THE NATURE OF BIOTROPHY IN *PHYTOPHTHORA INFESTANS*

P.C. Scheepens

THE NATURE OF BIOTROPHY
IN *PHYTOPHTHORA INFESTANS*

Stellingen

I

De groeisnelheid van *Phytopthora infestans* op basale voedingsmedia wordt door de biosynthese van aminozuren beperkt.

> H.R. Hohl, 1975: Phytopath. Z. 84, 18 - 33
> Dit proefschrift

II

De beperkte activiteit van het mitochondriale electronentransport van *Phytophthora infestans* is een aannemelijke oorzaak van zijn complexe voedings-eisen.

> Dit proefschrift

III

Het door Chakravorty en Shaw beschreven model voor de compatibele interactie tussen waardplant en obligate parasiet gaat uit van te veel onbewezen vóór-onderstellingen om geloofwaardig te zijn.

> A.K. Chakravorty en M. Shaw, 1977: Biol.
> Rev. 52, 147 - 179

IV

Hypersensitiviteit is geen primaire oorzaak van resistentie van planten tegen biotrofe parasieten.

> N. Doke, 1975: Physiol. Plant Pathol. 7,
> 1 - 7; M.C. Heath, 1976: Phytopathology 66,
> 935 - 936; Z. Kiraly, B. Barna, en T. Ersek,
> 1972: Nature 239, 456 - 458; S. Mayama,
> J.M. Daly, D.W. Rehfeld, en C.R. Daly,
> 1975: Physiol. Plant Pathol. 7, 35 - 47

V

Er bestaan goede gronden voor de conclusie, dat de vegetatieve fase van *Pythium* en *Phytophthora* diploid is.

> C.W. Dennett en M.E. Stanghellini, 1977:
> Phytopathology 67, 1134 - 1141; C.G. Elliott
> en D. McIntyre, 1973: Trans. Brit. Mycol.
> Soc. 60, 311 - 316; M. Long en N.T. Keen,
> 1977: Phytopathology 67, 675 - 677

VI

De term "genetische manipulatie" als aanduiding van recente ontwikkelingen in
de moleculaire biologie, stelt deze tak van wetenschap in een onverdiend
kwaad daglicht.

VII

De bewering van Halos en Huisman, dat de werking van het fungicide ethazol
berust op remming van het electronentransport tussen cytochromen b en c,
wordt onvoldoende door hun experimentele resultaten ondersteund.

P.M. Halos en O.C. Huisman, 1976:
Phytopathology 66, 158 - 164

VIII

Schimmels, die niet in vitro gekweekt kunnen worden, bieden weinig perspec-
tieven voor de biologische bestrijding van onkruiden in Nederland.

IX

Een goede leermeester kan zich in de gedachtenwereld van zijn leerlingen ver-
plaatsen; een slechte leermeester zal van zijn leerlingen het omgekeerde
eisen.

X

De benaming "waardevrije" wetenschap dient te worden vermeden, omdat deze kan
worden opgevat als een eufemisme van "waardeloze" wetenschap.

Alléén de allerwijsten en de allerdwaasten
veranderen nooit van mening

KUNG FU-TSE

Aan de nagedachtenis van mijn vader
Aan Rina

CONTENTS

INTRODUCTION

1. General

To control plant diseases caused by parasitic fungi the following
methods have been developed, which may be applied singly or in combinations:
sanitary and culture measures; breeding of plants for fungal resistance;
chemical, physical and biological control. These methods, however, all have
their disadvantages and shortcomings. Sanitary and culture measures and
physical and biological control are not universally applicable; they have
only been successful in a few limited cases. Breeding for resistance is only
possible if genes for resistance are available. Furthermore, it cannot give
solutions for acute problems, since breeding is a long lasting procedure.
The plant breeder is also confronted with the ability of many fungi to build
up new races which are compatible with the new varieties. Protective fungi-
cides only prevent infection at the treated area. Therefore, their applica-
tion has to be repeated several times during the season. They contribute
also significantly to the pollution of the environment. These disadvantages
are less applicable to most of the newly developed systemic fungicides. In
the control of plant diseases with systemic fungicides we are confronted with
other problems, for instance that of fungicidal resistance. The potential
ability of some fungi to develop resistance against several systemic fungi-
cides has been studied recently (VAN TUYL 1977).

For the reasons mentioned above, these methods for the control of fungal
plant diseases have to be refined, and new concepts have to be developed in
the future. In this respect, a broader insight in the basic processes in-
volved in the development of plant diseases and host resistance is indis-
pensable. We can agree with HEITEFUSS and WILLIAMS (1976) that "Studies on
the biochemistry and physiology of pathogens and pathogenesis no longer are
merely isolated academic exercises".

Invasion of the host by a parasite is characterized by four distinct
phases. These are: "recognition" of the host by the parasite; penetration by
means of chemical and physical forces; establishment of the parasite; meta-
bolic and ultrastructural changes in the host. The physiological plant
pathologist is concerned with the molecular and biophysical events during
these processes, and the factors inducing them. Of special interest are the
factors which determine whether a plant is resistant of susceptible to a
particular pathogen.

An interaction between host and parasite might be of a general nature,

1

representative for a distinct level of fungal specialization, or even spe-
cific for a particular combination of host and parasite. In this respect,
biochemical and physiological studies on obligate parasitism, representing
the highest level of fungal specialization, are of special interest. They
might contribute to a better understanding of the interaction between the
fungi involved and their hosts.

2. *Specialization of fungi: general ecological and physiological aspects*

The basic principle of symbiosis is one of nutrition involving two
living organisms. Symbiosis is mutualistic when it is to the benefit of both
partners; transport of nutrients is in two directions. When the advantage
taken by one partner is to the disadvantage of the other, the symbiosis is
no longer mutualistic but antagonistic: one organism has become parasitic to
the other. Every conceivable gradation is found between the parasitism in
which the parasite quickly destroys its victim and that in which parasite
and host mutually and permanenyly support one another (DE BARY 1887). This
precise description of the two extremes of symbiosis is still valid. Con-
troversies have only arisen when intermediate types were recognized. Re-
servations have to be made for all the systems that have been proposed in
the past to classify fungi according to their level of specialization (DE
BARY 1887, THROWER 1966, BRIAN 1967, LEWIS 1973). They have contributed,
however, to the delimitation of a set of criteria that describe different
levels of organization resulting from the interaction between two living
organisms.

The most essential criterion is probably, whether the parasite is necro-
trophic or biotrophic; the mechanism of parasites to invade their hosts, and
probably also the factors that determine susceptibility or resistance of the
host are of an entirely different nature in both cases. Necrotrophic para-
sites derive their nutrients from dead host cells which are usually killed
by the parasite in advance of penetration; biotrophic parasites derive their
nutrients from living host cells under natural conditions (LINK 1933). It
should be noted that the demarcation between these two categories is not al-
ways sharp, since some biotrophic fungi can become necrotrophic under certain
extreme environmental circumstances (cf. LEWIS 1973). Further distinctions
can be based on criteria such as host specificity, competitive saprophytic
ability, axenic culture, and several others.

2.1 Necrotrophic parasites

Unspecialized necrotrophic parasites appear as such whichever the criterion for specialization that is chosen (GARRETT 1970). Susceptible living tissue is colonized rapidly with the use of several tissue decomposing enzymes and toxins, and the fungus subsequently feeds itself on the dead tissue. The attributes that are used to invade the living host tissue serve as well to colonize dead substrates: these fungi are equally effective as saprophyte and parasite. Their host range is very large. Attack of the living host is mostly only successful in juvenile or senescent tissues in which the mechanisms for resistance are not yet or no longer fully expressed. GARRETT (1970) suggested that the most successful defense of the plant against these parasites is the production of phytoalexins. To this suggestion can be added that other fungitoxic compounds that are releazed upon decompartmentation of the host tissue may play a role as well.

The other extreme category of necrotrophs consists of the perthotrophic parasites. They can only colonize dead tissue that has been killed before: their saprophytic phase is restricted to survival on dead host tissue. Their host range is much smaller than that of the unspecialized necrotrophs; sometimes it is restricted to one species or cultivars of one species. Specialized necrotrophs must have developed mechanisms to attack host plants that have fully developed defense mechanisms. In general, the chemical and physical nature of these mechanisms of attack is still unknown. Thus far, only the production of host-specific toxins by some fungi could be directly related to their pathogenicity (PRINGLE and SCHEFFER 1964, SCHEFFER 1976).

2.2 Biotrophic parasites

The parasitism of biotrophic fungi is evolutionary more advanced than that of necrotrophic fungi (LEWIS 1973, 1974). BRIAN (1967) described and discussed six characteristic symptoms of diseases involving specialized fungi, where a balance between host and parasite is established at least temporally: i) intracellular penetration, ii) minimal tissue damage, iii) highly developed physiological specialization, iv) morphological disturbances in the host plant, v) formation of "green islands", vi) nuclear disturbances. With only a few restrictions, these symptoms are specific for the interaction of biotrophic parasites and their hosts. They are not necessarily found together in a particular interaction. Minimal tissue damage is a necessary prerequisite for the establishment of a prolonged feeding

3

relationship between a biotrophic parasite and its host; the plasmalemma of the host cell may be invaginated, it is never punctured. For this reason, biotrophic fungi have to repress the production of tissue decomposing enzymes and toxins that are disadvantageous to a harmonious relationship with their hosts. In other words, they have thrown away most of the equipment that is necessary to live as saprophytes.

The mechanisms which determine susceptibility or resistance of the host which are the basis for the extreme specialization commonly found with biotrophic fungi, are incompletely understood. It has been suggested that host specificity may be determined by the ability of these fungi to exploit succesfully the hormone balance of their hosts (LEWIS 1973, DALY 1976). It seems also plausible, but has not been proved, that the enhanced metabolic activity at the interface of host and biotrophic parasite is casued as well by fungal hormones of fungal metabolites that induce the production of such hormones in the plant.

BRIAN (1967) divided the biotrophic parasites into two categories, according to their ability to grow axenically. Those species which can be cultured axenically were termed ecologically obligate parasites, and those which cannot were called (physiologically) obligate parasites. This distinction was meaningful, since physiologically obligate parasites, without doubt, are evolutionary more advanced than ecologically obligate parasites. When BRIAN made this specification, physiologically obligate parasites comprised some species of the *Plasmodiophorales*, the *Peronosporaceae* and *Albuginaceae* within the *Peronosporales*, the *Erysiphales* within the *Ascomycetes*, and the *Uredinales* within the *Basidiomycetes*. However, as pointed out by GARRETT (1970), the distinction between these two categories has become blurred since the first report on the axenic culture of a rust fungus (WILLIAMS et al. 1966). Nevertheless, the term obligate parasite is still in use to designate a physiologically obligate parasite. Those rust fungi which can be cultured apart from their hosts are mostly included in this category (cf. SCOTT 1972, STAPLES and YANIV 1976). Moreover, one has to realize that only a few members of the *Uredinales* as yet can be cultured axenically. In no case, the axenic culture of an obligate parasite belonging to the downy and powdery mildew fungi has been reported. On the other hand, the ecologically obligate parasite *Phytophthora infestans* was considered an obligate parasite up to 1908, when CLINTON first succeeded in growing it on agar medium supplemented with lima bean extract.

GARRETT (1970) AND LEWIS (1973, 1974) pointed out that, in order to keep the invaded host tissue alive, biotrophic fungi ought to produce smaller

4

amounts of degradative enzymes than saprophytic and necrotropic fungi. There-
fore, they should mainly depend on relatively simple carbon sources. This
fact could be a reason for their reduced competitive saprophytic ability, but
it still does not explain why the axenic culture of many of these species has
proved to be so intricate, if not impossible. Until a few years ago the view
was held that the physiological dependency of obligate parasites upon their
hosts was due to mutations of the parasites which are lethal in the absence
of their hosts (the so-called metabolic lesion theory, cf. SCOTT 1972).
Therefore, it was believed that it should be impossible to cultivate repre-
sentatives of some fungal groups axenically. The axenic culture of some rust
fungi since then has cast much doubt on this idea. The factors which cause
the host dependency of obligate parasites are, no doubt, of a more subtle
nature. Mutations might have led to reduced activity, though not complete ab-
sence of some specific metabolic pathways in the fungus concerned.

3. Metabolic capacity of obligate parasites

In general, three different approaches have been made to gain infor-
mation on the metabolic capacity of obligate parasites:
1. Identification of substances moving from the host cell to the parasite,
   and their conversion products within the fungal partner.
2. Nutritional and biochemical studies on axenic rust cultures.
3. Studies on germinating spores.
Results from these studies suggest that the metabolic capacity of such fungi
must be larger than was originally believed.

EDWARDS and ALLEN (1966) studied the assimilation of $^{14}CO_2$ in healthy
and mildew-infected barley leaves. Their experimental results indicate that
sucrose is the main compound moving from the host to the parasite, which
then is quickly metabolized into other substances. The majority is converted
into mannitol, smaller amounts into other polyols, and aspartic and glutamic
acid. In similar experiments with rust-infected leaves, carbohydrates sup-
plied by the host were also converted into polyols by the fungus (LIVNE 1964,
PFEIFFER et al. 1969). With wheat stem rust, hexoses from the host tissue
could also be converted within the parasite into chitin and fatty acids
(PFEIFFER et al. 1969). JÄGER and REISENER (1969) have shown that in the
wheat/stem rust complex amino acids rather than proteins are moving from the
host cell to the parasite, and that they are subsequently incorporated into
fungal proteins. Their data further suggest that the parasite itself
synthesizes only negligible amounts of amino acids from glucose. However,

5

in subsequent experiments (REISENER et al. 1970) it turned out that within the fungus a substantial amount of alanine can be synthesized from glucose.

Nutritional studies on axenic rust cultures have revealed that, in general, they require a simple carbohydrate (e.g. glucose, sucrose, mannitol), organic nitrogen and sulphur, and some mineral salts (KUHL et al. 1971, HOWES and SCOTT 1972, COFFEY and ALLEN 1973, JONES 1973, BOSE and SHAW 1974). HOWES and SCOTT (1973) have shown that axenic cultures of *Puccinia graminis f.sp. tritici* cannot reduce sulphate, but can synthesize cysteine and methionine from ^{35}S-sulphide. The same authors (unpublished results, cited by SCOTT 1972) have demonstrated that this fungus can synthesize glutamic acid, lysine and arginine from glucose.

Studies on germinating spores are mostly concerned with nucleic acid and protein metabolism. With conidia of *Peronospora tabacina*, a small but significant incorporation of amino acids into proteins during the first hours of germination was demonstrated (HOLLOMON 1971). After the emergence of germ tubes, the total protein content declined. Both protein synthesis and germination of spores were prevented in the presence of cycloheximide. Dormant spores contained a stable messenger-RNA (HOLLOMON 1969). ^{3}H-uridine and ^{32}P-phosphate were mainly incorporated into soluble RNA and a heterodisperse RNA; in contrast to germinating conidia of *Neurospora crassa*, those of *P. tabacina* did not incorporate these substances into ribosomal RNA (HOLLOMON 1970). Therefore, it was concluded that protein synthesis - necessary for the germination of conidia - does not depend on synthesis of new m-RNA and r-RNA. This view is supported by the observation that inhibitors of RNA-synthesis abolished the incorporation of ^{3}H-uridine into RNA, but did not prevent germination of spores (HOLLOMON 1969). Results of studies on germinating uredospores of *Uromyces phaseoli* were essentially the same. An increase in protein content of 20-30% was demonstrated for the first three hours of germination (TROCHA and DALY 1970); this figure, however, is small when compared with that of germinating spores of saprophytes (cf. STAPLES et al. 1962, VAN ETTEN and BRAMBL 1968). No net synthesis of RNA was established for this period (TROCHA and DALY 1970). Therefore, it seems that in *U. phaseoli*, as in *P. tabacina*, protein synthesis during germination depends on polyribosomes and an m-RNA present in dormant spores (STAPLES et al. 1968, RAMAKRISHNAN and STAPLES 1970). However, in recent experiments, TÜCKHARDT (1976) could demonstrate incorporation of ^{32}P-phosphate into DNA, and low- and high-molecular-weight RNA including r-RNA during the first hours of germination of bean rust uredospores, although the total content of r-RNA declined during this period. Furthermore, it was shown by other authors that

formation of appressoria in vitro is accompanied with synthesis of m-RNA and DNA (RAMAKRISHNAN and STAPLES 1970, STAPLES and YANIV 1973). With STAPLES and YANIV (1976) we can conclude that "spores of the obligate parasites develop a competent machinery for the synthesis of protein required for germination, but induction of the changes required for growth evidently does not occur. In view of the successful culture of many rust fungi, it seems reasonable to suggest that continued growth is simply dependent on an adequate nutrition".

4. Aim and outline of the present study

Aim of this study was to uncover the biochemical basis for the inability of the ecologically obligate parasite *Ph. infestans* to grow on basal nutrient media. Results from this study might contribute to a better understanding of the factors that determine the physiological dependence of some groups of fungal parasites on their hosts.

Obligately parasitic fungi are able to synthesize nucleic acids, proteins and other cellular constituents from low-molecular-weight precursors, indicating that their biosynthetic machinery is essentially intact. This observation has led us to the assumption that, in some way, their intermediary metabolism is anomalous. A basic anomaly should prevent a rapid interconversion of low-molecular-weight substances; low concentrations of essential precursors should limit the synthesis of essential cellular constituents of the fungus concerned. In the parasitic phase, such an anomaly might be overcome in two possible ways: 1) a continuous supply of essential precursors by the host, and 2) complementation of the metabolic machinery by derepression of novel genes (cf. CHAKRAVORTY and SHAW 1977).

Ph. infestans seems a suitable object to provide experimental evidence for this hypothesis. On one hand, it can be regarded as an intermediate type between the facultatively and obligately parasitic members of the *Peronosporales*, (cf. YARWOOD 1956), and on the other good growth is provided on media supplemented with certain plant extracts. Furthermore, a direct comparison with related, less specialized species of the same family is feasible. Apart from *Ph. infestans*, two other members of the *Pythiaceae*, *Ph. erythroseptica* and *Pythium debaryanum*, were included in this study; they represent other levels of physiological specialization and biological adaptations within this family. Both fungi are soil saprophytes, *P. debaryanum* being able to attack many host plants, and *Ph. erythroseptica* having a smaller host range. In contrast to *Ph. infestans*, these two species are

7

necrotrophic when acting as parasites.

In a study of FEHRMANN (1971a, b), some evidence has been presented to substantiate that the energy metabolism of *Ph. infestans* might be disordered. For this reason, the present study of *Peronosporales* was concentrated on their mitochondrial electron transport.

In the first paper, the development of a method for the isolation of mitochondria from mycelium of *P. debaryanum* and *Ph. infestans* is described, and some basic characteristics of these organelles are compared. In the second paper, some anomalous effects of ADP on mitochondrial electron transport of *Ph. infestans, Ph. erythroseptica* and *P. debaryanum* are compared. This study was based on the previous observation that cell-free preparations of *P. ultimum, Ph. infestans* and other *Phytophthora* species differ in the response of NADH-oxidation to adenosine phosphates (FEHRMANN 1971b). In the third paper, an abbreviated pathway for the oxidation of NADH in mitochondria of *Ph. infestans* is described. The anomalies in the mitochondrial electron transport of the fungus, as compared with *Ph. erythroseptica* and *P. debaryanum*, are discussed with respect to its biotrophic character. To find a relationship between the nutritional requirements of *Ph. infestans* and its anomalous electron transport some specific nutritional requirements of the fungus were investigated. Results of these investigations are presented in the fourth paper.

REFERENCES

BOSE, A., and M. SHAW, 1974: Growth of rust fungi of wheat and flax on chemically-defined media. Nature 251, 646-648.
BRIAN, P.W., 1967: Obligate parasitism in fungi. Proc. Roy. Soc. London, Ser. B, 168, 101-118.
CHAKRAVORTY, A.K., and M. SHAW, 1977: A possible molecular basis for obligate host-pathogen interaction. Biol. Rev. 52, 147-179.
CLINTON, G.P., 1908: Report of the botanist: IV. Artificial cultures of *Phytophthora*, with special reference to oospores. Conn. Agric. Exp. Sta., Ann. Rept. 32, 891-907.
COFFEY, M.D., and P.J. ALLEN, 1973: Nutrition of *Melampsori lini* and *Puccinia helianthi*. Trans. Brit. Mycol. Soc. 60, 245-260.
DALY, J.M., 1976: Some aspects of host-pathogen interactions. In: Physiological Plant Pathology, ed. by R. Heitefuss and P.H. Williams, pp 27-50. Springer-Verlag, Berlin, Heidelberg, New York.
DE BARY, A., 1887: Comparative morphology and biology of the fungi, mycetozoa and bacteria. Clarendon Press, Oxford.
EDWARDS, H.H. and P.J. ALLEN, 1966: Distribution of the products of photosynthesis between powdery mildew and barley. Plant Physiol. 41, 683-688.
FEHRMANN, H., 1971a: Obligater Parasitismus phytopathogener Pilze. I. Vergleichende Wachstumsversuche mit *Phytophthora infestans* und verwandten Arten. Phytopath. Z. 70, 89-113.

