THE UNIVERSITY OF MANITOBA

STUDIES ON SOME ENZYME SYSTEMS INVOLVED IN NITROGEN METABOLISM OF WHEAT LEAVES

A THESIS

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ABSTRACT

The effect of benzimidazole and rust infection on wheat leaves led to quantitative changes in certain enzymes involved in amino acid metabolism. Benzimidazole increased theactivity of glutamic decarboxylase in detached wheat leaves. Rust infection increased the glutamic-aspartic transaminase activity but showed little effect on the alanine-glycine and alanine-glyoxalate transaminase. Pyridoxal phosphate increases the activity in rust infected leaves indicating that it is possibly utilized by the fungus in its nutrition. Transaminase activity is higher in induced resistant plants than in susceptible plants.

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INTRODUCTION

In studies of host parasite relations of higher plants, the nature of resistance and susceptibility to disease and of host-pathogen specificity has always presented intriguing problems in plant pathology. Plant breeders are continually developing new varieties of plants in order to cope with potential virulent strains of rust. Resistance and susceptibility of the new varieties is based on their genotype. A plant may be resistant because of its own genes for resistance or alternatively through lack of any of the genes for virulence in the parasite (Flor, 1946, 1947). Susceptibility then results from an interaction between specific gene products of the host and of the parasite. Person (1959) demonstrated that a gene-for-gene relationship is operative in host-parasite systems. He suggests that incomplete dominance in both host and parasite could possibly explain the range in host-parasite reaction that is found under natural conditions.

The course of action of resistant genes results in different metabolic patterns in the host plant. Since metabolic reactions are controlled by specific enzymes, the differences between susceptible and resistant plants may be reflected in their enzyme-coenzyme relationships. Smith (1959) has shown that the activities of glutamic decarboxylase, glutamic-aspartic transaminase and glutamic dehydrogenase are all profoundly altered in susceptible sunflower cotyledons as a result of rust infection. In one experiment, benzimidazole (a chemical that induces resistance in detached Khapli wheat leaves) was shown to prevent the deterioration of glutamic decarboxy-

lase which normally occurs in detached sunflower cotyledons. Although benzimidazole is unable to induce resistance in detached sunflower cotyledons, it has a profound effect on the rust reaction in detached Khapli wheat leaves.

Accordingly, this thesis consists of a study of the enzyme-coenzyme patterns in relation to (a) the effect of rust infection and (b) the effect of benzimidazole in wheat leaves.

LITERATURE REVIEW

The study on physiology of parasitism requires a full understanding of the alterations in certain metabolic pathways brought about by the interaction of host and parasite. In this review, the physiological aspects of host-parasite relationships will be described with particular emphasis on nitrogen metabolism.

BREAKDOWN OF RESITANCE BY CHEMICAL TREATMENT

The breakdown of resistance due to chemical treatments has facilitated the study of obligate parasites such as rusts in relation to their hosts. Khapli wheat (Triticum dicoccum Schubler) which has been used extensively in these studies, is normally resistant to wheat stem rust but can be made susceptible by various treatments. Several resistant varieties become less resistant to stem rust of wheat by treatment with growth inhibitors such as maleic hydrazide (Bromfield and Peet, 1954; Livingston 1953; Lyles et al. 1959; Samborski and Shaw 1957; Forsyth and Samborski 1958; Samborski et al. 1960). Induced susceptibility by maleic hydrazide is of obvious significance in studies on host parasite relations even though its precise action on higher plants or on the cereal rusts is not known. Perhaps it acts on the indoleacetic acid system (MacLachlan and Waygood 1956), as a gibberellin (Brian and Hemming 1957) by inhibi ting sulphydryl groups (Hughes and Spragg 1958), or in other ways depending on its concentration and the type of tissue concerned. (Audus and Thresh 1956). The breakdown of resistance has also been brought about

by spraying with DDT (dichlorodiphenyltrichloroethane) (Johnson, 1946; Bromfield and Emge 1958), the use of ionizing radiation (Schwinghamer 1957); and excesses of zinc, manganese and cobalt (Forsyth 1957).

Detached leaf culture has been used in many studies of obligate parasites (see review by Yarwood 1946). It was noted by Person et al. (1957), Samborski et al. (1958) and Wang (1959) that by simple detachment of the leaf and floating on water, susceptibility could be induced while benzimidazole in appropriate concentrations could retain the original rust reaction. The addition of glucose (Samborski et al. 1958) or 5,6-dimethylbenzimidazole (Wang 1959) could nullify the effect. The presence of cobalt ion in the benzimidazole/glucose solution could restore the resistant reaction while alone it had no effect. Nickel ions were found to have a pronounced inhibitory action in rust development (Wang et al. 1958) and are of economic importance in rust control.(Forsyth and Peterson 1958 and 1959).

A consistent feature associated with induced susceptibility of wheat plants is the accumulation of soluble nutrients in the leaf (Forsyth and Samborski 1958; Samborski et al. 1958; and Samborski and Shaw 1957 (IV)). Detached leaves when floated in water show a decrease in protein and an increase in amino acids, amides and alcohol soluble carbohydrates, while leaves floated in benzimidazole show little change in these fractions. Benzimidazole has been shown to act as an antipurine when used on microorganisms (Wooly 1944; Klotz and Mellody 1948) but may also act on other systems in higher plants. (Galston et al. 1953; Hilliman 1955). The action of benzimidazole in this detached leaf system however, may not be

identical to other systems since Wang (unpublished) has shown that the rate of incorporation of labelled glucose into nucleic acid is increased by the presence of benzimidazole. Therefore it is unlikely that benzimidazole acts as an antipurine.

Wang and Waygood (1959) showed that benzimidazole is capable of stimulating the formation of chlorophyll in detached leaves as well as preventing its destruction in light and in darkness. They speculate that benzimidazole either functions as or can take the place of a natural factor which is necessary for the biosynthesis of chlorophyll. Cobalt and 5,6-dimethylbenzimidazole constitute two major components of a vitamin B_{12} molecule which mediates both nucleic acid and protein metabolism in animal tissues and micro-organisms. (Batzer 1957; Dinning et al. 1958; Schlenk 1955; and Wagle et al. 1958). Wang (1959) suggests that benzimidazole or a derivative of it and cobalt may form a vitamin B_{12}-like factor in plants but as yet its natural occurrence has not been proved. The results of Samborski et al. (1958), Wang 1959, Wang et al. (1960) and unpublished observations by Wang, all point to the idea that benzimidazole maintains the physiology of the detached leaf as if it were attached.

A biochemical explanation of resistance and susceptibility would also be the explanation of the specificity displayed since the problems are the same. Among recent suggestions by workers on cereal rusts[15] the effect of hormone metabolism associated with nutrition of the host (Shaw and Hawkins 1958). Others have emphasized qualitative and quantitative nutritional relationships.

CARBOHYDRATE AND NITROGEN METABOLISM

The development of obligate parasites seems to be closely related to the carbohydrate metabolism of its host (Novikov 1937; Sempio 1942; Allen 1942 and Colatelo 1954). More recent attempts have been made to correlate changes in the rust reaction with changes in endogenous levels of substrate in the host (Forsyth and Samborski 1958; Samborski et al. 1958; Samborski and Shaw 1957; Shaw and Samborski 1956; Wang 1957).

Besides carbohydrate metabolism, nitrogen was generally thought to play an important role since it occupies a central position in nutrients which influence the disease disposition of the host (Doak 1931; Gassner 1931, 1933 and 1934; and Hassibrank 1939; Daly 1949).

Samborski and Shaw (1956 (II)) found that total weight remained constant in heavily rust-infected leaves of Little Club, but the total nitrogen content of heavily infected leaves of Khapli was lower than that of healthy leaves. This lower value of total nitrogen in the infected resistant Khapli was attributed to the rapid losses in dry weight and total nitrogen as a result of proteolysis, followed by transport of the soluble nitrogen to competing "sinks" in other parts of the growing plants. Wang (1957) using isotopic Nitrogen N^{15}, found that rust infection induces a systemic alteration in the nitrogen metabolism of the entire plant, the exact nature of this alteration depending on the disposition of the host to the parasite. He also suggested that the net protein increase in rust infected susceptible wheat could be the result of active enzyme synthesis. A change of protein metabolism in the host as a result of infection by a pathogen may also be accompanied by changes in the free amino acid level (Gessner

and Franke 1938). Samborski (1955) and Colotelo (1954) found a decrease in bound amino acids in rust infected leaves. Also found was a decrease in glutamic and aspartic acid in susceptible barley infected with mildew (Samborski 1955). However, inthe literature are reports of both increases (Drever and Larno 1954) and decreases (Fuchs and Rohringer 1955; Diener and Dekke 1954) of amino acids in infected tissue.

Colotelo (1956) concluded that an increase in amino acid content in infected leaves of Little Club was associated with protein synthesis. He also found a greater amount of ammonia in rust infected leaves of susceptible varieties at advanced stages of infection than in resistant ones. This could be a reduction in glutamine synthesis caused by an increased rate of protein synthesis and low carbohydrate content. Ammonia, which is formed by enzymic reduction of nitrate and during nitrogen fixation, is readily assimilated by plants in the biosynthesis of amino acids (Steward and Pollard 1957; Wood 1953).

ACCUMULATION OF METABOLITIES IN INFECTED TISSUE

In studies on alterations in host composition and metabolism as affected by parasitic infection, it has been ascertained that changes originate in the host cells. The classical respiratory studies of Allen and Goddard (1938) and Allen (1942) led to the view that it was the host rather than the fungus which contributed largely to the difference found when diseased and healthy plant tissues were compared.

Metabolites may be translocated into tissue attacked by an obligate parasite from surrounding healthy tissue. The movement may, however, take

place in reverse direction. Since rust infection depresses photosynthesis (Allen 1942; Kuprevicz 1947; Sempio 1950; Wang 1959) and increases respiration, the transport into infection zones would provide the necessary metabolites.

The degree of development of the parasite and the reaction of the host may be determined by the relative rates of transport into and utilization of metabolites within the infection zone (Shaw and Samborski 1956).

The accumulation of radioactive compounds at the sites of rust infection was first demonstrated by Gottlieb and Garner (1946). There is now a general agreement that an accumulation of various labelled compounds takes place at the infection sites of rust and mildew. (Shaw et al. 1954 and 1956; Yarwood and Jacobson 1950 and 1955; Wang 1960).

Many possible explanations have been put forward to explain the observed selective accumulation in tissues infected with obligate parasites. Shaw and Samborski (1956) attribute this to an active process in the host rather than in the parasite, while other reports (Staples and Ledbetter 1958) suggest that the reverse is true. Some discrepancy has been found in the distribution of radioactivity between primary and secondary leaves (Wang 1960). He attributes this to the difference in their physiological age and hence a difference in the rate of translocation. Also suggested was that actively growing regions of the fungus which have a relatively high rate of metabolism, especially of anabolism possess the highest concentration of the isotope.

Alterations in the level of endogenous indoleacetic acid following infection of cereal leaves with rust and mildew may play a role in regulating

the mobilization of metabolites at infections of obligate parasites (Shaw and Hawkins 1958).

Staples and Ledbetter (1958) have reported that much radioactivity was found in the young uredospores, their sporophores and the distal portions of the mycelium. Shaw and Samborski (1956) suggest that the inhibition of accumulation induced by agents such as dinitrophenol and sulfur is probably through its action on the fungus rather than on the host. However both dinitrophenol and anaerobiosis could interfere with the synthetic process of the fungus and the host as well as the process of translocation (Wang 1960). Thus it seems that fungal metabolic activity and growth of the fungus as well as the process of translocation play an important role in the apparent accumulation at infection sites.

Wang (1960) suggests that, since the difference in the labelling pattern between healthy and rusted tissue sections was mainly found in sugars, the fungus is capable of synthesizing part of its own carbohydrates and does not depend entirely on the host.

INHIBITION OF RUST DEVELOPMENT

Many workers have further investigated changes in the metabolic pathways using inhibitors of rust development. Shu and Tanner (1954) found that fluoride had no inhibitory effect on the respiration of rust spores which metabolize carbohydrates via the pentose phosphate pathway (Shu and Ledingham 1956). Samborski and Forsyth (1960) suggest that the probable inhibitory action on rust growth by fluoride is indirect and through its effect in carbohydrate metabolism in the host. They also show the inhibi-

tion of rust development by certain natural amino acids. Glycine reversed the inhibitory effect of histidine and methionine. Inhibitors can be reversed by other amino acids (Woods and Quacker 1958).

Thiosemicarbazide was also found to cause an inhibition which appeared to result from a change in the metabolism of the host (Fuchs and Bauermeister 1958). An outstanding effect on wheat plants was the marked increase in free amino acids. It is possible that inhibition of the rust was due to excessive levels of certain amino acids.

It has been suggested that an adequate level of essential amino acids is required for normal rust development (Samborski et al. 1958). Results now indicate that the effective concentrations of an amino acid must lie within certain limits relative to those of other amino acids (Samborski and Forsyth 1960). An amino acid may stimulate growth at one concentration and be inhibitory at another.

An explanation of inhbition of rust development by natural amino acids in bacteria was given by Cohen and Rickenberg (1956) in terms of competition for specific permeases. However, Samborski and Forsyth (1960) belive that this is reflected in abnormal amino acid systems by the fungal cell. The dual action of inhibition and nutrition is another possible explanation of the mechanism of resistance or susceptibility of a host to a race of rust. Samborski et al. (1960) indicate that both nutritional and inhibitory factors could be involved in resistance and specificity which conforms with the nutrition-inhibition hypothesis proposed by Garber (1956).

