CHANGES OF SOLUBLE LEAF PROTEINS AND ENZYME ACTIVITY IN TRITICUM AESTIVUM L. IN RESPONSE TO PLANT GROWTH REGULATORS, WATER STRESS AND PHENOLIC COMPOUNDS

By

SING-DAO TSAI

Bachelor of Science
National Taiwan University
Taipei, Taiwan
1961

Master of Science
National Taiwan University
Taipei, Taiwan
1965

Submitted to the Faculty of the Graduate College
of the Oklahoma State University
in partial fulfillment of the requirements
for the Degree of
DOCTOR OF PHILOSOPHY
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Thesis Approved:

Glenn W. Todd
Thesis Adviser

Edgar Basler

George V. Odell

John E Thomas

D Durham
Dean of the Graduate College

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CHAPTER I

GENERAL INTRODUCTION

In a living plant, water occurs in many states and is involved in all physiological processes. Because of the physical structure of water involving hydrogen- and dipole-dipole bonding that results in high internal pressure and high surface tension, water equilibria serve as important integrating forces in plant life.

The effect of water stress on plants is extremely complex and plants respond with many protective adaptations. Internal water stress affects growth by both direct and indirect mechanisms involving the alteration of water uptake, root pressure, seed germination, stomatal closure, transpiration, translocation, respiration, mineral relations, nitrogen metabolism, enzymatic activity, hormonal regulation and other processes.

Drought-tolerant plants are those able to endure lasting dehydration so the study of drought resistance was thus concentrated on the colloid-chemical properties of protoplasm and on metabolism. Dehydration in a plant causes such serious changes in nitrogen metabolism that protein synthesis is undoubtedly inhibited and catabolic processes become dominant (Shah and Loomis, 1965; Barnett and Naylor, 1966; Stutte and Todd, 1967). In addition, drought brought about changes in enzymatic activity which may have been due to either the inhibition of de novo synthesis of apoenzymes or the activation or

inactivation of latent enzymes as a response to the substances produced by dehydration in the plant cells.

The purpose of this study was to investigate the response of protein metabolism and of enzymatic activity to several growth regulatory chemicals applied to wheat plants subjected to water stress, and to test the reaction of several leaf enzymes to phenolic compounds. These efforts were made in hopes of finding methods to make use of such chemicals in cultural methods to increase drought resistance as well as yield of crops under arid conditions.

PART I

SOME EFFECTS OF GROWTH REGULATORY CHEMICALS ON THE CHANGES OF SOLUBLE PROTEIN IN WHEAT LEAVES SUBJECTED TO WATER STRESS

CHAPTER II

REVIEW OF LITERATURE

Properties of Leaf Protein

"Fraction 1 protein" (Singer, et al., 1952; Lyttleton and Ts'o, 1958; Pon, 1967) has been reported to comprise a large portion of the soluble protein of cytoplasm and chloroplasts from green leaves of higher plants. It consists of a protein of high molecular weight with a sedimentation coefficient of 16 to 18 Svedberg units (S). In other words, the cytoplasm in the cells of higher plant leaves is characterized by the presence of "fraction 1 protein." Wildman and Bonner (1947) showed that about 75% of the total proteins of spinach-leaf cytoplasm is the fraction 1 protein which could be purified to the extent of electrophoretical homogeneity. A similar report of a study made through ultracentrifugal analysis of green and blue-green algae and the purple sulfur photosynthetic bacterium Chromatium stated these all contained 18 to 21 S proteins, whereas in Rhodospirillum rubrum, the sedimentation coefficient was 6.2 S, and Rhodopseudomonas spheroides and R. palustris contained proteins that sedimented around 13 S (Anderson et al., 1968).

Thornber et al. (1965) and Trown (1965) have arrived at the conclusion that the ribulose diphosphate (RuDP) carboxylase activity of spinach beet is inseparable from fraction 1 protein. Trown (1965) determined the molecular weight of RuDP carboxylase as 515,000 by

sedimentation equilibrium. Its sedimentation coefficient was 18.57 S. He also concluded that fraction 1 protein is crude RuDP carboxylase which perhaps formed the protein moiety of protochlorophyll holochrome. Pon (1967) through physico-chemical studies suggested that 545,000 is the molecular weight of this main component in the fraction 1 protein of spinach chloroplasts. This major component of leaf protein has been confirmed to be present in the leaves of monocotyledons as well as in that of dicotyledons (Lyttleton, 1956).

Fraction 1 protein was synthesized in a particular plant parts. The results provided by Lyttleton (1956) suggested that fraction 1 protein would be found only in photosynthesizing tissue or in tissue which is capable of photosynthesis after it is exposed to light. He also demonstrated that the level of fraction 1 protein in relatively mature etiolated leaves was low, and disappeared from normal leaves on transfer to the dark and the fraction 1 protein was nil in the root tissues of wheat, rye-grass and in the leaves of albino rye-grass.

Using a variety of reagents, several investigators have shown that fraction 1 protein from spinach (Trown, 1965), and from Chinese cabbage (Haselkorn et al., 1965) can be dissociated into smaller fragments of around 3 S. Anderson et al. (1968) isolated three quite different molecular forms, namely 6 S, 13 S and 18 S of RuDP carboxylase from different species of photosynthetic bacteria. Whether they are indeed chemically similar or chemically unrelated proteins is not known. Haselkorn et al. (1965), using electron microscopic observation, indicated that the protein appears to be a cube with edges of about 120 $\mathring{A}$. The substructure can be seen in individual particles, consistent with a model composed of 24 subunits which had been thought to

attach to the RuDP carboxylase (Kreutz, 1965) and other photosynthetic enzymes.

Relation of Water to Protein

A single complex protein molecule is capable of binding some 20,000 water molecules (Stocker, 1960). "Bound water" was termed as the amount of water held at room temperature in an evacuated desiccator containing a desiccant but driven off in an oven at 80°C or 110°C respectively (Todd and Levitt, 1951). Levitt (1965) regarded bound water as the portion of water held by dry matter in equilibrium with a definite low vapor pressure. The greater part of the bound water is therefore due to solutes and colloids in the cell wall. Fisher (1965) proposed a theory to predict the amount of hydration possessed by any given protein based solely on the amino acid composition of that protein. The amount of water bound per g of protein was 0.28 g for an average calculated from 34 given individual proteins. This average value was almost constant for all proteins with quite different molecular weights. Fisher (1965), therefore, postulated that the shapes of protein molecules must vary tremendously, and that the variation must itself be some function of molecular weight to so exactly balance out the expected inverse relationship between molecular weight and surface area per unit weight. Walter (1955) concluded that the hydration of proteins is largely dependent upon the pH of the medium or the influence of electrolytes.

Metabolic Changes of Protein

The distribution of protein in the subcellular fractions of wheat

leaves was reported as 15% in debris and nuclei, 20% in chloroplasts, 2% to 4% in mitochondria and 60% in the supernatant fraction (Todd and Basler, 1965). Proteins in the supernatant fraction have been more extensively investigated. Soluble proteins in plant leaves subjected to moisture stress tend to be broken down from the large molecules into smaller ones, resulting in a decrease in the large molecular weight fraction, and an increase in the smaller molecular weight fraction (Stutte and Todd, 1967; Todd et al., 1970). There is a reduction in total amounts of protein (Todd and Basler, 1965; Stutte and Todd, 1969; Barnett and Naylor, 1966).

Enzyme-mediated metabolic processes are presumably controlled as directly as protein is by the water potential. Todd and Yoo (1964) found that various enzymes investigated lost their activities at different rates depending upon the degree of water stress. Enzymes saccharase and phosphatase lost their activities more rapidly than did a peptidase.

In some investigations of the nitrogen metabolism in higher plants, Chiball (1924 b) found that asparagine is one of the chief products of nitrogen metabolism in a mature runner bean leaf. The role of asparagine in the metabolism of the mature leaves appears to be one in which asparagine is the chief agent whereby nitrogen, in form suitable for resynthesis of protein, is conveyed from one part of the plant to another. Breakdown of proteins in plants occurs as a consequence of many agents such as wilting, detaching, diurnal rhythm and etc. Chibnall (1924 a) found diurnal variation of protein nitrogen in runner bean leaves. There was a loss of about 1.8% of protein nitrogen during the night time. The products of the protein decomposition have been

thought to be translocated to other parts of the plant. When a mature leaf of the runner bean was detached from the plant and kept with the cut end of the petiole in water or mounted in moist sand there was a rapid breakdown of protein in the blade, accompanied by an outflow of non-protein into the petiole. Under these conditions IAA treatment reduced the rate of protein breakdown in the leaf blade (Chibnall, 1954). It was noted that runner bean plants produced asparagine as the most prominent product of the protein metabolism. However, in these samples taken from the plants grown under drought conditions, the protein metabolism produced neither an increase in asparagine nor in free ammonia N, but a large increase in "amide N other than asparagine N." These plants did not set fruit (Chibnall, 1924 c). Barnett and Naylor (1966) found that water stress induced a higher degree of protein turnover, also a great increase in free proline and asparagine, but a decrease in glutamic acid and alanine levels. Macpherson (1952) also found a rapid accumulation of amides, particularly asparagine following a breakdown of protein in plants during wilting. The greatly increased amount of proline during water stress appeared to function as a storage compound in stressed plants (Barnett and Naylor, 1966). Kudrev (1967) reported that a large increase in free proline content and a smaller increase in free glutamic acid were found in wilted pumpkin plants.

Steric Changes of Protein

Some effects of water deficits on metabolic processes in a plant has been mentioned above. It may be questioned whether only a reduction of water potential by a few bars would be sufficient to cause

enough change in structure of proteins to modify enzyme action and in turn, to alter metabolic processes. However, Meyer and Gingrich (1961), applying an osmotic stress of one bar to one-half the roots of a wheat plant growing in solution culture (half of the root system being placed in a normal culture solution), surprisingly found an "initial physiological shock" which subsequently disturbed the metabolism of the entire plant. Kramer (1969) cited the works of Klotz and of Tanford to reveal that the removal of part of the water surrounding protein molecules may cause changes in configuration affecting permeability, hydration, viscosity, and enzyme activity. The "sulfhydryl-disulfide" hypothesis proposed by Levitt (1962) was used to explain drought, heat, pressure injuries, as well as frost injury, drought injury has been postulated as to be due to an unfolding and therefore a denaturation of the protoplasmic proteins. This results from the formation of intermolecular -S-S- bonds induced by the close approach of the protein molecules due to stress. Drought resistance is therefore inferred to be a resistance towards SH oxidation and SH $\rightleftharpoons$ SS interchange, and to prevent the formation of these intermolecular -S-S- bonds. Evidence to support this hypothesis was provided by Gaff (1966) who found that dehydration of protein from cabbage leaves caused a great decrease in amount of reactive sulfhydryl which was thought to be attributed to changes in configuration of the protein in the soluble fraction. Chen et al. (1964) also found that, in vitro, even low sucrose concentrations caused marked changes in enzyme activities and in the conformation of proteins.

Physiological Action of Plant Growth Regulators

To date, the information concerning the effects of water stress on the synthesis or transport of growth regulators which either accelerate or retard the protein synthesis or degradation is limited. Reports pertaining to antimetabolites in connection with plants subjected to water stress are also rare. Larson (1964) suggested that water stress inhibits auxin formation in the stem tips of trees reducing or cutting off the supply to the cambium and modifying cambial activity. Ben-Zioni et al. (1967) reported that in water-stressed disks of tobacco leaves the capacity to incorporate L-leucine-^{14}C into proteins was reduced by about 50% , and that this reduction of incorporation could be partially restored by pretreatment with kinetin prior to incubation. The optimal kinetin concentration for the maximal effect was ten-fold higher for stressed tissue than for the non-stressed tissue. Itai and Vaadia (1965); Itai et al. (1968) reported that there was significantly less cytokinin activity in the xylem exudate from roots of sunflowers subjected to water stress than in exudate from unstressed control plants, resulting in a decrease in the quantity of cytokinins reaching the shoot where a change in metabolism and the premature senescence of the plant occurred. Thus the primary effect of water deficit on the metabolic process in the leaves might be related to a change in quantity of endogenous hormones which are synthesized in the roots. Since the translocation of metabolites (Hartt, 1967) and of exogenously applied 2,4-D (Basler et al., 1961) was substantially decreased by water stress, the supply of cytokinins to the shoots might be reduced. There were many reports that roots supply gibberellins to the shoots (Jones and Lacey, 1968; Skene, 1967). The reduced synthesis and/or

accelerated inactivation of growth regulators such as cytokinins and gibberellins in the roots is probably an important factor in the reduction of growth observed in plants subjected to water stress. It might also be a factor in the rapid senescence of leaves on plants subjected to water stress. Experiments using radioactive techniques with ^{14}C-kinetin indicated that the mechanism of the inactivation caused by water stress involves a chemical transformation of the cytokinins (Itai and Vaadia, 1971).

CCC ((2-chloroethyl) trimethyl ammonium chloride) is a quaternary ammonium compound described as a growth retardant. The trimethyl quaternary ammonium cation is necessary for the activity (Tolbert, 1960). Wheat (Triticum aestivum L.) was sufficiently sensitive to soil drenches to serve as a bioassay plant for CCC (Tolbert, 1960). Halevy and Kessler (1963) reported that CCC-treated bean plants remained turgid for more than 42 days, while untreated plants were completely desiccated after 30 days. The fresh and dry weights of the treated plants were considerably higher than those of the untreated plants. Farah (1969) reported that CCC-treated wheat tillers were about 32% shorter than the untreated. Because the structure of the quaternary ammonium compounds is similar to certain cholinesterase inhibitors (Fries and McCarville, 1954), it suggested that dwarf plants which had been regarded as due to the shortage of GA were produced as the result of abnormal choline metabolism, and CCC was therefore considered to play a role as an antigibberellin in reducing the level of endogenous GA-like substances (Fontes and Ozbun, 1969).

It is finally noted that certain growth retarding or stimulating substances have had some effect in improving tolerance of plants to dry

soils (Halevy and Kessler, 1963; Halevy, 1964). These results from Halevy and Kessler (1963) suggest that both CCC and phosfon evidently increased the drought tolerance of Phaseolus vulgaris L.var. Brittle Wax. Similar results have been reported with wheat plants by El Damaty et al. (1965) and Miyamoto (1963).

CHAPTER III

MATERIALS AND METHODS

Plant Materials

Ponca, a winter wheat variety of Triticum aestivum L. was grown in a controlled environment chamber at 23 $\pm$ 2°C in 4" plastic pots with perlite as the supporting medium. Light intensity was maintained at 11,000 lux using Gro-lux lamps with a 14 hours light and 10 hours dark cycle. Two hundred seeds (germination rate around 90%) were sown in each pot containing perlite moistened with nutrient solution. The plants were watered regularly with the solution containing about 10^{-5} M to 10^{-4} M of growth regulators and other chemicals, namely 8.85 mg/l GA from Nutritional Biochemical Co. (NBC), 9.55 mg/l IAA from Sigma Chemical Co. and 8.50 mg/l kinetin from NBC, 22.0 parts per million of CCC and 17.2 mg/l cycloheximide from NBC respectively in each treatment. At fourteen days, plants were exposed to water stress. Water was withheld to provide experimental plants having a relative water content of about 65% to 80% (slightly wilted) and 20% to 60% (wilted). Samples selected for protein analysis were from the most recently mature leaves, the second leaf above the coleoptile of a plant, in order to obtain the leaves with approximately the same chronological age. Harvested leaves were temporarily kept in a plastic bag such that the original water status was maintained. One and one-half gm of leaf blades was sampled for each test.

Preparation of Soluble Protein in Supernatant Fraction

The harvested fresh materials were ground immediately with a pestle and mortar with cold Tris buffer (0.1 M pH 8.3). The homogenates were centrifuged for 30 min. at 27,000 x g. The supernatant was made up to a final volume of 4.5 ml containing 3% sucrose. All procedures through preparation of supernatants were carried out in a cold room at $0^{o}C$ to $4^{o}C$.

Separation of Soluble Protein

The dextran gel Sephadex G-100 was packed in a 1.4 cm x 88 cm column prepared by the procedure suggested by Pharmacia Fine Chemicals, Sweden (1966). A slurry was prepared by adding with mixing 10 gm of Sephadex G-100 to 100-120 ml 0.05 M phosphate buffer (pH 7.0) containing an antimicrobial agent (several drops of 0.05% sodium azide). Upon obtaining a homogenous suspension, the slurry was allowed to stand overnight to complete the hydration associated with the swelling of the beads. The trapped air was removed from the swollen slurry by vaccum. A layer of sand was placed on the top of the filter at the bottom of the column in order to produce a flat-topped filter to hold the gel particles. The slurry was then poured into the column with frequent stirring. After the packing was completed and the buffer level was dropped to within a few mm of the gel top, 0.5 ml of the supernatant was carefully placed on the top of gel column. The material was eluted with 0.05 M phosphate buffer (pH 7.0). The flow rate was controlled by a Polystaltic pump around 0.4 ml/min. Eluant passed through a continuously recording fluorometer (Turner Model III equipped with a

T-5 UV lamp; 254 nm interference excitation filter; emission filters of 7-60 and 5690), followed by a fraction collector. The recorder was set at 12.5 mV and at 0.1 inch per 1 minute of chart speed. Eluant was collected in 4.3 ml fractions. UV absorbance was measured at 280 nm with a Perkin-Elmer model 202 spectrophotometer.

A variety of known substances were also used as standards to check the resolving power of the column. Blue dextran 2,000 ($MW = 2 \times 10^{-6}$) from Pharmacia Fine Chemicals, Sweden, was assumed to move in the void volume (Vo). The following materials were selected to determine the elution volume (Ve): γ-globulin and tryptophan from Nutritional Biochemical Co., Cleveland, Ohio; bovine serum albumin, cytochrome C and glutathione from Sigma Chemical Co., St. Louis, Missouri.

Protein Determination

The fraction collected from Sephadex G-100 gel filtration was monitored UV light at 280 nm. The elution pattern profile was mainly composed of 2 peaks represented as fraction A and fraction B respectively (Figure 2). The area of each peak of UV absorbance on the same scale as Fig. 2 was determined by use of a polar planimeter (K & E 4236, Keuffel and Esser Co.). The tubes containing fraction A (45 to 50 ml) and fraction B (90 to 100 ml) were pooled respectively and a part of the solution was taken and the proteins were precipitated with 5% trichloroacetic acid. The amount of precipitated protein obtained from centrifugation for 10 min. at 6,000 x g was estimated by the Lowry Folin-Phenol method (1951) and calibrated with the standard curve which was based on Figure 1. The color development was allowed to proceed

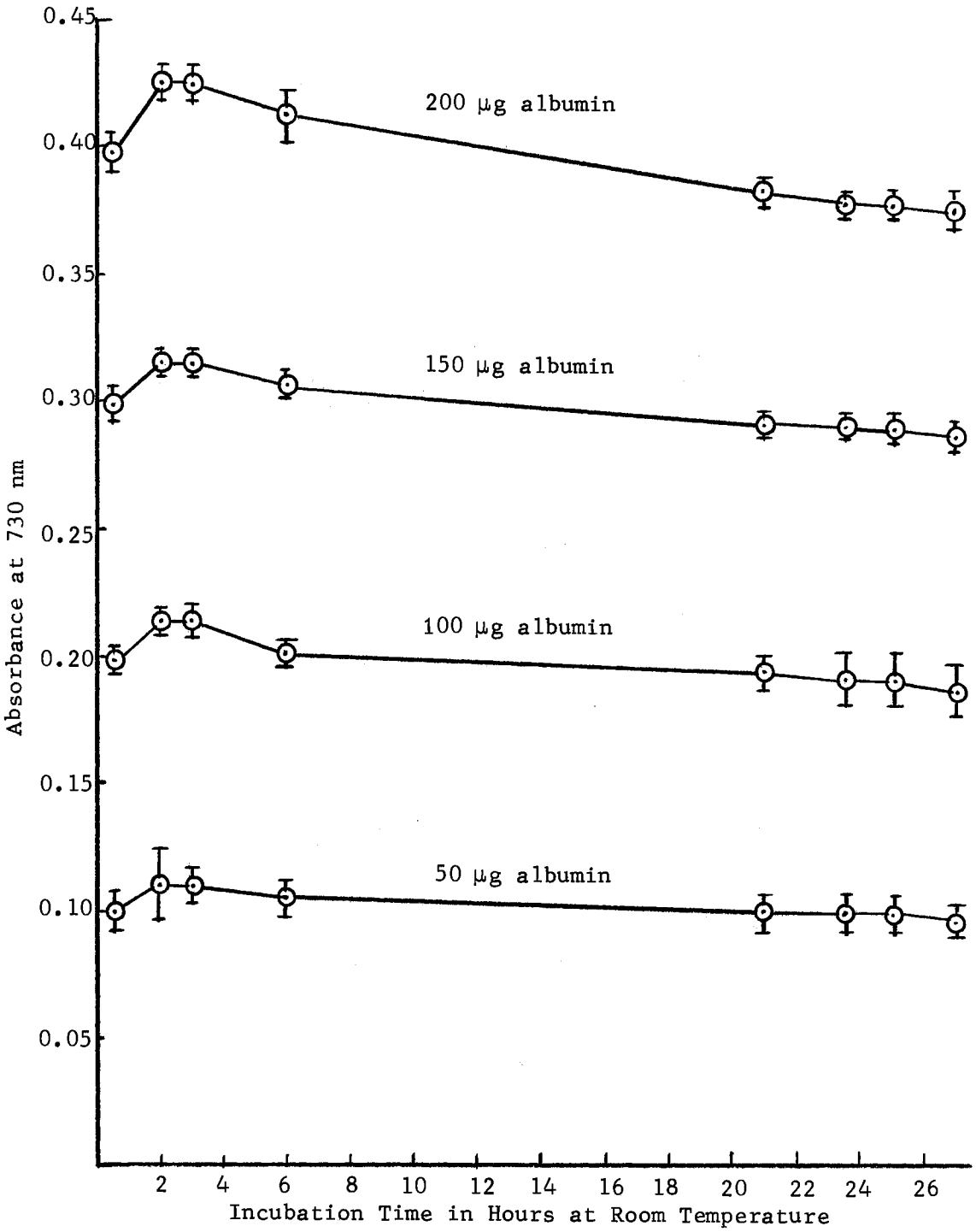


Figure 1. Folin-phenol Test of Color Development on Egg Albumin Versus Elapsed Time. Each point is the average of 2 replicates. Vertical lines indicate 2 standard error limits.

for 30 min. at room temperature in this investigation. Two replicates with two observations of each were done.

CHAPTER IV

RESULTS AND DISCUSSION

Leaf soluble protein separated into two large fractions with Sephadex G-100 gel filtration based on absorbance at 280 nm., namely fraction A and fraction B (Figure 2). The size of proteins in fractions A and B were estimated by comparison with a known protein elution profile as shown in Figure 3. Using blue dextran ($MW = 2 \times 10^{-6}$) which was assumed to move in the void volume (Vo), the elution volume (Ve) of the given substances was determined and the ratio Ve/Vo was plotted against log molecular weight as shown in Figure 4. This figure shows the Ve/Vo to be a linear function of the logarithm of the molecular weight. In comparing with Figure 2 and Figure 3, Fraction A was eluted with the void volume of the column equal to γ-globulin and therefore is presumed to have a molecular weight greater than 100,000, while fraction B, according to the elution volume, has a maximum molecular weight of approximately 12,000 compared to cytochrome C and also contains small peptides and amino acids or other aromatic compounds which would not be separated by using Sephadex G-100. A shift in quantity from fraction A to fraction B as shown in the wheat leaves subjected to water stress (Figure 2) indicated that a change(s) occurs in wheat leaf protein composition as the result of water stress. This change is probably due to the breakdown of larger molecular weight proteins into small molecular weight proteins, as was also found through the use

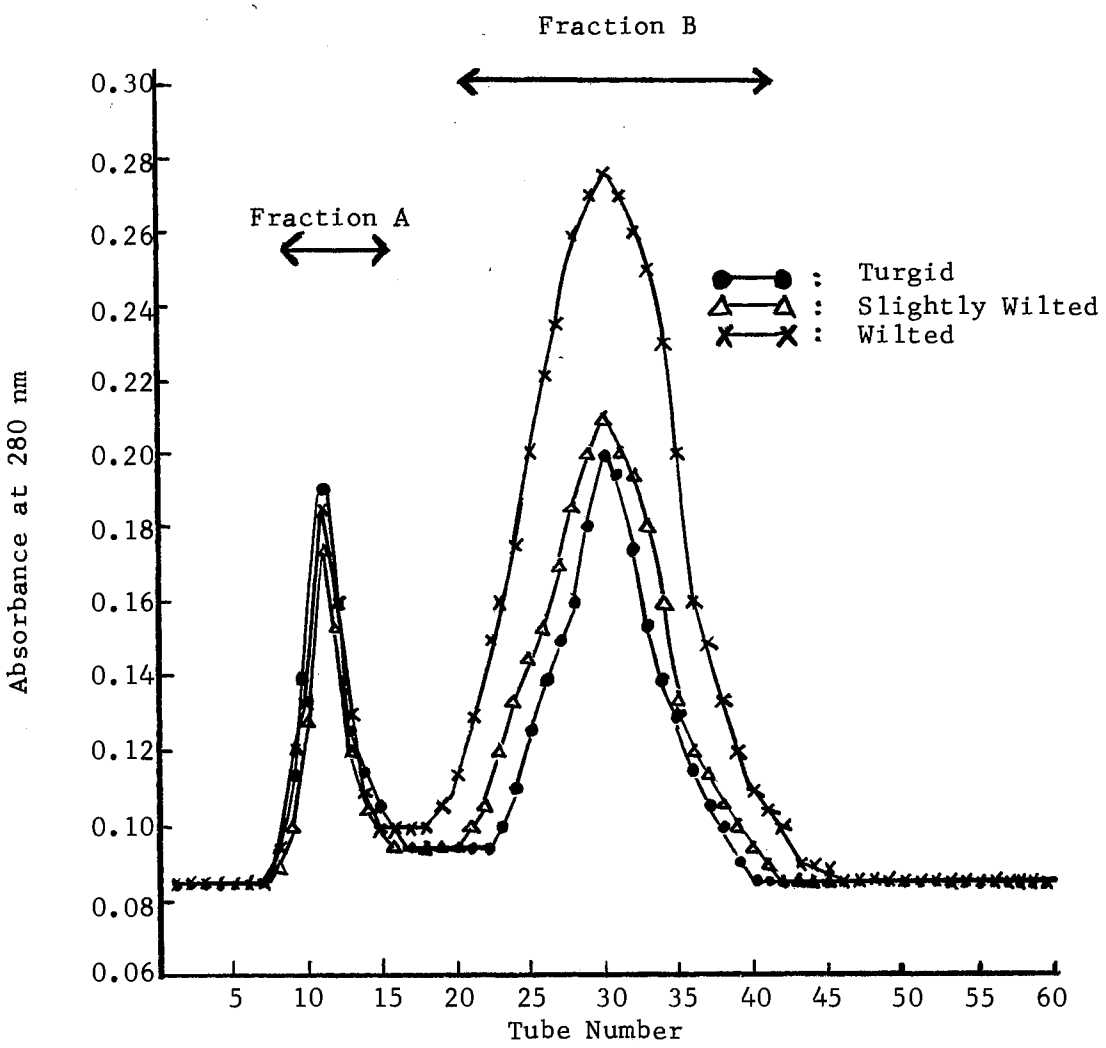


Figure 2. Elution Patterns of Wheat Leaf Supernatant Fraction from Sephadex G-100 1.4 cm x 88 cm Column Eluted with 0.05 M Phosphate Buffer pH 7.0. The amounts of materials used for each determination were 17.4 mg for turgid, 20.3 mg for slightly wilted and 33.4 mg for wilted, on the basis of dry weight.

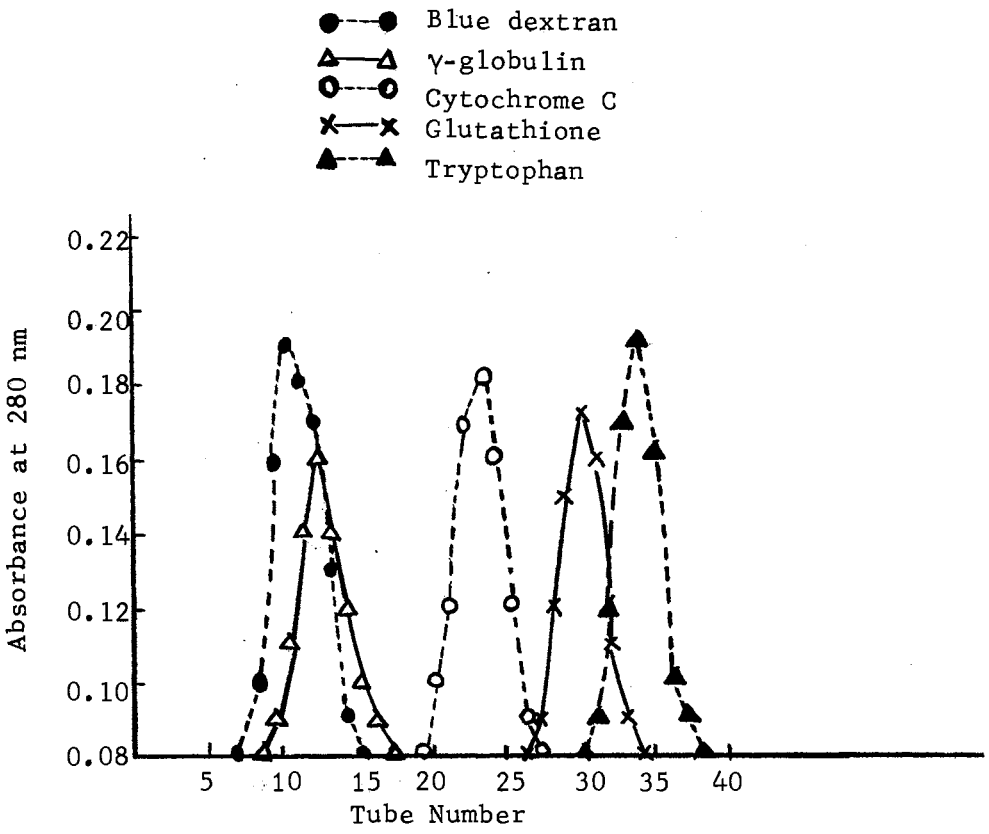


Figure 3. Elution Patterns of Known Components from Sephadex G-100 1.4 cm x 88 cm Column Eluted with 0.05 M Phosphate Buffer pH 7.0. 0.5 ml Aliquots of the Buffer Solution Containing 2 mg Blue Dextran, 2 mg γ-Globulin, 3 mg Cytochrome C, 2 mg Bovine Serum Albumin, 0.5 mg Tryptophan, 8 mg Glutathione were Loaded on the Top of the Column. The Flow Rate Was Set at 0.4 ml/min. The Marker Substances Were Monitored by the Absorbance at 280 nm.

