-Glucosidases
in Guanidine Hydrochloride

Strain	Date of Experiment	Concentration of GuHCl	Crude	"Y"	"W"
STA-4	11-16-73	3M	54.81	19.78	22.56
	2-8-74	3M	30.52	6.27	13.63
P-212	11-16-73	3M	1207.12	100.70	131.96
	2-8-74	3M	713.13	61.97	205.99
	2-12-74	3M	272.28	214.03	282.30
	2-15-74	3M	272.28	86.10	558.17
	2-26-74	4M	112.14	24.84	350.79
	3-7-74	4M	47.37	11.77	78.10
	3-8-74	4M	35.29	61.16	189.48
	3-28-74	4M	17.94	8.77	12.11
	4-23-74	4M	46.58	22.39	41.92
	4-25-74	4M	45.16	23.00	89.42
	4-30-74	4M	62.56	39.84	88.43
	5-9-74	4M	45.57	40.33	148.35
	3-21-74	5M	31.21	1.64	30.55
	3-26-74	5M	30.66	1.63	27.39
	4-2-74	5M	20.05	5.16	23.89
	4-4-74	5M	13.03	7.17	15.14
P-113	4-9-74	4M	56.03	3.37	80.29
	4-11-74	4M	23.99	.49	53.07
	4-16-74	4M	27.95	3.32	88.09
	4-18-74	3M	23.05	5.79	268.20
P-278 (Ethylene glycol)	6-24-74	4M	45.93	142.49	198.27
	6-25-74	4M	34.96	47.65	122.19
	6-26-74	4M	30.80	65.59	81.37
	6-27-74	4M	32.19	53.51	68.98
P-278 (GSCP)	6-19-74	4M	117.07	304.41	-
	6-25-74	4M	33.61	43.87	-
	6-26-74	4M	38.23	55.59	-
	6-27-74	4M	38.85	53.39	-

Effect of 4-5M Mann GuHCl on P-212 Conidial Aryl-β-Glucosidases

Although crude conidial washes in the presence of 4M GuHCl still exhibited considerable activation after an initial decline, "Y" and "W" preparations showed only slight activation, with "W" being activated more consistently than "Y". 4M GuHCl appeared to denature P-212 enzymes more rapidly than did 3M GuHCl, although complete inactivation was not always achieved by two hours incubation. P-212 "W" enzyme was more stable in the presence of 4M GuHCl than was P-212 "Y", as indicated by a comparison of half-times obtained for "Y" and "W" (Table 4). In comparison with values obtained from untreated samples, it is evident that in the presence of 4M GuHCl, the standard PNPG assay is only about 40% efficient.

When P-212 aryl-β-glucosidases were treated with 5M GuHCl, P-212 crude conidial washes behaved as with lower concentrations and "W" was sometimes very slightly activated. Conidial "Y" enzyme merely showed a rapid decline in the first few minutes of incubation and all P-212 "Y" activity had disappeared by the end of 15 minutes incubation. A comparison of the half-time values obtained for both "Y" and "W" indicates that P-212 "W" enzyme was much more stable than P-212 "Y" enzyme in the presence of 5M GuHCl.

Effect of 3-4M Mann GuHCl on P-113 Conidial Aryl-β-Glucosidases in Time

P-113 crude washes and P-113 "W" enzymes incubated with 4M Mann GuHCl exhibited a decline in activity which was sharp at first, but became more gradual as the incubation time increased. P-113 "Y" enzyme merely showed an extremely rapid decline. The most obvious difference between the inactivation curves of P-113 and P-212 was the complete absence of any

significant activation of P-113 enzymes in the time span used. P-113 "W" was much more stable than P-113 "Y" in the presence of 4M GuHCl, as "Y" activity had completely disappeared by 10 minutes incubation while "W" was still active after 2 hours incubation. The half-times of P-113 aryl-β-glucosidases appear in Table 4. In the presence of 3M Mann GuHCl, no significant differences were noted in the stability of P-113 crude washes or P-113 "W" enzymes, although P-113 "Y" did appear to be slightly more stable in 3M GuHCl than in 4M GuHCl (Table 4).

Investigation of GuHCl Inactivation Method

The data referred to above for the inactivation of P-212 and P-113 aryl-β-glucosidases at various concentrations of GuHCl are from experiments which were performed at room temperature and which produced relatively repeatable results for these strains for most concentrations of GuHCl. For some incompletely understood reason (or reasons), the 4M GuHCl inactivation curves of P-212 lacked repeatability. When these experiments were repeated, the major conclusions that could be drawn from the data did not differ from earlier experiments, yet the overall shape of the curves definitely did not coincide.