ENZYMES

The role of enzymes concerned with amino acid metabolism has been investigated by many workers.

Isolation of amino acid decarboxylases from micro-organisms and animal tissues (Sumner and Myrback 1951a and 1951b) and bacteria (Gale 1946) have been reported. Since the discovery of an enzyme which was specific for the decarboxylation of glutamic acid by Okunuki (1937) Schales et al. (1946) have shown that the enzyme is widely distributed in the plant kingdom. Active glutamic decarboxylases have been obtained from higher plants by Beevers (1951). Weinberg and Clendenning (1952), Kiraly and Farkas (1957), Rohrlich (1957), Linko and Milner (1959a and 1959b), Cheng et al. (1960) and Smith (1959). The enzyme is universally located in the cytoplasmic fraction of the cell.

Enzymic transaminase reactions were fist demonstrated by Braunstein and Kritzmann (1937) in muscle preparations. Trasamination was later shown to occur in pea plants by Virtanen and Laine (1938) and has now been described in a variety of higher plants (Wilson et al. 1954; Cook 1956; Cruickshank and Isherwood 1958). Only recently has a highly purified glutamic aspartic transaminase been obtained (Jenkins and Sizer 1957; Jenkins et al. 1959).

Transaminases catalysing amino group transfer from natural amino acids have been reviewed by Meister (1955). Although alanine participates in a number of transaminations (Sallach, 1955; Awapara and Seale 1952; Rowsell 1956; Rudman and Meister 1953 and Alternbern 1953) reports of a glyoxylate - alanine transaminase have been rare. Transaminase reactions with glyoxylate and other amino acids however, are wide spread in nature, Commarata and Cohen (1950) and Awapara and Seale (1952) reported an enzymic

transamination between glyoxylate and glutamate in animal tisue while Wilson et al. (1954) demonstrated similar activity in plant tissues. More recently Campbell (1956) observed a transamination of glyoxylate to glycine from alanine in Pseudomonas. An extract of aquatic fungus Blastocladiella emersonii was found by McCurdy and Cantino (1960) to mediate the rapid conversion of glyoxylate to glycine by amino group transfer from alanine. Kinetic studies were made on the purified enzyme and pyridoxal phosphate was not required.

Recent investigations on the control of metabolism by host enzymes show that respiration is stimulated in rust infected wheat (Kiraly and Farkas 1955) and bean tissue (Samborski and Shaw 1956). Person et al. (1958) found the respiration rate lower in treated leaves than in those floated on water. They also noted that the respiratory increase which normally follows detachment (James 1953) does not occur on benzimidazole,

Smith (1959) showed that rust infection is associated with alterations in the activities of certain enzymes involved in the metabolism of glutamic acid. The activity of glutamic decarboxylase from rust infected sunflower cotyledons is considerably decreased but can be reactivated by pyridoxal phosphate. In contrast, the activity of glutamic-aspartic transaminase from rust-infected tissue is markedly increased and is not significantly influenced by pyridoxal phosphate. The activity of these enzymes is not significantly altered in rust-infected resistant sunflower cotyledons. It is suggested that rust infection alters the degree of saturation of coenzyme on these enzymes.

SUMMARY

The many hypotheses proposed by various workers to explain the

phenomena of host parasite association and disease resistance have been made quite obvious in this review. If amino acids are essential to rust development it is possible that rust races may differ in their specific amino acid requirements and the evolution of their importance lends to more careful and extensive studies. The metabolic alterations as a result of infection may be related to the basic enzyme-coenzyme pattern of the cell. An examination of certain enzymes concerned in metabolism especially amino acid metabolism should, therefore, reveal characteristics of resistant and susceptible varieties in relation to obligate parasitism.

MATERIALS AND METHODS

CULTIVATION OF PLANT MATERIAL

Throughout most experiments, leaves of wheat, Triticum dicoccum Schubler var. Khapli, were used. The seeds were sown in a light soil in 6 inch pots and grown under greenhouse conditions. Primary leaves were excised at the early two-leaf stage (9 - 10 days) and used for enzyme preparations.

In experiments where benzimidazole was used, detached leaf culture techniques were employed. Leaves were floated in glass trays containing boiled distilled water (200 ml.) or on benzimidazole solution (100 p.p.m.). The trays were covered with Saranwrap (Dow Chemical Co.) and placed in a growth chamber at 21°C. under continuous illumination (650 ft. c.) for 3 days unless otherwise stated. The leaves were rinsed with distilled water prior to further treatment. Aseptic procedures were employed to minimize contamination by micro-organisms.

INOCULATION OF WHEAT LEAVES

Seedlings of Little Club wheat, Triticum compactum Host which was used as the susceptible host material were inoculated by rubbing a uredospore suspension of stem rust, Puccinia graminis (Pers.) f. sp. tritici. Erikss and Henn (race 15 B-1), on both the upper and lower surfaces. The plants were placed in a moist chamber (100% humidity) for 24 hours and grown for 7 days under greenhouse conditions until infection took place. Inoculated and uninoculated control plants were then detached and used for

the extraction of enzymes.

PROTEIN DETERMINATIONS

Protein determinations in crude extracts were made by classical micro-Kjeldahl procedures and a modification of the biuret method of Gornall et al. (1949), bovine serum albumin being used to obtain the standard curve. Total protein concentrations were determined by measuring the absorbance at 540 mμ.

In purified enzymes, protein was determined from the ratio of absorbance at 280 and 260 mμ according to Warburg and Christian (1941 - 42). Specific activities are expressed as enzyme units per mg. of protein.

ENZYME PREPARATION

Procedures followed in the isolation and purification of different enzymes are described below. Alterations to these methods will be given in the text with the appropriate experiments.

(a) Tris-Carbowax Method.

Khapli wheat leaves (previously described) were washed, chilled and ground with sand in a mortar with 1 gm. of tissue to 4 ml. of macerating medium which was prepared by adding Carbowax 4000 at the rate of 40 g. to 100 ml. 0.05 M Tris (tris (hydroxy methyl) aminomethane) pH 7.0. (Hagerman and Waygood, 1959). The brei was pressed through cheesecloth and the filtrate centrifuged at 3000 x g. for 2 minutes to remove the sand. The residue collected from centrifuging at 20,000 x g. for 10 minutes was resuspended by mechanical trituration in half the original volume of 0.05 M

Tris, 30% Carbowax pH 6 - 6.5. The final precipitate after this purification step was taken up in the same volume of .1 M phosphate pH 5.7. It was again centrifuged and the supernatent used as a source of crude enzyme. An aliquot was dialysed against distilled water overnight. All steps were carried out at 2°C.

(b) Phosphate Method.

Fifteen grams wheat leaves were washed, chilled and ground in a mortar with acid washed sand and 15 ml. of 0.2 M KH_2PO_4, pH 5.7. After squeezing through cheesecloth and centrifuging at 20,000 x g. for 10 minutes, the supernatant was adjusted to a final volume of 30 ml. This represented the crude enzyme.

Aliquots were then dialysed against 2 litres of distilled water at 2°C. for 16 hours and the final volume adjusted to 45 ml. This crude dialysed enzyme was then stored at -10°C.

The supernatant from the first step was brought to 30% saturation with ammonium sulfate by the addition of the solid salt with mechanical stirring. The extract was allowed to stand for 30 minutes, centrifuged at 20,000 x g. for 20 minutes and the precipitate was taken up in half the volume of 0.2 M phosphate pH 5.7. The supernatant solution was brought to 60% saturation with ammonium sulfate by the same technique. The resulting precipitate was collected by centrifugation at 20,000 x g. for 30 minutes and dissolved in half the original volume of 0.2 M phosphate. The ammonium sulfate concentration in this solution was reduced by overnight dialysis at 2° against 2 litres of distilled water. The precipitate which appeared during dialysis if any was discarded.

SPECTROPHOTOMETRIC METHODS.

The determination of glutamic-oxalacetic transaminase activity was made spectrophotometrically by measuring the zero order disappearance of DPNH in the presence of aspartate and ketoglutarate (Karmen, 1955) as outlined in Sigma technical bulletin No. 410. The reaction system contained: 1 ml. enzyme (with high malic dehydrogenase activity), 200 μM phosphate buffer, pH 7.5, 100 μM aspartate and 0.2 mg. reduced diphosphopyridine nucleotide (DPNH). After equilibrium 20 μM of α-ketoglutarate was added. Absorbancies at 340 mμ were measured in a Hilger spectrophotometer using a 1 cm. light path.

QUANTITATIVE DETERMINATION OF AMINO ACIDS

(a) Chromatography

The amino acid components of the transaminase reaction were determined quantitatively by means of one dimensional ascending paper chromatography adapted by Smith (1959) in this laboratory.

Reactions were terminated at the appropriate times with 0.5 ml. of 5% TCA (trichloracetic acid). The precipitate was removed by centrifugation and aliquots (5 μl) of the supernatent applied on Whatman No.1 filter paper. Separation was obtained by developing in the solvent phenol water (4 : 1) containing .05% 8-hydroxyquinoline. The presence of a chelating agent gave good separation of the amino acids as well as good reproducibility of the ninhydrin color. After 6 hours the chromatograms were removed and dried overnight at room temperature.

(b) Amino Acid Assay

Amino acids were located by spraying the chromatograms with 1%

ninhydrin in 95% ethanol and then heating for 30 minutes at 60°C. in an atmosphere saturated with ethanol vapor. Maximum color development took place within 2 hours at room temperature.

The individual colored spots were identified by their known Rf values, cut out and extracted with 5 ml. of 50% ethanol containing 0.025 M phosphate buffer (K_2HPO_4-KH_2PO_4, pH 6.5). Complete elution in most cases was accomplished in 4 to 6 hours but where the spots were intense, overnight extraction at 2°C. was satisfactory without any apparent loss of color.

The absorbances of the ninhydrin complexes were read at 570 mμ on a Hilger spectrophotometer using a 1 cm. light path.

Standard curves for each amino acid were obtained with solutions of known concentrations subjected to the above procedure.

QUALITATIVE DETERMINATION OF KETO ACIDS BY PAPER CHROMATOGRAPHY

(a) Extraction of Keto Acids.

Keto acids were separated and identified by paper chromatography of their 2-4-dinitrophenylhydrazones according to the modified method of Towers (1954) as adapted by Wang (1957). The transaminase reaction system was stopped by the addition of 1 ml. of 0.14% 2,4-dinitrophenylhydrazine (dissolved in 2 N HCl) to convert keto acids to the more stable phenylhydrazones. The protein precipitate was removed by centrifugation and discarded. The supernatent was diluted with approximately 10 ml. water and extracted twice in a separatory funnel with about 25 ml. ethyl acetate.

These extracts were combined, washed with water and the alkali soluble 2,4-dinitrophenylhydrazones then extracted twice with 1% aqueous sodium carbonate (25 ml.). The alkaline extracts (lower phase) were combined and immediately adjusted to pH 2 (approximately) with concentrated HCl (1 ml.), washed and re-extracted twice with ethyl acetate. After washing with water, the ethyl acetate was evaporated to dryness under an air jet at room temperature. The residue was redissolved in 95% alcohol to obtain a final volume of 1 ml.

(b) Assay of Keto Acids

An aliquot (10 µl) of the extract was spotted together with the known keto acid derivatives on Whatman No.1 paper and chromatographed in an ascending manner using tert-amyl alcohol, ethanol and water (9 : 1 : 4) as solvent. A small beaker containing NH_4OH was kept in the center of the solvent chamber. The chromatograms were developed overnight, dried at room temperature, and sprayed with 10% alcoholic sodium hydroxide. The keto acid derivatives were identified on the basis of their characteristic colors and known Rf values.

MANOMETRIC METHODS

The decarboxylation of glutamic and aspartic acid was followed manometrically by measuring CO_2 evolution in standard Warburg respirometers. The flasks contained the enzyme buffered at pH 5.7 with 0.1 M phosphate. Components of a standard reaction system in a total volume of 3.2 ml. were as follows: enzyme 1 ml; 0.1 M KH_2PO_4 pH 5.7 (150 µM) pyridoxal phosphate (2 µM).

After temperature equilibrium had been reached, 0.5 ml. (50 μM) of neutralized l (+) - glutamic acid was added from the side arm. Air or nitrogen (where specified) was used as a gaseous phase and readings were corrected with a control.

No correction was made for retention of carbon dioxide by the buffer since it is negligible at this pH. All experiments were performed at 30°C.

Specific activity is defined as Q_{CO_2} = μl CO_2/mg.protein/10 minutes.

EXPERIMENTAL RESULTS

GLUTAMIC DECARBOXYLASE ACTIVITY IN WHEAT LEAVES FLOATED ON WATER AND ON BENZIMIDAZOLE SOLUTION

(a) General Characteristics and Kinetics of Glutamic Decarboxylase.

The decarboxylation of amino acids is brought about by enzymes specific for the natural L-isomer of different amino acids. Pyridoxal phosphate functions as a prosthetic group in many of these enzymes. According to Schales (1951) in the first step of enzymatic decarboxylation, a condensation takes place between the aldehyde group of pyridoxal phosphate and the α amino group of the substrate. The reaction product, a substituted imino acid would lose CO_2 spontaneously. In the second step the amine would be liberated through hydrolysis or through displacement by a second mole of amino acid.

i. Identification of End Products of Glutamic Acid Decarboxylation.