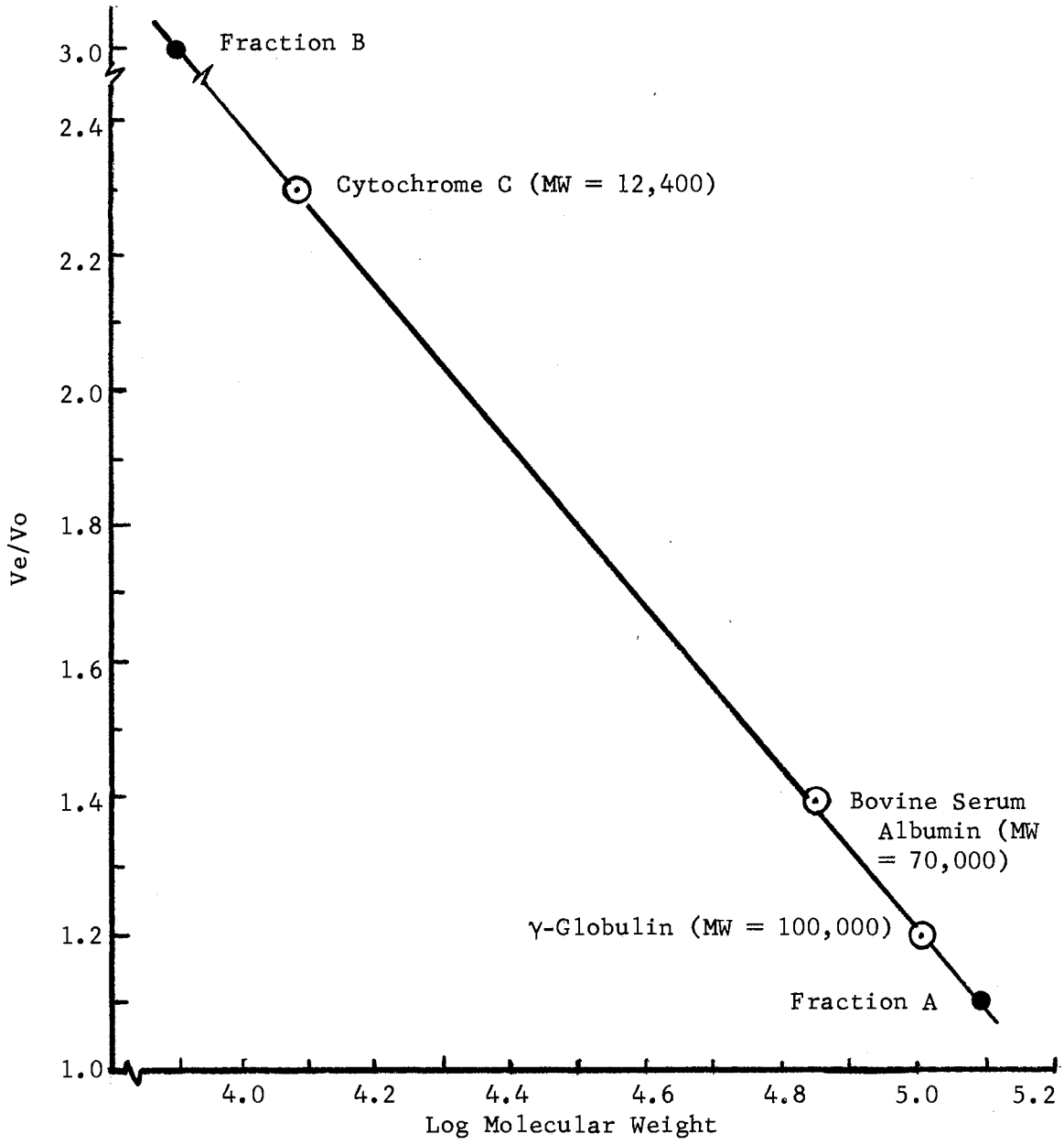


Figure 4. A plot of Ve/Vo against the molecular weight of known substances Ve = elution volume of the compound shown; Vo = elution volume for blue dextran. The molecular weight of fraction A protein was determined from its elution volume over void volume to be more than 100,000 while the molecular weight of fraction B protein is less than 12,400.

of electrophoretic analysis (Stutte and Todd, 1967), and into free amino acids (Chibnall, 1924; Barnett and Naylor, 1966; Kudrev, 1967; Kemble and Macpherson, 1951; Todd et al., 1970).

The results shown in Figs. 5a, 5b, and 5c are based on the Folin-phenol test of protein precipitated by 5% trichloroacetic acid in each fraction. They indicate that protein degradation occurs in all investigated tissues after they were subjected to water stress. Total protein content counted as the sum of proteins in fractions A and B is gradually decreased as the result of water stress. This decrease in protein content may be not only due to water stress but may also result from the normal processes of "aging." However, the change of protein content due to aging is smaller (Smillie and Krotkov, 1961) than that due to dehydration. Measurement of the area of fraction A and fraction B by planimetry of peak area shows changes as a function of water stress (Figure 6). In control plants not treated with chemicals, the peak area of fraction A decreased about 22% while fraction B increased 22% at 76% of RWC. At 43% RWC there was a 50% decrease in fraction A and a 40% increase in fraction B. In both CCC and kinetin treatments, only a slight increase of fraction B peaks and a small decrease of fraction A peaks were observed. Under severe drought conditions (29% RWC), there was only a 39% decrease in fraction A and no longer an increase in the fraction B of the CCC treatment. There was a 52% decrease in fraction A and a 40% increase in fraction B of IAA treatment at 53% of RWC, while in the GA treatment, there was a striking two fold increase in fraction B and about a 60% decrease in fraction A at 42% RWC. In the cycloheximide treated plants the percentage of the initial of fraction B area was markedly increased by

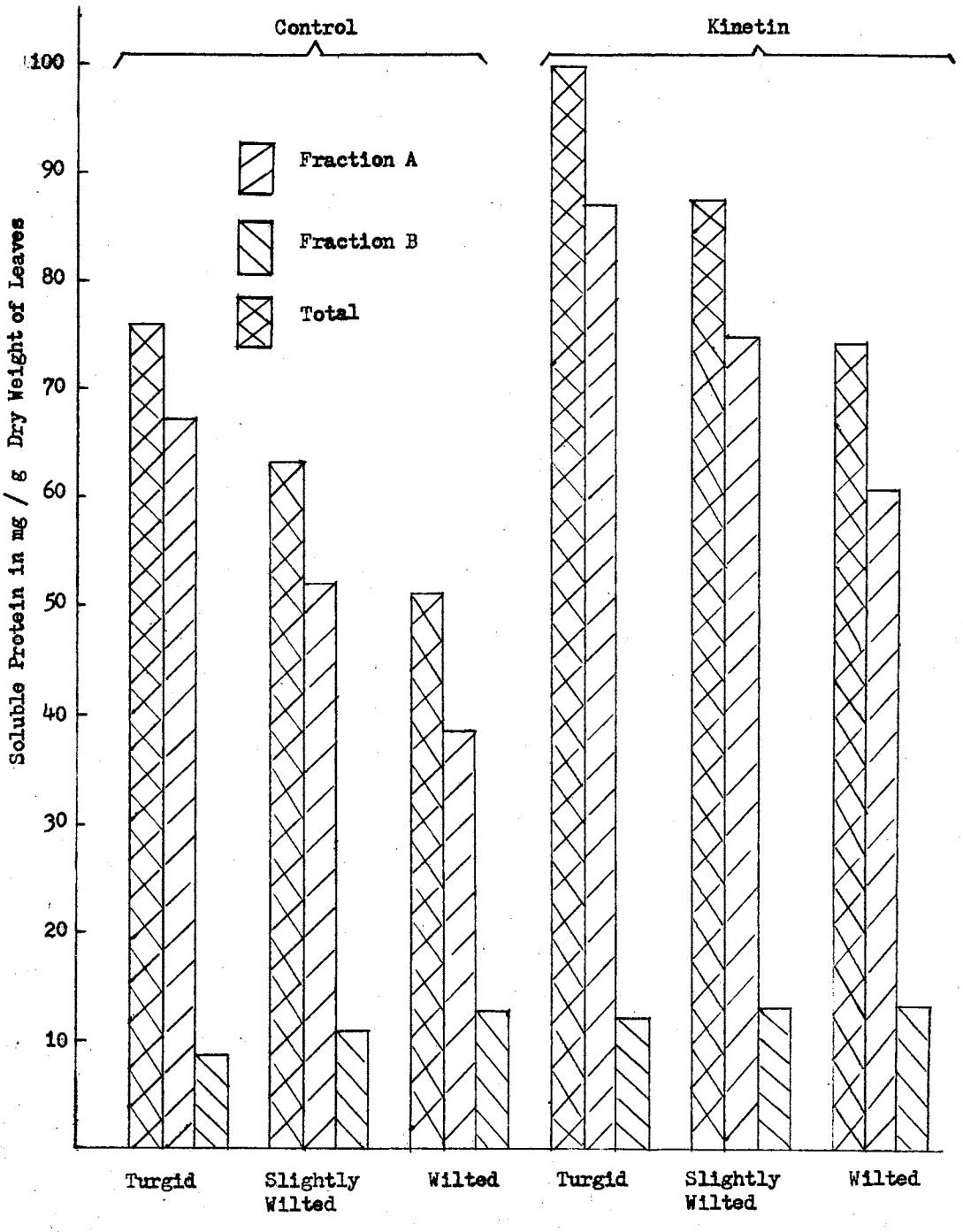


Figure 5a. Comparison of total proteins as the sum of fraction A and fraction B proteins in control and kinetin treatments under drought stress. Protein was precipitated with 5% TCA and then estimated by the Lowry Folin-phenol method.

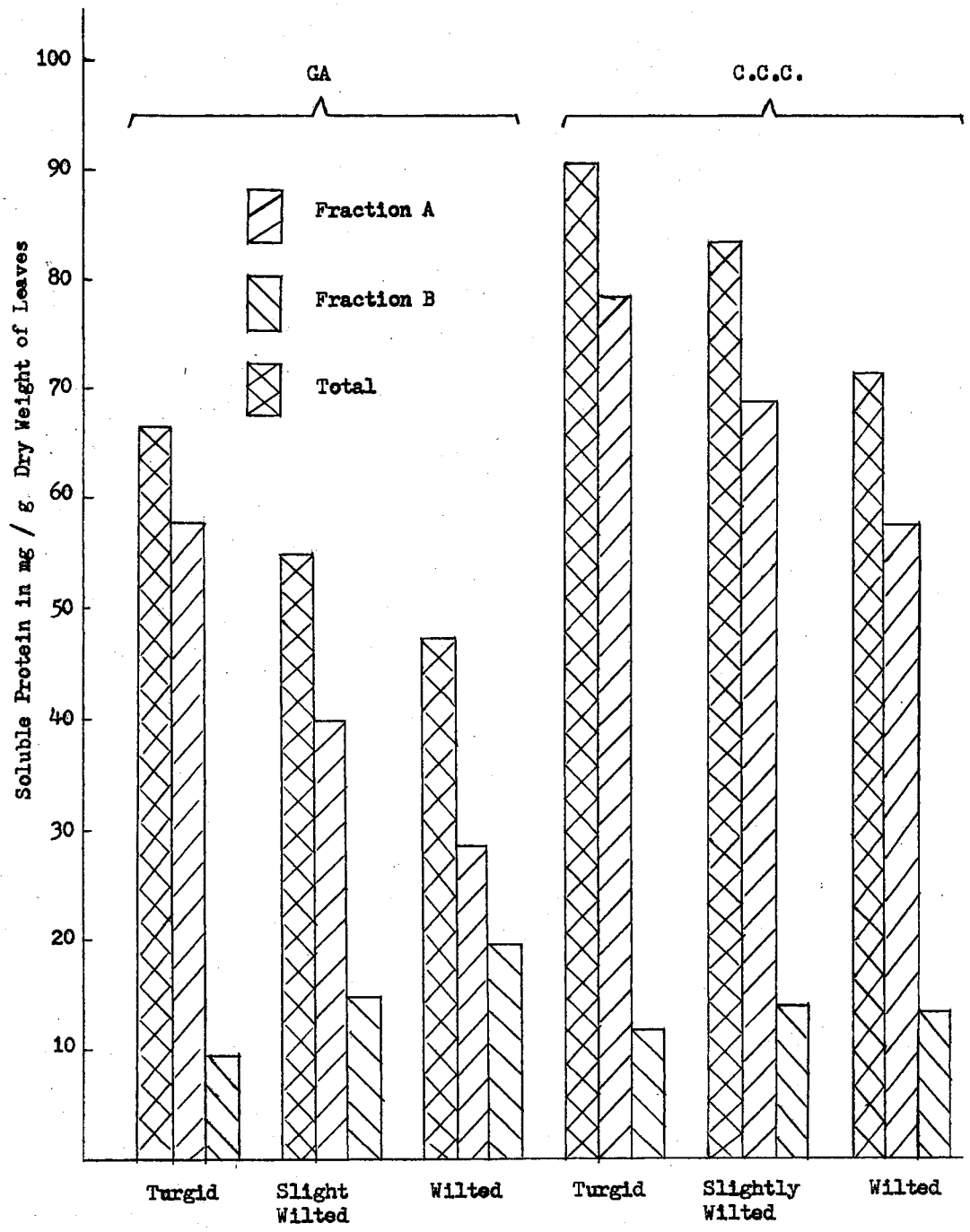


Figure 5b. Comparison of total proteins as the sum of fraction A and fraction B proteins in GA and CCC treatments under drought stress. Protein was precipitated with 5% TCA and then estimated by the Lowry Folin-phenol method.

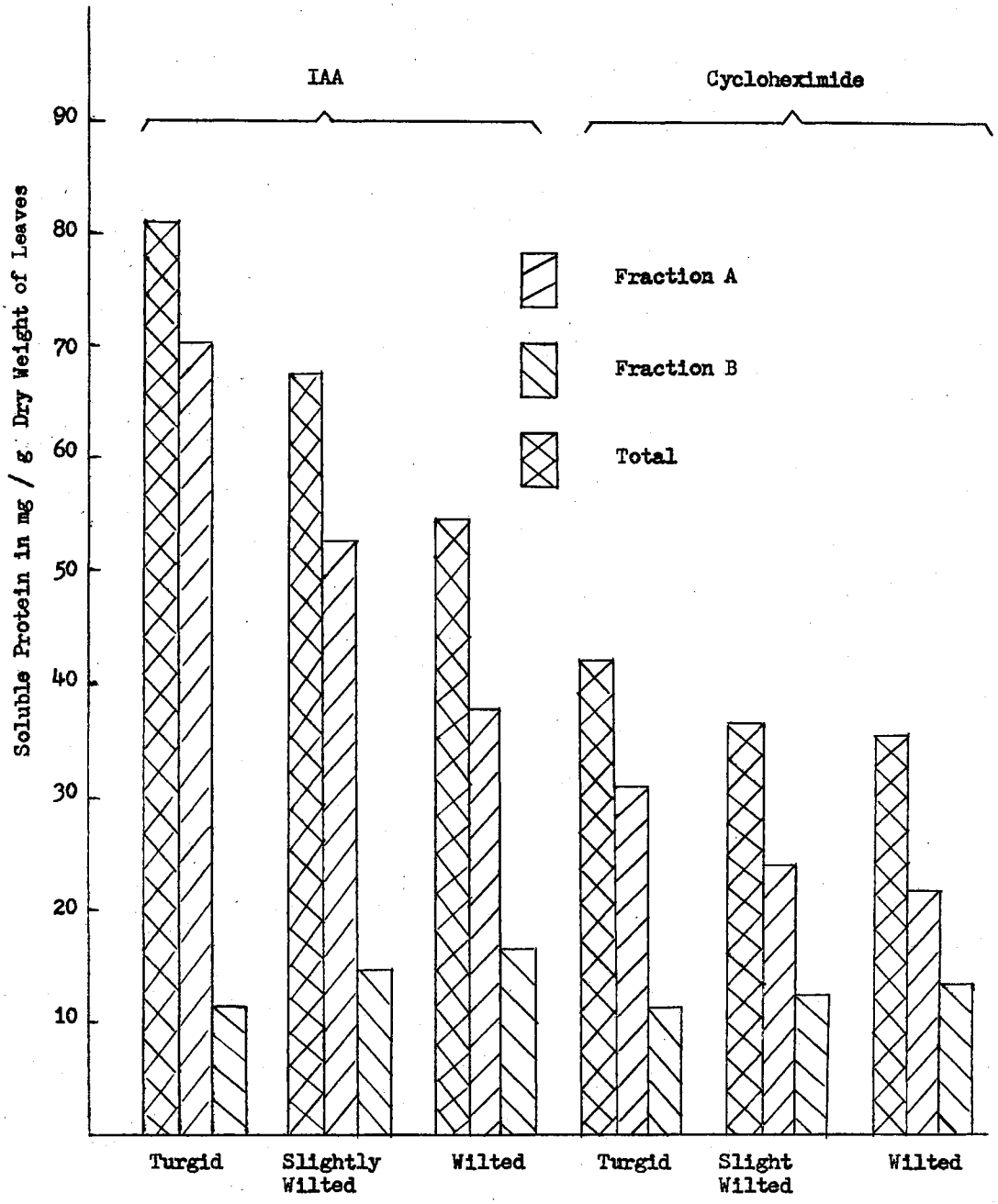
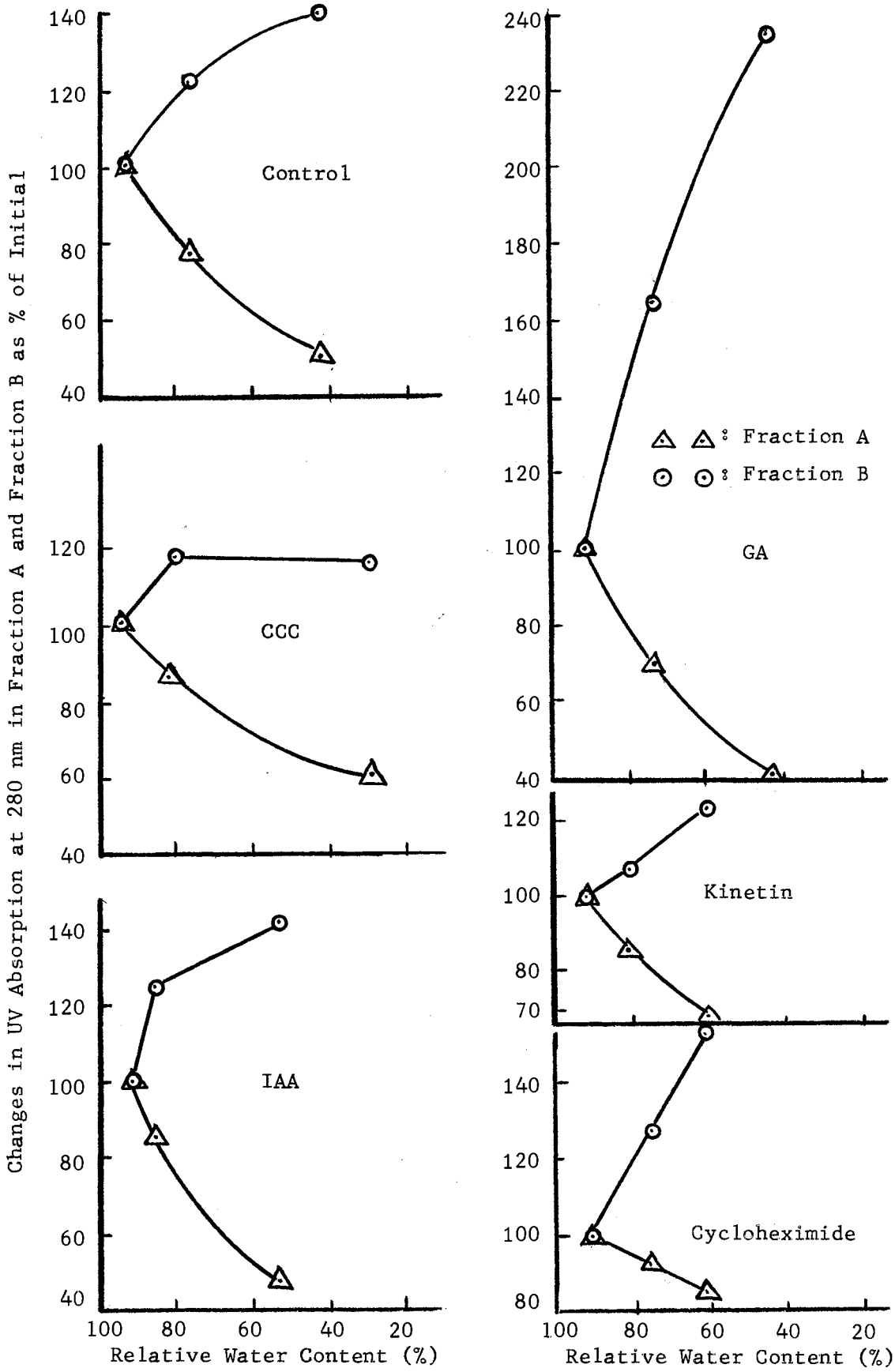


Figure 5c. Comparison of total leaf proteins as the sum of fraction A and fraction B proteins in IAA and cycloheximide treatments under drought stress. Protein was precipitated with 5% TCA and then estimated by the Lowry Folin-phenol method.

Figure 6. Changes as % of Initial in Fraction A and Fraction B by Growth Regulatory Chemicals in Different Moisture Status.


Changes in UV Absorption at 280 nm in Fraction A and Fraction B as % of Initial
Control
CCC
IAA
GA
Kinetin
Cycloheximide
△ △ : Fraction A
⊙ ⊙ : Fraction B
Relative Water Content (%)
Relative Water Content (%)

accompanying a slow decrease of fraction A area after a slight water stress.

In Figure 7, the soluble protein content (Folin-phenol determination) is plotted as a percentage of initial as a function of relative water content in various treatments. Again, the total proteins were decreased with increasing water stress. The GA treatment resulted in a markedly increased amount of fraction B protein accompanied by a drastic reduction of fraction A protein. The shifting of fraction A protein toward fraction B protein during water stress was retarded in CCC, kinetin and cycloheximide treatments as compared with control. IAA treatment did not change the patterns from that observed in controls not treated with chemicals.

However, in Figure 2 and Table I, the area of fraction B of UV absorbance was greater than fraction A. During water stress, the fraction B became greater and greater. Soluble protein precipitated with 5% TCA and measured by the Folin-phenol test, was much greater in fraction A than in fraction B (Figs. 5a, 5b, and 5c). This indicates that fraction B contains substances that are not precipitated with 5% TCA. In Figs. 5a, 5b, and 5c, on the other hand, the fraction A at turgid stage contains about 70% to 90% of total soluble proteins. Therefore, fraction A protein obtained from the Sephadex column is suggested to be the same as "fraction 1" protein which has been widely investigated (Singer et al., 1952; Lyttleton, 1956; Lyttleton and T'so, 1958; Thornber et al., 1955; Trown, 1965; Pon, 1967). Furthermore, recording fluorescent photometry showed only one large peak coinciding with the same retention time as fraction A determined with ultraviolet spectrophotometry, indicating that fraction A protein was highly

Figure 7. Changes of Protein Content Measured by Folin-Phenol Test on 5% TCA Precipitate in Fraction A and Fraction B as % of Initial by Growth Regulatory Chemicals in Different Moisture Status.

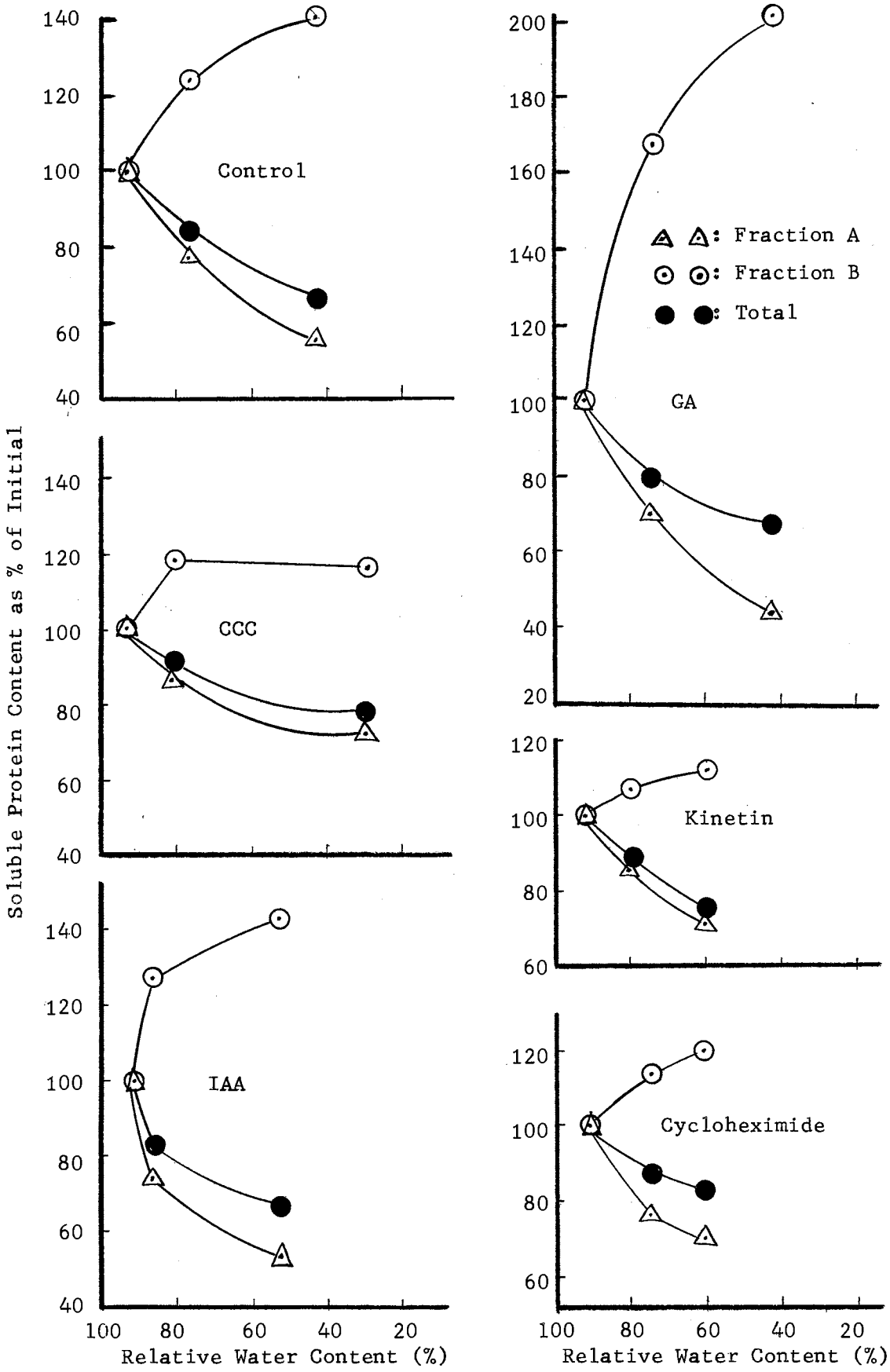

Soluble Protein Content as % of Initial
Control
CCC
IAA
GA
Kinetin
Cycloheximide
Fraction A
Fraction B
Total
Relative Water Content (%)
Relative Water Content (%)

TABLE I

CHANGES OF PEAK AREA BASED ON UV ABSORBANCE AT 280 nm IN BOTH FRACTION A AND FRACTION B BY GROWTH REGULATORY CHEMICALS DURING WATER STRESS. AREA MEASURED WITH POLAR PLANIMETER

Treatment	Relative Water Content	Dry Wt.	Fraction A Area in in^2 $Assay^{-1}$	Fraction B Area in in^2 $Assay^{-1}$	Fraction A Area in in^2 gm^{-1} DW	Fraction B Area in in^2 gm^{-1} DW	Fraction A % of Initial	Fraction B % of Initial
Control	93.57	17.386	0.55	1.37	31.63	78.80	100	100
	76.51	20.279	0.50	1.95	24.66	96.15	77.96	122.00
	43.51	33.394	0.53	3.69	15.87	110.50	50.17	140.20
IAA	90.73	20.317	0.68	2.08	33.47	102.37	100	100
	87.30	20.897	0.54	2.68	25.84	128.25	77.20	125.28
	53.27	26.724	0.43	3.85	16.09	144.06	48.07	140.72
Kinetin	92.26	16.654	0.74	1.81	44.43	109.26	100	100
	81.44	19.103	0.73	2.23	38.21	116.74	86.00	106.85
	60.03	19.942	0.60	2.64	30.09	132.39	67.72	121.17
CCC	94.60	18.152	0.72	1.75	39.67	96.41	100	100
	81.33	20.001	0.69	2.28	34.50	113.99	86.97	118.23
	29.32	40.241	0.95	4.54	23.80	112.83	59.95	117.02
GA	91.67	19.556	0.60	1.28	30.68	65.45	100	100
	73.82	22.509	0.48	2.45	21.32	108.85	69.49	166.33
	43.55	30.062	0.37	4.65	12.31	154.68	40.12	236.32
Cyclo-heximide	90.84	20.701	0.37	1.32	17.87	63.77	100	100
	75.18	21.084	0.35	1.72	16.60	81.57	92.89	127.91
	61.57	26.349	0.40	2.58	15.18	97.91	84.95	153.54

fluorescent. This large quantity of leaf proteins has been reported to include phosphoribo-isomerase, phosphoribulokinase, RuDP carboxylase (Haselkorn et al., 1965; Mendiola and Akazawa, 1964), and TPNH-dependent triosephosphate dehydrogenase (Heber et al., 1963). Invertase, on the other hand, showed a very high activity in fraction A but not in fraction B (Tsai and Todd unpb. observation), indicating that invertase is also involved in fraction 1 protein.

The results obtained show that in water stressed plants GA highly accelerated the decomposition of fraction A proteins and substantially increased the substances present in fraction B measured by UV absorbence. Drying brings about enzymatic changes which result in an increase in hydrolysis (Todd and Basler, 1965). Such hydrolytic enzymes including protease, phosphatase and β-glucanase (Briggs, 1963), α-amylase and ribonuclease (Chrispeels and Varner, 1967), protease (Jacobsen and Varner, 1967) were all induced by gibberellic acid through de novo synthesis (Jacobsen and Varner, 1967). GA caused a several-fold increase in protease in the wort of malted barley (Macey and Stowell, 1961). Accordingly, if the action of GA on the hydrolytic enzyme system in the leaves is similar to its action in seeds, the acceleration of protein degradation in wheat leaves under GA treatment might be the result of increased protease. However, some other workers stated that GA_3 retards senescence by delaying the decline in levels of chlorophyll, protein and RNA. The results from the present study do not agree with the latter statement.

The changes of protein components in the IAA treatment did not appear to be significantly different from those shown in the non-chemically treated control. Therefore, even though IAA at physiological

concentrations induces protein synthesis during growth (Parterson and Trewavas, 1967; Kudrev and Tyankova, 1966; Nooden and Thimann, 1963), this induction does not appear to occur in water-stressed plants. However, only leaves were followed and these are not actively growing like apical meristematic tissues.