FEHRMANN, H., 1971b: Obligater Parasitismus phytopathogener Pilze. II. Ver-
    gleichende biochemische Untersuchungen zum Energiestoffwechsel von
    Phytophthora-Arten. Phytopath. Z. 70, 230-262.
GARRETT, S.D., 1970: Pathogenic Root Infecting Fungi. Cambridge. University
    Press.
HEITEFUSS, R., and P.H. WILLIAMS, 1976: Preface of: Physiological Plant
    Pathology. Encyclopedia of Plant Physiology new series, Vol. 4. Springer-
    Verlag, Berlin, Heidelberg, New York.
HOLLOMON, D.W., 1969: Biochemistry of germination in *Peronospora tabacina*
    (ADAM) conidia: evidence for the existence of stable messenger RNA. J.
    Gen. Microbiol. 55, 267-274.
HOLLOMON, D.W., 1970: Ribonucleic acid synthesis during fungal spore germi-
    nation. J. Gen. Microbiol. 62, 75-87.
HOLLOMON, D.W., 1971: Protein synthesis during germination of *Peronospora
    tabacina* conidia. Arch. Biochem. Biophys. 145, 643-649.
HOWES, N.K., and K.J. SCOTT, 1972: Sulphur nutrition of *Puccinia graminis
    f.sp. tritici* in axenic culture. Can. J. Bot. 50, 1165-1170.
HOWES, N.K., and K.J. SCOTT, 1973: Sulphur metabolism of *Puccinia graminis
    f.sp. tritici* in axenic culture. J. Gen. Microbiol. 76, 345-354.
JÄGER, K., and H.J. REISENER, 1969: Untersuchungen über Stoffwechsel-
    beziehungen zwischen Parasit und Wirt am Beispiel von *Puccinia graminis
    var. tritici* auf Weizen. I. Aufnahme von Aminosäuren aus dem Wirtsgewebe.
    Planta 85: 57-72.
JONES, D.R., 1973: Growth and nutritional studies with axenic cultures of
    the carnation rust fungus, *Uromyces dianthi* (Pers.) Niessl. Physiol.
    Plant Pathol. 3, 379-386.
KUHL, J.L., D.J. MACLEAN, K.J. SCOTT, and P.G. WILLIAMS, 1971: The axenic
    culture of *Puccinia* species from uredospores: experiments on nutrition
    and variation. Can. J. Bot. 49, 201-209.
LEWIS, D.H., 1973: Concepts in fungal nutrition and the origin of biotrophy.
    Biol. Rev. 48, 261-278.
LEWIS, D.H., 1974: Micro-organisms and plants: the evolution of parasitism
    and mutualism. Soc. Gen. Microbiol. 24, 367-392.
LINK, G.K.K., 1933: Etiological phytopathology. Phytopathology 23, 843-862.
LIVNE, A., 1964: Photosynthesis in healthy and rust-affected plants. Plant
    Physiol. 39, 614-621.
PFEIFFER, E., K. JÄGER, and H.J. REISENER, 1969: Untersuchungen über Stoff-
    wechselbeziehungen zwischen Parasit und Wirt am Beispiel von *Puccinia
    graminis var. tritici* auf Weizen. II. Aufnahme von Hexosen aus dem Wirts-
    gewebe. Planta 85, 194-201.
PRINGLE, R.B., and R.P. SCHEFFER, 1964: Host-specific plant toxins. Ann. Rev.
    Phytopathol. 2, 133-156.
RAMAKRISHNAN, L., and R.C. STAPLES, 1970: Changes in ribonucleic acids during
    uredospore differentiation. Phytopathology 60, 1087-1091.
REISENER, H.J., E. ZIEGLER, and A. PRINZING, 1970: Zum Stoffwechsel des
    Myzels von *Puccinia graminis var. tritici* auf der Weizenpflanze. Planta
    92, 355-357.
SCHEFFER, R.P., 1976: Host-specific toxins in relation to pathogenesis and
    disease resistance. In: Physiological Plant Pathology, ed. by
    R. Heitefuss and P.H. Williams, pp. 247-269. Springer-Verlag, Berlin,
    Heidelberg, New York.
SCOTT, K.J., 1972: Obligate parasitism by phytopathogenic fungi. Biol. Rev.
    47, 537-572.
STAPLES, R.C., D. BEDIGIAN, and P.H. WILLIAMS, 1968: Evidence for polysomes
    in extracts of bean rust uredospores. Phytopathology 58, 151-154.
STAPLES, R.C., R. SYAMANADA, R. KAO, and R.J. BLOCK, 1962: Comparative bio-
    chemistry of obligately parasitic and saprophytic fungi. II. Assimilation
    of C^{14}-labeled substrates by germinating spores. Contrib. Boyce Thompson
    Inst. 21, 345-362.

STAPLES, R.C., and Z. YANIV, 1973: Synthesis of DNA in differentiating bean rust uredospores. Phytopathology 63, 207 (abstr.).

STAPLES, R.C., and Z. YANIV, 1976: Protein and nucleic acid metabolism during germination. In: Physiological Plant Pathology, ed. by R. Heitefuss and P.H. Williams, pp. 86-103. Springer-Verlag, Berlin, Heidelberg, New York.

THROWER, L.B., 1966: Terminology of plant parasites. Phytopath. Z. 56, 258-259.

TROCHA, P., and J.M. DALY, 1970: Protein and nucleic acid synthesis during germination of uredospores. Plant Physiol. 46, 520-526.

TÜCKHARDT, K.E., 1976: Nukleinsäurestoffwechsel ruhender und keimender Uredosporen des Bohnenrostes *Uromyces phaseoli*. Ph. D. Thesis, University of Göttingen, Germany.

VAN ETTEN, J.L., and R.M. BRAMBL, 1968: Protein synthesis during fungal spore germination. I. Characteristics of an in vitro phenylalanine incorporating system prepared from germinated spores of *Botryodiplodia theobromae*. Arch. Biochem. Biophys. 125, 13-21.

VAN TUYL, J.M., 1977: Genetics of fungal resistance to systemic fungicides. Ph. D. Thesis, University of Wageningen, The Netherlands.

WILLIAMS, P.G., K.J. SCOTT, and J.L. KUHL, 1966: Vegetative growth of *Puccinia graminis f.sp. tritici* in vitro. Phytopathology 56, 1418-1419.

YARWOOD, C.E., 1956: Obligate parasitism. Ann. Rev. Plant Physiol. 7, 115-142.

Institute of Plant Pathology and Plant Protection,
University of Göttingen

Mitochondrial electron transport in *Peronosporales*
I.   Isolation and properties of *Pythium debaryanum*
and *Phytophthora infestans* mitochondria

by
P.C. Scheepens and H. Fehrmann

With 6 Figures

INTRODUCTION

The aim of this study was to look for the biochemical basis for the
inability of the ecologically obligate parasite *Phytophthora infestans* to
grow on basal nutrient media. This might lead to a better understanding of
the factors which cause the dependency of obligate parasites upon their
hosts.

On the basis of morphological criteria, the *Peronosporales* within the
Oomycetes are divided into three families: the *Pythiaceae*, the
Peronosporaceae and the *Albuginaceae* (ALEXOPOULOS 1962). On the basis of
ecological, pathological and nutritional characteristics, different levels
of specialization can be recognized within the *Peronosporales*. Without
exception, all species of the *Peronosporaceae* and the *Albuginaceae* are
physiologically obligate parasites; until now all attempts at axenic
cultivation were unsuccessful. On the other hand, the *Pythiaceae* include
saprophytic as well as facultatively parasitic species with varying degrees
of competitive saprophytic ability, but no physiologically obligate para-
sites. They range from mainly saprophytic species, which are able to
parasitize plant tissue in a necrotrophic way, to mainly parasitic
species, the parasitism of which may be necrotrophic or biotrophic. *Ph.*
infestans represents the highest level of evolutionary development within
the *Pythiaceae*. According to BRIAN (1967) it is an ecologically obligate
parasite; in the terminology of LEWIS (1973) it is an obligately biotrophic
fungus. For growth and reproduction it depends on living host tissue. *In*
vitro, good growth is only possible on complex media (FEHRMANN 1971a, HOHL
1975), whereas most species of the same genus can be readily cultivated on
basal nutrient media. Apart from this nutritional peculiarity, specialization

is also documented by the comparatively small host range of *Ph. infestans*. It is confined to the genus *Solanum* and some related species within the *Solanaceae*. With *Pythium* and most other *Phytophthora* species the host range nearly always is quite large or at least larger than with *Ph. infestans*.

There seems to be only one principal difference between the parasitism of *Ph. infestans* and that involving physiologically obligate parasites of the *Peronosporales*: Translocatory sinks which are established in plants infected with obligate parasites belonging to the *Peronosporaceae* and the *Albuginaceae* (PERL et al. 1972, LEWIS 1974, THORNTON and COOKE 1974), do not occur in host tissue infected with *Ph. infestans*. FARRELL (1971) has shown that the accumulation of assimilatory products in infected potato leaves was mainly due to an increased rate of photosynthesis at and around the infection site and a retention of assimilates at this site rather than an influx of products from uninfected adjacent tissue.

Studies on the metabolic capacity of obligate parasites quite often have shown that their biosynthetic apparatus seems essentially intact. At least in some groups, a continuous supply of low-molecular-weight substances from the host seems the only prerequisite for good growth and sporulation of the parasite (cf. SCOTT 1972). This led us to the assumption that, in some way, their intermediary metabolism is anomalous. A basic anomaly should prevent a rapid interconversion of low-molecular-weight substances, thereby limiting the metabolic capacity of the fungus as a whole. To provide experimental evidence for this hypothesis, *Ph. infestans* seems to be a convenient subject, since on one hand it shares many characteristics with the obligately parasitic members of the *Peronosporales*, and on the other good growth is provided on media supplemented with certain plant extracts. Furthermore, a direct comparison with related, less specialized species, is possible. Apart from *Ph. infestans* we have included two other representatives of the *Pythiaceae, Ph. erythroseptica* and *Pythium debaryanum* which represent other levels of physiological specialization and biological adaptation within this family. Both fungi are soil saprophytes, *P. debaryanum* being able to attack many plants, and *Ph. erythroseptica* having a smaller host range. In contrast to *Ph. infestans* which is a biotrophic parasite, *Ph. erythroseptica* and *P. debaryanum* are necrotrophic when acting as parasites.

In a previous study, some first evidence has been presented to substantiate that the energy metabolism of *Ph. infestans* might be disordered (FEHRMANN 1971a, b). In order to get more detailed information on this subject, first of all it seemed necessary to develop a method for the isolation of intact mitochondria. In this paper, results of such efforts are

described for the isolation of mitochondria of *P. debaryanum* and *Ph. infestans*; moreover, some basic characteristics of these organelles are compared. In two subsequent publications, some more specific characteristics of *Ph. infestans, Ph. erythroseptica* and *P. debaryanum* mitochondria will be compared to emphasize the exceptional position of *Ph. infestans* within the *Pythiaceae*. In a fourth paper, the anomalous energy metabolism of *Ph. infestans* will be related to its nutritional requirements *in vitro*.

MATERIALS AND METHODS

Organisms and culture conditions:

P. debaryanum was kindly provided by Dr. M.A. de Waard, Laboratory of Phytopathology, Wageningen, The Netherlands. *Ph. infestans* was isolated from potato tubers. For two reasons this strain (02) was chosen for the bio-chemical experiments: i) its uniform growth on pea medium for many genera-tions, and ii) its inability to grow on basal media with only one or two amino acids present. For the last reason, effects we were looking for might be more pronounced in this strain than in strains with less distinct nutritional requirements. *P. debaryanum* was maintained on pea agar, *Ph. infestans* was cultivated on potato callus tissue to ensure that its vitality would remain unchanged. The callus tissue was generously supplied by Dr. G. Wolf from this laboratory. Plates of pea juice agar, as described by FEHRMANN (1971a), were inoculated at intervals of two weeks to provide the inoculum for the liquid cultures.

For mass cultures, Erlenmeyer flasks (300 ml) with 80 ml of nutrient so-lution each were inoculated and subsequently incubated at 20-22°C on a rotary shaker at 100 rev./min. One litre of nutrient solution contained 10 g glucose, 1 g KH_2PO_4, 500 mg $MgSO_4.7H_2O$, 50 mg $CaCl_2.2H_2O$, 5 mg $FeCl_3.6H_2O$, 0.1 mg thiamine, and pea juice, prepared by boiling 75 g of deep-frozen peas in 1 l of water for 15 min. and subsequent decanting. A fresh nutrient solution was prepared every week and stored in the dark. These conditions proved to be necessary for good growth of *Ph. infestans*, not for *P. debaryanum* (cf. HOLLOMON 1966). Mycelia of *P. debaryanum* and *Ph. infestans* were harvested after 3 and 7 days, respectively, when still in the logarithmic phase.

Attempts to isolate protoplast-like structures:

According to SIETSMA and DE BOER (1973), about 10 g of wet mycelium of

P. debaryanum was incubated for 3-4 hours at 22^OC in 30 ml of a medium containing 0.35 M KCl or 0.65 M sucrose, 0.02 M MOPS-KOH buffer, 0.03 M reduced glutathione, 0.2-1% (w/v) Helicase from *Helix pomatia*, 0.2-1% (w/v) cellulase from an *Oxyporus* spec., with a final pH of 6.5. In some experiments, also 1% (w/v) protease from *Streptomyces griseus* was added to the medium.

Isolation of mitochondria:

First, several methods used by other authors were compared, as described under "Results". Finally, the following method was adapted for our purposes: 20-30 g of mycelium (wet weight) was washed with ice-cold water on a Büchner funnel and homogenized for 15 sec. in a Waring blendor in a 10-fold volume of isolation medium. The isolation medium contained 0.45 M sucrose, 1 mM EDTA, 0.2% bovine serum albumin (BSA), and 0.02 M MOPS-KOH buffer, with a final pH of 7.4. The hyphae were disrupted with a grind-mill, an apparatus built in our laboratory after the original description of WEISS et al. (1970). The brei was squeezed through four layers of Monyl 30 gauze (30 μm mesh, Alphons Markert, Hannover, West Germany). The mitochondria were collected by fractionated centrifugation between 600 x g for 5 min. and 6,000 x g for 15 min. The mitochondrial pellet was carefully washed with about 15 ml of isolation medium to remove some fluffy material. The mitochondria were then re-suspended in isolation medium to give a final concentration of 40-60 mg of protein per ml.

The isolation procedure lasted for at the utmost 45 min. Subsequent experiments were completed within 2 hours. All steps of the isolation procedure were carried out in the cold $(0-4^O$C), and the mitochondrial suspensions were kept in an ice bath.

Analytical procedures:

Oxygen uptake was measured polarographically at 25^OC, using a Clark type electrode (Y.S.I., Ohio, USA) in a medium containing 0.45 M sucrose, 2.5 mM $MgSO_4 \cdot 7H_2O$, 10 mM KCl, 0.5 % BSA, 10 mM KH_2PO_4, and 15 mM MOPS-KOH buffer, with a final pH of 7.4. Protein was determined with the micro-biuret method according to ITZHAHI and GILL (1964), using BSA as standard.

Chemicals:

Cellulase (Merck, Darmstadt), Helicase (l'Industrie Biologique Francaise)

14

and protease (Sigma) were crude preparations containing several enzymes. All other chemicals were of analytical grade. ADP, NADH, α-oxoglutarate, pyruvate, and succinate were purchased from Boehringer, Mannheim; rotenone from EGA Chemie; antimycin A from Serva; morpholino propane sulphonic acid (MOPS) from Sigma; carbonyl cyanide, M-chlorophenylhydrazone (CCCP) from Calbiochem. All other chemicals were purchased from Merck, Darmstadt.

RESULTS

Degradation of the hyphal walls:

A successful isolation of mitochondria from microorganisms largely depends on the method used for the disruption of the cell walls. Preliminary experiments to dissolve the hyphal walls enzymatically were unsuccessful. The cell walls of the *Oomycetes* consist of an outer layer of amorphous glucan containing β-1,3 and β-1,6 linkages, and an inner layer of cellulose microfibrils and protein (NOVAES-LEDIEU et al. 1967, BARTNICKI-GARCIA 1969, HUNSLEY and BURNETT 1970, SIETSMA et al. 1975). Protoplast-like structures could be obtained from different *Pythium* and *Phytophthora* species, using extra-cellular enzyme preparations of *Streptomyces* species (BARTNICKI-GARCIA and LIPPMAN 1966, SIETSMA et al. 1967, JIMENEZ-MARTINEZ and NOVAES-LEDIEU 1969). A more promising approach was made by SIETSMA and DE BOER (1973), who were able to obtain "protoplasts" from *Pythium* PRL 2142 in a medium containing commercially available Helicase and cellulase. However, for large scale isolation of *P. debaryanum* "protoplasts" this method proved to be unsuitable. When a 4-h incubation of the hyphae in a medium containing Helicase and cellulase was followed by further mechanical disruption, mitochondrial preparations showed almost no respiratory activity.

Subsequently, different mechanical devices were tested including a motor-driven all-glass-homogenizer as used by WATSON and SMITH (1967), a roller mill as described by KAWAKITA (1970), a French press, and a grind-mill as desgined by WEISS et al. (1970). Of these instruments only the grind-mill proved to work satisfactory, as it provided reasonable yields of mitochondria which were only minimally damaged. Its only disadvantage was, that mitochondrial yield and quality varied much because of a fast abraison of the grinding wheels.

Fractionation of the mitochondrial suspensions:

In comparison to *Neurospora crassa* and *Saccharomyces carlsbergensis*

15

mitochondria (WEISS et al. 1970 CARTLEDGE and LLOYD 1972), isolated mitochondria of *P. debaryanum* and *Ph. infestans* were much larger (mean diameter about 1 μm) and less uniform in size and electron density. Therefore, their separation from hyphal fragments and other large cell constituents proved to be difficult. Commonly, these contaminations are removed by centrifugation at low gravities. In our case, however, even at a force as low as 1,000 x *g* a considerable fraction of the mitochondria was removed from the supernatant, whereas the supernatant still did contain some hyphal fragments. An improvement was made by squeezing the brei through a synthetic gauze beforehand, which was then followed by centrifugation at 600 x *g* for 5 min. The mitochondria were collected from the supernatant by subsequent centrifugation at 6,000 x *g* for 15 min. In several experiments this force proved to be sufficient since only a very small mitochondrial activity (measured as succinate respiration) remained in the supernatant fluid. The mitochondrial pellet was washed by dripping some isolation medium on it to remove some fluffy material which presumably contained severely damaged mitochondria. The mitochondrial fraction was suspended in a small volume of isolation medium by gentle homogenization with a glass rod.

Composition of the incubation medium:

Most constituents of the incubation medium were as used by other authors (cf. LLOYD 1974) with only minor modifications. The influence of pH on the respiratory activity of *P. debaryanum* mitochondria with succinate as substrate is shown in Fig. 1. Maximal activity was attained at pH 7.4, which then was always used for the incubation medium as well as for the isolation medium. The influence of BSA in the incubation medium on the "respiratory control ratios" of *P. debaryanum* mitochondria, again with succinate as substrate, is demonstrated in Fig. 2. It turned out that somewhat higher concentrations of BSA than described by other authors were necessary to obtain optimal respiratory control ratios. In further experiments, 0.5% BSA was added to the incubation medium.

Response of the mitochondria to ADP and the uncoupler CCCP:

Mitochondrial preparations of *P. debaryanum* and *Ph. infestans* showed an increase in respiration after addition of ADP; however, maximal rates were dependent on the concentration of the chemical, and were reached only after 2 min. Furthermore, when using low concentrations of ADP (0.1-0.3 mM),
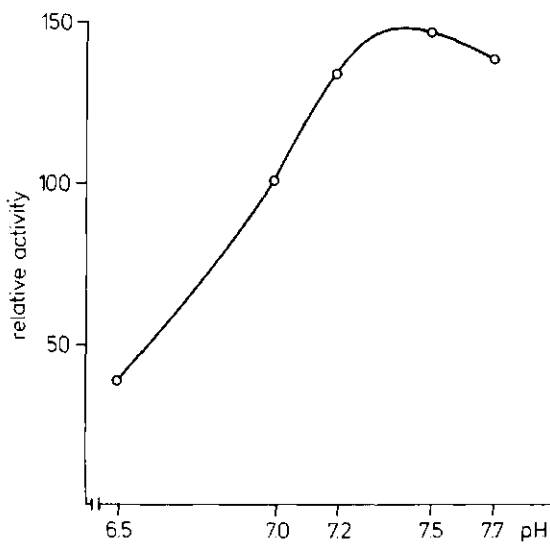
Fig. 1. Relative activity of mitochondria of *Pythium debaryanum* at different pH-values. Succinate, at a final concentration of 5 mM, was used as substrate. Activity at pH 7.0 was set at 100.
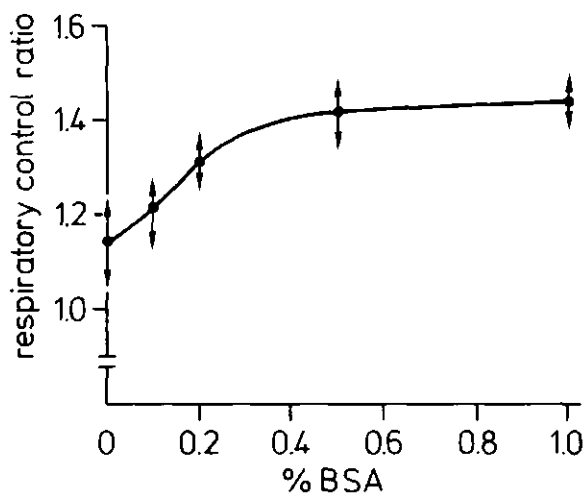
Fig. 2. Respiratory control ratios of *Pythium debaryanum* mitochondria at varying concentrations of bovine serum albumin (BSA). Succinate, at a final concentration of 5 mM, was used as substrate. Respiratory control ratios were determined as described in the text. The arrows indicate the standard error of the mean, calculated from four experiments.

