THE EFFECTS OF LEAF RUST INFECTION ON THE CONVERSION OF GLUCOSE-U-C^{14} INTO AMINO ACIDS AND SUGARS IN SEEDLING AND MATURE PLANTS OF PONCA WHEAT

By

DICK DON DAVIS

Bachelor of Science
University of Oklahoma
Norman, Oklahoma
1960

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1963

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Thesis Approved:

Glenn W. Todd
Thesis Adviser

Robert K. Gholson

A. M. Schlehuber

Harry Young, Jr.

Hellis A. Hanna (?)

J. H. Boggs
Dean of the Graduate School

592745

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CHAPTER I

INTRODUCTION

The nature of host-parasite metabolic interaction has been under study for a number of years by an increasing number of able investigators. Progress has been made in that a number of ways in which the physiology of diseased tissue differs from comparable healthy tissue are now known.

No clear-cut breakthrough has yet been recognized, however, that links up the various discrete facets of host-parasite interaction into a coherent pattern.

In the present study use has been made of experimental material that promised to have some unique advantages. The variety Ponca is one of a number of varieties of wheat that responds differently to leaf rust infection as the plant ages. Although susceptible to nearly all known physiological races as a young seedling, plants of the Ponca variety develop a high level of resistance to nearly all races prior to maturity. The metabolism of this variety changes at some stage of development, and this metabolic change is in some way correlated with a changed response to rust infection. Whether this change in host-plant metabolism involves a very basic active resistance response, or the elimination of a metabolic pathway vital to the nutrition of nearly all biotypes of the leaf rust fungus is not clear.

The ultimate development of the rust is known to be dependent upon a delicate physiological balance. In some cases the effect of moderate temperature changes in determining whether or not a rust infection will

proceed to the sporulation stage upon a given host indicates that enzyme systems are intimately involved.

It is apparently not known for certain at what stage of plant development the physiological balance is tipped in the direction of resistance or susceptibility. In fact it may well vary for different race-variety combinations.

The problem was attacked from several angles. (1) Some aspects of the intermediary metabolism of the healthy host plant at different stages of development were followed. (2) The metabolism of 6-day old infection sites were checked at these same stages of development of the host plant. (3) The metabolism of the infected tissue as the age of the infection increased was charted in three groups of plants, each representing a different stage of host-plant development.

The projected experimental design was quite simple. Plants of the Ponca variety were grown to maturity. At frequent intervals as the plants aged, a number of the plants were infected with leaf rust while another similar number served as uninfected controls. Methods of biochemical assay are available that could detect any gross differences in metabolic patterns in the two sets of plants. Carbohydrate and amino acid metabolism were arbitarily selected for this study.

By its very nature the experimental design contains one statistical weakness in that in a developmental study precludes exact replication.

Another apparent weakness that casts some shadow on the accuracy of the data is the fairly complex series of discrete biological and biochemical manipulations that the project involved. The accuracy of the data obtained at the conclusion depended partially upon the precision of each sequential step. Quite obviously a sequence of steps, each involving some experimental error at best, could contribute to a cumulative

error gross enough to be misleading.

These difficulties were recognized from the beginning, however, and ways to overcome them were planned carefully in advance. At certain intervals sufficient material was available for only one trial, but the potential gain seemed sufficient to warrant some risk.

The care that was taken in the experimental sequence will be described in the text.

CHAPTER II

REVIEW OF LITERATURE

Obligate parasites are pathogens that require a living host for their sustenance. They invade living tissue and maintain an intimate physiological contact with the cells of the host plant.

Electron micrograph studies have shown that the haustoria of wheat stem rust are surrounded by an encapsulation some 80 to 340 mu in thickness (24). This encapsulation contains particulate material (whether of host or parasitic origin is unknown) and is separated from the cytoplasm of the host by a thin membrane in which small vesicles sometimes form which protrude into the host cytoplasm.

Garber states (32): "The property of virulence with respect to the parasite assumes that the parasite is capable of utilizing the host environment as a growth medium and that the parasite can overcome the hosts' defense mechanisms." The study of parallel biochemical evolution in host-parasite relationships can thus be subdivided into two separate considerations: (1) nutritional interaction, and (2) resistance responses. The first implies merely that the host organism must serve as a substrate, and this could theoretically be quite simple. For example if the parasite were possessed of fairly complete enzyme systems, then one compound such as glucose might fill the substrate requirement. This may be the case with some facultative parasites.

Obligate parasitism apparently does not stem from the lack of simple substrates. Many substrates have been tested, but none have been

successful in promoting sustained growth on prepared culture media. Cultural attempts have been unsuccessful except in a few instances in which the virulence of the parasite was reduced in physiological changes that resulted in successful saprophytic existence.

From thousands of cultures of telial galls Cutter (15) claims to have found seven isolates of Gymnosporangium that will "survive continuous transfer on various synthetic media" while retaining the capacity to infect Pyrus.

Similar techniques were used on rust of Aristaema, Uromyces ari-triphylli (16) where numerous isolates produced five strains that could be maintained upon synthetic media, and which still retained their capability of infecting and producing viable spores on the host. Cutter (16) implies that the isolates that adapt to saprophytic culture diminish in pathogenicity. He states that they apparently change from obligate parasitism to a heterotrophic saprophytism in successive steps. Only rare cells of the rust undergo this transformation and those that do only retain some degree of ability to reinfect their host.

Yarwood (109) surmises that most of the attempts to culture obligate parasites on non-living media have not been recorded. Only a limited success has been obtained, although he notes that many obligates respond by restricted or enhanced germ tube growth when placed on media containing a variety of supplements.

The Role of Nutrition in Obligate Parasitism

The very existence of obligate parasites after millions of generations of selection pressure would seem to indicate that there exist some special advantages to the fungus in maintaining such an existence. Some support for this hypothesis comes from examining the conditions for opti-

mal susceptibility. Several lines of study (21,29) would indicate that host species support optimal growth of rusts when the environmental conditions bring about optimal growth in the host. It is as if the utilization of a living host possessed advantages to the fungus over and above the utilization of non-living organic substrates. Perhaps two actively metabolizing systems are better than one, if both of them can be controlled.

One possible advantage obtained in the utilization of a portion of a living plant would be the accumulation of growth substrates from sites distant from the point of infection, obtained through the vascular transport system. Shaw and Samborski (86) and Shaw (82) have rigorously established the existence of quantitative, metabolically dependent accumulation of C^{14} labelled glucose at the site of rust infections in wheat leaves. This is true also for compounds labelled with radiophosphorus. This latter phenomenon (the sump concept) would also operate for saprophytes.

Genetics and Physiology of Host Resistance

One fundamental basis of host resistance to parasitic organisms is the hypersensitive reaction. Essentially this amounts to a premature death of host cells, the walling off of the necrotic area, and the accumulation of phytotoxic and fungitoxic substances all occurring in rather rapid sequence in the tissue adjacent to where fungal hyphae have penetrated and started to grow (Muller, 58). The failure of this hypersensitivity reaction to occur upon fungal penetration is correlated with susceptibility in the host species and virulence in the parasite. When it does occur, the parasite is termed avirulent, and the host species resistant. Any host gene that produced a substance that would set in

motion a hypersensitive response upon contact and interaction with the fungus, would logically have a selective advantage if not otherwise detrimental to the host organism. Going one step further we might guess that the appearance of such a gene in high frequency in the host species would cause a positive selection pressure for genes in the fungus that would prevent the host gene substance from initiating the hypersensitive response. Upon such reasoning, Flor (27) in 1942 advanced the gene-for-gene hypothesis, assuming that for each gene for resistance in flax there was a corresponding gene for virulence in the population of flax rust. Later research has indicated, on the basis of statistical population and progeny studies, that gene-for-gene relationships do exist in a number of organisms.

Flor's original concept has been stated in more general terms by Person, et.al. (66): "A gene-for-gene relationship exists when the presence of a gene in one population is contingent on the continued presence of a gene in another population, and where the interaction between the two genes leads to a single phenotypic expression by which the presence or absence of the relevant gene in either organism may be recognized." Loegering and Powers (53) have further restricted the definition by recognizing that there are certain types of non-specific host resistance that can operate against an entire population of pathogens (i.e., tolerance, morphological exclusion, etc.).

Role of Respiration in the Disease Cycle

Significant among the changes which occur in host metabolism following infection with an obligate parasite, is a marked increase in the respiratory rate. According to Samborski, et. al.(75), the respiratory increase is rapid, perhaps doubling the rate in 24-48 hours. This high

rate is sustained in susceptible interactions; but in resistant interactions it falls off after the first few days even though the initial stimulus may have been higher. The respiratory rate in rust infected leaves of Khapli wheat (resistant) rose rapidly until about 5 days after infection then declined sharply. The respiratory rate in rust infected leaves of Little Club (susceptible) was considerably slower, but eventually greater. Dry weight and total nitrogen at rust infections in infected Khapli leaves declined at a steady, rapid rate. Both dry weight and nitrogen content increased at rust infection sites on susceptible Little Club. Micro-respirometer measurements show that colony centers of mildewed barley reach respiration rates of 7 to 10 times that of healthy tissue (9). Respiration of barley tissue from which the mildew had been removed was only 2 to 3 times as high as the controls. Low levels of mildew infection had a disproportionately large effect on the metabolism (photosynthesis and respiration) of barley leaves (52). The effect of mildew on yield of grain photosynthesis and respiration can be expressed as a constant (2.5-5) times the percentage of leaf area infected. (See Large and Doling, 51.)

Hackett has stated in a recent review (35) that it is generally accepted that the intracellular level of phosphate acceptor (ADP) controls the rate of respiration. High concentrations of ADP would tend to stimulate increased respiratory activity, while ATP formed during coupled phosphorylation would tend to check the rate increases. Pozsar and Kiraly (68) have pointed out the analogies between rust infection and dinitrophenol treatment in the breakdown of respiratory control in the infected tissues. Both tend to cause a pathologically high rate of respiration to occur. These authors indicate that both cases may be due to uncoupling of respiratory phosphorylation. If this hypothesis is true,

the mechanism through which it is achieved may possibly be oxidized phenols. Stem rust uredospores have been shown to possess polyphenoloxidase, and certain oxidized phenols (quinones) are efficient uncouplers.

Shaw and Samborski (85) tend to hold the view that some uncoupling of oxidative phosphorylation does occur in rusted tissues and that it occurs much earlier and at a higher rate in resistant varieties. Shaw (84) feels that rapid and extensive uncoupling is correlated with the restriction of synthesis and host cell degeneration that occur when resistance to the parasite is expressed. This is based on the observation by Shaw and Samborski (85) that DNP stimulated O_2 uptake in leaves of a stem rust susceptible variety considerably, while a rust resistant variety gave only a small response of short duration to DNP treatment.

Current theories concerning the chemical mechanism of susceptibility attach paramount importance to the control of oxidation and reduction processes in the host cell. Muller (58) has cited a large body of evidence showing how certain respiratory inhibitors applied to normally resistant host varieties will break down their resistance and allow avirulent races of obligate pathogens to attack them. Pyrocatechol, tyrosine, phenyl urethane, and KCN are capable of breaking down the resistance of potato strains to Phytophthora. On the basis of such evidence it is often assumed that host species maintain dynamic resistance mechanisms when their respiration proceeds normally. Kiraly and Farkas (47) advanced the theory in 1958 that, if the stimulation induced by the pathogen in the first few host cells parasitized is too intense, a premature physiological breakdown of the invaded cells will occur, resulting in the isolation of the pathogen necrosis. This theory has been supported experimentally by Kaul and Shaw (44) who measured a rise in the redox potentials of rust infected wheat leaves. This initial rise was halted and reversed in a

susceptible variety by the emergence of new poising systems in the reduced form. No such reversal occurred in a resistant variety, so the authors believe that the maintenance of an adequate reducing potential is correlated with a susceptible type of host-parasite interaction. They have postulated that a new respiration regulating enzyme is supplied by the rust.

Wheat and rust have been shown to be differentially sensitive to the respiratory inhibitors, fluoride and azide. Fluoride inhibits glycolysis in wheat apparently, but does not affect spore metabolism. Samborski and Forsyth (77) have noted that azide inhibited rust growth on wheat, and was non-phytotoxic. Similar results with wheat and mildew have been recorded (3).

White and Ledingham (105) have concluded after careful work with specific inhibitors, etc., that the electron transport system of the stem rust fungus is quite similar to higher plant systems containing pyridine nucleotides, flavoproteins, and functional cytochrome oxidase as well as cytochrome c.

Williams and Ledingham (107) obtained a mitochondria rich preparation from stem rust uredospores. This preparation responded to exogenous succinate, a-ketoglutarate, malate, citrate and isocitrate by measurable O_2 uptake in reduced Janus green B. The particle suspensions made no response to exogenous fumarate or pyruvate and succinate oxidation was inhibited by heat, malonate, and cyanide.

Since there are so many metabolic similarities, it is not unreasonable to consider the tissues of host and parasite as being in competition for the same metabolites. Shaw and Samborski (86) have shown that pustules of rust in heavily infected leaves compete with each other for nutrients. They have also hypothesized that the rust in-

fections compete with actively growing sites (i.e. meristems) in the host plant for nutrient substances. They came to this conclusion after observing that the resistance of Khapli wheat to stem rust was broken after treatment with maleic hydrazide or radiation in growth inhibiting doses. On the other hand, resistance was augmented by indoleacetic acid (76). Implicit in such reasoning is the concept that active growth constitutes resistance through competition for substrates. Along this same line, it has been noted that deficient nitrogen strengthens the resistance responses of the host, while an excess of nitrogen weakens it.

The fatty acid metabolism of host and parasite may be slightly divergent. Reisener, et al. (69) have recently shown that fat metabolism has a primary role in the respiration of stem rust uredospores. Valerate, butyrate, propionate and acetate all stimulate respiration. The longer chain valerate is much more effective than acetate, however.

The component fatty acids of a number of the rusts and smuts have been determined by Tulloch and Ledingham (100). They have found a long-chain fatty acid in these organisms that is rare in higher plants--cis-9-10-epoxyoctadecanoic acid. Although absent in the host, they found considerable amounts in the rusts.

An interesting finding with possible evolutionary significance was their discovery that the composition of the spore oils of four different Puccinia species parasitic on members of the Gramineae were quite similar overall, and distinctly different from the oil complement of 4 Puccinia species parasitic on members of the Compositae.

Certain resistant wheat varieties have been rendered susceptible by supplying low concentrations of maleic hydrazide (78). Races of leaf rust varied in their sensitivity to this chemical, but all of them were more sensitive than the stem rust races used and less sensitive than

wheat plants. Samborski, et al. (78) feel that the response of wheat and wheat rust to maleic hydrazide is an indication that both nutrition and inhibition may be involved in determining the reaction type. They have demonstrated that maleic hydrazide causes an accumulation of soluble nutrients in the wheat leaf and yet is fungitoxic at high concentrations even though it promotes susceptible reaction types at low concentrations.

When compared to uninfected controls, indoleacetic acid (IAA) levels are higher in infected susceptible leaves and lower in infected resistant leaves (81). Srivistava and Shaw (96) have found indications that flax rust is able to synthesize indoleacetic acid from tryptophan. This points to another unifying segment of metabolism of host and parasite, as well as an active agent possessed by the fungus that will affect many of the physiological functions of its host (e.g. nucleic acid metabolism, cell permeability, cellular elongation, etc.). Certain investigators (81) have theorized that manipulation of indoleacetic acid levels, perhaps by inhibiting decarboxylation of the auxin, might enable the parasite to direct the mobilization of substrates at the infection site.

Deverall and Daly (20) have criticized earlier work emphasizing the role of IAA-oxidase in rust-diseased tissues. They point out that the apparent rate of decrease in decarboxylation of indoleacetic acid in rusted tissues may not be due to repression of IAA-oxidase, but simply to lack of substrate. Their data show that exogenously supplied IAA ($C^{14}OOH$) was accumulated more slowly in rust-infected than in healthy tissue. It was only after sporulation was begun that the rates of IAA decarboxylation in rust-infected tissue fell significantly below diseased tissue. Various light treatments had a pronounced effect on the capacity of infected tissues to decarboxylate IAA expecially in the early

stages (2-3 days) when there was a significant increase in decarboxylation. Studies with blue, red and far red suggested a phytochrome type interaction with blue and far red generally stimulating the reaction and red being inhibitory. Manipulation of the light regime did alter infection intensity, but not enough in itself to control susceptibility or resistance in the response.

Metabolic Differences: Host vs. Parasite vs. Host-Parasite Complex

The respiratory quotient of germinating rust spores has been found to be 0.7, indicating a fat-metabolizing system (46). The respiratory quotient of both healthy and infected wheat leaves was found to be 1.0, indicating the respiration of carbohydrate substrates. Caltrider, et al. (11) have confirmed that rust uredospores metabolized endogenous reserves of fat during the first 12 hours of germination. Fatty acids decline from 14% to 9% of the spore dry weight apparently being converted to carbohydrate and protein during this period as measured by concurrent increases in the anthrone positive and ninhydrin positive fractions. B-oxidation of fatty acids (see Reisener, et al., (69) and the glyoxalate pathway may be active in uredospore metabolism as both isocitratase and malate synthetase are shown to be present in ungerminated uredospores of *Puccinia graminis* and *Uromyces phaseoli*. This pathway could account for the carbon skeletons reappearing as carbohydrates. At the same time, however, the Krebs cycle is probably also oxidizing part of the acetate to produce energy for the syntheses that occur. After the initial 12 hours of endogenous respiration, *Puccinia graminis* begins to utilize exogenously supplied succinate and glucose.

Daly, et al. (17) have made a thorough analysis of major carbohydrate metabolites occurring in healthy vs. rust infected leaves of bean

and safflower. In cotyledon tissue sections supplied with C^{14} labelled glucose, they found three compounds being actively synthesized in diseased tissue that were not detected in the controls. Using two-dimensional chromatography, they achieved good resolution of these compounds from the normally occurring hexoses and sucrose. By the use of spray reagents and hydrolysis tests in addition to Rf values compared to standards, one of these compounds was tentatively identified as trehalose. Another appeared to be a pentitol and co-chromatographed with arabitol. The third unknown was surmised to be a hexitol. Rf values of this compound were similar to but not identical to sedoheptulose and mannose, but did not give the qualitative color reactions of either, casting some doubt on a previous report by Jain and Pelletier (41).