The possibility that slight temperature differences could produce the observed variations was investigated by experiments in which a crude P-212 conidial wash was inactivated in a constant temperature water bath set at 25 C. Data points were obtained in triplicate. In the first experiment, the "standard method" described in the Methods was used in which a number of individual test tubes were utilized for the incubation of the enzyme and the GuHCl. The data obtained indicated that as the

incubation time in GuHCl was increased, the error involved in this method was increased. Wide variations were observed among the three data points. The lack of repeatability, therefore, was not totally due to the influence of temperature fluctuations in the laboratory environment.

The most critical factor in producing repeatable results proved to be a simple mechanical one. The mixing in all of the individual test tubes (30 per strain per experiment) was very likely not uniform. The modification of the experimental procedure described in the Methods in which the GuHCl and the enzyme solution were uniformly mixed in a 1:1 ratio was used to obtain much improved results. All further inactivation experiments were performed in a constant temperature water bath using this improved method.

The GuHCl reaction appeared to be extremely temperature dependent, as changing the temperature of the reaction from 25 C to 20 C greatly increased the stability of a P-212 crude conidial wash in the presence of 4M GuHCl. The shape of the inactivation curve and the rate of decline of P-212 enzymatic activity in 4 M at 20 C more closely approximated the curves obtained at "room temperature"; therefore, 20 C was perhaps closest to the temperature at which other GuHCl experiments were conducted.

The 4M GuHCl inactivation curves of P-212 aryl-β-glucosidases obtained by this improved method were much smoother and more "reasonable". P-212 "W" was still more stable than P-212 "Y" and an initial sharp decline was followed in each case by a more gradual decline; however, no activation effect was observed in P-212 crude conidial washes or purified "Y" and "W" preparations in the time span used. The absence of an "activation effect" might be due to one of several factors which will be discussed in a later section.

Effect of 4M GuHCl at 20 C on the Aryl-β-Glucosidases on P-278 Grown on 5% Ethylene Glycol

Eileen Hartis (unpublished) found that exotic strain P-278, which normally produces very little "W" (when grown on GSCP), will produce larger quantities of this isozyme when it was grown on GSCP containing 5% ethylene glycol. In order to obtain a sufficient P-278 "W" for inactivation experiments after partial purification by column fractionation, P-278 that had been grown on 5% ethylene glycol had to be used. It is not known at this time, however, how growth on ethylene glycol might affect the physical properties of "Y" and/or "W".

When.P-278 aryl-β-glucosidases were treated with 4M Mann GuHCl at 20 C, a relatively consistent pattern of inactivation was observed. A sharp decline in activity in the first minute of incubation was followed by a more gradual decline in all three enzyme preparations. P-278 "W" appeared to be more stable than P-278 "Y" (Table 4), but both isozymes exhibited the same gradual pattern of decline. No true activation effect was observed in these experiments, although some slightly elevated points in the curves suggested the possibility that activation may have occurred at a time not sampled in the experiment.

Effect of 4M Mann GuHCl at 20 C on the Aryl-β-Glucosidases of P-278 Grown on GSCP

As mentioned above, P-278 normally produces very little "W" and several attempts to purify enough "W" for inactivation experiments met with no success. Inactivation experiments were performed, however, using P-278 crude conidial washes and P-278 "Y" obtained from cultures grown on GSCP alone for purposes of comparison. The general inactivation pattern

observed in these enzyme preparations was quite similar to that of P-278 crude conidial washes and P-278 "Y" from ethylene glycol cultures. An initially sharp decline in activity was followed by a more gradual rate of inactivation. P-278 (GSCP) enzymes appeared to be slightly more succeptible to inactivation by 4M GuHCl than were P-278 (E.G.) enzymes (see Table 4), but this difference may not be significant.

Effect of 8M Urea on P-212 Conidial "Y" in the Presence and Absence of Various Reducing Agents

It was my intention when the chemical denaturation studies were begun to investigate not only GuHCl but also urea, SDS, and other commerically available denaturants. The inactivation effects of GuHCl proved to be so complicated, however, that it was decided to concentrate on GuHCl alone. One or two very preliminary experiments were conducted using 8M urea, however, and they are reported here to encourage further investigation in this area.

Purified P-212 conidial "Y" was incubated for 1 hour with 8M urea alone and with 8M urea in combination with 0.01M concentrations of each of the following three common reducing agents: Cleland's Reagent, 2-mercaptoethanol, and sodium thioglycolate. When samples from these reaction mixtures were separated by TLG on Biogel P-150, several interesting effects were noted. 8M urea alone did not appear to cause the conversion of P-212 "Y" to "W", but it did greatly deactivate "Y". 8M urea + 0.01M Cleland's caused slight "W" production from P-212 "Y", along with a slur of activity possibly representing other active enzyme pieces. 8M urea + 0.01M 2-mercaptoethanol and 8M urea + 0.01M sodium thioglycolate caused the production of "W" from P-212 "Y" with the concomitant

disappearance of "Y".   The results of one such experiment with urea are

shown in Figure 14.

