Plant Physiol. (1988) 88, 805–809
0032-0889/88/88/0805/05/$01.00/0

Hypoxic Stress Inhibits the Appearance of Wound-Response Proteins in Potato Tubers[1]

Received for publication April 3, 1988 and in revised form May 23, 1988

MICHAEL E. VAYDA* AND HOLLY J. SCHAEFFER[2]
Department of Biochemistry, University of Maine, Orono, Maine 04469

ABSTRACT

Potato (*Solanum tuberosum* L.) tubers respond to environmental stresses by alterations of macromolecular synthesis. In an aerobic environment tubers respond rapidly to wounding by synthesizing a set of proteins, the most prominent of which display apparent molecular weights of 78, 48, 38, and 31 kilodaltons. These proteins become intensely labeled by [^{35}S]methionine within 2 hours of wounding. The 78 kilodalton polypeptide has been identified by immunoprecipitation as phenylalanine ammonia-lyase. By contrast, tubers incubated in hypoxic conditions for a period as short as 1.5 hours exhibit significantly reduced incorporation of amino acids such that newly synthesized polypeptides are not detected. However, a second set of proteins is synthesized by wounded tubers after prolonged incubation in a hypoxic environment. One peptide of this set is precipitated by an antibody directed against aldolase; several of these proteins may be enzymes of glycolysis necessary for anaerobic metabolism. The results indicate that there is a complex regulatory mechanism which allows mature potato tubers to respond to changes in the environment.

Potato tubers are susceptible to the disease known as 'soft rot' when subjected to wet, hypoxic conditions (2, 7, 14, 18). The causative agents of this disease, soil bacteria of the genus *Erwinia*, secrete pectate lyases and a variety of other degradative, enzymatic activities that result in maceration of parenchymatous tissues (4, 18). The onset of soft rot correlates distinctly with the oxygen status of potato tubers in the storage environment or soil (2, 7). Alterations in bacterial metabolism under hypoxic conditions do not explain the occurrence of soft rot in this environment because purified preparations of pectate lyase from *Erwinia* cultures are sufficient to elicit the rapid decay of tuber tissues under low oxygen conditions (4, 14). Thus, the susceptibility of potato tubers to this disease is likely to result from a host response to hypoxic conditions which lowers tuber resistance to the pathogen (7, 14, 18). The purpose of this study is to determine what changes in tuber macromolecular metabolism occur during incubation in an oxygen-poor environment.

The host factors responsible for aerobic resistance to bacterial soft rot are not known. The most common method of entry by the *Erwinia* pathogen into tuber parenchyma is via bruises or wounds in the tuber surface (18). Such wounding events, the presence of pectic enzymes or the oligogalacuronate products of these activities, elicit the synthesis of phytoalexins, proteinase inhibitors, cell wall proteins, and key disease resistance enzymes of the phenylpropanoid and terpenoid pathways in several plant species including potato (1, 3, 6, 8, 20, 23, 25). Thus, it is not surprising that protein synthesis appears to be required to maintain resistance to pectolytic activities. Potato tubers pretreated with the protein synthesis inhibitor cycloheximide are susceptible to degradation by low concentrations of bacterial pectate lyases (27). Cycloheximide inhibits the synthesis of phenylalanine ammonia-lyase (26) and prevents the formation of the suberized layer that normally forms at tuber wound sites (27). Hypoxic treatment also prevents formation of a suberized layer at wounded tuber surfaces, even following incubation for several hours in air (W Butler, M Vayda, unpublished observations). Potato varieties which naturally produce low levels of phenylalanine ammonia-lyase exhibit a marked susceptibility to degradation by pectate lyase (27).