Comparative Biochemistry and Metabolism of Wheat and Rust

The metabolic versatility of photosynthetic higher plants is quite well known. Their ability to synthesize all of their own requirements utilizing only minerals taken up by the roots and gases absorbed from the air indicates the completeness of their enzymatic complement. More specialized studies have indicated the presence of glycolysis, the hexose shunt, the TCA cycle, and other ubiquitous metabolic processes in the wheat plant (10,11,18,19,23,97,98,105).

It is fairly well established that the host plant is furnishing carbon skeleton substrates to the parasite. Dickson, et al. (21) have found that excised leaves of etiolated or albino corn seedlings will not support growth of corn rust on water alone, but will support near optimal growth on sugar solutions, especially glucose. There is more involved than sugar substrates, however, for stem rust uredospores have been shown by Kasting, et al. (43) to synthesize 11 common amino acids upon sucrose

media in vitro.

It is not yet apparent where the metabolism of the rust is lacking. Shu, et al. (90) found that uredospores of wheat stem rust could utilize a number of different amino acids, sugars, and fatty acids in respiration. Of carboydrates tested (90) only uniformly labelled glucose and sucrose yielded more labelled respiratory CO_2 than D-mannitol-1-C^{14}. Uniformly labelled D-fructose and D-mannose-1-C^{14} were also utilized as were arabitol-1-C^{14}, glucitol-1-C^{14}, and ribose-1-C^{14} at low levels. Utilization of xylose-1-C^{14} and sedoheptulose-2-C^{14} was at very low levels.

Judging on the basis of CO_2 yield, glutamic acid is the amino acid most readily utilized by wheat stem rust uredospores; aspartic acid, isoleucine, leucine, alanine, arginine, and glycine as substrates gave fair yields of CO_2, while phenylalanine, threonine, proline, and lysine were broken down only slowly. Arginine and lysine are most efficiently incorporated into spore material. Histidine and tryptophan were not included in this study.

There is evidence that leaf rust spores contain succinoxidase, and can utilize exogenous succinate in a process that is competitively inhibited by malonate and that is cyanide sensitive. Staples (98) has identified aconitic, citric, fumaric, malic, and succinic acids in the respiring spores. This same investigator isolated the same organic acids from both healthy and infected wheat leaves. Apparently the TCA cycle is one unifying point in the metabolism of both species.

A common phenomenon that makes the gene-for-gene relationship hard to define and identify is the universal resistance response. Plants have mechanisms for excluding a broad spectrum of fungus invaders that are never associated with them as parasites (58). Plants from the arid areas of Australia have been shown to be hypersensitive to Phytophthora,

which is strictly a parasite of humid areas. If wheat leaves are exposed to 50°C. for 30-50 seconds, then Penicillium glaucum, a common saprophyte, is able to penetrate into the leaf and cause localized necrosis. In time, however, the wheat leaf regains its resistance and the fungus growth is stopped. When the resistance returns, histological changes occur in the surrounding tissue that are similar to those that occur when the leaf is penetrated by an avirulent rust race. Apparently then, specific resistance must be distinguished from generalized resistance if gene interaction studies are to be meaningful. This experiment seems to interpret resistance as an active process, and this concept is also supported by others. In 1940, Muller and Borger (59) found that parenchyma tissue of potato can be immunized against virulent races of the blight fungus by first inoculating it with an avirulent race.

Cruikshank (14) cites several cases in which non-specific fungitoxic compounds are formed as a result of physiological contact of host tissue with fungi that are not pathogenic to the host. For example when Monilinia fructicola--non-pathogenic on peas--was placed in contact with endocarp tissue of detached pea pods, a fungitoxin was produced. This compound was isolated and identified as 3-hydroxy-7-methoxy-4',5'-methylenedioxy-chromanocoumarane. It should be emphasized that this compound was isolated from material diffusing from the host tissue and not from macerated tissue. Apparently its formation was stimulated by metabolites diffusing from the fungus and is a generalized response to fungal contact and not in any way due to wounding. Some other fungitoxins are also believed to be formed by Orchis militaris, carrot and red clover as a response to substances diffusing from non-pathogenic fungi.

A number of species of fungi (14) non-pathogenic on carrot induced the formation of a fungitoxin when inoculated on carrot roots. This com-

pound has been isolated and identified as 3-methyl-6-methoxy-8-hydroxy-3, 4-dihydroxy-isocoumarin. This compound was not formed, however, when carrots were inoculated by Stemphylium radicinum which is pathogenic to this host.

Several species and races of Helminthosporium--all non-pathogenic to Malus species -- were found by Grijseels, et al. (33) to incite a hypersensitive response upon apple. These symptoms were indistinguishable from the response of a resistant selection of Malus atrosanguinea to races of Venturia inaequalis which are pathogenic to most species of apple trees. They suggested that the histological response of apple to invasion by Helminthosporium carbonum and Venturia inaequalis was quite similar in spite of the fact that the former is not pathogenic to apples.

A compound from white potato peel extracts that was fungitoxic to Helminthosporium carbonum proved to be an amino acid addition product of chlorogenic acid (12). It was broken down into chlorogenic acid, caffeic acid, isoleucine, methionine, phenylalanine, tryptophan, tyrosine, valine, and two other unidentified ninhydrin positive substances.

It is tempting to try to correlate these observations with the genetic nature of host resistance (dominant) and virulence of rust fungi (recessive) in which the dominant expression would indicate genes for the phytotoxin, and the recessive alleles in the parasite indicating the non-production of the diffusable metabolite--whatever it is--that stimulates the host to form a phytotoxin. If virulent races of fungi produced inhibitors to disable phytotoxic systems in the host, then virulence could be--and sometimes actually is--dominant.

Because of lack of necrosis and environmental interaction, these results are not strictly analogous to host parasite interactions with the

obligately parasitic rusts. The Ponca response, however, is more of a broad spectrum response than is usually the case. Two questions still remain as enigmas in the phytoalexin theory. (1) If successful parasitism depends upon production of a metabolite that inhibits the host phytoalexin-forming enzyme system, then why is virulence usually recessive to avirulence? (2) If recessive virulence means that the parasite refrains from forming certain metabolites that trigger the host phytoalexin forming system, then why does the presence of powdery mildew infections on the opposite side of the leaf enable wheat leaf rust to form a fully compatible interaction type on wheat varieties which are normally hypersensitive-immune to leaf rust?

The cases of toxins or phytoalexins listed above involve facultative parasites and may not be strictly comparable to cases involving obligates. However, Olein (63) has presented a sound case for a toxin in the wheat-stem rust interaction. By applying electric current to flecking wheat plants he was able to remove the necrotic area away from the region of mycelial growth toward the positive pole; presumably an electro-negative toxin is involved in producing the host cell necrosis As to time of toxin formation, no displacement occurred if the current was applied more than one day before flecking.

Sugar alcohols, especially sorbitol and mannitol, when applied to rust-infected detached wheat leaves caused blackening of the uredia (91). This discoloration was reversible and the uredia resumed normal development when the sugar alcohol was withheld and sucrose supplied to the leaf. Daly, et al. (17) have seen this as a possible competition for NADP by the sugar alcohol reductase, causing it to be in short supply insufficient to support extensive respiration by the hexose shunt, which is regarded as an integral feature of the metabolism of diseased tissue by a number of investigators.

investigators. Allen (2), Allen and Goddard (3), Kiraly and Farkas (46), Shaw and Samborski (85), and Daly, et al. (18) have indicated that although healthy wheat plants respire largely through the TCA cycle, infected plants tend to shift toward the hexose shunt pathway. This has been indicated by a lower C_6/C_1 ratio and malonate insensitive respiration in infected plants. To support this concept, Jain and Pelletier (41) have found a striking increase in sedoheptulose in heavily rusted plants, but this is questioned by Daly et al. (17). Millerd and Scott (57) cite Katz and Wood as demonstrating inadequacy in the C_6/C_1 of assaying the hexose shunt. These authors have examined the evidence pertaining to the great increase in respiration in resistant varieties and consider that this increase could be due to either uncoupling of phosphorylation or the operation of nonphosphorylating electron transport systems. They emphasize the importance of knowing the 'histological state' of the tissues analyzed, stating that the initial responses must be clearly distinguished from the degenerative responses that occur later.

Amino Acid Metabolism in Relation to the Disease Cycle

With reference to amino acid pools, Shaw and Colotelo (83) found that the relative ratios of specific free amino acids were quite similar in the varieties Little Club and Khapli. Little Club is highly susceptible while Khapli is highly resistant to most races of stem rust. They have also found an eventual increase (8-9-days after infection) in the concentration of aromatic amino acids. Since erythrose-4-phosphate is an intermediate in the pentose phosphate pathway and also a precursor of aromatic amino acids via the shikimic acid pathway, they point out a possible correlation between the increase in respiration through the pentose phosphate pathway and the increased levels of aromatic amino acids and

indole compounds. These same authors found the highest ratio (6.0) of serine relative to control levels at the 3-day stage of infection on the resistant species, Khapli. On the other hand, they found reduced levels of serine in both soluble and protein fractions of advanced (9 day) infections on susceptible Little Club.

Samborski and Forsyth (77) were able to inhibit rust growth with a number of compounds at non-phytotoxic levels. These compounds included several amino acid analogues (canavanine, p-fluorophenylalanine, ethionine) and certain concentrations of some naturally occurring amino acids (histidine, isoleucine, methionine, and serine). Certain pyrimidine analogues were effective inhibitors also (thymine, oxythymine, azathymine). They are quick to point out, however, that it is not clear whether the inhibition is due to a direct effect on the pathogen or through an indirect derangement of the host metabolism that prevents it from supporting parasitic growth. The inhibition by naturally occurring amino acids was felt to be due to competition (with other essential amino acids related to them in structure) for permease enzymes that facilitate entry into the fungus mycelium.

Nucleic Acid Metabolism and Protein Synthesis in Relation to the Disease Cycle

A striking advantage in obligate parasitism over saprophytism could reside in the utilization of the protein and nucleic acid forming potential already present in the host. Although obligate fungi have not yet been shown to exhibit viral type action, some interesting studies along this line have already appeared. Person (65) has shown that the haustoria, and more especially the vegetative hyphae from which they arise, give strong staining reactions for ribonucleic acid (RNA) in leaf rust and stem rust.

Rohringer and Heitefuss (70) have been able to incorporate P^{32} from orthophosphate into the RNA of both healthy and rusted wheat leaves. Purified RNA isolated from the rusted susceptible wheat leaves had 160-300% as much labelled RNA as the non-inoculated or the inoculated-resistant leaves. They found that RNA evidently builds up rapidly for a few days at the growing infection site and later declines as the infection matures and produces spores. The function of this RNA will have to await clarification in future studies. Rohringer, et.al (71) have recently demonstrated a dramatic increase in ribonuclease activity in infected wheat leaves. This increase occurs whether the host is resistant or susceptible. The next step will be to determine if the host plant contributes ribonucleotides as substrates for the synthesis of RNA in virulent strains of the fungus.

An increase of RNA content was found to occur in diseased leaves of barley concurrent with the appearance of mildew symptoms (55). This increase over comparable healthy controls increased until sporulation, then decreased rapidly. Guanylic acid content was observed to be particularly high at some infection stages.

Samborski, et al. (79) have recently demonstrated that rusted detached wheat leaves floated on benzimidazole showed a significantly reduced incorporation of glucose-U-C^{14} into soluble amino acids as opposed to non-inoculated controls. About an equal amount of label appeared as protein in each fraction, but incorporation of C^{14} into nucleic acid was about twice as high in the rusted tissues.

Rohringer, et al. (71) have investigated some striking changes in ribonuclease activity in wheat leaves inoculated with leaf rust. Specific activity doubled during the first two days of infection, dropped slightly during the third and fourth days; then rose to a maximum at the

6-7 day stage. The specific activity curves in compatible (susceptible) and hypersensitive (resistant) host-parasite interactions were similar. However, the activity level was constantly higher in the susceptible reaction.

A general depression of incorporation of cytidine, H^3, label into wheat leaf nuclei has been detected near rust infection sites (61). This reduced cytidine incorporation was apparently not due to reduced pool size. The effect was more intense in a susceptible variety than in a resistant one. Histologically, the effect extended some 100 μ beyond the mycelium of the fungus. On the other hand, Shaw and Srivastava (87) detected a four to five fold increase of adenine and another unidentified purine in wheat leaves infected by stem rust. The concentration of guanine, however, was essentially the same in both non-inoculated and rusted leaves. No evidence was obtained for the presence of a purine compound that would delay senescence in detached leaves as kinetin, benzimidazole or compounds similar to purines that are present in coconut endosperm.

Net protein synthesis has been shown in detached wheat leaves supplied with a solution containing nitrogen salts and kept in the light (37). Protein turnover was higher in detached leaves than in attached leaves in the dark. Their method of measurement was in turnover of compounds derived from $C^{14}O_2$, however, and they suggest the difference could be due to translocation. Newly synthesized sugars would be trapped in detached leaves.

Staples and Stahmann (99) found an alteration in the number of molecular forms or isozymes of 5 of 12 enzyme complexes studied in rusted vs. healthy bean leaves. Malic dehydrogenase, succinic dehydrogenase, acid, and alkaline phosphatase all had more isozymes in the rust-complex

than in comparable uninfected leaves.

Dilute solutions of kinetin and benzimidazole have been shown to enable detached leaves to retain their resistance, even though when floated on water detached wheat leaves become susceptible to rust infection (74,28). Samborski, etal. (78) had previously shown that protein loss in detached wheat leaves was impeded by benzimidazole. The ratio of soluble protein and free amino acids to total nitrogenous compounds usually rises rapidly after a leaf is detached, and leaves that are resistant become susceptible when removed from the plant. If these leaves are floated on benzimidazole solutions, however, this breakdown process is halted and the leaves remain resistant to rust infection. Addition of glucose to the solution will again break down the resistance unless it is countered by the addition of cobalt ion. This fact has caused Wang (102) to speculate that a vitamin B_{12} like factor may be involved in host resistance.

Benzimidazole forms a nucleotide structure as a portion of one of the vitamin B_{12} complex of molecules. Other benzimidazole analogs are involved in this particular complex along with adenine and hypoxanthine (30). This may mean that it is involved in nucleic acid metabolism as Dinning, et al. (22) suggest that vitamin B_{12} may be necessary for formate reduction in the methylation of uridine to form thymine.

The case for benzimidazole as a purine antagonist is indisputable. Wooley (108) showed that benzimidazole inhibition of growth in several yeasts and bacteria is negated by adenine and guanine, but by no other purines. Klotz and Mellody (49) have found benzimidazole inhibition to be completely reversed by nucleic acid, and the Lineweaver-Burk plots of their data strongly suggest competitive inhibition. Benzimidazole is not inhibitory to the growth of yeasts and bacteria if substituted in posi-

tion 2 (108). It was also noted that 2-hydroxybenzimidazole and glucobenzimidazole actually stimulated growth of Saccharomyces cerevisiae. Also, the effect of benzimidazole in retarding rust development on wheat was overcome by its dimethyl derivative (102).

Benzimidazole stimulates the formation of chlorophyll in detached, etiolated, wheat leaves, and it greatly retards the destruction of chlorophyll in detached green leaves both in light and in darkness (103). It also tends to preserve the same ratio of chlorophyll a/chlorophyll b, as is found in healthy, intact plants.

Role of Phenolic Compounds in Determining Disease Reaction

Rust resistant tissues of wheat normally form necrotic flecks a few days after fungal penetration. It has been known since the 1930's that these flecks contain phenolic pigments. Their presence on the scene, and the known phytotoxicity of oxidized phenolics, has stimulated experimentation designed to implicate these compounds as the basis of chemical resistance. Kiraly and Farkas are the leading investigators of recent years into the role of oxidized phenols in disease resistance. In a recent paper (47) they admit that the overall process of disease resistance may involve much more than the oxidation of phenols, but it is still believed important in the reaction scheme. High phenol concentration in the host plant, the activity of phenol oxidizing enzymes, the activity of reducing systems, and the stimulus for increased synthesis of aromatic compounds are all important facets of the net phenol balance. These authors (47, and see also Farkas and Ledingham, 25) also agree that phytotoxicity stems from oxidized phenols and that reducing systems are therefore important in maintaining the aromatics in reduced form. They have found that reducing systems (e.g. glucose-6-phosphate dehydrogenase)

are all more active in susceptible combinations. Some evidence (73) for a reducing (electron transport) chain consisting of triphosphopyridine nucleotide, glutathione, ascorbic acid, and ascorbic acid oxidase has been presented as the possible source for maintaining an adequate reducing potential in the parasitized cells. Evidence for a quinone reductase has been found in rust spores. An apparent dynamic resistance was found by Millerd and Scott (56) in 1955. They isolated a phenolic compound from extracts of wheat leaves infected with mildew, that was capable of producing mildew sensitivity in wheat varieties that are normally susceptible to mildew. Their results are discounted by Shaw (84), however, who contends that their treated tissues do not differ significantly enough from the controls.

The browning of host cells caused by increases in poly-phenol oxidation products may be described as a secondary companion phenomenon to the true defense reaction of host cells and not the central focal point of resistance. The polyphenoloxidase inhibitor 4-chlororesorcinol lowered the host resistance of apple to the scab disease and also inhibited oxidation of phloridzin to phloretin and other oxidation products which inhibit the germination of <u>Venturia</u> spores (62). The picture is confused, however, by the fact that the phloridzin and polyphenoloxidase content of resistant apple varieties is not significantly greater than susceptible varieties.

Vacuolar fragments appearing in diseased tissue of pine leaves are nearly always enclosed in newly synthesized membranes (7). This fragmentation response is felt to be correlated with the rapid rise in respiration in diseased tissue by presenting a vacuolar system with greatly increased surface area, and (judging by inclusions observed) different permeability properties. Boyer (6) has concluded that the early col-

lapse of the tonoplast with a resulting release of vacuolar phenols into the cytoplasm in young pine leaves did not affect the mycelium of blister rust sufficiently to account for the greater resistance of young leaves. He infers that mere correlation of total phenolic content between susceptible and resistant varieties of host species may be misleading.