Kinetin was used to suppress hydrolytic enzymes such as RNAse, DNAse and protease and was suggested to play a role in the retardation of senescence (Srivastava and Ware, 1965; Srivastava, 1968; Balz, 1966). Shaw et al. (1965) stated that kinetin delayed senescence by stimulating RNA and protein synthesis, which might be involved in the primary action of the hormone. This coincides with the results shown in Figures 5a, 6 and 7 as kinetin prevented the degradation of large molecular weight proteins into smaller ones during water stress. The differences in response to kinetin of stressed and unstressed control wheat leaves may be due to a lower endogenous level of cytokinins as a consequence of drying. This has been suggested by Itai and his colleagues (Itai and Vaadia, 1971; Itai et al., 1968; Ben-Zioni et al., 1967) that the normal supply of root cytokinins are important in shoot metabolism.

CCC has been used in a number of studies concerning plant water relations. Tolbert (1966 a), El Damaty et al. (1965) and Miyamoto (1963) have consistently shown that CCC-treated wheat plants were more resistant to drought than non-treated controls. Halevy and Kessler (1963) found that drought tolerance of bean plants was increased by CCC application. However, they did not elucidate the mechanism(s) of tolerance. The results from this experiment (Figure 5b, 6, 7 and Table I) show that CCC tends to reduce the destruction of fraction A

protein, maintaining a higher level of soluble protein. The mode of action of CCC in protein metabolism is not known. It is possible that the increase of drought tolerance in CCC-treated wheat plants is in part correlated with a capability of maintaining a higher level of soluble protein, through either slower protein degradation or higher synthetic capability under water stress.

Cycloheximide treatment results in a drastic decrease of protein content and an increase in the percentage of fraction B substances during water stress (Figure 6). It might be due to either an inhibitory effect of cycloheximide on the completion of nascent polypeptide chains or an inhibitory effect on the release of completed chains from the ribosome (Godchaux et al., 1967). The polysome formation requires simultaneous amino-acyl addition to a ribosome-messenger complex (Marcus and Feeley, 1966). In addition to breakdown of polysomes caused by water stress (Hsiao, 1970; Genkel et al., 1967), the inhibition of protein synthesis by cycloheximide would cause a lower protein content and possibly account for the increase of fraction B substances. Cycloheximide is thought to block protein synthesis. The results obtained probably indicate that protein shnthesis does continue in water stressed plants although at a reduced rate.

CHAPTER V

SUMMARY

Exogenous chemicals at concentrations of 10^{-5} M to 10^{-4} M were applied in the culture solution to a wheat cultivar, Ponca. After 14 days of growth, the plants were subjected to a certain degree of water stress. Leaves of the same chronological age were homogenized in buffer and the supernatant fractions were obtained from centrifugation at 27,000 x g for 30 min.

Through Sephadex G-100 column separation, two main fractions, namely fraction A and fraction B were observed at 280 nm. According to the Folin-phenol test on the 5% TCA precipitate from each fraction, the fraction A contained about 70% to 90% of the total buffer soluble protein. This fraction is probably equivalent to "fraction 1" protein. Fraction B contains lower molecular weight proteins and substantial amounts of non-protein substances. A quantitative shift from a decrease in amounts of the larger molecular weight fraction to an increase in the lower molecular weight fraction as the result of water stress was accelerated or retarded by pretreatments of various chemicals.

In contrast with untreated controls, GA promoted the degradation of fraction A proteins while substantially increasing the percentage of fraction B proteins during water stress; both kinetin and CCC contrarily retarded this change. No significant changes were caused by IAA

treatment. A lower level of total protein with a higher percentage of the Folin-phenol negative substances in fraction B was found. It thus suggests that both exogenously applied kinetin and CCC act as protectors against protein hydrolysis caused by water stress and are in part associated with the mechanism of drought tolerance. GA which accelerates protein degradation, may induce the susceptibility of plants to drought. Cycloheximide treated wheat leaves resulted in a low level of soluble protein.

PART II

CHANGES OF ENZYME ACTIVITY IN RESPONSE TO GIBBERELLIC ACID TREATMENT IN WHEAT PLANTS SUBJECTED TO WATER STRESS

CHAPTER VI

REVIEW OF LITERATURE

Protein-Water Relationship in a Living Cell

Proteins, the cell component highly correlated to life, whether in a contractile form or as functioning enzymes in a living cell are mostly associated with water. Therefore, the phrase "no water no life" has been put forth. In discussing the physical state of water in a living cell, Ling (1965) stated that the great complexity and variability of a living cell made the conclusion inevitable that the particular water structure orientated by proteins varied not only in different cells or in different regions of the cell but, in fact, in the same region of the same cell at different times. He suggested that more than 90% of the intracellular ions are adsorbed to cell proteins; dehydration per se, therefore, would not necessarily lead to the build up of very high concentration of ions in the cells. He claimed that the cell protection against frost injury by glycerol and sucrose might be due to reduction of ice formation. He also proposed that water is orientated in polarized multilayers on the surfaces of proteins in living systems. In describing the association of protein subunits with respect to the stability of the tobacco mosaic virus particle, Caspar (1963) stated that the specific folding of polypeptide chains used to be regarded as largely determined by hydrogen bonding but recently interest has shifted to the significant role of hydrophobic

or apolar bonding. Although no one type of interaction is likely to determine the conformation of a protein, it does appear that the apolar bonding will, in general, account for a larger fraction of the free energy difference between the "unfolded" and "folded" conformation than the hydrogen bonding. The hydrophobic bond would be weakened by a decrease in dielectric constant of the medium because of the greater solubility of the non-polar groups under these conditions, but an increase in the ionic strength of the medium would strengthen the hydrophobic bonding by decreasing the solubility of the nonpolar groups. Gates (1964) claimed that the changes of physiological activities under water deficits were attributed to the changes in protoplasmic hydration. Klotz (1958) in discussing the effects of hydration on the spatial configuration of the protein components of the protoplast, postulated that the ice-like ordering of water molecules changed into a co-ordinated lattice about the protein molecules during hydration. Such hydration envelopes may be close to 20% by weight of the protein molecules. Fisher (1965) calculated the first monomolecular layer of water which was strongly bound to protein as 0.28 grams water per gram protein for 34 individual proteins. This calculated value also corresponds to about 20% of the final amount of water adsorbed by a protein. Stocker (1960) reported that a single complex protein molecule could bind around 20,000 molecules of water to its polar groups, particularly hydroxyl, amino and amide groups. Removal of part of the water surrounding protein molecules may cause changes in configuration affecting permeability, hydration, viscosity and enzyme activity (Kramer, 1969). Gaff (1966) also reported that dehydration of protein from cabbage leaves caused changes in amount of reactive sulfhydryl which could be

attributed to changes in configuration of the protein. Relatively small changes in osmotic potential caused marked changes in protein structure and enzyme activity (Chen et al., 1964).

Drought Injury and Tolerance

The large cells encountered in many higher plants oftentimes contain immense vacuoles. In the opinion of Iljin (1957), the cause of desiccation injury is considered to be a mechanical rupture of the protoplast. Stocker (1960) found that the degrees of changes in the plant protoplast during drying were dependent upon the different types of plants. Russian works cited by Stocker (1960) and Gates (1964) revealed that changes in the colloid-chemical state of the protoplast during water deficits might intensify the hydrolytic breakdown of protein in the plant cell; upon dehydration, the protoplasmic structures lost their abilities to bind invertase and other enzymes, so that these enzymes became more active and stimulated intensive hydrolytic decomposition. Todd and Basler (1965) pointed out that drought injury or death is probably due to breakdown of synthetic machinery rather than the clumping or coagulation of protoplasm, and that the tissues which are less sensitive to moisture stress probably have much less active hydrolytic enzymes. Therefore, a higher percentage of large molecular weight soluble protein is retained under water stress in hardier wheat varieties than in susceptible ones (Stutte and Todd, 1967). Drought hardening was then considered as an involvement in strengthening the water-protein bindings, and as an increase in structural viscosity as well as a decrease in pore permeability (to water and urea) (Stocker, 1960). Mechanisms of drought tolerance that have been proposed include

accumulation of "bound water" by protein (Todd and Levitt, 1951), increased water retention capacity (Sandhu and Lande, 1958; Salim et al., 1969), a decrease of oxidation of protein -SH groups to -S-S- groups (Levitt, 1962), protection by sugars, salt balance, etc. (Stocker, 1960) and by chlorocholinechloride and kinetin application (Tsai and Todd, see Part I), an increase in the ratio of organic to inorganic phosphorus, and a more "stable" nitrogen metabolism (Parker, 1968).

Changes in Enzyme Activity with Water Stress

It has been known that protein production by a living cell is dependent on nucleic acid bound to proteins following the central dogma: DNA duplication $\xrightarrow{\text{transcription}}$ RNA $\xrightarrow{\text{translation}}$ protein synthesis. All known enzymes contain protein. Any stress-induced damage to this synthetic machinery will lead to an alteration of the endogenous level of enzymes. The nucleic acid content of the wheat leaf supernatant fraction was drastically reduced with increasing water stress (Todd and Basler, 1965). Kessler (1961) claimed that water deficits impair the nucleic acid metabolism which is intimately connected with protein synthesis. Gates and Bonner (1959) concluded that the block to net RNA synthesis under water stress was caused by more rapid destruction than synthesis of RNA. This connection with RNAse activity has been found with wheat leaves by Todd and Yi (1971) that both RNAses having maximal activities at pH 5.0 and pH 5.8 are definitely increased as a result of water stress. Similar results with tomato leaves were reported (Dove, 1967). A change in the RNA composition of an increase in the ratio of guanine and cytosine to adenine and uracil was found during water stress (Stutte and Todd, 1968) especially with the most

rapid increase from 0 to 20% water loss (Kessler and Frank-Tishel, 1962).

Todd (1966) stated that "many enzymes are quite stable toward drying when in the pure state; this would suggest that if some enzyme is destroyed by the drying process it is unusually susceptible toward drying or may be inactivated by the multitude of other small molecular weight substances left behind as the cell dries, e.g., salts, coenzymes, substrates, etc. . . ." Additionally the phenolic compounds which might participate in the regulation of enzyme action during water stress will be discussed in Part III. Another growth inhibitor "inhibitor-B" or abscisic acid was substantially increased with wilting (Wright, 1969), which might cause the changes in enzyme activity during water stress.

A speculation about the correlation between the stability of enzymes and their sulfhydryl group(s) as well as the molecular size was postulated by Levitt (1966). He concluded that the heat stable enzymes, in general, are characterized by lower molecular weight (i.e., below 100,000), the heat-labile by higher molecular weight; the heat-stable enzymes are free of SH groups, the heat-labile all possess SH groups. Many of the factors associated with dehydration tolerance are often associated with heat hardiness (Levitt, 1962).

Reduction of nitrate to nitrite catalyzed by nitrate reductase is critically important in the nitrogen metabolism of plants. Accumulation of nitrate in respect to decrease of nitrate reductase activity during moisture stress was found (Mattas and Pauli, 1965; Younis et al., 1965). Huffaker et al. (1970) found that 58% of the nitrate reductase activity was lost during a 4 day stress period where relative water content of

both stressed and control barley leaves were approximately the same, i.e., 95.5% and 96.8% respectively; nitrite reductase showed a tendency to decrease; phosphoenolpyruvate carboxylase activity decreased 1 day later in the stress period by about one-half the activity of nitrate reductase; phosphoribulokinase and ribulose-1,5-diphosphate carboxylase were very slightly affected by water stress. However, working with pea plants, Darbyshire (1971) found that IAA oxidase activity was increased 16% of the control during water stress, and was increased 62% after rewatering. He speculated that the rewatering brought about the resumption of active meristematic growth and caused the resynthesis of auxin which led to the induction of IAA oxidase synthesis in the tissues. On the other hand, the specific activity of cytochrome oxidase of mitochondria isolated from dehydrated roots of maize was increased four and half-fold over the control; enzyme activity was also found in the supernatant fraction from dehydrated materials but not in the supernatant from those moistened over distilled water (Nir, 1970).

CHAPTER VII

MATERIALS AND METHODS

Preparation of Plant Materials

Wheat (Triticum aestivum L.) cultivars Kanking, which is relatively drought hardy and Ponca, which is relatively drought susceptible were obtained from the Agronomy Department, Oklahoma State University, and grown in 30 cm x 50 cm trays with vermiculite under controlled environmental conditions with 14 hours photoperiod with 11,000 lux light intensity and 20°C at night and 25°C at day temperature. Fourteen-day-old plants were initially subjected to moisture stress. Seventy-five leaf blades were collected from the most recently mature leaf of each plant, of approximately similar chronological age, and used for each assay.

Materials used for electrophoresis were obtained from the plants which were grown as above. The leaf blades and the crown tissues (i.e., basal portion of the stem about 2 cm in length above the seed) at a similar age were harvested. Harvested samples were placed in plastic bags, in order to maintain the internal moisture unchanged, and homogenized as soon as possible.

Ponca plants treated with GA and cycloheximide were grown in the same culture solution as above, containing 10^{-4} M GA_3 or 10^{-3} M cycloheximide from Calbiochem, Los Angeles respectively. A 1.5 gram fresh weight portion of leaves with approximately similar chronological

age was harvested for each test. Two replicates with two observations of each were analyzed.

Determination of Relative Water Content (RWC)

Eight leaves were sampled at random from the harvested leaves which were used for each assay. About 2.5 to 3.0 cm in length from the tip of each sampled leaf was cut off and used for relative water content measurement. The method of the measurement was introduced by Weatherley (1950) and modified by Todd et al. (1962). Samples for measuring RWC were weighed immediately after harvesting with a precision balance (Federal Pacific Electric Co., Newark, N. J.).

Relative water content (RWC) was calculated as follows:

$$RWC = \frac{\text{fresh wt.}-\text{dry wt.}}{\text{turgid wt.}-\text{dry wt.}} \times 100\%$$

Preparation of Crude Enzymes

After weighing, the fresh materials were cut into small pieces in a container with 0.2 M Tris-HCl buffer (pH 7.4) containing 8% polyvinylpyrrolidone (PVP). This was ground three times with an Omni mixer at full speed for 1 min., with 1 min. interval between grindings. During the grinding process the preparation was kept in an ice bath. The slurry was passed through 2 layers of nylon stocking. The filtrate was then centrifuged at 27,000 x g for 30 min. at $4^{\circ}C$. The supernatant was brought to 80% saturation with ammonium sulfate and allowed to stand for 2 hours at $4^{\circ}C$. The precipitate was collected by centrifugation at 27,000 x g for 10 min. For preparing glucose-6-phosphate dehydrogenase (G-6-PD), the precipitate was redissolved in 0.05 M

phosphate buffer pH 7.4 to make a 5 ml final volume. For indoleacetic acid (IAA) oxidase assay, the precipitate was then resuspended in 0.05 M phosphate-citrate buffer pH 4.0 to make a 5 ml of final volume.

Glucose-6-Phosphate Dehydrogenase (G-6-PD) Assay

In the first experiment, "G-6-PDH Stat-pack" prepared by Calbiochem Co., containing substrates and coenzyme in an appropriate buffer solution was used. 2.5 ml of the prepared assay mixture was placed in a quartz cuvette and 0.1 ml of enzyme extract was added for each assay. Activity was determined by measuring the change of NADP to NADPH at 340 nm with a Perkin-Elmer Spectrophotometer, Model 202 for 10 min. The reaction was followed at one minute intervals after the enzyme extract was added (Figure 8). The enzyme activity was expressed as the change of absorbance in 10 min. per 10 mg dry weight. In the second experiment, 3.0 ml total volume of the mixture containing 0.5 ml of glucose-6-phosphate (0.01 M) from Sigma Co., 0.3 ml of NADP (0.002 M) from Sigma Co., 0.5 ml of $MgCl_2$ (0.02 M), 1.6 ml of Tris buffer (0.01 M) pH 7.2 and 0.1 ml of enzyme extract was used for each assay. The readings were taken 30 seconds after enzyme addition and for a total of 10 min. The enzyme activity was expressed as μ moles of NADP reduced in 10 min. per 10 mg dry weight in terms of the calibration curve shown in Figure 9.

IAA Oxidase Assay

In the first experiment, 3.5 ml of the reaction mixture consisted of 0.1 ml of IAA (10.0 mM), 0.1 ml of $MnCl_2$ (2.6 mM), 0.1 ml of 2,4-dichlorophenol (DCP) (2.6 mM) in 3.1 ml of 0.005 M phosphate-citrate

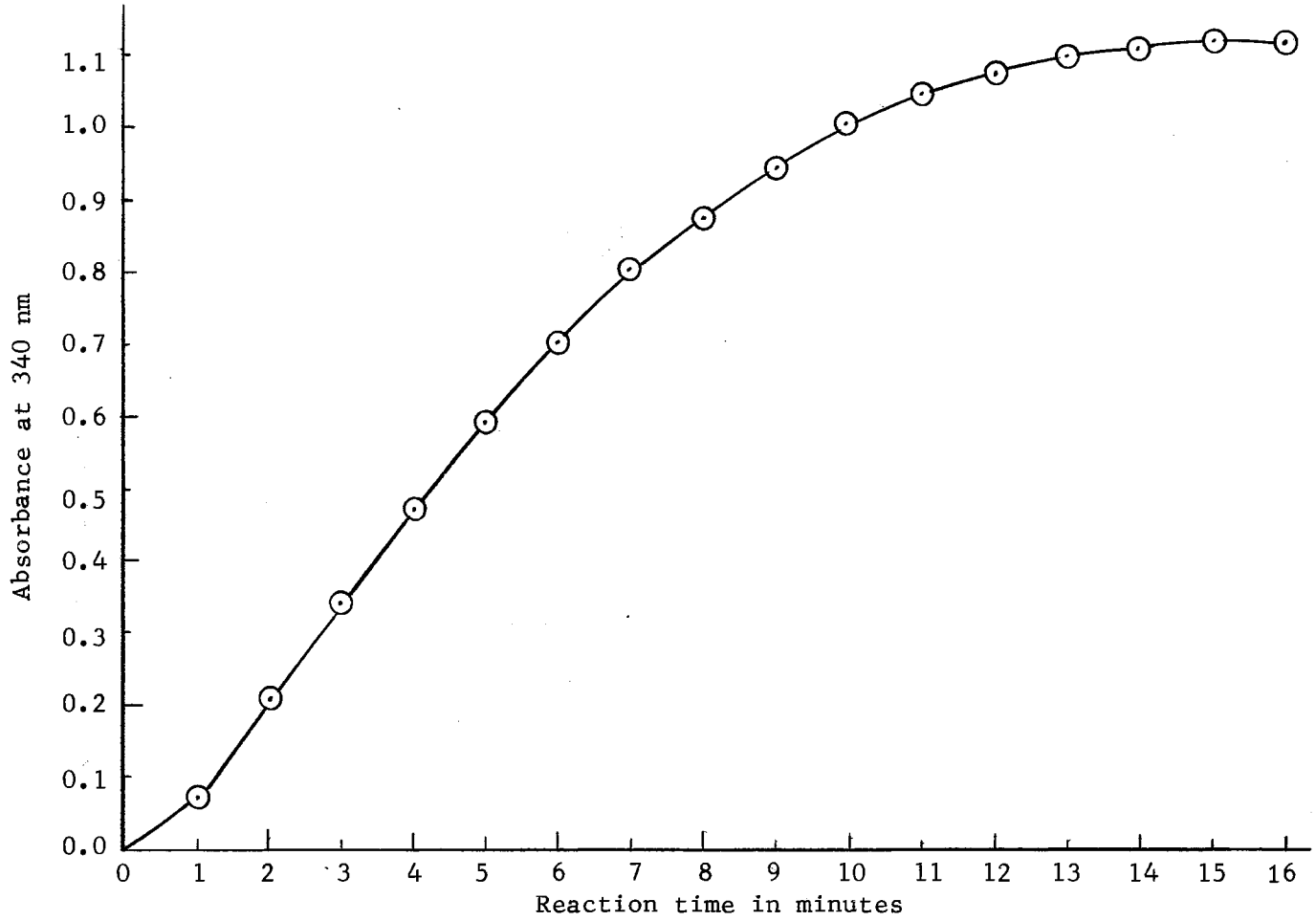


Figure 8. Assay of Glucose-6-Phosphate Dehydrogenase. The curve shows the enzyme activity as a function of incubation time. After 11 minutes the reaction rate slows down drastically. The conditions are those of the assay described in the text.

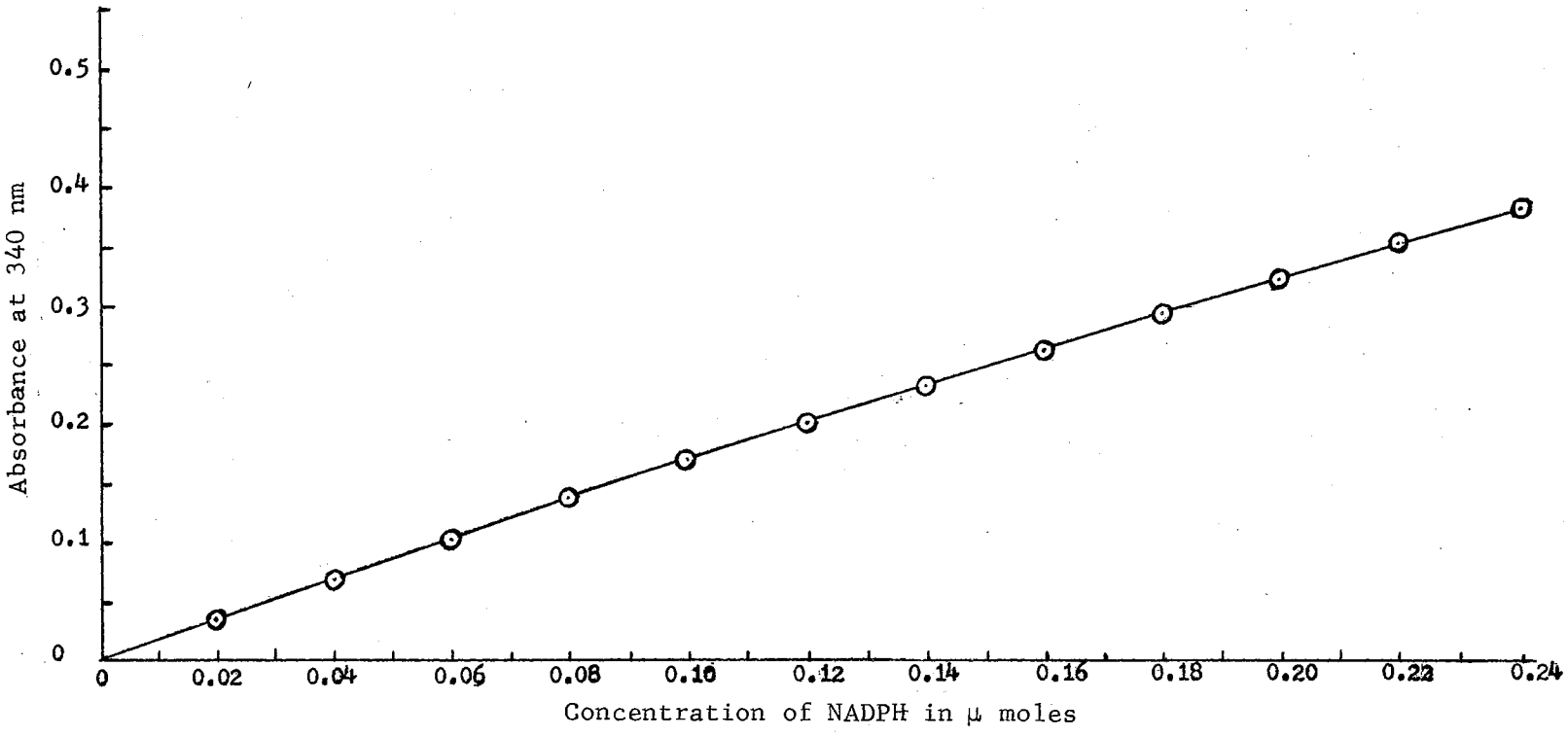


Figure 9. Typical Standard Curve for NADPH Determination

buffer pH 4.0 and 0.1 ml of enzyme extract. The mixture was incubated at room temperature for 2 hours, then 0.5 ml of the reacted mixture was placed in a tube along with 0.5 ml of deionized water and 2.0 ml of Salkowski reagent, which consisted of 3.0 ml of 0.5 M $FeCl_3$, 100 ml of deionized water and 60 ml of concentrated H_2SO_4. In the second experiment, 13 ml of reaction mixture contained 0.5 ml of IAA, 0.5 ml of $MnCl_2$, 0.5 ml of DCP with the same concentration as above, and 0.1 ml enzyme in 11.4 ml of buffer as above, then a 1.0 ml aliquot was taken to react with 2 ml of Salkowski reagent. The color development was allowed to proceed for 1 hour and the absorbance was then determined with a Perkin-Elmer Spectrophotometer, Model 202 at 530 nm. The enzyme activity was expressed as μ moles IAA destroyed per 2 hours per 10 mg dry weight in terms of the calibration curve shown in Figure 10.

Electrophoretic Method

The acrylamide gels were prepared according to the instructions provided by the Buchler Instrument, Inc., Fort Lee, N. J. A 3.3% acrylamide of upper gel with Tris-glycine buffer pH 8.9 was matched by a lower gel in a 3/16" x 3" glass tube containing 7.5% acrylamide with Tris-HCl working buffer pH 8.4. Fifty μl of the supernatant which was obtained from a 27,000 x g centrifugation of the homogenate, containing 3% sucrose, was placed on the top of the upper gel. A current of 1.25 milliamperes per tube was applied until the sample entered the stacking gel after which the current was increased to 2.5 milliamperes per tube for the remainder of the run. The leading buffer front marked by the dye, bromphenol blue was followed visually and the run was terminated when this front had reached a distance of about 1 mm from

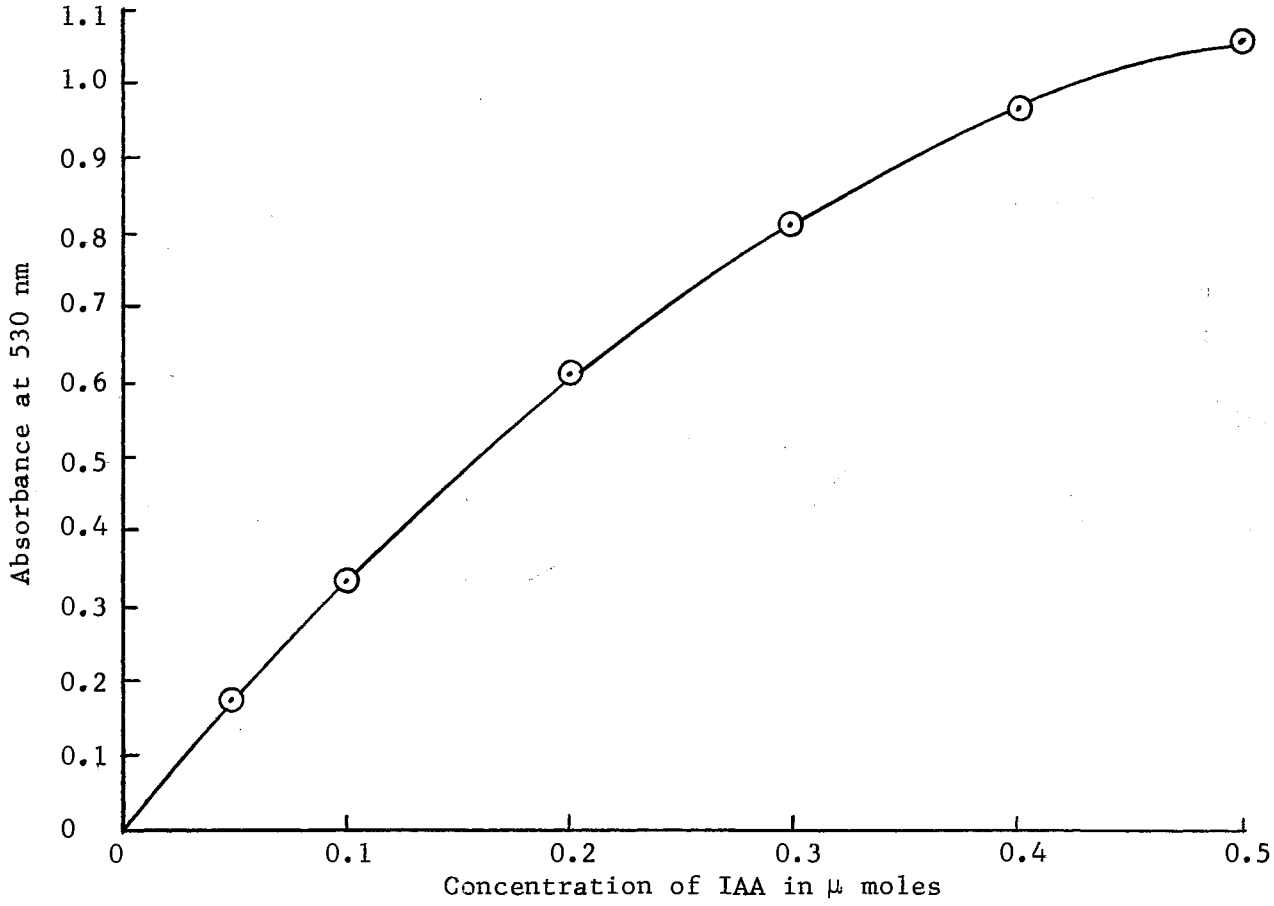


Figure 10. Typical Standard Curve for IAA Determination with Salkowski Reagent

the bottom of the gel column. After electrophoresis, the gel was removed by using a syringe and stained in a specific staining solution.

Total proteins were stained with 1% amido black. The excess dye was destained electrophoretically in 7% acetic acid.

Iron-containing proteins were stained by immersing the gels in a freshly prepared solution containing 0.2 ml of 30% H_2O_2, 0.2 gram benzidine and 0.5 ml of glacial acetic acid in 100 ml water. The characteristic blue bands, which fade to dark brown upon storage in water, formed within 30 min.

Peroxidase isozymes were located by immersing the gels for 30 min. in 0.05 M acetate buffer pH 5.4 containing 0.01 M o-dianisidine followed by 5 min. in 0.01 M H_2O_2. The bands showed immediately as orange red.

Glucose-6-phosphate dehydrogenase was located by immersing gels for 1 hour in a 20 ml of Tris-glycine buffer (2.9 g Tris; 0.6 g glycine; 0.18 ml HCl per liter; pH 8.1) solution containing 0.01 M glucose-6-phosphate, 15 mg nicotinamide-adenine dinucleotide phosphate (NADP), 10 mg nitroblue tetrazolium and 0.5 mg phenazine methosulfate. The purple color of the bands is characteristic of dehydrogenase activity.

Malic dehydrogenase and succinic dehydrogenase were located by immersing the gels in the same above solution used for staining the glucose-6-phosphate dehydrogenase except the substrates and cofactors were changed; 0.01 M malic acid and 15 mg nicotinamideadenine dinucleotide (NAD) for malic dehydrogenase and 0.01 M succinate and 15 mg flavin adenine dinucleotide (FAD) for succinic dehydrogenase.