Figure 14. Thin-layer Gel Filtration of Purified P-212 "Y" Incubated with 8M Urea and Various Reducing Agents.
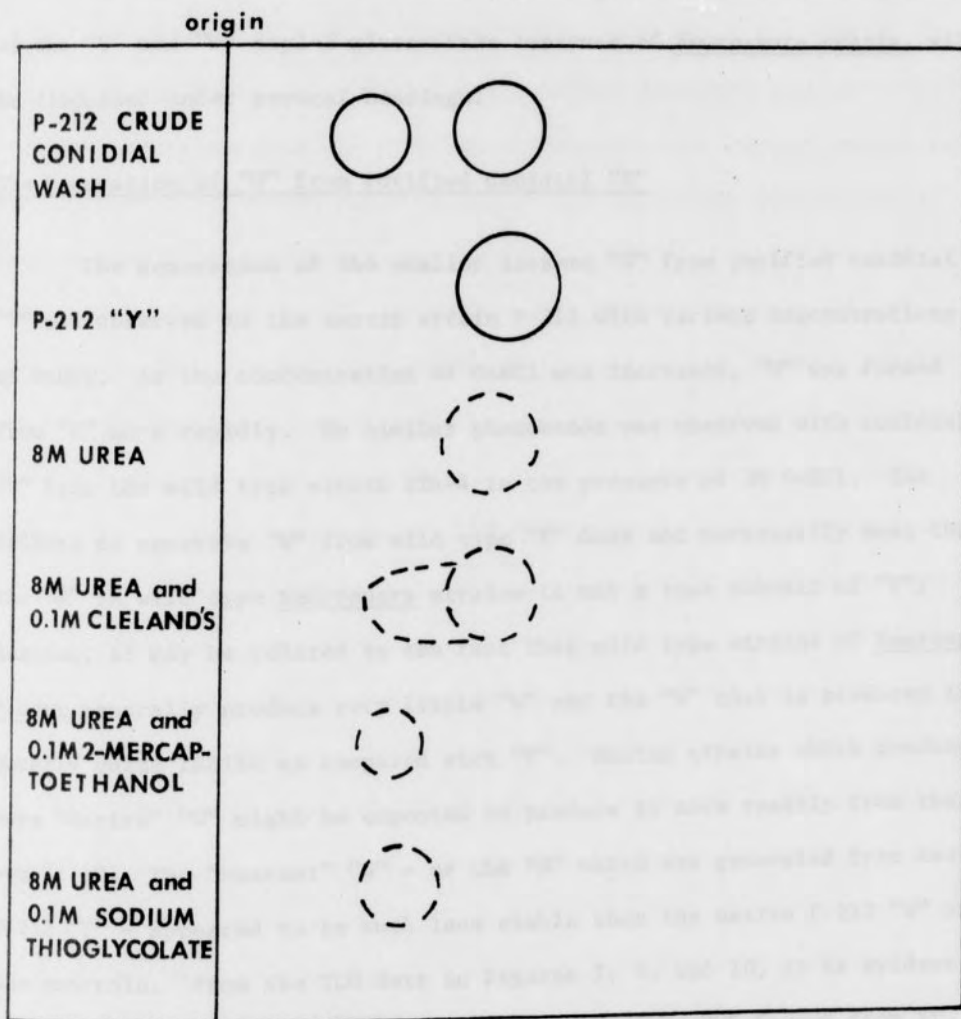
DISCUSSION

The research in this thesis, involving the chemical denaturation of the "Y" and "W" aryl-β-glucosidase isozymes of Neurospora crassa, will be discussed under several headings.

The Generation of "W" from Purified Conidial "Y"

The generation of the smaller isozyme "W" from purified conidial "Y" was observed in the exotic strain P-212 with various concentrations of GuHCl. As the concentration of GuHCl was increased, "W" was formed from "Y" more rapidly. No similar phenomenon was observed with conidial "Y" from the wild type strain STA-4 in the presence of 3M GuHCl. The failure to generate "W" from wild type "Y" does not necessarily mean that the "W" in wild type Neurospora strains is not a true subunit of "Y"; instead, it may be related to the fact that wild type strains of Neurospora crassa generally produce very little "W" and the "W" that is produced is usually quite labile as compared with "Y". Exotic strains which produce more "native" "W" might be expected to produce it more readily from their own "Y's". The "nascent" "W" - or the "W" which was generated from exotic P-212 "Y" - appeared to be much less stable than the native P-212 "W" of the controls. From the TLG data in Figures 7, 9, and 10, it is evident that P-212 "Y" is also more stable than the nascent "W" formed from it. It is not completely understood why P-212 "Y" does not disappear completely as "W" is produced, but both the inactivation of and subsequent decline in "Y" activity indicate that P-212 "Y" is dramatically affected by exposure to GuHCl in other ways.