17

respiration rates did not decrease when the ADP should have been exhausted, or upon addition of oligomycin, an inhibitor of oxidative phosphorylation. From these observations we must conclude that our mitochondrial preparations did not show true respiratory control as defined by CHANCE and WILLIAMS (1956). As is shown in Fig. 3 maximal respiration rates of *P. debaryanum* mitochondria with succinate as substrate were attained in the presence of 2 mM ADP. Respiration could also be stimulated by CCCP, an uncoupler of
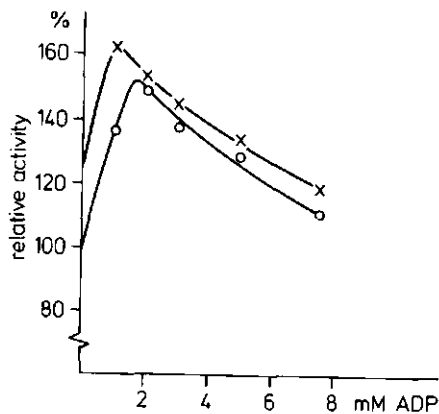
Fig. 3. Influence of the uncoupler CCCP on the relative activity of *Pythium debaryanum* mitochondria at different concentrations of ADP. Succinate, at a final concentration of 5 mM, was used as substrate. Activities were measured in the absence (o-o), or presence of 20 µM CCCP (x-x). The control without ADP and CCCP was set at 100.

oxidative phosphorylation, at 20 µM. In the presence of CCCP maximal respiration rates were also reached when 2 mM ADP was simultaneously added. A response of the mitochondria to ADP and CCCP was only observed when the mitochondria were carefully prepared in the presence of BSA (cf. Fig. 2). From the above observations we must conclude that oxidative phosphorylation was partially uncoupled from respiration; In other words, the mitochondria were not functionally intact. Electron micrographs of mitochondrial suspensions revealed that their structural integrity was largely preserved, although some damage to the outer membranes had occurred. This was true for

both *P. debaryanum* and *Ph. infestans* mitochondria.

Mitochondrial activity and sensitivity to respiration inhibitors:

The electron transport system and the sites of action of the respiratory inhibitors used in this study are schematically represented in Fig. 4. This Figure also shows a specific, TMPD-mediated by-pass of the antimycin A-sensitive site (see below).
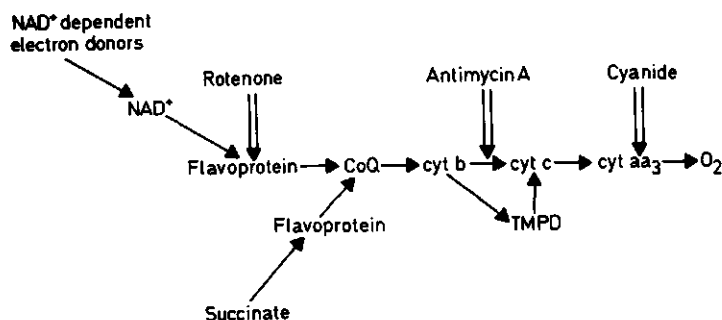
Fig. 4. Schematic representation of the electron transport chain illustrating the sites of action of the inhibitors used in this study and the TMPD-mediated by-pass of the antimycin A-sensitive site.

The response of *P. debaryanum* mitochondria to varying concentrations of the respiratory inhibitors antimycin A and cyanide is shown in Figures 5 and 6, respectively. The sensitivity of the succinate respiration to antimycin A was comparable to that described for other organisms (SLATER 1967). Cyanide could almost completely block the succinate as well as the NADH respiration, although the concentrations which were necessary for maximal inhibition were remarkably high. At 1 mM of the chemical, activities were still 15-20% of the control.

Respiratory rates with different substrates, sensitivity of respiration to the inhibitors rotenone, antimycin A and cyanide, and "respiratory control ratios" are presented in the Tables 1 and 2. Respiratory control is here defined as the ratio of the respiratory rate in the presence of substrate and ADP to that in the absence of the phosphate acceptor.

Respiration rates of *P. debaryanum* mitochondria were lower than described for mitochondria of other fungi (cf. LLOYD 1974). Respiration with pyruvate
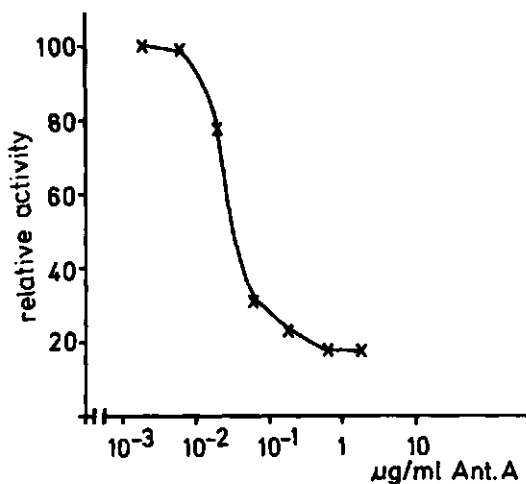
Fig. 5.

Influence of antimycin A on the oxygen uptake of mito-chondria of *Pythium debaryanum*. Cuvettes contained 1.2 mg of mitochondrial protein per ml. Succinate, at a final concen-tration of 5 mM, was employed as substrate.
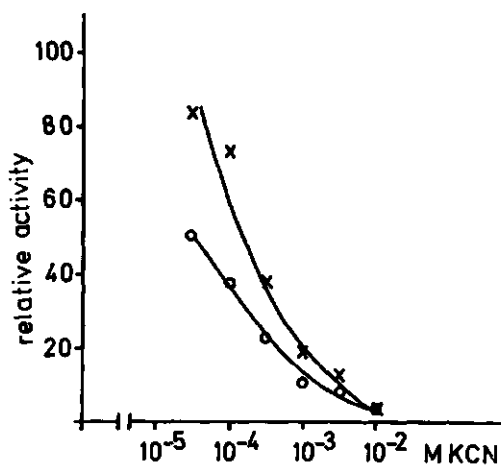
Fig. 6.

Influence of cyanide on the oxygen uptake of mitochondria of *Pythium debaryanum*. 0.74 mM NADH (o-o), or 5 mM succinate (x-x) were used as substrates.

plus malate and with α-oxoglutarate, respectively, as substrates was strongly inhibited by antimycin A and cyanide, but only about 60% by rotenone. Respiration with succinate as substrate was almost insensitive to rotenone, but sensitive to antimycin A and cyanide. Respiration with NADH as substrate was almost completely inhibited by antimycin A and cyanide, but only partially by rotenone. As compared with *P. debaryanum*, respiration rates of *Ph. infestans* mitochondria - especially with succinate as substrate - were much lower. Oxygen uptake with pyruvate plus malate and α-oxoglutarate, respectively, as substrates was also sensitive to rotenone, antimycin A and cyanide, that with succinate was sensitive to antimycin A and cyanide, but insensitive to rotenone. Respiration of NADH was sensitive to cyanide, but

20

Table 1. Activity of *Pythium debaryanum* mitochondria and sensitivity of
respiration to various inhibitors; respiratory control ratios.
The mitochondrial suspension was added to the incubation medium to give a
final mitochondrial protein concentration of 1.5-2.5 mg/ml. After 3 min. of
incubation the reaction was started by the addition of pyruvate (5 mM) plus
malate (4 mM), α-oxoglutarate (5 mM), succinate (5 mM) or NADH (1 mM), and
the oxygen uptake recorded. Subsequently, ADP (1 mM) was added. Rotenone
(25 µM), antimycin A (2.5 µM) or cyanide (10 mM) were then added to the
cuvettes (final concentrations in parentheses). 1 µg electron corresponds
with $\frac{1}{4}$ µmole of O_2.

Substrate	Oxygen uptake, µg electrons/min. g of protein				Respiratory control ratio
	Control	+ Rotenone	+ Antimycin A	+ Cyanide	
Pyruvate + Malate	32	14	< 3	< 3	1.6
α-Oxogluta- rate	28	7	< 3	< 3	3.2
Succinate	82	68	< 3	< 3	1.4
NADH	138	64	18	< 3	1.1

Table 2. Activity of *Phytophthora infestans* mitochondria, and sensitivity of
respirations to various inhibitors; respiratory control ratios.
The mitochondrial suspension was added to the incubation medium to give a
final mitochondrial protein concentration of 1.5-3.5 mg/ml. Further
additions were as described under Table 1.

Substrate	Oxygen uptake, µg electrons/min. g of protein				Respiratory control ratio
	Control	+ Rotenone	+ Antimycin A	+ Cyanide	
Pyruvate + Malate	23	8	6	< 3	1.7
α-Oxogluta- rate	18	4	< 3	< 3	2.6
Succinate	28	26	4	< 3	1.3
NADH	76	70	66	< 3	1.0

- in contrast to *P. debaryanum* - almost insensitive to both rotenone and

antimycin A. This rotenone and antimycin A-insensitive respiration of *Ph.*

infestans mitochondria will be discussed more comprehensively in another

paper (SCHEEPENS and FEHRMANN 1977).

As demonstrated in Tables 1 and 2, the oxygen uptake of *Ph. infestans*

mitochondria was smaller than that of *P. debaryanum* mitochondria, irre-

spective of the substrate employed. This fact suggests that there is a narrow-pass for the electron flux in the respiratory chain of *Ph. infestans* as compared with *P. debaryanum*. Results from another experiment led to the conclusion that this narrow-pass might be located at the cytochrome(s) b level. Through addition of the artificial electron mediator, N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to the reaction mixture the second site of phosphorylation - at the cytochrome b level - can be selectively by-passed (LEE et al. 1967). Electrons are then taken up from cytochrome(s) b by TMPD and from here directly transferred to cytochrome c, in this way circumventing the antimycin A-sensitive site (cf. Fig. 4). As is shown in Table 3, TMPD greatly enhanced the respiration of succinate in the presence

Table 3. Respiration of succinate of *Pythium debaryanum* and *Phytophthora infestans* mitochondria in the presence or absence of antimycin A and TMPD. Mitochondrial suspensions were added to the incubation medium containing 1 mM ADP to give a final mitochondrial protein concentration of 1.5-2.0 mg/ml. After 3 min. of incubation, succinate (5 mM), antimycin A (2.5 μM), and TMPD (0.3 mM) were successively added to the cuvettes; after addition of each compound oxygen uptake was recorded for some minutes (final concentrations in parentheses).

Successively added compound	Oxygen uptake, μg electrons/min. mg of protein	
	P. debaryanum	*Ph. infestans*
Succinate	82	28
+ Antimycin A	10	4
+ TMPD	107	67

of antimycin A with both *P. debaryanum* and *Ph. infestans* mitochondria. As compared with the rates in the absence of antimycin A and TMPD, however, respiration of succinate increased by only 30% in the case of *P. debaryanum*, but by 140% with *Ph. infestans* mitochondria. Thus, the narrow-pass in the electron transport chain of *Ph. infestans* seems to be located in the region of cytochrome(s) b.

The relatively high respiration rate of *Ph. infestans* mitochondria with NADH as substrate is possibly due to the presence of a special shunt which enables the electrons from NADH to be transferred directly to cytochrome c, thereby circumventing the narrow-pass in the main electron transport chain (SCHEEPENS and FEHRMANN 1977).

DISCUSSION

   Mitochondrial preparations of *Ph. infestans* and *P. debaryanum* did not
show true respiratory control as defined by CHANCE and WILLIAMS (1956):
Respiration did not return to the state 4 when added ADP should have been
exhausted. This might be due to the relatively high ATP-ase activity of the
mitochondrial preparations (SCHEEPENS, unpublished results). Moreover, the
uncoupler CCCP only slightly stimulated respiration in the absence of ADP.
The fact that a substantial part of added NADH was oxidized by mitochondria
of *P. debaryanum* via the rotenone-sensitive respiration system indicates
that the inner membrane system had become leaky upon isolation. However,
both electron micrographs of mitochondrial suspensions, and the fact that
mitochondrial respiration was stimulated upon addition of ADP suggest that
the structural integrity of the mitochondria was mainly preserved, and that
the quality of the mitochondrial preparations of *P. debaryanum* and *Ph.
infestans* was throughout comparable.
   The respiration rate of succinate in *Ph. infestans* mitochondria was much
slower than in those of *P. debaryanum*. Upon addition of the electron mediator
TMPD, succinate respiration of *Ph. infestans* mitochondria was greatly
enhanced, whereas with *P. debaryanum* only a slight stimulation was observed.
These results indicate that, as compared with *P. debaryanum*, a narrow-pass
is present in the mitochondrial electron transport chain of *Ph. infestans*
which limits the electron flux, probably in the cytochrome c-directed,
antimycin A-sensitive region of cytochrome(s) b, which can be selectively
by-passed with TMPD. Respiration rates with pyruvate plus malate, and α-
oxoglutarate, respectively, were about the same as the respiration rate of
succinate with *Ph. infestans* mitochondria, indicating that they were limited
by the same narrow-pass. On the other hand, with *P. debaryanum* mitochondria
respiration rates of pyruvate plus malate and α-oxoglutarate were much lower
than that of succinate.
   Respiration of added NADH by *P. debaryanum* mitochondria was almost
completely inhibited upon addition of antimycin A, which was not the case
with *Ph. infestans*. As is indicated by the results presented in Table 2, and
is discussed more extensively in another paper (SCHEEPENS and FEHRMANN 1977),
in *Ph. infestans* mitochondria added NADH was mainly oxidized via a rotenone-
and antimycin A-insensitive shunt, thereby circumventing the postulated
narrow-pass in the main electron transport system. Energetically, the assumed
shunt is quite disadvantageous: Upon oxidation of NADH, ADP could be oxida-
tively phosphorylated at one site only.

Cyanide completely inhibited respiration of *all* substrates used in *P. debaryanum* as well as in *Ph. infestans* mitochondria. Apparently, a cyanide-insensitive, respiratory pathway as described for some higher plants, algae and fungi (SHERALD and SISLER 1970, KAWAKITA 1971, LAMBOWITZ et al, 1972, BAHR and BONNER 1973, GLEASON 1974, GRANT and HOMMERSAND 1974) is not present in these species.

From the results presented in this paper one can conclude that an electron transport system similar to that of other organisms is also present in *P. debaryanum* and *Ph. infestans*. This view is supported by the work of GLEASON and UNESTAM (1968), who measured the cytochrome content in representatives of the *Oomycetes* including *P. debaryanum*. In contrast to other organisms, however, only one type of cytochrome c could be detected. Cytochromes a and b were absent in dormant oospores of *Ph. capsici*, whereas mycelium of this fungus contained the usual set of cytochromes (RIBEIRO et al. 1975). Further-more, the results seemed to confirm a previous postulation that the energy metabolism of *Ph. infestans* is disordered at the level of cytochrome c or a preceding step of the electron transport system (FEHRMANN 1971a, b). A comparison of some specific characteristics of mitochondria and the possible concequences of the limited electron flux for the synthetic capacity of *Ph. infestans* will be presented in some following papers.

SUMMARY

A method was developed to isolate intact mitochondria of *Pythium debaryanum* and *Phytophthora infestans*, and some general characteristics of these organelles of both fungi were compared. The structural integrity of the mitochondria was essentially preserved, although oxidative phosphorylation was partially uncoupled.

Results from studies on the respiration of different substrates, and the sensitivity of respiration to various inhibitors indicated that in *P. debaryanum* as well as in *Ph. infestans* an electron transport system similar to that described for other organisms was present. Cyanide-resistant respiration, as described for some higher plants, algae and fungi, was absent in the fungi tested.

In *Ph. infestans*, respiration rates with the substrates pyruvate plus malate, α-oxoglutarate, and especially that with succinate, were much lower than in *P. debaryanum*. Evidence is presented that a narrow-pass, located in the cytochrome(s) b region limits the electron flux in the electron transport chain of *Ph. infestans*. Addition of the artificial electron mediator TMPD to

the reaction mixture enhanced respiration by about 140% in the case of *Ph. infestans*, but only by 30% with *P. debaryanum*.

The experimental results indicate that an alternative pathway for the oxidation of externally added NADH exists in mitochondria of *Ph. infestans*, which circumvents the postulated narrow-pass. Whereas NADH-oxidation was blocked by cyanide in both species, this process was strongly inhibited by antimycin A only in *P. debaryanum* mitochondria, but not in those of *Ph. infestans*. This fact suggests that NADH reduced cytochrome c directly in the latter fungus.

ZUSAMMENFASSUNG

Mitochondrialer Elektronentransport in *Peronosporales*
I. Isolierung und Charakterisierung der Mitochondrien von
Pythium debaryanum und *Phytophthora infestans*

Es wurde eine Methode zur Isolierung intakter Mitochondrien aus *Pythium debaryanum* und *Phytophthora infestans* entwickelt, und einige allgemeine Charakteristika dieser Organellen beider Pilze wurden miteinander verglichen. Die strukturelle Zusammensetzung der Mitochondrien war im wesentlichen erhalten; allerdings war die oxydative Phosphorylierung meist teilentkoppelt.

Untersuchungsergebnisse zur Atmungsaktivität mit verschiedenen Substraten und spezifischen Hemmstoffen deuteten daraufhin, dass sowohl in *P. debaryanum* als auch in *Ph. infestans* eine Atmungskette vorhanden war wie sie auch für andere Organismen beschrieben wurde. Ein cyanid-unempfindlicher Nebenweg der Atmung fehlt bei *Ph. infestans* und *P. debaryanum*.

In Mitochondrien von *Ph. infestans* war die Atmungsaktivität bei Verwendung von Pyruvat plus Malat, α-Ketoglutarat, vor allem aber von Succinat als Substrat bedeutend niedriger als in solchen von *P. debaryanum*. Es ergaben sich Hinweise dafür, dass in der Atmungskette von *Ph. infestans* ein Engpass für den Elektronenfluss im Bereich der Cytochrome b existiert. Zusatz von TMPD zum Reaktionsgemisch beschleunigte die Atmung bei *Ph. infestans* um 140%, dagegen bei *P. debaryanum* nur um 30%.

Die Resultate deuten ferner daraufhin, dass zugefügtes NADH bei *Ph. infestans* über einen alternativen Weg oxydiert werden kann, der den postulierten Engpass im Cytochrom b-Bereich umgeht. Während die NADH-Oxydation bei beiden Arten durch Cyanid blockiert wurde, wurde dieser Prozess nur bei *P. debaryanum* stark durch Antimycin A gehemmt, nicht dagegen bei *Ph. infestans*. Diese Tatsache weist auf eine direkte Reduktion von Cytochrom c durch

NADH bei *Ph. infestans*.

REFERENCES

ALEXOPOULOS, C.J., 1962: Introductory Mycology, 2nd edition. Wiley, New York
and London.

BAHR, J.T., and W.D. BONNER, 1973: Cyanide-insensitive respiration. I. Steady
states of skunk cabbage spadix and bean hypocotyl mitochondria. J. Biol.
Chem. 248, 3441-3445.

BARTNICKI-GARCIA, S., 1969: Cell wall differentiation in the *Phycomycetes*.
Phytopathology 59, 1065-1071.

BARTNICKI-GARCIA, S., and E. LIPPMAN, 1966: Liberation of protoplasts from
the mycelium of *Phytophthora*. J. Gen. Microbiol. 42, 411-416.

BRIAN, P.W., 1967: Obligate parasitism in fungi. Proc. Roy. Soc. London,
Ser. B 168, 101-118.

CARTLEDGE, T.G., and D. LLOYD, 1972: Subcellular fractionation by differential
and zonal centrifugation of aerobically grown glucose-de-repressed
Saccharomyces carlsbergensis. Biochem. J. 126, 381-393.

CHANCE, B., and G.R. WILLIAMS, 1956: The respiratory chain and oxidative
phosphorylation. Adv. Enzymol. 17, 65-134.

