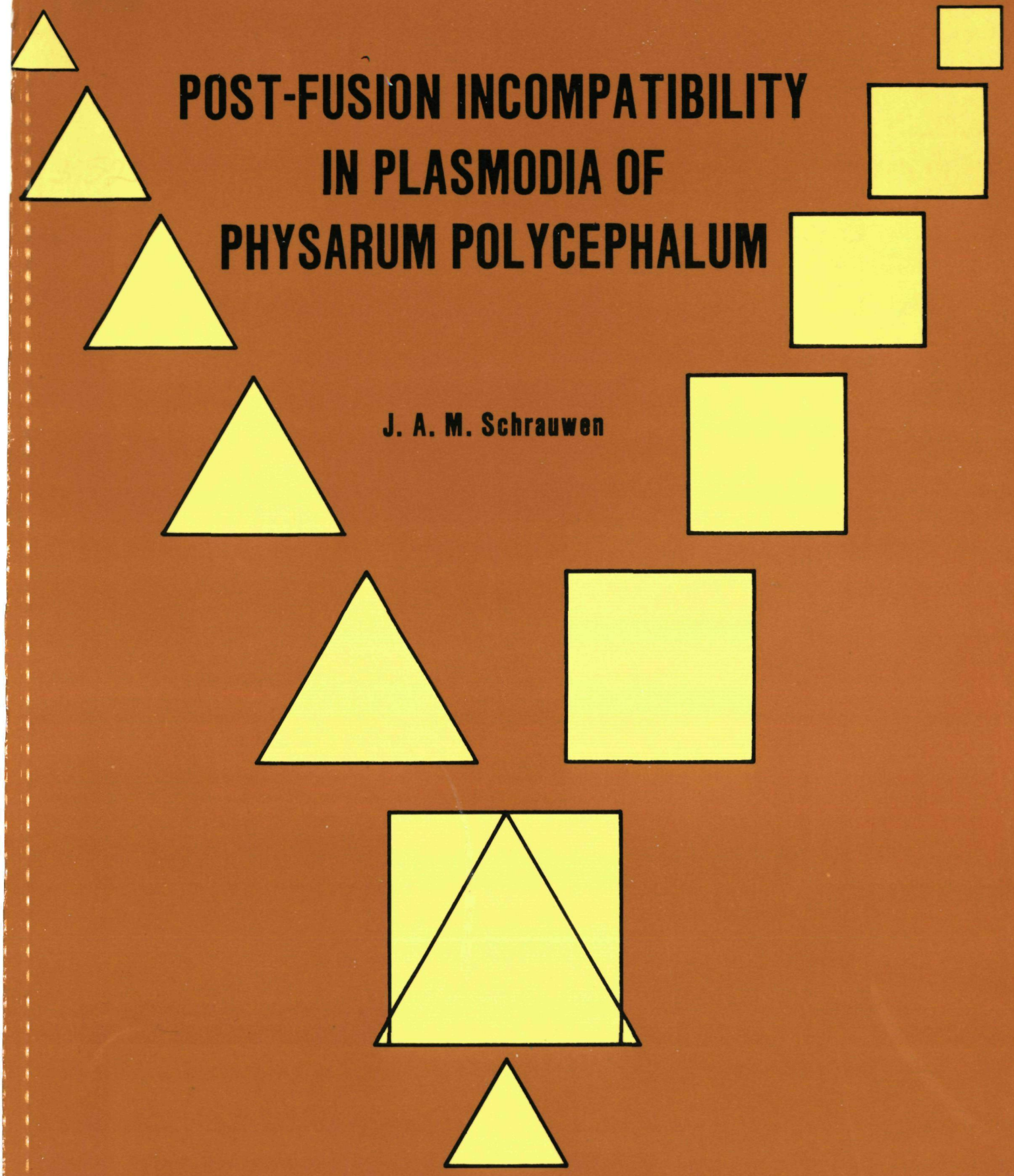
POST-FUSION INCOMPATIBILITY IN PLASMODIA OF PHYSARUM POLYCEPHALUM

J. A. M. Schrauwen



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Een onderzoek naar de physiologische veranderingen
in een heterokaryon plasmodium van
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Lia
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CONTENTS

1 GENERAL INTRODUCTION

The investigation of cellular interactions has made an extensive growth during the last decades. Topics in this field of research include:

a) inhibition of pollentube-growth or -development after self pollination in higher plants (Linskens and Kroh, 1967; Linskens 1975 a and b, 1981; de Nettancourt, 1984).

b) rejection of transplanted cells or organs in animals (Williamson, 1980)

c) allergic reactions (Howlet and Knox, 1984)

d) effect of non–adhesion after grafting of plants (Brabec, 1965; Yeoman, 1984)

e) elimination of cells or tissues: -after transporting the living material into another environment by exudates or toxins already present (bloodtransfusion, yeast) -after fusion and mixing of cytoplasms of two different cells (fungi,Carlile and Gooday, 1978; Paramecium,Tsukii and Hiwatashi, 1983; plantprotoplasts, Negrutiu et al, 1983)