Miscellaneous Compounds Correlated With Resistance and Susceptibility of Various Hosts to Obligate and Facultative Parasites

Mukherjee and Shaw (60) cite two critical unpublished studies as showing stem rust infection to cause a striking increase in RNA in wheat leaves. DNP treatment was found to increase the Pi/Po ratio in infected as well as uninfected leaves. They felt that this response eliminated the possibility of the uncoupling of phosphorylation being the major cause of the early respiratory rise in diseased tissue. A possible coupling with the pentose phosphate path was postulated as lipid synthesis results in the oxidation of NADPH.

Chlorophyll could be a competitor for NADPH with the hexose shunt. Benzimidazole has been shown (103) to prevent chlorophyll breakdown in addition to its role in preventing protein breakdown. Scott and Smillie (80) have found the increase in respiration to be concurrent with a decrease in photosynthesis.

Pantothenate has been found to increase the severity of stem rust on three varieties of wheat (38). It apparently was able to cause the breakdown of host resistance. The concentration of this vitamin necessary to favor optimum growth of the parasite is not known, but apparently is much higher than normally occurs in the host tissue.

Futtrell and Berry (31) observed that 10^{-1} and 10^{-2}M Na-propionate,

when added to the nutrient solution of wheat plants in pots before infecting them with rust, reduced the size of the resulting pustules.

Two strains of Helminthosporium gramineum differing in pathogenicity responded differently to the addition of amino acids to culture media (93). Basic differences in amino acid metabolism could be connected with pathogenicity on various varieties of apples.

Van Andel (101) found that DL-serine, DL-threonine,-L-threo-B-phenylserine, and DL-histidine markedly increased the resistance of cucumber seedlings to Cladosporium cucumerinum. These compounds could be applied to any part of the plant, and as long as 24 hours after the plants were sprayed with a suspension of fungus conidia, they still gave considerable protection. In vitro tests showed that 0.4% D-serine prevented growth of C. cucumerinum on nutrient agar plates. This concentration was non-phytotoxic. DL-serine also gave protection to broad bean against Botrytis fabae and to tomato against Phytophthora infestans.

Williams and Boone (106) could not change the reaction of inoculated apple leaves to Venturia inaequalis from resistant to susceptible by supplying the leaf with amino acid solutions.

The D and DL isomers of phenylalanine greatly increased the resistance of 7 apple varieties to Venturia inaequalis (50). Exogenously supplied solutions of the L isomer had no effect.. It was possible to obtain an increase in resistance to apple scab in one selected variety by infusing the D isomers of several naturally occurring amino acids into apple leaves, especially D-alanine and D-γ aminobutyric acid.

Mutant strains of Venturia inaequalis were variable in their pathogenicity on apple(5,45). Mutants requiring biotin, nicotinic acid, inositol, and pantothenic acid still produced lesions. Mutants requiring choline and histidine produced fleck symptoms in all cases,

while various mutants requiring purines, pyrimidines, and methionine showed no symptoms in some cases, flecks in others. A large number of arginine requiring mutants and two proline requiring mutants produced no symptoms at all.

It should be pointed out that the appearance of the fleck symptom does not necessarily indicate greater virulence than those showing no symptoms because of the association of fleck symptoms and the hypersensitive resistance response.

By properly supplementing apple leaves with the required substance, Kline, et.al. (48) found that a number of auxotrophic non-pathogenic mutants of *Venturia inaequalis* could become pathogenic. Supplements of choline, riboflavin, uracil, arginine, histidine, and methionine added to apple leaves allowed non-pathogenic mutants, that had lost the capacity to synthesize these nutrients, to develop a severe infection. Supplements of adenine, hypoxanthine, and guanine failed to allow mutants deficient in these nutrients to regain their pathogenicity. All mutants could grow on apple leaf sap indicating that the required nutrients were in the apple leaf. Supplements added to the sap caused little or no increase in growth of most of the mutants. Two striking exceptions were histidineless and methionineless isolates. The latter almost doubled its rate of growth when leaf sap was supplemented and the histidineless mutant increased its growth 11 fold upon addition of exogenous histidine. This mutant had the lowest rate of growth on unsupplemented leaf sap, but the highest on supplemented.

CHAPTER III

MATERIALS AND METHODS

Plant Materials

All plants of Ponca wheat (CI 12128) used in the experiment were the increase of a single plant (designated as selection 5212) selected from seed stocks of the Department of Botany and Plant Pathology, Oklahoma Agricultural Experiment Station, in 1962. Plants from this seed were selfed for two generations to furnish progeny with maximum possible homozygosity. This was deemed essential for the success of the experiment. It was hoped to obtain plants that would give identical responses to the same stimuli including: rust infection, vernalization, temperature, etc. Allowing for slight environmental variations, the plants were in fact observed to be almost identical in their responses. In only one case was an aberrant reaction noted. In the final rust infection one plant appeared to be slightly more susceptible than other plants treated similarly, in that the rust sporulated more quickly. In all other tests the plants used in the various treatments were selected at random from the bulk population, and no other aberrant response was noted.

In order to test for metabolic changes due to age and vernalization, 48 seeds of Ponca selection 5212 were planted singly in 4-inch pots in the fall. All but two germinated and produced healthy plants. Healthy and rust-infected leaves of these plants were collected at 6 weeks of

age. At this time the plants were showing the large susceptible pustules characteristic of seedlings. At about 9 weeks of age these plants were divided into two groups; one of these was subjected to 5 weeks vernalization, while the other was kept at 70 degrees F. Later these plants were placed in a growth chamber and kept there until the vernalized plants headed out and matured grain. Samples were also taken at this time (23 weeks from seed).

These same plants were used in the age of infection experiment, and made up lots 2 and 3 of the plants described below. A more detailed chronology of the growth and development of these plants is presented in Table III of the appendix.

Three different lots of plants (with different environmental backgrounds, but carrying the same Ponca selection 5212 genetic background) were used to check the metabolic responses occurring at various stages of infection. Leaf samples were collected for analysis from each lot at 0 days (uninfected controls), 2 days, and 6 days following inoculation. No visible symptoms were apparent on the 2-day collections; the 6-day collections were in the full fleck to incipient pustule stage.

The three lots of seedlings were as follows:

1. Seedlings, planted February 14, 1964, inside the growth chamber. Generalized growing conditions: Day temperature, 78 degrees F. for 13 hours; night temperature, 64 degrees F. Light to a maximum of 1500 ft. candles intensity at midday with lower intensities simulating dawn and twilight supplied for 14 hours daily. More than 85% of the lighting was supplied by gro-lux fluorescent bulbs; the remainder by incandescent bulbs. Relative humidity usually 50%.
2. Older Non-Vernalized Plants. Planted November 5, 1963, in

greenhouse room thermostatically controlled at 70 $\pm$ 2 degrees F. Low intensity natural illumination supplemented by low level intensity fluorescent and incandescent lamps. Day length set at 12 hr. Moved to growth chamber on February 18, 1964. Conditions thereafter identical to Lot 1.

3. Vernalized Plants. Planted November 5, 1963, and grown under environmental conditions identical to Lot 2 until January 9, 1964. Vernalized from January 9, 1964, to February 18, 1964, by subjecting the plants to a 10-hour day at low light intensity (500 ft. candles or less) at 52 degrees F., and night temperatures of 38 degrees F. Removed to growth chamber on February 18, 1964. Growth conditions from this date on were identical to Lot 1. For a more detailed account of the growth record on Lots 2 and 3 refer to Table III of the appendix.

Plants from all three lots were inoculated inside the growth chamber overnight on March 10-11, 1964. It was necessary to use warm water inside the inoculation chambers, and to set the growth chamber control at 95% humidity in order to keep the leaves moist enough for germination and infection to take place. Infection severity was fairly uniform and approximately 25% on the modified Cobb scale.

The leaf rust pathogen used in the experiment was identified as _Puccinia recondita_ Rob. ex. Deam. f. spl _tritici_ Erikss, physiologic race 105-NA61-14 (13.92). Its reaction on the international and 1961 supplemental differentials is listed on Table IV of the appendix. This rust culture was obtained from the Department of Botany and Plant Pathology, Oklahoma Agricultural Experiment Station; and fresh spores were maintained throughout the duration of the experiment by periodic increases. Purity was maintained by using the variety Wesel (resistant to a number of common

races, but fully susceptible to 105-14) as the host variety for population increase, and by keeping the culture well isolated from possible contamination. Each time the culture was increased, the possibility of contamination was checked by the reaction of the bulk population on the rust differentials mentioned above. The rust culture was maintained in highly purified form, with no more than a trace of contamination showing up on any set of the differentials.

Incubation Procedure

Only healthy looking green leaves were selected for incubation with the C^{14} labelled sugar. The selected leaves were cut with a razor blade and placed in 10 ml. erlenmeyer flasks. About 20 leaves was the maximum number that could be effectively incubated in a single flask. The cut ends were submerged in water containing 7.5uc [1] of glucose-U-C^{14}. The amount of water in the flask varied from 1-3 ml. according to the total fresh weight of the detached leaves making up the sample. Samples varied from 0.65 to 2.65 grams fresh weight. Enough water was added so that the leaves would take up most of the water through the open xylem elements and disperse it throughout the leaf during the 4 to 5 hour incubation period. Water uptake through the xylem was accelerated by placing the samples in a growth chamber with high light intensity. (1500 ft. candles) and low relative humidity (10%) to increase transpiration.

After 4 to 5 hours of transpiration-translocation, some 25 to 40% of the labelled sugar was incorporated into leaf tissue as estimated by counting aliquots of liquid remaining in the flasks after the leaves had

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[1]Only 6uc used in both healthy and rusted samples from 6-week old seedlings.

been removed.

The leaves were rinsed thoroughly to wash off excess sugar solution, placed in labelled vials and immediately lyophilized at -40 degrees C. After 24 to 48 hours of lyophilization, the samples were stored in a dessicator above fresh $CaCl_2$.

Extraction Procedure

It was originally intended to analyze for possible effects of rust infection on chlorophyll and/or carotenoid metabolism. For this reason the first two steps in the extraction procedure were done at 35 degrees C. at low light intensity to protect the pigments against deterioration by photolytic or oxidative chemical reactions. The remaining steps were all carried on at room temperature.

(1) Five ml. of an effective combination of lipid solvents (A 2:2:1 mixture of 95% ethanol, chloroform, and absolute ether) was added to the sample, allowed to stand for 48 hours, then decanted into an amber glass bottle.

(2) A second extraction was made with another 5 ml. of the same solvent mixture. After 48 hours this extract was decanted off, combined with the first extract, and stored at -10 degrees Centigrade. The leaves appeared largely colorless after these two steps, but traces of pigments still remained in spots.

(3) 15 ml. 95% ethanol was added and the vial containing the sample placed on a shaker for six hours. This extract was decanted off and combined with 7.5 ml. of hexane.

(4) 15 ml. 60% ethanol was added, the sample vial again agitated on the shaker for six hours, then the extract decanted and combined with the 95% ethanol extract and hexane mixture.

(5) The sample was finally extracted with 10 ml. of 10% ethanol, shaken at room temperature for three hours. The three ethanolic extracts and added hexane were then placed in a separatory funnel, 60 ml. distilled water was added and the mixture allowed to stand until most of the remaining pigments diffused into the upper hexane layer, leaving the alcohol-water layer almost clear. The alcohol water layer containing the soluble amino acids, sugars and various other components was drained into an evaporative flask, and evaporated to dryness in preparation for further purification of the extract on ion exchange resin columns. These five extraction steps, although relatively mild, were fairly thorough in removing soluble labelled compounds from the leaves as the residue from one sample failed to yield as much as 10% more label after being boiled in 20% ethanol.

Separation of the Extract into Cationic, Anionic, and Non-Polar Fractions

Ion exchange resins were used to accomplish this separation. The Dowex 50 cation exchange resin was prepared and used according to the procedure outlined by Plaisted (67). Preparation and use of the Dowex 1 anion resin was by the method of Wang (104).

The dried extract was taken up in 5 ml. of fresh deionized water. The flask was rinsed with 1 ml. deionized water. These two aqueous extracts were combined and adsorbed on the Dowex 50 column. The organic acids and sugars were then washed through the column with two 15 ml. portions of fresh deionized water, while the amino acids remained bound on the resin. The eluent was then placed on a 1.4 x 15 cm. bed of Dowex 1, and the solution allowed to pass through the bed by gravity flow. It was then rinsed with one resin bed volume of fresh deionized water, and

the eluent containing the non-polar sugars collected.

Considerable difficulty was experienced in holding all of the amino acid fraction on the Dowex 50 resin bed while the sugars and organic acids were being flushed through. It was found to be absolutely necessary to adjust and hold the pH very close to 7, and to keep the carrier solvent free of ethanol. Sharp drop in pH or the presence of ethanol depressed ionization enough to allow significant amounts of the amino acids to pass through with the organic acids and sugars. The pH was held up to a satisfactory level by using only fresh deionized water as the solvent, and by forcing the air entering the system to flow over a trap of 5N NaOH. In addition, a large excess of resin was used, approximately 5X the exchange capacity of the largest sample. Milliequivalents of exchangeable amino acid were calculated from data published by Shaw & Colotelo (83) that gave a good approximation of the mg. of soluble amino acids per gram of dry tissue in wheat plants.

After being flushed through the Dowex 1 column, the non-ionic fraction containing the sugars was first evaporated to dryness in a flash evaporator to remove traces of acid that might be present. The residue was then redissolved in 5 ml. deionized water (3 ml. + 2 ml. rinse), and then lyophilized to dryness. Finally, the residue was taken up in a volume of deionized water suitable for spotting (0.5 ml. per gram fresh wt. of the original sample).

Sugar Chromatography

Full size (18½ x 22½ inch) sheets of Whatman No. 1 paper, with the spot placed 3 inches from the edge of the paper, were used for two dimensional chromatographic separation of the sugars. From 10 to 40 (usually 20) lambdas of the concentrated sample solution were used for

each spot. Perhaps the best of many solvents tried was the 52:32:16 volumetric mixture of n-butanol, ethanol, and deionized water. This solvent had been used to good effect in prior experiments by Daly, et al. (17) and was used as the first irrigating solvent along the long axis of the paper. After the first solvent the paper was dried for 18 hours. The lower edge of the paper was serrated with pinking shears, and the solvent allowed to drip from the edge until ribose, the fastest moving sugar in all solutions tried, had moved some 40 to 45 centimeters from the origin. This required about 62.5 ml./sheet and a run of 56 to 64 hours. Solvent 2 was a miscible mixture of benzene, n-butanol, pyriding, deionized water, and benzyl alcohol in the volumetric proportions: 0.25:5:3:2.6:0.75. This solvent was developed after considerable experimentation, and was distinguishable from solvent 1 in that the disaccharides, oligosaccharides, xylose, and ribose tended to have higher Rg values in this solvent.

Amino Acid Chromatography

The amino acid fraction was also chromatographed on full size sheets of Whatman No. 1 paper with the spot placed 3 inches from the edges. After extensive experimentation, the following solvent system proved to be quite satisfactory. Solvent 1, used for the first run along the short axis of the paper, was composed of: n-butanol, benzyl alcohol, 23N formic acid, glacial acetic acid, and deionized water (12:6:2.5:2:4 by volume).

About 53 ml. per sheet were used and the solvent front allowed to approach near the edge of the sheet, the run requiring some 16 to 21 hours. Following development with the first solvent, the papers were dried from 7 to 9 hours. This drying time was found to be quite critical, for a residue was deposited by the first solvent that was useful in re-

ducing the streaking of histidine, arginine, and glutamic acid in the phenolic solvent that followed. Fisher certified liquid phenol and deionized water in the ratio (v/v) of 4:1 were used for the second solvent. To 500 ml. of this solvent 35 mg. of 8-hydroxyquinoline and 0.5 ml. of .25N NaOH stock solution were added. Then the papers were irrigated with 50 ml. per sheet of this solvent. Some 21 to 30 hours were required for the visible band of residue left from the first solvent to move down near the bottom of the paper, as solvent 2 was allowed to drip from the serrated lower edge. The fastest migrating amino acid, proline, travelled near the leading edge of the band of residue so that the proper time to remove the chromatograms could be accurately judged by inspecting from time to time through the glass side panels of the chromatograph chamber.

All solvents used in chromatography were mixed just prior to using. Amino acid chromatographs were equilibrated about 7 hours with the appropriate solvent. Such equilibration was not found to be necessary for the extremely lengthy sugar runs.

A stainless steel chamber, with glass side panels for viewing the solvent front and a glass top panel with holes for insertion of the funnel to fill the trays, was used throughout the experiment. This model, designed to accomodate 10 full size sheets with descending direction of solvent flow, was manufactured by Research Specialties Co.

Identification of Amino Acids

The innovation of a new solvent system as previously described made it necessary to thoroughly analyze the movement of each amino acid. This was done as follows: (1) Amino acid standards were spotted singly 1" apart in a line and developed with solvent 1. This paper was then sprayed with ninhydrin, and the relative movement of the amino acids

tabulated (See appendix, Table V). As expected, the Rf values in this solvent did not differ markedly from those listed for butanol-acetic acid-water (4:1:1) as listed by Smith (93) and Fruton and Simmonds (30). There were some differences, however. (2) These same amino acids were spotted in a line on a blank paper that had been developed in solvent 1. These spots were allowed to migrate in solvent 2. Then after drying and spraying with ninhydrin, the amino acids were tabulated as to their relative movement in solvent 2.

As might be expected, when the amino acids were spotted together, the Rf values were somewhat affected.

However, the ones that moved most rapidly when spotted alone apparently continued to move the farthest from the origin when present in mixtures or plant extracts. While Rf values were modified, the relative order of separation apparently was never changed in either solvent.

For comparative purposes reference chromatograms were run with each set of analyses. One reference sheet was spotted with all the amino acid standards. The others were spotted with several amino acids that differed widely in their rate of migration in both solvent systems.

Special care had to be used in identifying the amino acids. Several difficulties were encountered. Specific color tests (See Smith, 93) with reagents other than ninhydrin were only about 50% successful. This was probably due to the low concentrations used and/or interfering residues left from the developing solvents. The sulfanilic acid test for tyrosine (95) was never successful because of the high background color due to traces of phenol left in the paper. This persisted even after 4 rinses in absolute ethanol or 95% ethanol-ether 1:1. Color tests for histidine were only slightly better. Some measure of success was obtained with the Ehrlich test for tryptophan.