The synthesis of proteins in potato tubers exposed to hypoxic conditions has not been examined previously. Many other plants exhibit marked metabolic changes when subjected to hypoxic conditions which include specific alterations in the program of protein synthesis (15, 21), changes in the stability of cytoplasmic mRNA species (9), and induced transcription of several nuclear genes (9, 25). The experiments described here indicate that the usual proteins synthesized by potato tubers in response to wounding are not apparent during periods of hypoxic stress; this may provide a partial explanation for the loss of resistance of potato tubers to soft rot under these conditions.

MATERIALS AND METHODS

Potato Stocks. Potato tubers (*Solanum tuberosum* L. cv Russett Burbank) were rinsed briefly in tepid, soapy water to remove soil debris, surface sterilized with 5% hypochlorite (v/v), dried and incubated at ambient temperature (20°C) for 16 to 24 h prior to experimental use. Tubers wounded immediately after storage at 4°C incorporate less than 10% of the [^{35}S]methionine label that is incorporated by tubers preincubated at room temperature overnight.

Wound Induction and Hypoxic Conditions. Wounds were inflicted by insertion of a plastic tip for the p200 micropipette (USA Plastics) into a whole tuber to a depth of approximately 2.5 cm. Tubers were incubated with 0.25 or 0.5 mCi of [^{35}S] methionine (>800 Ci/mM, New England Nuclear), added to the wound either immediately or at a specified time after wounding as indicated in the Figures. Hypoxic conditions were established by incubating tubers in an argon atmosphere, generated by bubbling argon gas through dH_2O under a sheet of Saran Wrap draped over tuber surfaces.

Sample Preparation. Approximately 5 g of tuber tissue immediately surrounding the wound site was removed using an apple corer. This 1 cm diameter cylinder contained greater than 90% of the label added. The sample was ground using a Sybron

[1]M.E.V. supported by U.S. Department of Agriculture grant GAM 8700996, Maine Agricultural Experiment Station Grant ME08402, and the University of Maine Faculty Research Fund.

[2]Present address: Department of Genetics, Michigan State University, East Lansing, MI 48824.

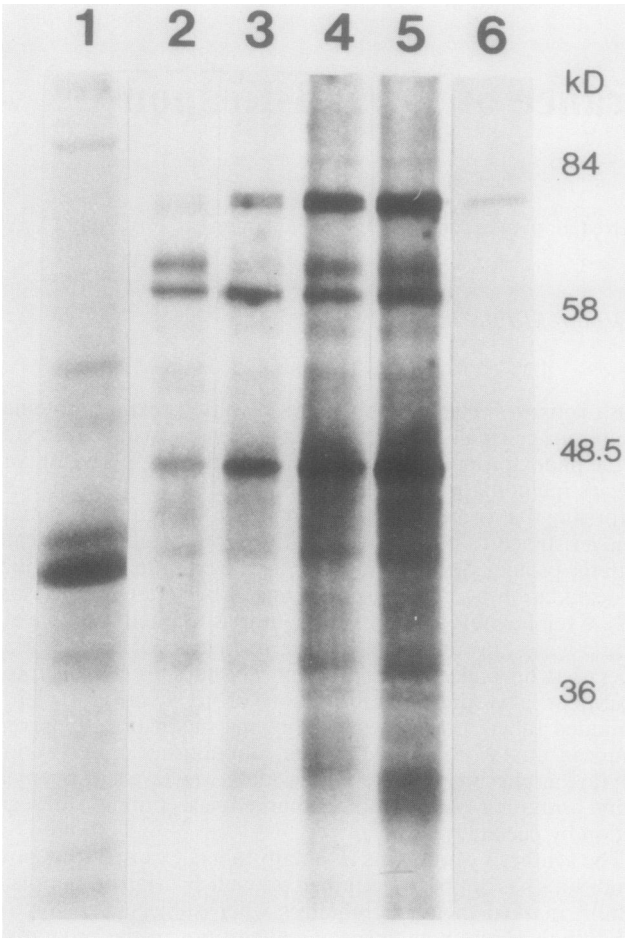

FIG. 1. Fluorograph of polypeptides synthesized by potato tubers incubated under aerobic conditions in the presence of 0.5 mCi [^{35}S] methionine for 3 h. Lane 1, tuber polypeptides visable upon staining with Coomaisse blue; lanes 2, 3, 4, and 5, polypeptides of tuber extracts to which label was presented at the time of wounding or 3, 6, or 9 h after wound induction, respectively; lane 6, polypeptides immunoprecipitated by an antibody against phenylalanine ammonia-lyase from an extract of an aerobic potato tuber, labeled by incubation for 1 h with 0.25 mCi [^{35}S]methionine presented 18 h after wounding.