CHAPTER VIII

RESULTS AND DISCUSSION

Changes of Glucose-6-Phosphate Dehydrogenase Activity with Water Stress

Changes of G-6-PD activity in the leaves of both wheat cultivars, Ponca and Kanking which were subjected to moisture stress are shown in Figure 11. Activity was substantially decreased during the initial stress period. A 48% reduction of activity in Kanking and 38% reduction in Ponca occurred after 2 days of water stress, whereas after 6 days of drought, approximately 66% of the activity had been lost in Kanking and 52% in Ponca as compared to turgid control leaves. In Figure 12, a similar result obtained from the second experiment shows that a 40% reduction of G-6-PD activity occurred in Ponca leaves at about 65% relative water content, without chemical treatment, and a 46% reduction at the severe stress of 21% RWC. Pretreatment with GA (10^{-4} M) prior to dehydration gave a higher level of G-6-PD whereas a lower level of enzyme was present following cycloheximide (10^{-3} M) treatment. However, neighter chemical protected this enzyme from reduction in activity as a result of water stress. An attempt was then made to determine how these chemicals affected *in vitro* G-6-PD activity. This result shown in Table II indicates that neither GA nor cycloheximide had any effect on *in vitro* enzyme activity at the levels used. Thus, in contrast to control, non-chemical treatment, the higher

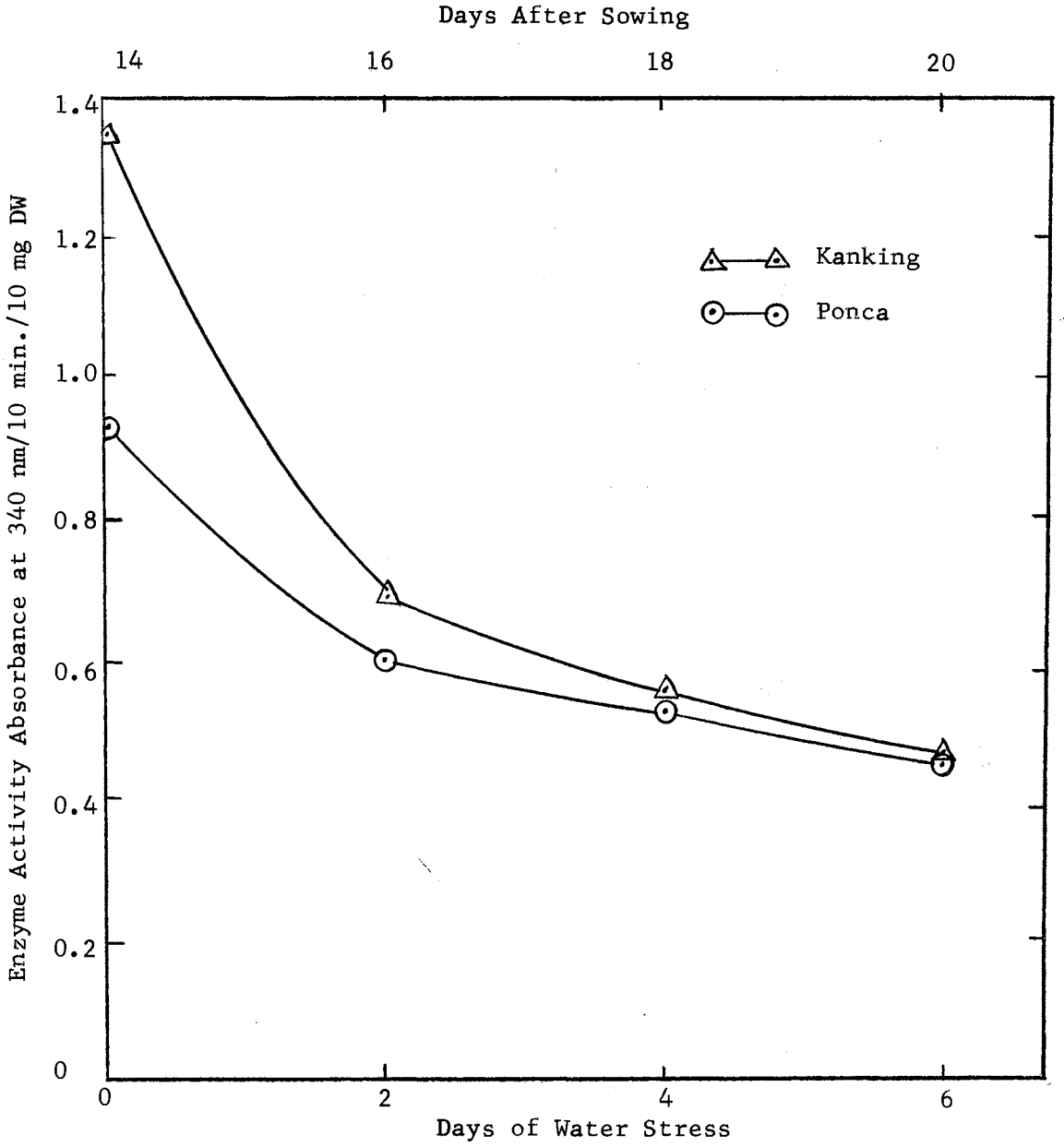


Figure 11. Activity of Glucose-6-Phosphate Dehydrogenase Against Days of Water Stress. Each point represents the average of two to three observations from the same sample.

Figure 12. The Effect of GA and Cycloheximide on Changes in the Activity of Glucose-6-Phosphate Dehydrogenase in Wheat Cultivar Ponca Leaves Subjected to Water Stress. Each observed value is a mean of duplicate samples, and the spread is shown by the vertical line through points.

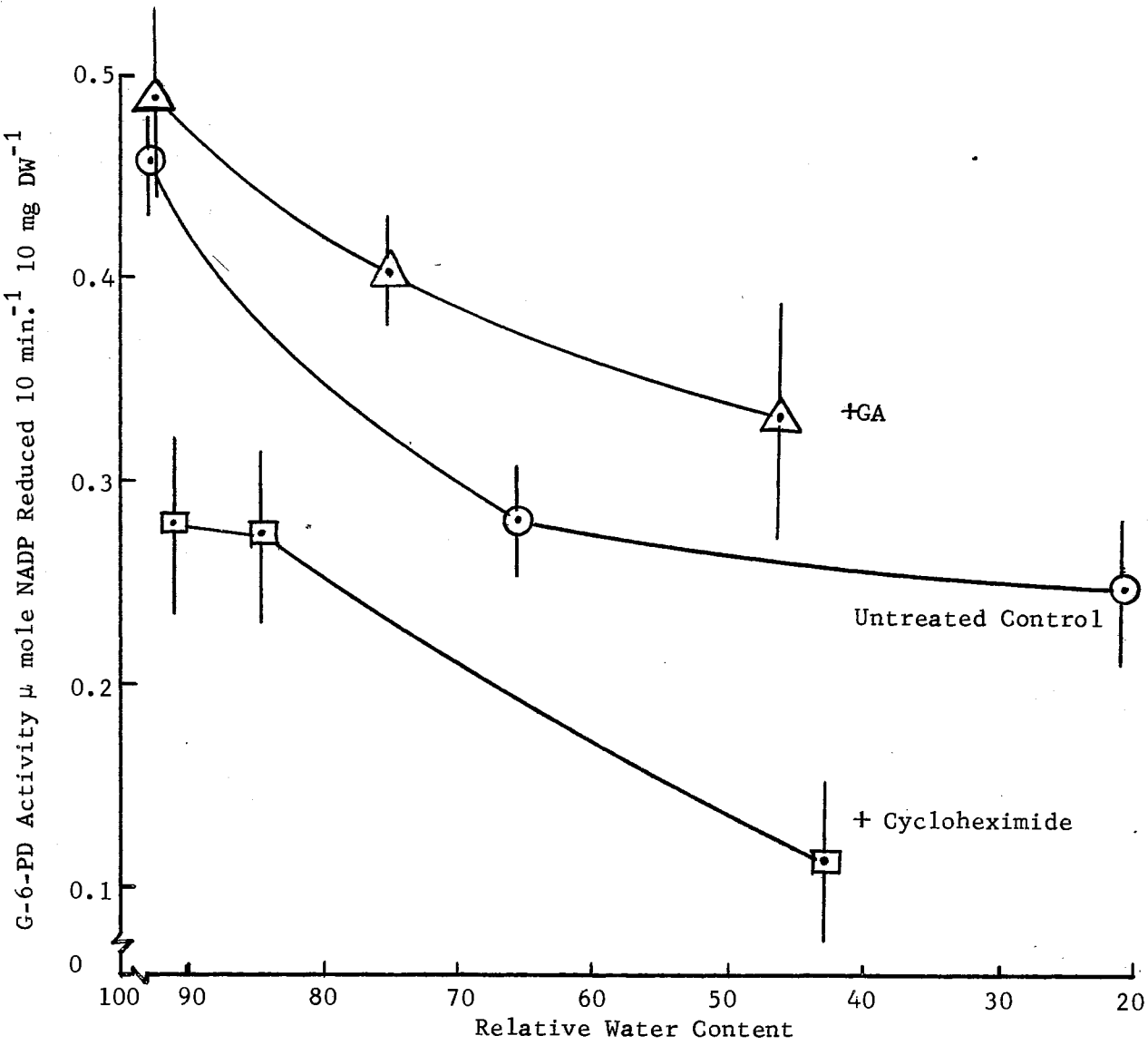

G-6-PD Activity μ mole NADP Reduced 10 min.$^{-1}$ 10 mg DW^{-1}
0.5
0.4
0.3
0.2
0.1
0
100
90
80
70
60
50
40
30
20
Relative Water Content
+GA
Untreated Control
+ Cycloheximide

TABLE II

EFFECTS OF GIBBERELLIC ACID AND CYCLOHEXIMIDE IN VITRO ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE OF WHEAT LEAVES

Addition	Enzyme Activity μ mole NADP Reduced $10\ min.^{-1}$, $10\ mg^{-1}$	Relative Activity
GA		
None	0.238	100
1.9×10^{-5} M	0.238	100
3.9×10^{-5} M	0.244	103
5.8×10^{-5} M	0.230	97
9.7×10^{-5} M	0.238	100
CYCLOHEXIMIDE		
None	0.254	100
1.3×10^{-4} M	0.254	100
2.7×10^{-4} M	0.254	100

enzyme activity after GA treatment and the lower after the cycloheximide treatment are presumably due to changes in de novo synthesis of enzyme protein stimulated by GA but inhibited by cycloheximide. GA has been widely accepted as a plant growth hormone that regulates nucleic acid synthesis within a cell (Bamberger, 1971; Jarvis et al., 1968; Osborne, 1965 and etc.) and cycloheximide has been regarded as an effective inhibitor of protein synthesis (Key et al., 1967). The mechanism of inhibition of cycloheximide relates possibly to a direct inhibition of peptide bond formation and to some impairment of the termination or release mechanism (Godchaux et al., 1967). In Figures 11 and 12, G-6-PD activity does not decrease linearly with a reduction of relative water content, but decreases most rapidly at the initial stage of water stress. This indicates that G-6-PD is extremely susceptible to water stress. However, G-6-PD is a key enzyme which catalyzes the oxidation of glucose-6-phosphate to D-gluconolactone-6-phosphate in the pentose phosphate shunt which has been reported to be present in higher plants (Gibbs, 1952). There was a corresponding decrease in the rate of respiration found in the mitochondria whose membranes had been changed by water stress (Miller et al., 1971). A similar decrease in the respiratory rate (O_2 consumption) was found in the root cells from drought stressed Zea mays plants (Nir et al., 1970). Also a severe water stress reduced CO_2 output to the extent of 50% in wheat seedlings (Kaul, 1966). These findings are parallel to the result presented in this study that G-6-PD activity was decreased as the result of water stress. Stocker (1960) postulated that protoplasmic transformations occurred in the reaction or initial phase during water stress, whereas G-6-PD activity was lost at the initial stage of water

stress. Thus, the loss of G-6-PD activity could be related to the protoplasmic transformation at an initial drought stage. On the other hand, the dissociation of polyribosome into monosomes which was substantially enhanced by water stress (Hsiao, 1971), was also caused by energy-depleting conditions, namely anaerobiosis and dinitrophenol treatment (Lin and Key, 1967). Although the shift from polyribosome dissociation into monosomes can be blocked by cycloheximide (Lin and Key, 1967; Hsial, 1971), G-6-PD activity in cycloheximide-treated wheat leaves (Figure 12) does not seem to be protected from the reduction caused by water stress. Even in the case of GA treatment, G-6-PD activity decreased with increasing water stress. The reduction in activity of this enzyme in response to water stress might therefore be due to the breakdown of enzyme protein in the wheat leaves.

Working with G-6-PD from Dutch elm disease fungus, Asante and Neal (1964) clarified that SH group(s) is the essential group(s) for G-6-PD activity. The oxidation of sulfhydryl into disulfide caused by water stress (Levitt, 1962) might therefore, be in part correlated with this reduction phenomenon.

Some other small molecular weight compounds located in their specific compartments within a cell may be changed in location upon dehydration. Phenolic compounds, for example, may be released from vacuoles to come in contact with an enzyme system in the cytoplasm after the tonoplast has been broken down. Another example, the "inhibitor-B" named by Bennet-Clark and Kefford (1953) which has been identified as abscisic acid (ABA) was proportionally increased with wilting in wheat leaves (Wright, 1969). These substances may be also related to the changes of G-6-PD activity during water stress.

Changes of IAA Oxidase Activity with Water Stress

Young growing tissues have lower levels of IAA oxidase and aged non-growing tissues have higher levels of this enzyme (Galston and Dalberg, 1954). The endogenous level of IAA oxidase then might be closely parallel to the senescence of plant tissues.

IAA oxidase activity of wheat plants are presented as a function of time from the last watering (Figure 13) and as a function of relative water content (Figure 14). At 3 days of water stress there was a 44% increase in enzyme activity compared with the turgid control in the cultivar Ponca leaves and an increase of about 40% activity in the cultivar Kanking leaves (Figure 13). After 5 days of drought, there was still about a 20% increase in activity over controls in both varieties. After 7 days of drought stress, there was a decrease of about 2% IAA oxidase activity compared with the initial values shown in Ponca leaves and a decrease of 10% in Kanking leaves. In Figure 14, there was a 45% increase in IAA oxidase activity in non-chemical treated Ponca leaves at 65% relative water content, while there was a 5% decrease in activity under severe drought condition with only 21% RWC. Moreover, the addition of GA to the turgid control Ponca leaves caused a 20% increase in IAA oxidase activity compared with the untreated turgid controls. At 75% RWC, GA sharply enhanced the IAA oxidase activity about two-fold over the non-chemical treated leaves at the same water content. In other words, the activity of IAA oxidase was consistently higher in the GA-treated tissues than in the untreated and such difference sharply increased along with increasing water stress up to around 75% RWC. The enzyme activity in both GA treated and untreated Ponca leaves became gradually decreased, when the

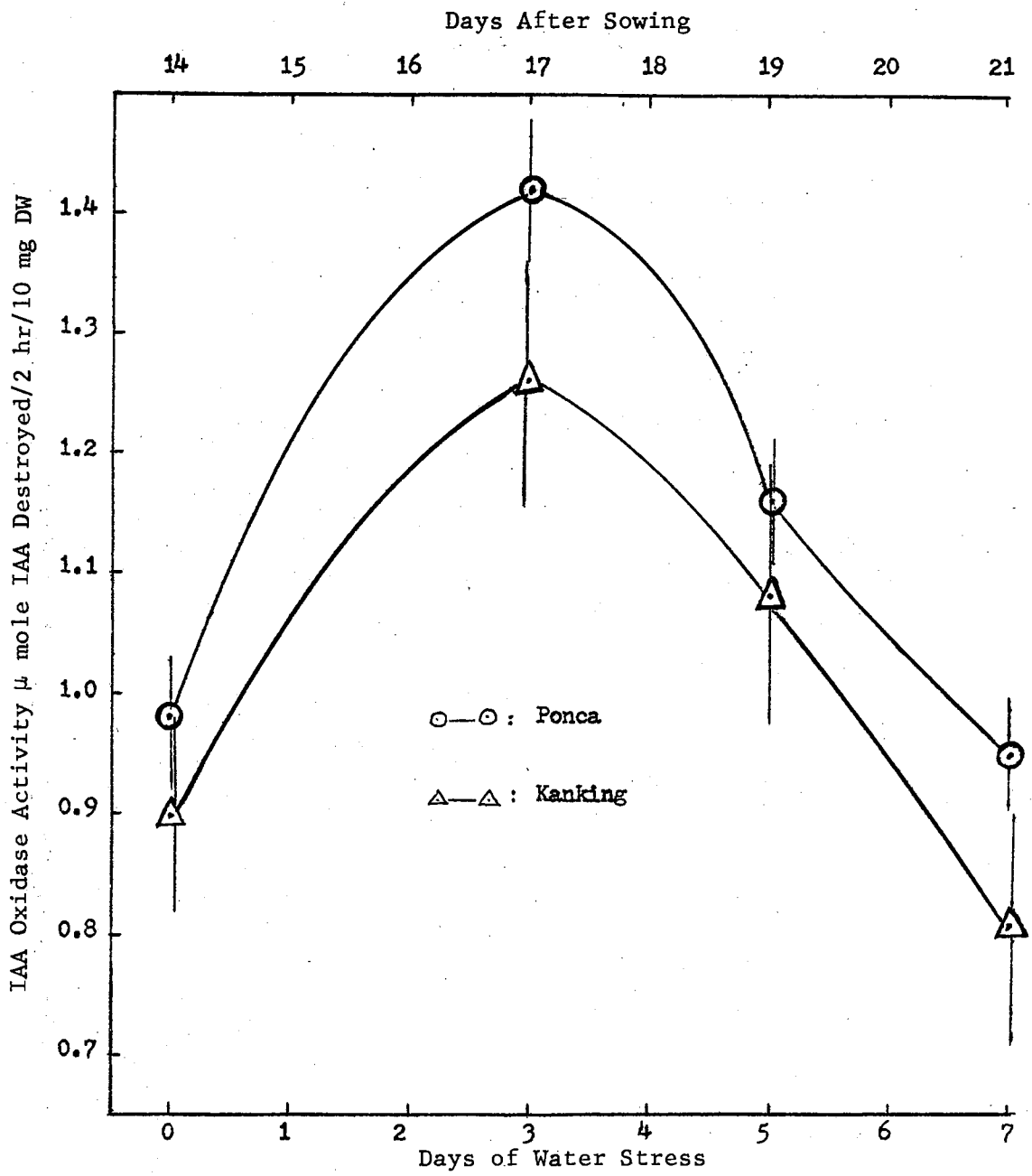
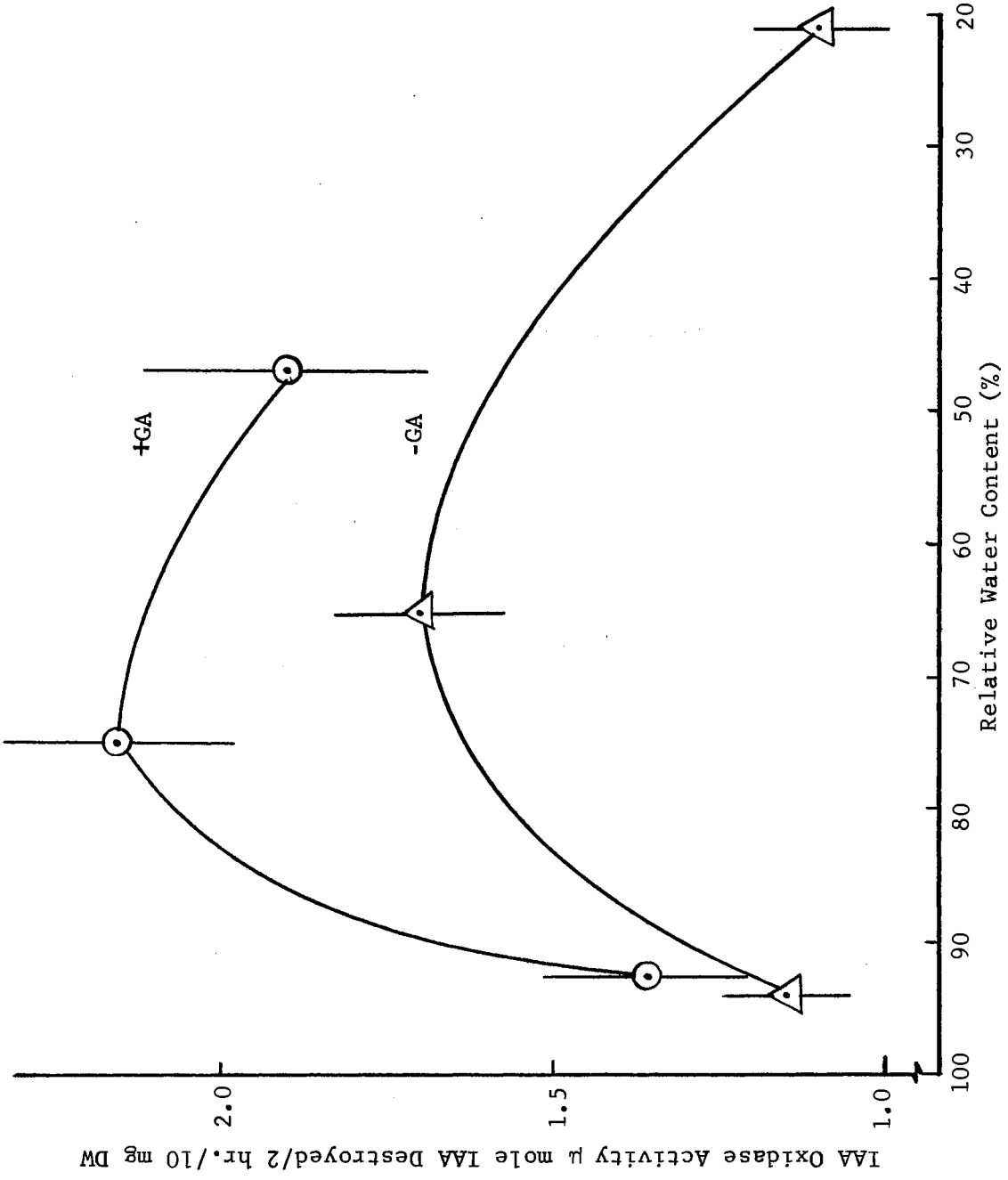


Figure 13. Changes of IAA Oxidase Activity in Wheat Cultivars Ponca and Kanking Leaves Under Different Moisture Status. Each point is a mean of duplicate samples, and the spread is shown by the vertical line through points.

Figure 14. The Effect of GA on Changes in the Activity of IAA Oxidase in Wheat Cultivar Ponca Leaves Exposed to Water Stress. Each point is an average of duplicate samples, and the spread is shown by the vertical line through points.


IAA Oxidase Activity µ mole IAA Destroyed/2 hr./10 mg DW
2.0
1.5
1.0
100
90
80
70
60
50
40
30
20
Relative Water Content (%)
+GA
-GA

relative water content was less than 75%.

In general, GA is known to be able to increase the level of diffusible auxin in plant tissues (Kuraishi and Muir, 1963), but the question of how gibberellin promotes such increases in IAA oxidase has not been settled. The results obtained from this study (Figure 14) as well as reported recently by Bolduc et al. (1970) and by Ockerse et al. (1970) showing an increase in the level of IAA oxidase after GA_3 treatment are further evidence against an IAA-sparing action of GA. Lee (1971) pointed out that oxidation of IAA by the GA-promoted IAA oxidase isozymes might lead to formation of intermediates and products more active than IAA in stimulating growth. That such highly active substances might be involved is supported by the findings of Tuli and Moyed (1969) that certain products of enzymatic oxidation of IAA were more active as auxins than IAA itself. In brief, GA enhances IAA oxidase activity by which some products or intermediates of the oxidation of IAA become more active than IAA in stimulation of plant growth.

The level of IAA as a substrate of IAA oxidase may be changed by moisture stress. The auxin levels were highest in Anastatic spp and Helianthus spp at 30% up to 60% to 80% of the water-holding capacity of the soil (Hartung and Witt, 1968). At higher and lower soil water contents the auxin level was diminished. An inverse relationship between auxin content and IAA oxidase activity is rational to explain the changes in endogenous IAA levels. Kinetin and acetamidoflourene increase the level of IAA by decreasing IAA oxidase activity (Jain et al., 1969). Nevertheless, IAA oxidase activity from Figures 13 and 14 was substantially increased by drought in both cultivars. This

result is mostly consistent with the finding of Darbyshire (1971), even though different types of plant materials were used.

With regard to the changes of nitrogen compounds in response to water stress, many investigators (Barnett and Naylor, 1966; Shah and Loomis, 1965) have found that after hydrolysis of proteins some free amino acids actually decrease but some others increase. Free proline concentration dramatically increased to 10 to 125 times its control value as the result of moisture stress (Barnett and Naylor, 1966). Free proline functions as a storage compound during water stress and may be of physiological significance in terms of adaptation to drought. Chemically, both proline and hydroxyproline are secondary amines, with a similar structure. They do not have a peptide-bond hydrogen to contribute to hydrogen bonds such that a polypeptide chain must be terminated during synthesis or interrupted at each proline or hydroxyproline residue in the protein. Proline is the normal precursor for protein-bound hydroxyproline in plants (Cleland and Olson, 1967), and possibly hydroxylated into hydroxyproline in vivo (Yip, 1964). Perhaps the increased proline as well as hydroxyproline might be related to the IAA oxidate activity enhanced by water stress. Recent work done by Whitmore (1971) indicates that hydroxyproline can restore IAA oxidase activity. A hydroxyproline-rich glycoprotein, extensin, linked by the hydroxy groups of hydroxyproline in a glycosidic linkage with arabinose is present in the primary cell walls of flowering plants as well as in lower green plants (Miller and Lamport, 1968; Lamport and Miller, 1971). It increases markedly in the cell wall of the Alaska pea epicotyl during the transition of rapidly elongating tissue into non-elongating, mature tissue (Cleland and Karlsnes, 1967). This

increase of hydroxyproline in protein may be a factor in the cessation of cell elongation in connection with the enhancement of IAA oxidase activity. On the other hand, peroxidase being able to catalyze the oxidation of IAA (Shin and Nakamura, 1962) is more active when its structure is composed of more glycosidic hydroxyproline molecules. For example, an isoperoxidase from horse radish anionically migrated at pH 8.5 in starch gel electrophoresis, showed a higher activity when it contained a greater proportion of hydroxyproline-o-glycosidic linkages (Liu and Lamport, 1968). This evidence is consistent with Whitmore's work (1971) and would provide one of the possibilities for interpreting the results shown in Figures 13 and 14, that the IAA oxidative capacity was changed with increasing drought in connection with increasing proline as the result of water stress (Barnett and Naylor, 1966; Chen et al., 1964).

A functional role of IAA oxidase in vivo remains uncertain. However, the application of GA to intact plants dramatically increased IAA oxidase (Figure 14; Bolduc et al., 1970; Ockerse et al., 1970; Lee, 1971). IAA oxidase extracted from the stressed Ponca leaves was examined in vitro after additions of either GA or cycloheximide. In Table III, neither compound influenced in vitro activity. Furthermore, the application of actinomycin D and cycloheximide to callus tissues inhibited the GA-induced enzyme activity (Lee, 1971). It therefore, seems likely that RNA and protein synthesis is involved in the mechanism of IAA oxidase enhancement. GA-dependent increased IAA oxidase activity is therefore, proposed to be the result of action of GA at the level of RNA through synthesis (Filner and Varner, 1967; Jacobsen and Varner, 1967), stabilization (Gayler and Glasziou, 1969), stimulation

TABLE III

EFFECT OF GA AND CYCLOHEXIMIDE IN VITRO
ON IAA OXIDASE EXTRACTED
FROM WHEAT LEAVES

Addition	Enzyme Activity μ moles IAA Destroyed 2 hr^{-1}, 10 mg^{-1} DW	Relative Activity
None	1.3650	0
GA:		
2.2×10^{-5} M	1.4370	+ 0.0720 ns*
2.2×10^{-4} M	1.2965	- 0.0685 ns
CYCLOHEXIMIDE:		
7.7×10^{-5} M	1.4080	+ 0.0430 ns
7.7×10^{-4} M	1.3390	- 0.0260 ns

* LSD at 5% = 0.1133

of the utilization of previously synthesized RNA (Yuang and Mann, 1967), and/or to be the result of inactivation of IAA oxidase inhibitor(s) in the cells with the addition of GA.

Differential Properties of Drought Susceptibility and Resistance

No single factor has yet been found that would provide a mechanism(s) for drought resistance. Since plant tissues develop more IAA oxidase activity as they age, the endogenous level of IAA oxidase might be an indicator of vitality per se. In Figure 13, the drought susceptible cultivar Ponca leaves contain a higher level of IAA oxidase than the drought hardier cultivar Kanking leaves do. This evidence might offer an explanation of the reported differences in drought resistance of these two cultivars.

In Figure 15, both cultivars Kanking and Ponca were grown in two growth chambers. According to the measurement of relative water content, Kanking always showed a higher percentage of relative water content than Ponca after any given period of water stress. This implies that the cultivar Kanking has a higher capability of water retention in the plant cells and cultivar Ponca has a lower water retention ability. This finding confirmed the results of Salim and Todd (1965) and Salim et al. (1969) that a reduction of transpiration and an increase of water use efficiency under water stress is a factor involved in drought resistance, and that water retention of intact plants following exposure to low vapor pressure was correlated with survival. It is therefore, suggested that the different water retention

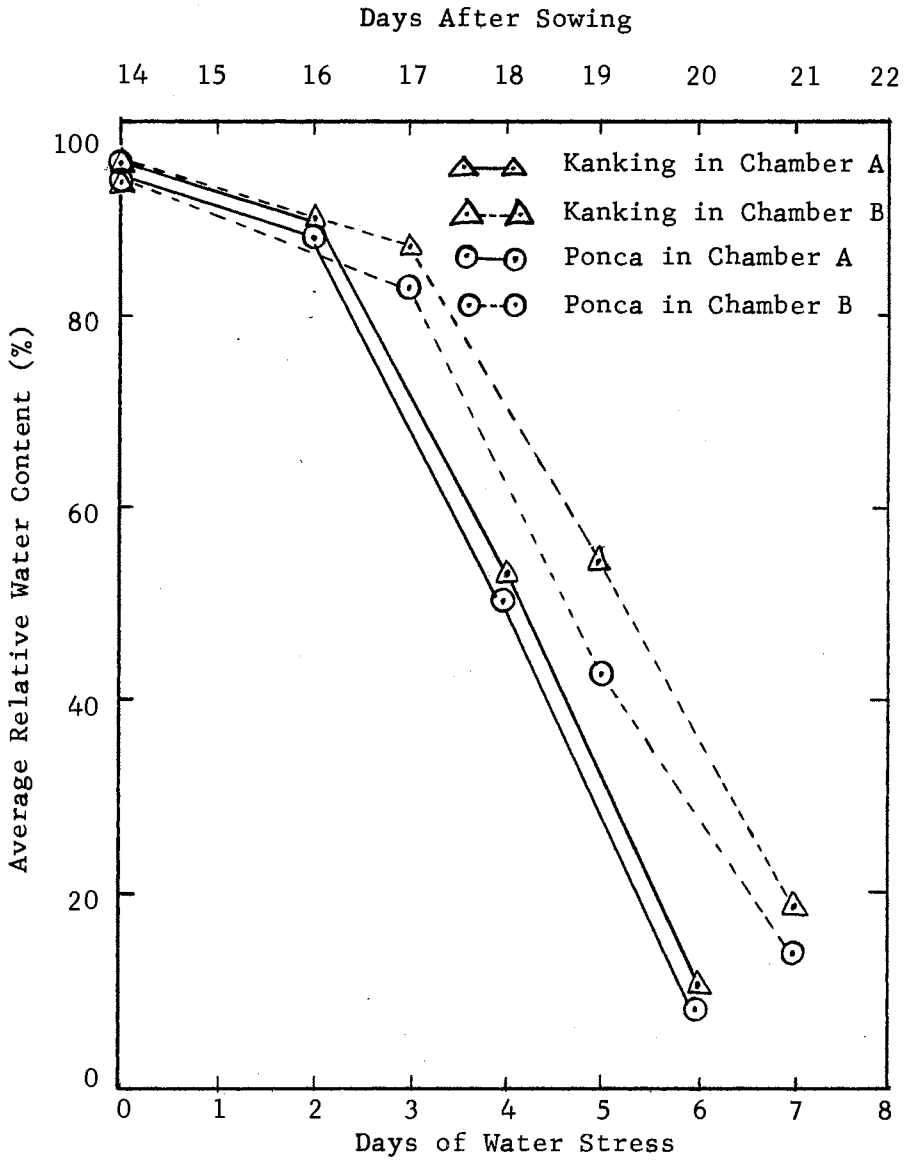


Figure 15. Average Percentage of Relative Water Content in the Second Leaves of Kanking and Ponca Grown Under Two Different Conditions After Exposure to Moisture Stress (Mean of 8 Samples). (Chamber A was equipped with higher light intensity and higher temperature, so that the plants in chamber A probably had a higher transpiration rate than those in chamber B).

ability in different cultivars used in this study might be positively correlated with their difference in resistance to drought.