FARREL, G.M., 1971: Localization of photosynthetic products in potato leaves
infected by *Phytophthora infestans*. Physiol. Plant Pathol. 1, 457-467.

FEHRMANN, H., 1971a: Obligater Parasitismus phytopathogener Pilze. I. Ver-
gleichende Wachstumsversuche mit *Phytophthora infestans* und verwandten
Arten. Phytopath. Z. 70, 89-113.

FEHRMANN, H., 1971b: Obligater Parasitismus phytopathogener Pilze II. Ver-
gleichende biochemische Untersuchungen zum Energiestoffwechsel von
Phytophthora-Arten. Phytopath. Z. 70, 230-262.

GLEASON, F.H., 1974: Cyanide-insensitive respiration in *Saprolegnia*. Mycolo-
gia 66, 73-76.

GLEASON, F.H., and T. UNESTAM, 1968: Cytochromes of aquatic fungi. J. Bacte-
riol. 95 1599-1603.

GRANT, N.G., and M.H. HOMMERSAND, 1974: The respiratory chain of *Chlorella
protothecoides*. II. Isolation and characterization of mitochondria. Plant
Physiol. 54, 57-59.

HOHL, H.R., 1975: Levels of nutritional complexity in *Phytophthora*: Lipids,
nitrogen sources and growth factors. Phytopath. Z. 84, 18-33.

HOLLOMON, D.W., 1966: Reducing compounds and the growth of *Phytophthora
infestans*. J. Gen. Microbiol. 45, 315-324.

HUNSLEY, D., and J.H. BURNETT, 1970: The ultrastructural architecture of the
walls of some hyphal fungi. J. Gen. Microbiol. 62, 203-218.

ITZHAHI, R.F., and D.M. GILL, 1964: A micro-biuret method for estimating
proteins. Anal. Biochem. 9, 401-410.

JIMENEZ-MARTINEZ, A., and M. NOVAES-LEDIEU, 1969: Protoplastos de hongos
Oomycetos. Microbiol. Espan. 22, 209-218.

KAWAKITA, M., 1970: Studies on the respiratory system of *Aspergillus oryzae*.
II. Preparation and properties of mitochondria from mycelia. J. Biochem.,
Tokyo 68, 625-631.

KAWAKITA, M., 1971: Studies on the respiratory system of *Aspergillus oryzae*.
III. Properties of mitochondria from mycelia grown in the presence of
antimycin A. J. Biochem., Tokyo 69, 35-42.

LAMBOWITZ, A.M., E.W. SMITH, and C.W. SLAYMAN, 1972: Electron transport in
    Neurospora mitochondria. Studies on wild type and poky. J. Biol. Chem.
    247, 4850-4858.
LEE, C.P., G.L. SOTTOCASA, and L. ERNSTER, 1967: Use of artificial electron
    acceptors for abbreviated phosphorylating electron transport: Flavin-
    -cytochrome c. In: S.P. COLOWICK and N.O. KAPLAN, Methods in Enzymology
    X, 33-37. Academic Press, New York and London.
LEWIS, D.H., 1973: Concepts in fungal nutrition and the origin of biotrophy.
    Biol. Rev. 48, 261-278.
LEWIS, D.H., 1974: Micro-organisms and plants: The evolution of parasitism
    and mutualism. Soc. Gen. Microbiol. 24, 367-392.
LLOYD, D., 1974: The Mitochondria of Microorganisms. Academic Press, New
    York and London.
NOVAES-LEDIEU, M., A. JIMENEZ-MARTINEZ, and J.R. VILLANUEVA, 1967: Chemical
    composition of hyphal walls of *Phycomycetes*. J. Gen. Microbiol. 47,
    237-245.
PERL, M., Y. COHEN, and J. ROTEM, 1972: The effect of humidity during darkness
    on the transfer of assimilates from cucumber leaves to sporangia of
    Pseudoperonospora cubensis. Physiol. Plant Pathol. 2, 113-122.
RIBEIRO, O.K., G.A. ZENTMYER, and D.C. ERWIN, 1976: Cytochromes in the
    mycelium and oospores of *Phytophthora capsici*. Phytopathology 66, 172-174.
SCHEEPENS, P.C., and H. FEHRMANN, 1977: Metabolic anomalies as a possible
    cause of biotrophy: Abbreviated electron transport in *Phytophthora
    infestans*. Physiol. Plant Pathol. 11, 71-78.
SCOTT, K.J., 1972: Obligate parasitism by phytopathogenic fungi. Biol. Rev.
    47, 537-572.
SHERALD, J.L., and H.D. SISLER, 1970: Antimycin A-resistant respiratory
    pathway in *Ustilago maydis* and *Neurospora sitophila*. Plant Physiol. 46,
    180-182.
SIETSMA, J.H., J.J. CHILD, L.R. NESBITT, and R.H. HASKINS, 1975: Hyphal walls
    of *Pythium acanthicum*. J. Gen. Microbiol. 86, 29-38.
SIETSMA, J.H., and W.R. DE BOER, 1973: Formation and regeneration of proto-
    plasts from *Pythium* PRL 2142. J. Gen. Microbiol. 74, 211-217.
SIETSMA, J.H., D.E. EVELEIGH, R.H. HASKINS, and J.F.T. SPENCER, 1967:
    "Protoplasts" from *Pythium* sp. PRL 2142. Can. J. Microbiol. 13, 1701-1704.
SLATER, E.C., 1967: Application of inhibitors and uncouplers for a study of
    oxidative phosphorylation. In: S.P. COLOWICK and N.O. KAPLAN, Methods
    in Enzymology X, 48-57. Academic Press, New York and London.
THORNTON, J.D., and R.C. COOKE, 1974: Changes in respiration, chlorophyl
    content and soluble carbohydrates of detached cabbage cotyledons following
    infection with *Peronospora parasitica*. Physiol. Plant Pathol. 4, 117-125.
WATSON, K., and J.E. SMITH, 1967: Oxidative phosphorylation and respiratory
    control in mitochondria of *Aspergillus niger*. Biochem. J. 104, 332-339.
WEISS, H., G. VON JAGOW, M. KLINGENBERG, and T. BUECHER, 1970: Character-
    ization of *Neurospora crassa* mitochondria prepared with a grind-mill.
    Eur. J. Biochem. 14, 75-82.

Address of the authors: Mr. P.C. SCHEEPENS and Prof. Dr. H. FEHRMANN, Institut
für Pflanzenpathologie und Pflanzenschutz der Universität, Grisebachstrasse 6,
D-3400 Göttingen-Weende. Requests for reprints should be addressed to the
second author.

Institute of Plant Pathology and Plant Protection,
University of Göttingen

Mitochondrial electron transport in *Peronosporales*
II.   Anomalous effects of ADP on the electron transport of
Phytophthora infestans, Phytophthora erythroseptica
and *Pythium debaryanum*

By
P.C. Scheepens and H. Fehrmann

with 6 Figures

INTRODUCTION

In a preceding paper (SCHEEPENS and FEHRMANN 1978), the development of a method for the isolation of structurally intact mitochondria from mycelium of *Pythium debaryanum* and *Phytophthora infestans* was described, and some general characteristics of these organelles of both species were compared. It was shown that, as compared with *P. debaryanum*, the electron flux through the main electron transport chain of *Ph. infestans* mitochondria is limited. Evidence was presented that the bottle-neck in the electron transport chain of *Ph. infestans* is located in the region of cytochrome(s) b.

In an earlier study it was shown that the oxidation of added NADH by cell-free preparations of *P. ultimum, Ph. infestans* and other *Phytophthora* species can be influenced by adenosine phosphates, especially by ADP (FEHRMANN 1971). The effect of ADP ranged from a strong inhibition in *Ph. infestans* to a stimulation in *Ph. erythroseptica*. From these results one might conclude that - beyond its function in oxidative phosphorylation - ADP could play an additional role in the regulation of the electron transport. Since the properties of submitochondrial particles, especially with respect to their response to phosphate and phosphate acceptor, can differ from those of intact mitochondria (cf. GREGG and LEHNINGER 1963, LENINGER and GREGG 1963), the first aim of the present study was to reproduce the observed effects of ADP with isolated mitochondria. Secondly, it was of interest to look for the site(s) of action of ADP, and especially to see whether the cytochrome b region - the location of the narrow-pass in the electron transport chain of *Ph. infestans* - was involved.

MATERIALS AND METHODS

Culture conditions for *P. debaryanum* and *Ph. infestans* were described previously (SCHEEPENS and FEHRMANN 1978). *Ph. erythroseptica* was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. Mycelium was grown under the same conditions as used for *P. debaryanum* and *Ph. infestans*, and harvested after 4 days.

Isolation of mitochondria from mycelia was also described earlier (SCHEEPENS and FEHRMANN 1978).

Oxygen uptake was recorded polarographically using a Clark type electrode. A mitochondrial suspension containing 2.3-4.8 mg of protein was incubated in a medium with a final pH of 7.4 containing 0.45 M sucrose, 2.5 mM $MgSO_4 \cdot 7H_2O$, 10 mM KCl, 10 mM KH_2PO_4, 15 mM MOPS-KOH buffer (morpholinopropane sulphonic acid), 0.5% (w/v) bovine serum albumin (BSA), and an amount of ADP as indicated under each Figure, to give a final volume of 1.35 ml. After 3 min. of incubation the reaction was started by addition of 15 µl of the substrate concerned.

Cytochrome c reduction was measured spectrophotometrically at 550 nm with a Zeiss PM 6 spectrophotometer. Mitochondrial suspensions were incubated in the medium described above, to give a final concentration of 0.3-1.0 mg of mitochondrial protein per ml. After 3 min. of incubation, KCN (1.3 mM) and cytochrome c from horse heart (0.1% w/v) were supplied to the medium (final concentrations in parentheses); subsequently, the reaction was started by addition of the substrate concerned.

To measure the activity of succinate dehydrogenase, mitochondria (0.6 mg of protein per ml) were incubated in the medium described above. After 3 min., KCN (10 mM), succinate (5 mM) and methylene blue (1 mM) were added to the medium (final concentrations in parentheses). Oxygen consumption as the result of spontaneous re-oxidation of the reduced carrier was measured polarographically.

The results presented in the Figures are mean values of at least three different mitochondrial preparations. Activities were expressed as percentage of the controls without ADP. ADP-concentration of the stock solutions was estimated spectrophotometrically at 260 nm, using a millimolar extinction coefficient of 14.5 (PULLMAN 1967). Reduced coenzyme Q_6 was prepared according to RIESKE (1967). All chemicals used for the biochemical experiments were of analytical grade.

RESULTS

Results on the influence of different concentrations of ADP on the
succinate respiration of isolated mitochondria of *Ph. infestans* and *P.
debaryanum* are presented in Fig. 1.
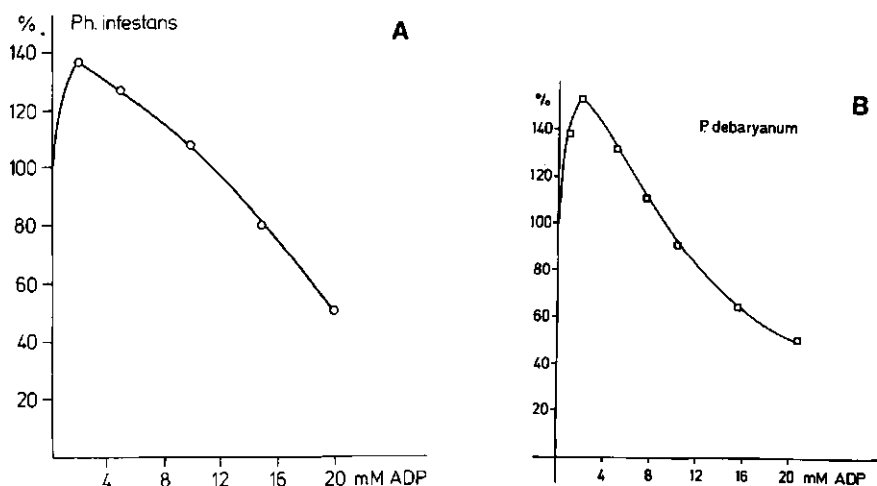
Fig. 1. Effect of ADP on oxygen uptake by isolated mitochondria of
Phytophthora infestans (A) and *Pythium debaryanum* (B), using succinate (5 mM)
as substrate.

With both fungi, respiration was stimulated by low concentrations of ADP.
For *P. debaryanum* this stimulation was demonstrated to be due to a coupling
of respiration to oxidative phosphorylation (SCHEEPENS and FEHRMANN 1978).
Maximal respiration rates were obtained at about 2 mM ADP. At higher
concentrations of ADP respiration rates declined for both species, finally,
at 20 mM, being 30-40% of the maximal rates. This inhibition of respiration
with succinate as substrate is comparable to the inhibition of NADH-oxidation
by ADP in an earlier study (FEHRMANN 1971).

In subsequent experiments, the influence of ADP on the activities of
different segments of the respiratory chain was estimated in order to locate
the site(s) involved in the inhibition by ADP. In addition to *Ph. infestans*
and *P. debaryanum*, *Ph. erythroseptica* was included in this study. The
influence of ADP on the reduction rate of cytochrome c with succinate as
substrate is shown in Fig. 2. For *Ph. infestans* and *P. debaryanum*, the
curves were very similar to those obtained with oxygen as electron acceptor,

Fig. 2.

Effect of ADP on cytochrome c reduction by isolated mitochondria of *Phytophthora infestans* (A), *Phytophthora erythroseptica* (B) and *Pythium debaryanum* (C), using succinate (5 mM) as substrate.

indicating that the site of action of ADP is localized between succinate dehydrogenase and cytochrome c. The influence of ADP on cytochrome c reduction with succinate as substrate was approximately the same for the three tested fungi. Former investigations have shown that in the case of *Ph. erythroseptica* NADH-oxidation by cell-free preparations is stimulated at fairly high concentrations of ADP, the maximum rate being obtained at 8 mM of the latter substance (FEHRMANN 1971). This finding is not paralleled by the results demonstrated in Fig. 2 B.

The influence of ADP on activity of succinate dehydrogenase of *P.*

debaryanum is presented in Fig. 3. The activity of this enzyme was only slightly inhibited at high concentrations of ADP. Therefore, it is unlikely
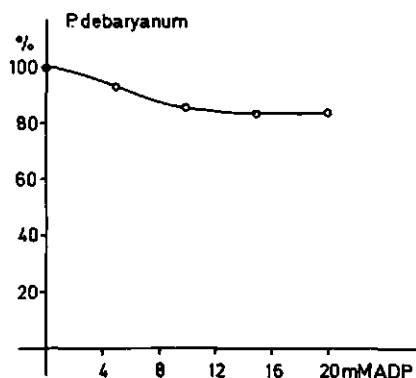
Fig. 3.

Effect of ADP on activity of succinate dehydrogenase in mitochondria of *Pythium debaryanum*, using succinate (5 mM) as substrate.

that the site of action of ADP is localized on this enzyme. This view is supported by the results presented in Fig. 4. Reduction rates of cytochrome c, using reduced coenzyme Q_6 as substrate, were slightly promoted at low
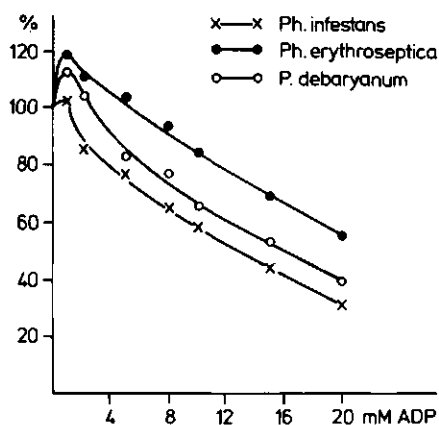
Fig. 4.

Effect of ADP on reduction of cytochrome c by isolated mitochondria of *Phytophthora infestans* (x-x), *Phytophthora erythroseptica* (●-●), and *Pythium debaryanum* (o-o), using reduced coenzyme Q_6 (1 mM) as electron donor.

concentrations of ADP, but inhibited at higher concentrations. This inhibition was most pronounced in the case of *Ph. infestans*, less with *P. debaryanum*, and least with *Ph. erythroseptica*. The activity of this segment of the respiratory chain was inhibited to about the same extent as that of succinate oxidase and succinate-cytochrome c reductase.

Cytochrome c reduction by NADH was also inhibited by ADP, as is shown in Fig. 5. The extent of inhibition was approximately the same for the three fungi tested. In a preceding paper (SCHEEPENS and FEHRMANN 1977) it was
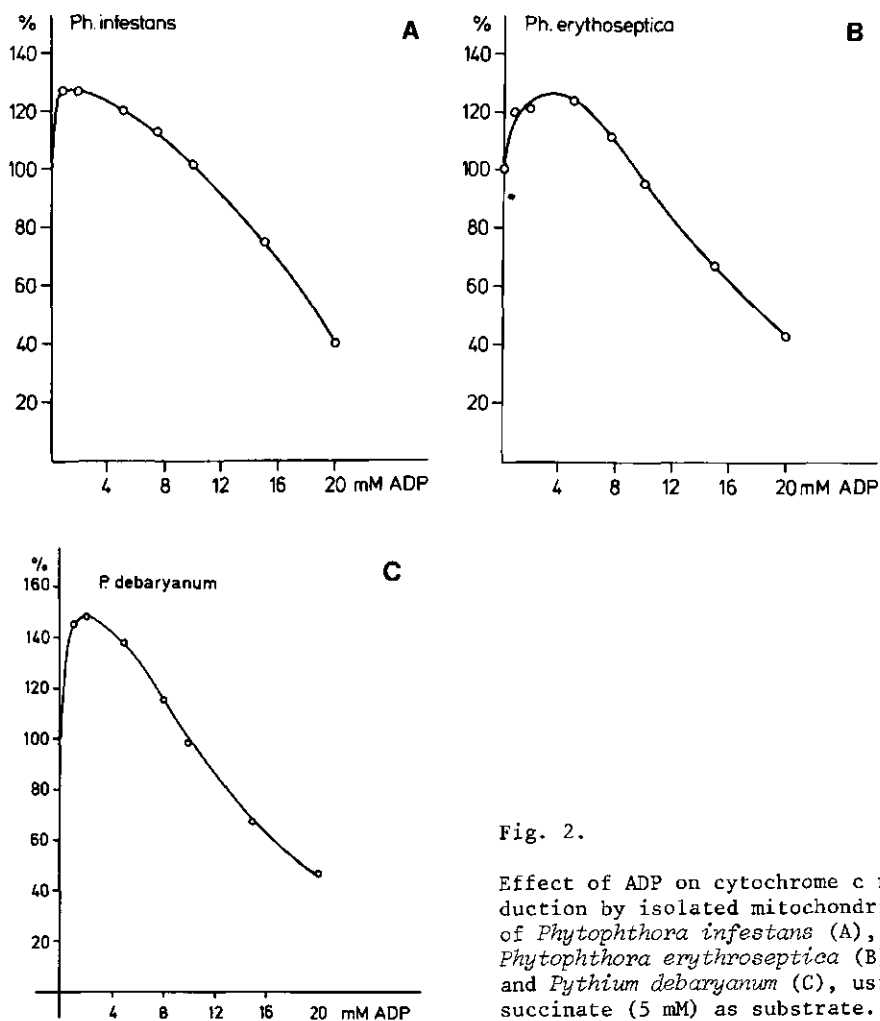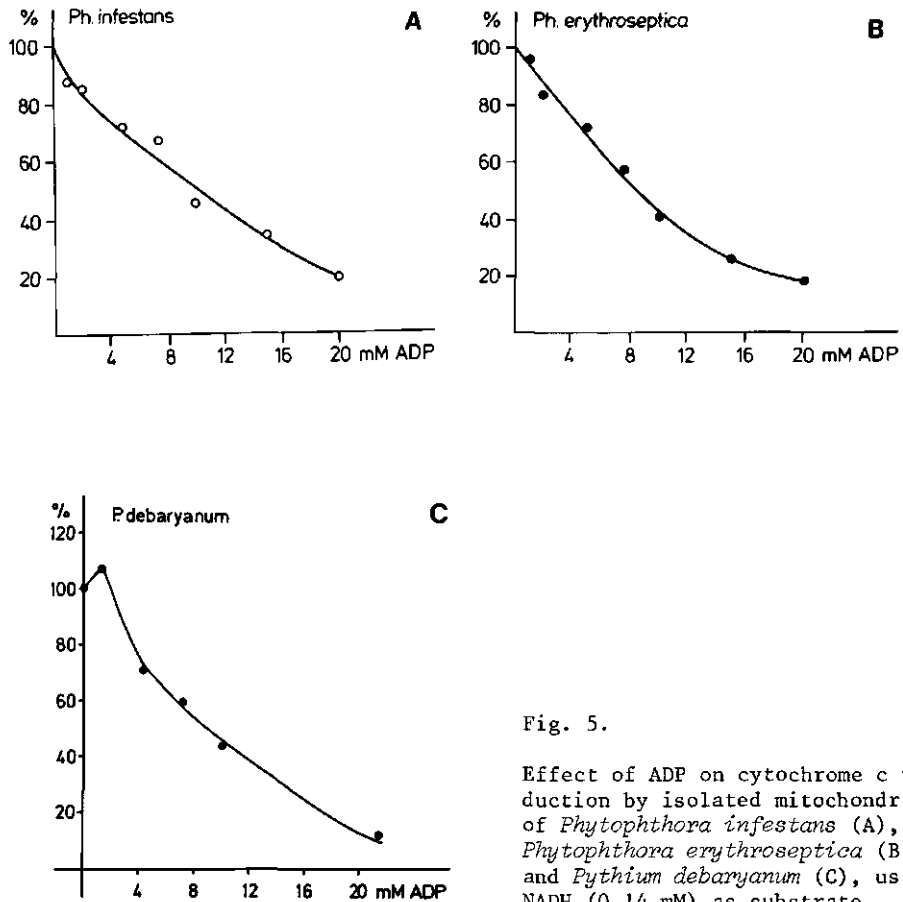

Fig. 5.