The electrophoresis results depicted in Figure 6 show that with increased incubation in GuHCl, the streaks of activity produced from P-212 "Y" gradually appear to approach the mobility of "W". It is possible that, as the "Y" enzyme is being unfolded, positive charges are uncovered in the molecule which tend to "neutralize" a portion of the negative charges of the "Y" enzyme, making its mobility more like that of "W". Although charge effects are probably responsible to a certain extent for the "intermediate" stages observed, the thin-layer data (see Figure 7) tend to support the idea that the production of "W", rather than a charged-induced modification of "Y", is responsible for the observed effect.

The GuHCl dissociation studies performed with purified P-212 "Y" are of considerable interest since an exhaustive survey of the literature did not reveal any direct parallels. Although dissociation studies are common (i.e., Apella and Markert, 1961), the treatment of a purified isozyme in an attempt to generate a smaller isozyme believed to be a subunit of it has not been reported to the knowledge of this investigator. One study has been done by Liu and Lamport (1973) in which isozymes of horseradish peroxidase were generated from preexisting forms of the enzyme by incubation at 4 C in solutions with pH's of 7 or higher. The generated peroxidase isozymes are formed irreversibly, however, even after retitration of the extracts to a lower pH.

It would be of considerable interest to explore the effect of other denaturing agents on the generation of "W" from "Y". Some agents that could be used are urea, sodium doedecyl sulfate, ethylene glycol, high or low pH, or such solvent systems as formic acid and 1.0M NaCl. The experiment with urea and various reducing agents briefly referred to in the

results section would be a convenient point of departure. Another fascinating possibility for future work is a denaturation-renaturation experiment with P-212 "Y".

Detection of the "New" Intermediate

When P-212 "Y" was incubated with 4M GuHCl at 4 C or with 5M GuHCl and 0.005M 2-mercaptoethanol at room temperature, a "new" enzymatic activity with a mobility on TLG between that of "Y" and "W" was observed (Figures 9 and 10). This intermediate had not been previously observed when experiments were conducted at room temperature or when Cleland's Reagent was used as the reducing agent. Some discussion of the factors influencing the appearance and thin-layer position of this intermediate is included in the results section, but much more remains to be explained about this new phenomenon. One possible reason why the intermediate had not been observed previously may be that it is quite unstable under standard experimental conditions and, as such, does not remain active long enough to be detected; however, it is stable enough to be detected under conditions which serve to promote its stability. The protection of most enzymes by low temperatures is a well documented and generally accepted fact and there is some evidence that the reducing environment provided by 2-mercaptoethanol stabilizes P-212 "Y" and "W" more than that provided by Cleland's Reagent. These facts serve to support the idea that the intermediate could have been formed in other experiments, yet was too unstable to be detected.

If the above consideration is true, it is possible that this new enzyme activity represents a true intermediate step in the denaturation

of "Y" to "W". Such an intermediate complex, formed by the partial dis-
sociation of aspartate transcarbamylase by mercurials, has been found by
Evans and his coworkers (1974). The specific activity of the intermediate
is essentially the same as that of the native enzyme and native aspartate
transcarbamylase can be reconstituted from it. The significance of find-
ing such a stable intermediate is in elucidating the overall mechanism
of the reaction. A two-state process, in which no stable intermediate
between the native and the denatured state is found in significant quan-
tities, would be eliminated by the finding of such an intermediate.

On the other hand, this new intermediate may be an example of the
previously described incorrectly folded molecules which are not on the
direct pathway between native and denatured states of enzymes. Ikai and
Tanford (1971) believe that such incorrectly folded molecules could be
a general feature of the time course of protein denaturation processes.
Even if this second alternative is actually the case, the mechanism of
denaturation of Neurospora aryl-β-glucosidases by GuHCl would still be
more complicated than a two-state process. In fact, the mechanism would
very likely be even more complex than if the new intermediate actually
represents a true step in the denaturation of the enzyme, since an incor-
rectly folded molecule must be unfolded before it can be transformed to
the native state. Much remains to be discovered about this intermediate.
A good detailed kinetic study of the system would aid in determining
which of these two alternatives represents the actual picture. It would
also examine the possibility that the intermediate is an experimental
artifact or merely a relatively infrequent phenomenon in the denaturation
of these enzymes by GuHCl.

Inactivation Studies

Denaturation Kinetics Although no "proper" determination (see Laidler and Bunting, 1973) of the kinetics of the denaturation of "Y" and "W" by GuHCl was attempted in these studies, some general conclusions can be drawn on the basis of the inactivation curves obtained from PNPG assays of GuHCl-treated preparations. The most interesting of the strains investigated was the exotic strain P-212, and, since it was also the strain used in the dissociation studies with purified conidial "Y", it will be used as a general model for discussion.