Electrophoretic Survey

Proteins and enzymes were isolated from wheat cultivars Kanking and Ponca leaves exposed to water stress and separated with 7.5% polyacrylamide gel electrophoresis in an anionical gel system. The bands with different kinds of color density in each gel column represent the different relative amounts of stained protein or active isoenzymes. The 50 μl aliquot of each supernatant applied to each gel column contained different amounts of original plant sample on a dry weight basis, the drier the plants the more material being contained in the aliquot. The control turgid Ponca leaves had more than 90% relative water content. Slightly wilted and wilted represent the leaves containing around 65% and around 40% RWC respectively. Changes in electrophoretic mobility reflect either a molecular weight change or a change in the net charge of the protein molecule which occurs when the substituted amino acid carries a charge different from that of the one it replaces at a given pH.

The results shown in Figure 16 demonstrate that total soluble proteins positively stained by Wool black are changed in composition with increasing dehydration. The increasing number of bands and the decreasing thickness of bands indicate a change of large soluble molecular weight protein into smaller molecular weight proteins occurs as the wheat leaves were exposed to drought. These data closely coincide with those data described both in Part I and in those done by Stutte and Todd (1967). Changes in Fe-containing protein by water

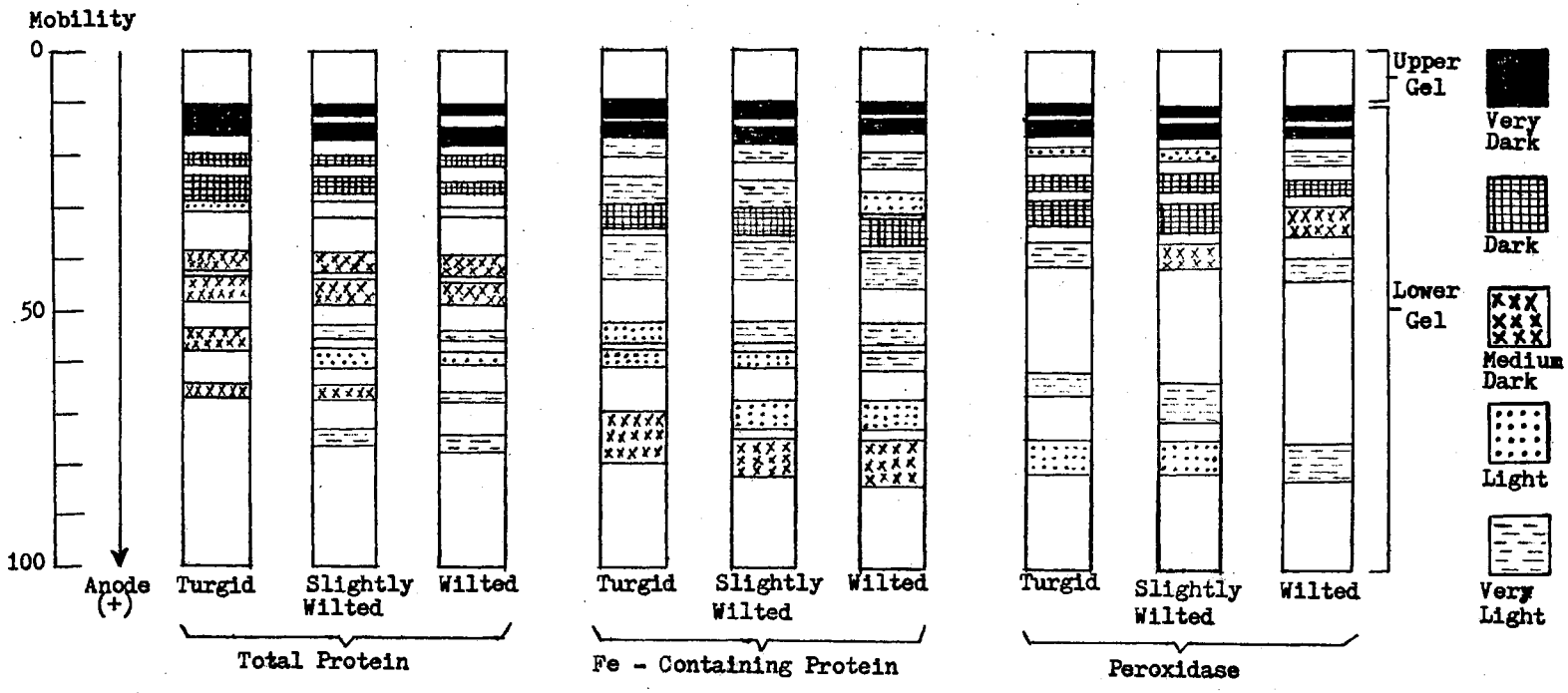


Figure 16. Typical Polyacrylamide Gel Electrophoretic Patterns from Ponca Leaves

stress were not significant except in wilted leaves. Peroxidase in slightly wilted leaves was increased with respect to one isozyme that changed from very light band on the column to medium dark, one dark band and one light band becoming thick. After wilting, there were changes of one light band into very light, one dark band into very light, and one band was missing. All of these changes are regarded as decreasing enzyme activity in comparison with the control. The changes of peroxidase in relation to water stress approximately paralleled that of IAA oxidase activity affected by drought (see Figures 13 and 14). Both activation and inactivation of the enzymes IAA oxidase and peroxidase possibly follow the same mechanism(s) during water stress. Dehydrogenases (Figure 17) appeared to be decreased relatively in proportion to the degree of drought. G-6-PD and succinic dehydrogenases were reduced quantitatively rather than qualitatively during water stress. Malic dehydrogenase decreased in quantity during initial water stress but there was an increase in quality during the wilted stage. The decreases of these dehydrogenases are suggested to be at least in part correlated with respiratory changes which are generally decreased by drought (Kaul, 1966; Miller et al., 1971; Nir et al., 1970). The reduction of G-6-PD activity shown in the electrophoretic zymogram pattern is consistent with the results shown in Figures 11 and 12.

Isoenzymes are produced in several molecular forms. Malic dehydrogenase in either leaf or crown tissues of both cultivars appeared to be composed of two isozymes (Figure 18). In Figure 19, G-6-PD showed tissue specific as well as an intra-specific characteristics. There were three G-6-PD isozymes in both leaf (Figure 19) and

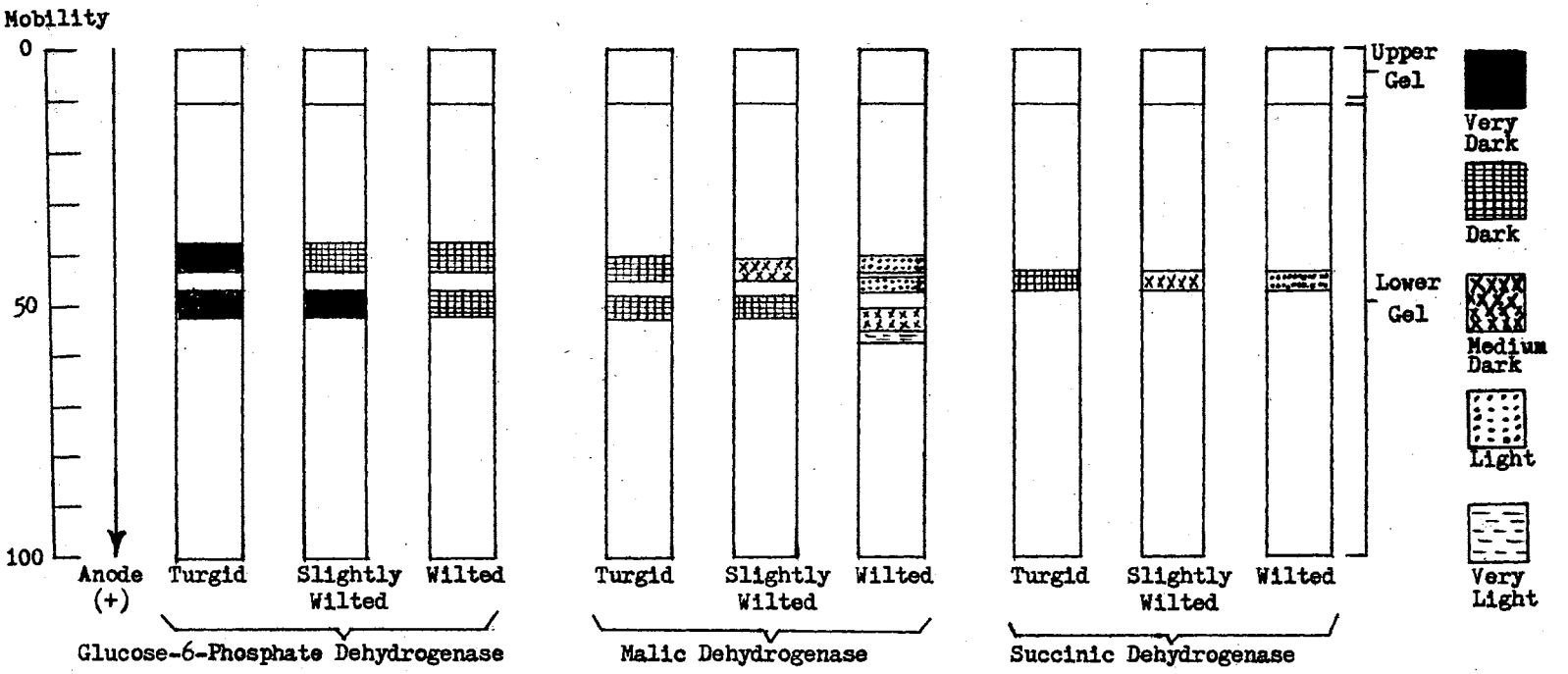


Figure 17. Typical Polyacrylamide Gel Electrophoretic Zymogram Patterns from Ponca Leaves, Compared on the Basis of Fresh Weight

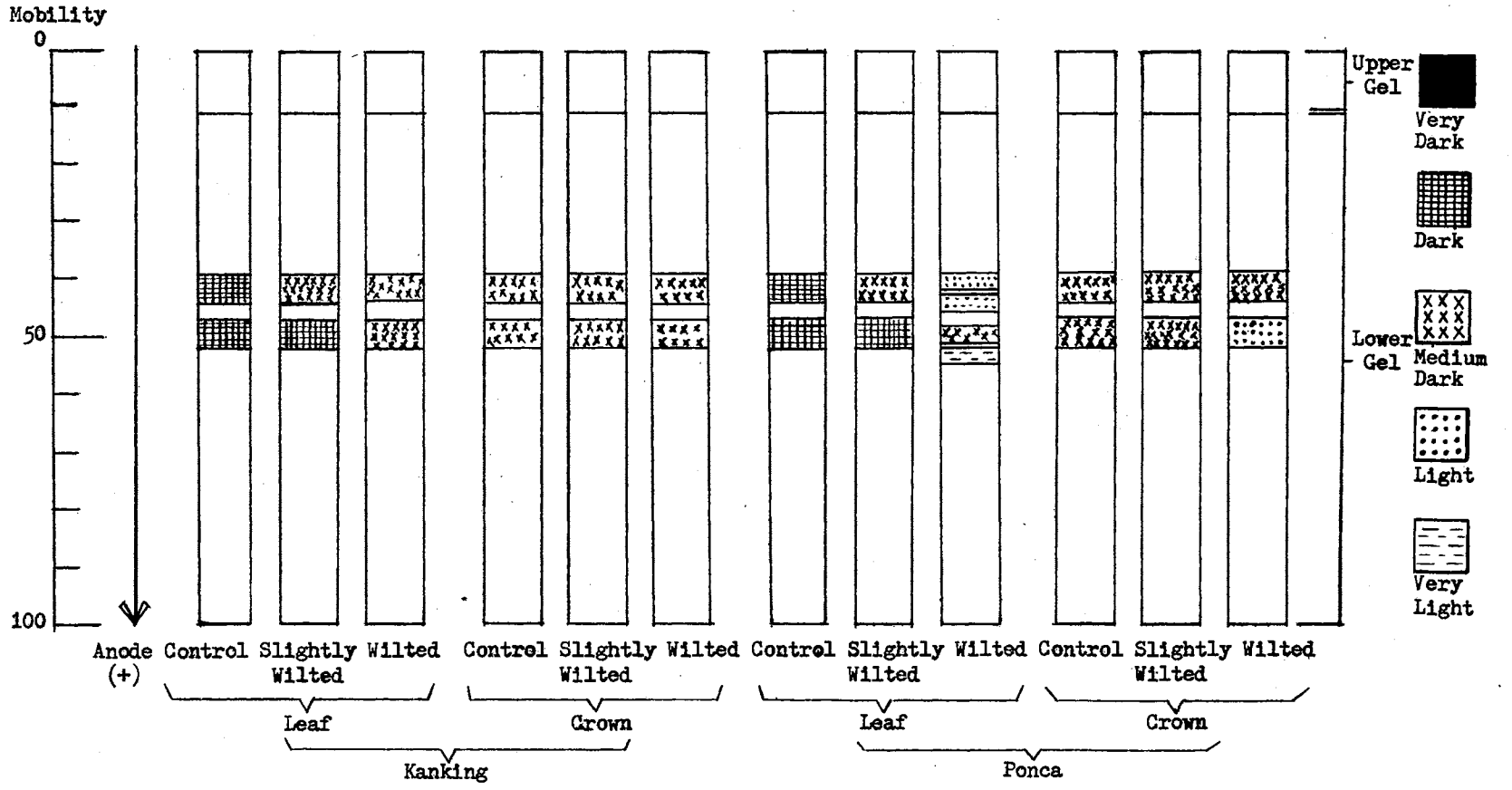


Figure 18. Typical Polyacrylamide Gel Electrophoretic Zymogram Patterns of Malic Dehydrogenase, Compared on the Basis of Fresh Weight

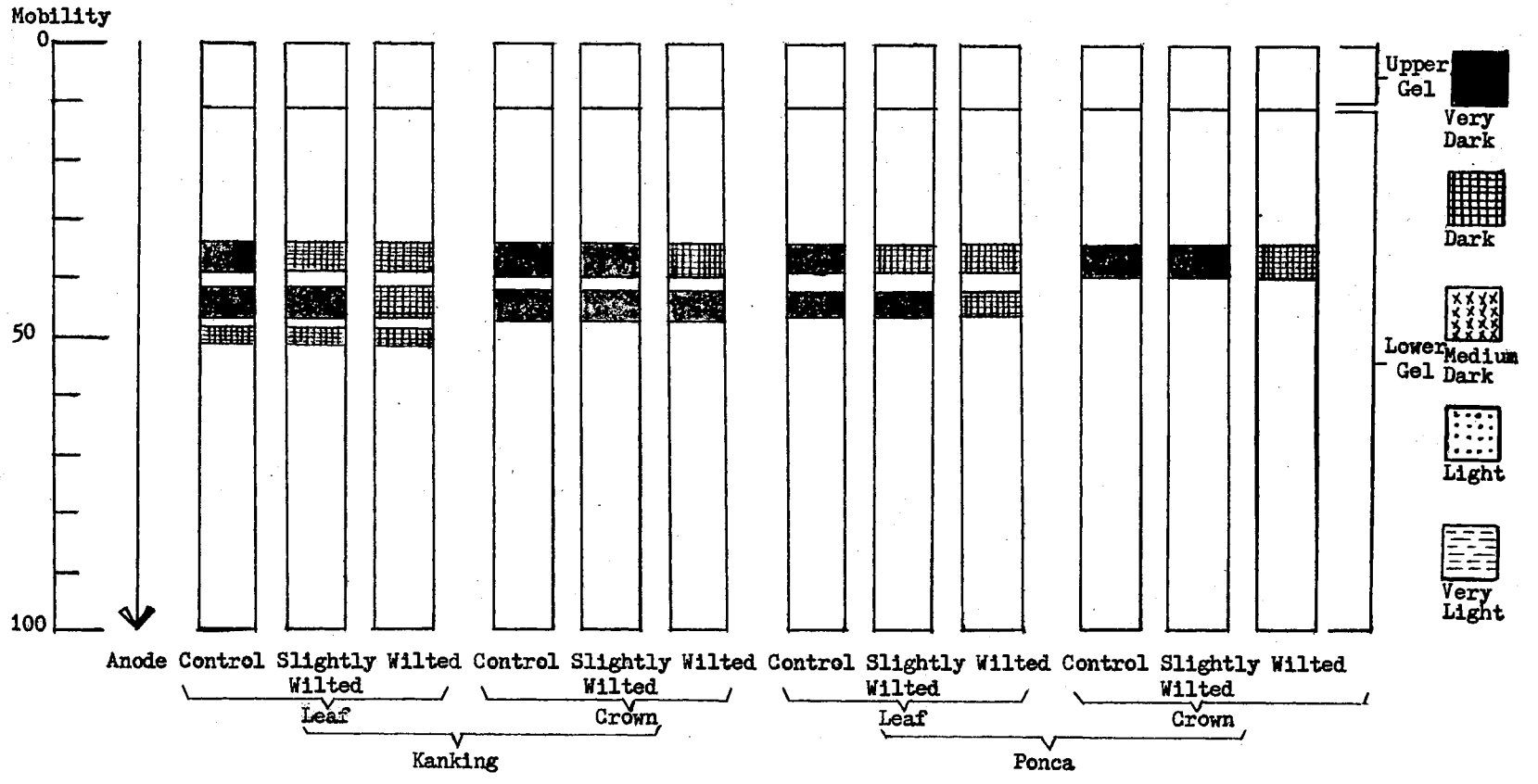


Figure 19. Typical Polyacrylamide Gel Electrophoretic Zymogram Patterns of Glucose-6-Phosphate Degydrogenase, Compared on the Basis of Fresh Weight

root (Figure 20) and only two isozymes in the crown tissues of the cultivar Kanking, while there appeared to be two G-6-PD isozymes in leaf and only one isozyme in the crown tissues of the cultivar Ponca. This polymorphism of G-6-PD, characterized by tissue-specificity, corresponds to the ontogenesis of the enzyme during differentiation and development of wheat plants (Scandalios, 1969).

GA treatment qualitatively changes G-6-PD in the leaf of Kanking by increasing one isozyme which seems to be separable from the low mobility component present in untreated leaves (Figure 20). The mechanism of the GA-dependent induction of isoenzyme probably involves in the primary action of GA as mentioned previously. However, GA treatment quantitatively but not qualitatively enhanced the enzyme activity in Ponca leaves and cycloheximide, as opposed to GA action, suppressed G-6-PD activity compared with the untreated materials observed in electrophoretic zymogram patterns (Figure 21). This promotes a conclusion that the G-6-PD is characteristic of a cultivar, can be organ-specific under genetic control, but can be changed quantitatively in activity upon exogenous application of GA.

Several investigations on the effect of plant growth regulators on peroxidase activity have been reported. At least seven peroxidase isozymes separable by electrophoresis are stable and an eighth isozyme is present as the tissue elongated and aged which is repressed by IAA (5×10^{-5} M) application (Ockerse et al., 1966). There were eight isoperoxidases separable by electrophoresis in coleoptiles of oats and one of them was repressed by IAA or 2,4-D application and the other one was induced by similar treatment (Stuber and Levings, 1969). Thus, each isoperoxidase responds to some optimal level of hormone.

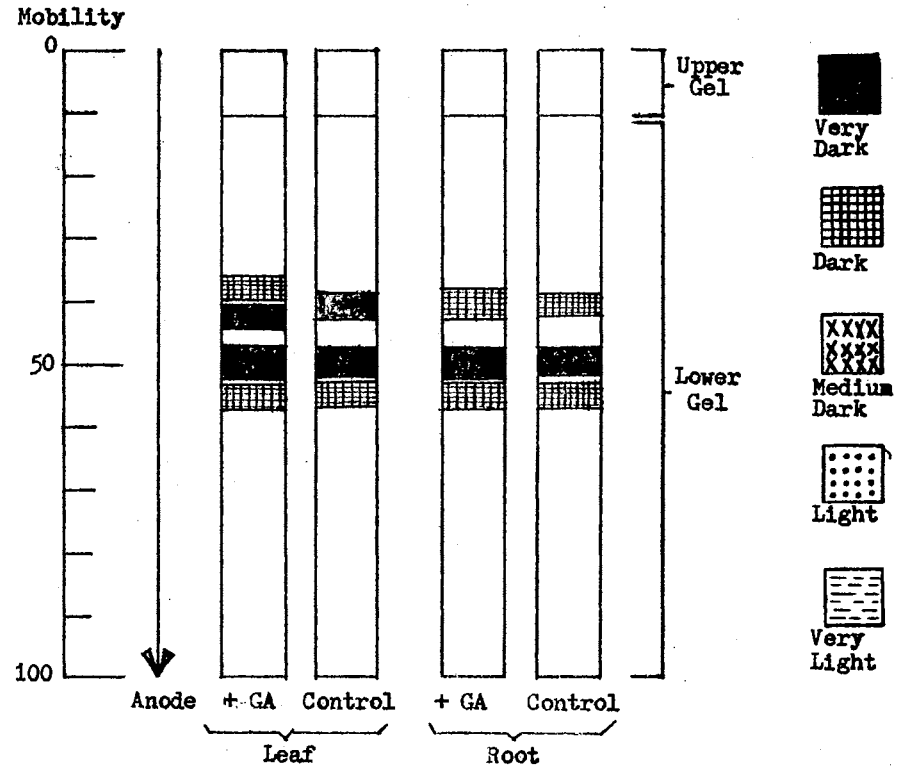


Figure 20. Typical Zymogram Pattern Indicates the Effect of GA on the Change of G-6-PD Activity in Kanking

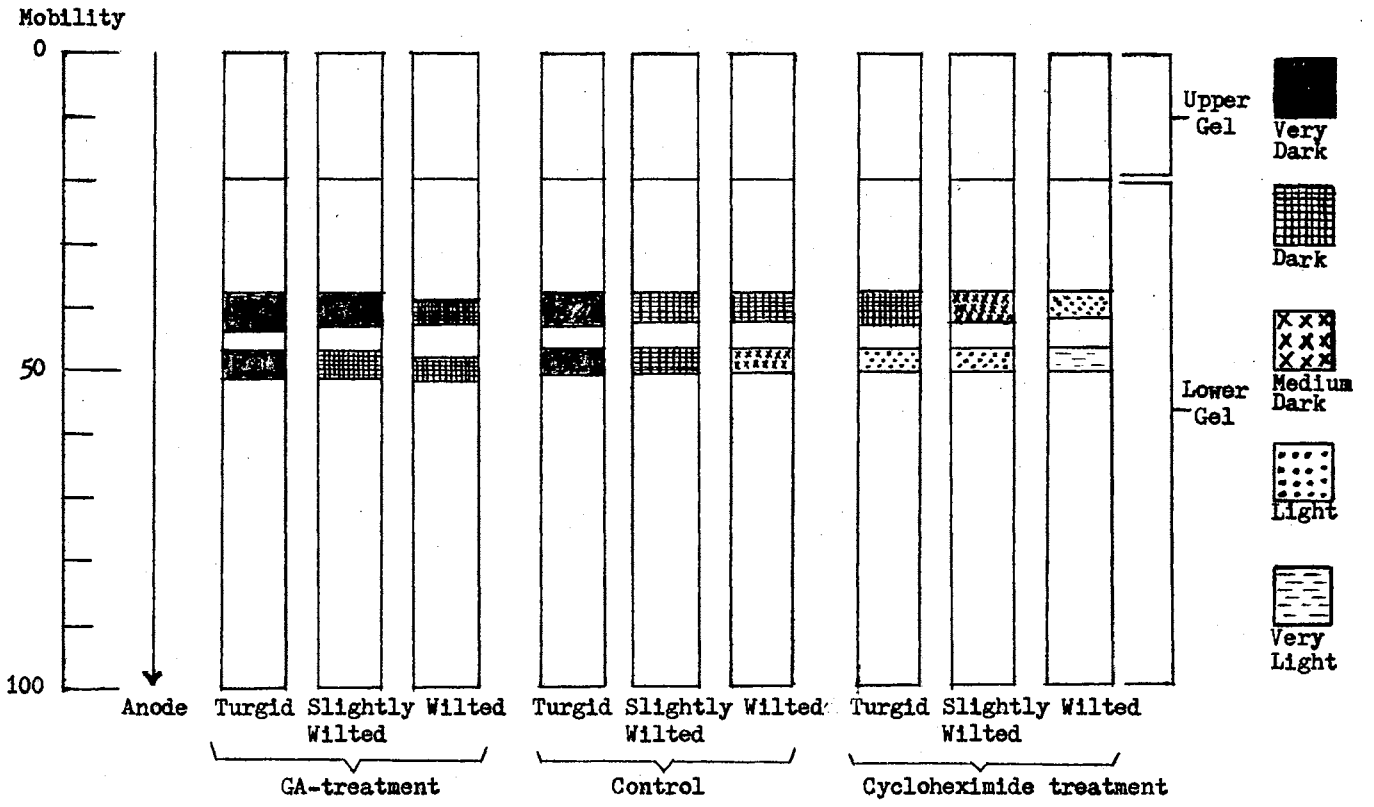


Figure 21. Typical Zymogram Pattern Indicates the Effect of Growth Regulatory Chemicals on the Change of G-6-PD Activity in Ponca Leaves Subjected to Water Stress

On the other hand, application of GA substantially enhances the amount of peroxidase activity in barley as a linear relationship to the logarithm of the GA concentration (Harmey and Mudray, 1968). Conversely, the GA_3 treatment of the elongated pea stem decreased soluble protein and inhibited the rise in peroxidase activity, while the application of GA_3 to young internodes led to a persistent depression in peroxidase activity (Birecka and Galston, 1970).

The results from the present investigation shown in Figure 22 indicate that there are at least eight isoenzymes involved in the zymogram patterns. Moreover, GA treatment shows that the third and the seventh isozyme counted from the top of gel column possess higher color density than the control after water stress. Thus, the overall enzyme activity is higher after GA treatment than the untreated control. During water stress, the sixth and seventh isozymes appear to be higher in activity in the slightly wilted leaves than those in the turgid, while the fifth, sixth and seventh isozymes at the wilted stage decrease the color density as compared with turgid plants. These results imply that water stress slightly enhanced peroxidase activity after an initial water stress in both GA treated and untreated control plants but declined in activity at the wilted stage, and that the changes of enzyme activity are mostly quantitative rather than qualitative. This agrees with the observations of Birecka and Galston (1970).

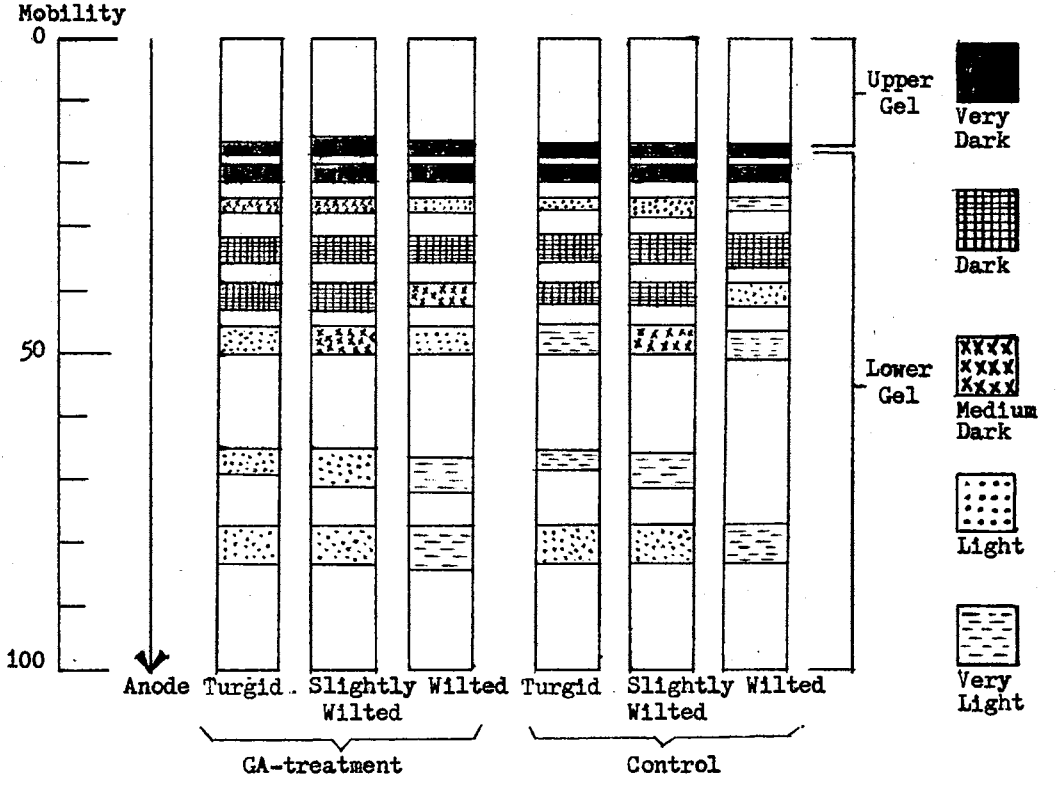


Figure 22. Typical Zymogram Pattern Indicates the Effect of GA on the Change of Peroxidase Activity in Ponca Leaves Exposed to Water Stress

CHAPTER IX

SUMMARY

Glucose-6-phosphate dehydrogenase showed a drastic decrease in enzyme activity during initial drying stages of wheat cultivars Kanking and Ponca. GA treatment caused a rise of endogenous enzyme level but had no effect on the reduction of activity caused by water stress. The endogenous level of G-6-PD appeared to be higher in Kanking than in Ponca leaves. From the observation of electrophoretic zymogram patterns, there are three isozymes of G-6-PD found in the blades and the roots and only two in the crown tissues of Kanking, while two isozymes of G-6-PD are found in the blades and only one in the crown tissues of Ponca. This intra-cultivar variation as well as organ-specificity of G-6-PD perhaps was the main cause for the different endogenous levels of enzyme in the two wheat cultivars. GA treatment induced one extra isozyme in Kanking leaves but did not cause such an induction in Ponca. It was found that the decrease of G-6-PD activity during water stress was due to a quantitative change rather than a qualitative one. Water stress caused decreases in malic dehydrogenase and succinic dehydrogenase activity.