It was possible to identify tentatively most of the labelled compounds through co-chromatographing with known standards or by careful comparison to reference chromatographs, of which several were included with each run.

One constant feature was the order of migration, although absolute distance travelled was somewhat variable. Some of the amino acids migrated about the same distance whether spotted alone or in a mixture. The absolute movement of others was slowed considerably when spotted in a mixture of 4 or more amino acids (See Table V).

For this reason the reference mixtures were more reliable for identifying some unknowns, notably histidine, than the reference chromatographs with the amino acids spotted singly.

Absolute distances migrated were compared to the data for amino acid standards on separate sheets developed simultaneously with the samples for one criterion of identification. The position of each spot relative to alanine which was present in all samples was also taken into consideration. Alanine was given an arbitrary value of 1.0. Ra values refer to movement relative to alanine. The spots appearing in all of the 48 hour infected samples made nearly identical patterns and probably represented the same array of compounds. The same was true for compounds appearing in the uninfected controls.

Resolution of the compounds under study was always sharp and complete with the exception of one compound of the 6-day infected plants occurring in the leucine region. This could be either leucine, isoleucine, or phenylalanine.

Some of the compounds could be identified easily, for example aspartic acid and alanine which were well separated from all others. This was true also for the spot which has been designated as unknown II, and for a spot

designated as unknown III, which appeared in the 48 hour-infected samples. Identification of cysteine, lysine, histidine, serine, glutamic acid, and glycine, however, required more careful inspection, Despite the fact that all of these compounds could be shown to be resolved from the others (See Table I), distance migrated in the solvents varied from sheet to sheet, and especially between sets of chromatographs run at different times. This variation was great enough to allow for overlapping in Ra values when the values obtained on all sheets in all sets were plotted on a single composite chromatograph (Figure 1).

The treatments showed a large variation in the specific amino acids that were actively synthesized during the incubation with labelled glucose. In order to locate and resolve the active sites on each chromatograph, a radioautograph was made of each one as follows: The chromatographs were cut to 12 x 15 inches in size, leaving only ½ inch of paper behind the origin line for both dimensions of solvent flow. This size was sufficient to encompass the entire array of amino acids, although the fast-moving leucines were always near the corner opposite the origin. Four orientation marks were placed along the origin lines with radioactive ink, and the chromatographs were placed in paper folders with 12 x 15 inch sheets of Kodak no-screen X-ray film in the dark room. Paper envelopes of brown wrapping paper were folded tightly against the edges of the folder so that chromatograph paper and film were locked in their original position relative to each other. These envelopes were sealed with tape and placed between layers of beaverboard or plywood to keep them flat. Finally, these layers were placed in a box which was securely wrapped to keep out light and stored in the darkroom for an exposure of 3 weeks.

The film was developed in accordance with the directions given on Kodak X-ray film developer.

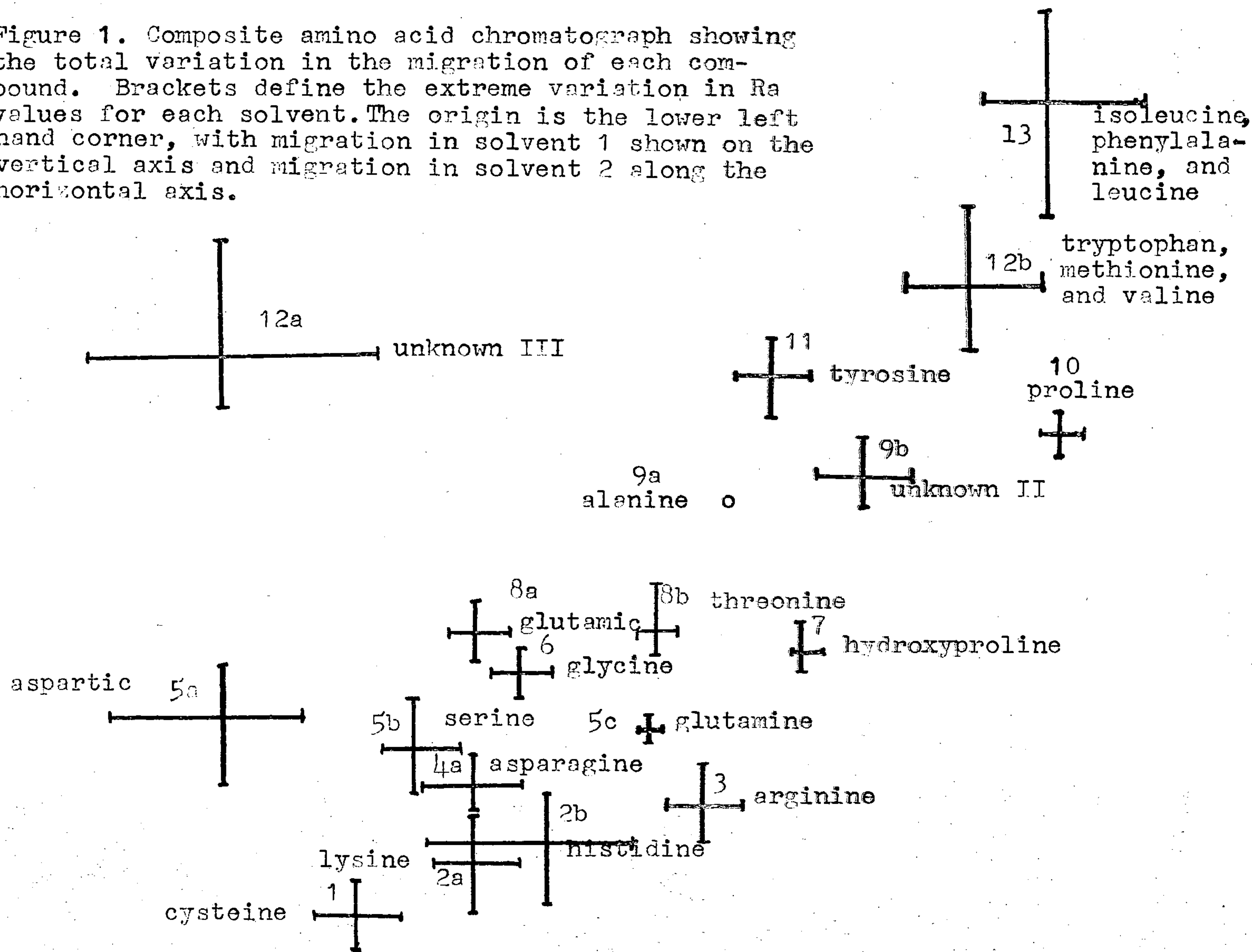

Figure 1. Composite amino acid chromatograph showing the total variation in the migration of each compound. Brackets define the extreme variation in Ra values for each solvent. The origin is the lower left hand corner, with migration in solvent 1 shown on the vertical axis and migration in solvent 2 along the horizontal axis.

After developing, the sites where the film had been exposed to beta radiation from considerable concentrations of C^{14} appeared as dark spots. By realigning the orientation marks on the chromatograph with their corresponding spots on the film, the active sites on the chromatograph could be located and outlined, and their relative activity fairly well estimated by the amount of darkening of the film. (See Figure 2).

Assay of Radioactivity

Sugars were assayed using a R.E.A.C. gas flow G.M. counter with a 2-inch window.

The amino acids spots were cut out of the paper and placed in vials containing toluene, PPO, and POPOP. These were counted for 10 minutes in a Nuclear-Chicago liquid scintillation counter. Each sample was counted at wide and narrow window ranges and an efficiency estimate made on the sample according to the ratio of narrow range window count/wide range window count. The figures presented in the tables have been corrected to 100% efficiency by comparison of the ratio as calculated above to a graph supplied by the manufacturer to show the percent efficiency at various numerical values of the ratio.

Detection of Sugars

Since both solvent fronts were allowed to overrun the edge of the paper for many hours, the relative movement of the sugars was based on Rg ratio, in which the distance of the center of the glucose spot from the origin was arbitrarily designated as 1.0. Identification of the various spots following the development of the chromatograms was done by overspotting with sugar standards.

Three reagents were used to detect sugars on the chromatograms.

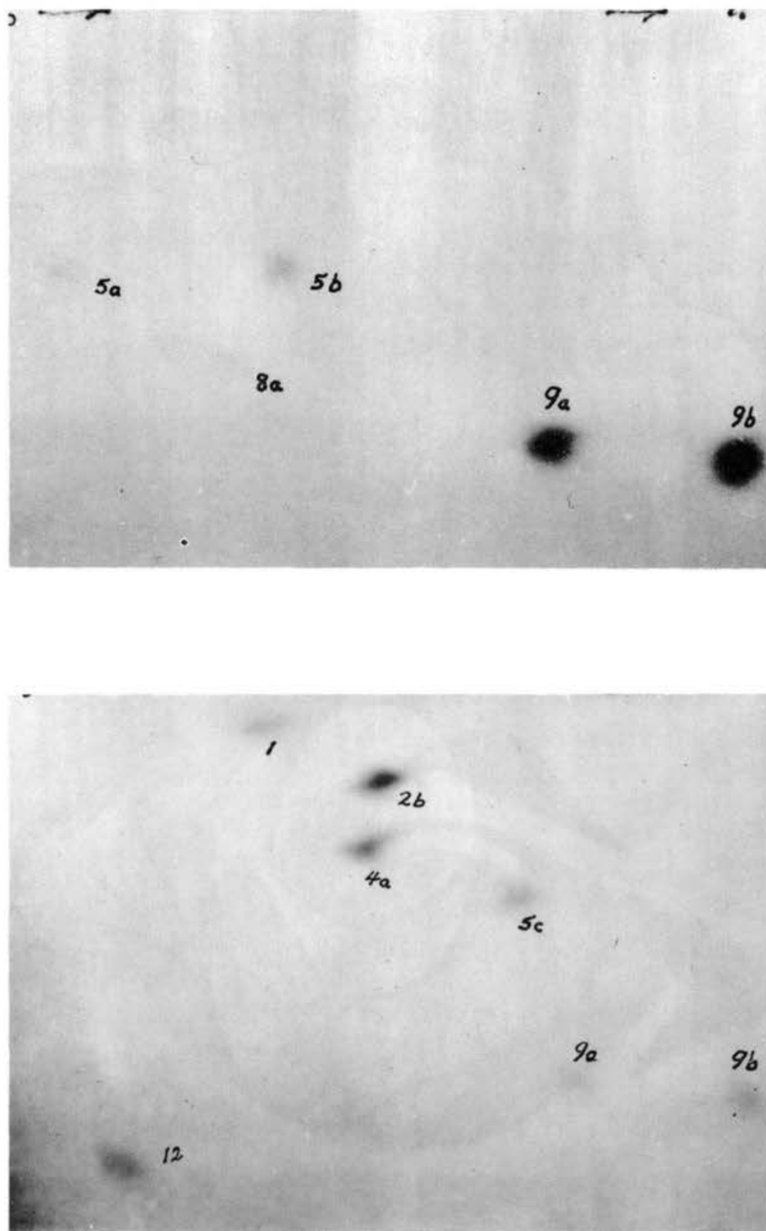

Figure 2. Photographs of the radioautographs that revealed the active sites on the chromatographs of amino acid extracts from vernalized healthy leaves of Ponca wheat (top), and vernalized leaves bearing two day old rust infections (bottom). Note that each chromatograph shows three spots that do not appear on the other, and that the sites of major activity are different.

Before counting, the papers were sprayed with aniline phthalate to locate glucose and pentoses, then with p-anisidine. The latter reagent located fructose, sucrose, and a number of oligosaccharides. Certain reference chromatograms spotted with standard solutions of trehalose and arabitol were dipped in $AgNO_3$ solution and then sprayed with NaOH according to the procedure given by Hough (40). This treatment revealed several oligosaccharides, trehalose, and arabitol, which would not react with the two routinely used reagents. Reference chromatograms spotted with standard solutions of sucrose, glucose, fructose, arabinose, xylose, and ribose were included in every set.

Figure 3 shows the minimum resolution obtained with this solvent system. Its chief weakness lies in the inability to separate fructose and arabinese. Other components are well resolved.

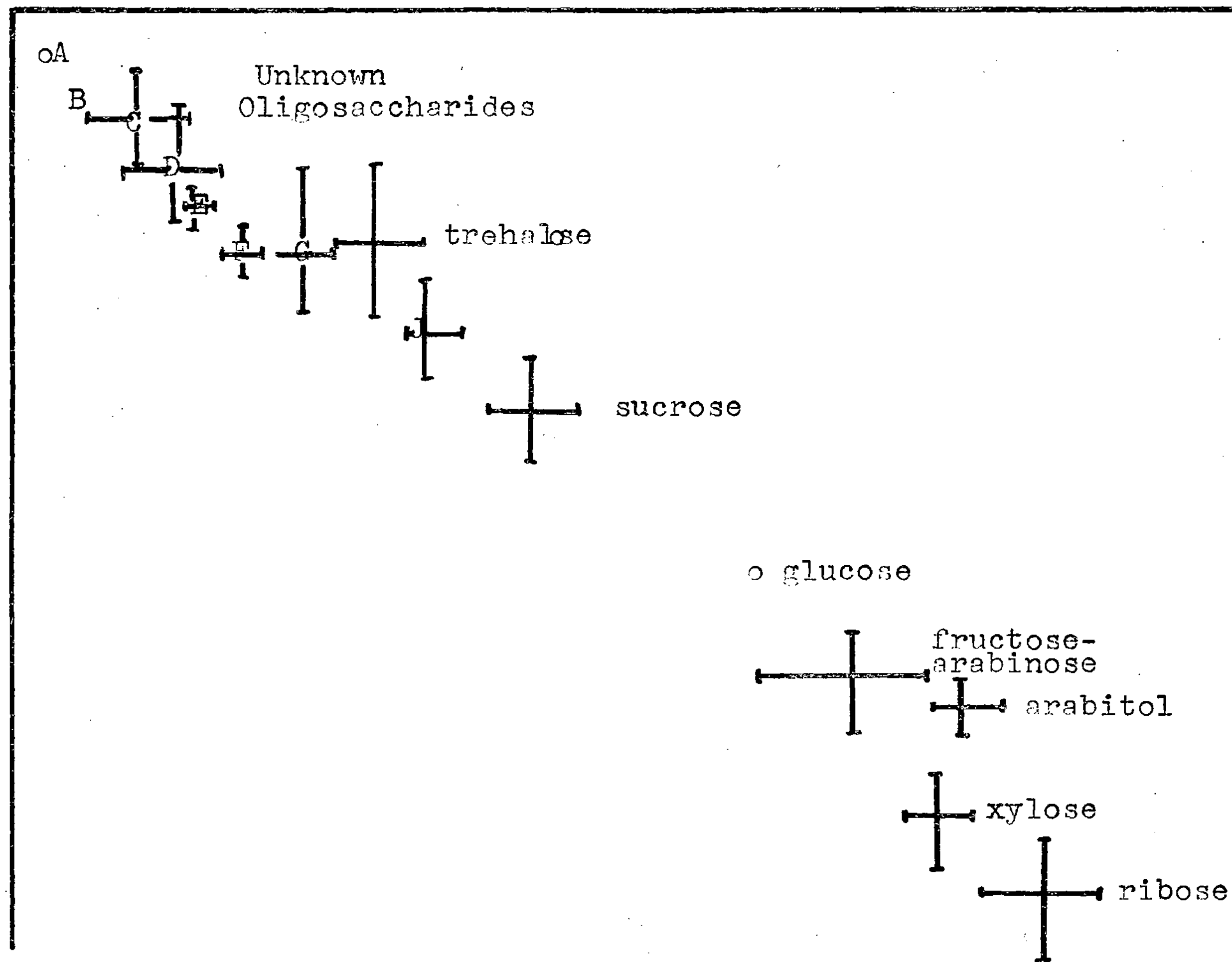

Figure 3. Composite sugar chromatograph showing the total variation in migration of each compound. Brackets define the extreme fluctuation in Rg values. The origin is at the upper left hand corner, with migration in solvent 1 shown on the vertical axis and migration in solvent 2 along the horizontal axis.

CHAPTER IV

RESULTS

Changes in Intermediary Metabolism of the Host-Parasite Interaction Attributable to Aging and/or Vernalization of the Wheat Host

From many years of field observations it is known that aging and/or vernalization are correlated with physiological changes in plants of Ponca wheat that confer a high degree of physiological resistance to leaf rust development. Table I shows how inbred plants of Ponca selection 5212 exhibited the same general response when grown to maturity under controlled conditions of temperature range, day length, and light intensity. In an attempt to determine the cause of this response, the age of infection was held constant at 0 and 6 days (designated as "H" and "R" respectively in Tables II and III) in all samples selected for the age-vernalization study.

Figure 4 shows the trends in amino acid labelling due to age and vernalization, as the plants used in this study were vernalized in the time between the collection of the 6-week and 19-week samples. Figure 5 shows the effect of age alone where none of the plants were vernalized. The 4-week and 8-week samples are not strictly comparable to the others, as these seedlings were grown in the spring in the growth chamber and subjected to long days, while the other samples were taken from seedlings started in the greenhouse in the fall under 12-hour days.

At comparable ages the spring-grown seedlings showed a higher degree of resistance (See Table I). This probably accounts for erratic data from

TABLE I

RUST REACTION OF RACE 105-14 ON SEVERAL GROUPS

OF PLANTS OF PONCA SELECTION 5212

Treatment [a]	Number of Observations Plants	Leaves	Mean Reaction Type [b]
	As Scored on December 15 and January 4		
6-S1	c		2.75
	As Scored on March 21 11 days after inoculation		
4-S2	73	99	2.51
19-V	8	42	1.90
19-S1	9	35	1.00
	As Scored on April 28 15 days after inoculation		
8-S2	5	d	1.28
23-S1	4	d	1.20
23-V	11	58	1.30

a - Treatments coded as follows:

(1) Numerals: 4, 6, 8, 19, or 23 = age of plants in weeks

(2) Letters: SS1 = fall planted seedlings
S2 = spring planted seedlings
V = fall planted seedlings subjected to 5 weeks vernalization

(3) Letters (Used in subsequent tables):
H = healthy or uninfected leaves
R = leaves supporting 6 day old rust infections
R-2d = leaves having 2 day old rust infections

b - Reaction types scored as follows:

0 = immune
0;= very highly resistant
1 = highly resistant
2 = resistant
3 = susceptible
4 = highly susceptible

In averaging the reactions on the plants, 0; was scored as 0.3, 1- as 0.7, 1+ as 1.3, etc. Where more than one type of reaction occurred on the same leaf, it was scored as the mean (e.g. 0;-2 was tallied as 1.2).

c - Reaction type uniform on all plants

d - Reaction type uniform on all leaves of each plant

4 to 8 weeks. In order of decreasing susceptibility these treatments run: 6, 4, 8, 19. The 19-week old seedlings show definite departures from trends in the three samples from young plants.