All these interaction phenomena are characterized by initial "recognition" events, discussed already in 1877 by Darwin in a botanical context:..... "It may be said that the two pollens and the two stigmas mutually recognize each other" cited according Heslop–Harrison and Linskens, 1984.
All recognitions between the participating tissues or cells are determined genetically. It results in a non–acceptance of one cell type. This rejection reaction can de effected either by preformed compounds in one partner, or produced after the first contact (fungi,Schrauwen, 1984; Carlie and Gooday, 1978; Lane, 1981;allergy, Howlett and Knox, 1984).
This non–acceptance or non–tolerance of cells which in principle should be able to co–exist, is named incompatibility. The incompatibility phenomena occur in prokaryotes and eukaryotes and are widely spread in higher plants, (de Nettancourt, 1984; Heslop–Harrison and Linskens, 1984) and fungi (Esser and Blaich, 1973; Carlile and Gooday, 1979).
In these systems incompatibility is controlled by 1 or 2 genes mostly, with multiple alleles (de Nettancourt, 1984; Lane, 1981; Linskens and Kroh, 1967; Carlile and Gooday, 1979; Schrauwen, 1984).
In the animal body a direct contact between cell membranes is common. In plants the stiff cell wall however is a limiting factor for interaction studies between cells. Slime moulds provide a good experimental system for the study of cell fusion and their consequences. The lack of exgastic cell walls in slime molds make this organism as an excellent model for the investigation of incompatibility reactions. The slime mould can be cultured under laboratory conditions as a microorganism on defined or semi–defined media in the haploid as well as in the diploid form (Chaper 2, Fig 1). In our studies the diploid form could be obtained as microplasmodia in shake culture and as macro or surface plasmodia when cultured in petri dishes (Daniel and Baldwin, 1964; Schiebel, 1973). Several properties such as synchronous mitosis and differentiation, of *P.polycephalum* have evoked an intensive interest in this organism. This has resulted in well documentated observations on the physiological processes (Aelen et al, 1983; Hardman et al, 1982; Mohberg and Rusch, 1974; Turnock et al, 1981) and genetics (Anderson and Holt, 1981; Dee, 1973) in this slime mould. As in other slime moulds, the cytoplasm of *P. polycephalum* is surrounded by a plasma membrane and a slime layer, thin at the border of a well growing one day old plasmodium cultured on growth medium (Huttermann, 1973; Rusch, 1970). This unique envelope system of *P.polycephalum,* together with the genetical and physiological knowledge described above, makes this slime mould an excellent model for both studies on interactions between cells after fusion and their contact.

Somatic fusion

When two macroplasmodia of *P.polycephalum* approach each other they fuse if the plasmodia are of the same age and have identical *fus* (fusion) genes (Carlile, 1976; Carlile and Dee, 1967; Cooke and Dee, 1975; Collins, 1979; Lane, 1981; Schrauwen, 1984).

Contact of two plasmodia of different strains not followed by fusion may result in the growth of the plasmodia along or even over each other.
Fusion of two plasmodia of different strains may be followed by a post–fusion reaction that destroys the fused plasmodium partially (gedeeltelijk) and sometimes totally (Carlile and Dee, 1967; Collins, 1979; this thesis). The failure to fuse is indicated as fusion–incompatibility and the destruction with post-fusion incompatibility. Both reactions are genetically determined and are associated to heterogenetic incompatibility (Esser and Blaich, 1973; Carlile and Gooday, 1978; Lane, 1981; Schrauwen, 1984). The number of loci that govern fusion are about 13 (Collins, 1982; Schrauwen, 1984). Interactions after fusion are controlled by multiple pairs of alleles, "dominant" and "recessive" (Carlile, 1976; Carlie and Gooday, 1978). This means two plasmodia of *P.polycephalum* fuse and form a heterokaryon if their fusion loci are identical. This fusion may be followed by the destruction of a part or of the total heterokaryon, called lysis or the lethal reaction, if the composition of the *let* (lethal) alleles is different (Schrauwen, 1982). In the absence of sufficient nutrients or with great differences in plasmodial size of the two strains at the moment of fusion no lethal reaction appears. However, the fused heterokaryon obtains the properties of one strain within 48 hours after fusion irrespective of the occurrence of a partial lysis reaction (Carlile, 1972). The plasmodium that survives after the fusion of two different plasmodia, with a partial or without a self-destruction reaction, is described as the killer strain; the other is called the sensitive strain.
The properties of the killer or sensitive strain is dependent on the set of *let* alleles of the two mating types. The strain with major dominant *let* alleles will be the killer. This means that a strain can be the killer type in one fusion reaction and the sensitive type after fusion with another strain.
The lethal reaction is expressed in *P.polycephalum* 4–6 hours after fusion.
The properties of *P.polycephalum* summerized above make the slime mould an excellent model–system for incompatibility studies at the molecular level.
This thesis describes a number of studies on somatic incompatibility with this model organism.

Cellular interactions in slime moulds

For a better understanding of the genetical background about the cellular interactions in slime moulds, we reviewed articles including those on the life cycle of *P.polycephalum.*
The fusion of two incompatible plasmodia is shown in three figures.