Effect of ADP on cytochrome c reduction by isolated mitochondria of *Phytophthora infestans* (A), *Phytophthora erythroseptica* (B), and *Pythium debaryanum* (C), using NADH (0.14 mM) as substrate.

shown that in mitochondria of *Ph. infestans* the major fraction of externally added NADH is oxidized via an antimycin A-insensitive pathway which apparently is connected with the main electron transport chain in the region of cytochrome c. Thus, the inhibition of NADH-oxidation by ADP at high concentrations of the chemical probably means that its site of action is located at or close to cytochrome c. This view is supported by the results presented in Fig. 6, demonstrating the influence of ADP on the oxidation of

33

characterize this narrow-pass and to elucidate its significance for the intermediary metabolism.

Previously, it was speculated that an inhibition of respiration by ADP might explain the limited growth rate of *Ph. infestans* (FEHRMANN 1971). This view was supported by results from experiments with cell-free extracts. We must conclude that this hypothesis is not tenable any more. In this paper it was shown that the influence of ADP on mitochondrial respiration in fact was very similar to that previously described for the influence of ADP on NADH-oxidation by submitochondrial systems of *Ph. infestans*. However, with isolated mitochondria of *Ph. infestans, Ph. erythroseptica* and *P. debaryanum* the extent of inhibition was almost identical in all three fungi. Moreover, for mitochondrial respiration of *Ph. erythroseptica* we were unable to reproduce the pattern of stimulation by ADP which was demonstrated in the earlier study on NADH-oxidation by cell-free extracts. Although mitochondrial respiration of *Ph. infestans, Ph. erythroseptica* and *P. debaryanum* was inhibited by ADP, the concentration needed to produce a pronounced effect was too high to be of any physiological significance.

Further research is necessary to elucidate the fundamental metabolic disorder(s) which render obligately plant parasitic fungi unable to grow and reproduce apart from living host tissue. In this respect, the finding of a narrow-pass for the electron transport in the respiratory chain of the ecologically obligate parasite *Ph. infestans* may provide some new stimulus.

SUMMARY

The influence of ADP on mitochondrial respiration of *Phytophthora infestans, Phytophthora erythroseptica* and *Pythium debaryanum* was compared.

Respiration of succinate was stimulated by low concentrations of ADP in the three fungi, which was due to the coupling of oxidative phosphorylation to respiration. Higher concentrations of the chemical were inhibitory. The extent of inhibition was about the same in the three species. By employing specific inhibitors and different combinations of substrates and electron acceptors, the site of inhibition by ADP could be localized in the cytochrome c region of the respiratory chain. For several reasons, however, a physiological significance of this inhibition by ADP seems doubtful:

1. Concentrations of ADP required for a significant inhibition of respiration were relatively high.
2. Respiration of cysteine, a substance which stimulates growth of *Ph. infestans*, was also inhibited by ADP.

36

3. Respiration of *Ph. erythroseptica* and *P. debaryanum*, which grow well on basic nutrient media, was inhibited by ADP in the same way as that of *Ph. infestans*.

ZUSAMMENFASSUNG

Mitochondrialer Elektronentransport in *Peronosporales*
II. Anomale Einwirkung von ADP auf den Elektronentransport in *Phytophthora infestans, Phytophthora erythroseptica* und *Pythium debaryanum*.

Der Einfluss von ADP auf die Atmung isolierter Mitochondrien von *Phytophthora infestans, Phytophthora erythroseptica* und *Pythium debaryanum* wurde verglichen.

Die Succinat-Atmung der drei Testpilze wurde bei relativ niedrigen ADP-Konzentrationen stets in gewissem Ausmass stimuliert. Diese Förderung beruht auf der Kopplung der oxydativen Phosphorylierung zur Atmungskette. Bei höheren ADP-Konzentrationen war die Atmung der drei Pilze stets und in vergleichbarem Ausmass gehemmt. Durch Einsatz spezifischer Hemmstoffe und die gezielte Kombination verschiedener Substrate und Elektronen-Akzeptoren konnte der Ort der ADP-Hemmung eingegrenzt werden: Er befindet sich im Bereich des Cytochrom c. Aus verschiedenen Gründen ist jedoch eine physiologische Bedeutung dieser ADP-Hemmung fraglich:

1. Für eine nennenswerte Atmungshemmung waren relatif hohe ADP-Konzentrationen erforderlich.
2. Auch die Cystein-Atmung wurde durch ADP gehemmt; das Wachstum von *Ph. infestans* wird jedoch bei Gegenwart von Cystein in vollsynthetischen Agarnährböden gefördert.
3. Die hemmende Wirkung des ADP war bei den untersuchten Pilzen etwa gleich gross; aus theoretischen Ueberlegungen müsste sie aber vor allem bei *Phytophthora infestans* besonderes ausgeprägt sein.

REFERENCES

FEHRMANN, H., 1971: Obligater Parasitismus phytopathogener Pilze. II. Vergleichende biochemische Untersuchungen zum Energiestoffwechsel von *Phytophthora*-Arten. Phytopath. Z. __70__, 230-262.

GREGG, C.T., and A.L. LEHNINGER, 1963: Dependence of respiration on phosphate and phosphate acceptor in submitochondrial systems. II. Sonic fragments. Biochim. Biophys. Acta 78, 27-44.

GUTMAN, M., and N. SILMAN, 1974: An energy independent interaction of ATP with the mitochondrial NADH-dehydrogenase: Competitive inhibition. FEBS Letters 47, 241-243.

LEHNINGER, A.L., and C.T. GREGG, 1963: Dependence of respiration on phosphate and phosphate acceptor in submitochondrial systems. I. Digitonin fragments. Biochim. Biophys. Acta 78, 12-26.

PULLMAN, M.E., 1967: Measurement of ATP-ase, ^{14}C-ADP-ATP, and ^{32}P$_i$-ATP exchange reactions. In: S.P. COLOWICK and N.O. KAPLAN, Methods in Enzymology X, 57-60, Academic Press, New York and London.

RIESKE, J.S., 1967: Preparation and properties of reduced coenzyme Q-cyto-chrome c reductase (complex III of the respiratory chain). In: S.P. COLOWICK and N.O. KAPLAN, Methods in Enzymology X, 239-245. Academic Press, New York and London.

SCHEEPENS, P.C., and H. FEHRMANN, 1977: Metabolic anomalies as a possible cause of biotrophy: Abbreviated electron transport in *Phytophthora infestans*. Physiol. Plant Pathol. 11, 71-78.

SCHEEPENS, P.C., and H. FEHRMANN, 1978: Mitochondrial electron transport in *Peronosporales*. I. Isolation and properties of *Pythium debaryanum* and *Phytophthora infestans* mitochondria. Phytopath. Z. (in press).

Address of the authors: Mr. P.C. SCHEEPENS and Prof.Dr. H. FEHRMANN. Institut für Pflanzenpathologie und Pflanzenschutz der Universität, Grisebachstrasse 6, D-3400 Göttingen-Weende.
Requests for reprints should be addressed to the second author.

`

Metabolic anomalies as a possible cause of biotrophy:
abbreviated electron transport in *Phytophthora infestans*

P.C. Scheepens and H. Fehrmann

Institute of Plant Pathology and Plant Protection,
University of Goettingen, Germany (GFR)

The mitochondrial electron transport in *Phytophthora infestans* was compared with that of *Phytophthora erythroseptica* and *Pythium debaryanum*. The three species represent increasing levels of biological specialization and ecological adaptation within the *Peronosporales*. The oxidation of endomitochondrially generated NADH, and especially that of succinate in mitochondria of *Ph. infestans* was much slower than in the other two species; obviously, there exists a narrow pass in the main electron transport chain, presumably in the region of cytochrome(s) b. However, in *Ph. infestans* mitochondria an additional pathway was operating, which permitted the oxidation of externally added NADH at a much higher rate. In this case, the electrons from NADH were introduced directly into the cytochrome c region of the main electron transport chain, thereby circumventing the postulated narrow-pass. Until now, such a pathway was not described for any other fungus. It was sensitive to cyanide, but insensitive to rotenone and antimycin A. In *Ph. erythroseptica* and *P. debaryanum*, this pathway was also present, but it did not contribute much to the oxidation of NADH.
    From this study, a low energy yield and thereby a shortage of precursors for important biosynthetic processes are proposed as the cause for the inability of *Ph. infestans* to grow on basic nutrient media *in vitro*.

INTRODUCTION

The biochemical interactions between obligately parasitic fungi and the host tissue, once the parasite has established itself successfully, has drawn much attention in recent phytopathological research. So far a number of fungi hitherto considered to be obligate parasites have been cultured axenically (29, 36).

The *Peronosporales* within the *Oomycetes* are known for their high ecological and physiological variability (7, 13). On one hand, the *Peronosporales* include aquatic and soil-inhabiting species, which are strict saprophytes or facultative parasites with a relatively large host range; on the other hand, this oder includes highly specialized obligate parasites. The physiological variability is also reflected in biochemical characteristics. For instance, the lipoamide dehydrogenase of *Pythium ultimum* differs

considerably from that of *Phytophthora erythroseptica* (10, 11).

For several reasons, *Phytophthora infestans* can be considered an intermediate type between facultative parasites such as most *Pythium* and *Phytophthora* species, on one hand, and physiologically obligate parasites such as the downy mildew fungi, on the other (37). According to BRIAN (3), *Ph. infestans* is an ecologically obligate parasite: Due to its very low saprophytic competitive ability, it cannot survive in soil under natural conditions. For growth and reproduction it depends on living host tissue; the parasitism is biotrophic (24, 25, 31, 32, 33). The host range is confined to a few *Solanum* species. In defined culture media the growth rate is rather limited. However, nutrient media supplemented with certain plant extracts provide good growth of the fungus *in vitro*.

The close relationships in the biology of *Ph. infestans* to physiologically obligate parasitism, its pronounced specialization and the limited growth of the fungus on defined media led us to the assumption, that it in some way is anomalous in metabolism. A fundamental anomaly then could act as a disorder, limiting the metabolic capacity as a whole and causing its high dependency on living host tissue. Previously, it was postulated that the energy metabolism of *Ph. infestans* is disordered (8, 9). The present study is again devoted to this problem. Certain characteristics of the electron transport in isolated mitochondria of *Ph. infestans* are compared with those in mitochondria of *Pythium debaryanum* and *Ph. erythroseptica*. Both fungi are soil saprophytes, *P. debaryanum* being able to attack many host plants, whereas *Ph. erythroseptica* has a smaller host range.

The results are discussed with respect to the biotrophic character of *Ph. infestans*.

MATERIALS AND METHODS

Organisms and culture conditions

Ph. infestans was isolated from potato tubers. *Ph. erythroseptica* was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands. *P. debaryanum* was kindly provided by Dr. M.A. de Waard, Laboratory of Phytopathology, Wageningen, The Netherlands.

Ph. infestans was maintained on potato callus tissue to ensure its vitality. The callus tissue was generously supplied by Dr. G. Wolf from this laboratory. Plates with pea juice agar (8) were inoculated at intervals of two weeks with mycelium from the tissue cultures to provide the inoculum for

liquid cultures. *Ph. erythroseptica* and *P. debaryanum* were maintained on pea juice agar. For mass cultures, Erlenmeyer flasks (300 ml) with 80 ml of nutrient solution were inoculated and incubated at 20-22°C on a rotary shaker at 100 rpm. One litre of nutrient solution contained 10 g glucose, 1 g KH_2PO_4, 200 mg $MgSO_4 7H_2O$, 50 mg $CaCl_2 . 2H_2O$, 5 mg $FeCl_3 . 6H_2O$, 0.1 mg thiamine, and pea juice (75 g of deep-frozen peas boiled in 1 l of water for 15 min. and subsequently decanted). Mycelia of *Ph. infestans, Ph. erythroseptica* and *P. debaryanum* were harvested after 7, 4 and 3 days respectively, while still growing in the logarithmic phase.

Isolation of mitochondria

The washed mycelia were homogenized in a Waring blender in a medium containing 0.45 M saccharose, 1 mM EDTA, 0.2% bovine serum albumin and 0.02 M MOPS buffer with a final pH of 7.4. The hyphal walls were disrupted with a grind-mill, an apparatus described by WEISS et al. (35). After squeezing the brei through four layers of a synthetic gauze (30 μm mesh), the mitochondria were collected by fractionated centrifugation between 600 *g* for 5 min. and 6,000 *g* for 15 min.

Analytical procedures

Oxygen consumption was measured polarographically, using a Clark type of electrode (Y.S.I., Ohio, USA). Cytochrome c reduction was determined spectro-photometrically at 550 nm with a Zeiss PM 6 spectrophotometer. The extinction coefficient used for cytochrome c (reduced-oxidized) was 18.5 x $10^6 M^{-1} cm^{-1}$ (5). Activities of acid phosphatase and glucose-6-phosphatase, respectively, were measured with the methods described by BERGMEYER (2). The results presented in the tables are mean values of at least four different mitochondrial preparations (± S.E.M.). Protein was determined according to ITZHAHI & GILL (17), using bovine serum albumin as standard. All chemicals used for the biochemical experiments were of analytical grade.

RESULTS

Electron micrographs of mitochondrial suspensions revealed that the organelles of the three species were of globular shape after isolation (mean diameter about 1 μm). The structural integrity seemed essentially to be preserved, although some damage to the outer membranes was noticed. The

activities of acid phosphatase and glucose-6-phosphatase, used as markers
for the presence of lysosomal and microsomal contaminations, respectively,
were very low in the mitochondrial preparations compared to the original
homogenates. However, respiratory control ratios were rather low, and
addition of the uncoupler carbonyl cyanide, m-chlorophenylhydrazone (CCCP)
only slightly stimulated respiration. These observations indicate that the
mitochondria were mostly uncoupled. More detailed information on their
properties will be presented elsewhere (28).

Respiratory inhibitors are useful tools in elucidating different
mitochondrial electron transport pathways. The sites of action of the
respiratory inhibitors rotenone, antimycin A and cyanide are shown in Fig. 1.
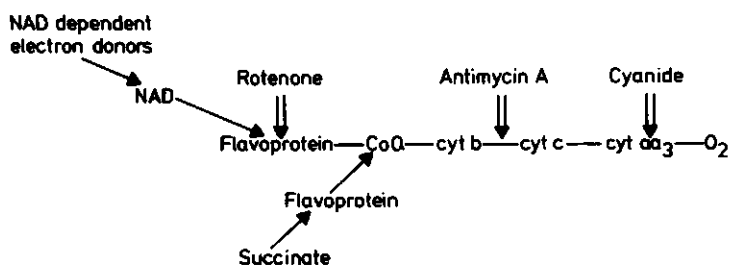
Fig. 1. Schematic presentation of the electron transport system illustrating
the sites of action of the inhibitors used in this study.

First the influence of rotenone and antimycin A on the reduction rate of
cytochrome c was investigated. Rotenone inhibits the oxidation of endo-
mitochondrial NADH without affecting the oxidation of succinate; antimycin A
blocks the electron transport between cytochromes b and c (5). α-Oxoglutarate
and pyruvate which generate an endomitochondrial pool of NADH, and also free
NADH and succinate were employed as substrates. The results are presented in
Table 1. As expected, in all cases the oxidation of succinate was insensitive
to rotenone but sensitive to antimycin A. The oxidation of α-oxoglutarate and
pyruvate plus malate was inhibited by rotenone and antimycin A in the three
fungi tested. With respect to the oxidation of added NADH, however, remark-
able differences in the influence of the inhibitors were observed between
the three species. In *P. debaryanum* and *Ph. erythroseptica* mitochondria, both
rotenone and antimycin A were able to inhibit the oxidation of NADH, but in
Ph. erythroseptica to a smaller extent than in *P. debaryanum*. In *Ph.*

42

Table 1. Influence of rotenone and antimycin A on the cytochrome c reduction of *Ph. infestans, Ph. erythroseptica* and *P. debaryanum* mitochondria.
To the reaction mixture (pH 7.4) containing 0.45 M saccharose, 2.5 mM MgSO$_4$.7H$_2$O, 10 mM KCl, 0.5% BSA, 1 mM ADP, 10 mM phosphate, 15 mM MOPS buffer, plus 25 μM rotenone or 2.5 μM antimycin A in methanol, respectively, mitochondrial suspension was added to give a final concentration of 0.3 to 1.0 mg mitochondrial protein. Control cuvettes contained the same amount of methanol. After 3 min. incubation time, KCN (1.3 mM) and cytochrome c from horse heart (0.1%) were supplied to the medium and subsequently the reaction was started by the addition of α-oxoglutarate (5 mM), pyruvate (5 mM) plus malate (4 mM), succinate (5 mM) or NADH (0.5 mM) as the respective substrate (final concentrations in parentheses). One μg electron corresponds with 1 μmol of reduced cytochrome c.

| | Added substrate | | | |
	α-Oxo- glutarate	Pyruvate + malate	Succinate	NADH
Cytochrome c reduced:				
(μg electron/min/g protein)				
Ph. infestans	3	6	9±1	34±6
Ph. erythroseptica	5±1	6±1	11±1	21±2
P. debaryanum	6±1	8±2	27±3	24±3
Residual activity after				
addition of rotenone (25 μM):				
Ph. infestans	< 1	2	9	32
Ph. erythroseptica	< 1	2	12	11
P. debaryanum	< 2	4	22	6
Residual activity after				
addition of antimycin A (2.5 μM)				
Ph. infestans	< 1	2	2	28
Ph. erythroseptica	< 1	< 1	< 1	10
P. debaryanum	< 1	< 1	< 1	4

infestans, however, both substances were almost inactive in blocking the oxidation of added NADH. From these results it became evident that in *Ph. infestans* and in *Ph. erythroseptica* an additional pathway for the oxidation of NADH was present. Conclusions about the relative importance of this pathway are only possible when maximal oxidation rates are measured. These requirements were not met with cytochrome c as electron acceptor, since respiration rates were much higher (c.f. Table 2).

In table 2, respiration rates, and the influence of rotenone, antimycin A and cyanide on respiration, are presented; succinate and NADH were employed as substrates. It is assumed that, in this case, maximal respiratory activities were measured. Activity of NADH-respiration in the presence of antimycin A, presented as percentage of the control without inhibitors (given

43

Table 2. Influence of rotenone, antimycin A and cyanide on the oxygen uptake
of *Ph. infestans*, *Ph. erythroseptica* and *P. debaryanum* mitochondria.
Mitochondrial suspension was added to the reaction medium described under
Table 1, initially without inhibitors, to give a final mitochondrial protein
concentration of 1.5 to 3.2 mg/ml. After the addition of NADH (1 mM) or
succinate (5 mM), the oxygen uptake was recorded. Rotenone (25 uM), antimycin
A (2.5 µM) or cyanide (10 mM) were then added to the same cuvettes to measure
the extent of inhibition (final concentrations in parentheses). One µg
electron corresponds with 0.25 µmol of O_2.

	µg Electrons/min/g protein		% of control
	With succinate	with NADH	with NADH
Oxygen uptake: control			
Ph. infestans	28±8	76±18	
Ph. erythroseptica	56±10	74±12	
P. debaryanum	82±14	138±18	
+ Rotenone (25 µM):			
Ph. infestans	26	70	
P. debaryanum	68	64	
+ Antimycin A (2.5 µM):			
Ph. infestans	4	66	(86)
Ph. erythroseptica	4	14	(20)
P. debaryanum	10	18	(13)
+ Cyanide (10 mM):			
Ph. infestans	< 3	< 3	
Ph. erythroseptica	< 3	< 3	
P. debaryanum	< 3	< 3	

in parentheses), thus indicates directly the magnitude of the rotenone- and
antimycin-resistant NADH-shunt. In *P. debaryanum* mitochondria, NADH-
respiration could be blocked almost completely by antimycin A, indicating
that the antimycin-resistant NADH shunt plays only a minor role in this
fungus. However, inhibition by rotenone was far from complete. Therefore, a
rotenone-insensitive, antimycin-sensitive NADH shunt, as described for many
higher plants and other fungi (12, 18, 19, 21, 34, 35), was probably also
present in *P. debaryanum*. In *Ph. erythroseptica* mitochondria also, most of
the NADH was respired via the antimycin-sensitive electron transport system.
The extent of the rotenone-insensitive respiration of NADH was not determined
for this species. In *Ph. infestans* mitochondria, however, rotenone and anti-
mycin A were again almost inactive in blocking the NADH respiration. The
presence of an antimycin-sensitive site in this fungus was demonstrated with
succinate as substrate. Therefore, we can conclude, that NADH is mainly
oxidized via an abbreviated pathway, thereby circumventing the rotenone- and

antimycin-sensitive sites.