Carbon dioxide evolution was measured manometrically and the end product, γ-amino-butyric acid was identified by parallel runs and co-chromatography with synthetic acid which gave the same Rf value. This agrees with published results on glutamic decarboxylation in other plants (Beevers 1951; Naylor and Tolbert 1956).

ii. Coenzyme Requirements and pH relationships.

The enzymatic decarboxylation of glutamic acid, catalysed by

dialysed wheat leaf extracts was stimulated by the addition of cofactor pyridoxal phosphate. There was, however, considerable activity in the absence of a cofactor (Figure 1).

The effect of coenzyme upon initial reaction velocity was tested with varying concentrations of pyridoxal phosphate added to the system. The velocity of the reaction increases with the pyridoxal phosphate concentration up to about 4 µg. (Figure 2). At this level the reaction velocity is about 50% greater than that observed in the absence of added coenzyme.

A first order equation is followed only if pyridoxal phosphate is added in excess. In its absence, a time activity curve (Figure 6) is characterized by a rapid decrease in reaction velocity with time.

Highest activity for glutamic acid decarboxylase has been shown in previous investigations (Schales et al. 1946; Hood 1954 and Smith 1959) to be at pH 5.7 - 5.8.

iii. Effect of Substrate Concentrations

The variation of initial reaction velocities with different concentrations of glutamate was determined in phosphate buffer pH 5.7 used for routine assays. A typical set of progress curves for the decarboxylation of variable amounts of glutamic acid (3 - 40 µM) is shown in Figure 3. Distinctive features which should be noted are firstly, the lag phase which preceeded decarboxylation probably due to the incomplete removal of endogeneous inhibitors and secondly, the rates of CO_2 evaluation increase linearly with increasing concentrations of glutamic acid up to

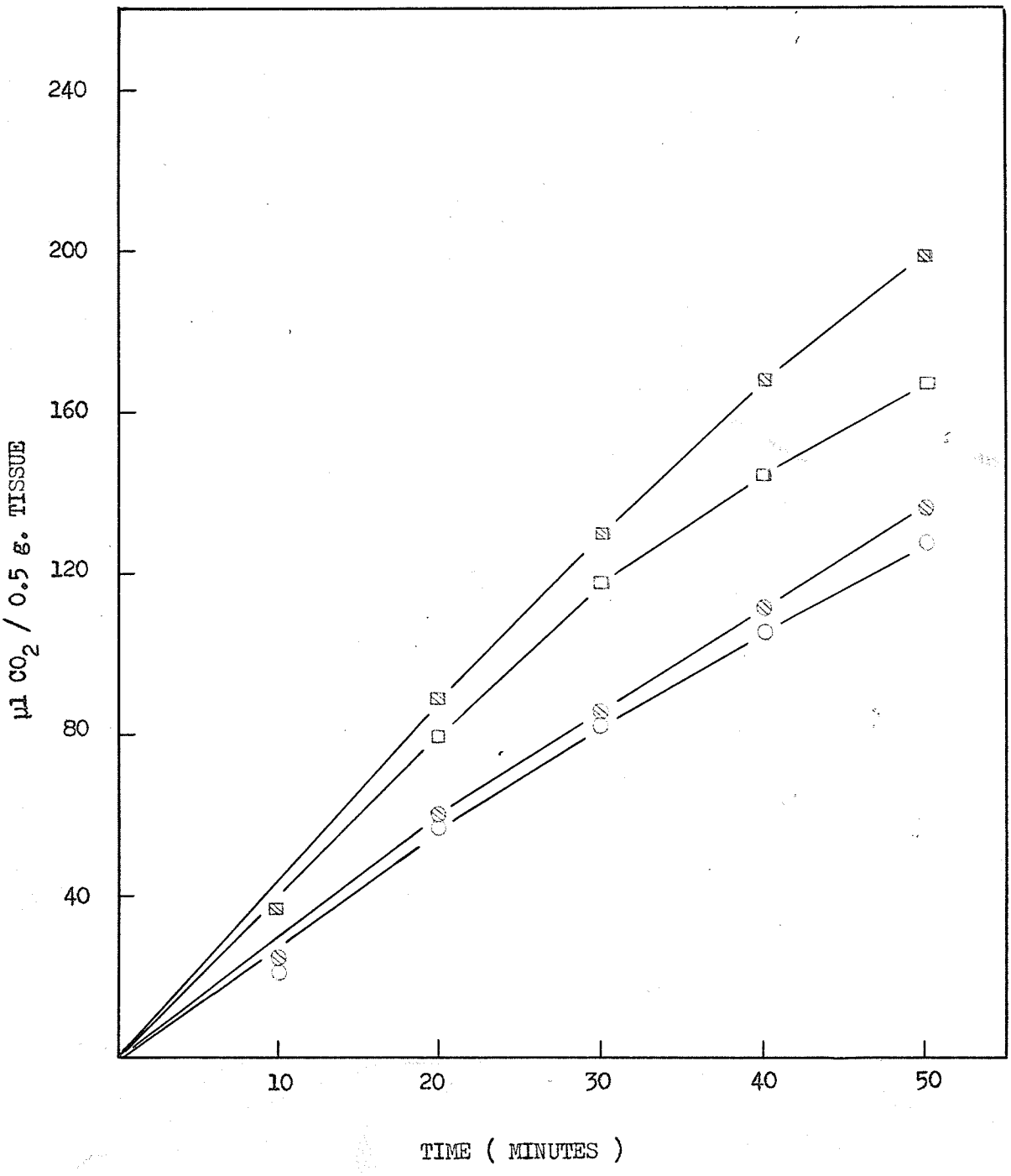

240
200
160
120
80
40
µl CO_2 / 0.5 g. TISSUE
10
20
30
40
50
TIME (MINUTES)

Figure 2. Pyridoxal phosphate concentration curve.

Standard reaction system.

Rate is μl CO_2/ ml. enzyme/ minute

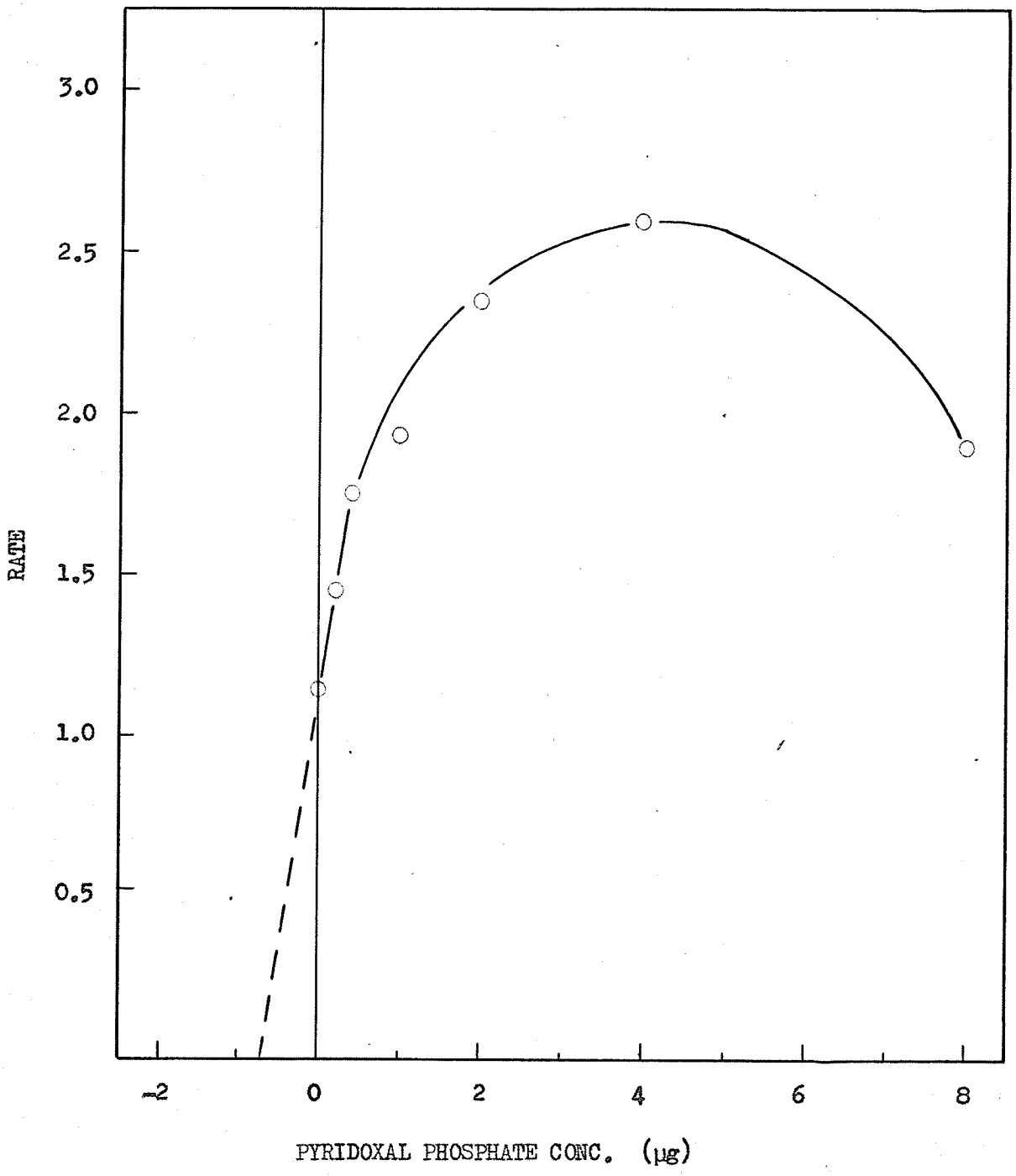

3.0
2.5
2.0
1.5
1.0
0.5
RATE
-2
0
2
4
6
8
PYRIDOXAL PHOSPHATE CONC. (µg)

Figure 3. Progress curves for the decarboxylation of variable amounts of glutamic acid.

Standard reaction system with the following concentrations of substrate.

A 3 μM

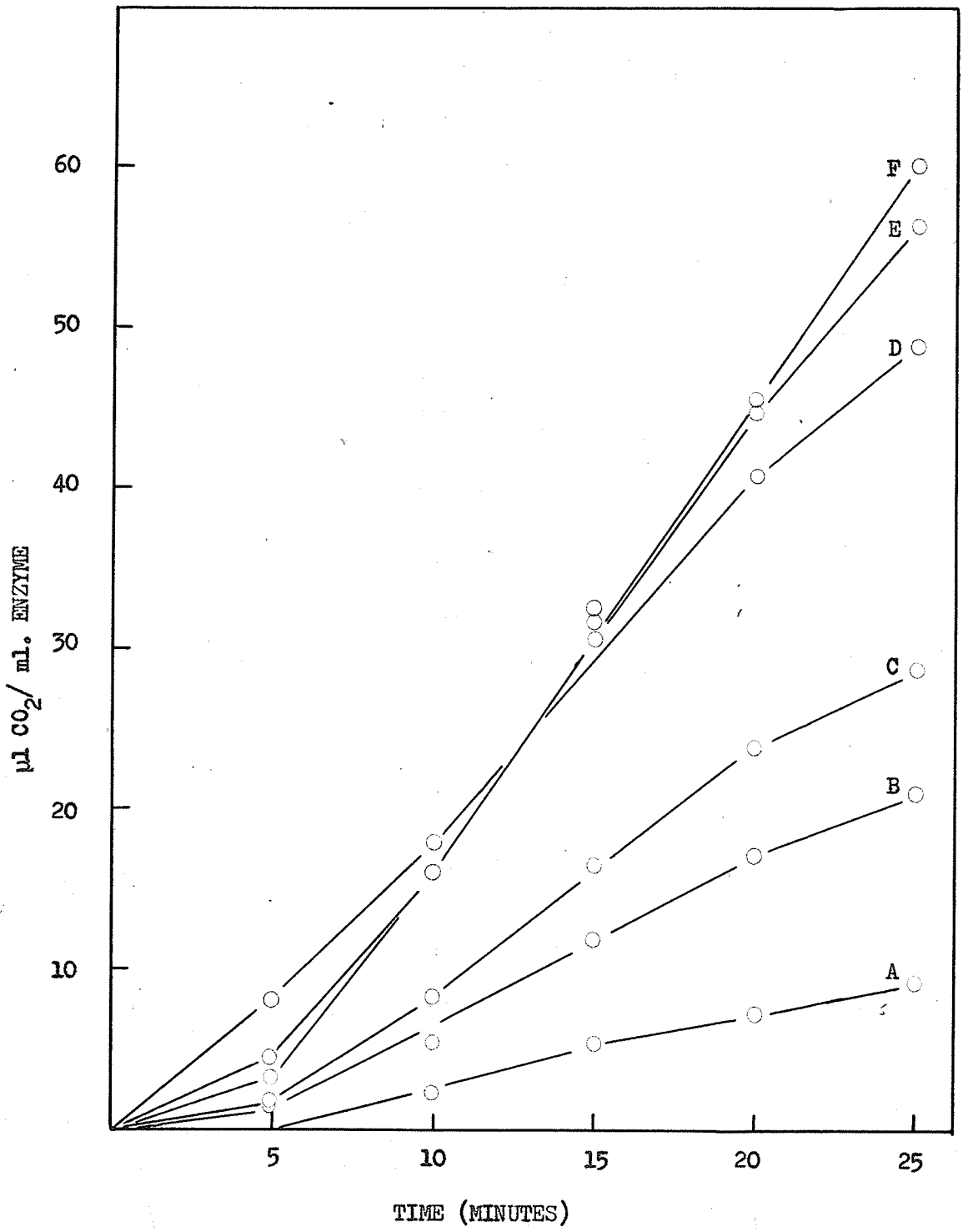
B 6 μM

C 10 μM

D 15 μM

E 30 μM

F 40 μM


60
50
40
30
20
10
µl CO_2/ ml. ENZYME
F
E
D
C
B
A
5
10
15
20
25
TIME (MINUTES)

about 4.0×10^{-2} M. (Figure 4).