At the end of the 23rd week the unvernalized plants still score slightly lower in mean reaction type than the vernalized plants. The data indicate that the vernalized plants did not quite attain the level of resistance that is often found in field grown plants. As scored in Table I the older unvernalized plants actually decrease in resistance during the last four weeks of the experiment. It can also be noted in the vernalized plants (Figure 3) that the trends in amino acid synthesis from the 6th to 19th weeks run counter to the trend of the 19th to the 23rd week. On the surface it appears that the maturing plants revert to a "young seedling" pattern of amino acid synthesis, and this seems to defy explanation.

The sugar chromatographs revealed many oligosaccharides. Examination of the R_g values in the appendix shows that several of these unknown compounds appear consistently and are probably not simply artifacts. No attempt was made to identify this array, however, as none of them seemed to be directly correlated with reaction type.

Trehalose, arabitol, xylose, and ribose were clearly resolved with the system used; but with two possible exceptions they were always missing from the non-ionic extracts.

Changes in Intermediary Metabolism of the Host-Parasite Interaction Attributable to Age of the Developing Infection

In an effort to probe into the biochemical basis determining whether or not a leaf rust infection site on a leaf of Ponca will develop into a

susceptible type interaction an attempt was made to follow sequences of changes in sugar and amino acid intermediary metabolism.

The results of the tests are presented in Tables II and III and the variation of some of the major compounds shown in Figures 4, 5, 6, and 7. The most noticeable changes in intermediary metabolism were found in comparing amino acid synthesis at various stages of infection. Figure 6 shows the effect of the age of infection on amino acid metabolism in detached leaves of three different treatments of Ponca selection 5212. Each sample consisted of some 8 to 12 leaves of the three treatments. The 19-week old seedlings showed the highest degree of resistance to leaf rust infection, with considerable chlorosis and a type 2 reaction. The 19-week old plants following 5 weeks of vernalization appeared to be more susceptible, while the 4-week old seedlings exhibited fully susceptible type 4 reactions.

It should be kept in mind that the percentages shown of each compound at each stage of infection may be totally unrelated to their actual concentration in the amino acid pool. The figures relate to the relative amount label from exogenously supplied glucose-U-C^{14} that was converted to specific compounds during the four-hour incubation period. In other words, it was a quantitative measure of synthetic activity at a specified time, under specified conditions, with each specified time and condition measured independently of all others.

Independence of the measurements was assured by two factors: (1) each sample consisted of a different group of leaves, and (2) each sample was incubated with labelled glucose for only four hourse

The data shown in Figure 5 clearly demonstrate that the age of the infection can have effect on the metabolic differences that are detected

TABLE II

ASSAY OF LABEL IN THE CATIONIC FRACTION SITES OF ACTIVITY ON AMINO ACID CHROMATOGRAPHS

Set 1

Samples	cys 1	hist 2b	Unk I 4a	asp 5a	ser 5b	gmn 5c	gly 6	glu 8a	ala 9a	Unk II 9b	Unk III 12	leu 13	Total
19-NV-R	134[b] (8.2)	753 (45.6)				434 (26.2)			14 (0.8)	308 (18.6)			1643 (99.4)
19-NV-R-2d	250 (4.9)	1245 (24.6)	1867 (36.9)			1307 (25.8)			89 (1.8)		301 (5.9)		5059 (99.9)
19-NV-H		241 (10.3)	412 (17.6)	86 (3.7)	269 (11.5)				433 (18.4)	900 (38.5)			2341 (100.0)
4-S2-R-2d	330 (10.3)	1400 (43.8)	545 (17.0)			343 (10.7)			168 (5.3)	252 (7.8)	157 (4.9)		3195 (99.8)
6-S1-R		140 (4.6)		408 (13.4)	173 (5.7)		420 (13.8)	639 (21.0)	601 (19.7)	244 (8.0)		425 (14.0)	3050 (100.2)
19-V-R	259 (7.4)	1021 (29.4)	609 (17.5)			301 (8.7)		157 (4.5)	596 (17.1)	483 (13.9)	52 (1.5)		3478 (100.0)
Set 2													
4-S2-H	2	245 (8.2)	112 (3.3)	41 (1.3)	433 (14.8)				1203 (40.2)	953 (32.0)			2987 (99.8)
8-S2-H		304 (18.0)	212 (12.6)		166 (9.9)	89 (5.3)			295 (17.5)	614 (36.5)			6680 (99.8)

Continued

TABLE II - Continued

Set 2 (Continued)

Samples	cys 1	hist 2b	Unk I 4a	asp 5a	ser 5b	gmn 5c	gly 6	glu 8a	ala 9a	Unk II 9b	Unk III 12	leu 13	Total
6-S1-R		145 (4.1)		436 (12.5)	183 (5.2)			1207 (34.4)	567 (16.2)	307 (8.8)		655 (18.7)	3500 (99.9)
6-S1-H		137 (4.4)	54 (1.7)	209 (6.7)	572 (18.2)		154 (5.1)	168 (5.4)	1470 (47.2)	359 (11.5)			3123 (100.2)
4-S2-R		355 (15.4)	210 (9.1)	56 (2.4)	198 (8.6)	118 (5.1)		184 (8.0)	628 (27.2)	563 (24.3)			2312 (100.1)
8-S2-R		282 (11.8)	258 (10.9)	49 (2.1)		139 (5.8)	205 (8.6)	52 (2.2)	338 (14.2)	736 (30.9)		322 (13.5)	2381 (100.0)
23-V-R		466 (15.6)	189 (6.3)	227 (7.6)	241 (8.0)	133 (4.4)		263 (8.8)	641 (21.4)	504 (16.8)		333 (11.1)	2997 (100.0)
Set 3													
19-V-H					210 (22.1)				324 (34.1)	418 (43.9)			952 (100.1)
23-V-H				228 (6.2)	423 (11.5)			241 (6.5)	1284 (34.8)	1310 (35.6		200 (5.4)	3686 (100.0)
19-V-H					105 (19.2)				197 (35.9)	246 (44.9)			548 (100.0)

Continued

TABLE II - Continued

Set 3 (Continued)

Samples	cys 1	hist 2b	Unk I 4a	asp 5a	ser 5b	gmn 5c	gly 6	glu 8a	ala 9a	Unk II 9b	Unk III 12	leu 13	Total
19-V-R-2d	212	659	570		126	450			193	136	164		2510
	(8.5)	(26.3)	(22.7)		(5.0)	(17.9)			(7.7)	(5.4)	(6.5)		(100.0)
23-V-H				129	244				632	644			1649
				(7.8)	(14.8)				(38.3)	(39.1)			(100.0)
19-V-R-2H	446	1339	1161		189	783			379	228	279	4804	4804
	(9.3)	(27.9)	(24.2)		(3.9)	(16.3)			(7.9)	(4.8)	(5.8)		(100.0)

[a]For explanation of treatments, see Table I on page 41.

[b]Top figure is actual count. Figure in parenthesis is the percent of the total for the particular sample.

TABLE III

ASSAY OF LABEL IN NON-POLAR FRACTION SITES OF ACTIVITY ON SUGAR CHROMATOGRAPHS

Set 1

Samples	Group A: Unknown Oligosaccharides								
	A	B	C	D	E	F	G	H	I
6-S1-R[a]	27[b] (0.2)		50 (0.4)	189 (1.6)					
6-S1-H	68 (1.7)	5 (0.1)					16 (0.4)		76 (1.9)
8-S2-H	183 (8.9)			134 (6.5)		49 (2.5)	98 (4.8)		20 (1.0)
19-NV-R-2d	2 (0.2)	(1.7	238 (2.0)	414 (3.5)	250 (2.1)	28 (0.2)	806 (6.8)		
19-V-R-2d	2 (0.2)	17 (1.7)		37 (3.6)			72 (7.0)		
8-S2-R	75 (1.2)		44 (0.7)	97 (1.6)	124 (2.0)	107 (1.7)	90 (1.4)	190 (3.0)	87 (1.4)
23-NV-R	725 (7.2)	392 (3.9)		382 (3.8)		227 (2.3)	318 (3.2)		120 (1.2)
4-S2-R	90 (3.4)	28 (1.1)	51 (1.9)	103 (3.9)		46 (1.7)	120 (4.5)		

Continued

TABLE III - Continued

Set 1 (Continued)

Samples	Group A: Unknown Oligosaccharides								
	A	B	C	D	E	F	G	H	I
4-S2-R-2d	105 (1.5)		121 (1.7)	48 (0.7)	137 (2.0)	39 (0.6)	1040 (15.0)		40 (0.6)
23-V-R	2	8	21	42		51	53		42

Samples	Group B: Mono and Disaccharides					
	Sucrose	Glucose	Fructose	Arabitol	Xylose	Ribose
6-S1-R	3626 (30.2)	4322 (36.0)	3623 (30.1)		9 (0.1)	9 (0.1)
6-S1-H	1676 (43.0)	643 (16.5)	1002 (25.7)		329[c] (8.4)	73 (1.9)
8-S2-H	575 (28.0)	543 (26.4)	354 (17.2)		4 (0.2)	
19-NV-R-2d	2550 (21.4)	4256 (35.6)	3102 (26.0)			
19-V-R-2d	337 (32.6)	299 (28.9)	269 (26.0)			
8-S2-R	590 (9.5)	1190 (19.1)	3647 (58.4)			6 (0.1)
23-NV-R	2043 (20.3)	2965 (29.4)	2957 (29.3)	21 (0.2)	12 (0.1)	13 (0.1)

Continued

TABLE III - Continued

Set 1 (Continued)

Samples	Group B: Mono and Disaccharides Sucrose	Glucose	Fructose	Arabitol	Xylose	Ribose
4-S2-R	1209 (45.4)	583 (21.9)	423 (15.9)		6 (0.2)	4 (0.2)
4-S2-R-2d	3241 (46.7)	1284 (18.5)	869 (12.5)			10 (0.1)
23-V-R	656	2068	1889	3		

Set 2

Samples	Group A: Unknown Oligosaccharides A	C	E	F	Group B: Mono and Disaccharides Sucrose	Glucose	Fructose	Xylose	Ribose
19-V-R			215 (1.2)	2160 (12.0)	6813 (37.8)	5272 (29.3)	3513 (19.5)	15 (0.1)	15 (0.1)
19-NV-R	585 (4.8)		1090 (8.9)	978 (8.0)	3478 (28.4)	3839 (31.4)	2271 (18.6)		

Set 3

Samples	Group A: Unknown Oligosaccharides A	B	C	D	E	F	G	H
23-V-H	164 (2.6)		164 (2.6)	172 (2.7)	153 (2.4)	189 (3.0)		16 (0.3)

Continued

TABLE III - Continued

Set 3 (Continued)

Samples	A	B	C	D	E	F	G	H
			Group A: Unknown Oligosaccharides					
23-V-H	83		89	65	65	90		
23-V-R	50 (1.1)	41 (0.9)		68 (1.5)	86 (1.9)	127 (2.8)	65 (1.4)	74 (1.6)
8-S2-H	31 (1.3)		165 (6.7)	62 (2.5)	66 (2.7)	127 (5.2)		

Samples	Sucrose	Glucose	Fructose	Arabitol	Xylose	Ribose
		Group B: Mono and Disaccharides				
23-V-H	3492 (54.9)	1172 (18.4)	821 (12.9)	10 (0.2)	4 (0.1)	
23-V-H	1762	589	414			
23-V-R	727 (15.9)	2172 (47.9)	2970 (25.5)	9 (0.2)	7 (0.2)	2 (0.04)
8-S2-H	797 (32.7)	712 (29.2)	479 (19.7)			

[a]For explanation of treatments, see Table I on page 41.

[b]Top figure is the actual count. Figure in parenthesis is the percent of the total for the particular sample.

[c]Negative reaction to aniline phthalate given by this spot.

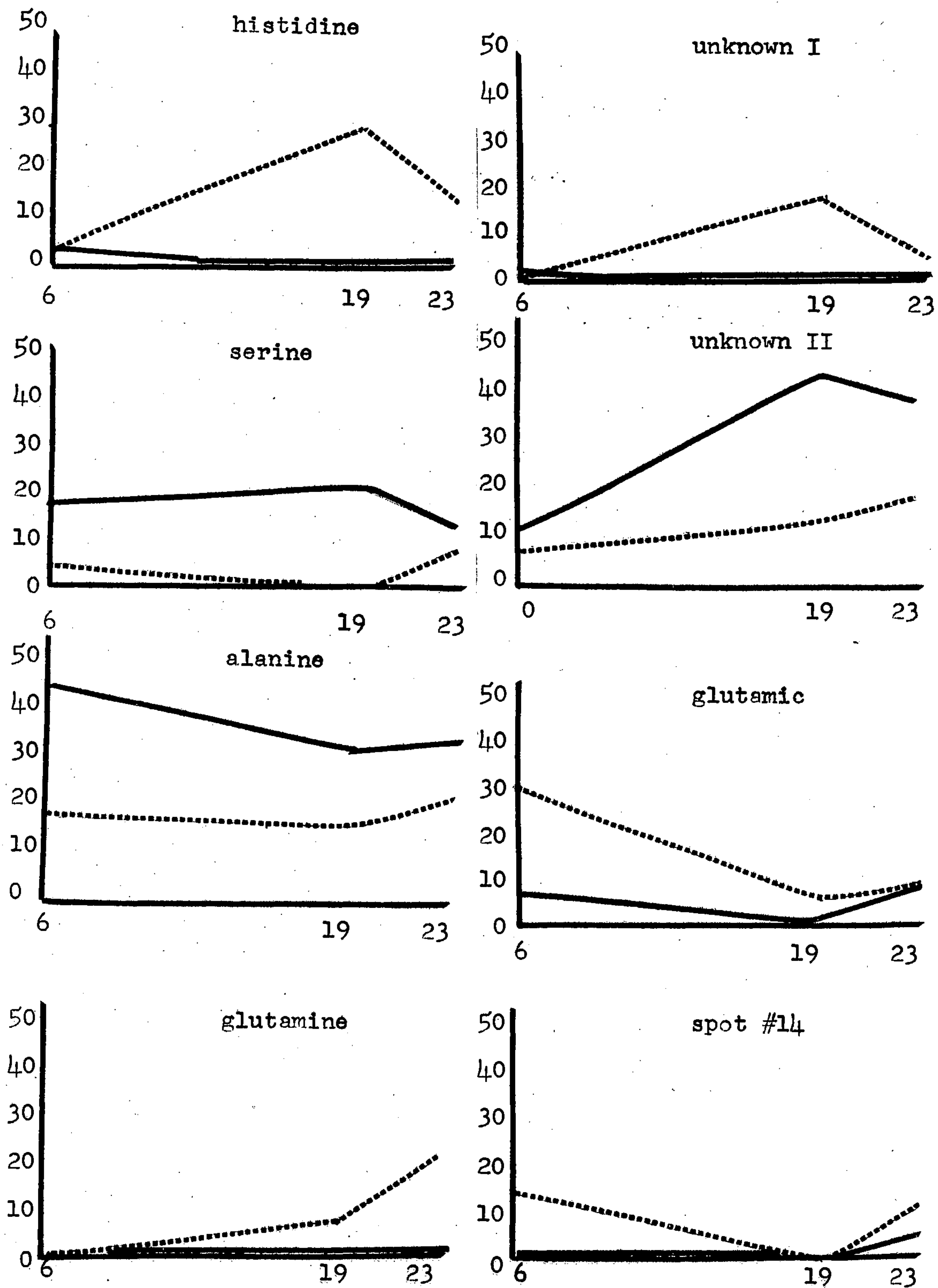

Figure 4. Effect of plant age and vernalization upon the relative incorporation of C^{14} into amino acids. Ordinate: percent of total fraction. Abscissa: age of wheat host in weeks. Symbols: Rusted leaves.........; healthy leaves———.