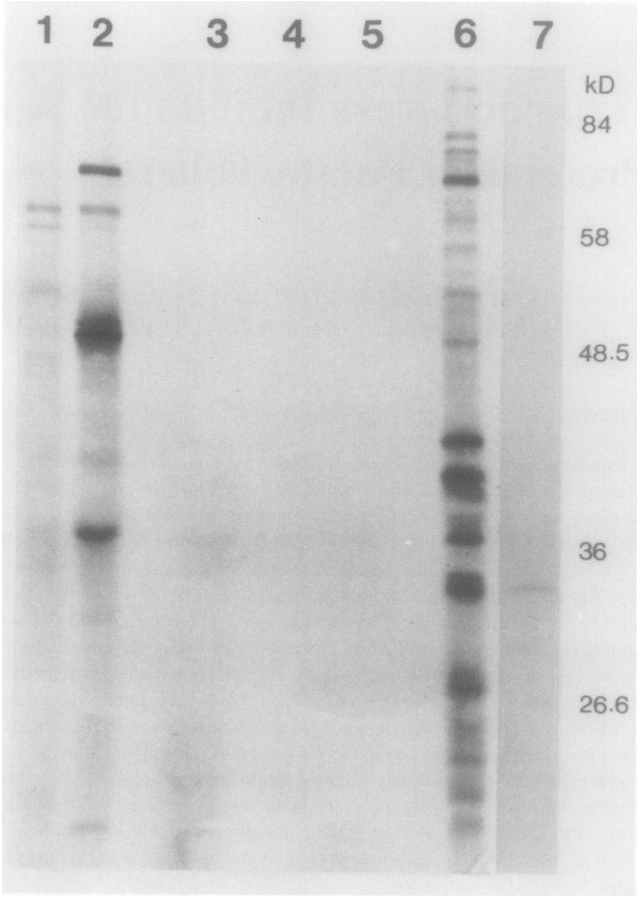

FIG. 2. Fluorograph of polypeptides synthesized by aerobic and hypoxic tubers in a 1 h incubation with [^{35}S]methionine. Tubers maintained in the air were incubated with 0.5 mCi [^{35}S]methionine for 1 h presented at the time of wounding (lane 1) or 18 h after wound induction (lane 2). Tubers incubated in a moist, argon atmosphere for 1.5, 4, or 25 h before wounding (lanes 3, 4, and 5, respectively) were labeled with [^{35}S]methionine for 1 h, presented at the time of wounding. Lane 6, extract of a wounded, hypoxic tuber maintained in the argon atmosphere for 25 h and incubated for 1 h with 0.5 mCi [^{35}S]methionine presented 19 h after wounding. Lane 7, polypeptides immunoprecipitated by an antibody against fructose-1,6-bisphosphate aldolase from the 19 h wounded, 25 h hypoxic tuber extract.