Partially purified enzyme (1 mg./ml.) and a final pyridoxal phosphate concentration of 1×10^{-5} M gave CO_2 outputs that were virtually linear after the initial lag phase. The increment from 5 to 10 minutes (Figure 3) after CO_2 evolution had commenced were chosen as the most reproducable feature of the progress curve. The glutamic acid concentration curves were approximately hyperbolic (Figure 4) and by the maximum velocities obtained, the Michaelis constant was estimated to be 8.5×10^{-3} M at pH 5.7 and 30°C. in air.

(b) Studies on the Inhibition of Glutamic Decarboxylase.

Preliminary experiments with crude extracts gave somewhat lower activity than dialysed preparations. It was found that some inhibitors were present in the crude extracts as shown in Table I. There is a decrease of 46% in water treated leaves and a decrease of 24% in benzimidazole treated leaves with the addition of boiled enzyme.

TABLE I

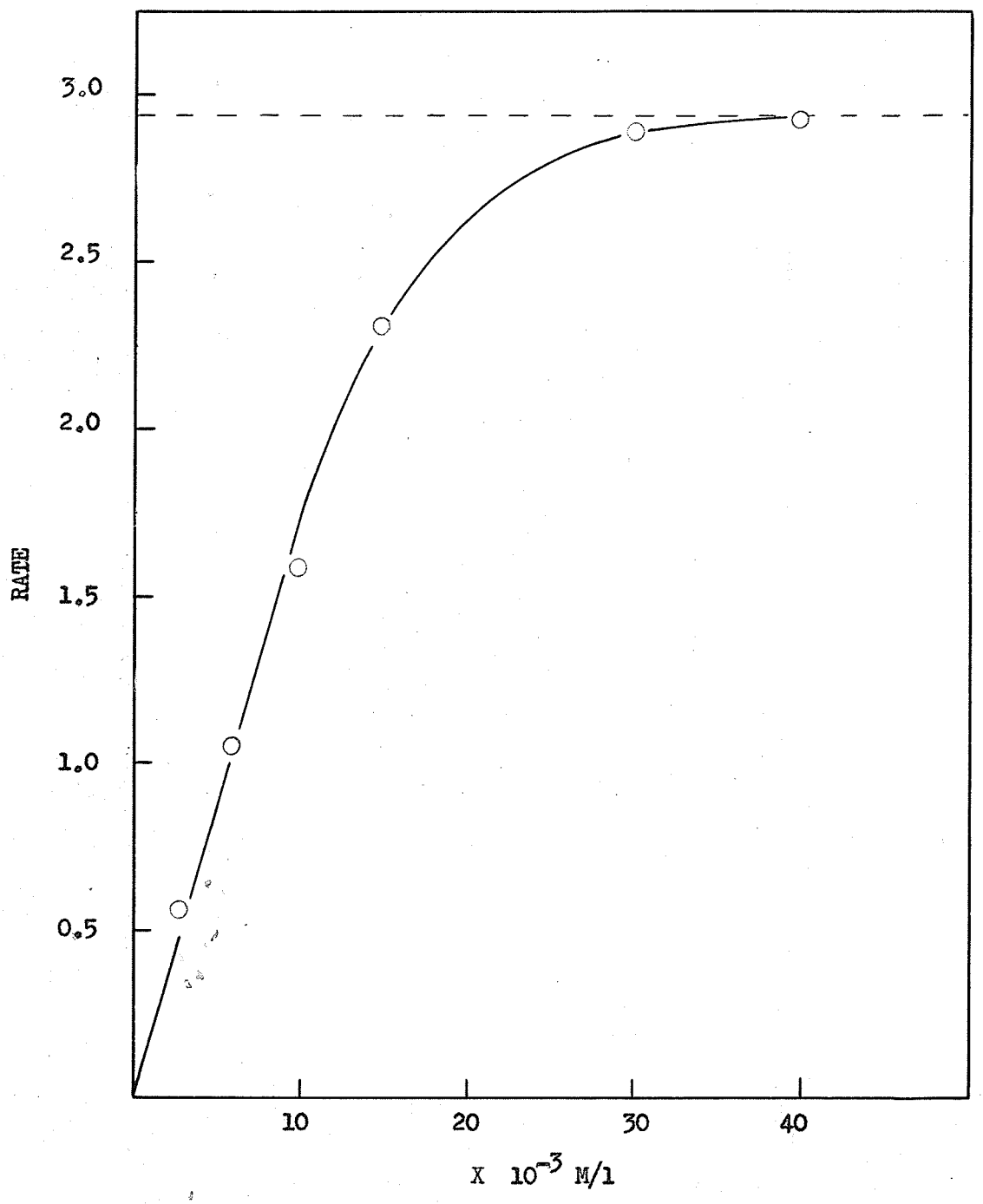
The Effect of Boiled Enzyme on Glutamic Decarboxylase Activity in Wheat Leaves.

	Q_{CO_2}		
	-PP	+PP	+Boiled Enzyme *
Floated on water	81	99	44
Floated on 100 p.p.m. benzimidazole	66	80	50

* 0.75 ml. of boiled crude enzyme, pH 5.7 was added to the standard reaction system to a total volume of 3.2 ml.

Figure 4. The effect of substrate concentration on glutamic decarboxylase.

Standard reaction system with dialyzed enzyme. Rates are μl CO_2/ ml. enzyme/ minute


3.0
2.5
2.0
1.5
1.0
0.5
RATE
10
20
30
40
X 10⁻³ M/l

The natural inhibitors could be dialysed out and in all later experiments, dialysed preparations by the phosphate method were used. Figure 1 shows that in crude enzymes, pyridoxal phosphate have very little effect whereas in dialysed extracts there is a greater increase in activity. The small effect of dialysis indicates that the coenzyme is firmly bound to the protein.

(c) The Effect of Benzimidazole on Glutamic Decarboxylase in Khapli Wheat Leaves.

Leaves floated on benzimidazole showed a glutamic decarboxylase activity 80% higher than the water control as shown in Figure 5. A time progress curve for water and benzimidazole floated leaves compared to those immediately detached is shown in Figure 6. It should be noted that with added pyridoxal phosphate, the decarboxylase activity in all three systems showed the same increase.

Experiments show that in the presence of pyridoxal phosphate, the activity of the carboxylase was increased in both the water and benzimidazole treated leaves. This may be due to an unsaturation of coenzyme or a combination of less coenzyme plus an increase in apoenzyme. Very little difference was found between benzimidazole and water treated leaves when no pyridoxal phosphate was added (Table II).

At low concentrations of glutamate, the rate of CO_2 evolution was greater in the water control than in benzimidazole treated leaves (Figure 7) whereas at higher substrate concentrations, the reverse is true. (Figure 6). Perhaps at higher concentrations, the substrate has overcome

Figure 5. Glutamic decarboxylase activity in crude undialyzed enzyme.

Standard reaction system with pyridoxal phosphate.

O - extract from leaves floated on water

Δ - extract from leaves floated on benzimidazole

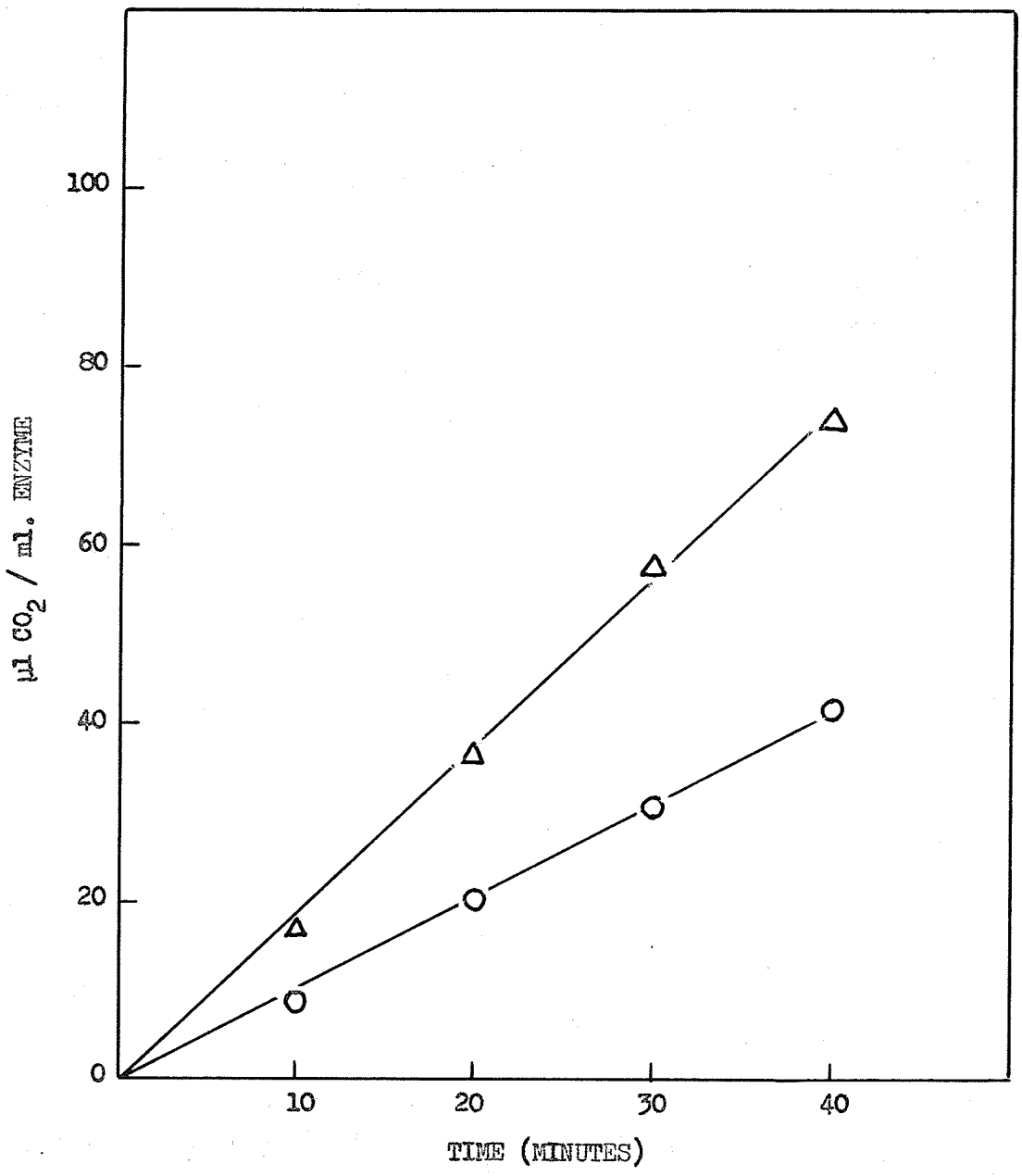

100
80
60
40
20
0
µl CO_2 / ml. ENZYME
10
20
30
40
TIME (MINUTES)

Figure 6. Effect of pyridoxal phosphate (PP) on glutamic acid decarboxylase.

Standard reaction system with dialyzed enzymes.