IAA oxidase activity increased substantially at low water deficit. This elevation of activity with increasing dehydration was lost when the leaves became wilted. The application of GA at 10^{-4} M resulted in a substantial increase of IAA oxidase activity. The higher

endogenous level of IAA oxidase in Ponca than in Kanking correlates with the drought hardiness of both cultivars, suggesting that the mechanism(s) of drought hardiness may be due at least in part to difference in the level of IAA oxidase.

Most of the peroxidase isoenzymes showed a change of activity in quantity rather than in quality with changing moisture status in wheat leaves. GA slightly increased isoperoxidase activity in quantity but not in quality.

Finally, higher water retention capability was found in Kanking than in Ponca under the circumstances investigated, suggesting that a possible correlation between drought hardiness and water retention ability exists.

PART III

SOME EFFECTS OF PHENOLIC COMPOUNDS ON ENZYME ACTIVITIES

CHAPTER X

REVIEW OF LITERATURE

Secondary Metabolites

Many biological compounds such as nucleic acids, proteins and the aromatic amino acids, phenylalanine and tyrosine are absolutely essential for life and have been regarded as primary metabolites. There are many other substances found in vascular plants in small amounts that are often referred to as secondary compounds. Some of these secondary compounds are responsible for the taste and smell of food products; others are used in medicine and technology.

There is a wide range of the known secondary metabolites. Some of the most important compounds have been classified into several groups (Kretovich, 1966).

(1) Aliphatic organic acids, e.g., acetic acid, glycolic acid, glyoxylic acid, α-ketoglutaric acid, L-malic acid and etc. are important in plant metabolism, and are extensively used in the food industry.

(2) Aromatic compounds including a wide variety of nitrogen-free phenylpropane derivatives occur both in the free state and as esters, glycosides and tannins; many are of importance in medicine and food manufacturing processes.

(3) Glucosides are those compounds whose aglucones or non-sugar portions are linked with sugar, e.g., glucovanillin composed of D-glucose and the aglycone, vanillin by a glycosidic linkage. They are

responsible for the taste and aroma of some food products from plants and are also used medicinally.

(4) Tannins are readily converted into brown or red materials by oxidizing enzymes, and are responsible for some of the taste qualities of wines, tea, coffee, and cocoa.

(5) Essential oils are volatile substances found in many plants, and are used in the perfume industry.

(6) Rubber and gutta-percha which are both obtained from laticiferous plants, have numerous industrial uses.

(7) Alkaloids are nitrogenous heterocyclic compounds many of which elicit physiological responses in animals; many are used in medicine.

(8) Growth stimulants, antibiotics, and phytonicides which are toxic to micro-organisms.

Phenolic compounds are characterized by their structural formulas containing one benzene ring. Many natural phenolic compounds have at least one hydroxyl group linked as an ether, ester or glycoside rather than in a free form. Either ethers or esters are less soluble in water than the parent phenols while the glycosides are more water-soluble (Robinson, 1963).

Biosynthetic Pathways

The biosynthesis of a great variety of plant aromatic compounds are mostly derived via 5-dehydroquinic acid (Robinson, 1963). This acid is formed by the condensation of 2-phosphoenolpyruvic acid and D-erythrose-4-phosphate, which are intermediates of the glycolytic pathway and pentose phosphate shunt respectively.

Figure 23 illustrates the conversion of 5-dehydroshikimic acid to protocatechuic acid and gallic acid which is then glucosidically linked to form polygalloyl glucose as the parent compound of many, if not all, the hydrolyzable tannins. The main pathway of the formation of cinnamic acid and p-coumaric acid from phenylalanine and tyrosine respectively does not go through the corresponding keto and hydroxyl acids but occurs by one-step elimination of the amino group catalyzed by deaminases. Monocots have both enzymes, tyrosine deaminase and phenylalanine deaminase, and can synthesize p-coumaric acid from both tyrosine and phenylalanine, but dicots have only phenylalanine deaminase, and are therefore unable to make p-coumaric acid (or compounds derived from it) from tyrosine (Koukol and Conn, 1961; Neish, 1960). o-coumaric acid formed by hydroxylation of cinnamic acid may first be hydroxylated and then go on to form hydroxycoumarin; or the parent coumarin may be made first and then hydroxylated (Robinson, 1963). This type of hydroxylation may be either non-enzymatic or due to a peroxidase system (Buhler and Nason, 1961). Figure 24 illustrates how aromatic compounds are derived from p-coumaric acid through hydroxylation and methylation. The structure of lignin is not yet known. The formation of lignin has been suggested to follow the non-enzymatic polymerization of the enzyme-mediated free radical of coniferyl alcohol with other molecules (Freudenberg, 1959). Shimada et al. (1970) found that S-adenosylmethionine-catechol o-methyl-transferase (EC 2.1.1.6.) from the bamboo and poplar (angiosperm) was able to be metaspecific for 3,4-dihydroxy-, 3,4,5-trihydroxy- and 3,4-dihydroxy-5-methoxy-cinnamic acids. The rate of formation of sinapic acid from 5-hydroxyferulic acid in the <u>in vitro</u> reaction was

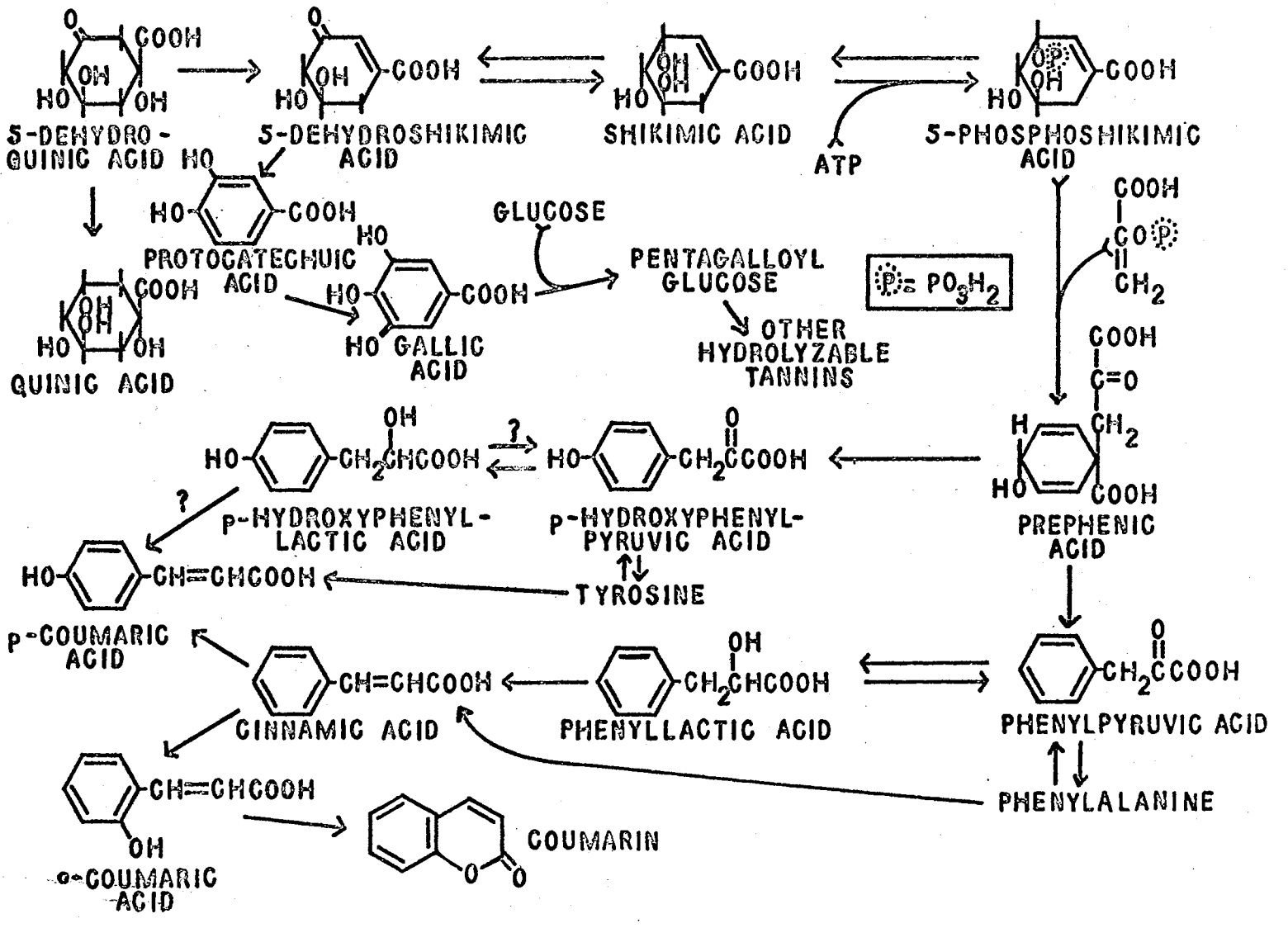


Figure 23. Aromatic Compounds Derived from 5-Dehydroquinic Acid (after Robinson, 1963)

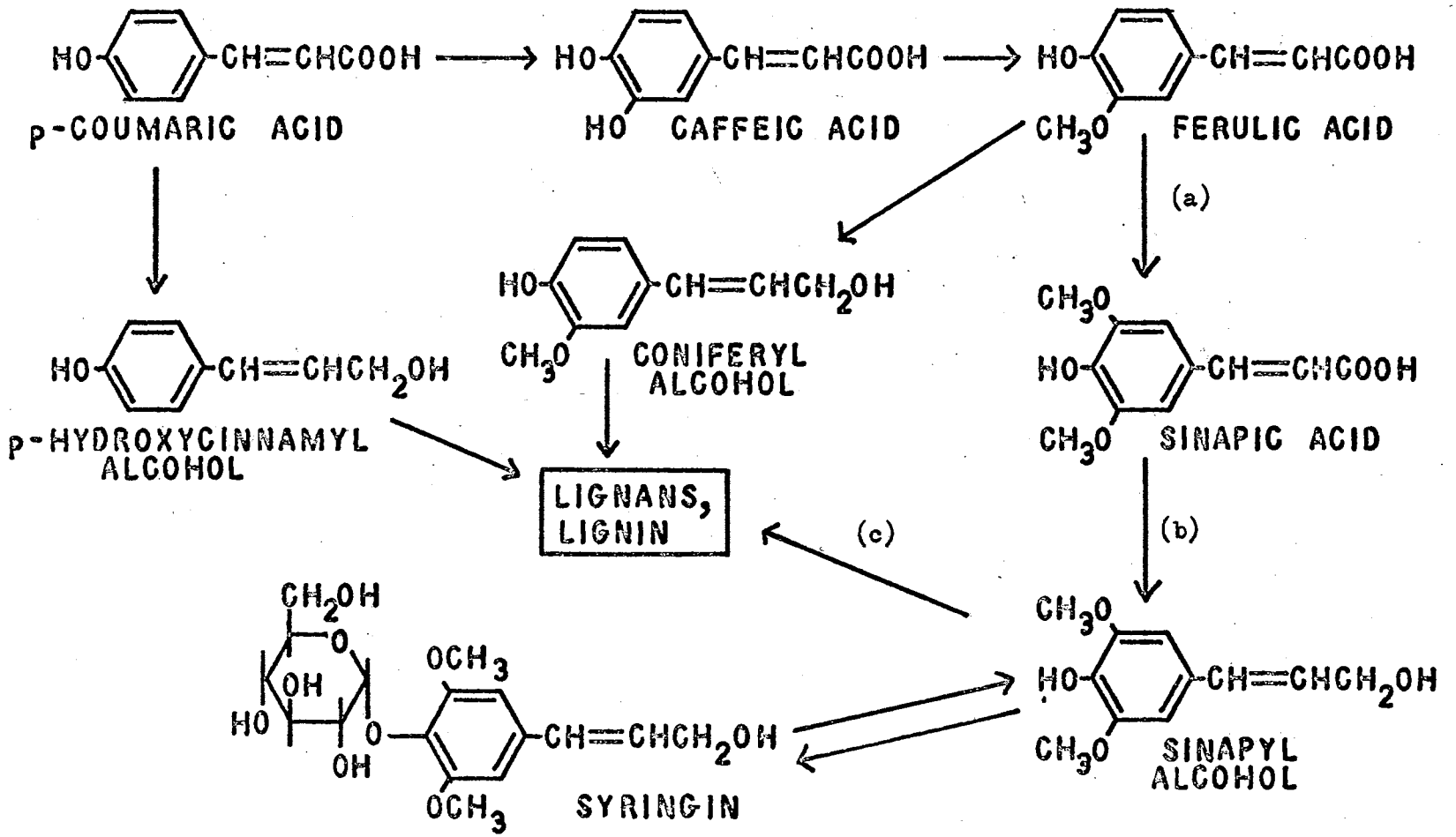


Figure 24. Aromatic Compounds Derived from p-Coumaric Acid (after Robinson 1963)

found to be greater than that of ferulic acid from caffeic acid. However, the enzyme from the sliced tissue of Ginkgo biloba (gymnosperm) did not methylate 3,4,5-trihydroxy-cinnamic and 3,4-dihydroxy-5-methoxycinnamic acids but only 3,4-dihydroxy-cinnamic acid. In connection with the biosynthesis of lignins in different plants, Shimada et al. (1970) noted that the lignins of gymnosperms largely contain guaiacyl units whereas the lignins of angiosperms contain both syringyl and guaiacyl groups and suggested that gymnosperm might lack one or more of the series of enzymes controlling the reactions (a), (b), (c), (Figure 24). Therefore, gymnosperm o-methyltransferase might methylate only caffeic acid in turn to produce guaiacyl lignin, while angiosperms might have two o-methyltransferases; one for caffeic acid and the other for 5-hydroxyferulic acid, resulting in the synthesis of an additional form of syringyl lignin.

Chlorogenic acid is extremely widely distributed among higher plants (Sondheimer, 1964; Hanson, 1966; Steck, 1968). The chemical structure of chlorogenic acid is 3-o-caffeoyl-D-quinic acid. Generally, there are some isomers present such as neochlorogenic acid (5-o-caffeoyl-quinic acid), analogous coumaroyl and feruloyl compounds. This class of compounds has been collectively termed "chlorogenoids" which may include not only quinic derivatives but also shikimic and other closely allied acids (Steck, 1968). Gamborg (1967) using radioactive precursors, showed that quinic acid and caffeic acid could serve as the direct precursors of chlorogenic acid in potato tuber cells, but there was no indication whether the labeling of caffeic acid was due to metabolism or resynthesis of caffeic acid. Hanson (1966) working with potato tuber slices, demonstrated that the caffeoyl

derivatives were formed by the hydroxylation of the p-coumaroyl derivatives. However, Steck (1968) proposed a scheme for the metabolic pathways (Figure 25) of chlorogenic acid in tobacco, and suggested that there were two pathways operative in this synthesis. The major route suggested was: cinnamic acid⟶ p-coumaric acid⟶ p-coumaroylquinic acid⟶ chlorogenic acid, and the secondary route: cinnamic acid⟶ p-coumaric acid⟶ caffeic acid⟶ chlorogenic acid. Possible routes to the biosynthesis of catechol in Gaultheria were proposed by Towers et al. (1965) as shown in Figure 26 for establishing the relationship of the phenolic compounds to one another in plants.

Phenolic Compounds Isolated From Wheat Plants

As shown in Figure 23, it is apparent that the intermediates of the phenylalanine and tyrosine pools can serve as precursors of a wide variety of aromatic compounds. Deamination of both phenylalanine and tyrosine leads to the production of cinnamic acid and p-coumaric acid respectively by means of the specific enzymatic catalysis. However, not all plant species can convert the tyrosine pool intermediates into the phenolic cinnamic acids. Neish (1961) found the enzyme catalyzing deamination of L-tyrosine, i.e., tyrase, to be present in substantial amounts in some grasses (sorghum, barley, rice, wheat, oats, corn and sugar cane), but not in some legumes (pea, lupine, alfalfa and white sweet clover). However, the conversion of the intermediates of the phenylalanine pool to cinnamic acid can probably take place in both dicotyledons and monocotyledons (Koukol and Conn, 1961; Neish, 1960). Hydroxylation of cinnamic acid then leads to the

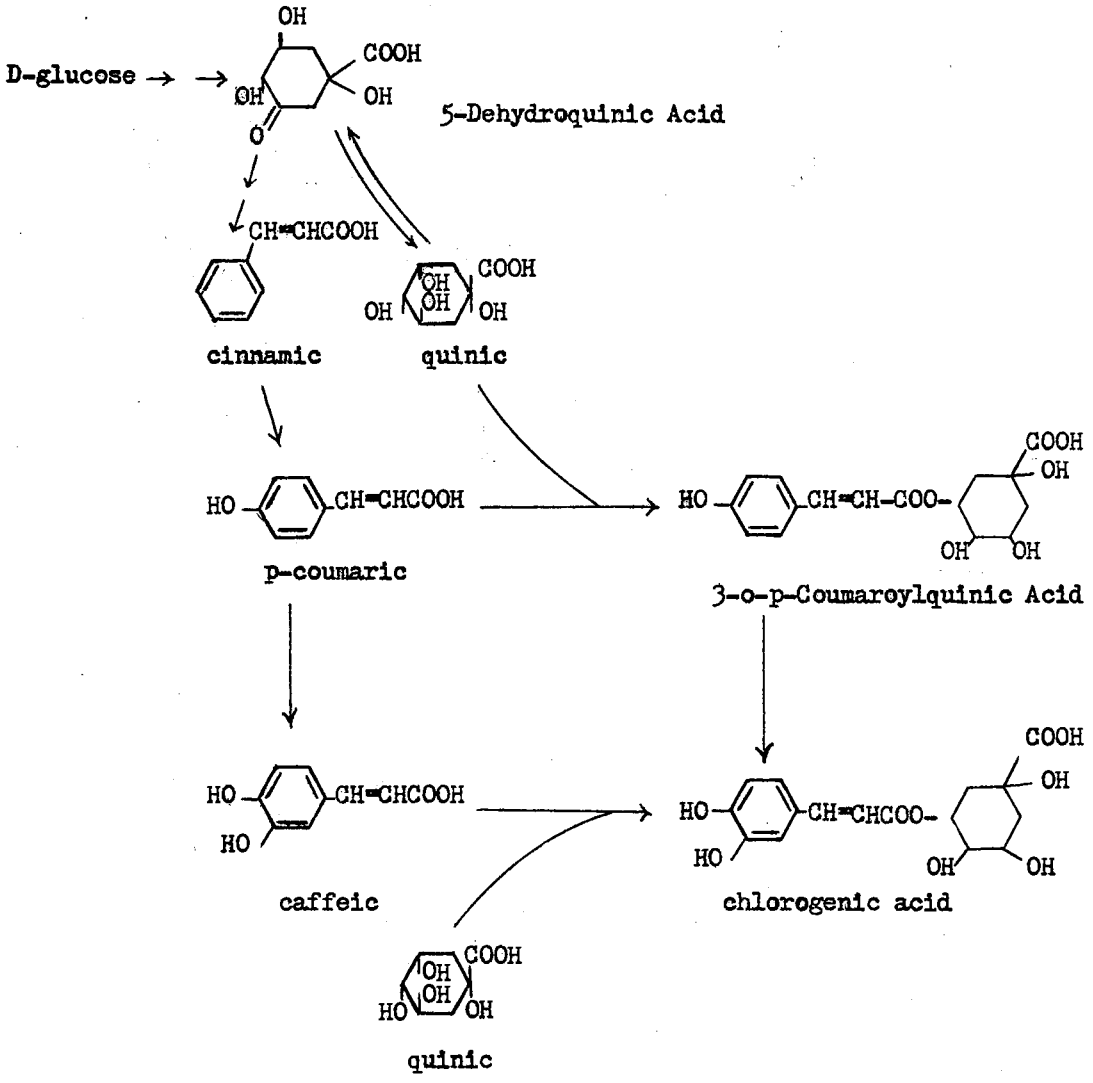


Figure 25. Suggested Pathway for the Formation of Chlorogenic Acid (after Gamborg 1967 & Steck 1968)

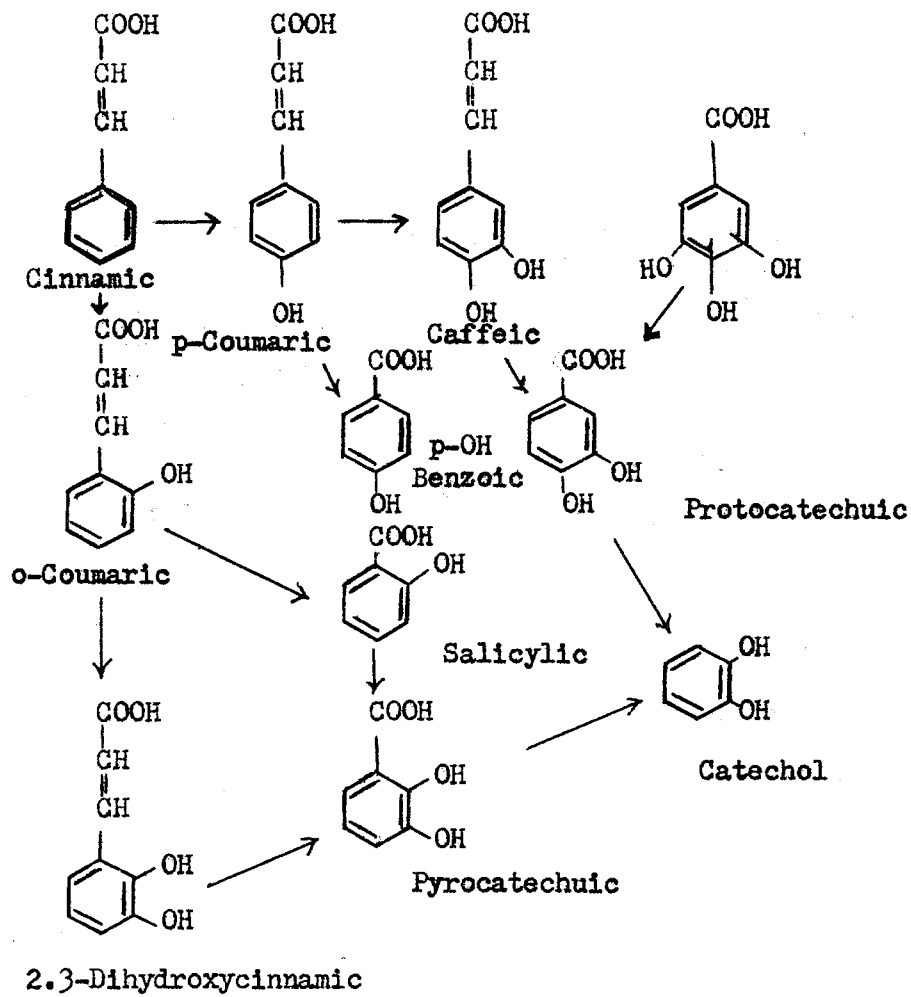


Figure 26. Possible Routes to the Biosynthesis of Catechol (after Towers et al 1966)

formation of the phenolic cinnamic acids. Reduction of these acids to cinnamyl alcohols, followed by oxidative polymerization of the alcohols, is probably the major pathway in the formation of lignin (Figure 24).

Each step of a metabolic process is controlled by its specific enzyme. A relatively small number of qualitative changes in enzyme constitution during the course of evolution might thus have resulted in the production of the wide array of the secondary metabolic substances which are found in the plant kingdom. The phenolic acids in wheat plants have been examined both qualitatively and quantitatively by several investigators. El-Basyouni and Towers (1963) found that large amounts of phenolic acids were present in a bound form by ester or glycosidic linkages; only p-coumaric and ferulic acids could be detected in a free state; most p-coumaric, ferulic, caffeic, sinapic, p-hydroxybenzoic, vanillic and syringic acids occurred as alkali- and acid-hydrolyzable derivatives in ethanolic extracts of wheat tissues. Guenzi and McCalla (1966) confirmed that ferulic, p-coumaric, syringic, vanillic and p-hydroxybenzoic acids were largely isolated from wheat and some other grasses; p-coumaric acid was present in a much greater concentration than the other acids. Nevertheless, the endogenous level of phenolic acids in wheat plants were changed due to aging and some other factors. Phenolic acids in the ethanol-soluble fractions reached a maximum concentration 9 days after germination, then fell off with increasing age reaching a minimum after 4 to 5 weeks (El-Basyouni and Towers, 1964). A study was made on the changes of phenolic compounds in response to water stress in wheat plants (Tsai and Todd, 1972 in manuscript).

Activation and Inhibition of Enzymes

The presence of phenolic compounds in many types of plants indicates that they have an important role in plant metabolism and may be directly or indirectly correlated with growth processes in plants. Endogenous phenolic compounds are rapidly oxidized to form quinones, condensed tannins and brown pigments during extraction of plant tissues. The oxidation of phenols is catalyzed by phenoloxidase but only occurs to any appreciable extent when tissue is homogenized (Anderson, 1968). The products of the phenoloxidase reaction powerfully inhibit plant enzymes (Jones et al., 1965 ; Loomis and Battaile, 1966; Slack, 1966; Anderson and Rowan, 1967) and subcellular organelles (Hulme et al., 1964 & 1964; Lieberman and Biale, 1965; Verleur, 1965; Stokes et al., 1968). The intracellular localization of phenoloxidase is still controversial (Anderson, 1968).

Quinones, the oxidation products of phenolic compounds, condense readily with free sulfhydryl groups (Steck, 1966) and terminal α-amino and imino groups of proteins and less readily with the ε-amino group of lysine (Loomis and Battaile, 1966; Mason and Peterson, 1955). It follows that quinones readily react with proteins and inhibit enzymes. Hulme et al. (1964) and Jones et al. (1965) suggested that a loss of soluble enzyme protein occurs by co-precipitation of protein with polymerized forms of oxidized phenolics. The degree of inactivation, however, may vary with the activity of phenoloxidase and the concentration of endogenous phenolics, the types of phenolics and the characteristics of the specific enzyme. Although many facts are known about the inhibitory activity of different phenolic compounds, the chemical and biochemical aspects of their modes of action are obscure. Some

investigators proposed the idea that para- and ortho-diphenols exhibit their strong inhibition when they convert to the corresponding quinones. The oxidized quinones may also form a condensation product with IAA (Leopold and Plummer, 1961). Therefore, as a general rule, those phenols which are difficultly oxidized to quinones act as activators or are inert to IAA destroying enzymes i.e., IAA oxidase and peroxidase, while ortho- and para-dihydric phenolics may exert inhibitory influences. However, the concentration at which a compound is tested and the presence of other activators may be of critical importance. For example, Gortner and Kent (1958) found that in the absence of other activators, ferulic acid stimulated the oxidation of IAA by IAA oxidase from pineapple. If p-coumaric acid was present ferulic acid at a somewhat higher concentration had an inhibitory effect. Therefore, a model that IAA oxidase possesses two active sites competitive for either a cofactor or a substrate was proposed by Gortner and Kent (1958), in which one particular active site must be occupied by the IAA substrate and the other by the phenolic cofactor for oxidation to take place. At higher concentrations, p-coumaric acid saturates both active sites so that the enzyme becomes inactive. Scopoletin inhibits the oxidation of IAA apparently by a competitive action (Andreae, 1952; Imbert and Wilson, 1970). Catechol, hydroquinone, pyrogallol, scopoletin, and riboflavin have been reported to be the competitive inhibitors of IAA oxidation (Waygood et al., 1956). In addition, IAA oxidase was competitively inhibited by guaiacol (Goldacre, 1951) and by chlorogenic acid (Rabin and Klein, 1957). Ferulic acid isolated from maize inhibited an IAA oxidase from maize (Gelinas and Postlethwait, 1969). Caffeic acid inhibited IAA oxidase in a noncompetitive fashion, but quinic acid,

the other moiety of chlorogenic acid was inactive (Robin and Klein, 1957).

Different enzymes may have a different number as well as different properties of the active site in each enzyme molecule. For example, IAA oxidase possesses two active sites in its reaction with substrates and cofactor respectively, and each active site can be competitively occupied by inhibitors, e.g., ferulic acid (Gortner and Kent, 1958) and chlorogenic acid (Robin and Klein, 1957). The results from those investigators indicate that the stimulators of IAA oxidase include manganous ions, resorcinol, p-coumaric acid and the analogues of 2,4-dichlorophenol, while the other dihydric phenols and pyrogallol all act as inhibitors (Waygood et al., 1956). On the other hand, catechol and gallic acid slightly inhibited G-6-PD and some other dehydrogenases, while tannic acid, and chlorogenic acid completely inhibited G-6-PD activity (Firenzuoli et al., 1969). G-6-PD has also been reported to be completely inhibited by oxidized chlorogenic acid at 10^{-4} M (Alberghina, 1964). Kinetic studies of potato tuber phenolase, Macreae and Duggleby (1968) suggested that this enzyme possesses two sites responsible for the hydroxylation of monophenols and the oxidation of o-diphenols, the former site was attached with copper atoms losing activity more rapidly during isolation due to a twist or a spread of the protein residue. Both p-coumaric acid and ferulic acid inhibit potato tuber phenolase by competitive type action using p-cresol as substrate, and by non-competitive and mixed type of inhibition using chlorogenic acid as a substrate. Similar results of Walker (1969) showed that p-coumaric and ferulic acid powerfully act as competitive inhibitors of catechin oxidation but as non-competitive inhibitors

for the oxidation of chlorogenic acid by apple phenolase. This reveals that the type of inhibition depends on the substrate. They also showed that o-coumaric acid acted as a non-competitive inhibitor for both substrates.

On the other hand, invertase which has been suggested to be located in the cytoplasm or vacuoles of grape cells may be coagulated with tannin to form a tannin-protein complex and/or a protein-tannin-cell wall complex (Hawker, 1969). This complex might be the one which was regarded as the insoluble invertase by Arnold (1966). Loomis and Battaile (1966) reported that both phenols and tannins present in grapes could combine with enzyme proteins reversibly by hydrogen bonding and irreversibly by oxidation followed by covalent condensations. Working with potato tuber invertase, Schwimmer et al. (1961), and Pressey (1966) concluded that an endogenous inhibitor of invertase was present. Frost et al. (1968), Schwimmer et al. (1961), and Pressey (1966) regarded the enzyme-inhibitor complex as highly dissociable, while Kern and Natale (1958) and Ewing and MaAdoo (1971) thought that this enzyme-inhibitor complex was not highly dissociable. This mysterious inhibitor of potato tuber invertase has not been to date identified. Pyrocatechol at 10^{-2} M applied to the foliage of sugar beet significantly inhibited invertase and phosphatase but stimulated sucrose synthetase and sucrose phosphate synthetase (Singh and Wort, 1970).