polytron homogenizer at top speed for 60 s in 2 mL of a buffer comprising 25 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 50 mM β-mercaptoethanol, and 2.5 mM PMSF.[3] It is essential to maintain the sample chilled in an icebath during homogenization to avoid gel formation. Large debris was removed by filtration through one layer of cheesecloth and Miracloth. Filtrate was analyzed for total counts recovered and TCA precipitable counts incorporated, then stored frozen at −20°C. The filtrate was adjusted to 0.1% SDS (w/v), incubated at 50°C for 5 min, and centrifuged at 1500 rpm in an IEC Clinical centrifuge for 5 min to remove insoluble starch. Labeled proteins were resolved by electrophoresis through 11 or 12.5% SDS-polyacrylamide gels as described previously (12, 17, 24). Gels were subjected to fluorography using EnH^{3}ance (New England Nuclear) as directed by the supplier, and Kodak X/AR-5 film. Filtrates without SDS were incubated with antiserum prepared against bean phenylalanine ammonia-lyase or maize fructose-1,6-bisphosphate aldolase, kindly provided by Dr. R. Dixon and Dr. P. Kelley, respectively. Antigen-antibody complexes were precipitated by adsorption to formalin-fixed *Staphylococcus aureus* cells (Immunoprecipitin, Bethesda Research Labs, Bethesda, MD). Labeled polypeptides were eluted by boiling in the presence of 1% SDS, and were resolved by PAGE (12) and fluorography.

[3]Abbreviations: PMSF, phenylmethylsulfonyl fluoride.

RESULTS

Mature potato tubers respond to wounding by synthesizing several additional polypeptides. Wounded tubers incubated for 3 h at ambient temperature in the presence of 0.5 mCi [^{35}S] methionine synthesize the labeled proteins shown in Figure 1. Several protein species, namely those migrating with apparent mol wt of 78, 48, 38, 31, and 10 kD are more highly labeled when [^{35}S]methionine is presented to tubers 3, 6, or 9 h after wounding (Fig. 1, lanes 3, 4, and 5, respectively) than when label is presented immediately upon wounding (Fig. 1, lane 2). None of these prominent proteins comigrate with the abundant tuber proteins evident by staining gels with Coomassie blue (Fig. 1, lane 1). These abundant polypeptides, which range from 90 to 5 kD, correlate with tuber polypeptides described previously (16, 17) that are known to accumulate greatly during tuberization.