▣ leaves immediately detached plus PP

□ leaves immediately detached without PP

◍ leaves floated on water, plus PP

○ leaves floated on water, without PP

◬ leaves floated on benzimidazole solution plus PP

△ leaves floated on benzimidazole solution without PP

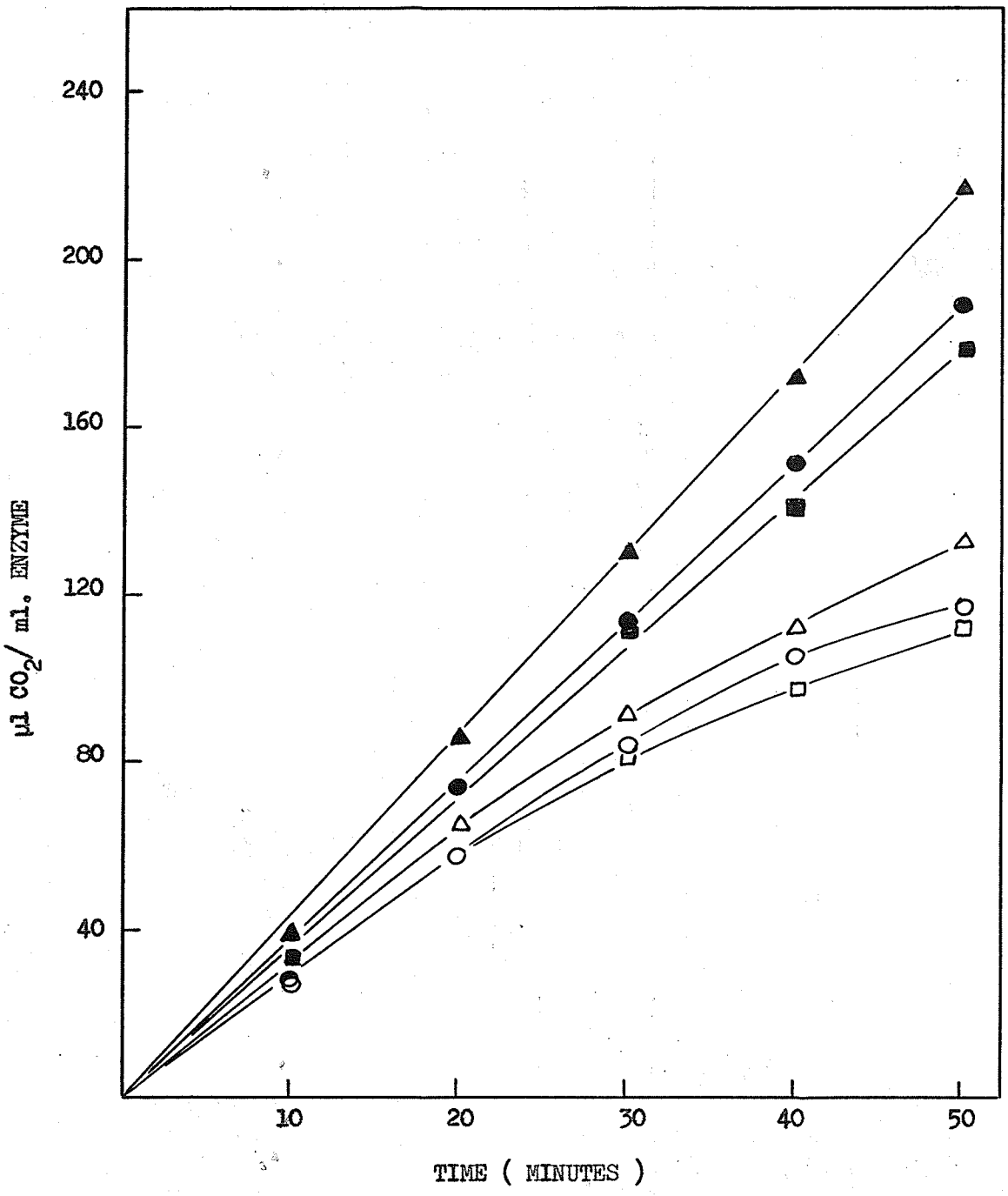

240
200
160
120
80
40
µl CO_2/ ml. ENZYME
10
20
30
40
50
TIME (MINUTES)

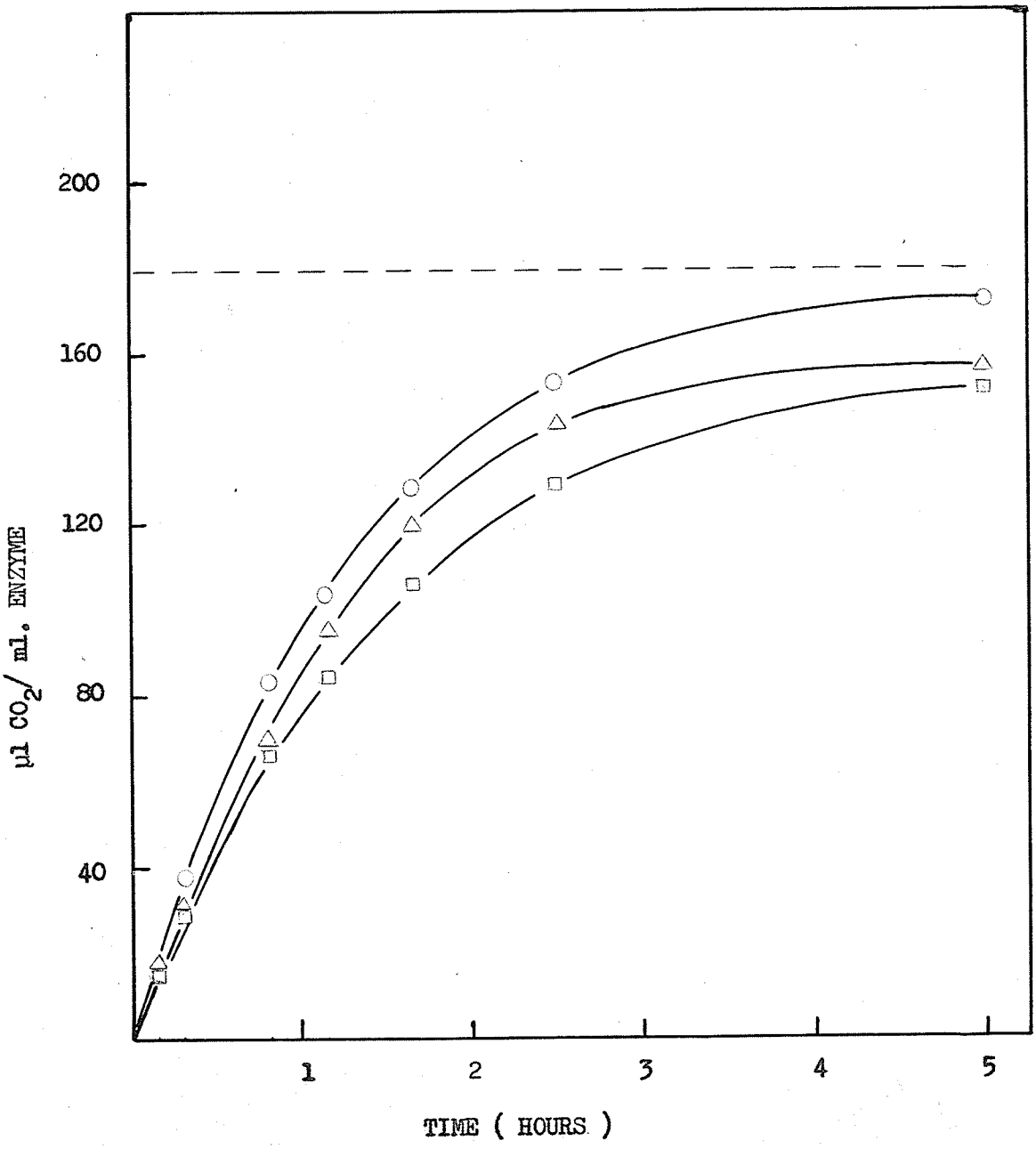
Figure 7. Time activity curves/ or glutamic decarboxylase with limiting substrate concentration.

Standard reaction system with dialyzed enzyme plus pyridoxal phosphate.

□ extracts from leaves immediately detached

○ extracts from leaves floated on water

△ extracts from leaves floated on benzimidazole


200
160
120
80
40
µl CO2 / ml. ENZYME
1
2
3
4
5
TIME (HOURS)

an inhibitory effect in the benzimidazole treated leaves. Smith (1959) reported a competitive type of inhibition in rusted sunflower cotyledons which can be displaced by extraction in an excess of glutamic acid. No attempts were made to isolate the inhibitor.

TABLE II

The Effect of Benzimidazole on Glutamic Decarboxylase in Wheat Leaves.*

Treatment	Q_{CO_2} -PP	Q_{CO_2} +PP
Immediately detached	29	36
Floated on Water	32	41
Floated on 100 p.p.m. Benzimidazole	33	43

* Standard reaction system.
Q_{CO_2} = µl CO_2/mg. protein/10 minutes.

(d) General Characteristics of Aspartic Decarboxylase.

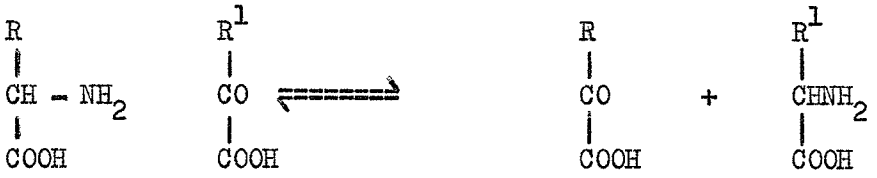
During the studies with glutamic acid decarboxylase, it was found that dl-aspartic acid could be decarboxylated in the presence of dialyzed wheat leaf extracts. Since reports show only 3 amino acid decarboxylases to be present in plants, glutamic acid decarboxylase (previous references) α methylene-L-glutamic decarboxylase in peanut, pepper and barley (Rowden and Done 1953) and methionine decarboxylase in cabbage (Mendel 1959) further studies were desirable.

Aspartic acid decarboxylase activity was found in dialyzed extracts from leaves floated on water and on 100 p.p.m. benzimidazole plus 0.1% aspartate (Figure 8). Aspartic acid was added to the solution in an attempt to induce the decarboxylase. Other experiments, however, showed similar activity in the absence of this acid. Pyridoxal phosphate increased the activity by over 50% in both transaminations.

By comparison, decarboxylase activity was much higher with glutamic acid as a substrate than with aspartic acid. In the presence of pyridoxal phosphate, glutamic decarboxylase activity was increased by 97% in leaves floated on benzimidazole + aspartate compared to the water control. This is a much greater increase than that normally found in experiments with benzimidazole alone.

GLUTAMIC-ASPARTIC TRANSAMINASE ACTIVITY IN WHEAT LEAVES

The process of transamination in which an amino group is transferred from one molecule to another can be expressed in the following manner.



The reaction is reversible and requires pyridoxal phosphate as a coenzyme.

(a) Transamination to a α ketoglutaric acid.

An enzyme associated with dialyzed wheat leaf extracts was found to be capable of catalyzing the transfer of the amino group from glutamic acid to oxalacetic acid by the following equation:

Figure 8. Decarboxylase activity with glutamic and aspartic acids as substrates.

Standard reaction system with dialyzed enzyme.

⊙ leaves floated on water, glutamic acid + PP

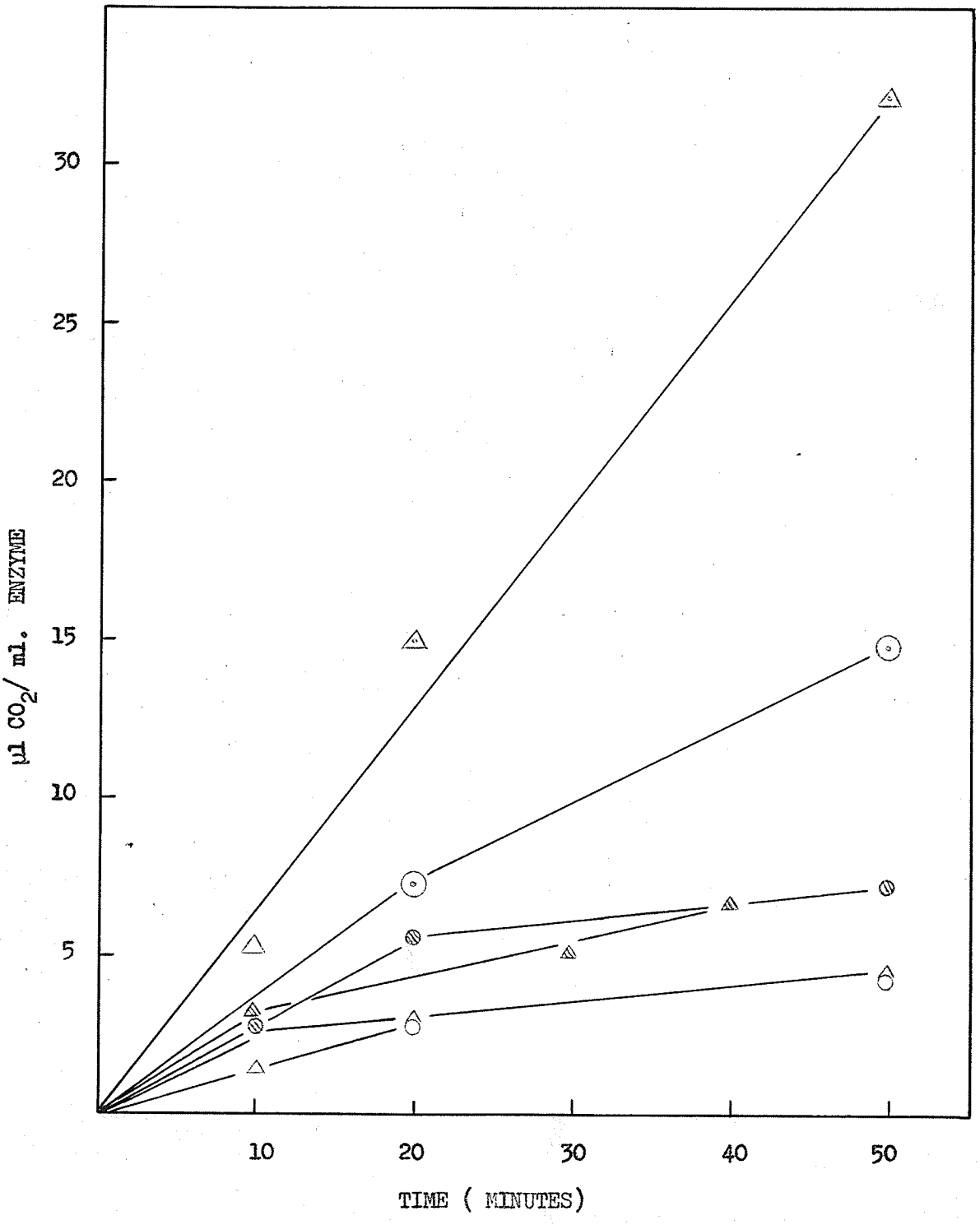
△ leaves floated on benzimidazole + aspartate+ glutamic acid + PP

⊘ leaves floated on water, aspartic acid + PP

○ leaves floated on water, aspartic acid - PP

⧋ leaves floated on benzimidazole + aspartic acid; aspartic acid + PP

△ leaves floated on benzimidazole + aspartic acid: aspartic acid - PP


30
25
20
15
10
5
µl CO_2/ ml. ENZYME
10
20
30
40
50
TIME (MINUTES)

l - glutamic acid + oxalacetic acid $\rightleftharpoons$ α ketoglutaric acid + aspartic acid

Pyridoxal phosphate is involved and is considered to be firmly bound to the enzyme (Meister 1957). The mechanism of reaction proposed by Jenkins et al. (1959) is that pyridoxal phosphate is bound to the enzyme through the phosphate and also through an aldehydic Schiff base with an amino group on the enzyme.