The lethal reaction as a function of fusion and

In many systems secrations are responsible for a killer-like reaction. Experiments to check the possible presence of comparable exudates in plasmodia of *P. polycephalum* are described in Chapter 3.
A destructive reaction can be obtained only after the fusion of a sensitive and killer plasmodium in the heterokaryon. Therefore, special attention has been paid to events occuring during the 6 hour period between the moment of fusion and the lysis reaction. This is the time period during which non–tolerance comes to expression. The genetical control of the post fusion–incompatibility reaction suggests the involvement of transcription and translation of genes in the post–fusion interaction processes. To obtain insight into these metabolic processes, ihibitors of RNA (Fouquet et al, 1975) and protein (Nations et al, 1974) synthesis are excellent tools with which to test the involvement of these macromolecules. Results of such experiments and their consequences are discussed in Chapter 3.

DNA as an inetermediair and target of the incompatibility processes.

In addition to translation (Chapter 3), transcription may also be a part of the series of reactions that enters into the lethal reaction. Minor (small) modifications at one or both DNA strands of the incompatible plasmodia prior to fusion may show the influence of DNA at the lethal reaction (Funderud et al, 1978). Results of experiments that describe the possible effect of DNA from both plasmodia on the lethal reaction are shown in Chapter 4.
Fusion of sensitive and killer plasmodia having great differences in size causes no lethal reaction (Carlile, 1972). However, the heterokaryon of killer and sensitive changed in a plasmodium with the properties of killer within 48 hours. There is a change of phenotype as well as the capacity to summon a lethal reaction after fusion with a sensitive plasmodium (Chapter 3). This suggests that in the heterokaryon the DNA of the sensitive strain has disappeared. To check this suggestion DNA strands of sensitive and killer plasmodium were marked in different manners before fusion. At different moments after fusion of both treated plasmodia, the DNA composition of the heterokaryon can be monitored after isolation of total DNA from the fused plasmodium. The results of this experiments are discussed in Chapter 4.

Membranes as a site of the incompatibility reaction

Lysis reactions in mammalian cells demonstrate comparable symptoms as these observed in heterokaryotic plasmodia of *P.polycephalum*. Though the mechanism of the reaction in mammalian cells is not clear, a disturbance of the bilayer appears to be involved in the lysis reaction (Lachman, 1983). Comparison of the turnover or synthesis of membranes in fused plasmodia, homokaryons as well as heterokaryons, might indicate the involvement of membranes in the post–fusion incompatibility reaction. Phospholipids are the main components of the lipid bilayer and their synthesis and turnover can be measured by the incorporation of phosphate. The results of such measurements are described and discussed in chapter 5.

Changes in properties of the heterokaryon

Results of experiments described in Chapter 3,4 and 5 suggest the involvement of proteins in the sensitization for the lethal reaction of the heterokaryon. Qualitative changes of proteins can be measured by gel electrophoresis and are described in Chapter 6.
The synthesis of different metabolic components in the heterokaryon suggests the presence of a number of intermediates in charge of the interactions in the heterokaryon. Different methods used to test the "formed" intermediates in plasmodia of *P.polycephalum* are described and discussed in Chapter 6.

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2 CELLULAR INTERACTIONS IN SLIME MOULDS

14 Cellular Interaction in Plasmodial Slime Moulds

J. A. M. SCHRAUWEN

14.1 Introduction

During this century progress in biology has been greatly facilitated by the use of model organisms which may be relatively unimportant in nature but which are good systems for the study of specific problems. The interaction between genetically different cells is a fundamental biological problem (e.g. transplantation of organs, blood transfusion, grafting of plants), and an excellent model organism for studying such problems is the myxomycete *Physarum polycephalum*, which can be grown rapidly on simple media in the laboratory; biochemical and genetical work on *P. polycephalum* has been carried out in many laboratories. Genetical work has also been carried out with a second species, *Didymium iridis*, which, however, has not been grown in pure culture on soluble media and therefore is less suitable for physiological and biochemical investigations. The life cycle and features of myxomycetes have been described extensively (ASHWORTH and DEE 1975, COLLINS and BETTERLEY 1982, DEE 1982, GOODMAN 1980, HAUGLI et al. 1980, LANE 1981, and OLIVE 1975). A brief description of the life cycle follows and is summarized in Fig. 1. The plasmodium, a mass of protoplasm containing up to several million nuclei, is not subdivided into cells and is surrounded by a plasma membrane and slime. The lack of the rigid outer wall gives the plasmodium a high flexibility, which is unique for such large cells. Starvation of a plasmodium may give rise to a sclerotium, a resting phase, which under suitable conditions will produce a plasmodium again. In the presence of light, a starving plasmodium forms sporangia. During this process the diploid nuclei undergo meiosis and haploid spores are finally released. When a spore germinates it produces a uninucleate amoeba, which may form a flagella and gives rise to a flagellate. In the absence of nutrients the amoebae or flagellates will develop into a haploid dormant stage, the thick-walled cyst. The fusion of two mating-compatible amoebae results in a zygote with a diploid nucleus. By growth and by coalescence with other zygotes a tiny plasmodium is formed. The remaining haploid amoebae may then be engulfed and digested. Finally in culture the plasmodium may grow to cover an entire Petri dish. A unique feature of a plasmodium is the shuttle protoplasmic streaming at rates of up to 1.5 mm s^{-1} (BRITZ Chap. 2.3, Vol. 7, this Series) with reversals within a minute. This shuttle streaming causes a balanced distribution of metabolic products over the whole plasmodium. When two plasmodia meet this can be followed by fusion and complete mixing or by non-fusion and growth of both plasmodia alongside each other. Fusion may be followed by a post-fusion incompatibility reaction. For *P. polycephalum*, sexual fusion