Cyanide completely inhibited respiration in all three species, although the required concentration for maximal inhibition was higher than described for other organisms (5). Apparently, a cyanide-insensitive pathway as described for some higher plants, algae and fungi (1, 14, 16, 20, 21, 22, 30) was not present in these species.

DISCUSSION

In animal mitochondria, the inner membrane system is impermeable to NADH (4). Presumably, this is also the case with mitochondria from other sources, as externally added NADH is predominantly oxidized via a rotenone-insensitive pathway (23, 26). In *P. debaryanum* and *Ph. erythroseptica* mitochondria, however, a substantial part of added NADH was oxidized via the rotenone-sensitive electron transport system. This would mean that, in these species, either an efficient substrate-linked shuttle system is present, or the inner membrane system has become leaky upon isolation. In the latter case, oxidation rates of pyruvate plus malate and α-oxoglutarate are probably underestimated because of some leakage of cofactors of the respective dehydrogenases.

From the results presented in this paper one can conclude that in all three species succinate and endomitochondrially generated NADH were oxidized via an electron transport pathway similar to that described for other fungi, higher plants and animals. This view is supported by the work of GLEASON & UNESTAM (15), who measured the cytochrome content in representatives of the *Oomycetes* including *P. debaryanum*. In contrast to other organisms, however, only one type of cytochrome c could be detected. Furthermore, in *Ph. capsici* the usual set of cytochromes was present (27). In *Ph. infestans*, however, the activity of this main electron transport system, as especially reflected by the respiration of succinate, was much lower than in *Ph. erythroseptica* and *P. debaryanum*. The dye tetramethyl-p-phenylene-diamine (TMPD) is able to transfer electrons directly from cytochrome b to cytochrome c in the presence of antimycin A; the second phosphorylation site of the respiratory chain is thereby circumvented (5). Succinate respiration in *Ph. infestans* mitochondria is greatly enhanced upon addition of TMPD (28). Therefore, the narrow pass in the electron transport chain is probably located in the cytochrome b region. Externally added NADH was rapidly oxidized via an abbreviated electron transport pathway in *Ph. infestans*. At least two facts indicate that this is of mitochondrial origin and that it is associated with the main electron

transport chain. First of all, it could be demonstrated with both cytochrome c and oxygen as electron acceptors. Moreover, this pathway was sensitive to cyanide, but not to antimycin A. For the last reason, it should be connected with the main electron transport chain in the cytochrome c region. In mung bean mitochondria as well, an antimycin A resistant NADH-cytochrome c reductase was demonstrated (6). However, this is associated with the outer mitochondrial membrane and - in contrast to the one in *Ph. infestans* - is not connected with the main electron transport chain itself. If the antimycin A resistant respiration in *Ph. infestans* was due to an artefact of isolation, a comparable magnitude of this pathway should be expected for *Ph. erythroseptica* and *P. debaryanum*. However, although this pathway obviously was present too in these two species, here most of the added NADH was respired via the antimycin-sensitive electron transport system.

A narrow pass in the respiratory chain of any organism could act as a disorder when related to the metabolic requirements of this organism. Due to the limited electron flux, only limited amounts of ATP can be oxidatively phosphorylated from ADP. Furthermore, reactions coupled to the electron transport chain, i.e. the interconversion of intermediates of the citric acid cycle, should proceed only slowly. Under normal circumstances such a disorder could be lethal for the organism involved. The experimental results presented in this paper suggest that there exists such a narrow pass in the electron transport chain of *Ph. infestans*. However, the presence of an additional electron transport pathway in this fungus could to some extent overcome this disorder. Although this pathway allows the synthesis of as a maximum one mole of ATP per mole NADH, metabolic interconversions could proceed somewhat faster (c.f. ref. 18 and 26). Some specific factors from peas, which promote growth of *Ph. infestans in vitro* (MIESKES & FEHRMANN, to be published), have no or only slightly stimulatory effects on mitochondrial respiration. Their nature suggests that they are merely involved in the intermediary metabolism of the fungus. By this means, on the basis of an anomaly in energy metabolism the capacity of the fungus for autonomous growth and reproduction *in vitro* is limited and dependent on certain constituents of the medium. In its parasitic phase, the fungus should be supplemented best by biotrophic nutrition from the host cell. In other words: The alternative pathway of NADH-oxidation *and* the narrow pass in the cytochrome b region of the main electron transport chain should modify the intermediary metabolism of the pathogen. Good growth can be guaranteed only when key substances are supplied from the host *in vivo* or added to the nutrient medium *in vitro*.

46

REFERENCES

1. Bahr, H.T. & Bonner Jr., W.D. (1973). Cyanide-insensitive respiration.
   I. The steady states of skunk cabbage spadix and bean hypocotyl mito-
   chondria. The Journal of Biological Chemistry 248, 3441-3445.
2. Bergmeyer, H.U. (1970). Methoden der enzymatischen Analyse, 2nd edition.
   Verlag Chemie, Weinheim, GFR.
3. Brian, P.W. (1967). Obligate parasitism in fungi. Proceedings of the
   Royal Society of London, Series B, 168, 101-118.
4. Cederbaum, A.I., Lieber, C.S., Beattie, D.S. & Rubin, E. (1973).
   Characterization of shuttle mechanisms for the transport of reducing
   equivalents into mitochondria. Archives of Biochemistry and Biophysics
   158, 763-781.
5. Colowick, S.P. & Kaplan, N.O. (1967). Methods in Enzymology, Volume X:
   Oxidation and Phosphorylation. Academic Press, New York and London.
6. Douce, R., Manella, C.A. & Bonner Jr., W.D. (1973). The external NADH
   dehydrogenases of intact plant mitochondria. Biochimica et Biophysica
   Acta 292, 105-116.
7. Erwin, D.C., Zentmyer, G.A., Galindo, J. & Niederhauser, J.S. (1963).
   Variation in the genus *Phytophthora*. Annual Review of Phytopathology
   1, 375-396.
8. Fehrmann, H. (1971). Obligater Parasitismus phytopathogener Pilze. I.
   Vergleichende Wachstumsversuche mit *Phytophthora infestans* und ver-
   wandten Arten. Phytopathologische Zeitschrift 70, 89-113.
9. Fehrmann, H. (1971). Obligater Parasitismus phytopathogener Pilze. II.
   Vergleichende biochemische Untersuchungen zum Energiestoffwechsel von
   Phytophthora-Arten. Phytopathologische Zeitschrift 70, 230-262.
10. Fehrmann, H. (1977). Comparison of lipoamide dehydrogenase of *Pythium
    ultimum* and *Phytophthora erythroseptica*. Phytopathologische Zeit-
    schrift (In press).
11. Fehrmann, H. & Veeger, C. (1974). Lipoamide dehydrogenase of the
    phytopathogenic fungi *Pythium ultimum* and *Phytophthora erythroseptica*:
    Differences in conformation and kinetics. Biochimica et Biophysica
    Acta 350, 292-303.
12. Fukami, M.H., Light, P.A. & Garland, P.B. (1970). Changes of mitochondrial
    $NADH_2$ oxidation pathways in *Torulopsis utilis* grown on acetate. FEBS
    Letters 7, 132-134.
13. Gäumann, E. (1964). Die Pilze, 2nd edition. Verlag Birkhäuser, Basel.
14. Gleason, F.H. (1974). Cyanide-insensitive respiration in *Saprolegnia*.
    Mycologia 64, 73-76.
15. Gleason, F.H. & Unestam, T. (1968). Cytochromes of aquatic fungi. Journal
    of Bacteriology 95, 1599-1603.
16. Grant, N.G. & Hommersand, M.H. (1974). The respiratory chain of *Chlorella
    prototaecoides*. II. Isolation and characterization of mitochondria.
    Plant Physiology 54, 57-59.
17. Itzhahi, R.F. & Gill, D.M. (1964). A micro-biuret method for estimating
    proteins. Analytical Biochemistry 9, 401-410.
18. Jagow, G. von, & Klingenberg, M. (1970). Pathways of hydrogen in mito-
    chondria of *Saccharomyces carlsbergensis*. European Journal of Bio-
    chemistry 12, 583-592.

19. Kawakita, M. (1970). Studies on the respiratory system of *Aspergillus oryzae*. II. Preparation and some properties of mitochondria from mycelia. Journal of Biochemistry, Tokyo 68, 625-631.

20. Kawakita, M. (1971). Studies on the respiratory system of *Aspergillus oryzae*. III. Properties of mitochondria from mycelia grown in the presence of antimycin A. Journal of Biochemistry, Tokyo 69, 35-42.

21. Kull, U. (1972). Die mitochondriale Atmungskette höherer Pflanzen. Naturwissenschaftliche Rundschau 25, 65-68.

22. Lambowitz, A.M., Smith, E.W. & Slayman, C.W. (1972). Electron transport in *Neurospora* mitochondria: Studies on wild type and poky. The Journal of Biological Chemistry 247, 4850-4858.

23. Lloyd, D. (1974). The Mitochondria of Microorganisms. Academic Press, New York and London.

24. Meyer, G. (1939). Zellphysiologische und anatomische Untersuchungen über die Reaktion der Kartoffelknolle auf den Angriff der *Phytophthora infestans* bei Sorten verschiedener Resistenz. Arbeiten der Biologischen Reichsanstalt 23, 97-133.

25. Mueller, K.O., Meyer, G. & Klinkowski, M. (1939). Physiologisch-genetische Untersuchungen über die Resistenz der Kartoffel gegenüber *Phytophthora infestans*. Die Naturwissenschaften 27, 765.

26. Palmer, J.M. (1976). The organization and regulation of electron transport in plant mitochondria. Annual Review of Plant Physiology 27, 133-157.

27. Ribeiro, O.K., Zentmyer, G.A. & Erwin, D.C. (1976). Cytochromes in the mycelium and oospores of *Phytophthora capsici*. Phytopathology 66, 172-174.

28. Scheepens P.C. & Fehrmann, H. Mitochondrial electron transport in *Peronosporales*. I. Isolation and properties of *Pythium debaryanum* and *Phytophthora infestans* mitochondria. Phytopathologische Zeitschrift (in press).

29. Scott, K.J. (1972). Obligate parasitism by phytopathogenic fungi. Biological Reviews 47, 537-572.

30. Sherald, J.L. & Sisler, H.D. (1970). Antimycin A-resistant respiratory pathway in *Ustilago maydis* and *Neurospora sitophila*. Plant Physiology 46, 180-182.

31. Tomiyama, K. (1955). Cell physiological studies on resistance of potato plants to *Phytophthora infestans*. II. The death of the intracellular hyphae in the hypersensitive cell. Annals of the Phytopathological Society of Japan 19, 149-154.

32. Tomiyama, K. (1956). Cell physiological studies on resistance of potato plants to *Phytophthora infestans*. IV. On the movement of cytoplasm of the host cell induced by the invasion of *Phytophthora infestans*. Annals of the Phytopathological Society of Japan 21, 54-62.

33. Tomiyama, K. (1957). Cell physiological studies on the resistance of potato plants to *Phytophthora infestans*. VII. Growth of intracellular hyphae of *Phytophthora infestans* in the living potato plant cells which are resistant or susceptible to the infection of them. Annals of the Phytopathological Society of Japan 22, 129-133.

34. Watson, K. & Smith, J.E. (1967). Oxidative phosphorylation and respiratory control in mitochondria from *Aspergillus niger*. Biochemical Journal 104, 332-339.

35. Weiss, H., Jagow, G. von, Klingenberg, M. & Bücher, T. (1970). Characterization of *Neurospora crassa* mitochondria prepared with a grind-mill. European Journal of Biochemistry 14, 75-82.

36. Wolf, F.T. (1974). The cultivation of rust fungi upon artificial media. Canadian Journal of Botany 52, 767-772.

37. Yarwood, C.E. (1956). Obligate parasitism. Annual Review of Plant Physiology 7, 115-142.

Institute of Plant Pathology and Plant Protection
University of Göttingen

Cultivation of *Phytophthora infestans* on defined
nutrient media

By

P.C. Scheepens and H. Fehrmann

with 6 Figures

INTRODUCTION

Phytophthora infestans hardly grows on basal nutrient media; in a previous
study attempts were made to find the biochemical basis for this phenomenon
(SCHEEPENS and FEHRMANN 1977, 1978a, b). It was demonstrated that, as compared
with *Ph. erythroseptica* and *Pythium debaryanum*, the mitochondrial electron
transport system of *Ph. infestans* is anomalous. It was suggested that this
anomaly might be the primary cause of general metabolic disorders, thereby
limiting the synthetic capacity of the fungus as a whole.

In the present study, some specific nutritional requirements of *Ph.
infestans* were investigated in order to find out whether they could be re-
lated to its anomalous electron transport.

Until the beginning of this century, *Ph. infestans* was considered an
obligate parasite. In 1908, CLINTON first succeeded in cultivating the fungus
axenically by employing a medium supplemented with lima-bean extract. Extracts
of other plants serve as well (cf. HENNINGER 1959). Many attempts have been
made to cultivate the fungus on chemically defined media. In course of time,
the nature of many growth-promoting factors has been elucidated.

Like other *Phytophthora* species, *Ph. infestans* depends on thiamine for
growth (PAYETTE and PERRAULT 1944, RONCADORI 1965). Sterols are necessary for
oospore induction in all members of the *Pythiaceae* (HENDRIX 1964, 1965, 1970).
They also stimulate vegetative growth to some extent, especially in liquid
cultures; for *Ph. infestans*, the same phenomenon was established by LANGCAKE
(1974). Growth of *Ph. infestans* is stimulated by phospholipids as well, but
to a smaller extent than that of other *Phytophthora* species (HOHL 1975). The
stimulating effect of phospholipids is probably due to their unsaturated
fatty acid moiety. Growth of *Ph. infestans* can also be stimulated to some

degree by reducing compounds such as ascorbate or cysteine (HOLLOMON 1966, FEHRMANN 1971, CUPETT and LILLY 1973).

Much attention has been paid to the nitrogen sources. Nitrate proved to be a poor nitrogen source, ammonium salts being somewhat better if added to a properly buffered medium (RONCADORI 1965); however, organic nitrogen sources are definitely better than any inorganic nitrogen source (HALL 1959, HENNINGER 1959, FEHRMANN 1971). However, to achieve growth rates comparable to those on media supplemented with certain plant extracts, amino acid mixtures have to be added to the medium (HOHL 1975).

The present study was mainly devoted to the nutrition of *Ph. infestans*, using mixtures of several amino acids, the composition of which was based on chemical analysis of growth-promoting substances from peas (MIESKES and FEHRMANN, unpublished results). Some attention was also paid to phospholipids. In general, the results were in agreement with those of HOHL (1975).

MATERIALS AND METHODS

Microorganisms and cultural conditions:

Ph. infestans strains 02 and 04 were isolated from potato tubers. Strain 02 was the same one used for the biochemical experiments described elsewhere (SCHEEPENS and FEHRMANN 1977, 1978a, b); it was maintained on potato callus tissue. Strain 04 was kept on pea-juice agar slants under mineral oil. Isolates of races 0 and 4 of *Ph. infestans* originating from the Biologische Bundesanstalt, Braunschweig, Germany, were kindly provided by Dr. G. Wolf from this laboratory. *Ph. erythroseptica* was obtained from the Centraalbureau voor Schimmelcultures, Baarn, The Netherlands.

Inoculum for growth experiments was derived from three- and six-day-old cultures of *Ph. erythroseptica* and *Ph. infestans*, respectively, on pea juice agar. Slices of agar medium with mycelium were punched out with a 5 mm cork borer from the margin of the colonies and then placed with the mycelium downwards on the surface of freshly prepared agar plates. The plates were incubated in the dark at 20°C. Growth was expressed in mm as linear increase in diameter of the colonies. Each figure represents the mean value of six replicates.

Agar media and their constituents:

Pea juice agar was prepared by boiling 150 g of deep-frozen peas in 1 l

of water for 15 min. and subsequent decanting. 5 g Glucose and 20 g agar were then added to the pea-juice. Defined media always contained 20 g glucose, 1 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, 0.1 g $CaCl_2.2H_2O$, 10 mg EDTA, di-sodium salt, 0.1 mg thiamine, 10 mg $FeSO_4$, 1 ml of a stock solution of trace elements (SCHROPP 1951), and 20 g agar per litre, supplemented with varying sources of nitrogen. The final pH of the media was 6.5. In some experiments, 200 mg of a mixture of soya-bean phospholipids (Sigma) was added per litre of nutrient medium.

The following nitrogen sources were used:
a) Peptone, acid hydrolyzed (Evans), 1000 mg/l
b) A purified fraction from peas, containing several amino acids, 500 mg/l. This fraction was hydrolyzed for 5 h in 6 N HCl at $110^{o}C$ before use
c) Glutamine, 700 mg/l
d) Three mixtures of 13 different amino acids each, 1000 mg/l. Their composition (cf. Table 1) was based on chemical analysis of the fraction mentioned under (b) (MIESKES and FEHRMANN, unpublished results); instead of glutamic and aspartic acid which are present in peas, their respective amides were used. In one experiment, single amino acids or combinations of certain amino acids were omitted from the medium. The total concentration was always maintained at 1000 mg/l by replenishing the amount of omitted amino acids with the same amount of glutamine.

Table 1: Composition of amino acid mixtures (I, II, III) for the cultivation of *Phytophthora infestans*. The mixtures were added to a defined agar medium to give a final concentration of 1 g/l.

Amino acid	Concentration (mg/l):		
	I	II	III
Alanine (ala)	82	45	82
Glycine (gly)	8	37	8
Valine (val)	30	59	30
Leucine (leu)	9	65	9
Serine (ser)	18	52	18
Lysine (lys)	17	73	17
Threonine (thr)	90	60	90
Isoleucine (ile)	21	65	21
Histidine (his)	17	78	17
γ-Amino butyric acid (aba)	102	51	102
Arginine (arg)	270	87	150
Asparagine (asn)	19	180	240
Glutamine (gln)	317	148	214

Unless stated otherwise, chemicals were purchased from Merck, Darmstadt, Germany.

RESULTS

Comparison of different nitrogen sources:

From the analysis of growth promoting substances from peas it became evident that a fraction containing several amino acids, when added to the basal nutrient medium, supported growth of strain 02 of *Ph. infestans* approximately to the same extent as pea-juice (MIESKES and FEHRMANN, unpublished results). To provide further evidence for the assumption that only amino acids were responsible for the stimulation of growth, this fraction from peas was replaced by a defined mixture of amino acids. This mixture I contained 13 amino acids which were present at approximately the same ratio as in peas. After its addition to the medium, growth of four strains of *Ph. infestans* was compared with that on pea-juice agar. The results are given in Fig. 1. With the four strains of *Ph. infestans* the growth rates in the logarithmic phase were about the same on pea-juice agar and on the defined medium with added amino acids. In all cases, however, the lag phase was longer on the defined medium, with strain R4 even much longer.
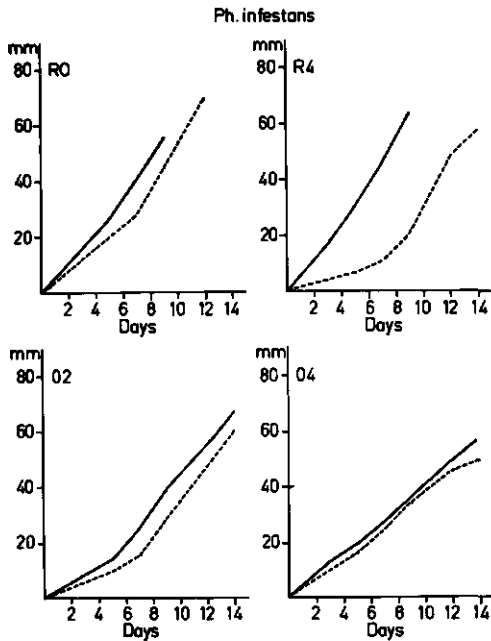

Fig. 1.

Growth of four strains of *Phytophthora infestans* on pea-juice agar (——) and on a defined medium containing amino acid mixture I (---).

In the same experiment, the effect of other nitrogen sources on the growth of the four strains of *Ph. infestans* was tested. The linear growth

52

rates of the four strains on these media, relative to that on control plates
(= 100%) with pea-juice agar after 9 days, are summarized in Table 2.

Table 2: Relative growth of four *Phytophthora infestans* strains on nutrient
media with different nitrogen sources; growth compared with that on pea-juice
agar (= 100%). Plates were incubated for 9 days.