The inactivation curves of P-212 aryl-β-glucosidases in 3M GuHCl shown in Figure 12 can be conveniently divided into three major portions. The first portion, comprising the difference between the untreated and "simultaneous addition" optical density values (and slightly farther in many cases), can be considered the initial decline. Since it occurs so rapidly and has an effect of considerable magnitude, this initial decline is very likely due to some sort of inhibition at or distortion of the active site of the enzyme. The second division includes the ascending portion of the curve. This has been referred to previously as the "activation effect" and was also observed in the dissociation studies with purified conidial "Y". The activation may result from the exposure of buried active sites of the enzyme as it is being unfolded. This disaggregation of the subunits of the enzyme may uncover active sites which are sometimes even more active than the ones that are normally exposed on the surface of the three-dimensional structure of the enzyme. Hayashi, Minami, and Hata (1972) observed a similar effect with yeast pro-proteinase C after brief exposure to low concentrations of several protein denaturants,

including GuHCl and urea. From their results, it was concluded that the denaturants rearrange the quarternary structure of the proenzyme and lead to unmasking of the active site.

As mentioned earlier, the "activation effect" was not uniformly observed with P-212, especially at higher concentrations and under constant temperature conditions. It was not observed at all in strain P-113 or in strain P-278. The failure to observe a significant activation effect in all cases may be due to one or more of the possibilities listed below.

1. The activation effect observed with P-212 inactivated at room temperature may be an experimental artifact. Although this certainly has to be considered as a possibility, it is one that can very likely be rejected due to the amount of experimental data which tend to suggest some sort of activation of these enzymes. The fact that the activation effect was observed in TLG and electrophoresis experiments also tends to eliminate this possibility.

2. The activation effect occurs under the conditions of the experiment at a time not sampled by the time sequence used. This suggestion is a very appealing one and very likely plays a larger part in the failure to observe the activation effect under all conditions. The apparent absence of an activation effect at higher concentrations may be that it occurs almost instantaneously, somewhere between "0 incubation time" (simultaneous addition) and 1 minute incubation (late addition), a time span not sampled in my experiments. This possibility could be easily tested with the aid of a "continuous sampler" that would remove samples to be assayed from the reaction mixture every second or so.

3. The activation effect is caused by and/or is extremely dependent upon small temperature fluctuations in the surrounding laboratory environment. This possibility appears very likely when one considers the fact that an activation effect was always observed with strain P-212 when the experiment was performed at room temperature, but it was not observed when the experiment was performed at a constant temperature of 20 C. If the activation effect was dependent upon slight temperature fluctuations, the absence of any significant temperature fluctuations (as in a constant temperature water bath) might greatly affect its occurrence.

The third and final portion of the curve can be considered the "second decline". It comprises that descending part of the curve that begins after the activation effect has occurred. The reason for the second decline is probably very similar to that for the activation effect. As the molecule continues to be unfolded and disaggregated, additional active sites are being exposed. They are, however, less active than the ones which are normally exposed on the surface of the three-dimensional structure and are much less active than the "activated sites". In addition, as some molecules of the enzyme are being destroyed, the specific activity of the entire system goes down, even though complete inactivation of all enzyme molecules has not yet occurred. In some cases, as for example with P-212 "Y" and "W" in 5M GuHCl and P-113 "Y" in 4M GuHCl, complete inactivation of the enzyme does result.

As mentioned previously, no attempt was made in these studies to determine the reaction order of the denaturation of Neurospora aryl-β-glucosidases by GuHCl. It is safe to assume, however, that this process does not follow simple first order kinetics. The possible discovery of

at least one stable intermediate in the denaturation of "Y" suggests that a two-state system, in which only the native and the fully denatured protein appear in significant amounts, does not strictly apply in this case. Further investigation into the equilibrium kinetics of the reaction, perhaps using optical rotation or difference spectroscopy, would be of immeasurable value in elucidating the mechanism of the denaturation of Neurospora aryl-β-glucosidases by GuHCl.

Stability of "Y" versus "W" From the inactivation curves and the half-time values (Table 4), it can be concluded that, in all strains and all concentrations of GuHCl used, the aryl-β-glucosidase isozyme "W" was more stable than the larger isozyme "Y". The difference in stability ranged from slight (as in wild type strain STA-4) to considerable (as in exotic strain P-113) with several gradations in between. This finding was in direct contrast to what was expected at the beginning of these studies and is somewhat difficult to explain when one considers the relative abundance of "Y" in most strains as compared with the smaller amount of "W" in those strains. The stability of the "W" itself may be correlated with the amount of "W" that is generally produced by the strain. For example, STA-4 produces very little "W" and its "W" is only slightly more stable than its "Y". On the other hand, P-212 produces large amounts of "W" and its "W" is significantly more stable than its "Y", particularly at low concentrations of GuHCl. That the situation is probably much more complicated is illustrated by the behavior of strain P-278, which normally produces almost no detectable "W" but can be "induced" to produce more "W" when it is grown on media containing low levels of ethylene glycol (Hartis, unpublished data). The "W" produced by ethylene glycol cultures is more