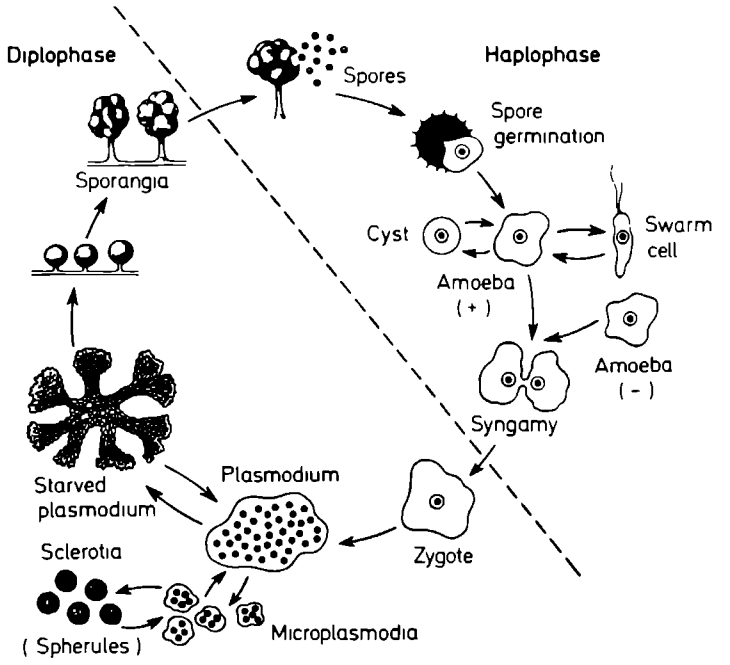

Fig. 1. Life cycle of *Physarum polycephalum*. (HÜTTERMANN 1973)

between amoebae, somatic fusion of plasmodia and the post-fusion incompatibility reaction are controlled genetically by different sets of genes. This offers an opportunity to study interactions between cells biochemically and genetically with both the haploid and the diploid phases of a single species.

These interactions will be discussed mainly for *P. polycephalum* and to a lesser extent for *D. iridis* and other myxomycetes.

14.2 Fusion Between Amoebae

During its life cycle, myxomycetes alternate between two morphologically distinct vegetative phases, the microscopic uninucleate amoebal phase and the macroscopic plasmodial phase (Fig. 1). In the normal life cycle of the slime mould two haploid amoeba undergo cell and nuclear fusion, giving rise to a diploid multinucleate plasmodium.

In some strains the step from amoeba to plasmodium occurs without sexual interaction. This can be shown by counting the chromosomes or estimating the amount of nuclear DNA in nuclei of amoebae and plasmodia. If the plasmodial phase is initiated by sexual fusion of amoebae, the amount of DNA per nucleus is twice as much in the plasmodia as in the amoebae, whereas asexual initiation does not cause a change in the nuclear DNA content.

DEE (1960) demonstrated that a single spore of *P. polycephalum* gives a clone of amoebae, which cannot on their own give rise to plasmodia. She concluded that the fusion of amoebae is controlled by a pair of alleles at a mating-

type locus, designated *mt* with alleles *mt-1* and *mt-2*. It is now known that the mating-type locus is multiallelic (Dee 1966) and at least 13 mating types of *P. polycephalum* and 12 of *D. iridis* could be demonstrated (Collins and Bitterley 1982).

So far each new diploid plasmodial isolate from nature has given amoebae showing two new mating types, based on two further alleles at the mating-type locus (Carlile and Gooday 1978, Collins 1979). Amoebae differing at the mating-type locus can fuse to form a plasmodium. The series of processes involved in the plasmodial development is, however, still largely unknown (Dee 1982).