Rapid advances have resulted from the development of chromatographic methods of analysis as a means of exploring the pathways of cellular metabolism. Many methods however have been developed for quantitative measurements of transaminase activity. The present work employs both spectrophotometric and chromatographic techniques.

Glutamic aspartic transaminase activities were assayed by the measurement of aspartate formed. Crude dialysed extracts from Khapli wheat leaves were prepared by the phosphate method buffered at pH 8.0. The reaction mixture consisted of 170 µM .1M KH_2PO_4 pH 8.0, 10 µM glutamic acid, 3 µM pyridoxal phosphate (freshly prepared), 10 µM α ketoglutaric acid and 1 ml. of transaminase preparation to a final volume of 3.0 ml. The mixture was incubated at 30°C. for 20 minutes before the addition of substrate. Control experiments with boiled enzyme (5 minutes) were also carried out. After 30 minutes (except where otherwise stated), 0.5 ml. of 5% trichloracetic acid was added to the system and the precipitated proteins removed by centrifugation. Amino acids were then determined quantitatively by paper chromatography.

i. Reversal of Reaction

The reaction was found to be reversible and measurements were

made spectrophotometrically with DPNH. No other studies were made other than to show that the enzyme is operating in the reverse direction.

In the reverse direction glutamic-aspartic transaminase activity was highest in water treated leaves (Figure 9).

(b) General Characteristics and Kinetics of Glutamic Aspartic Transaminase.

Preliminary experiments showed that glutamic aspartic transaminase was not being influenced by side reactions involving the formation or destruction of any of the reactants. Loss of glutamic acid to γ amino-butyric acid did not make significant changes in the reaction since decarboxylase activity at the high pH of 8 is barely noticeable.

i. Isolation and Purification of the Enzyme.

Early studies on the intracellular localization of transaminase enzymes by Wilson et al. (1954); Cruickshank and Isherwood (1958) and Smith (1959) suggest that the bulk of transaminase activity is in the cytoplasmic fraction of the cell. Freebairn and Remmert (1957) found active glutamic aspartic transaminase in the mitochondrial fraction of cauliflower while more recent reports by Bone and Fowden (1960) suggest that in Mung bean, the enzyme is predominantly located in mitochondria. They do not rule out however, the possibility of distribution at other sites within the cell.

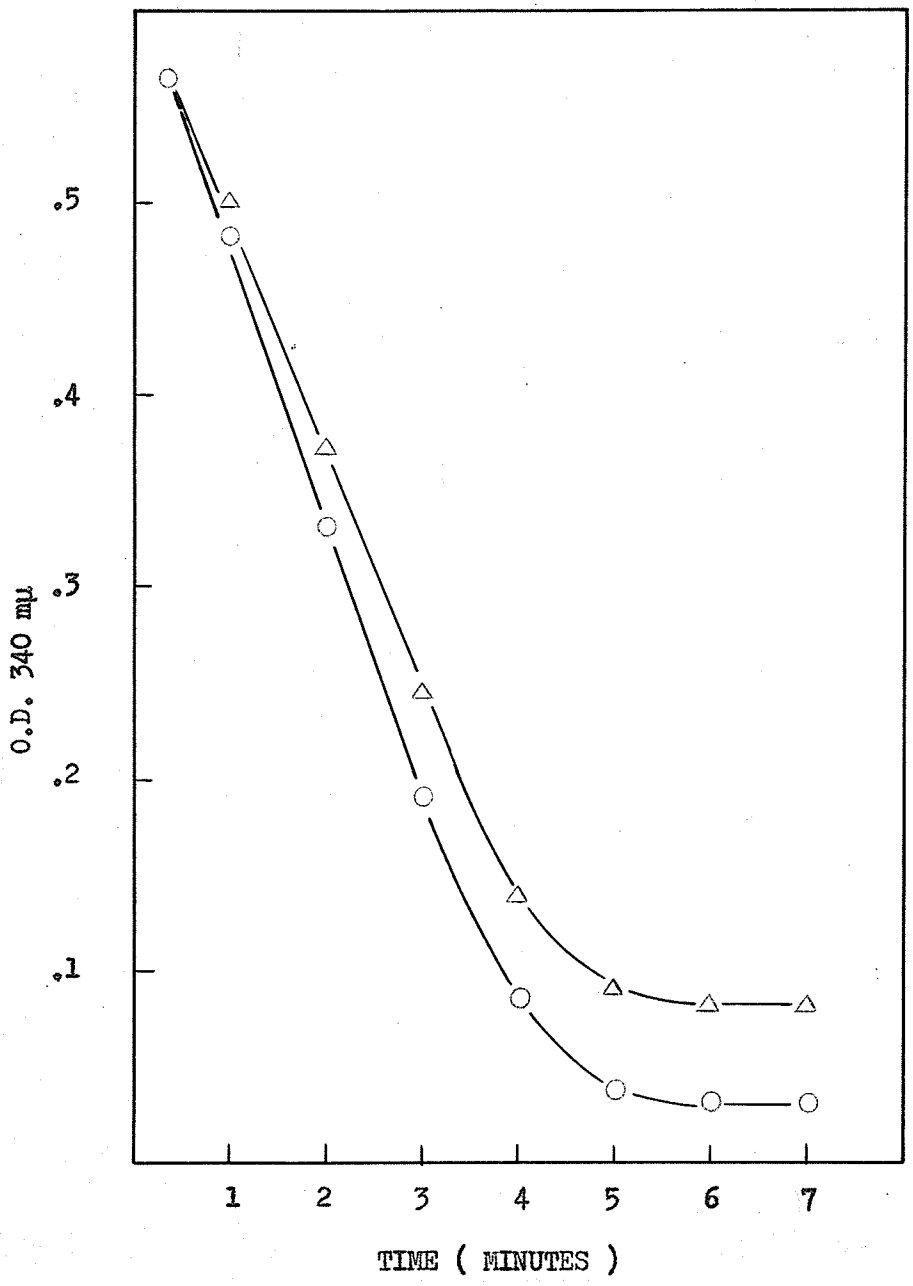
Throughout most experiments crude dialyzed extracts were assayed

Figure 9. Reverse reaction of glutamic-aspartic transaminase.

Conditions as described in spectrophotometric methods.

O - extract from leaves floated on water

Δ - extract from leaves floated on benzimidazole solution


.5
.4
.3
.2
.1
O.D. 340 mμ
1
2
3
4
5
6
7
TIME (MINUTES)

for glutamic-aspartic transaminase activity. Since comparisons between different treatments of the leaves was made, purifications were not desirable. However, in the study of some of the kinetics of the enzyme, partial purifications were made. Ammonium sulfate at various concentrations was found to be an effective precipitating agent. The phosphate method is at a pH of 8.0 instead of 5.7.

ii. pH Relationships.

The activity of glutamic aspartic transaminase is highest at pH 8.3 - 8.5 as shown by recent investigations of Cruickshank and Isherwood (1958; Cook (1956) Jenkins et al. (1959) and Smith (1959). The activity of the enzyme decreases below pH 8.0 while an extensive breakdown of oxaloacetate to pyruvate occurs above pH 8.5. The reaction mixture was well buffered at pH 8.0 and subsequent experiments were carried out at this pH.

iii. Coenzyme Requirements.

The coenzyme pyridoxal phosphate was found to increase the glutamic aspartic transaminase activity as shown in Table III. The present finding that enzymatic activity is increased by adding pyridoxal phosphate is consistent with the evidence previously presented by Umbreit and Gunsalus (1945), Roberts and Frankel (1957a and b) and Shukuya and Schwert (1960). After dialysis the enzyme still retains high activity and it is presumed that the binding of coenzyme to apoenzyme is very firm.

The resolution of pyridoxal phosphate from the apoenzyme has been carried out by Holzer et al. (1958) in yeast and also by Smith (1959) in sunflower cotyledons. No attempts were made to resolve the coenzyme in these studies.

(c) The Effect of Benzimidazole on Glutamic-Aspartic Transaminase Activity in Wheat Leaves.

The rates of transamination of the forward reaction in the absence of pyridoxal phosphate showed practically no difference in the enzyme activities in leaves floated on water and on benzimidazole solution. (Table III). When calculated on a percentage transamination basis, approximately 27% had been transaminated in each case during the first 10 minutes of the reaction, while $Q_T(N)$ values were 22.4 and 21.6 for the enzymes from water and benzimidazole treated leaves respectively.

The addition of pyridoxal phosphate had a very marked effect on both transaminase activities but benzimidazole treated leaves showed a greater increase. On a percentage transamination basis, the activity of the extract from leaves floated on water was increased to 29% during the first 10 minutes while the activity from benzimidazole treated leaves was increased to 35%. The $Q_T(N)$ values are now increased to 25.0 and 27.6 respectively. Benzimidazole appears to increase the glutamic aspartic transaminase activity in wheat leaves by 10%.

(d) Influence of Rust Infection on Glutamic-Aspartic Transaminase Activity in Wheat Leaves.

In this experiment healthy and rust-infected Little Club wheat

TABLE III

Glutamic-Aspartic Transaminase Activity in Detached Khapli Wheat Leaves Floated on Water and on Benzimidazole Solution

Treatment	Glutamic Acid Transaminated (μ moles)		$Q_T(N)$*	
	-PP	+PP	-PP	+PP
Floated on Benzimidazole	3.6	4.6	21.6	27.6
Floated on Water	3.4	3.8	22.4	25.0

* $Q_T(N) = \frac{\mu M \text{ Amino acid transaminated}}{\text{mg. protein X hours.}}$

PP = pyridoxal phosphate

Reaction System= Enzyme .1 ml.; pyridoxal phosphate 3 μM; glutamic acid 13 μM; oxalacetic acid 13 μM, pH 8.0 for 10 minutes at 30°C.

leaves were used for the extraction of enzymes. In the absence of pyridoxal phosphate, the rate of transamination was slightly higher in rust-infected than in healthy leaves (Table IV). The presence of 3μM pyridoxal phosphate in the system did not alter the activity in healthy tissue but showed a marked increase in rust-infected tissue.

ALANINE-GLYCINE TRANSAMINASE ACTIVITY IN WHEAT LEAVES

Studies on the transaminase systems in wheat leaves led to the discovery of an alanine-glycine transaminase which has not been reported in plants. Further work on this enzyme was desirable and the kinetics were studied.

TABLE IV

Transaminase Activity in Healthy and Rust-Infected Susceptible Wheat Leaves (var. Little Club)*

Substrates	Rate (μmoles reaction product)			
	Healthy		Rust-Infected	
	-PP	+PP	-PP	+PP
Glutamic acid + oxalacetic acid	9.5	9.4	9.9	11.2
Alanine + Glyoxylate		9.5		9.5

* Standard system

(a) General Characteristics and Kinetics of Alanine-Glycine-Transaminase.

The present investigation has demonstrated a highly active transaminase from dialyzed wheat leaf extracts that catalyzes the following reaction:

$$\text{L-alanine} + \text{glyoxylic acid} \xrightleftharpoons{\text{pyridoxal phosphate}} \text{glycine} + \text{pyruvate}$$

Along with enzymic activity, there is a slight non enzymic transamination which is more pronounced at a higher pH.

i. Isolation and Purification of Alanine-Glycine Transaminase.

Dialyzed wheat leaf extracts were found suitable for most experiments, however a partial purification by ammonium sulfate fractionation was desirable for kinetic studies. The phosphate method of preparing the enzyme was used, the only difference being a change in buffer

from pH 5.7 to pH 8.0. Preliminary experiments showed that most impurities were removed in dialyzing against distilled water overnight. The 30 - 60% ammonium sulfate fraction was employed wherever a purified enzyme was desirable. A unit of transaminase was defined as the amount of enzyme catalyzing production of 1 ;M glycine/ 10 minutes; specific activity, the number of units per mg. protein.

ii. Identification of End Products.