stable than the "Y" (see Table 4), possibly indicating that the unstable "W" produced by the strain on standard media has undergone a change in the presence of ethylene glycol that renders it more stable. This possibility has not been adequately investigated since no purified "W" from P-278 grown on GSCP has been obtained for GuHCl inactivation experiments. Any definitive statement about this unusual behavior would depend on the results of such experiments.

Effect of GuHCl Concentration The effect of GuHCl concentration was best illustrated by the inactivation curves of P-212 since this strain was inactivated at three different concentrations of GuHCl. The extent of the initial decline increased slightly with increasing concentration of GuHCl, a difference of approximately 0.10 optical density unit for 3M GuHCl as compared with 0.20 and 0.30 for 4M and 5M, respectively. As mentioned previously, the activation effect was most pronounced at 3M GuHCl and appeared to be absent in all but the crude conidial washes at 4M and 5M GuHCl. The possible reasons for this have been mentioned earlier in the discussion of the activation effect itself. The most obvious effect of increasing the concentration of GuHCl was the extent of inactivation of the enzymes. At low GuHCl concentrations, the second decline was very slight; while at higher GuHCl concentrations, both P-212 "Y" and "W" were completely inactivated. This result is easily explained in terms of traditional theories of solution chemistry (i.e., A denaturant at high concentration is more effective than at low concentration.). P-212 "Y" was most sensitive to changes in GuHCl concentration, while the aryl-β-glucosidases in the crude conidial wash of P-212, as might be expected due to the heterogenous nature of the wash, were least sensitive to these molarity changes.

Strain Differences   A comparison of the inactivation curves of
STA-4, P-212, P-113, and P-278 revealed wide variation in their response
to GuHCl.   Space limitations do not permit a complete enumeration of these
differences here, but some general conclusions can be drawn from these
curves.   Since only one standard laboratory wild type strain was investigated,
a general statement concerning all wild types is impossible, but it is
obvious that, for wild type strain STA-4 at least, wild type aryl-β-gluco-
sidases appear to be much more sensitive to denaturation by GuHCl than are
exotic enzymes and are rapidly inactivated by low (3M) concentrations of
the denaturant.   Although a great deal of variation is observed among the
three exotics tested, these strains in general appear to possess aryl-β-
glucosidases (both "Y" and "W") that are somewhat resistant to inactivation
by GuHCl.   The variation among these strains is most pronounced in the
behavior of their "Y" enzymes.   It is probable that, although the genes
responsible for the formation of the aryl-β-glucosidase isozymes were very
likely evolved before the genes that govern the characters that set these
three strains apart, selection has been operating on the genes that control
the stability of the two isozymes in different ways in these three strains.
Since denaturation by GuHCl is an artificial state which these enzymes
would never encounter in nature, it is difficult to speculate on the adap-
tive significance of the difference in stability between the two isozymes
which is observed in these strains.   Perhaps the thermal denaturation
studies to be reported by Eileen Hartis will shed more light on this fas-
cinating question.

It is apparent that much remains to be learned about the chemical
inactivation characteristics of these enzymes.   There are numerous other

denaturing solvent systems which could be used to investigate this problem.
Denaturation studies with P-278 and high concentrations of ethylene glycol
would be of particular interest in view of its effect on this strain at
very low concentrations.

There are many reducing agents other than the two that I used, such
as cysteine, glutathione, sodium thioglycolate, 2,3-dimercaptopropanol,
and dithioerythritol (DTE, an isomer of Cleland's), that might influence
the stability and the state of aggregation of these enzymes in GuHCl. As
mentioned earlier, denaturation-renaturation studies with both aryl-β-
glucosidase isozymes would be of immeasurable value in determining the
overall denaturation kinetics of these enzymes and would also shed light
on how these molecules are aggregated to produce the native enzyme.