14.2.1 *Physarum polycephalum*

Considerable attention has been given to the "amoebal plasmodial transition". Mutagenic treatment of amoebae can yield strains showing different defects in plasmodial development and lead to a better understanding of the processes involved. In 1960 Dee described the genetics of mating compatibility in a single locus, strain *mt*. Subsequently, many isolates from diverse geographic regions were studied by different groups. Their results, reviewed by Carlile and Gooday (1978) and Collins (1979) demonstrate that each isolate carries distinct *mt* alleles. At least 13 alleles are known (Collins 1979). So far most results of fusion experiments with mutants demonstrate that mutations affecting plasmodial development are located at or near the mating-type locus. Recent results suggest the involvement of other, unlinked loci in the development of diploid plasmodia from heterothallic amoebae. Adler and Holt (1975) found that cells heterozygous for the *mt* locus, especially those carrying the *mt*-h allele (which can permit haploid amoebae to develop into plasmodia by apogamy), developed more rapidly into plasmodia than cells homozygous at the *mt* locus. They suggested that the mating-type locus is a regulatory locus and that the role of the *mt* locus is to control the differentiation of zygotes into plasmodia. Dee (1978) identified in strains of *P. polycephalum* from different laboratories two alleles of a gene, *rac* (rapid crossing locus), unlinked to the *mt*-locus. Youngman et al. (1979) studied the rate and extent of plasmodium formation in mating tests involving pairs of largely isogenic amoebal strains compatible for *mt* alleles. They observed a systematic variability, plasmodia formed either rapidly and extensively or slowly and inefficiently, with a difference in rate of formation of more than a thousandfold. Their genetic experiments show the presence of a locus that affects amoebal cell fusion. Youngman et al. (1979) designated the new compatibility locus *mat B* and renamed the original *mt* locus *mat A*. The earlier described *rac* locus (Dee 1978) seems to be comparable with *mat B*. Kirouac-Brunet et al. (1981) confirmed these results, and found in each natural plasmodial isolate of *P. polycephalum* studied so far two different alleles for each *mat B* and each *mat A* locus. The presence of 13 alleles at each locus, *mat A* and *mat B*, has been demonstrated. In nature the probability of mating between amoebae increases with the presence of a large number of alleles for the *mat B* as well as for the *mat A* locus.

In an attempt to clarify the specific function of both mating-type genes YOUNGMAN et al (1981) carried out fusion experiments in mixtures of amoebae different in respect to their mating alleles They found that mixtures of haploid amoebae could either remain haploid or develop into stable diploid amoebae or into diploid amoebae that become plasmodia The proportion of cells which developed to a certain stage was determined by the composition of the mating alleles in the amoebae mixture. The data of Table 1 demonstrate a high frequency of diploid amoebae or plasmodia obtained from different mixtures YOUNGMAN et al. (1981) concluded that *mat A* and *mat B* separately regulated two discrete stages of mating. In the first stage, haploid amoebae fuse in pairs, with a specificity determined by *mat B* genotype, to form diploid zygotes. The differentiation of these zygotes into plasmodia is regulated by *mat A* and is unaffected by *mat B*. Microcinematographic studies of amoebal mixtures illustrated that cell fusion is extensive in *mat B* heterothallic mixtures, regardless of *mat A* genotypes and is very rare in *mat B* homoallelic mixtures (HOLT et al 1980). Further support for the *mat A* and *mat B* mating theory came from the analysis of mutations that affect differentiation. These results demonstrated that nearly all such mutations map very close to *mat A*, and indeed most have been inseparable from *mat A* by recombination (ADLER and HOLT 1977, ANDERSON and DEE 1977, ANDERSON and HOLT 1981, DAVIDOW and HOLT 1977, GORMAN et al 1979, HONEY et al. 1979, SHINNICK and HOLT 1977, SHIPLEY and HOLT 1982). As in other organisms the formation of a zygote can be brought about in different ways (BERGFELD 1977).

Table 1. The result of interaction between haploid amoebae as a function of the mating-type alleles (Based on the results of YOUNGMAN et al 1981)

Composition of mating-type alleles in haploid amoebae mixtures		Frequency of amoebae used	Formed plasmodia	Diploid amoebae
mat A	*mat B*	(%)		(%)
Unlike	Unlike	10	Many	–[a]
Like	Unlike	10[b]	None	5
Like	Like	0 1[b]	None	<0 1
Unlike	Like	0 1	Few	–[a]

[a] Diploid amoebae heterozygous for *mat A* cannot easily be isolated because they develop readily into plasmodia

[b] When *mat A* alleles alike, some fused amoebae separate without nuclear fusion and therefore do not give rise to diploid amoebae

In myxomycetes the possibilities of formation of plasmodia are:

a) Non-heterothallic or clonal formation of plasmodia
   1. Apogamic - after fusion of amoebae no karyogamy occurs and the haploid condition will be preserved. Plasmodia formed in this way should be termed haploid facultative apomicts (COLLINS and BETTERLEY 1982).

2 A transition from haploid to diploid state is involved with the plasmodium formation This could happen by chromosome replication without nuclear division or after fusion of two like amoebae followed by karyogamy This latter is homothallic mating

b) Heterothallic mating or formation of plasmodia by mating between different haploid clones

A diploid plasmodium develops by a two-stage process which consists of fusion (syngamy) of amoebae carrying different *mat A* alleles and the differentiation (development) of the resulting diploid zygotes into plasmodia In contrast, a haploid plasmodium (Cooke and Dee 1975, Adler and Holt 1975, Mohberg 1977) will be formed without change in ploidy, and apparently without amoebal fusion (Anderson et al 1976)

Clonal development of plasmodia (selfing) that seems to be apogamic is genetically regulated and is at a moderate rate (one plasmodium from every ten amoebae) in the presence of a special allele of *mat A*, designated *mat* A_h (was *mt h*) In contrast "heterothallic" alleles of *mat A* also self, but at exceedingly low frequencies (less than one plasmodium from 10^8 amoebae) (Wheals 1970, 1973, Adler and Holt 1977, Youngman et al 1977) Mutations closely linked to *mat A* that affect plasmodial development have been demonstrated (Anderson 1979, Honey et al 1979, Carlile and Gooday 1978) and can be subdivided into