Nitrogen source:	Relative growth rate, %			
	Phytophthora infestans strain:			
	02	04	RO	R4
Glutamine	5	64*	40	6
Peptone	18	–	18	13
Hydrolyzed fraction from peas	9	54	15	–
Amino acid mixture I	69	95	74	26

*
very sparse mycelium

The strains reacted in a different way to the various nitrogen sources, but
the relative growth rate was always highest with strain 04, followed by
strains RO, 02 and R4, in that order. On the medium containing glutamine as
the only nitrogen source, the linear growth rate of strains 04 and RO was
still quite reasonable. With strain 04, however, the mycelial mat was very
thin. Strains 02 and R4 did hardly grow on this medium. In contract to the
mixture of amino acids, the hydrolyzed fraction from peas supported growth of
two strains only poorly; on this medium, only strain 04 grew moderately. This
observation indicates that the amides glutamine and asparagine which were
de-aminated during hydrolysis were necessary for two strains, and favourable
for the third one (strain RO was not tested). Peptone was included in this
study as it greatly enhances growth of some rust fungi (G. WOLF, personal
communication). However, it proved to be a poor nitrogen source for the three
tested strains of *Ph. infestans*. Here, one should realize that the employed
Evans' peptone was acid-hydrolyzed and therefore deficient in the amides
mentioned (cf. FOUDIN and WYNN 1972). With trypsinized peptone the situation
might be different.

Effect of phospholipids on the growth of Ph. infestans:

Recently, it has been demonstrated that several species of *Phytophthora*,
including *Ph. infestans*, show a remarkable response in growth upon addition
of phospholipids to the nutrient medium (HOHL 1975). With some species, but

not with *Ph. infestans*, there also seemed to be a kind of interaction between the nitrogen source and the phospholipids: they grew well on nitrate nitrogen provided that phospholipids were present in the medium. Therefore, before any further effects of mixtures of amino acids were studied, the response in growth of three strains of *Ph. infestans* to phospholipids was determined. For comparison, *Ph. erythroseptica* was included in this study. The growth rate of the fungi on pea-juice agar, on a defined medium containing amino acid mixture I, and on the latter medium with added soya-bean phospholipids was compared. The results are presented in Fig. 2. With *Ph. erythroseptica* and
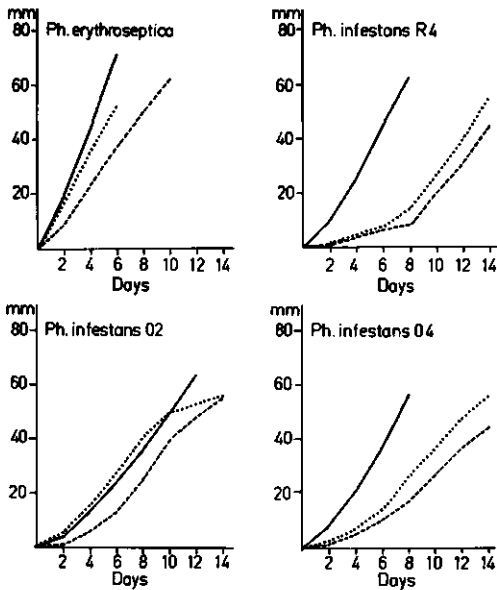
Fig. 2.

Growth of *Phytophthora ery-throseptica* and three stains of *Phytophthora infestans* on pea-juice agar (——), a defined medium containing amino acid mixture I (---), and the latter medium supplemented with soya-bean phospholipids (...).

the three tested strains of *Ph. infestans* phospholipids stimulated growth when added to the medium with amino acids. Mostly, stimulation was highest in the earlier growth phases and became less pronounced in the logarithmic phase. With strain 02 of *Ph. infestans*, the growth rate on the medium with added phospholipids was even higher than that on pea-juice agar during the first 8 days. Compared with the previous experiment (cf. Fig. 1), strain 04 showed a somewhat higher growth rate on pea-juice agar; relative growth rates on the defined media, therefore, were only seemingly much smaller in this experiment.

Comparison of different mixtures of amino acids:

Growth of three strains of *Ph. infestans* on basal medium supplemented

with phospholipids and amino acid mixture I was compared with that on the same medium, but supplemented with the same amino acids, some of them at other concentrations (amino acid mixtures II and III, cf. Table 1). Mixture II contained 0.5 mM of each amino acid except for asparagine and glutamine which were present at concentrations higher than 0.5 mM. Mixture III was composed like mixture I except for a higher concentration of asparagine and lower concentrations of arginine and glutamine. The experiment was carried out with strains 02, 04 and R4, and linear growth was measured after 10, 12 and 14 days, respectively. The results are prsented in Table 3. Mixtures II and III did not differ significantly from each other with respect to their

Table 3: Growth of *Phytophthora infestans* on defined media containing mixtures of 13 amino acids at different concentrations.

Amino acid mixture:	Linear mycelium growth, mm:		
	Strain 02 (10 days)	Strain 04 (12 days)	Strain R4 (14 days)
I	49	55	48
II	59***	28**	31***
III	62***	30*	30***

*, **, ***, significantly different from the mean growth rate on the medium containing mixture I at P = < 0,05, 0,01 and 0,001 respectively (*t* test).

ability to support growth of the three strains of *Ph. infestans*. Differences in growth between the medium containing amino acid mixture I on one hand, and mixtures II and III on the other, were significant in all cases. With strain 02, growth on the latter media was better than on the medium containing mixture I, with strains 04 and R4 the opposite was true.

   A problem remaining to be solved was, whether any of the amino acids in the mixtures was an essential growth factor for any of the strains of *Ph. infestans*. Therefore, growth of three strains of the fungus on basal medium supplemented with amino acid mixture III and phospholipids was compared with that on the same medium containing fewer amino acids. According to their supposed pathways of biosynthesis, amino acids were omitted alone or to-gether with others which have the same precursor (cf. MAHLER and CORDES 1971). Linear growth was measured after 6, 12 and 14 days for strains 02, 04 and R4, respectively. The results are presented in Table 4. With strain 02, a significant reduction of growth occurred only in the absence of γ-amino butyric acid plus glutamine. Strain 04 showed a decreased growth in the

absence of alanine, glycine, valine plus leucine, and in the absence of γ-amino butyric acid plus arginine. With strain R4, growth was decreased in

Table 4: Growth of *Phytophthora infestans* on a medium containing a mixture of amino acids and on the same medium with amino acids as indicated omitted (cf. Table 1).

Amino acids omitted from mixture III:	Linear mycelium growth, mm of strain:		
	02 (6 days)	04 (12 days)	R4 (14 days)
None	31	30 [*]	30 [*]
aba, arg	31	43 [*]	38 [*]
ala, gly, val, leu	29	43 [*]	26
his	32	31	18 [**]
ser	30	23	12 [*]
aba, gln	22 [***]	28 [**]	14 [*]
ile, lys, thr	31	11 [**]	11 [***]

*, **, ***, significantly different from the mean growth on the medium containing the complete mixture III at P = < 0.05, 0.01 and 0.0001 respectively (*t* test).

the absence of serine, and in the absence of γ-amino butyric acid plus glutamine. Growth of this strain was enhanced when γ-amino butyric acid plus arginine were omitted from the medium.

From the results presented in Tables 3 and 4 it became evident that maximal growth of *Ph. infestans* on defined nutrient media depended on the presence of some specific amino acids in the medium, but also on their concentration ratio. The optimal composition of the medium, however, was different for the three strains tested.

DISCUSSION

Results from this study again substantiate that a successful cultivation of *Ph. infestans* on defined nutrient media largely depends on appropriate nitrogen sources. These requirements were met by addition of amino acid mixtures to a basal medium containing glucose, some mineral salts, trace elements and thiamine. On this medium, however, the initial growth rate of the four strains of *Ph. infestans* was lower than on pea-juice agar. With one of these strains, R4, it was demonstrated that an initial growth rate comparable to that on pea-juice agar could only be achieved when in addition several other fractions from peas were supplemented to the medium (MIESKES

and FEHRMANN, unpublished results). One constituent of these fractions was identified as ascorbic acid, which is probably important to provide a high redox potential of the medium (cf. CUPPETT and LILLY 1973). HOHL (1975) reported that in his experiments some strains of *Ph. infestans* were auxotrophic for some purine bases. Probably, these are also present in pea-juice, and might stimulate growth of some of our strains. The initial growth rate of our strains was also promoted by soya-bean phospholipids; in this way, the growth rate of one strain (02) was even higher than on pea-juice agar.

A more detailed study on the requirements of *Ph. infestans* for amino acids revealed that they all are more or less dispensable. Evidently, to a certain extent any amino acid is interchangeable by others, but apparently this phenomenon depends on the tested strain. There was no indication for any auxotrophy for a specific amino acid. This fact would mean that the fungus is able to synthesize all amino acids by itself. In most organisms, inorganic nitrogen - in the case of nitrate after several reduction steps - is primarily incorporated into glutamic acid which in turn serves as the nitrogen source for the other amino acids (FOWDEN 1967, MAHLER and CORDES 1971, PATEMAN and KINGHORN 1976, and many others). The carbon skeletons are supplied via the common pathways of intermediary metabolism. Since *Ph. infestans* requires several amino acids apart from glutamic acid or glutamine, this fact probably means that the synthesis of their direct precursors proceeds only slowly.

It has been suggested that, as a consequence of the low respiratory activity of *Ph. infestans*, reactions which depend very much on energy metabolism should also proceed slowly (SCHEEPENS and FEHRMANN 1977). Thus, they should result in the synthesis of insufficient amounts of some amino acid precursors. On this basis, it is plausible that the fungus depends on *several* amino acids at a time which can be replaced by others to some extent. Possibly, amino acids can also be replaced by other intermediary products that are synthesized via the common pathways of intermediary metabolism. For further confirmation of this hypothesis, however, more detailed investigations on the intermediary metabolism of *Ph. infestans* are necessary.

SUMMARY

Growth rates of four strains of *Phytophthora infestans* on a synthetic

medium containing glucose, some mineral salts, thiamine and a mixture of 13 amino acids, were initially lower than those on pea-juice agar, but during the subsequent logarithmic phase they became approximately the same on both media. Addition of other nitrogen sources such as glutamine, acid-hydrolyzed peptone, or a hydrolyzed fraction from peas to the basal medium resulted only in limited growth of these strains.

The initial growth rate of three strains of *Ph. infestans* increased by addition of soya-bean phospholipids to the basal medium with amino acids; with one strain it was even higher than on pea-juice agar.

No auxotrophy for any amino acid could be observed for any of the three strains tested. Omitting certain amino acids from the medium hardly affected growth, while omitting others reduced growth to a large extent. These effects varied with different strains. The growth rates were also dependent on the concentration ratio of the amino acids in the mixture. The results suggest that it is not possible to divise a fully synthetic nutrient medium meeting all requirements of every strain of *Ph. infestans*.

The high nutritional demands of *Ph. infestans* are most likely due to a limited activity of some pathways of intermediary metabolism, especially that of the citric acid cycle. From this point of view, the limited acitvity of the mitochondrial electron transport chain could be the primary cause of the synthesis of only insufficient amounts of some amino acids, possibly also of other low-molecular-weight compounds such as phospholipids.

ZUSAMMENFASSUNG

Wachstum von *Phytophthora infestans*
auf definierten Nährmedien

Die Wachstumsraten von vier Stämmen von *Phytophthora infestans* auf einem Medium, das neben Glucose, einigen Mineralsalzen und Thiamin, ein Gemisch von 13 Aminosäuren enthielt, waren zunächst geringer als jene auf Erbsensaftagar. Sie wurden während der anschliessenden logarithmischen Phase letzteren aber vergleichbar. Zusatz von Glutamin, säurehydrolysiertem Pepton oder einer hydrolysierten Fraktion aus Erbsen, die verschiedene Aminosäuren enthielt, ermöglichte nur ein beschränktes Wachstum des Pilzes.

Die anfängliche Wachstumsrate der drei getesteten Stämme von *Ph. infestans* konnte durch die zusätzliche Zugabe von Phospholipiden aus Sojabohnen noch gesteigert werden. Bei einem Stamm war das Wachstum dann sogar besser als auf Erbsensaftagar.

Keine der getesteten Aminosäuren war für *Ph. infestans* essentiell im Sinne einer spezifischen Auxotrophie. Das Fehlen gewisser Aminosäuren im Medium beeinflusste das Wachstum kaum, während das Fehlen anderer Aminosäuren das Wachstum stark beeinträchtigte. Diese Einwirkungen variierten je nach geprüftem Stamm. Die Wachstumsraten waren auch von der relativen Konzentration der einzelnen Aminosäuren im Medium abhängig. Die Ergebnisse deuten daraufhin, dass es nicht möglich ist, ein für diese Pilzart allgemeingültiges vollsynthetisches Nährmedium zu entwickeln, das die Ernährungsansprüche aller Stämme gleichermassen voll erfüllt.

Die hohen Nährstoffanforderungen von *Ph. infestans* können durch eine geringe Aktivität einiger Wege des Intermediärstoffwechsels, speziell des Zitronensäurezyklus, einleuchtend erklärt werden. Die geringe Aktivität der mitochondrialen Atmungskette dürfte die primäre Ursache dafür sein, dass bestimmte Aminosäuren und womöglich auch andere Verbindungen wie Phospholipide in unzureichendem Masse vom Pilz synthetisiert werden können.

REFERENCES

CLINTON, G.P., 1908: Report of the botanist: IV. Artificial cultures of *Phytophthora*, with special reference to oospores. Conn. Agric. Exp. Sta., Ann. Rpt. 32, 891-907.

CUPPETT, V.M. and V.G. LILLY, 1973: Ferrous ion and the growth of twenty isolates of *Phytophthora infestans* in synthetic media. Mycologia 65, 67-77.

FEHRMANN, H., 1971: Obligater Parasitismus phytopathogener Pilze. I. Vergleichende Wachstumsversuche mit *Phytophthora infestans* und verwandten Arten. Phytopath. Z. 70, 89-113.

FOUDIN, A.S., and W.K. WYNN, 1972: Growth of *Puccinia graminis f. sp. tritici* on a defined medium. Phytopathology 62, 1032-1040.

FOWDEN, L., 1967: Aspects in amino acid metabolism in plants. Ann. Rev. Plant Physiol. 18, 85-106.

HALL, A.M., 1959: The culture of *Phytophthora infestans* in artificial media. Trans. Brit. Mycol. Soc. 42, 15-26.

HENDRIX, J.W., 1964: Sterol induction of reproduction and stimulation of growth of *Pythium* and *Phytophthora*. Science 144, 1028-1029.

HENDRIX, J.W., 1965: Influence of sterols on growth and reproduction of *Pythium* and *Phytophthora* spp. Phytopathology 55, 790-797.

HENDRIX, J.W., 1970: Sterols in growth and reproduction of fungi. Ann. Rev. Phytopathology 8, 111-130.

HENNINGER, H., 1959: Versuche zur Kultur verschiedener Rassen von *Phytophthora infestans* (Mont.) de By. auf künstlichen Nährböden. Phytopath. Z. 34, 285-306.

HOHL, H.R., 1975: Levels of nutritional complexity in *Phytophthora*: Lipids, nitrogen sources and growth factors. Phytopath. Z. 84, 18-33.

HOLLOMON, D.W., 1966: Reducing compounds and the growth of *Phytophthora infestans*. J. Gen. Microbiol. 45, 315-324.

LANGCAKE, P., 1974: Sterols in potato leaves and their effects on growth and sporulation of *Phytophthora infestans*. Trans. Brit. Mycol. Soc. 63, 573-586.

MAHLER, H.R., and E.H. CORDES, 1971: Biological Chemistry, 2nd ed., Harper and Row, New York, Evanston, San Francisco, London.

PATEMAN, J.A., and J.R. KINGHORN, 1976: Nitrogen metabolism. In: The Filamentous Fungi. Vol. II. Biosynthesis and Metabolism, pp. 159-237. Ed. by J.E. Smith and D.R. Berry. Academic Press, New York and London.

PAYETTE, P.A., and C. PERRAULT, 1944: Action de la thiamine sur le *Phytophthora infestans* (Mont.) de Bary, Can. J. Res., Sect. C, 22, 127-132.

RONCADORI, R.W., 1965: A nutritional comparison of some species of *Phytophthora*. Phytopathology 55, 595-599.

SCHEEPENS, P.C., and H. FEHRMANN, 1977: Metabolic anomalies as a possible cause of biotrophy: Abbreviated electron transport in *Phytophthora infestans*. Physiol. Plant Pathol. 11, 71-78.

SCHEEPENS, P.C., and H. FEHRMANN, 1978a: Mitochondrial electron transport in *Peronosporales*. I. Isolation and properties of *Pythium debaryanum* and *Phytophthora infestans* mitochondria. Phytopath. Z. (in press).

SCHEEPENS, P.C., and H. FEHRMANN, 1978b: Mitochondrial electron transport in *Peronosporales*. II. Anomalous effects of ADP on the electron transport of *Phytophthora infestans, Phytophthora erythroseptica* and *Pythium debaryanum*. Phytopath. Z. (in press).

SCHROPP, W., 1951: Methodenbuch, Band VIII, "Der Vegetationsversuch". 1. Die Methodik der Wasserkultur höherer Pflanzen. Neumann Verlag, Berlin.

Address of the authors: Mr. P.C. SCHEEPENS and Prof.Dr. H. FEHRMANN, Institut für Pflanzenpathologie und Pflanzenschutz, Grisebachstrasse 6, D-3400 Göttingen-Weende. Requests for reprints should be addressed to the second author.

Obligate fungal parasites grow and reproduce only in association with living host plants and cannot be cultured axenically. As is known from the literature, they are able to synthesize nucleic acids, proteins and other cellular constituents from low-molecular-weight precursors. However, their synthetic capacity is only limited when compared with that of saprophytic and facultatively parasitic fungi. From such observations some authors have suggested that derepression of genes is necessary for continued growth of the obligate parasite (BRIAN 1972, CHAKRAVORTY and SHAW 1977, and others). Substances that act as derepressors should move from host to parasite. SCOTT and MACLEAN (1969) suggested that the development of rust colonies from individual sporelings *in vitro* is associated with a change in the expression of the rust genome with consequent reorientation of rust metabolism. However, no substantial proof of these hypotheses has been given; the nature of substances that could act as derepressors is still unknown. Alternatively, the limited synthetic capacity of obligate parasites might be explained on the basis of an insufficiently operating intermediary metabolism, resulting in the synthesis of insufficient amounts of essential precursors for biosynthetic processes. In the latter case, growth of obligate parasites is supported by a supply of low-molecular-weight substances; synthesis of new proteins is not absolutely required. In the present study, results are presented to substantiate that this supposition might be true for the biotrophic fungus *Phytophthora infestans*.

FEHRMANN (1971a,b) found indications that the energy metabolism of *Ph. infestans* might be disordered. For that reason, this study was focussed on mitochondrial electron transport. *Ph. erythroseptica* and *Pythium debaryanum*, representing lower levels of specialization within the *Peronosporales*, were included in this study.

A method was developed for the isolation of mitochondria from mycelium of the three fungi. With this method the characteristic structure of these organelles was preserved, although part of the outer membranes was damaged. At least two facts indicate that their functional integrity was not fully preserved: oxidative phosphorylation was partly uncoupled from respiration, and the inner membrane system was permeable to NADH. Uncoupling of oxidative phosphorylation was deduced from the observation that stimulation of respiration upon addition of ADP was smaller than in mitochondria of other fungi (cf. LLOYD 1974), and that respiration rates did not decline when the added ADP should have been exhausted. Furthermore, respiration was only

slightly stimulated upon addition of the uncoupler CCCP. Permeability of the mitochondrial inner membranes to NADH was suggested by the observation that a substantial part of this compound was oxidized via the rotenone-sensitive electron transport system in *P. debaryanum* and *Ph. erythroseptica*. Mitochondrial inner membranes of animals, plants and some fungi have been shown to be impermeable to NADH (CEDERBAUM et al. 1973, LLOYD 1974, PALMER 1976). Therefore, it is likely that mitochondria of *P. debaryanum* and *Ph. erythroseptica* have become "leaky" upon isolation. It would be meaningless to compare energy-dependent reactions of the three fungi such as oxidative phosphorylation or oxidation-reduction kinetics of cytochromes. However, the comparable quality of mitochondrial preparations of the three fungi would justify the study of other characteristics such as measuring electron transport rates and determining the presence of additional respiratory pathways.

As compared with mitochondria of *P. debaryanum* and *Ph. erythroseptica*, those of *Ph. infestans* showed two exeptional features: a low activity of the main electron transport system and the presence of an alternative pathway for the oxidation of NADH. Mitochondrial respiration of *Ph. infestans* was greatly enhanced upon addition of the artificial electron transport mediator TMPD; in the presence of this chemical, electrons selectively bypass the antimycin A-sensitive, cytochrome c-directed region of cytochrome(s) b. This fact indicates that the narrow-pass in the respiratory chain is located in the cytochrome b region which is circumvented upon addition of TMPD. On the other hand, respiration of *P. debaryanum* mitochondria was only slightly stimulated upon addition of this dye.