Stability of "Nascent" "W" versus "Native" "W"

One of the most intriguing results of these denaturation studies
was the observed difference in stability of the native "W" enzyme and what
we have chosen to call "nascent" "W". Native "W" is much more stable in
the presence of GuHCl than is the native "Y", but "W" which is generated
from "Y" - nascent "W" - is much less stable than the "Y" from which it
was produced. It is possible that, as Jermyn (1962) found with the β-
glucosidases in Stachybotrys atra, "W" (and to a lesser extent, "Y") is
surrounded by "sugar coatings" - polysaccharides in association with the
three-dimensional structure of the native enzyme molecule. A heavy
coating of polysaccharide would perhaps tend to make native "W" resistant
to the effects of GuHCl. The color of concentrated conidial washes which
contain good "W" activity is often quite yellow which tends to support

the conclusion that carbohydrates are associated with the presence of "W".
If this is indeed the case, the "W" formed from the "Y" would not be pro-
tected as the native "W" might be. Even if "Y" were to be surrounded by
a polysaccharide coat, the "W" generated from it might not be protected
on all sides of the molecule by these "sugar coatings" and would thus be
more succeptible to attack by GuHCl. A way in which this hypothesis might
be tested is to treat "Y" and "W" with an agent capable of disrupting or
destroying a polysaccharide coat, such as the enzyme chitinase or one of
the deaminases. If these pre-treated enzymes were then exposed to GuHCl,
differences in their response to the agent, as compared with appropriate
control samples, might be noted. Although polysaccharide coatings may be
found to be an incorrect assumption, some mechanism similar to it must be
the proper explanation since the difference in stability is undoubtedly
correlated with some structural difference arising from the difference in
the way the two "Ws" are formed.

Possible Isozyme Relationship of "Y" and "W"

On the basis of the chemical denaturation studies described in this
thesis, I would like to propose a tentative hypothesis regarding the exact
isozyme relationship of "Y" and "W". The successful generation of "W"
from P-212 "Y" under a variety of conditions of incubation with GuHCl
suggests that "W" is at least in part a subunit of "Y". As mentioned in
the introduction, there are two ways in which "W" could be a subunit of
"Y". First, "Y" could be a homopolymer composed of a number of identical
subunits, in this case, a number of "Ws". Second, "Y" could be a hetero-
polymer composed of two or more types of subunits, of which "W" is one.

The second alternative is possible since an additional enzymatic activity, which has a mobility on TLG less than that of "W", has been occasionally observed in TLG experiments. It will be further described by Eileen Hartis. This activity, designated "V", is not observed with any regularity and has been noted in only one or two of the many TLG runs I made. When it is observed, however, it has good activity, indicating that the failure to observe it regularly is probably not related to a high degree of lability under the conditions of the experiment. The possibility can not be eliminated that "W" and "V" participate coordinately in determining the structure of "Y" in a manner similar to the α and β subunits of tryptophan synthetase described by Goldberg, Yanofsky, and their coworkers (1966) in which two αs and two β_2s (a dimer composed of two β subunits) combine to form the native enzyme. Two "W" molecules and two dimers, each composed of two "V" molecules, might combine to form "Y". The two "Vs" together might form a complex that has a molecular weight that is identical, or nearly so, to that of "W". This complex would thus be indistinguishable from "W" on TLG plates. The infrequent observation of "V" itself may be due to the fact that the dimer is not easily dissociated. A heteropolymeric structure composed of "W" and an additional, as yet unobserved, subunit of identical molecular weight is also possible, but, at this point, it can be rejected on the basis of the lack of electrophoretic and TLG evidence for such an additional molecule.

I have also chosen to reject the possibility that "V" is a bonafide subunit of "Y" and to propose that "Y" is a homopolymer composed of two identical subunits, designated "W", each with a molecular weight of approximately half that of the molecular weight of "Y" which has been

previously determined to be 168,000 (Eberhart and Beck, 1970). This

possibility appears likely since "W" has a mobility on TLG that is very

close to that of cellobiase ("X") which is known to have a molecular weight

of 80,000 (Eberhart and Beck, 1970). This hypothesis could be proved or

disproved by the results of C-terminal amino acid analysis of the native

"Y" enzyme. If a single type of amino acid is revealed by such experi-

ments, a homopolymeric structure for "Y" would indeed be a likely pos-

sibility (Amelunxen, Noelken, and Singleton, 1970). In addition, the

determination of the exact size of "W" by ultracentrifugation in a sucrose

density gradient or by comparative gel filtration with compounds of known

molecular weight would also yield more information on whether or not this

structure is feasible. The existence of "V" could even fit into this

interpretation if "W" is considered to be a dimer composed of two "Vs".

"Y" could then be visualized as a tetramer composed of four identical,

enzymatically active monomers. The infrequent observation of "V" might

once again be due to a high degree of stability of the dimer which would

render it very difficult to dissociate. Treatment of a highly active

preparation of "W" with high concentrations of GuHCl (or of an even more

effective denaturant) or exposure to ion exchange resins would determine

if "V" could be generated from "W" and, thus, if "W" could be a dimer

composed of two "Vs".

From the denaturation studies reported in this thesis, however, it

is possible to conclude that "Y" may have a structure similar to the one

shown in Figure 15, depicting a dimeric molecule composed of two "Ws" which

are associated in the native state with a carbohydrate moiety which aids

in protecting these enzymes from attack by chemical denaturants.