1 Mutants with a decreased rate of differentiation into plasmodia (Cooke and Dee 1975, Davidow and Holt 1977, Anderson 1979, Anderson and Dee 1977, Wheals 1973), like *apt* (amoebal plasmodial transition), *npf* (non-plasmodial formers) and the strain CLd (Colonial Leicester delayed)

2 Mutants with an increased rate of differentiation into plasmodia (Adler and Holt 1977, Shinnick and Holt 1977, Gorman et al 1979, Anderson and Holt 1981), designated. *gad* (greater asexual differentiation) in which an increase in the frequency of plasmodial formation has been demonstrated

Plasmodial development in these strains can be a tool for a better understanding of the initiation of amoebal-plasmodial transition and can clarify the role of syngamy. For a long time the latter was considered to be a key step in the initiation of plasmodial development Gorman et al (1979) studied untreated as well as mutagenized amoebal cultures which are stable for the selfing mutation and which are able to develop into plasmodia within a clone These investigators found an extremely low frequency of spontaneous mutation to selfing, and concluded that a highly specific type of genetic alteration is involved, and that point mutations within a single gene affecting selfing are unlikely The haploid level of DNA (Adler and Holt 1977, Gorman et al 1979, Mohberg 1977, Cooke and Dee 1975) of plasmodial nuclei in selfing mutants suggests that these strains no longer require syngamy to initiate differentiation

Subsequent complementation and recombination experiments were performed with CL (Colonia Leicester) strains characterized by decreased differentiation of plasmodia (Wheals 1973, Anderson and Dee 1977). Honey et al (1979) obtained 21 differentiation mutants (designated *dif*) from CL by NMG (N-methyl-N-nitro-N-nitrosoguanidine) mutagenesis and concluded that the mutants fall into two complementation groups, which are closely linked to,

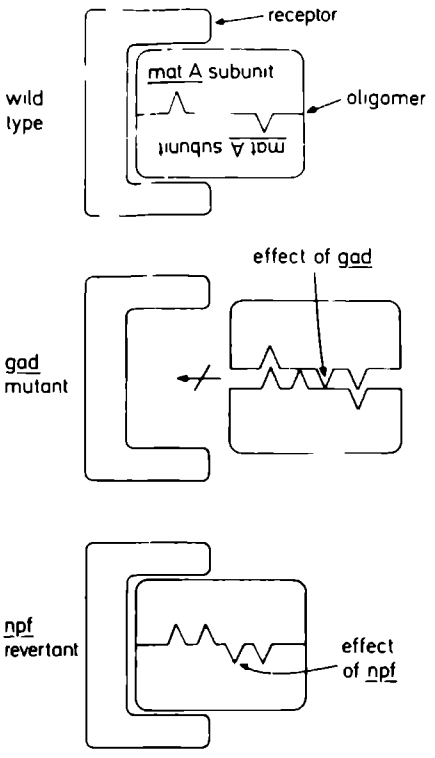

Fig. 2. A self-recognition model of *mat A* function and the effects of *gad* and *npf* mutations for fusion between amoebae of *P. polycephalum*. (ANDERSON and HOLT 1981)

or allelic at the *mat A* locus. The authors have proposed a repressor-operator model in which the *mat A* gene codes for a repressor molecule that acts at a nearby operator on the chromosome. The operator is adjacent to a structural gene, *dif*, whose expression is prevented by the interaction of the *mat A* product with the operator. Fusion of amoebae with mutants, like *npf* and revertants of *gad*, could cause a modulation of the repressor-operator hypothesis. These experiments can be performed with great efficiency between strains carrying different *mat B* alleles, without affecting the specificity of the complementation (HOLT et al. 1980, YOUNGMAN et al. 1981, ANDERSON 1979). ANDERSON and HOLT (1981) carried out fusion experiments with amoebae isolated which were revertants of selfing (*gad*) mutants and in which different alleles for *mat B* were introduced to facilitate complementation. They tested the repressor-operator model of HONEY et al. (1979) with their complementation results, and concluded that this model did not fit the facts. For the interacting system of amoebae of *P. polycephalum*, they suggested a simple model in which *mat A* and mostly *gad* and *npf* mutations are concerned with a single structural gene. In their model (Fig. 2) the structural gene makes a *mat A* product, which affects plasmodium formation by interacting at a receptor site in the cell and the specificity of the reaction is based on self-recognition. The postulated *mat A* product, a subunit of an oligomeric protein, would be active only if constructed from the subunits of a single *mat A* allele. The postulated *mat A* product would be inactive due to the inaccurate fit of allele-specific regions on unlike subunits. Experiments with strains of other origins are necessary to test this model.