The mechanism of action of the inhibitory substances in reducing the enzyme activity is controversial. It appears to be due to either the formation of a protein-phenolic complex resulting in decreasing the solubility of the enzyme proteins (Sander, 1965; Hulme et al., 1964)

or the phenolic compounds directly affecting the soluble enzymes (Alberghina, 1964; Firenzuoli et al., 1969). Thus, many protecting agents have been employed in order to improve efficiency of enzyme extraction. They include polyvinylpyrrolidone (PVP), thiols, ascorbate, bovine serum albumin (BSA), and reducing agents such as cysteine, thioglycollate, metabisulphite, dithionite, diethyldithiocarbamate (DIECA), and etc.

Phenolic compounds as well as the other secondary metabolites are thought to be located in the vacuoles of cells. Thus, little oxidation of phenolics occurs in intact plant cells because the phenolics are spatially separated from phenoloxidase by the tonoplast. The tonoplast is a single layer of membrane and exhibits semipermeable properties and serves as a barrier between cytoplasm and vacuolar sap. In aging, or as the result of some environmental stresses, e.g., drought stress, a breakdown of the tonoplast may occur with the resultant mixing of the vacuolar sap with the cytoplasm (Shaw and Manocha, 1965; Varner, 1961). Anatomical evidence showed that mild water stress resulted in increased deposition of lignin. With more severe stress there was disorganization of cells of the wheat leaf (Ridley and Todd, 1966). Thus, some crucial enzymes are proposed to be tested <u>in vitro</u> in response to the addition of phenolic compounds which have been known to be major components in wheat plants.

CHAPTER XI

MATERIALS AND METHODS

Sources of Enzymes

Partially purified IAA oxidase was extracted from winter wheat cultivars Kanking and Ponca (Triticum aestivum L.). Plants were grown in a controlled environment with 14 hours of 11,000 lux light illumination daily at 25 $\pm$ 2°C temperature. About 75 leaves were harvested for each sample and homogenized with 0.2 M Tris-HCl buffer containing 8% polyvinylpyrrolidone (PVP). The homogenization was done with an Omni mixer at full speed for 1 min., with 1 min. interval between grindings for 3 times. The homogenate was kept in cold during grinding. The slurry was passed through two layers of nylon stocking. The filtrate was then centrifuged at 27,000 x g for 30 min. at 4°C. The supernatant was brought to 80% saturation with ammonium sulfate and allowed to stand for 2 hours at 4°C. The precipitate obtained from centrifugation at 27,000 x g for 10 min. was then resuspended in 0.05 M phosphate-citrate buffer pH 4.0 to make a 5 ml of final volume.

Crude glucose-6-phosphate dehydrogenase was obtained by the same procedures described above, except the precipitate obtained with 80% ammonium sulfate saturation was resuspended in 0.05 M phosphate buffer pH 7.4 to make a 5 ml of final volume. Purified G-6-PD from Torula yeast with a capacity of 325 μ moles of nicotinamide adenine dinucleotide

phosphate reduced per min. per mg protein was obtained from Sigma Chem. Co.

Crude invertase was extracted from the leaves of wheat cultivar Ponca. Three grams of fresh leaves were ground in a mortar and pestle with 0.01 M acetate buffer pH 4.5. To facilitate grinding, small amounts of pure sand were added. The homogenate was centrifuged at 27,000 x g for 30 min. at 4°C. The supernatant fraction was then used for enzyme assay. Purified enzyme from yeast was purchased from Calbiochem, Los Angeles, California.

Enzyme Assays

IAA oxidase: The assay mixture contained 0.1 ml of IAA (10.0 mM), 0.1 ml of $MnCl_2 \cdot 4H_2O$ (2.6 mM), 0.1 ml of 2,4-dichlorophenol (2.6 mM) in 0.005 M phosphate-citrate buffer pH 4.0 and 0.2 ml of partially purified enzyme to make 3.5 ml total volume. At both zero time and 2 hours after incubation at room temperature, a 0.5 ml aliquot was placed in a test tube with 0.5 ml deionized water and 2.0 ml Salkowski reagent. The Salkowski reagent consisted of a mixture of 15 ml of 0.5 M $FeCl_3$, 500 ml distilled water, and 300 ml of concentrated H_2SO_4 (Meudt and Gaines, 1967). The color was allowed to develop for 1 hour and the absorbance was then determined with a Perkin-Elmer Spectrophotometer, Model 202, at 530 nm. Simultaneously, 0.1 ml of various phenolic compounds at 16 mM were added by replacing the same volume of buffer solution in the 3.5 ml reaction mixture. The enzyme activity was expressed as μ moles IAA destroyed in 2 hours per 10 mg dry weight in terms of calibration curve shown in Figure 10 (in Part II).

G-6-PD: In a quartz cuvette, 2.5 ml of G-6-PDH "Stat-Pack"

solution prepared by Calbiochem Co., was added with 0.5 ml to 1.0 ml of ferulic acid, p-coumaric acid, o-coumaric acid, and syringic acid at either 2 mM or 4 mM concentrations. The reaction was initiated by the addition of 0.1 ml enzyme extract. Absorbance readings were made every minute for 10 minutes. The spectrophotometric method was calibrated against the curve in Figure 9 to establish the amount of NADPH produced during the reaction. Additionally, a 0.5 ml of 10,000-fold diluted purified enzyme G-6-PD was added to 2.5 ml assay mixture either without or with the phenolic compounds ferulic acid (6.7×10^{-4} M) or o-coumaric acid (3.3×10^{-4} M) The assay mixture contained 0,5 ml of glucose-6-phosphate (0.01 M), 0.3 ml of NADP (0.002 M), 0.5 ml of $MgCl_2$ (0.02 M), and 0.1 M Tris buffer pH 7.2 (Mukerzi and Ting, 1968). The readings were taken every 30 seconds after enzyme addition for a total of 10 minutes.

Invertase: 10 ml of 6.5% sucrose in 0.01 M acetate buffer pH 4.5 was placed in a tube either without or with 1 ml of phenolic compounds at various concentrations and allowed to stand in a water bath at $32^{\circ}C$ for 15 minutes. One ml of crude enzyme was then added to make a 12 ml total volume for assay. The reaction was allowed to proceed for 30 minutes. Two ml aliquot of the reaction mixture was mixed with 2 ml of 0.1 N NaOH which was used to stop the reaction. For kinetic study of invertase, 0.5 ml of enzyme solution containing 20 µg purified yeast invertase was added to the mixture containing 5 ml of sucrose in 0.01 M acetate buffer pH 4.5 and 0.5 ml of either distilled water or 0.61 M p-coumaric acid. The reaction was allowed to stand at $35^{\circ}C$ for 10 minutes. One ml of the assay mixture was mixed with 1 ml of 0.1 N NaOH stopping the enzyme reaction. Invert sugar was

determined by dinitrosalicylic reagent introduced by Sumner (1935 and 1925) and Todd (1968). In brief, one ml aliquot to be tested for reducing sugars was added with 3 ml dinitrosalicylic reagent in a test tube and allowed to stand for 5 minutes in boiling water. The tube was cooled with running water and diluted with distilled water up to 25 ml. Absorbance was measured with a spectrophotometer. The amounts of invert sugar were calculated in terms of a calibration curve shown in Figure 27.

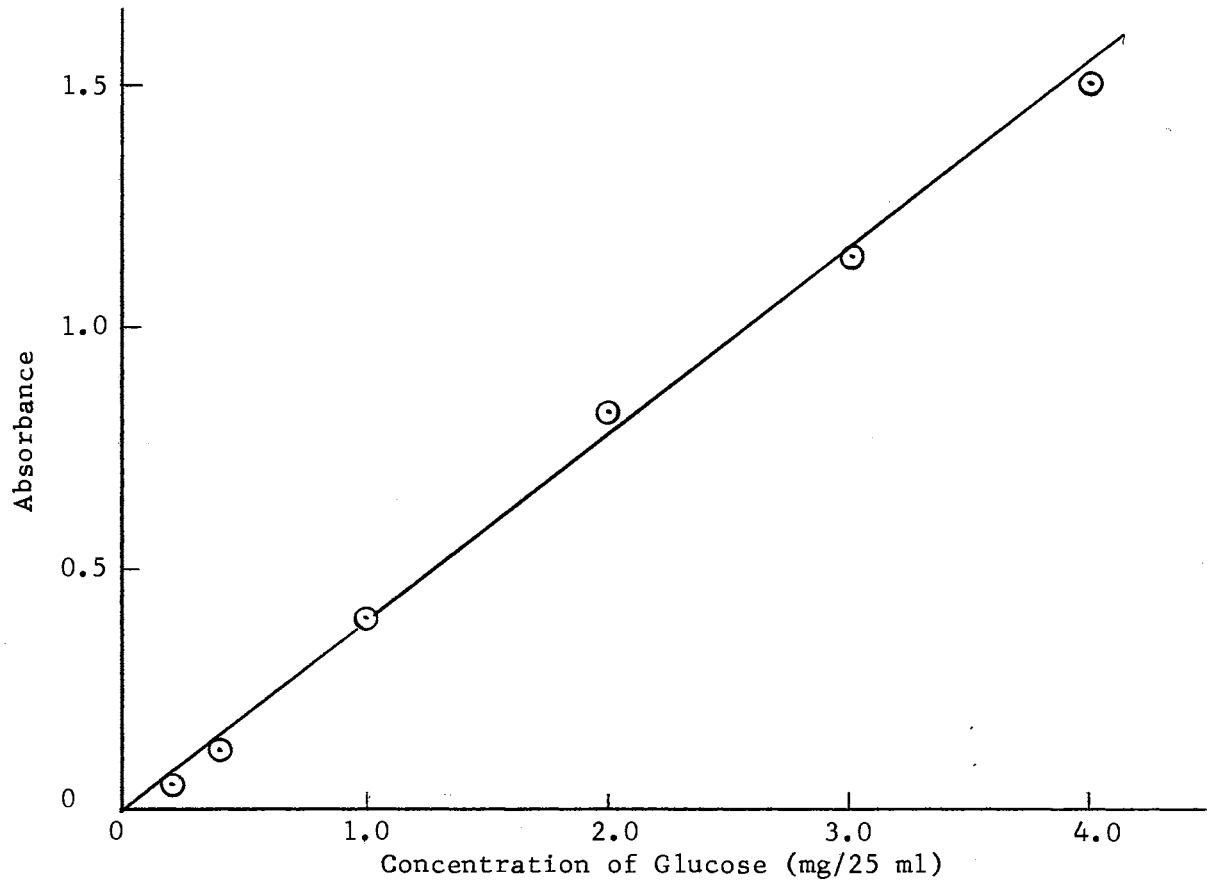


Figure 27. Standard Curve for Reducing Sugar Determination with Dinitrosalicylic Reagent

CHAPTER XII

RESULTS AND DISCUSSION

Phenolic compounds may either play a role as a coenzyme or as an enzyme inhibitor, depending on their concentrations. p-coumaric acid, a major phenolic compound in wheat leaves, is a typical example of a phenolic compound which activates IAA oxidase at low concentration but inhibits the same enzyme at high concentration (Gortner and Kent, 1958). The results from the investigation presented in Table IV show that o- or p-coumaric acid at 0.457 mM causes no significant inhibition of IAA oxidase which has been extracted from either Kanking or Ponca cultivars. At the same concentration, caffeic acid, ferulic acid and syringic acid show a highly significant inhibition of IAA oxidase. Under these assay conditions, the assay mixture contained about 0.3 mM IAA as the substrate of IAA oxidase, 0.075 mM dichlorophenol as coenzyme, 0.075 mM $MnCl_2$ as activator, and the additional compounds at 0.457 mM. o- and p-coumaric acids do not seem to compete with substrate and coenzyme which have been linked to the two active sites on the enzyme apoprotein as proposed by Gortner and Kent (1958). The inhibition by ferulic, caffeic, and syringic acids occurred probably as a competition for either one or both of the active sites with substrate and coenzyme.

G-6-PD catalyzes the oxidation of glucose-6-phosphate to D-gluconolactone-6-phosphate coupled with the reduction of NADP to NADPH. It has been reported that phenolic compounds as well as tannin

TABLE IV

EFFECT OF PHENOLIC COMPOUNDS AT 0.457 mM
ON IAA OXIDASE ACTIVITY OF
WHEAT LEAVES

Source of Enzyme	Phenolic Compounds at 0.457 mM	Relative Activity %	Percentage Inhibition	LSD
Kanking	Control	100	--	
	Caffeic acid	0	100^{**}	$\bar{D}_{5\%} = 8.4420$
	Ferulic acid	2.3	97.7^{**}	
	Syringic acid	4.6	95.4^{**}	$\bar{D}_{1\%} = 11.5641$
	p-Coumaric acid	95.4	4.6^{ns}	
	o-Coumaric acid	98.2	1.8^{ns}	
Ponca	Control	100	--	
	Caffeic acid	1.5	98.5^{**}	$\bar{D}_{5\%} = 5.1705$
	Ferulic acid	0.8	99.2^{**}	
	Syringic acid	4.8	95.2^{**}	$\bar{D}_{1\%} = 7.0827$
	p-Coumaric acid	98.9	1.1^{ns}	
	o-Coumaric acid	103.1	-3.1^{ns}	

substantially inhibit G-6-PD and some other dehydrogenases in vitro (Firenzuoli et al., 1969; Hulme et al., 1964; Goldstein and Swain, 1965; Alberghina, 1964). Primary examination of G-6-PD extracted from wheat leaves was made with the addition of several phenolic compounds. The results in Table V and Figure 28 show that ferulic, p-coumaric, o-coumaric, and syringic acids decreased G-6-PD activity as a percentage of the control at all concentrations from 0.323 mM to 0.556 mM for the last three compound; from 0.323 mM to 0.646 mM for ferulic acid. From these results, it was of interest to determine the mechanism of inhibition. Commercial enzyme G-6-PD from Torula yeast was used for this purpose. Under these assay conditions, the enzyme activity against enzyme concentration showed a linear relation illustrated in Figure 29. A 10,000-fold dilution of enzyme was chosen for kinetic studies. G-6-PD activity was inhibited by ferulic acid at 6.7×10^{-4} M and by o-coumaric acid at 3.3×10^{-4} M concentrations as shown in Figures 30 and 31; Figures 32 and 33. From the double reciprocal plots of reaction rates (μ moles NADP reduced in 10 min.) and substrate concentrations, it appears possible that both ferulic acid and o-coumaric acid acted as competitive inhibitors with NADP and were non-competitive inhibitors with glucose-6-phosphate. G-6-PD apoprotein is composed of two polypeptide chains of 51,000 molecular weight with similar physical properties as indicated by sedimentation equilibrium studies. These two-chain apoenzymes can further dimerize to yield the final NADP-complex consisting of two apoenzyme half-molecules each composed of two subunits (Yeu et al., 1969). An allosteric property of the NADP-enzyme tetramer may be involved in the reaction in the present studies. Phenolic compounds play a role in competing with the coenzyme NADP

TABLE V

EFFECT OF PHENOLIC COMPOUNDS ON GLUCOSE-6-PHOSPHATE DEHYDROGENASE

Compound	0.323 mM		0.556 mM		0.646 mM	
	ΔA_{340} in 10 min.	% Inhibition	ΔA_{340} in 10 min.	% Inhibition	ΔA_{340} in 10 min.	% Inhibition
Ferulic acid	0.130	50.0	--	--	0.030	88.5
p-Coumaric acid	0.110	57.7	0.065	69.8	--	--
o-Coumaric acid	0.165	36.5	0.110	48.8	--	--
Syringic acid	0.225	13.5	0.170	20.9	--	--
H_2O	0.260	0	0.215	0	0.260	0

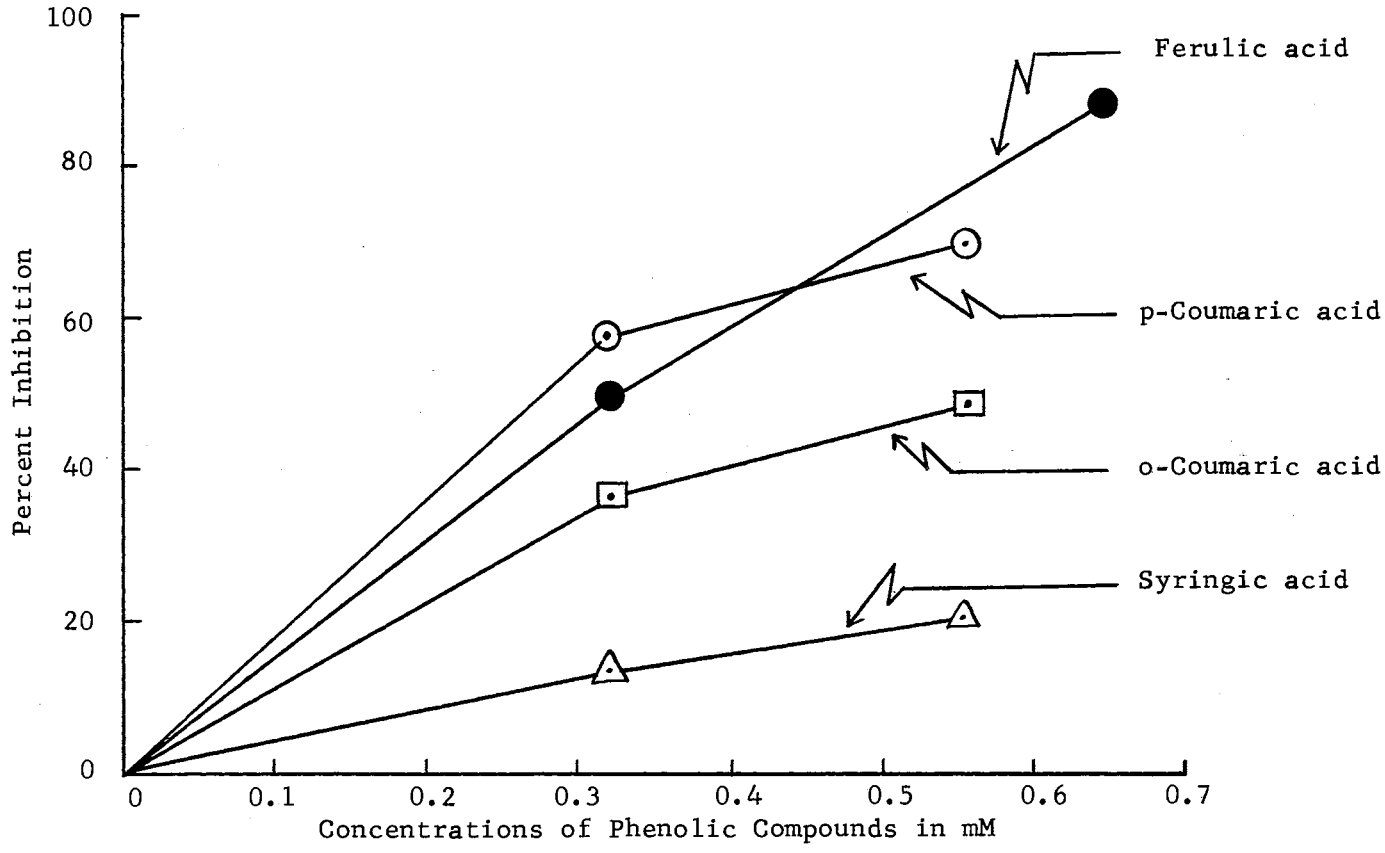


Figure 28. Percent Inhibition of Glucose-6-Phosphate Dehydrogenase by Various Phenolic Compounds. Composition of Assay Mixture Described in Text.

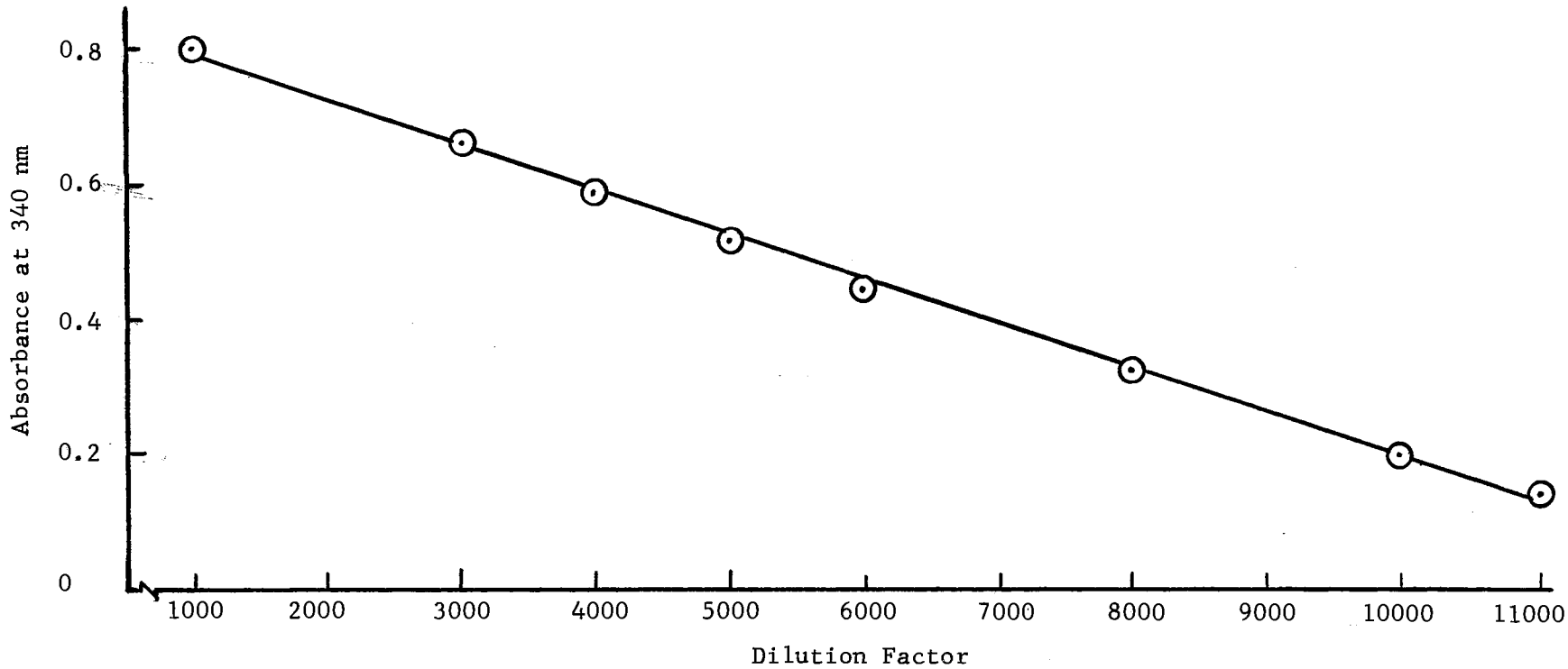


Figure 29. The Activity of Glucose-6-Phosphate Dehydrogenase as a Function of Enzyme Concentration. 0.5 ml of diluted enzyme was added to a cuvette with 3 ml mixture containing 0.5 ml of 0.02 M $MgCl_2$, 0.3 ml of 0.002 M NADP, 0.5 ml of G-6-PD 0.01 M and 1.2 ml of 0.1 M Tris buffer pH= 7.2.

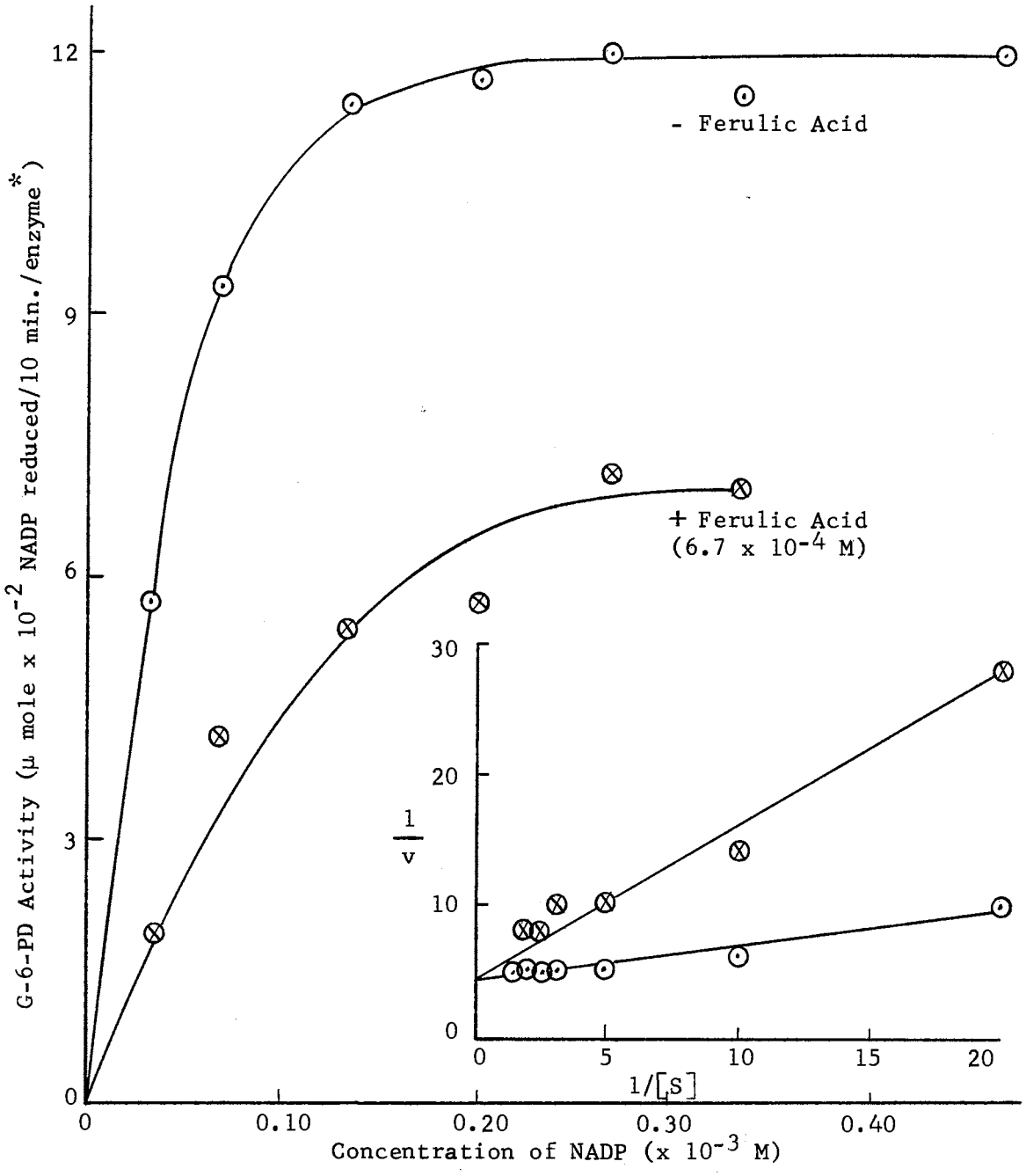


Figure 30. The Activity of Glucose-6-Phosphate Dehydrogenase as a Function of NADP Concentrations in the Presence and Absence of Ferulic Acid, and the Double Reciprocal Plot

*Enzyme Equivalent to 0.5 ml of 10,000-fold Diluted Yeast G-6-PD.

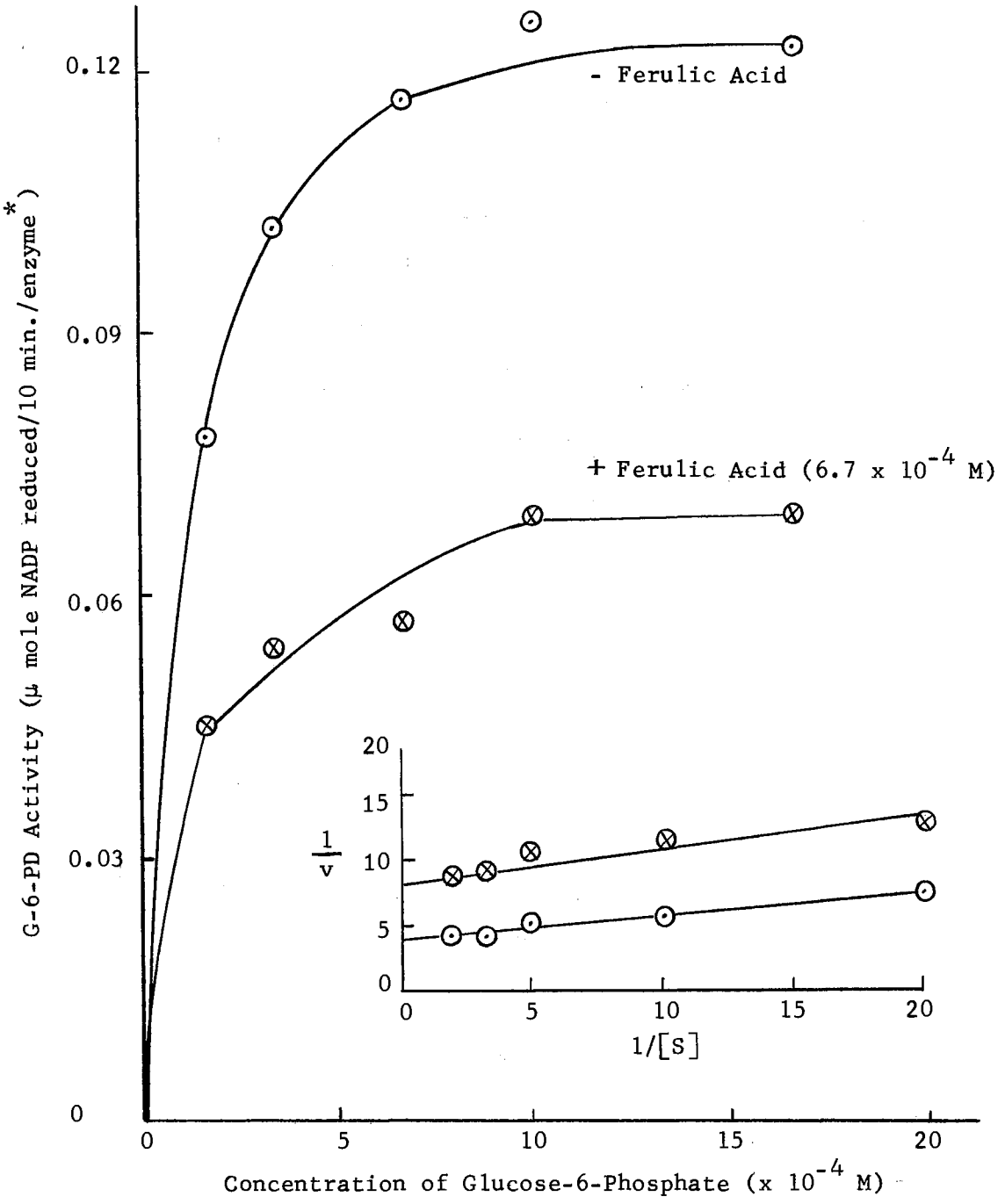


Figure 31. The Activity of Glucose-6-Phosphate Dehydrogenase as a Function of Glucose-6-Phosphate Concentrations in the Presence and Absence of Ferulic Acid, and the Double Reciprocal Plot

*Enzyme equivalent to 0.5 ml of 10,000-fold diluted yeast G-6-PD in each assay.