Figure 15. Hypothetical Isozyme Relationship of <u>Neurospora</u> Aryl-β-
Glucosidase Isozymes "Y" and "W".
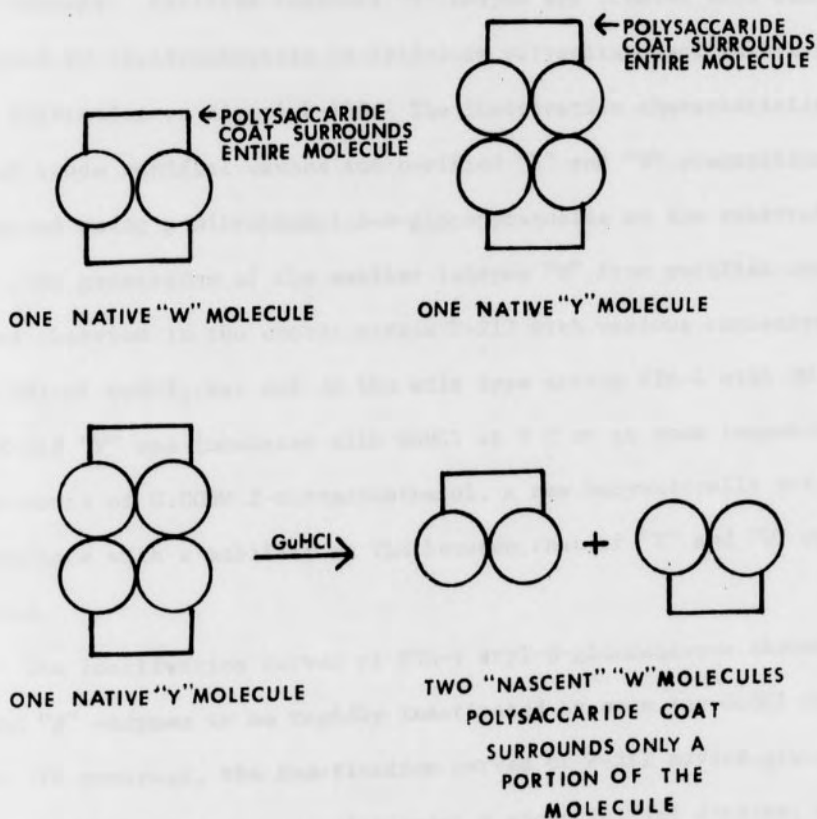
SUMMARY

The effect of guanidine hydrochloride (GuHCl) on the stability and the state of aggregation of <u>Neurospora</u> aryl-β-glucosidase isozymes "Y" and "W" was studied in one wild type and several exotic strains by two major methods. Purified conidial "Y" enzyme was treated with GuHCl and separated by electrophoresis on cellulose polyacetate and thin-layer gel (TLG) filtration on Biogel P-150. The inactivation characteristics in time of crude conidial washes and purified "Y" and "W" preparations were determined using p-nitrophenyl-β-D-glucopyranoside as the substrate.

The generation of the smaller isozyme "W" from purified conidial "Y" was observed in the exotic strain P-212 with various concentrations (2 to 5M) of GuHCl, but not in the wild type strain STA-4 with 3M GuHCl. When P-212 "Y" was incubated with GuHCl at 4 C or at room temperature in the presence of 0.005M 2-mercaptoethanol, a new enzymatically active intermediate with a mobility on TLG between that of "Y" and "W" was observed.

The inactivation curves of STA-4 aryl-β-glucosidases showed both "Y" and "W" enzymes to be rapidly inactivated by even low GuHCl concentrations. In contrast, the inactivation curves of P-212 aryl-β-glucosidases indicated that these enzymes, following a sharp initial decline, were reactivated in the presence of low GuHCl concentrations. At higher GuHCl concentrations, the initial decline was followed by a more gradual one. Crude conidial washes and purified "W" from exotic strain P-113 exhibited a pattern of inactivation in 4M GuHCl similar to that observed with P-212

at higher concentrations of GuHCl, as did the aryl-β-glucosidases of exotic strain P-278. P-113 conidial "Y" was completely deactivated by even brief exposure to 3-4M GuHCl. When the half-times of Neurospora aryl-β-glucosidase isozymes "Y" and "W" in GuHCl were determined using linear regression analysis, the calculated values showed that, for all strains and all concentrations of GuHCl used, the smaller isozyme, "W", was more stable than the larger isozyme.

From these studies it appeared that "W" generated from "Y" (called "nascent W") is less stable than is the "native W" from conidial washes. A possible explanation for this effect is presented. The GuHCl inactivation characteristics of these enzymes are discussed in light of current theories of protein denaturation and a theory as to the exact isozyme relationship of "W" and "Y" is proposed on the basis of the research described in this thesis.

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