Exogenous NADH - in contrast to succinate and endomitochondrially generated NADH - was rapidly oxidized by mitochondria of *Ph. infestans*. Oxygen consumption and cytochrome c reduction with NADH as substrate were insensitive to rotenone and antimycin A; oxygen consumption with this substrate was sensitive to cyanide. These facts indicate that exogenous NADH is oxidized via an abbreviated electron transport pathway which is connected with the cytochrome c region of the main electron transport chain, thereby circumventing the postulated narrow-pass. This NADH-shunt was found to be also present in *P. debaryanum* and *Ph. erythroseptica*, but here it did not contribute much to the oxidation of NADH. Thus far, it has not yet been described for any other fungus. If its demonstration was due to an artifact of isolation, a comparable order of magnitude should be expected in the two other species.

As a consequence of the low respiratory activity reactions that are directly coupled to respiration should also proceed slowly, e.g. those of

oxidative phosphorylation and the citric acid cycle. Then, only limited amounts of ATP could be synthesized from ADP, and only small amounts of essential precursors could be synthesized from the citric acid cycle. The presence of the additional electron transport pathway in *Ph. infestans* could to some extent overcome this disorder. Although this shunt allows the synthesis of maximally 1 mole ATP per mole NADH, metabolic interconversions could proceed somewhat faster than in its absence under certain conditions. One of these conditions is, that mechanisms are present for the transport of reducing equivalents from the mitochondria to the cytoplasm. The presence of such shuttle mechanisms has been demonstrated in animals, higher plants and the yeast *Saccharomyces carlsbergensis* (VON JAGOW and KLINGENBERG 1970, CEDERBAUM et al. 1973, PALMER 1976). However, too little is known about the intermediary metabolism of *Ph. infestans* for definitive statements in this direction. Only the presence of the abbreviated pathway in a fungus which could need it (while a narrow-pass is present in the main electron transport chain) suggests that it could play a functional role in a modified intermediary metabolism. The possible role of abbreviated, non-phosphorylating electron transport pathways in green plants has been discussed by PALMER (1976). Since these organisms can derive ATP from photosynthesis, the most important function of mitochondria in green tissues is probably not the conservation of energy, but the synthesis of carbon skeletons for biosynthetic purposes. The same might be true for obligate parasites, and also for *Ph. infestans*, which might derive energy-rich compounds from their host plants.

In a study of FERHMANN (1971b) it was shown that the oxidation of NADH by cell-free preparations of *P. ultimum*, *Ph. infestans* and other *Phytophtora* species can be influenced by adenosine phosphates, especially by ADP. The effects of ADP on NADH-oxidation ranged from a strong inhibition in *Ph. infestans* to a stimulation in *Ph. erythroseptica*. From these results one might conclude that ADP might play a role - beyond its function in oxidative phosphorylation - in the regulation of electron transport. In this respect, it was of interest to look for the site(s) of action of ADP, especially to see whether the cytochrome b region - localized as a narrow-pass in the electron transport chain of *Ph. infestans* - was involved.

Mitochondrial respiration of the three fungi tested with succinate as substrate was stimulated by low concentrations of ADP. This stimulation was demonstrated to be based on the coupling of oxidative phosphorylation to respiration. High concentrations of the chemical were inhibitory. The pattern of inhibition was almost the same for the three fungi. By employing specific inhibitors and different combinations of substrates and electron

acceptors the site of inhibition by ADP could be localized in the cytochrome c region of the respiratory chain. With each combination of electron donor and acceptor inhibition was about the same for the three fungi. Since *Ph. erythroseptica* and *P. debaryanum* grow well on basal nutrient media, it is highly improbable that this anomalous inhibition of respiration by ADP is the cause of the reduced capacity of *Ph. infestans* to grow on these media. Because of the relatively high concentrations of this chemical needed for a significant inhibition, it is doubtful whether it has any physiological significance at all.

In a physiological study, some specific nutritional requirements of *Ph. infestans* were investigated in order to find out whether they could be related to the anomalous electron transport of this fungus.

Four strains of the fungus grew well on a defined nutrient medium containing only low-molecular-weight substances: glucose, some mineral salts, thiamine and a mixture of several amino acids. However, growth rates in the lag phase were lower than those on a medium supplemented with pea juice. Growth rates could be somewhat improved when a mixture of phospholipids was added to the medium containing amino acids. It has been demonstrated by other authors, that other substances such as sterols, ascorbate, and cysteine, can also slightly stimulate growth of this fungus (HOLLOMON 1966, FEHRMANN 1971a, CUPPETT and LILLY 1973, HOHL 1975 and others). In general, growth stimulation by these compounds was more pronounced in the lag phase than during the logarithmic phase.

A more detailed study on the requirement of *Ph. infestans* for amino acids revealed that they were all more or less dispensable. However, some of them could be omitted from the medium without affecting growth, while omitting others reduced growth to a large extent. In agreement with HOHL (1975) we can conclude that it is more important that several amino acids are present in the medium than some special ones. This fact would mean that the fungus is able to synthesize all amino acids by itself, but rates of biosynthesis are low when no proper precursors are added to the medium. Since some amino acids can evidently act as precursors of others, it is likely that the synthesis of their carbon skeletons is a growth-limiting factor when the fungus is cultivated on basal nutrient media. Theoretically, it could also be possible that transaminations are rate limiting in the synthesis of amino acids, but in view of the fact that several of them are necessary for good growth, this supposition is not likely.

The complex nutritional requirements of *Ph. infestans* can likely be explained on the basis of an anomalous electron transport. The narrow-pass in

the main electron transport chain and the alternative pathway for NADH-oxidation should modify the intermediary metabolism of the pathogen. Good growth can only be guaranteed when certain key substances (amino acids, phospholipids) are supplied by the host *in vivo* or added to the nutrient medium *in vitro*. However, the results do not exclude the presence of other growth-limiting steps in the metabolism of this fungus. For further confirmation more detailed investigations, especially on the intermediary metabolism of *Ph. infestans*, are necessary. Nevertheless, the results indicate that it should be possible for *Ph. infestans* to grow *in vivo* and *in vitro* without its metabolic machinery being drastically changed. In view of the fact that some rust fungi - until recently considered to be obligate parasites - can now be cultivated on defined nutrient media, it is not unlikely that this conception can also be true for these fungi and other obligate parasites. For that reason, this work might stimulate studies on the nature of obligate parasitism in the future.

REFERENCES

BRIAN, P.W., 1972: The metabolic background of disease resistance. In: "The way ahead in plant breeding". Proc. 6th Congr. Eucarpia, Cambridge 1972.

CEDERBAUM, A.I., C.S. LIEBER, D.S. BEATTIE, and E. RUBIN, 1973: Characterization of shuttle mechanisms for the transport of reducing equivalents into mitochondria. Arch. Biochem. Biophys. 158, 763-781.

CHAKRAVORTY, A.K., and M. SHAW, 1977: A possible molecular basis for obligate host-pathogen interaction. Biol. Rev. 52, 147-179.

CUPPETT, V.M., and V.G. LILLY, 1973: Ferrous ion and the growth of twenty isolates of *Phytophthora infestans* in synthetic media. Mycologia 65, 67-77.

FEHRMANN, H., 1971a: Obligater Parasitismus phytopathogener Pilze. I. Vergleichende Wachstumsversuche mit *Phytophthora infestans* und verwandten Arten. Phytopath. Z. 70, 89-113.

FEHRMANN, H., 1971b: Obligater Parasitismus phytopathogener Pilze. II. Vergleichende biochemische Untersuchungen zum Energiestoffwechsel von *Phytophthora*-Arten. Phytopath. Z. 70, 230-262.

HOHL, H., 1975: Levels of nutritional complexity in *Phytophthora*: Lipids, nitrogen sources and growth factors. Phytopath. Z. 84, 18-33.

HOLLOMON, D.W., 1966: Reducing compounds and the growth of *Phytophthora infestans*. J. Gen. Microbiol. 45, 315-324.

LLOYD, D., 1974: The Mitochondria of Microorganisms. Academic Press, New York and London.

PALMER, J.M., 1976: The organization and regulation of electron transport in plant mitochondria. Ann. Rev. Plant Physiol. 27, 133-157.

SCOTT, K.J., and D.J. MACLEAN, 1969: Culturing of rust fungi. Ann. Rev. Phytopathol. 7, 123-146.

VON JAGOW, G., and M. KLINGENBERG, 1970: Pathways of hydrogen in mitochondria of *Saccharomyces carlsbergensis*. Eur. J. Biochem. 12, 583-592.

SUMMARY AND CONCLUSIONS

Phytophthora infestans, being an intermediate type between the facultative and obligate parasites among the *Peronosporales* (class *Oomycetes*), was used to study the factors which are responsible for the inability of obligate parasites to grow axenically. In a comparative study on mitochondrial electron transport of *Ph. infestans*, *Ph. erythroseptica* and *Pythium debaryanum*, three species representing decreasing levels of specialization within the *Peronosporales*, the exceptional position of *Ph. infestans* was emphasized (papers I, II and III). The anomalous electron transport of the fungus is possibly the primary cause of general metabolic disorders, resulting in its complex nutritional requirements as described in paper IV.

A method for the isolation of mitochondria from mycelium of *P. debaryanum* and *Ph. infestans* was developed. With this method, the structural integrity of the organelles of both species was essentially preserved; however, oxidative phosphorylation was partially uncoupled, indicating that mitochondrial function was not fully intact.

Results from studies on respiration and cytochrome c reduction, using various substrates and specific inhibitors, indicate that in *Ph. infestans* as well as in *Ph. erythroseptica* and *P. debaryanum* mitochondria, an electron transport system similar to that described for other organisms was present. Cyanide-resistant respiration, as described for some higher plants, algae and fungi, was absent in these species.

In *Ph. infestans* mitochondria respiration rates with the substrates pyruvate plus malate, α-oxoglutarate, and especially succinate, were much lower than in *Ph. erythroseptica* and *P. debaryanum*. Evidence was presented that a narrow-pass, located in the cytochrome(s) b region, limited the electron flux in the electron transport chain of *Ph. infestans*. Addition of the artificial electron transport mediator N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) to the reaction mixture enhanced respiration by about 140% in the case of *Ph. infestans*, but only by 30% with *P. debaryanum*.

In *Ph. infestans* an additional pathway was found to operate which permitted the oxidation of externally added NADH at a much higher rate than that of pyruvate plus malate, α-oxoglutarate and succinate. Its demonstration with both cytochrome c and oxygen as electron acceptors, its insensitivity to rotenone and antimycin A, and its sensitivity to cyanide indicate that this pathway is of mitochondrial origin, and connected with the main electron transport chain in the cytochrome c region, bypassing the postulated narrow-pass. This pathway was also found to be present in *Ph. erythroseptica* and

P. debaryanum mitochondria, but it did not contribute much to the oxidation of NADH in these species.

Respiration of *Ph. infestans, Ph. erythroseptica* and *P. debaryanum* mitochondria with succinate as substrate was stimulated by low concentrations of ADP. This stimulation was due to the coupling of oxidative phosphorylation to respiration. Higher concentrations of the chemical were inhibitory. The extent of inhibition was about the same in the three species. By employing specific inhibitors of mitochondrial respiration and different combinations of substrates and electron acceptors, the influence of ADP on several segments of the electron transport chain of the three fungi was measured. In this way, the site in inhibition by ADP was localized in the cytochrome c region of the electron transport chain. Because of the relatively high concentrations of ADP that are necessary for a significant inhibition and the similarity of the inhibition pattern in the three fungi tested, this anomalous inhibition of respiration by ADP is not likely related to the inability of *Ph. infestans* to grow on basal nutrient media.

Linear growth rates of four strains of *Ph. infestans* on a defined nutrient medium containing glucose, some mineral salts, thiamine and a mixture of 13 amino acids were initially lower than on pea-juice agar, but during the subsequent logarithmic phase they became approximately the same on both media. Addition of other nitrogen sources such as glutamine, acid-hydrolized peptone, or an hydrolyzed fraction from peas to the basal nutrient medium resulted only in limited growth of these strains.

The initial growth rate of three strains of *Ph. infestans* was increased by addition of soya-bean phospholipids to the basal medium plus amino acids; with one strain it was even higher than on pea-juice agar.

No auxotrophy for any amino acid could be observed for any of the three strains tested. Omitting certain amino acids from the medium hardly affected growth, while omitting others reduced growth to a large extent. These effects varied with different strains of the fungus. Growth rates were also dependent on the concentration ratio of the amino acids. The results suggest that it is not possible to devise a synthetic nutrient medium meeting all the requirements of every strain of *Ph. infestans*.

The demand of *Ph. infestans* for several amino acids at a time which can to some extent be replaced by others, is likely due to a limited activity of some pathways of intermediary metabolism. From this point of view, the limited activity of the mitochondrial electron transport could be the primary cause of the fungus' inability to synthesize sufficient amounts of some amino acids, possibly also of other low-molecular-weight compounds such as phos-

67

pholipids. Good growth of the fungus is only guaranteed when such substances are provided from the host *in vivo* or added to the nutrient medium *in vitro*.

SAMENVATTING

Phytopthora infestans, die als een overgangsvorm tussen de facultatief
en obligaat parasitaire schimmels binnen de *Peronosporales* (klasse *Oomycetes)*
kan worden beschouwd, werd als toetsschimmel gebruikt om na te gaan, welke
faktoren een autonome groei van obligaat parasitaire schimmels *in vitro* ver-
hinderen. In een vergelijkende studie over het mitochondriale electronen-
transport van *Ph. infestans, Ph. erythroseptica* en *Pythium debaryanum*, die
in deze volgorde afnemende specialisatieniveaus binnen de *Peronosporales*
vertegenwoordigen, werd de uitzonderlijke positie van *Ph. infestans* in deze
groep benadrukt (Publicaties I, II, en III). Het abnormale electronentrans-
port van de schimmel is mogelijk de primaire oorzaak van andere storingen in
zijn metabolisme, resulterend in de complexe voedingseisen, zoals die in
publicatie IV worden beschreven.

Er werd een methode ontwikkeld voor het isoleren van mitochondrien uit
mycelium van *P. debaryanum* en *Ph. infestans*. Met deze methode bleven de
structurele eigenschappen van deze organellen in hoofdzaak behouden; het ge-
deeltelijk ontkoppeld zijn van de oxydatieve fosforylering duidde er evenwel
op, dat hun functie gedeeltelijk gestoord was.

Resultaten van studies over de ademhaling en de cytochroom c reductie bij
gebruikmaking van verschillende substraten wezen erop, dat zowel in mito-
chondrien van *Ph. infestans* als van *Ph. erythroseptica* en *P. debaryanum* een
electronentransport systeem aanwezig was, dat vergelijkbaar was met dat van
andere organismen. Cyanide-resistente ademhaling, zoals die bij enige hogere
planten, algen en schimmels is gevonden, ontbrak bij deze schimmels.

In mitochondrien van *Ph. infestans* waren de ademhalingsintensiteiten met
de substraten pyruvaat plus malaat, α-ketoglutaraat, maar vooral succinaat,
veel geringer dan in die van *Ph. erythroseptica* en *P. debaryanum*. Toevoeging
van N,N,N',N'-tetramethyl-p-phenyleendiamine (TMPD) - een stof, die de
electronen rechtstreeks van cytochroom b naar cytochroom c overdraagt - aan
het reactiemengsel verhoogde de mitochondriale ademhalingsintensiteit van
Ph. infestans met 140%, die van *P. debaryanum* slechts met 30%. Dit wijst er-
op, dat bij *Ph. infestans,* in vergelijking met *P. debaryanum,* het electronen-
transport ter hoogte van cytochro(o)m(en) b stagneert.

In *Ph. infestans* was een extra weg aanwezig voor de oxydatie van toege-
voegd NADH, hetgeen zowel met zuurstof als met cytochroom c als electronen-
acceptor werd vastgesteld; hij was ongevoelig voor rotenon en antimycine A,
maar gevoelig voor cyanide. Deze feiten wijzen erop, dat deze weg van mito-
chondriale oorsprong is en ter hoogte van cytochroom c met de hoofdketen

verbonden. De plaats in de hoofdketen, waar het electronentransport stagneert, wordt hierbij vermeden. Deze weg was ook aanwezig in mitochondrien van *Ph. erythroseptica* en *P. debaryanum*, maar droeg hier slechts in geringe mate bij tot de oxydatie van NADH.

De ademhalingsintensiteit van mitochondrien van *Ph. infestans*, *Ph. erythoseptica* en *P. debaryanum* met succinaat als substraat werd door lage ADP-concentraties gestimuleerd. Deze stimulering was het gevolg van de koppeling van de oxydatieve fosforylering aan het electronentransport. Hogere concentraties van deze stof remden de ademhaling. De mate van remming was bij de drie getoetste schimmels ongeveer hetzelfde. Door het gebruik van specifieke remstoffen, en verschillende combinaties van substraten en electronenacceptoren werd de invloed van ADP op verschillende segmenten van de ademhalingsketen nagegaan. Op deze wijze werd vastgesteld, dat de plaats waar ADP zijn remmende werking uitoefent, ter hoogte van cytochroom c was gelocaliseerd. Omdat relatief hoge ADP-concentraties nodig waren om een duidelijke remming van de ademhaling te veroorzaken, en omdat het remmingspatroon van ADP bij alle drie toetsschimmels ongeveer gelijk was, is het onwaarschijnlijk, dat deze ongewoonlijke remming door ADP verband houdt met het onvermogen van *Ph. infestans* tot autonome groei op basale voedingsmedia.

De lineaire groeisnelheid van vier stammen van *Ph. infestans* op een gedefiniëerd voedingsmedium, dat naast glucose, enige minerale zouten en thiamine een mengsel van 13 aminozuren bevatte, was aanvankelijk geringer dan op erwtesap-agar. Gedurende de logarithmische fase waren de groeisnelheden op de beide media ongeveer gelijk. Toevoeging van andere stikstofbronnen aan het medium, zoals glutamine, in zuur gehydrolyseerd pepton, of een gehydrolyseerde fractie uit erwten, leidde slechts tot geringe groei van deze stammen.

De initiële groeisnelheid van drie stammen van *Ph. infestans* werd verhoogd door toevoeging van fosfolipiden uit sojabonen aan het medium met aminozuren; bij één stam was zij zelfs groter dan op erwtesap-agar.

In geen der getoetste stammen van *Ph. infestans* kon een auxotrofie voor enig aminozuur worden vastgesteld. Het weglaten van sommige aminozuren uit het medium beïnvloedde de groei nauwelijks, terwijl het weglaten van andere de groei in sterke mate reduceerde. De effecten variëerden met de gebruikte stam. De groeisnelheden waren ook afhankelijk van de verhouding, waarin de aminozuren werden toegediend. De resultaten wijzen erop, dat het niet mogelijk is een synthetisch voedingsmedium samen te stellen, waarop alle stammen van *Ph. infestans* optimaal groeien.

De gelijktijdige behoefte van *Ph. infestans* aan meerdere aminozuren, die

tot op zekere hoogte door andere kunnen worden vervangen, wordt mogelijk ver-
oorzaakt door een begrensde activiteit van enige wegen van het intermediair
metabolisme. Vanuit dit oogpunt zou de begrensde activiteit van het electro-
nentransport de primaire oorzaak kunnen zijn van het feit, dat de schimmel
niet in staat is bepaalde aminozuren, mogelijk ook andere laag-moleculaire
verbindingen zoals fosfolipiden, in voldoende hoeveelheden te synthetiseren.
Goede groei van de schimmel is alléén mogelijk, als zulke stoffen *in vivo*
via de waardplant worden verkregen, of *in vitro* aan het voedingsmedium wor-
den toegediend.

CURRICILUM VITAE

Petrus Carolus Scheepens werd op 18 januari 1946 te Vleuten geboren. In 1964 behaalde hij het H.B.S.-B diploma aan het Sint Bonifatius Lyceum te Utrecht. Hierna werden twee jaren achtereenvolgens op een chemisch laboratorium en op het ouderlijke groenteteeltbedrijf doorgebracht. In 1966 begon hij zijn studie aan de Landbouwhogeschool te Wageningen, waar op 23 januari 1973 het doctoraal-examen in de richting planteziektenkunde, met als hoofd-vak de fytopathologie, en de bijvakken biochemie (verzwaard) en erfelijk-heidsleer, met lof werd behaald.

Vanaf 16 april 1973 tot 1 juni 1977 was hij als wetenschappelijk mede-werker verbonden aan het "Institut für Pflanzenpathologie und Pflanzenschutz" van de universiteit te Göttingen, daarna tot 1 december 1977 als promotie-assistent bij de vakgroep Fytopathologie van de Landbouwhogeschool te Wageningen.

Sedert 1 december 1977 is hij als wetenschappelijk onderzoeker verbonden aan het Centrum voor Agrobiologisch Onderzoek te Wageningen.