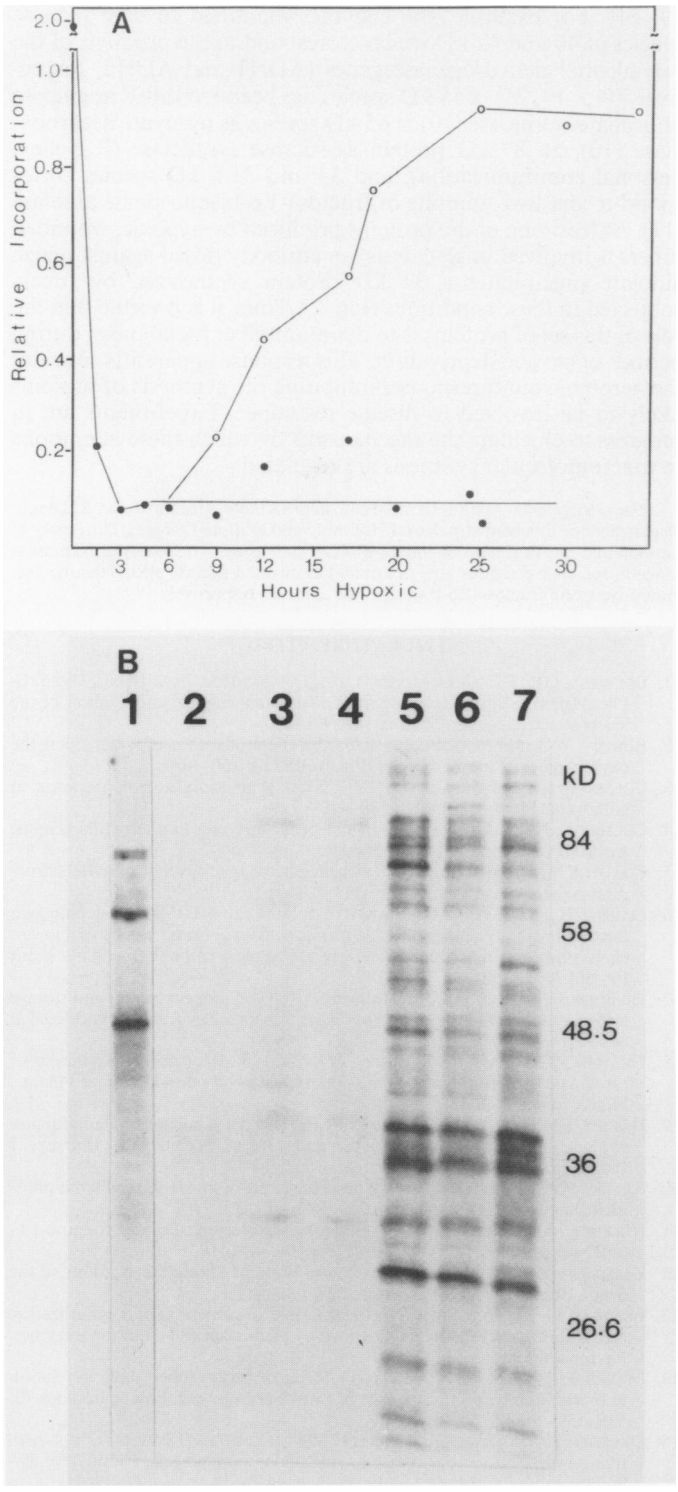

FIG. 3. A, Label incorporated by tubers incubated under hypoxic conditions, incubated for 2 h in the presence of 0.5 mCi [^{35}S]methionine presented at the time of wounding (●); label incorporated by a second set of tubers maintained under hypoxic conditions for 6 h, wounded and incubated for 2 h with 0.5 mCi [^{35}S]methionine at the various times indicated (○). Relative incorporation is calculated as TCA-precipitable cpm/total counts × 100. B, Fluorograph of labeled polypeptides synthesized by hypoxic tubers when incubated for 2 h with [^{35}S]methionine presented at various times after wounding. Lane 1, labeled polypeptides synthesized by an aerobic tuber 3 h after wounding. Tubers were incubated in an argon atmosphere for 6 h, wounded and labeled by hypoxic tion with 0.5 mCi [^{35}S]methionine at the time of wounding (lane 2) or 3, 6, 9, 12, or 24 h after wound induction (lanes 3, 4, 5, 6, and 7, respectively).

The major protein species, the patatins, migrate as broad bands of 40 and 42 kD; the modified, more slowly migrating form of this protein serves as a variety-specific marker (16). The amount and relative proportions of these abundant tuber proteins do not change when tubers are wounded or subjected to hypoxic conditions (data not shown).

The mobility of the highly labeled polypeptides compares well to wound response proteins of other plant species. In order to determine whether any of these proteins were indeed related, labeled tuber extracts were incubated with an antiserum raised against a major wound-response protein of bean, phenylalanine ammonia-lyase (PAL). A 78 kD polypeptide was immunoprecipitated by this antiserum only, and comigrated with one of the intensely labeled proteins of wounded potato tubers (Fig. 1, lane 6). The proteins which are synthesized in response to wounding apparently are not synthesized by unwounded tubers. These proteins are not evident in samples of tubers labeled with [^{35}S] methionine for short periods of 1 h or less at the time of wound induction (Fig. 2, lane 1), nor is the 78 kD polypeptide immunoprecipitated from these samples with anti-PAL serum (data not shown). The major, newly synthesized, labeled proteins begin to accumulate within 2 h after the wounding event, and continue to be synthesized by tubers incubated with [^{35}S]methionine 20 h after wounding (Fig. 2, lane 2). These results indicate that mature tuber tissues respond to wounding by synthesizing a novel set of proteins, at least one of which is a key enzyme in a pathogen-resistance pathway.