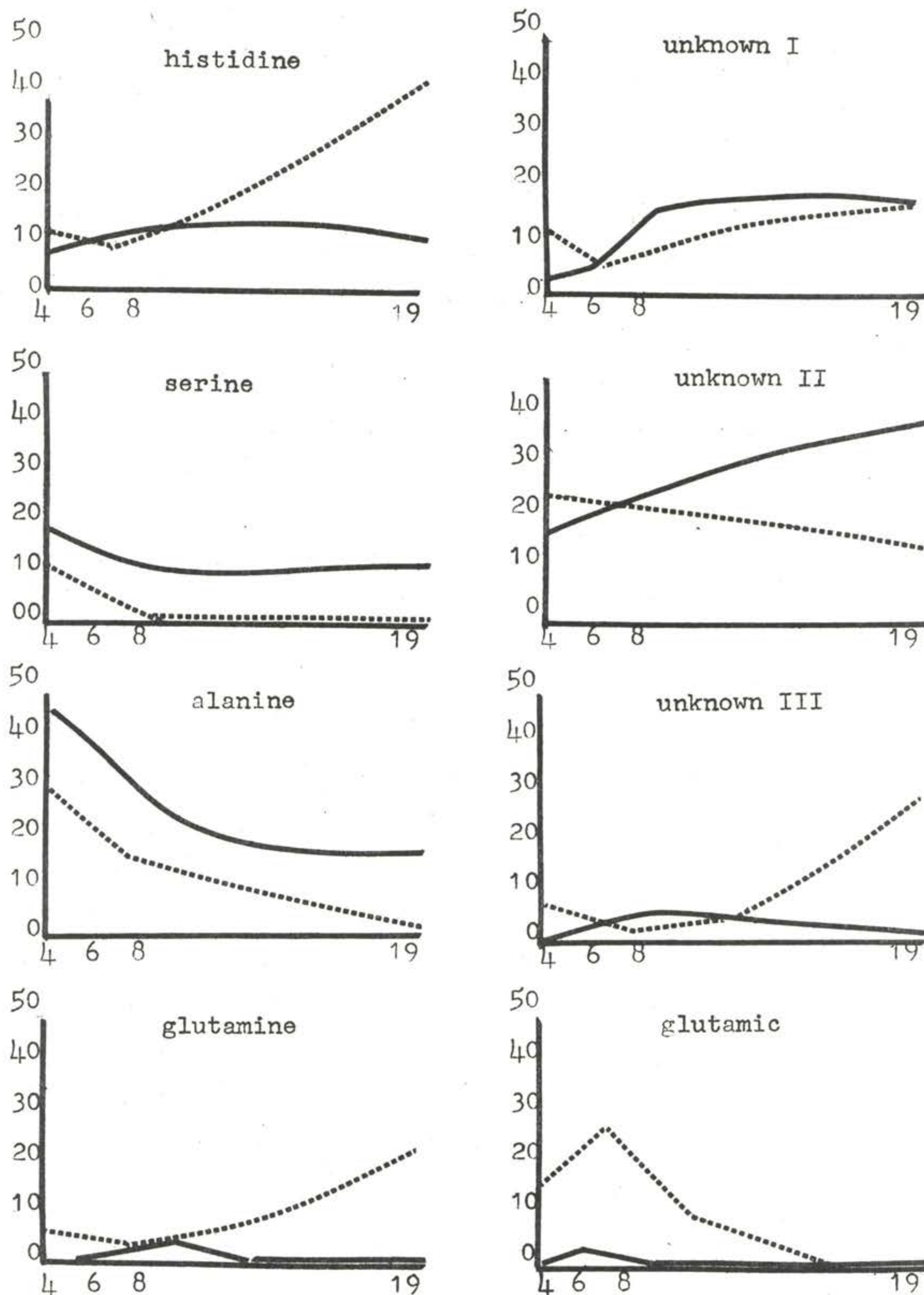

Figure 5. Effect of plant aging in the absence of vernalization upon the relative incorporation of C^{14} into certain amino acids. Ordinate: percent of total fraction. Abscissa: age of wheat seedlings in weeks. Symbols: Rusted leaves.........; healthy leaves——— .

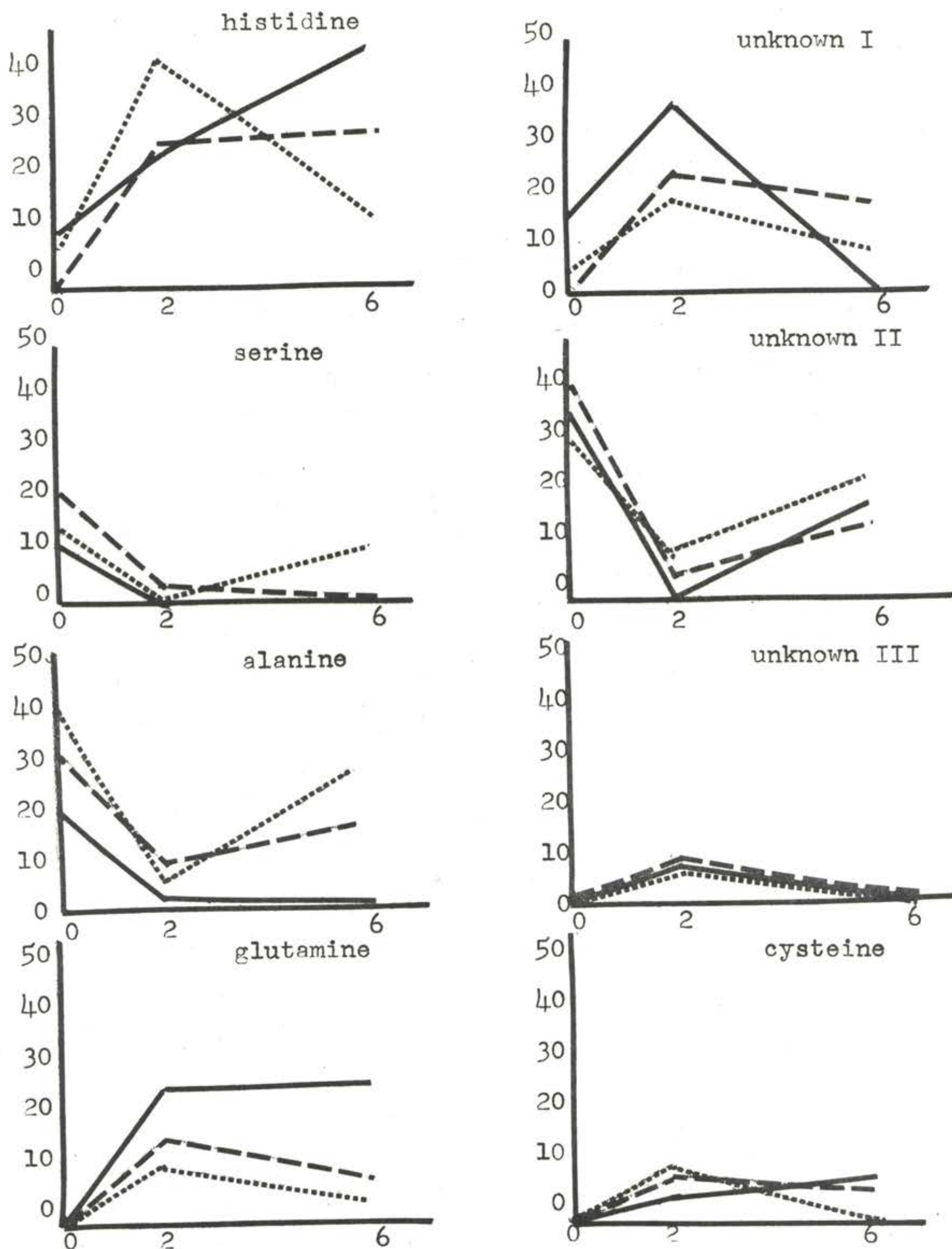

Figure 6 . Relative incorporation of C^{14} into various amino acids in leaves of Ponca wheat after infection by leaf rust. Ordinate: Percent of total fraction in each amino acid. Abscissa: Days after infection with Puccinia recondita, race 105-14. Symbols: 19 week old seedlings ———; 19 week old vernalized plants- — —; 4 week old seedlings ....

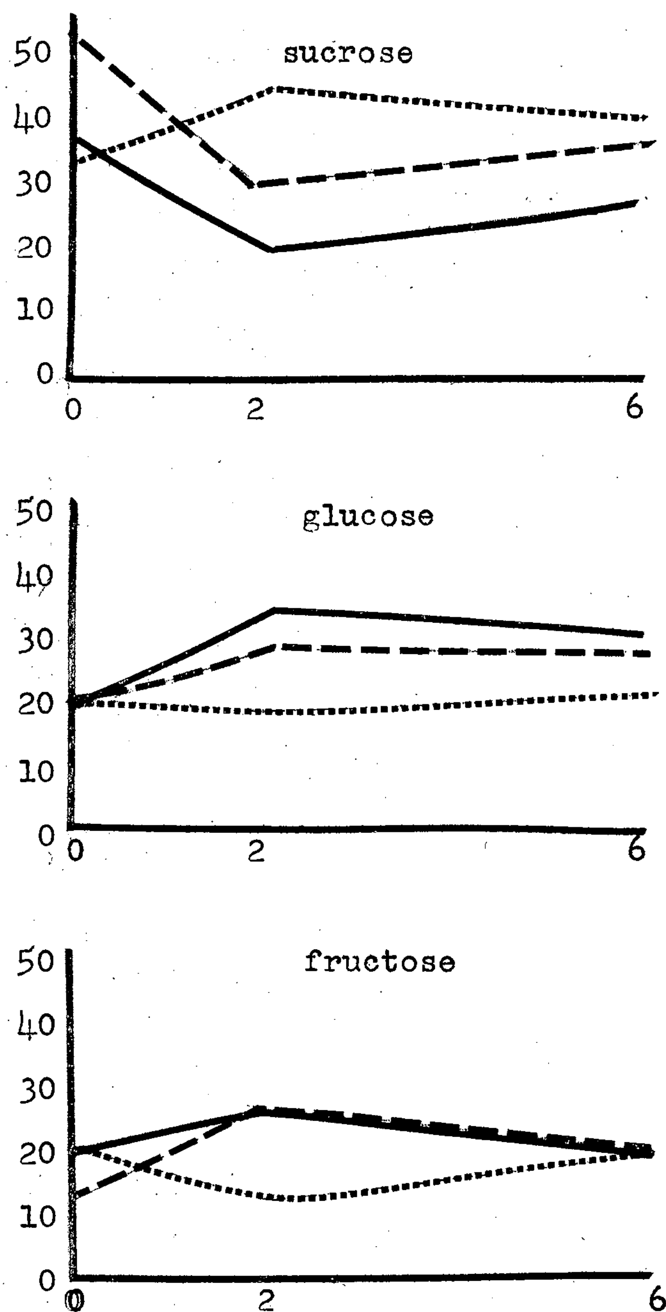

Figure 7. Relative incorporation of C^{14} into individual sugars in leaves of Ponca wheat after infection by leaf rust. Ordinate: Percent of total sugar fraction in each sugar. Abscissa: Days after infection with Puccinia recondita, race 105-14. Symbols: 19 week old seedlings ———; 19 week old vernalized plants — — —; 4 week old seedlings ········.

when comparing rusted leaves to healthy controls (0 days). It could be noted at a glance that in the case of most of the amino acids in all three treatments, there is a sharp contrast in levels of synthesis after 2 days of host-parasite interaction. In most cases, however, at the time when the fleck symptom is expressed (6 days) these changes have moderated or even reverted to a level that approximates that in the control.

A similar change and reversion pattern can be noted in the major sugars in Figure 6.

Since such striking changes occur as the infection ages, the data comparing synthesis in healthy leaves vs. leaves with 6-day old infections is of doubtful value, except to show the effect of aging and vernalization on the intermediary metabolism of Ponca wheat.

Of the three treatments represented in Figures 5 and 6, the synthetic patterns in the 4-week old seedlings show a very strong tendency to revert to control levels (0 days) at the fleck stage (6 days). This tendency is less pronounced in the 19-week old vernalized plants, while the 19-week old non-vernalized plants show a continuous drift away from the control level in five cases (cysteine, serine, histidine, glutamine, and alanine). There was a difference in degree of reaction type as mentioned previously (See Table I). In this respect comparative resistance was correlated with drift away from control levels.

Comparing the sugar metabolism of the three treatments, the highly susceptible 4-week old seedlings seem to show contrasting trends to both groups of older plants.

It would not be possible to point out which of the increases or decreases in synthetic rates, if any, were correlated with resistance and which of them are merely due to the generalized host-parasite interaction.

Due to unexplained darkening during lyophilization, the true controls

for the effect of age of infection on sugar metabolism were lost. In their place were substituted the best samples available. For the two unvernalized treatments the mean of the 6-week and 8-week old healthy seedlings was used. These two agree fairly well. For the vernalized plants the 23-week old vernalized sample was used. This may be misleading in this instance, for during the 19th to 23rd weeks the plants grew from rosette to the heading stage. Figure 8 indicates that earlier trends in sugar metabolism were reversed during these last four weeks.

In Tables II and III the data showing the amount of label detected in various sugars and amino acids for each sample are grouped into sets. Each set represents a group of samples chromatographed at the same time and under identical conditions. These tables can be compared to Tables I and II of the appendix to determine how the identifications of the various compounds were partially determined on the basis of the R_a value (or movement in each of the two solvents relative to alanine).

The R_a tables show the great similarity of movement within sets of chromatographs developed at the same time. Figure 1 shows the larger range of values for each compound considering all three sets. Here it can be noted that a number of the compounds are rather well resolved even when the variation due to comparison of sheets run at different times is allowed to remain (e.g. alanine, cysteine, aspartic, glutamine, unknowns II and III). Even in these cases the fact that certain compounds in the samples have identical R_a values to well resolved standards is not implied to constitute proof of identity. It merely means that in the absence of evidence to the contrary, it is most logical to tentatively identify components of the sample with the most commonly occurring amino acids with which their chromatographic properties correspond. Even so,

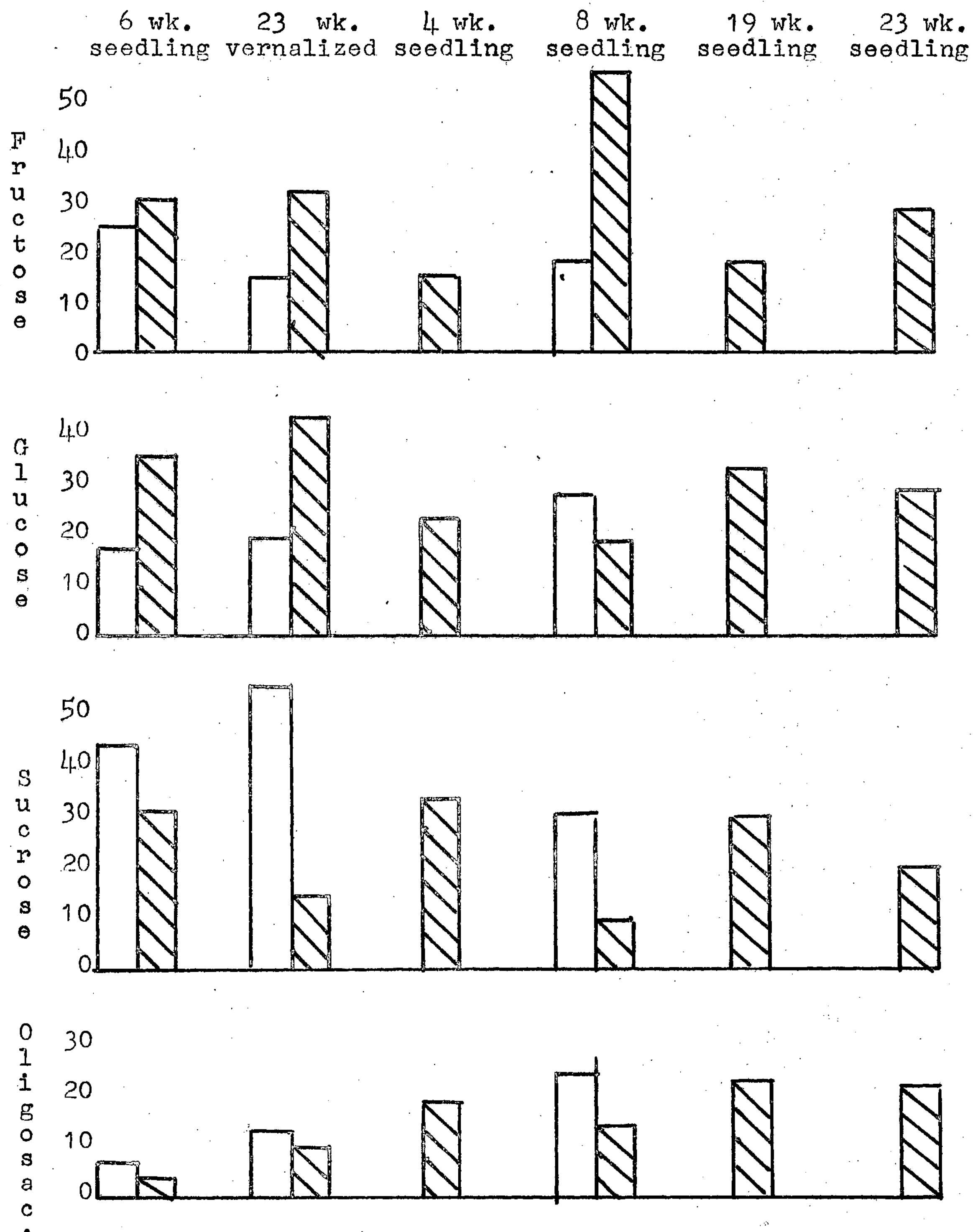

Figure 8. Fluctuation in relative amounts of C^{14} label incorporated in the various sugar fractions due to different age and vernalization treatments of Ponca selection 5212. Unshaded bars = healthy leaves; shaded bars = rust infected leaves.

some of the components of the sample mixtures posed problems even as to tentative identification. Lysine and histidine, for example, may streak or have erratic values in the phenolic solvent. In general the proper aliquot for good resolution was too low to give a positive sulphanilic acid test. In the last set, however, success was finally obtained in co-chromatographing a proper size aliquot of histidine standard with some of the samples. The standard was found to co-chromatograph quite well with the spot that had been identified as histidine and one unmistakably positive sulphanilic test was obtained. In addition, an unlabelled ninhydrin positive spot was detected in the position predicted for lysine. An unlabelled ninhydrin positive spot was also detected in the position corresponding to the threonine standard.

The spot labelled 4a,(Unknown I) is an enigma. It corresponds most directly to the asparagine standard in R_a values. The ninhydrin spot obtained, however, was not brown. It is close enough to serine to be easily mistaken for it if serine were not present. Both of them were present in one sample, however, and on rather shaky ground it is assumed to be distinct. It is quite possible that some of these spots may in fact be serine.

Unknown II was first thought to be tyrosine, but consistently failed to correspond accurately to the tyrosine--or any other--of the 19 standards used.

Unknown III is truly unique, and this compound merits further attention. It seems clearly to be associated with the establishment of the parasite. It was detected only in the leaves with 2-day old infection sites. Of some 450 amino compounds listed by Smith (94) only a very few of them move faster in acidic butanol than in phenol. One compound, 3-OH-anthranilic, seemed on paper to have somewhat similar properties,

but did not co-chromatograph with unknown III.

CHAPTER V

DISCUSSION

Rowell, Loegering and Powers (72) have emphasized the necessity of using appropriate materials for studies to clarify the biochemical nature of the dual response of a host plant and an invading parasite to each other. In their entirety these responses govern the infection type, whether susceptible or resistant. Host plant lines isogenic for specific loci governing resistance and strains of the parasite isogenic for the corresponding genes governing virulence are regarded as optimal material to use in searching for biochemical entities that govern resistance or susceptibility. Isogenic systems would help to keep the background variation at a minumum and allow attention to be focussed on the "distinct biochemical process" which controls reaction type.

These authors admit to the possibility that the critical interaction governing infection type may set in motion a sequence of biochemical steps. These biochemical steps and the critical interaction which sets them in motion may be different in the various levels of reaction type.