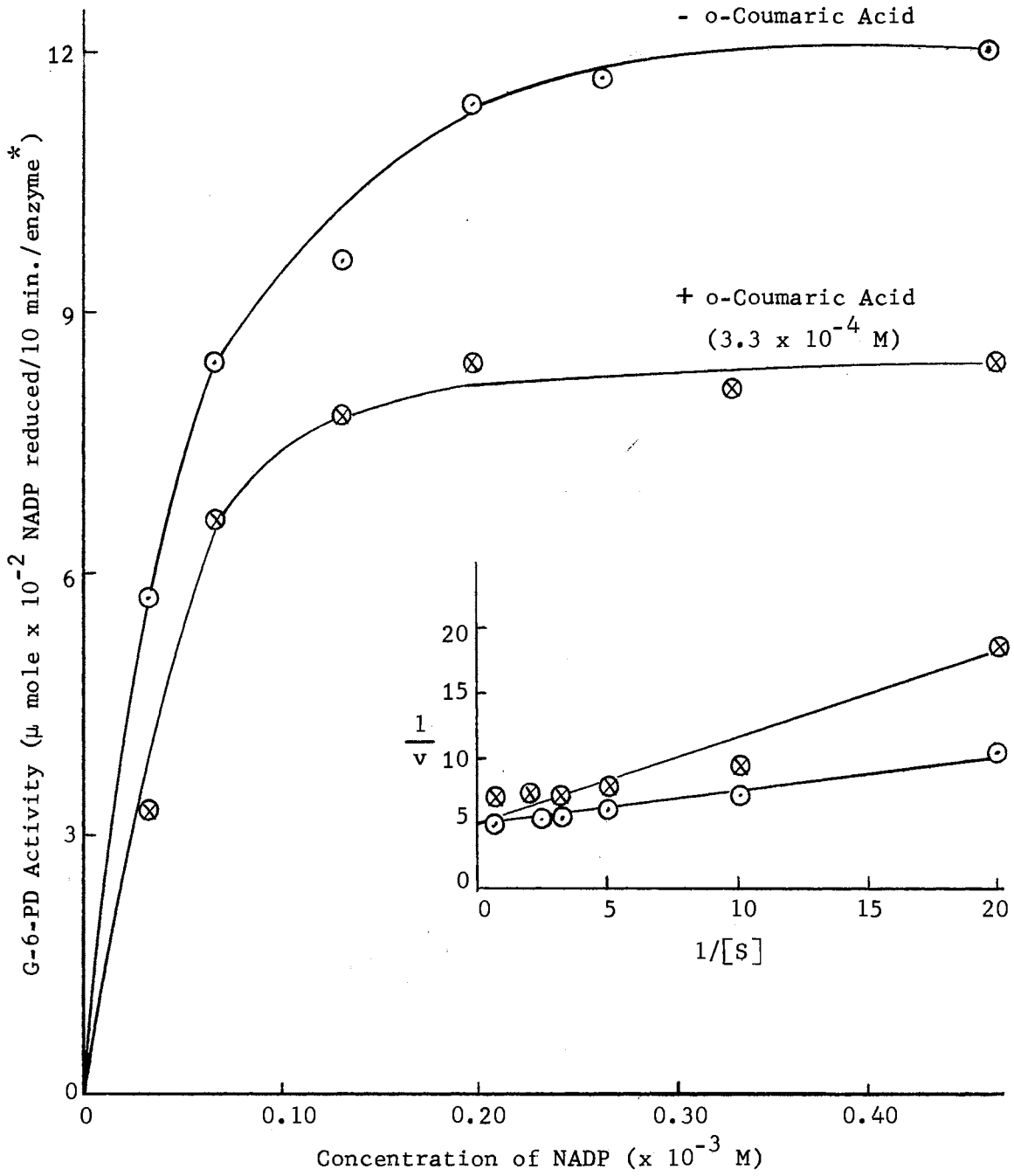


Figure 32. The Activity of Glucose-6-Phosphate Dehydrogenase as a Function of NADP Concentrations in the Presence or Absence of o-Coumaric Acid and the Double Reciprocal Plot

*Enzyme equivalent to 0.5 ml of 10,000-fold diluted yeast G-6-PD.

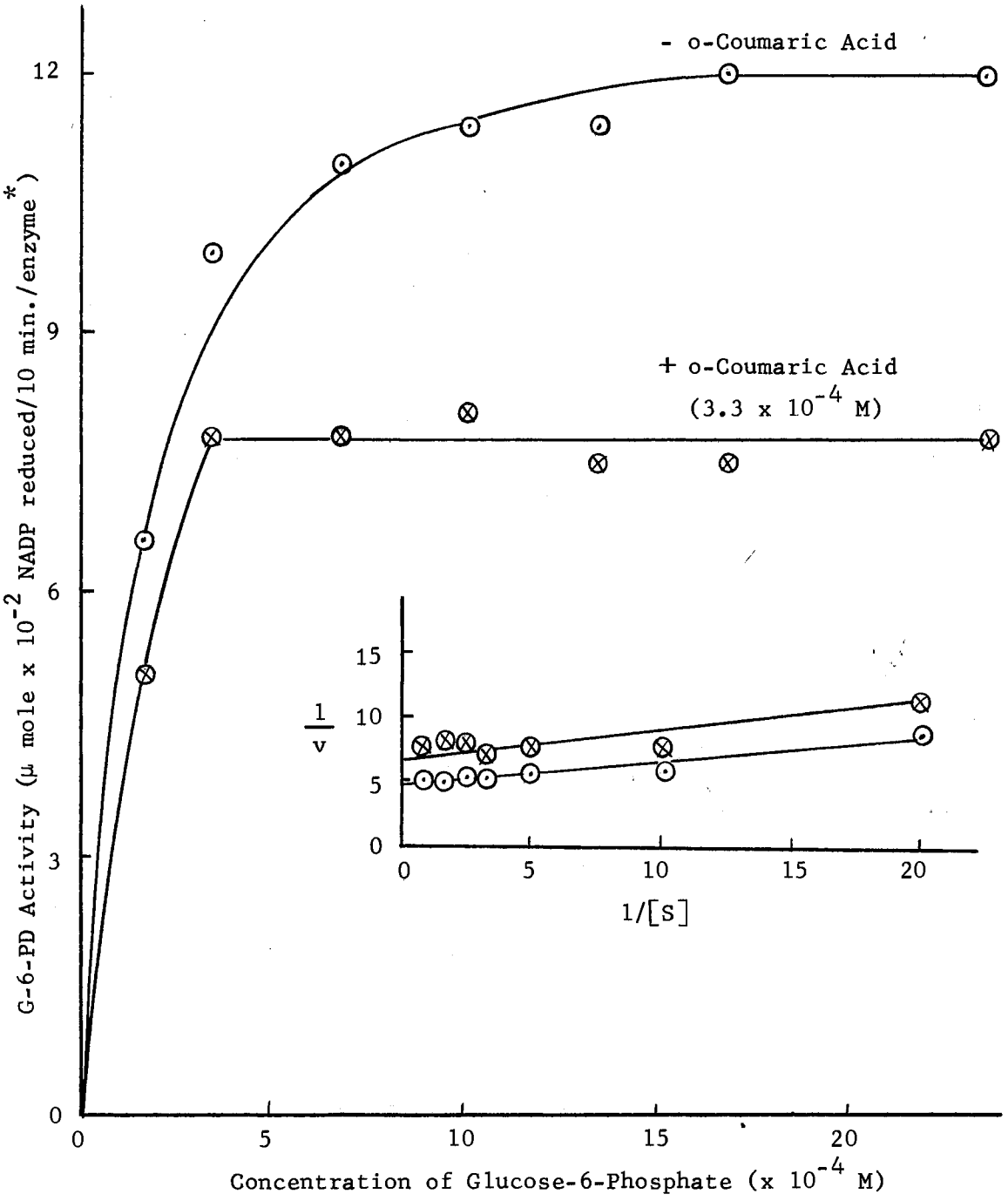


Figure 33. The Activity of Glucose-6-Phosphate Dehydrogenas as a Function of Glucose-6-Phosphate Concentration in the Presence and Absence of o-Coumaric Acid, and the Double Reciprocal Plot

*Enzyme equivalent to 0.5 ml of 10,000-fold diluted yeast G-6-PD.

at one active site and to irreversibly bind to the second active site which normally binds with the substrate, glucose-6-phosphate. The inhibition of enzyme activity due to the blockage of the NADP active site can be overcome by the addition of more NADP, while non-competitive inhibition of G-6-PD by the phenolic compounds used is irreversible through substrate addition.

Earlier work on G-6-PD by Chefurka (1956) and Asante and Neal (1964) indicated that many sulfhydryl reagents were able to influence enzyme activity. They suggested that the G-6-PD enzyme requires free SH groups for activity. The oxidation of SH group(s) into disulfide groups results in a loss of enzymatic activity (Levitt, 1962). Quinones can act as an oxidizing agent and they are products of the oxidation of phenolics (Alberghina, 1964; Smith and Lester, 1961). Furthermore, oxidized chlorogenic acid as well as p-hydroxymercuri-benzoate, the typical -SH group inhibitor, were completely inhibitory to G-6-PD. The inhibitory effect of oxidized chlorogenic acid on the enzyme was prevented by NADP (Alberghina 1964). This indicates that -SH group(s) are very important for G-6-PD activity. Since a high affinity of sulfhydryl compounds for quinones was found (Alberghina, 1964; Slack, 1966), a decrease of G-6-PD activity in the presence of phenolic compound might therefore, be attributed to the inactivation of the -SH group(s). Another mechanism for the inhibition might be that phenolic compounds either directly combine with protein to form a protein-phenolic complex (Hulme et al., 1964; Jones et al., 1965) which would inactivate many enzymes by altering their tertiary structure (Sanderson, 1965) or bond with $-NH_2$ or = NH groups on the apoenzyme (Loomis and Battaile, 1966; Alberghina, 1964).

β-D-fructofuranoside fructohydrolase (EC 3.2.1.26) (trivial name β-fructofuranosidase—formerly known as invertase, sucrase, saccharase etc.) was extracted from wheat leaves or from yeast (Calbiochem) for the following investigation. At first, invertase extract from wheat leaves in the presence of o-coumaric acid from 8.3×10^{-4} M to 33.2×10^{-4} M concentration was inhibited proportionally from 22.2% to 74.1% of the control (Table VI). For further studies, yeast enzyme was employed. It was found that p-coumaric acid at 5×10^{-2} M inhibited 74.2% of invertase activity (Table VI). The percentage of inhibition calculated from Table VI against concentration of o-coumaric acid shows approximately a linear relation (Figure 34). Previous reports of inhibition of invertase by coumaric acids has not been found. However, pyrocatechol sprayed on the foliage of mature sugar beet significantly reduced invertase activity subsequently extracted from the leaves (Singh and Wort, 1970).

For kinetic study, 20 μg of purified yeast invertase was chosen to be used for each assay. The result as shown in Figure 35 indicates the inhibition can be partially overcome by an addition of sucrose (Figure 35). A Lineweaver-Burk plot of the reciprocal of the reaction rate against the reciprocal of the concentration of substrate indicates that p-coumaric acid is a competitive inhibitor. The Km (Michaelis constant) obtained from this study is 26.7 mM at pH 4.5 which agrees with the results of Josephson and later Myrback and Bjorklund cited by Arnold (1965) showing that 26 mM for a Km value was constant between pH 4.0 and pH 8.0 for yeast β-fructofuranosidase and 129 mM was found at pH 2.0. Additionally, an acid invertase (optimum pH 4.5) partially purified from the root of carrot had a Km of 56 mM (Ricardo and Rees,

TABLE VI

INHIBITION OF INVERTASE BY PHENOLIC COMPOUNDS

Concentration of Inhibitors	Convert Sugar Produced	Inhibition Percentage
o-coumaric acid*		
None	3.24 mg	0
8.3×10^{-4} M	2.25	22.2
16.6×10^{-4} M	1.68	48.1
24.9×10^{-4} M	1.44	55.6
33.2×10^{-4} M	0.84	74.1
p-coumaric acid**		
None	3.72	0
5.0×10^{-2} M	0.96	74.2

*Test on crude enzyme extracted from Ponca leaves in the amount of enzyme equivalent to 0.21 gm FW in 30 min.

**Test on the yeast enzyme containing 20 γ per assay.

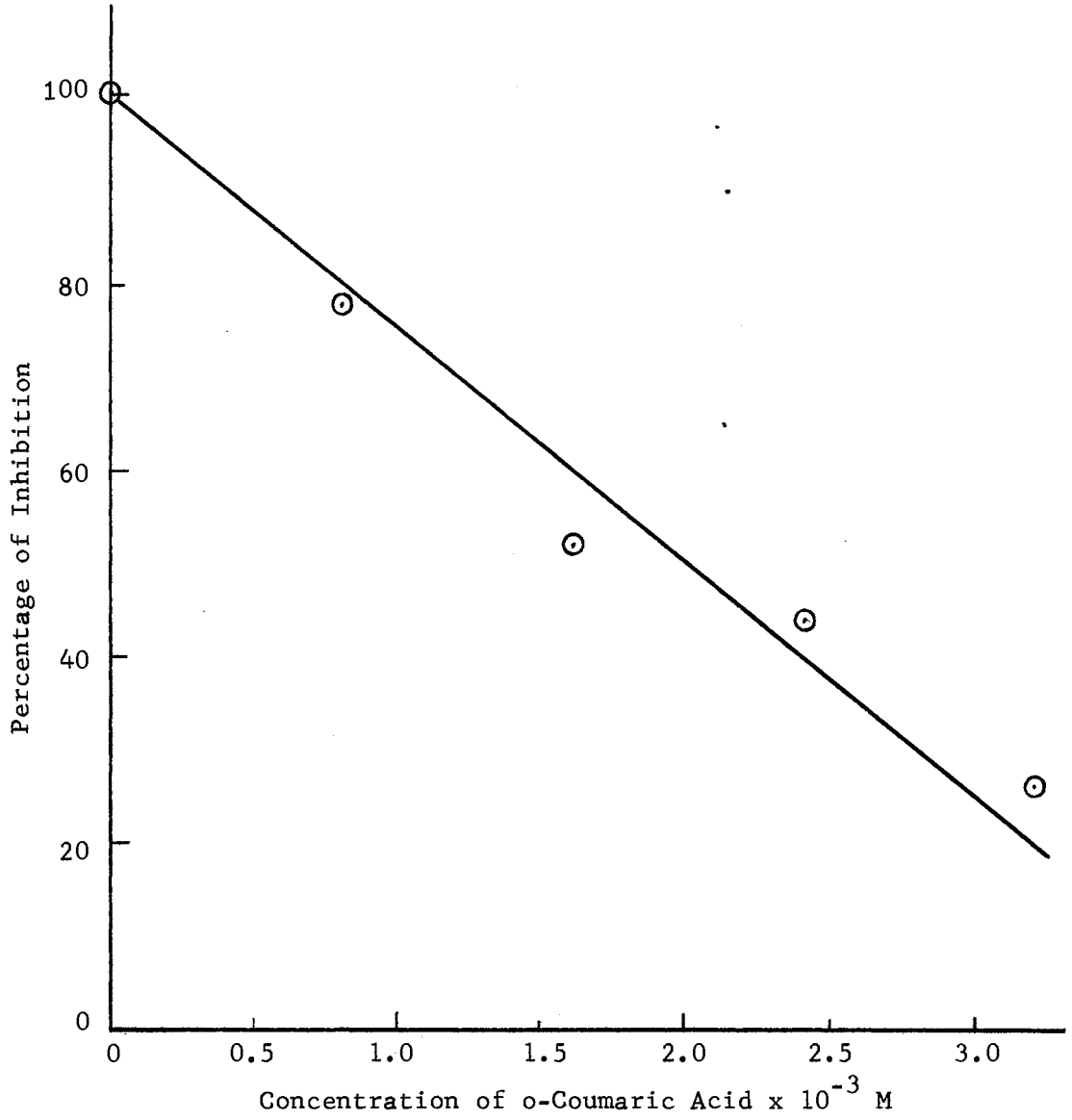
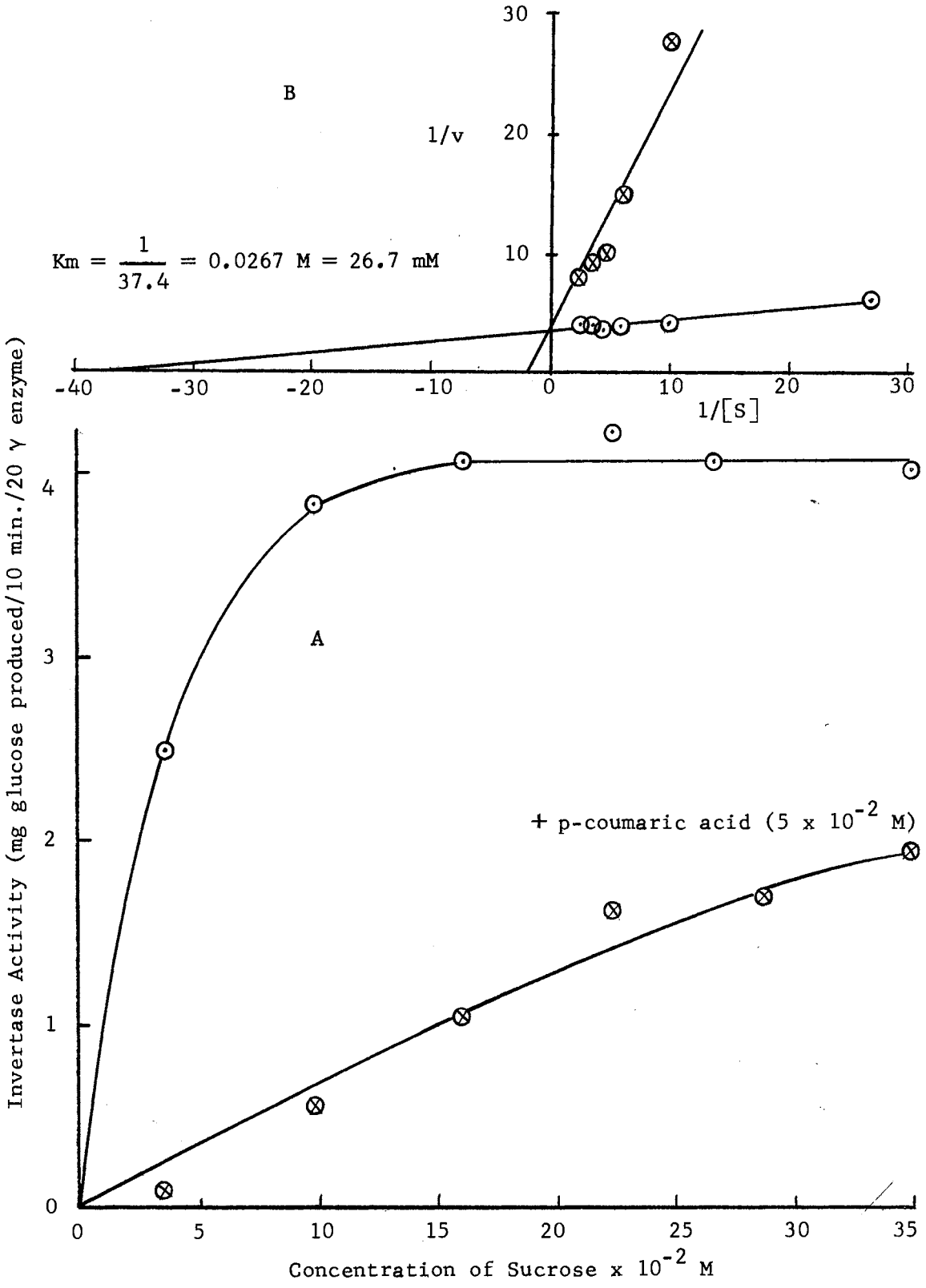


Figure 34. Percent Inhibition of Invertase as a Function of Concentrations of o-Coumaric Acid

Figure 35. A. Invertase Activity as a Function of Sucrose Concentration in the Presence and Absence of Phenolic Compound (5×10^{-2} M)

B. Double Reciprocal Plot of the Activity of Invertase in the Presence and Absence of Phenolic Compound


B
1/v
30
20
10
$Km = \frac{1}{37.4} = 0.0267\ M = 26.7\ mM$
-40
-30
-20
-10
0
10
20
30
1/[S]
A
+ p-coumaric acid (5×10^{-2} M)
Invertase Activity (mg glucose produced/10 min./20 γ enzyme)
0
1
2
3
4
0
5
10
15
20
25
30
35
Concentration of Sucrose x 10^{-2} M

1970). The Km value for other invertases were 4 mM for sucrose and 22 mM for raffinose with grape berries (Arnold, 1965); 4 mM for sucrose with wheat leaves (Ward, 1953); 6 mM with maize radicle (Hellebust and Forward, 1962); and 13 mM and 25 mM respectively for sugar-cane acid and neutral invertase (Hatch et al., 1963). With the enzyme from Phaseolus vulgaris, the Km value was 2.4 mM on sucrose and 14 mM on raffinose (Cooper and Greenshields, 1964). It is noted that the Km for the bean β-fructofuranosidase acting on sucrose is about seven fold smaller than that for the yeast enzyme (Kuhn's work cited by Cooper and Greenshields, 1964). Thus most of the invertases from higher plants possess a higher affinity for the substrate than that from yeast. The comparisons of Km values among different sources of enzymes, may have involved a possibility that invertases with different chemical structures from different sources are responsible for different properties of catalysis.

Iodoacetamide is well known as an inhibitor of enzymes which rely on a free -SH group for their activity. In many cases, iodoacetamide at 10 mM was inhibitory to invertase obtained from either grape berries (Arnold, 1965) or bean plants (Cooper and Greenshields, 1964). This indicates that an -SH group(s) is involved in the enzyme activity. Phenolic compounds such as o-coumaric acid, p-coumaric acid (see Table VI and Figure 34), pyrocatechol (Singh and Wort, 1970) affecting the changes of enzyme activity may be in part correlated with the functional -SH group(s) inactivated but it may be more important in competing with sucrose for the substrate-binding site (Fig. 35). However, in potato tuber, an inhibitor (Schwimmer et al., 1961; Pressey, 1966) which showed non-competitive inhibition to invertase is not the

same type as p-coumaric acid. This unique inhibitor obtained from potato tuber through partial purification has been reported as a protein with about 17,000 molecular weight which did not inhibit yeast, Neurospora and several other plant invertases but completely inhibited potato tuber invertase (Pressey, 1967). This evidence confirms the speculation that invertases from different sources have different enzyme properties.

CHAPTER XIII

SUMMARY

Cytologically the vacuoles of a cell contain many secondary metabolites separated from the cytoplasm by a single layer of tonoplast. Relatively large quantities of phenolic compounds that change with age or with senescence would be released to attack enzyme systems in the cytoplasm when the tonoplast in the cells is broken down by dehydration or some other agents. Those phenolic compounds found in wheat leaves are mainly p-coumaric acid, ferulic acid, syringic acid, vanillic acid, and p-hydroxybenzoic acid. Several of these were used to test the response of enzymes including IAA oxidase, G-6-PD and invertase in vitro.

Caffeic acid, ferulic acid and syringic acid at 0.547 mM powerfully inhibited IAA oxidase, while at the same concentration, p-coumaric and o-coumaric acids did not affect the activity significantly.

G-6-PD extracted from wheat leaves appeared to be inhibited to a certain extent by ferulic acid, p-coumaric acid, o-coumaric acid, and syringic acid. Kinetic studies on yeast G-6-PD showed that both ferulic acid and o-coumaric acid acted similarly as competitive inhibitors of the coenzyme-linking active site as well as the role of non-competitive inhibitor of the substrate-linking site. It was suggested that G-6-PD is an allosteric enzyme which can be attacked by inhibitors at several different sites and that its functional -SH group(s) might

be inactivated by phenolic compounds.

Inhibition of invertase extracted from wheat leaves was proportionally increased with increasing concentrations of o-coumaric acid from 8.3×10^{-4} M to 3.3×10^{-3} M. Also about 74% of yeast invertase activity was inhibited by 5×10^{-2} M of p-coumaric acid. The data from the kinetic studies showed that p-coumaric acid was a competitive inhibitor with sucrose in yeast invertase. The Km value of 26.7 mM obtained in this investigation indicates a low affinity between sucrose and invertase.

Thus, the results support the concept that phenolic compounds released from the vacuole may participate in acceleration of senescence by inhibiting the activity of cell enzymes.

CHAPTER XIV

GENERAL SUMMARY AND CONCLUSIONS

Water stress brings about marked changes in metabolic processes. In response to water stress protein catabolism increases over anabolism. The higher molecular weight proteins were broken down into the smaller molecular weight proteins and other hydrolytic products so that the percentage of high molecular weight proteins was decreased and the percentage of small molecular weight proteins was increased as the result of water stress. This shifting from protein construction to protein destruction may possibly be regulated by endogenous plant hormones which have been shown to change their activity during water stress. Using exogenous growth regulatory chemicals in the culture medium, it was found that both kinetin and CCC were capable of maintaining high levels of the large molecular weight proteins; GA acted in an opposite manner and dramatically accelerated the degradation of large molecular weight proteins. IAA treatment did not affect the breakdown normally caused by water stress; cycloheximide application obviously resulted in a low protein content in wheat leaves. It suggests that the application of kinetin and CCC might be able to decrease the susceptibility of wheat plants under drought conditions, while the application of GA might bring about more susceptibility of wheat plants to drought.

Water stress also brought about changes in enzyme levels. Glucose-6-phosphate dehydrogenase was found to be very susceptible to drought

stress. Endogenous levels of glucose-6-phosphate dehydrogenase were higher in Kanking leaves than in Ponca leaves, which may be positively correlated with the number of isoenzymes in the same tissues. Electrophoretic zymogram patterns of glucose-6-phosphate dehydrogenase indicated that there are three isozymes in the leaf blades and only two in the crown tissues of Kanking, while there are two isozymes in the leaf blades and only one in the crown tissues of Ponca. This intra-specific variation and organ-specificity of G-6-PD is compatible with the belief that the synthesis of isoenzymes in tissues is under genetic control during plant differentiation or development. According to the electrophoretic observations, the reduction of G-6-PD activity as the result of water stress is probably a result of quantitative rather than qualitative changes in enzyme.

The application of GA and cycloheximide to wheat cultivar Ponca resulted in an increase of G-6-PD activity in the GA treatment and a decrease in cycloheximide treatment. However, GA and cycloheximide in vitro did not affect the enzyme activity. Moreover, both chemicals applied to the plants did not protect the enzyme from reduction of activity caused by water stress. It is, therefore, suggested that the differences of G-6-PD activity in the treatments are due to the changes of internal levels of enzyme by the regulation by GA and cycloheximide on the de novo synthesis system.

On the other hand, IAA oxidase activity was found to be appreciably increased at initial stage of water stress and then decreased at greater water deficits. The application of GA at 10^{-4} M resulted in a consistently higher level of IAA oxidase than the untreated control during water stress.

The higher endogenous level of IAA oxidase in Ponca than in Kanking is suggested to be in part involved in the difference in drought tolerance between the two cultivars.

Internal water retention capability was also found to be higher in Kanking than in Ponca under several degrees of water stress. This is positively correlated with the known drought hardiness of both cultivars.

Compartmentalization of the secondary metabolites which are separated by the tonoplast from the cytoplasm is of considerable importance in an active cell. From the review of literature, it appears that the vacuoles of the mature plant leaves contain relatively large quantities of phenolic compounds that change in both quantity and quality with age or when plants are exposed to drought stress. Breakdown of the tonoplast membrane during drought stress would release the phenolic compounds into the cytoplasm. The effect of these compounds on several cytoplasmic enzymes was examined. IAA oxidase was almost completely inhibited by caffeic acid, ferulic acid and syringic acid at 0.457 mM, but not significantly changed by p-coumaric acid and o-coumaric acid. G-6-PD from wheat leaves was inhibited with ferulic acid by 50% and 88% at concentrations of 0.323 mM and 0.646 mM respectively. It was also substantially inhibited by p-coumaric acid, o-coumaric acid and syringic acid. Based on kinetic studies, both phenolic compounds, ferulic and o-coumaric acid inhibit the reaction catalyzed by G-6-PD. This inhibition was shown to be competitive for NADP and non-competitive for glucose-6-phosphate. It is, thus, suggested that G-6-PD is an allosteric enzyme that can be attacked by inhibitors from several directions causing the inactivation of the enzyme.

o-Coumaric acid tested *in vitro* was found to be an effective inhibitor to the invertase which was isolated from wheat leaves. The inhibition was proportionally increased with increasing the concentration of inhibitor from 8.3×10^{-4} M to 3.3×10^{-3} M. p-Coumaric acid at 5×10^{-2} M inhibited the yeast invertase activity by 74%. A kinetic investigation of the inhibition mechanism showed that p-coumaric acid is a competitive inhibitor with sucrose to the enzyme. The determined Km value was 26.7 mM for sucrose as substrate with enzyme commercially prepared from yeast. The affinity of the yeast enzyme for substrate is quite lower than that of higher plant invertases given in the literature.

Thus, the senescence of plant tissue caused by water stress might be due to *in vivo* inhibition by phenolic compounds.

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VITA

Sing-dao Tsai

Candidate for the Degree of

Doctor of Philosophy

Thesis: CHANGES OF SOLUBLE LEAF PROTEINS AND ENZYME ACTIVITY IN TRITICUM AESTIVUM L. IN RESPONSE TO PLANT GROWTH REGULATORS, WATER STRESS AND PHENOLIC COMPOUNDS

Major Field: Botany

Biographical:

Personal Data: Born at Chia-yi, Taiwan, October 23, 1937, the son of Mr. and Mrs. Pi Tsai.

Education: Graduated from Provincial Chia-yi Agricultural Vocational High School, Chia-yi, Taiwan, 1956; received the Bachelor of Science degree from National Taiwan University, Taipei, Taiwan, in June, 1961 and the Master of Science degree from the same University, in June, 1965; completed requirements for the Doctor of Philosophy degree at Oklahoma State University, in May, 1972.

Professional Experience: Taught in grade school, 1956-1957; served as a second lieutenant (ROTC) in Chinese Army, 1961-1962; Agronomist in Dept. of Agricultural and Forestry, Taiwan Provincial Government, 1962-1963; research associate in Taiwan Agricultural Research Institute, Taipei, Taiwan, 1965-1966; graduate teaching assistant, summer, 1968, graduate research assistant, 1968-1970 and research technician, 1970-1972, at the Department of Botany, Oklahoma State University.

Member: Chinese Agronomy Association; Society of Sigma Xi.