The major objective of these experiments was to determine what response, if any, tubers display when subjected to the hypoxic conditions correlated with the loss of resistance to bacterial infection. Incorporation of [^{35}S]methionine by tubers is decreased 10-fold within 1.5 h and 20-fold within 4 h of incubation in a hypoxic environment whether wounded aerobically (not shown) or wounded at the time [^{35}S]methionine is presented (Fig. 2, lanes 3 and 4; Fig. 3A). Labeled proteins are not detected in tubers incubated under hypoxic conditions for 25 h prior to presentation of label at the time of wounding (Fig. 2, lane 5). Thus, this response to hypoxia apparently overrides the aerobic wound response.

Further, there appears to be a secondary hypoxic response which requires wounding for its induction. More than 15 major, [^{35}S]methionine labeled polypeptides, ranging in apparent mol wt from greater than 110 kD to less than 15 kD, are synthesized by hypoxic tubers that were wounded and maintained in hypoxic conditions for an additional 19 h before the presentation of [^{35}S] methionine label (Fig. 2, lane 6). At least one novel protein is present in this set of polypeptides. A labeled protein of 34 kD is immunoprecipitated by an antibody raised against a component of maize fructose-1,6-bisphosphate aldolase (Fig. 2, lane 7). This polypeptide is not immunoprecipitated from labeled extracts of wounded tubers maintained in aerobic conditions (data not shown). Conversely, the 78 kD protein is not immunoprecipitated from hypoxic, wounded tuber extracts using the anti-PAL antiserum (data not shown). In contrast to the the rapid aerobic wound response which occurs within 2 h of wounding, labeled proteins are only detected in hypoxic tubers with a lag period of 3 to 6 h after wounding. The proteins synthesized by hypoxic tubers appear to accumulate after wounding in two steps: a subset of the polypeptides is apparent 3 to 6 h after wounding (Fig. 3B), which do not, in general, constitute the most prominently labeled proteins synthesized by hypoxic tubers 9 to 24 h after wounding. This complete set of polypeptides continues to be synthesized by tubers under hypoxic conditions for at least

28 h after wounding, but their appearance is blocked by the addition of 100 μg/mL α-amanitin at the time of wounding (data not shown). In summary, wounded tubers respond to hypoxia with an initial inhibition of protein synthesis or amino acid incorporation, followed by the appearance of a new set of polypeptides.

DISCUSSION

The inhibition of wound response protein synthesis is likely to partially explain why tubers lose resistance to disease in hypoxic environments. These newly synthesized polypeptides display a half-life of only about 4.5 h under either aerobic or hypoxic conditions (M Vayda, H Schaeffer, unpublished observations). If these components do play a role in plant defense mechanisms, the inhibition of their synthesis would render tubers sensitive to disease within hours of exposure to hypoxic conditions. At least one of these proteins appears to be phenylalanine ammonia-lyase (Fig. 1), a key enzyme of the phenylpropanoid pathway (1, 3). The identity of the other proteins synthesized by aerobic tubers in response to wounding is currently not known. However, the mol wt of the proteins observed is consistent with several other known wound response proteins. Genes known to be expressed by several different plant tissues in response to wounding in addition to phenylalanine ammonia-lyase (78 kD) (1, 3, 27) include polyphenol oxidase (13), chitinase (30 kD) (22), chalcone synthase (20), proteinase inhibitors I and II (12.5 and 8.1 kD, respectively) (8), the hydroxyproline-rich glycoprotein extensin (23), cell wall protein p33 (33 kD) (M Tierney, personal communication), and a glycine-rich cell wall protein (5). RNA encoding several of these proteins accumulates in tubers in response to wounding (L Cook, M Vayda, unpublished observations). Phytoalexins and phenolic compounds elicited by several plant species in response to wounding or the presence of pathogens might be involved in the hypersensitive reaction which limits fungal or bacterial growth (3). Chlorogenic acid and other phenolic compounds which are prevalent in potato tubers have been implicated as inhibitors of bacterial enzyme activities. Similarly, polyphenol oxidase, which oxidizes the phenolic compounds in suberin to initiate a nonspecific free radical-mediated polymerization of proteins at the wound site has also been implicated in aerobic disease resistance (13). Other host factors either reversibly block pectolytic activity directly, or somehow render the substrates for these enzymes unavailable for attack. The inability to synthesize aerobic wound response proteins, due to inhibition by either cycloheximide (26, 27) or hypoxic stress is likely to be sufficient to render tubers susceptible to disease.