In the wheat variety Ponca, it is possible to utilize lines that are isogenic for resistance and susceptibility by merely analyzing the disease interaction on the same plant, first as a susceptible seedling and later as a resistant mature plant. This resistance which occurs has been found to be due to a single major gene(110) which is presumably inactive in the young seedlings.

There is little doubt that the mature plant resistance found in

Ponca wheat is physiological in nature. Hart (36) has described a type of morphological mature plant resistance of certain varieties of wheat to to stem rust. In these varieties, broad, coalesced strands of collenchyma in the stems can restrict the size and shape of developing stem rust pustules. However, the leaves of all varieties studied apparently had enough rust susceptible tissue to allow the pustules to develop virtually unrestricted. If mature plant leaf morphology is not a barrier to stem rust development, it should be no hindrance to the development of smaller leaf rust pustules.

The plants used in the experiment did not develop the degree of resistence that is sometimes seen in the field in cool, moist seasons. On the other hand, Ponca has been observed to lose its resistance in the field when subjected to combined heat and moisture stress. Johnson and Newton (42) have listed a number of wheat varieties that, although resistant at 70 degrees F., begin to show induced susceptibility when subjected to a mean temperature of 75 degrees F., and lose most or all of their resistance at 80. The conditions of this experiment were not severe enough (72 degrees F. mean temperature, 50% relative humidity) to cause a breakdown in resistance. Thus it was probably not the failure to obtain an adequate level of resistance that defeated the major objective, but rather the lack of ability to predict the age when the onset of resistance would occur.

The main emphasis of the study was focussed upon finding qualitative differences in the intermediary metabolism of the wheat host that were associated with maturity and generalized leaf rust resistance. This was done by periodically infecting a portion of the plants with leaf rust and detaching the leaves for analysis after the infection had progressed for 6 days (about 144 hours). The primary object of the search was to

find a metabolic constituent whose presence, absence, or change in concentration correlated with the onset of the incompatibility or hypersensitive resistance interaction.

The most interesting aspect of the sugar studies are the compounds that are conspicuously missing. Recent studies by Daly, et al. (17) have shown extremely high labelling in trehalose and a pentitol, possibly arabitol. Standard solutions of both these compounds were chromatographed along with the sugar samples and were adequately resolved. With one possible minor exception, however, there was never any significant activity recorded at either site.

One possible explanation of this discrepancy could be in that these studies involved different rust organisms. Daly was working with the rusts of bean and safflower--*Puccinia phaseoli* and *P. catharmi*.

Another possibility which seems more likely to account for the differences would be the contrast in fungus development. Daly used the sporulation stage, while these studies refer to the fleck stage and in three instances even earlier stages. In all probability major segments of metabolic activity may be successively altered as the fungus penetrates the leaf, passes through the critical period of establishing (or failing to establish) a compatible relationship with the host, vegetatively proliferates through the leaf, and finally assimilates the large body of substrates necessary for spore formation.

It was reasoned that the point in the infection cycle where the scales were just tipped in the direction of the ultimate disease response would be the most fruitful stage to analyze.

There was some reason to believe that the host-parasite interaction during the first 6 days had already produced the reaction type determinants. The studies of Allen (4) have shown that the interaction be-

tween the haustoria of the invading fungus and the cells of the host has produced distinctive cytological changes. The difference in cytomorphology of cells that are compatible with the fungus (susceptible) and those that are hypersensitive (resistant) to fungal invasion is well delineated 6 days after infection, when the fleck symptom is fully expressed. Forsyth and Samborski (28) have shown that in McMurachy wheat, which has a gene for stem rust resistance whose expression is dependent upon temperature, the reaction type is fixed after fleck symptoms have appeared and can no longer be altered by temperature manipulations. Their observations would tend to place the point at which the reaction type becomes fixed and irreversible at about 5 days (115-123 hours). On the basis of personal observations as well as studies such as these, it is felt that meaningful data that might lead to the discovery of the most critical metabolic interaction in determining reaction type will have to be taken at an early stage of the infection cycle. Studies of sporulating infections and germinating spores may produce more knowledge of the gross intermediary metabolism of the parasite, but to date they have not revealed the determinants of reaction type. This information should logically come from "*in situ*" studies made at the critical time.

Since it was believed that there is a "critical" period in the infection cycle at which time the reaction type was fixed, metabolic pool size was essentially ignored. Attention was focussed upon short term (4 hour) spans in the infection cycle. Only compounds being actively synthesized during this short incubation period were subject to analysis.

According to autoradiographic studies by Shaw and Samborski (86), two to four hours was sufficient time for labelled substances fed to excised infected leaves to become localized at the infection sites. This

was true for several classes of compounds, inorganic ions (Ca^{45} and P^{32}), organic acids, sugars and sugar derivatives (including glucose), and even for phytotoxic and fungitoxic phenols such as protocatechuic acid.

Since pre-existent pool size is being disregarded, it can be assumed that a major portion of the activity being assayed in this study is occurring at the site of infection. Pool size has been ignored so that attention can be focussed on the major metabolic activity occurring at a specific stage on infection irrespective of the activity before infection, or prior activity during the course of infection. In addition, this helps prevent significant activity in minor components from being obscured.

In all cases the shift in intermediary amino acid metabolism was most severe at 2 days and somewhat moderated at 6 days. The 2-day stage is probably near the critical period at which the reaction type hangs in the balance,(See Shaw and Colotelo, 83, and Forsyth, 28), while at six days the reaction type is fixed. In each treatment there is a rise in relative synthesis of histidine, cysteine, glutamine, and two other compounds at the two day stage. One of these latter (4a in Figure 1 and Table II) could be either asparagine or serine. It has been tentatively labelled as asparagine, but may quite well be serine in some or many instances.

In view of the possible correlation of serine with host resistance and fungistasis as reported by Shaw and Colotelo (83), Van Andel (101), and Samborski and Forsyth (77), this needs to be examined further. Serine appears to be somewhat of of a common denominator of resistance; that is, increases in serine are often correlated with host resistance and/or fungistasis. This is not universally true, however, since it apparently is not a determinant of reaction type in Venturia-Malus inter-

action.

Both histidine and serine have been found to inhibit rust at non-phytotoxic levels by Samborski and Forsyth (77). Shaw and Colotelo (83) also found a dramatic rise in serine at the 3-day stage of resistant interactions on Khapli wheat. That these changes are correlated with resistance is suggested by the fact that there is a sharp drop in relative activity in all four of these compounds at the 6-day stage in 4-week old seedlings. This corresponds with the establishment of a fully susceptible reaction type.

At this time it is not clear just what the function of serine might be in the disease cycle. Some of the amino acids offer a number of interesting possibilities. Histidine, or one closely resembling it in certain properties, is one of two amino acids being synthesized at an extremely high rate at the critical 48 hour stage in the infection cycle. Whether this buildup is vital to the establishment of the fungus or is part of the hosts' generalized resistance response that is eventually negated by the fungus, remains to be determined. Some evidence for both lines of reasoning are current in the literature. Possible resistance is indicated by the findings of fungistatic properties at non-phytotoxic levels by Samborski and Forsyth (77) and Van Andel (101). On the other hand, naturally occurring histidine deficiency severely limits the growth of Venturia upon apple extracts (48). Addition of histidine supplement allows the fungus to grow well upon this medium. This situation does not involve obligate parasitism and may not be analogous to host-pathogen interactions in rust diseases.

Whether antagonistic or beneficial to the pathogen, histidine metabolism has some interesting interactions with other segments of intermediary metabolism.

Histidine is formed from the same precursors as the purines, and in this sense histidine synthesis is in competition with purine synthesis. Nucleic acids and their precursors have a vital role in the enhanced synthesis associated with the proliferation of the fungus.

One possible active defense mechanism might lie in the de-repression of histidine synthesis. Non-repressible mutant enzymes have resulted in stoppage of purine synthesis in bacteria. Shedlevsky and Magasanik (88) suggest that the adenine moiety phosphoribosyl ATP is enzymatically cleaved between N_1 and C_6 to produce an intermediate which in subsequent steps is enzymatically converted (with the addition of ammonia) to imidazole glycerol phosphate and 5-amino 1-(5-phosphoribosyl)-4-imidazolecarboxamide. Histidine controls this cycle by inhibiting the enzyme that causes the condensation of ATP and PRPP.

The failure of the histidine inhibition to operate in this cycle can cause adenine deficiency if there is a mutation preventing the subsequent reconversion of 5-amino 1-(5 - phosphoribosyl)-4-imidazolecarboxamide to inosine monophosphate and subsequently to AMP through a succinyl-AMP intermediate.

Shedlovsky and Magasanik (89) quote unpublished data stating that histidine mutants of Escherichia coli that lack the enzyme cleaving PRPP-ATP into imidazole glycerol phsophate and 5-amino 1-(5 - phosphoribosyl)-4-imidazolecarboxamide could be incited to produce phage DNA only in media supplemented by histidine or adenine, while other amino acid auxotrophs could be incited to produce phage DNA in an unsupplemented medium.

Perhaps temporary paralysis of certain feedback inhibition systems could derange the host metabolism sufficiently to check the pathogen. Non-repressible enzymes or enzyme inhibitors could function to create a local metabolic imbalance. If the local imbalance became severe

enough, it could result in local fungistasis and host cell necrosis before the imbalance would register in markedly changed composition of metabolite pools in the leaf tissue as a whole.

Some fascinating aspects of the role of divalent cations in rust diseases have been presented by Wang (102). Cobalt ion induces chlorosis in detached wheat leaves at extremely low concentrations, and chlorosis is intimately associated with hypersensitive resistance in the host. This ion also functions to activate peptidases and arginase and forms part of the vitamin B_{12} molecule which functions in nucleic acid synthesis. Histidine binds cobalt and other divalent ions in stable chelates.

Another distinctive feature of the disease symptoms incited by obligate parasites such as rusts and mildews is the pathologically high rate of respiration. Histidine possibly links in this aspect also, for Boyer et.al. (8) have found a presumed intermediate in oxidative phosphorylation which after exhaustive chromatographic, hydrolytic, and color tests appeared to be very similar or identical to 3-phosphoryl histidine. Supplementary experiments indicated that this compound, while apparently bound to a mitochondrial protein, serves as an intermediate between iP and ATP. If this hypothesis is correct, a rapid buildup in histidine to meet the rising demand for this intermediate is a possibility. The occurrence of the rapid rise in respiration does roughly coincide with the peak histidine synthesis. Resistant varieties characteristically respond to rust invasion with higher initial respiratory rise. This possibly leads to partial uncoupling and host cell deterioration--consequently hypersensitive resistance. Perhaps the concentration of the phosphoryl intermediate may serve to prevent uncoupling to such an extent that cell degeneration ensues. If so, then the moder-

ated respiratory rise as found in compatible interactions would be a source of more usable chemical energy (ATP) if enough intermediate phosphate acceptor were available.

In short, since histidine metabolism touches so many vital points, it would be well to explore it further.

Spot 12a is an unknown compound present in detectable amounts only at the critical 48 hour stage in infection. It is apparently lacking in the controls and 6-day old infections. It was ninhydrin positive, but no other color reagents nor ultra violet examination gave any clue to its identity. Its chromatographic properties, however, were distinctive. Of 450 odd amino compounds listed by Smith (93), only a very few migrate significantly faster in acidic butanol than in phenol. Only one compound had Rf values in these two solvents approximating the unknown, and this was 3-OH-anthranilate. This compound, however, did not cochromatograph with the unknown. Still, the anthanilate family of compounds appears to be the group most likely to furnish an identity.

Drawing upon his own data (83) and that of Farkas and Kiraly (26), Shaw (84) has emphasized the role of glutamate and glutamine in the metabolism of rust infected tissue. He suggests that the utilization of ATP in glutamine synthesis could contribute to an increase in respiration rate.

It is noteworthy that glutamine is the only compound that shows a completely consistent trend to increase in rusted tissue of Ponca wheat as the plants age. This is true of both the vernalized and unvernalized plants.

Conversely, alanine and another unidentified compound (unknown II) decrease sharply from the 0 to 2 day stage of infection. They rise again thereafter, and approach the control levels at the 6 day stage.

One exception is alanine synthesis in the most resistant treatment (19-S1), where it remains at a low level.

CHAPTER VI

SUMMARY

1. Plants of an inbred line of Ponca wheat were grown to maturity under controlled environmental conditions. Samples of healthy and rust infected leaf tissues supporting 6-day old infections of wheat leaf rust were collected at various stages in the growth of the wheat plants. These samples were incubated with glucose-U-C^{14} and subsequently analyzed to reveal the pattern of conversion of the labelled substrate into soluble amino acids and sugars.

2. The plants used in the experiment did develop mature plant resistance, approaching the high level of resistance that is usually expressed by this variety of wheat when grown in the field. There was no apparent change in the pattern of C^{14} labelling with the gradual onset of mature plant resistance.

3. Significant concentrations of label in trehalose or arabitol were not found as expected in the infected tissues. If extensive synthesis of these compounds does occur in wheat leaf rust infections, it is apparentlyddelayed until after the flecking (6 day) stage of development.

4. Analyses were made of 2- and 6-day old rust infections as compared with the healthy controls. Wheat leaves having 2-day old infections showed patterns of amino acid synthesis that were strikingly different from either the control or the 6-day old infections. These metabolic patterns were similar for young seedlings and $3\frac{1}{2}$ month old vernalized

plants.

5. It was postulated that 2-day old infections are near the critical period in rust development in which the reaction type has not been irreversibly determined and host resistance mechanisms are still operative.

6. Amino acids most actively synthesized at the 2-day infection stage have been tentatively identified as histidine, asparagine (or serine), cysteine, glutamine, and two compounds that do not correspond to any of 19 common protein amino acids. One of these unknowns has unique chromatographic properties and is found only in 2-day old infections. Any or all of these compounds could possibly be involved in reaction type. The departure from the control levels (0 days) in synthetic rate is most striking in the most resistant treatment, so it is possible that higher levels of these compounds tend to confer resistance.

7. Sugar metabolic rates also show changes at the 2-day infection stage. There is a tendency for more of the C^{14} label to appear in glucose and fructose and less in sucrose in the more resistant treatments, while the most susceptible treatment shows more label in fructose at the expense of sucrose. None of the changes are striking.

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APPENDIX

EXPERIMENTAL CHRONOLOGY

November 5, 1963	48 seeds of Ponca selection 5212 planted singly in 4" pots, and placed inside three small chambers of transparent plastic in greenhouse room thermostatically controlled at 70 ± 2 degrees F.
November 10	Seedlings emerged.
November 22	Supplementary lighting (fluorescent and incandescent) of about 250 ft. candles supplied and day length set at 12 hours.
November 27	Plants appearing quite spindly; leaves quite long (to 15").
December 5	Plants sturdier, color good, leaves still very long (to 18").
December 10	First sign of tillering.
December 15	Rust reaction uniforly 2+ to 3.
December 26	Plants supplied with 47 ml. standard strength Hoagland nutrient solution.
December 27	Growth differential noted between the plant groups in the 3 plastic chambers. Three of the most advanced plants in the large group were position-interchanged with 3 of the least advanced plants of the small group.
January 4, 1964	Rust reaction still definitely 2+ to race 105-NA61-14; no pustules on plants inoculated with race 9.

January 9	36 plants removed to vernalization room. Day length reduced to 10 hours of low (less than 500 ft. candles) intensity light. Day temperature 52 ± 2 degrees F., night temperature 38 ± 2 degrees F. Continual check kept on the thermograph chart.
January 13	Reaction of 16 non-vernalized plants (NV) which were kept at 12-hour day length at 70 degrees F. still generally 0; to 1- to race 9 and 2+ to 3 to race 105-NA61-14 (one aberrant 1+ pustule on leaves inoculated with the latter). Extensive tillering noted on this group.
January 20	Extensive tillering noted on plants undergoing vernalization. Vernalized plants still susceptible to 105-NA61-14.
January 31	Vernalized plants still mildly susceptible to 105-NA61-14.
February 13	50 seeds of selection 5212 planted in ten 12-inch pots and placed in Scherer-Gillett growth chamber.
February 18	Some resistant fleck reactions noted on vernalized plants following infection with 105-NA61-14. On this date both the vernalized and non-vernalized plants were moved to the Scherer-Gillett plant growth chamber. Newly planted February 13 seedlings of selection 5212 just beginning to emerge.
February 19	Spring planted seedlings emerge.

TABLE IV

REACTION OF RUST CULTURE USED IN EXPERIMENTS ON THE STANDARD WHEAT LEAF RUST DIFFERENTIAL VARIETIES

International Differentials	Reaction	
	Type[a]	Class
Malakof	4	S
Carina	0;-2	R
Brevit	2+	R
Webster	0;-2	R
Loros	4	S
Mediterranean	4	S
Hussar	4	S
Democrat	4	S
Supplemental Differentials		
Lee	4	S
Westar	4	S
Sinvalocho	4	S
Waban	0;	R

[a]Numerical scale of severity of reaction type defined as follows:

0 = no visible symptoms
0;= necrotic flecks
1 = slight sporulation in necrotic lesions
2 = moderate sporulation in necrotic lesions
3 = moderate to heavy sporulation in lesions showing some chlorosis
4 = heavy sporulation with little or no chlorosis occurring in the host tissue.

TABLE V

R_a VALUES OF AMINO ACID STANDARDS

	Set 3 (1 dimensional)	Set 3 (2 dimensional)	Set 2	Set 1
cysteine	.19[a]		.15	.07
	.97		.55	.50
lysine	.42		.29	.20
	.74		.70	.66
histidine	.30	.29	.29	.20
	.93	.64	.86	.81
aspartic	.55	.60	.54	.43
	.29	.25	.40	.42
asparagine	.42			
	.71			
glutamic	.80		.77	.70
	.49		.66	.69
serine	.55	.54	.55	.42
	.55	.59	.62	.63
glycine	.66		.65	.63
	.69		.75	.71
alanine	1.0	1.0	1.0	1.0
	1.0	1.0	1.0	1.0
arginine	.46		.40	.31
	.92		.95	1.0
threonine	.82		.77	.70
	.83		.90	.93
tryptophan	1.46	1.43		
	1.26	1.31		
tyrosine	1.22		1.22	1.31
	1.02		1.10	1.08
phenylalanine	1.65		1.74	1.90
	1.36		1.36	1.43

Continued

TABLE V - Continued

	Set 3 (1 dimensional)	Set 3 (2 dimensional)	Set 2	Set 1
leucine	1.72		1.74	1.88
			1.36	1.43
methionine	1.42		1.43	1.52
	1.30	1.29	1.29	1.35
valine	1.41	1.40	1.43	1.53
		1.30	1.29	1.27
hydroxyproline	.68	770	.67	.62
		1.15	1.13	1.15
proline		1.13		1.16
	1.43	1.47		1.43
isoleucine		1.63	1.68	1.95
	1.27	1.40	1.39	1.42

[a]Top figure refers to movement in solvent 1; bottom figure refers to movement in solvent 2.