Pyruvate was identified as a product of the enzymatic reaction by chromatography of its 2,4-dinitrophenylhydrazone and was readily distinguishable from the dinitrophenyl-hydrazones of glyoxalate, oxalacetate and ketoglutarate. Glycine and alanine were determined quantitatively by chromatography as outlined in Methods and Materials. They were detected with ninhydrin and measured at 570 mμ. Alanine-glycine transaminase activity was determined at 30°C. in 1.5 ml. assay mixtures containing sodium glyoxylate monohydrate, 10 μM L-alanine, 10 μM phosphate buffer pH 8.0, 170 μM; and pyridoxal-5-phosphate 3μM. After 30 minutes (except where specified), the reaction was stopped by the addition of 0.25 ml. of 5% TCA and the precipitate removed by centrifugation. Where keto acids were determined, 1 ml. of 2,4-dinitrophenyl hydrazine solution was added to stop the reaction. A control was necessary to correct for a small amount of non-enzymatic activity which occurs at this pH.

iii. Reaction Rate.

The rate of glycine formation is directly proportional to

enzyme concentration up to about 2 units. A time course of the reaction is shown in Figure 10. The equilibrium of the transamination is far in the direction of glycine production and was not reached even after 100 minutes. The reaction proceeded only to about 50% conversion while the reverse reaction yielding alanine was detectable only under certain conditions. A true equilibrium therefore was not obtained. It was thought that perhaps phosphoenol-pyruvate was the immediate substrate and 12μM were added to the system instead of pyruvate. The results were negative except that a small amount of alanine was formed when potassium fluoride in a final concentration of 10^{-3} M was added. McCurdy and Catino (1960) showed that pyruvate had an inhibitory effect upon the enzyme which probably explains why the reverse reaction was inoperative most of the time.

It was desirable to see whether a condensation took place between the two substrates to produce oxalacetic acid. No conclusive results were obtained probably because of the rapid decomposition of exalacetate to pyruvate at this pH.

iv. Coenzyme and pH Requirements.

In common with other transaminases the alanine-glycine system requires the co-factor, pyridoxal phosphate. Activity was increased upon the addition of 3 μM pyridoxal phosphate to the system. There is however, considerable activity without added cofactor indicating that pyridoxal phosphate is firmly bound to the enzyme (Meister 1954).

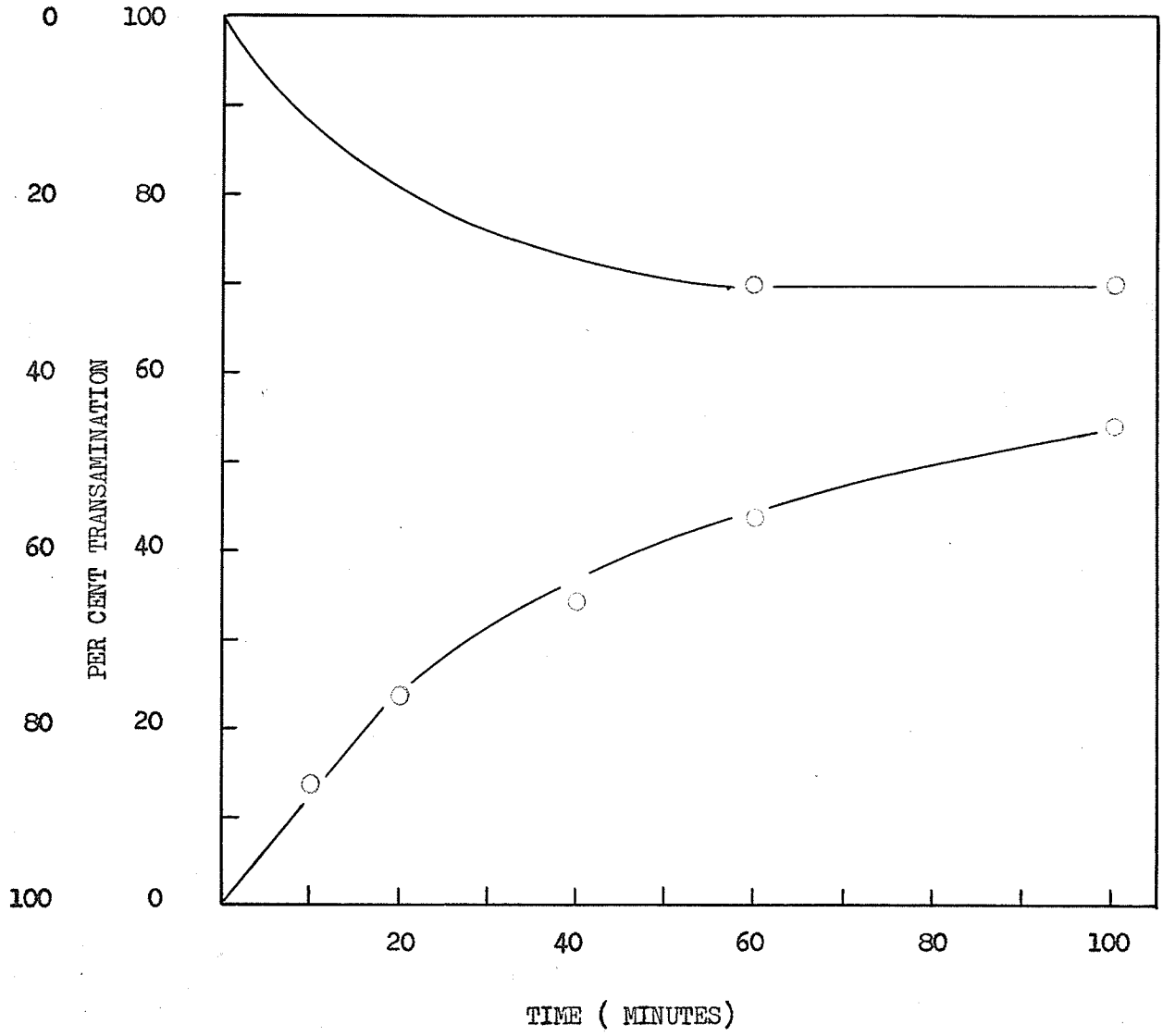
The activity of alanine-glycine transaminase was found to be highest

Figure 10. Progress curves for forward and reverse reactions of alanine-glycine transaminase.

Standard reaction system.

Ordinate numbers on the right show % T measured by glycine formation.

Ordinate numbers on the left show %T measured by alanine formation and glycine disappearance.


0
20
40
60
80
100
100
80
60
40
20
0
PER CENT TRANSAMINATION
20
40
60
80
100
TIME (MINUTES)

at about pH 8.5 (Figure 11) which agrees favorably with the finding of McCurdy and Catino (1960). Routine assays were however, carried out at pH 8.0 in order to minimize non-enzymic transamination.

v. Substrate Concentration Curve.

The alanine concentration curves determined from the initial velocities of the transamination reaction with varying amounts of substrate were approximately hyperbolic. By the use of a Lineweaver-Burke plot of 1/V vs 1/S (Figure 12) the Michaelis constant was estimated to be 3.1×10^{-3} M at pH 8.0: this agrees favorably with the K_m from other plant extracts by Jenkins, et al. (1959).

vi. Enzyme Concentrations

The effect of enzyme concentration on alanine-glycine transamination with optimum cofactor is shown in Figure 13. A standard system with enzyme concentration of 0.1 - 2 ml. was used.

(b) Effect of Benzimidazole on the Alanine-Glycine Transaminase in Wheat Leaves.

An experiment was carried out on enzymes prepared from leaves floated on water and on benzimidazole solution. Results were calculated on a basis of the amount of glycine formed. Table V shows that in the absence of cofactor there is an increase of 17.5% in benzimidazole treated leaves compared to the water control. In the presence of pyridoxal phosphate the increase was 24%.

Figure 11. The effect of pH on alanine-glycine transaminase.

Standard reaction system.

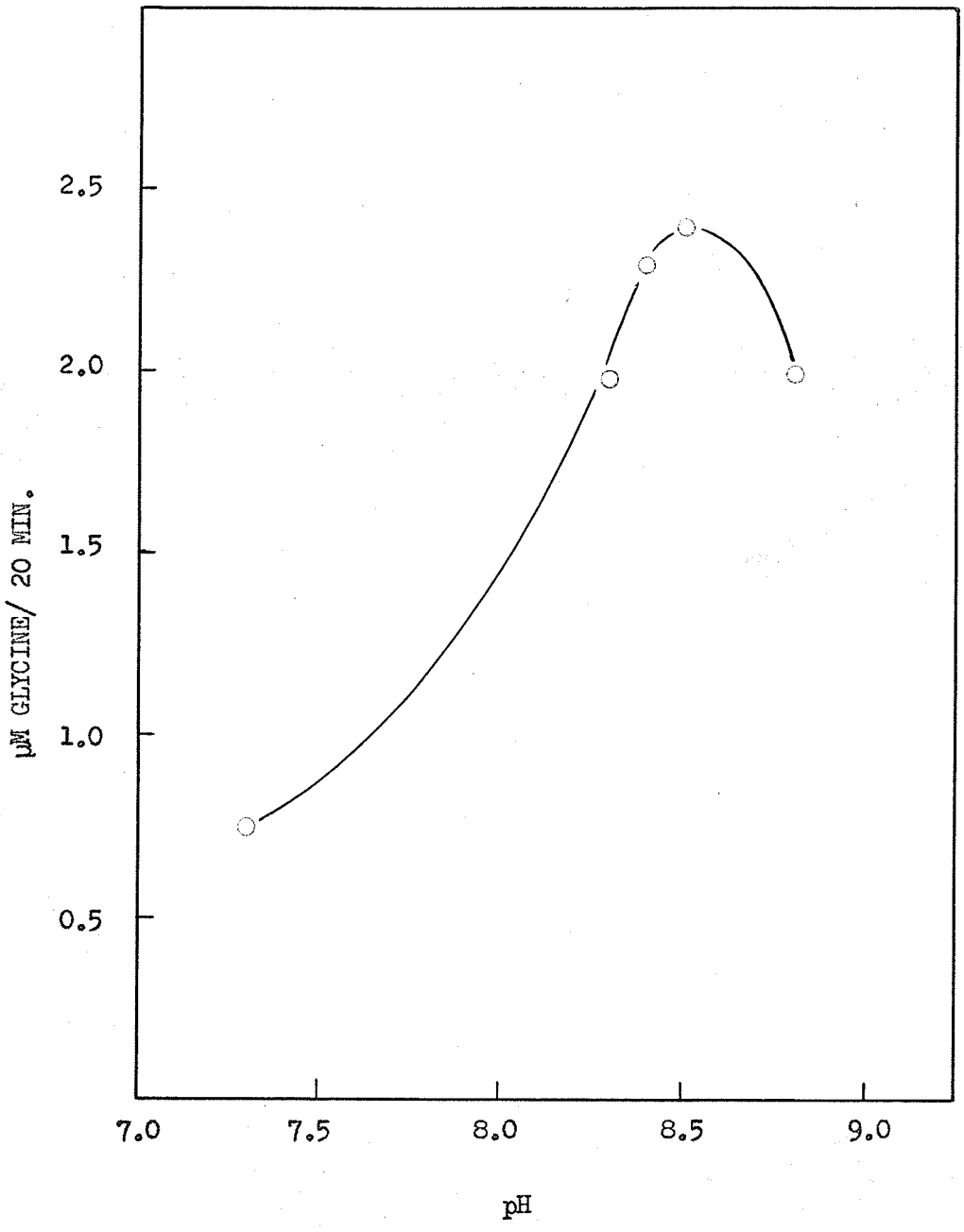

2.5
2.0
1.5
1.0
0.5
μM GLYCINE/ 20 MIN.
7.0
7.5
8.0
8.5
9.0
pH

Figure 12. Substrate concentration curve for alanine-glycine transaminase.

Standard reaction system with dialyzed enzyme plus pyridoxal phosphate.