The influence of the environment or the syngamy process has been studied Clonally produced plasmodia do not form at temperatures above 28 °C with amoebal mutants of *P polycephalum* carrying *npf A* allele, but in appropriate crosses plasmodia can be derived at such high temperatures with the *npf A* mutants (ANDERSON and DEE 1977) Zygote formation is affected by pH in a mutant carrying gene *imz* (ionic modulation of zygote formation), and SHINNICK et al (1978) suggested that this is due to an amoebal surface component involved in syngamy Comparable results with an optimum rate of mating at pH 5 were described earlier (COLLINS and TANG 1977) The presence of $MgSO_4$ in the culture medium enhanced the efficiency of mating between strains carrying different *mat A* and *mat B* alleles (SHINNICK et al. 1978, YOUNGMAN et al 1981). Also the amoebae themselves affect the environment with extracellular products PALLOTTA et al (1979), SHIPLEY and HOLT (1982) demonstrated that sexual plasmodium formation is induced by products secreted by non-mating amoebae. This occurs when the non-mating amoebae are combined directly with the mating cells, as well as when the non-mating amoebae and the mating cultures are separated only by filters of 0 2 µm pore size Similar results indicating that diffusible substances secreted by growing amoebae activated the differentiation of amoebae into plasmodia by the asexual process were described earlier (YOUNGMAN et al. 1977). Growing bacteria commonly used as a food source in the culture medium of amoebae (GOODMAN 1980), inhibit the mating process between amoebae by raising the pH of their surroundings (YOUNGMAN et al 1981) This decrease in rate of amoebal fusion may be caused by a change of the amoebal surface (JACOBSON 1980)

14.2.2 *Didymium iridus*

As with *Physarum polycephalum,* interaction between amoebae of *Didymium iridis* has been a subject of research in several laboratories and the results have been discussed in extensive reviews (CARLILE and GOODAY 1978, COLLINS 1979, COLLINS and BETTERLEY 1982) Plasmodium initiation of *D. iridis* will therefore be surveyed briefly with special attention to the differences between *D iridis* and *P polycephalum*

The number of plasmodial isolates of *P polycephalum* studied are limited (about 8) but *D iridis* has been sampled so extensively that 43 amoebal isolates have been obtained (COLLINS and BETTERLEY 1982) As for other myxomycetes, plasmodium initiation in *D iridis* can be divided into two classes *heterothallic* and *nonheterothallic*. For *D iridis* a heterothallic mating has been shown to be based on a one-locus, multiple allelic system (COLLINS and BETTERLEY 1982) The mating characteristics for the non-heterothallic isolates may be either homothallic or apomictic The studies of interaction between amoebae of *D iridis* have focussed on its usefulness in understanding speciation and evolutionary relationships in the myxomycetes, whereas with *P polycephalum* studies on the genetical fine structure of the mating locus have been performed with a view to elucidating physiological mechanisms. The results with *D iridis* demonstrated that 12 amoebal isolate formed plasmodia only when mated and hence

were heterothallic, and 31 isolated were non-heterothallic (COLLINS and BETTERLEY 1982). As in *P. polycephalum* (for strains carrying *mat* A_h), in *D. iridis* some amoeba strains have been shown to produce plasmodia in clones as well as in appropriate crosses. Clonally developed plasmodia possess haploid nuclei (THERRIEN and YEMMA 1975, THERRIEN et al. 1977) and in their spore progeny only one mating type is present (COLLINS and LING 1968), which suggests that clonally formed plasmodia have developed apogamically, and it seems that true meiosis does not occur prior to the formation of spores (COLLINS and BETTERLEY 1982). The ploidal state of myxoflagellates and plasmodia can be determined from the DNA level of their nuclei. The results of many DNA measurements (THERRIEN and YEMMA 1974, THERRIEN et al. 1977, COLLINS 1980, MULLEAVY and COLLINS 1979, 1981) suggest a conversion of $n \rightarrow 2n$ for the sexual cycle, either heterothallic or homothallic and a constant level of $n \rightarrow n$ or $2n \rightarrow 2n$ for the apomictic non sexual cycle. Studies (COLLINS 1980) with subpopulations of *D. iridis* demonstrated that the DNA level in spores displayed in line A corresponded to n, haploid, and in line B to 2n, diploid. The heterothallic line A was spontaneously derived from the apomictic line B. These findings demonstrate that non-heterothallic apomictic isolates spontaneously can shift from a non-sexual to a sexual life-cycle mode. An explanation for this phenomenon, perhaps a result of mutation as in *P. polycephalum* (DEE personal communication) has not been found, but the occurrence of apomictic strains of *D. iridis*, ploidal level of 2n throughout, can be explained by the absence of meiosis during the development of the spore. Studies of the influence of the environment on sexual mating of amoebae of *D. iridis* are rather difficult to perform and thus the evidence is poor. Treatment of amoebae with proteases, prior to and at various times after mixing (SHIPLEY and ROSS 1978), indicate that the induction of the fusion process by a diffusible inducer is controlled by a protease-sensitive process, but the recognition and fusion of amoebae are governed by a mechanism insensitive to protease. SHIPLEY and HOLT (1980) demonstrated that exponentially growing amoebae become competent to fuse only after the cell density reaches a critical value and that a dense culture of amoebae can induce fusion competence in a sparse clonal culture when the two cultures are separated by a filter (0.2 μM pores). Plasmodium initiation in other slime moulds has not been studied so extensively, buth the results that have been obtained are reviewed by CARLILE and GOODAY (1978) and COLLINS (1979).