TABLE VI

AMINO ACID CHROMATOGRAPHS

R_a VALUES OF SPOTS LOCATED BY RADIOAUTOGRAPHY

Set 1

Samples Analyzed		Treatment[a]					
Spot Location	Possible Identity	19-NV-R	19-NV-R-2d	19-NV-H	4-S2-4R-2d	6-S1-R	19-V-R
1	cysteine	.08[b] .58	.10 .56		.09 .55		.08 .58
2b	histidine	.18 .69	.25 .66	.21 .68	.25 .68	.21 .65	.24 .66
5a	aspartic			.54 .40		.53 .41	
4a	unknown I (asparagine?)		.42 .69	.39 .69	.41 .70		.44 .69
6	glycine					.67 .66	
5b	serine			.53 .64		.51 .62	
8a	glutamic					.65 .63	.78 .60
5c	glutamine	.54 .92	.57 .91		.60 .88		.59 .91
9a	alanine	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0
9b	unknown II	1.07 1.20	1.07 1.20	1.17 1.21	1.04 1.19	1.10 1.22	1.08 1.22
12	unknown III		1.40 .54	1.	1.41 .53		1.50 .46
13a b c	leucine, isoleucine, or phenylalanine					1.89 1.34	

Continued

TABLE VI - Continued

Set 2

Samples Analyzed		Treatment[a]						
Spot Location	Possible Identity	4-S2-H	8-S2-H	6-S2-R	6-S2-H	4-S2-R	8-S2-R	23-V-R
2b	histidine	.23	.25	.20	.25	.22	.25	.25
		.70	.74	.67	.75	.72	.73	.70
5b	serine	.58	.57	.53	.55	.55		.56
		.67	.66	.64	.64	.66		.65
4a	unknown I	.42	.43		.42	.42	.44	.42
	(asparagine?)	.71	.73		.72	.72	.71	.73
6	glycine				.63			
					.72			
8a	glutamic			.77	.75	.74	.79	.75
				.67	.64	.66	.65	.67
5a	aspartic	.63		.56	.56	.55	.56	.59
		.46		.41	.43	.42	.43	.42
5c	glutamine		.59			.57	.57	.59
			.91			.94	.93	.90
9a	alanine	1.0	1.0	1.0	1.0	1.0	1.0	1.0
		1.0	1.0	1.0	1.0	1.0	1.0	1.0
9b	unknown II	1.07	1.08	1.02	1.03	1.09	1.08	1.05
		1.21	1.24	1.22	1.20	1.24	1.21	1.21
13a	leucine,			1.68				
b	isoleucine, or			1.32				
c	phenylalanine							

Continued

TABLE VI - Continued

Set 3

Samples Analyzed		Treatment[a]					
Spot Location	Possible Identity	19-V-H	23-V-H	19-V-H	19-V-R-2d	23-V-H	19-V-R-2d
1	cysteine				.13 .47		.11 .47
2a	lysine	.24[b] .46	.28 .49				.25 .48
2b	histidine		.29 .65	.29 .65	.26 .64		.24 .66
5a	aspartic		.59 .26			.61 .26	
4a	unknown I (asparagine?)				.42 .63		.40 .64
5b	serine	.59 .58	.58 .59	.57 .62	.56 .61	.61 .58	.54 .59
8a	glutamic		.79 .52				
5c	glutamine		.78 .85		.56 .90		.52 .89
9a	alanine	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0	1.0 1.0
9b	unknown II	1.06 1.26	1.05 1.29	1.01 1.29	1.06 1.28	1.02 1.28	1.05 1.29
13a	tryptophan		1.46 1.30	1.41 1.34		1.51 1.33	
12	unknown III				1.20 .19		1.22 .22
13b c	leucine, isoleucine, or phenylalanine		1.59 1.16				

[a]For explanation of treatment symbols, see Table I, p. 41.
[b]See footnote on Table V, p. 92.

TABLE VII

AMINO ACID CHROMATOGRAPHS: ABSOLUTE MIGRATION MEASURED IN CENTIMETERS

Group I

Spot	Possible Identity	19-NV-H	19-NV-R-2d	19-NV-R-6d	6-S-R-6d	19-V-R-2d	19-V-R-6d	Std. 1	Std. 2	Std. 3	Std. 4
11	cysteine		1.3[b] 16.0	1.0 15.9		1.2 15.5	1.0 16.0			0.8 13.5	
2a	lysine									2.5 17.8	
2b	histidine	2.5 19.0	3.2 18.8	2.2 18.9	2.6 18.0	3.4 19.3	3.2 18.3			2.5 22.0	
3	arginine								3.8 27.0		
5a	aspartic	6.3 11.3			6.6 11.4						5.2 11.3
5b	serine	4.5 19.4	5.5 19.5		6.3 17.3	5.6 19.8	5.7 19.0				5.1 17.2
5c	glutamine										
6	glycine				8.3 18.3			7.7 19.1			
7	hydroxy-proline										7.6 31.3

Continued

TABLE VII - Continued

Group I (Continued)

Spot	Possible Identity	19-NV-H	19-NV-R-2d	19-NV-R-6d	6-S-R-6d	19-V-R-2d	19-V-R-6d	Std. 1	Std. 2	Std. 3	Std. 4
8a	glutamic	6.2 18.0			9.3 17.5		10.2 16.5		8.5 18.8		
8b	threonine		7.4 25.8	6.7 25.3		8.2 24.7	7.7 25.2		8.5 25.2		
9a	alanine	11.7 28.0	13.0 28.4	12.3 27.5	12.4 27.8	13.7 28.2	13.1 27.7	12.2 27.1			
10	proline							14.2 38.7			
11	tyrosine	13.1 34.0	14.0 34.1	13.2 32.9	13.7 33.9	14.2 33.5	14.2 33.7			16.0 29.3	
12a	unknown		19.5 15.3			19.3 14.9	19.6 12.7				
12b2	methionine								18.6 36.5		
12b3	valine										18.7 34.5
13a	phenylalanine									25.2 38.7	
13b	isoleucine								25.8 38.5		

Continued

TABLE VII - Continued

Group I (Continued)

Spot	Possible Identity	19-NV-H	19-NV-R-2d	19-NV-R-6d	6-S-R-6d	19-V-R-2d	19-V-R-6d	Std. 1	Std. 2	Std. 3	Std. 4
13c	leucine							23.0 38.8			
13d	unknown						23.5 37.2				

Group II

		4-S-H	4-S-R-2d	8-S-H	8-S-R-6d	6-S-H	6-S-R-6d	23-V-R-6d	Std. 1	Std. 2
1	cysteine								2.6 15.4	
2a	lysine								4.9 19.5	
2b	histidine	3.6 18.8	3.2 18.6	3.9 20.5	4.3 19.8	4.6 20.4	3.1 17.9	4.1 19.2	5.0 24.0	
3	arginine									6.8 26.3
5a	aspartic	10.0 12.3	8.2 11.0		9.5 11.8	10.3 11.9	8.8 10.9			9.3 11.2
5b	serine	6.6 18.9	6.2 18.7	6.9 20.3	7.5 19.2	7.7 19.9		6.9 20.2		9.4 17.1

Continued

TABLE VII - Continued

Group II (Continued)

Spot	Possible Identity	4-S-H	4-S-R-2d	8-S-H	8-S-R-6d	6-S-H	6-S-R-6d	23-V-R-6d	Std. 1	Std. 2
5c	glutamine									
6	glycine		8.2 17.2	9.0 18.5	9.6 17.9	10.2 17.8	8.3 17.1	9.2 17.9	11.1 20.8	
7	hydroxy-proline									11.5 31.5
8a	glutamic	9.2 17.5	11.0 17.2		13.4 17.8	13.9 17.7	12.0 17.8	12.4 18.4		13.1 18.3
8b	threonine		8.4 24.5	9.4 25.4	9.7 25.2			9.7 24.8		13.1 25.0
9	alanine	15.9 26.7	14.8 26.0	15.9 27.9	16.9 27.2	18.5 27.7	15.7 26.8	16.5 27.6	17.1 27.8	
10	proline									20.4 39.2
11	tyrosine	17.0 32.4	16.2 32.3	17.2 34.5	18.2 32.8	19.0 33.4	16.0 32.8	17.3 33.5	20.9 30.4	
12b1	tryptophan									
12b2	methionine									24.5 35.9
12b3	valine									24.5 35.9

Continued

TABLE VII - Continued

Group II (Continued)

Spot	Possible Identity	4-S-H	4-S-R-2d	8-S-H	8-S-R-6d	6-S-H	6-S-R-6d	23-V R-6d	Std. 1	Std. 2
13a	phenylalanine								29.7 37.7	
13b	isoleucine									28.7 38.6
13c	leucine								29.7 37.7	
13	unknown				29.1 38.8		26.3 35.3	27.8 35.9		

Group III

								Co-chromatograph Standards			
	Possible	23-V-H		19-V-R-2d		19-V-H		Std.	23-V-H	19-V-R-2d	19-V-H
Spot	Identity	a	b	a	b	a	b	1	b	b	b
1	cysteine			2.0 13.4	2.5 13.4						
2a	lysine	5.3 14.2		4.6 13.7							
2b	histidine			4.5 18.8	5.1 18.4				5.6 18.6	6.0 19.0	5.4 18.2
5a	aspartic	11.0 7.5	11.4 7.5					10.3 7.1			

Continued

TABLE VII - Continued

Group III (Continued)

Spot	Possible Identity	23-V-H a	23-V-H b	19-V-R-2d a	19-V-R-2d bb	19-V-H a	19-V-H b	Co-chromatograph Standards: Std. 1	Co-chromatograph Standards: 23-V-H b	Co-chromatograph Standards: 19-V-R-2d b	Co-chromatograph Standards: 19-V-H b
5b	serine	10.9 16.9	11.1 17.1	7.5 18.1	8.4 18.2	11.2 16.7	10.6 17.2	10.1 16.9			
7	hydroxy- proline							13.1 32.8			
8a	glutamic	14.6 14.9		10.0 16.8	11.1 17.5						
8b	threonine			9.6 25.1	11.1 25.8						
9	alanine	18.6 28.7	18.3 29.3	18.6 28.3	19.8 28.7	19.1 28.7	18.5 27.8				
10	proline							21.2 42.1			
11	tyrosine	19.5 36.9	18.7 37.5	19.6 36.5	21.0 36.7	20.2 36.1	18.7 35.9				
12a	unknown			22.7 6.1	23.7 5.4						
12b1	tryptophan	29.6 33.3							27.6 37.6	29.6 33.3	26.0 37.3

Continued

TABLE VII - Continued

Group III (Continued)

Spot	Possible Identity	23-V-H a	23-V-H b	19-V-R-2d a	19-V-R-2d b	19-V-H a	19-V-H b	Co-chromatograph Standards: Std. 1	Co-chromatograph Standards: 23-V-H 2	Co-chromatograph Standards: 19-V-R-2d 3	Co-chromatograph Standards: 19-V-H 4
12b3	valine							26.3 37.4			

[a]For explanation of treatment symbols, see Table 1, p. 41.

[b]Migration expressed in centimeters: top figure represents movement due to solvent 1. Bottom figure represents movement due to solvent 2.

TABLE VIII

Rg VALUES OF KNOWN SUGARS IN A REPLICATED REFERENCE SET OF CHROMATOGRAPHS

No.	Trehalose	Sucrose	Glucose	Fructose	Arabinose	Arabitol	Xylose	Ribose
1	.53[a]	.76	1.00	1.16	1.17	1.21	1.41	1.56
	.53	.68	1.00	1.15	1.16	1.30	1.29	1.40
2	.53	.77	1.00		1.19	1.25	1.41	
	.53	.69	1.00		1.14	1.30	1.29	
3	.50	.75	1.00		1.20	1.25	1.43	
	.55	.71	1.00		1.13	1.29	1.30	
4		.77	1.00	1.16			1.39	
		.68	1.00	1.17			1.33	
5	.51	.77	1.00		1.21	1.26	1.43	
	.52	.70	1.00		1.16	1.29	1.27	
6		.77	1.00	1.15	1.18		1.39	
		.64	1.00	1.09	1.14		1.23	
7		.75	1.00	1.16				
		.67	1.00	1.16				
8		.76	1.00	1.17			1.43	
		.70	1.00	1.16			1.28	
9	.50	.73	1.00	1.13		1.19	1.39	
	.50	.68	1.00	1.17		1.32	1.31	

[a]See footnote on Table V, p. 92.

TABLE IX

Rg VALUES OF DISCRETE CHROMATOGRAPH SPOTS LOCATED WITH

ANILINE PHTHALATE AND P-ANISIDINE SPRAY REAGENTS

Set 1

Sample	Unknown Oligosaccharides						
	A	B	C	D	E	F	G
6-S-R[a]				.29[b]		.43	
				.21		.34	
6-S-H			.22				.50
			.09				.37
8-S-H				.24		.40	.45
				.18		.30	.36
19-NV-R-2d			.23	.31	.37	.45	.53
			.10	.18	.23	.31	.38
19-V-R-2d		.15		.25			.45
		.12		.18			.39
8-S-R			.23	.33		.44	.52
			.12	.21		.30	.38
23-NV-R				.29		.41	.50
				.19		.30	.38
4-S-R				.30		.45	.52
				.18		.31	.37
4-S-R-2d				.26	.33	.40	.49
				.18	.22	.29	.37
23-V-R	.08	.18	.26	.31	.43	.43	.49
	.03	.09	.14	.21		.31	.39

Treatment	Unknown H	Sucrose	Glucose	Fructose	Xylose	Ribose
6-S-R		.79	1.0	1.14		1.63
		.70	1.0	1.14		1.44
6-S-H		.66	1.0	1.16	1.45	1.69
		.69	1.0	1.17	1.27	1.38

Continued

TABLE IX - Continued

Set 1 (Continued)

Treatment	Unknown H	Sucrose	Glucose	Fructose	Xylose	Ribose
8-S-H		.74	1.0	1.14		1.58
		.67	1.0	1.15		1.47
19-NV-R-2d		.80	1.0	1.15		1.53
		.69	1.0	.99		1.50
19-V-R-2d		.74	1.0	1.15		1.58
		.68	1.0	1.14		1.42
8-S-R	.66	.80	1.0	1.16		1.63
	.54	.69	1.0	1.14		1.38
23-NV-R		.79	1.0	1.15	1.38	1.58
		.68	1.0	1.16	1.31	1.45
4-S-R		.79	1.0	1.14	1.38	1.52
		.68	1.0	1.15	1.31	1.41
4-S-R-2d		.77	1.0	1.13	1.40	1.61
		.68	1.0	1.16	1.32	1.42
23-V-R	.66	.78	1.0	1.13	1.40	
	.55	.69	1.0	1.18	1.27	

Set 2

	Unknown Oligosaccharides								
Treatment	C	D	E	F	H	I	Sucrose	Glucose	Fructose
19-V-R	.23	.30	.35	.43			.76	1.0	1.22
	.14	.20	.24	.34			.64	1.0	1.19
19-NV-R	.20	.35		.41			.77	1.0	1.29
	.14	.26		.33			.65	1.0	1.23

Continued

TABLE IX - Continued

Set 3

Treatment	Unknown Oligosaccharides				
	C	D	F	G	Trehalose
23-V-H	.13		.25	.34	
	.20		.34	.41	
23-V-H	.14	.18	.26	.28	
	.21	.28	.35	.43	
Standard Solutions					.41
					.55
23-V-R	.17		.26	.34	.43
	.23		.34	.41	.48
8-S-Hq	.17	.21	.30	.34	
	.20	.26	.36	.41	
8&S-R			.29	.37	.44
			.34	.42	.50

Treatment	Unknown H	Sucrose	Glucose	Fructose	Xylose	Arabitol
23-V-H		.64	1.0	1.22		
		.71	1.0	1.16		
23-V-H		.67	1.0	1.17	1.45	
		.76	1.0	1.17	1.32	
Standard			1.0		1.52	1.28
			1.0		1.32	1.26
8-S-H		.66	1.0	1.20		
		.72	1.0	1.17		
23-V-R	.50	.65	1.0	1.22		
	.57	.69	1.0	1.15		
8-S-R	.53	.67	1.0	1.21		
	.58	.71	1.0	1.14		

[a]For explanation of treatment symbols, see Table I, p. 41.

[b]See footnote on Table V, p. 92.

VITA

Dick Don Davis

Candidate for the Degree of

Doctor of Philosophy

Thesis: THE EFFECTS OF LEAF RUST INFECTION ON THE CONVERSION OF GLUCOSE-U-C^{14} INTO AMINO ACIDS AND SUGARS IN SEEDLING AND MATURE PLANTS OF PONCA WHEAT

Major Field: Plant Physiology

Biographical:

Personal data: Born at Hobart, Oklahoma, August 7, 1933. Son of Boyd Clinton and Alice Ione Davis.

Education: Attended elementary through first year of high school at Mountain Park, Oklahoma; attended last two years of high school at Hobart, Oklahoma; entered Oklahoma Christian College in 1951, completing only one year of study there; attended Altus Junior College for one semester in 1957; entered the University of Oklahoma in September, 1957, completing requirements for the Bachelor of Science degree in August, 1960; entered Oklahoma State University in September, 1960, completing work for the Master of Science degree in June, 1963, and the Doctor of Philosophy degree in July, 1965.

Professional Experience: Graduate Research Assistant, Department of Botany and Plant Pathology, Oklahoma State University, 1960-62. Assistant Professor, Department of Agronomy, New Mexico State University, 1964-65.

Date of Final Examination: July, 1965.