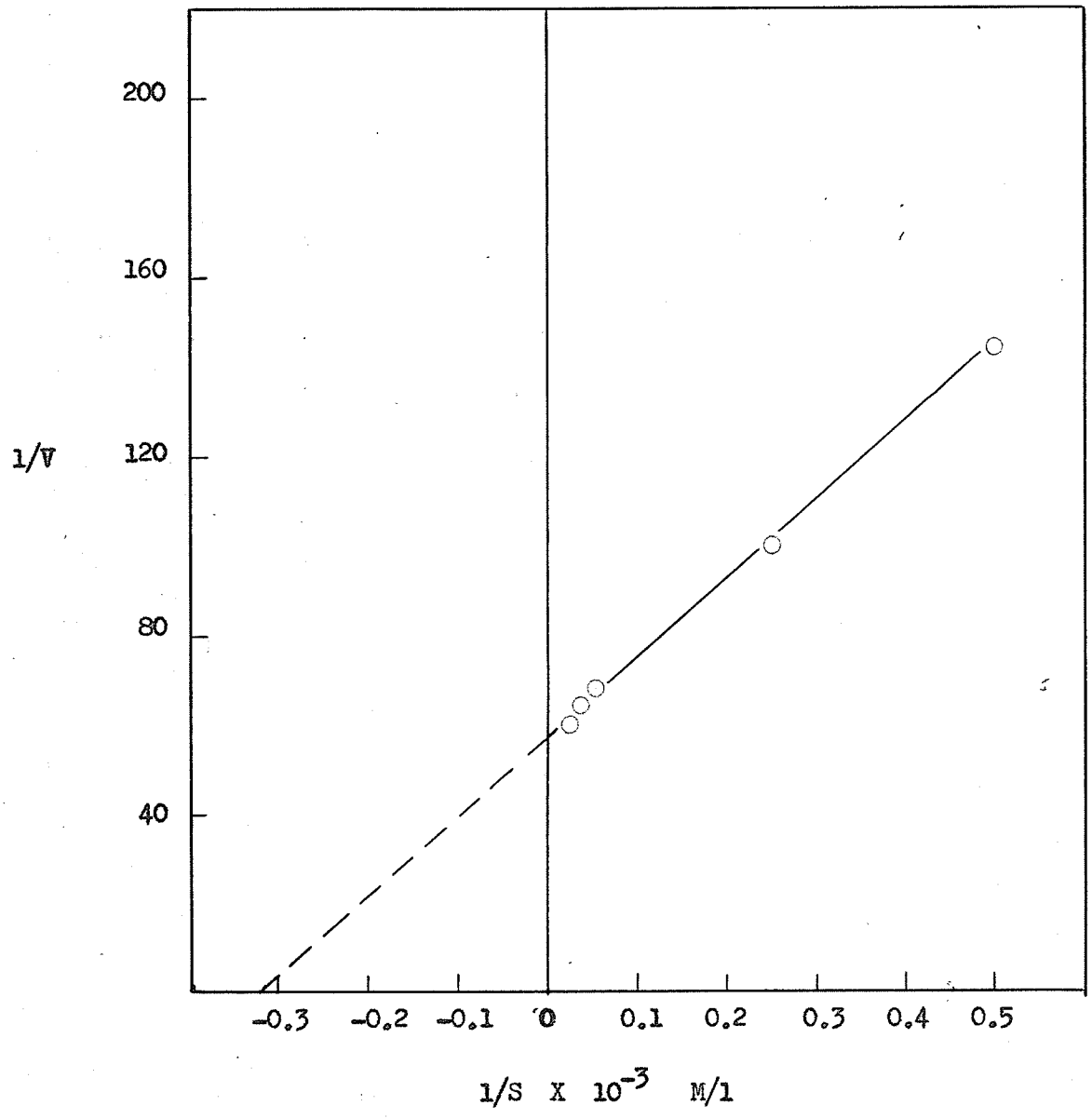

200
160
120
80
40
1/V
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
0.4
0.5
1/S X 10^-3 M/l

Figure 13. The effect of enzyme concentration on alanine-glycine transaminase.

Standard reaction system with dialyzed enzyme and pyridoxal phosphate.

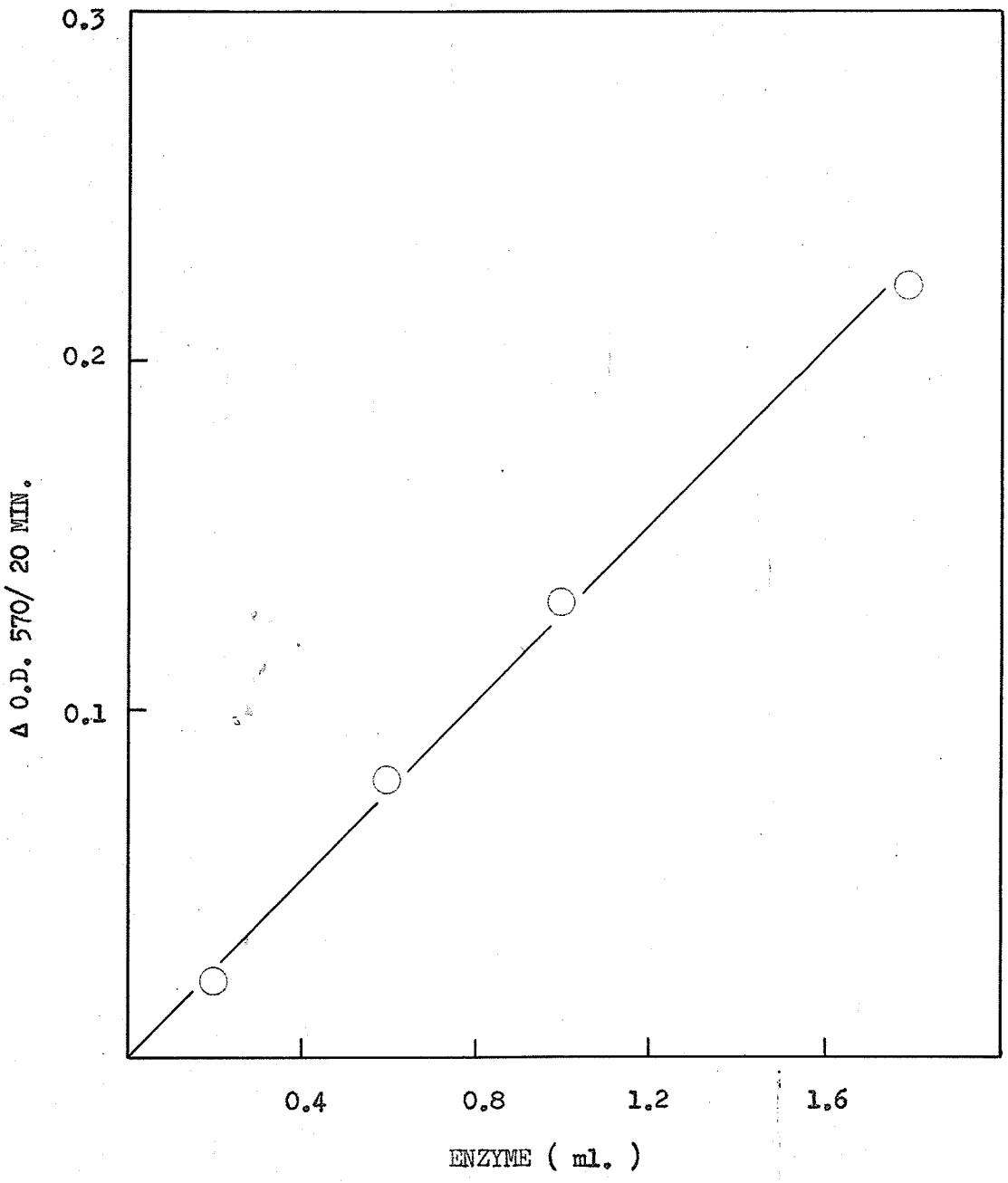

0.3
0.2
0.1
Δ O.D. 570/ 20 MIN.
0.4
0.8
1.2
1.6
ENZYME (ml.)

TABLE V

Effect of Benzimidazole on the Alanine-Glycine Transaminase Activity in Wheat Leaves

Treatment	μM Glycine Formed	
	-PP	+PP
Leaves floated on water	4.0	4.2
Leaves floated on Benzimidazole	4.7	5.2

(c) The Influence of Rust Infection on Alanine Glycine Transaminase in Wheat Leaves.

An experiment was carried out using rusted and non rusted Little Club wheat leaves as a source of transaminases. Table IV compares the effect of rust with and without added pyridoxal phosphate. Even though there was some variation in the results, the average figures in both rusted and non rusted tissue are the same.

(d) Substrate Specificity.

Incubations were carried out with alanine and the keto acids glyoxylate, α ketoglutarate and oxaloacetate. The products were identified by paper chromatography.

In addition to the alanine and glyoxalate activity, an enzymic transamination between alanine and α ketoglutarate was detectable but not with oxaloacetate. Further work is necessary to determine if this is due to the same or a different enzyme.

Assays of transaminase activity between glutamic and oxaloacetate

and alanine + glyoxalate were carried out to test their specificity. If two different enzymes are involved the rate of aspartic acid formation should be proportional to the rate of glycine formation in different enzyme preparations. From Table VI it can be seen that in most cases the ratio of glutamic + oxaloacetate to alanine + glyoxalate is the same, indicating that the reactions are probably catalyzed by the same enzyme.

TABLE VI

Specificity of Transaminases

Fraction	Alanine + Glyoxalate (μM glycine formed)[1]	Glutamin + Oxaloacetate. / Alanine + glyoxalate
Crude extract	4.9	.64
Dialyzed	2.5	.53
2 Carbowax	2.4	.17
3 Acetone powder	1.0	.57

1. Standard reaction system with pyridoxal phosphate.

2. Final pH of 8.0

3. Prepared according to the method of Hageman and Waygood (1959).

DISCUSSION

The main concern in this thesis has been in a study of the enzyme-coenzyme relationships in wheat leaves effected by benzimidazole and rust infection. The study was undertaken with certain enzymes involved in amino acid metabolism.

Most of the investigations referred to in the literature have been concerned with qualitative and/or quantitative changes in nitrogenous compounds. Although important, these observations do not explain the direct cause of these alterations. Since metabolic reactions are controlled by specific enzymes, the differences in metabolic activity may be reflected in enzyme-coenzyme relationships. For this reason alterations in the activities of certain enzymes were studied in relation to rust - infection and the effect of benzimidazole.

The results of these experiments have shown that alterations do exist in the activities of glutamic decarboxylase, glutamic-aspartic and alanine-glycine transaminases. Pyridoxal phosphate was required in all the reactions studied. By the large number of metabolic reactions involving pyridoxal phosphate (Meister, 1957), it appears that this vitamin is of vast importance in amino acid metabolism. Wheat leaf extracts, after dialysis, still showed considerable activity without coenzyme indicating that pyridoxal phosphate is firmly bound to the enzyme (Meister, 1957).

Glutamic decarboxylase activity is increased in extracts from leaves floated on benzimidazole solution compared to the water control. Both

treatments however, showed higher activity than immediately detached leaves. Upon the addition of pyridoxal phosphate, the activities in all systems increased the same amount. At no time was the activity of extracts from benzimidazole treated leaves lower than those from immediately detached. These findings are in accord with those by Smith (1959) who showed that benzimidazole increases the level of glutamic decarboxylase in sunflower cotyledons. Benzimidazole has maintained the amount of apoenzyme but had little effect on the level of the coenzyme. Wang (1959), Samborski et al. (1958) and Wang et al. (1960), state that benzimidazole maintains the physiology of the detached leaf as if it were attached. It appears that these findings add to the above evidence, however, in this case, the level of a particular enzyme is maintained.

The discovery of an aspartic decarboxylase which also requires pyridoxal phosphate as a coenzyme will probably cast some light on metabolic pathways operating in the plant. The increase in glutamic decarboxylase activity when aspartate was added to the benzimidazole solution may not be of importance at present. However, as the problems are solved, this may lead to a clearer explanation of resistance and susceptibility in plants.

Transaminases play a significant role in cellular metabolism. By extensive interconversion of amino acids, a wide array of amino acids may be formed. By means of transamination reactions it is possible to obtain from glutamic acid almost all of the known amino acids present in the cell.

A significant finding in this study is that rust infection altered

the activity of glutamic aspartic transaminase. An increase in activity was observed in rust infected leaves when pyridoxal phosphate was added. There was no change with added coenzyme in healthy leaves. Smith (1959) however found that the addition of pyridoxal phosphate had little or no effect on the activity of the enzyme from rust infected sunflower cotyledons. From our findings it appears that pyridoxal phosphate is utilized by the fungus in its nutrition as suggested by Farkas and Kinaly (1958) for wheat stem rust.

The transamination of alanine + α ketoglutarate was demonstrated in wheat leaves. The enzyme showed low activity compared to the other transaminases. An attempt to show transamination between alanine and α ketoglutarate by Smith (1959) was unsuccessful.

High activity was obtained in extracts with alanine + glyoxalate as substrates. Enzymological studies in host-parasite relations have never before involved the study of alanine-glyoxalate transaminase. Rust infection did not appear to affect the transaminase activity, but conclusions cannot be drawn due to the limited amount of work done with rust infected tissue.

It appeared that alanine-glycine transaminase is similar to the glutamic-aspartic transaminase in wheat leaves. Perhaps it is one of two glutamic-oxalacetic transaminases present in plants (Fleischer, 1960). Since the reverse reaction is operative only under certain conditions, more work should be carried out in this field. Benzimidazole increased the activity of the alanine-glycine transaminase. The difference between benzimidazole treated and water treated leaves was more pronounced in the

presence of pyridoxal phosphate.

Throughout this study, benzimidazole was used to induce resistance into the leaves once they were detached. If benzimidazole treatments are truly represented by resistant plants then we can conclude that in resistant plants the transaminase activity is higher than in susceptible plants. Similarly the decarboxylase activity is high in resistant and low in susceptible plants. It is hoped that this comparative study of host enzyme systems will contribute some knowledge to the elucidation of basic biochemical differences in resistant and susceptible plants.

CONCLUSION

Studies on the basic enzyme-coenzyme relationships in detached leaves of Khapli wheat have led to quantitative changes in the enzyme patterns.

Benzimidazole increased the level of glutamic decarboxylase leaf extracts. It has maintained the amount of apoenzyme but did not control the level of the coenzyme. An aspartic decarboxylase was detected which requires pyridoxal phosphate as a coenzyme.

Rust infection increased the glutamic-aspartic transaminase activity but showed little effect on the alanine-glycine transaminase. It appears that pyridoxal phosphate is utilized by the fungus in its nutrition.

Transaminations with alanine + α ketoglutarate and alanine + glyoxalate were noted. The alanine-glycine transaminase and glutamic-aspartic transaminase appeared to be the same enzyme.

Transaminase activity is higher in resistant plants and lower in susceptible ones.

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