It is not known whether the failure to detect labeled aerobic wound response proteins under hypoxic conditions is due to decreased amino acid uptake, a larger pool size of methionine, or a direct inhibition of protein synthesis. We suspect that the latter case is true because the typical oxidation of phenolics is not observed when hypoxia stressed tubers are returned to aerobic conditions (W Butler, M Vayda, unpublished observations). Further, the RNA species which encode known wound response proteins that accumlate in the cytoplasm of tubers wounded in the air decrease in hypoxic tuber tissues (L Cook, M Vayda, unpublished observations). These observed responses of potato tubers are similar to the marked metabolic changes exhibited by other plant species when subjected to anaerobic conditions (19, 21). Maize root cells, for example, synthesize only 20 proteins at dramatically elevated levels, in contrast to the many proteins synthesized by roots in air (15, 21). Indeed, protein synthesis is not detected in maize leaf cells subjected to anaerobic conditions (15). Of the anaerobically induced maize root proteins that have been identified, all are enzymes which participate in glycolysis or other aspects of carbohydrate metabolism necessary to maintain energy production under anaerobic conditions (10, 11, 15, 19, 21). For example, the enzymes identified to date include species of 40 and 42 kD which correspond to the products of the two alcohol dehydrogenase genes (ADH1 and ADH2, respectively) (15, 19, 21), a 55 kD species has been identified as glucose phosphate isomerase (10), a 65 kD species as pyruvate decarboxylase (10), an 87 kD protein as sucrose synthetase (P Kelley, personal communication), and 33 and 31.5 kD species correspond to the two subunits of fructose-1,6-bisphosphate aldolase (11). At least one of the proteins produced by hypoxic, wounded tubers is involved in glycolysis: an antibody raised against maize aldolase precipitates a 34 kD protein synthesized by tubers subjected to these conditions (Fig. 2). Thus, it is possible that the role of this set of proteins is to maintain tuber metabolism during periods of oxygen deprivation. This response apparently overides the aerobic wound response, inhibiting the synthesis of proteins likely to be involved in disease resistance. Experiments are in progress to elucidate the mechanisms by which these alterations in macromolecular synthesis are regulated.

Acknowledgments—The authors wish to express their gratitude to Dr. R. Dixon, Department of Biochemistry, Royal Holloway and Bedford Colleges, University of London and Dr. P. Kelley, School of Biological Sciences, University of Nebraska-Lincoln, for their generous gifts of antibodies directed against phenylalanine ammonia-lyase and fructose-1,6-bisphosphate aldolase, respectively.

LITERATURE CITED

1. Bolwell GP, CL Cramer, CJ Lamb, W Schuch, RA Dixon 1986 L-Phenylalanine ammonia-lyase from *Phaseolus vulgaris*: modulation of the levels of active enzyme. Planta 169: 97–107
2. Burton WG, MJ Wigginton 1970 The effect of a film of water upon the oxygen status of a potato tuber. Potato Res 13: 150–186
3. Collinge DB, AJ Susarenko 1987 Plant gene expression in response to pathogens. Plant Mol Biol 9: 389–410
4. Colmer A, NT Keen 1986 The role of pectic enzymes in plant pathogenesis. Annu Rev Phytopathol 24: 383–409
5. Condit CM, RB Meagher 1987 A gene encoding a novel glycine-rich structural protein of petunia. Nature 323: 178–181
6. Davis KR, GD Lyon, AG Darvill, P Albersheim 1984 Host pathogen interactions XXV: endopolygalacturonic acid lyase from *Erwinia carotovora* elicits phytoalexin accumulation by releasing plant cell wall fragments. Plant Physiol 74: 52–60
7. De Boer SH, A Kelman 1978 Influence of oxygen concentration and storage factors on susceptibility of potato tubers to bacterial soft rot. Potato Res 21: 65–80
8. Graham JS, G Hall, G Pearce, CA Ryan 1986 Regulation of synthesis of proteinase inhibitors I and II mRNAs in leaves of wounded tomato plants. Planta 169: 399–405
9. Hake S, PM Kelley, WC Taylor, M Freeling 1985 Coordinate induction of alcohol dehydrogenase 1, aldolase and other anaerobic RNAs in maize. J Biol Chem 260: 5050–5054
10. Kelley PM, M Freeling 1984 Anaerobic expression of maize glucose phosphate isomerase I. J Biol Chem 259: 673–677
11. Kelley PM, M Freeling 1984 Anaerobic expression of maize fructose-1,6-diphosphate aldolase. J Biol Chem 259: 14180–14183
12. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
13. Leatham GF, V King, MA Stahmann 1980 *In vitro* protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. Phytopathology 70: 1134–1140
14. Maher EA, A Kelman 1983 Oxygen status of potato tuber tissue in relation to maceration by pectic enzymes of *Erwinia carotovora*. Phytopathology 73: 536–539
15. Okimoto R, MM Sachs, EK Porter, M Freeling 1980 Patterns of polypeptide synthesis in various maize organs under anaerobiosis. Planta 150: 89–94
16. Park WD 1983 Tuber proteins of potato: a new and surprising molecular system. Plant Mol Biol Rep 1: 61–66
17. Park WD, C Blackwood, GA Mignery, MA Hermodson, RM Lister 1983 Analysis of the M_r 40,000 tuber glycoprotein of potatoes. Plant Physiol 71: 156–160
18. Perrombelon MC, A Kelman 1980 Ecology of the soft rot Erwinias. Annu Rev Phytopathol 18: 361–387
19. Ricard B, B Mocquot, A Fournier, M Delseny, A Pradet 1986 Expression of alcohol dehyrodgenase in rice embryos under anoxia. Plant Mol Biol 7: 321–329
20. Ryder TB, CL Cramer, JN Bell, MP Robbins, RA Dixon, CJ Lamb 1984 Elicitor rapidly induces chalcone synthase mRNA in *Phaseolus vulgaris* cells at the onset of phytoalexin defense response. Proc Natl Acad Sci USA 81: 5724–5728

21. SACHS MM, M FREELING, R OKIMOTO 1980 The anaerobic proteins of maize. Cell 20: 761–767
22. SCHLUMBAUM A, F MAUCH, U VOGELI, T BOLLER 1986 Plant chitinases are potent inhibitors of fungal growth. Nature 324: 365–367
23. TIERNEY ML, JE VARNER 1987 The extensins. Plant Physiol 84: 1–2
24. VAYDA ME, A ROGERS, SJ FLINT 1983 The structure of nucleoprotein cores released from adenovirions. Nucleic Acids Res 11: 441–460
25. VAYDA ME, M FREELING 1986 Insertion of the maize transposable element *Mu1* into the first intron of Adh1 interferes with transcription elongation but does not disrupt chromatin structure. Plant Mol Biol 6: 441–454
26. ZUCKER M, MM EL ZAYAT 1968 The effect of cycloheximide on the resistance of potato tuber discs to invasion by a fluorescent *Pseudomonas* sp. Phytopathology 58: 339–344
27. ZUCKER M, L HANKIN 1970 Physiological basis for a cycloheximide induced soft rot of potatoes by *Pseudomonas fluorescens*. Ann Bot 34: 1047–1062