14.2.3 Conclusions Concerning Interactions Between Amoebae of Myxomycetes

In the conversion of the tiny uninucleate amoebae into a plasmodium that contains millions of nuclei and may cover many square centimeters many factors are involved.

First of all there is the environmental component which may make the amoebae capable of fusion. The influence of pH, presence of divalent cations, bacterial food source and chemical composition of the culture medium are external factors which have been demonstrated to affect the fusion of amoebae of many myxomycetes (COLLINS 1982). One "internal" factor in *D. iridis* as

well as in *P. polycephalum* is a substance which induces rapid fusion, and is produced by a dense exponentially growing population of amoebae (SHIPLEY and HOLT 1980, 1982, YOUNGMAN et al. 1977). Whether this substance is a factor that mediates cell–cell recognition, as "golaptins" do in *Dictyostelium discoideum* and *Polyspondylium pallidum* (HARRISON and CHESTERON 1980), is unknown.

Genetic studies in *P. polycephalum* have demonstrated the function of two loci, *mat A* and *mat B*, in controlling plasmodium formation from amoebae. For the sexual process two discrete stages have been described. The formation of diploid zygotes after fusion of amoebae in pairs is determined by the *mat B* genotype and the differentiation of zygotes into plasmodia is controlled by *mat A* and unaffected by *mat B* (YOUNGMAN et al. 1981). In *D. iridis* the plasmodial development from amoebae, either apomictic or sexual, appears to be under control of one locus, the mating locus. The manner in which genetical factor(s) exert their effect is unknown, but for *P. polycephalum* a model has been proposed (Fig. 2).

14.3 Somatic Incompatibility Between Plasmodia

There is a striking difference between the research on the fusion between amoebae and between plasmodia. The work of the former topic is more genetical, whereas that on the latter is also ultrastructural and biochemical. After the germination of spores from a myxomycete a dense amoebal population develops, which gives rise to many small plasmodia. These fuse with each other to yield large plasmodia. When two large plasmodia of different origin come into close proximity the events that follow depend on the individual genetic composition of each plasmodium. Related studies on somatic incompatibility reactions will now be discussed. Earlier reviews are those of CARLILE (1973), CARLILE and GOODAY (1978), and COLLINS (1979).

14.3.1 *Physarum polycephalum*

If two plasmodia come into contact they may fail to fuse, either due to a genetical difference or to a difference in age even if they have an identical genetical composition. The former reaction is somatic fusion incompatibility (CARLILE and GOODAY 1978). The latter may be caused by the thick slime layer, which is developed by an old plasmodium, so that young and old plasmodia cannot come in close proximity. If fusion of plasmodia of different strains does occur, a series of processes may result in a lethal reaction which may destroy the fused plasmodium, partly or completely, a few hours later. The events which lead to the elimination of one or both plasmodia is known as the post-fusion incompatibility reaction. These two forms of somatic incompatibility were already known in 1967 (CARLILE and DEE).

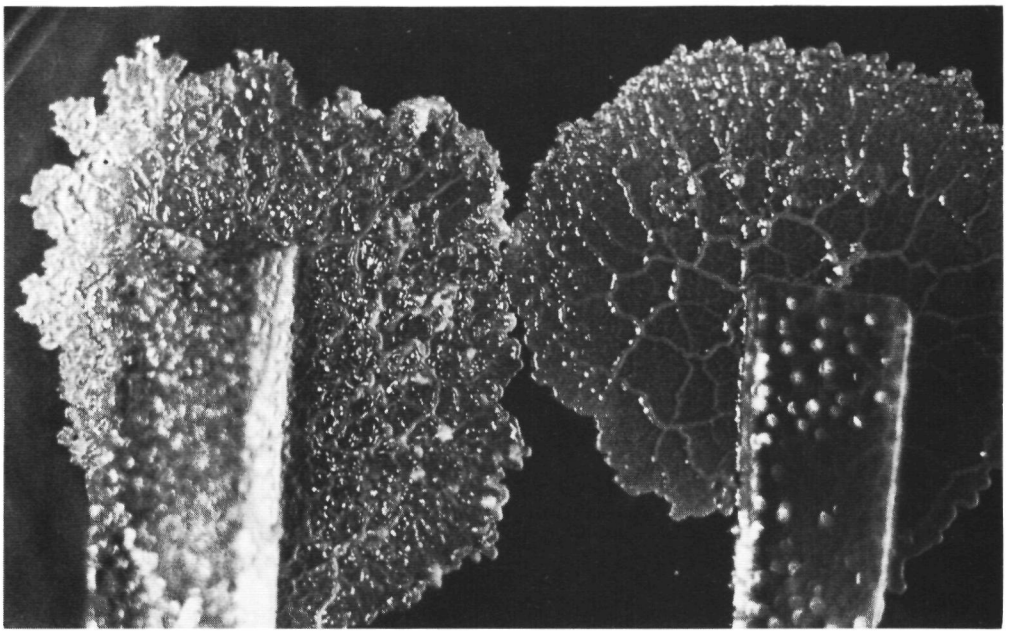

Fig. 3. Plasmodia of strains 15 and 29 of *P. polycephalum* grown out on agar medium in a 9-cm Petri dish met each other

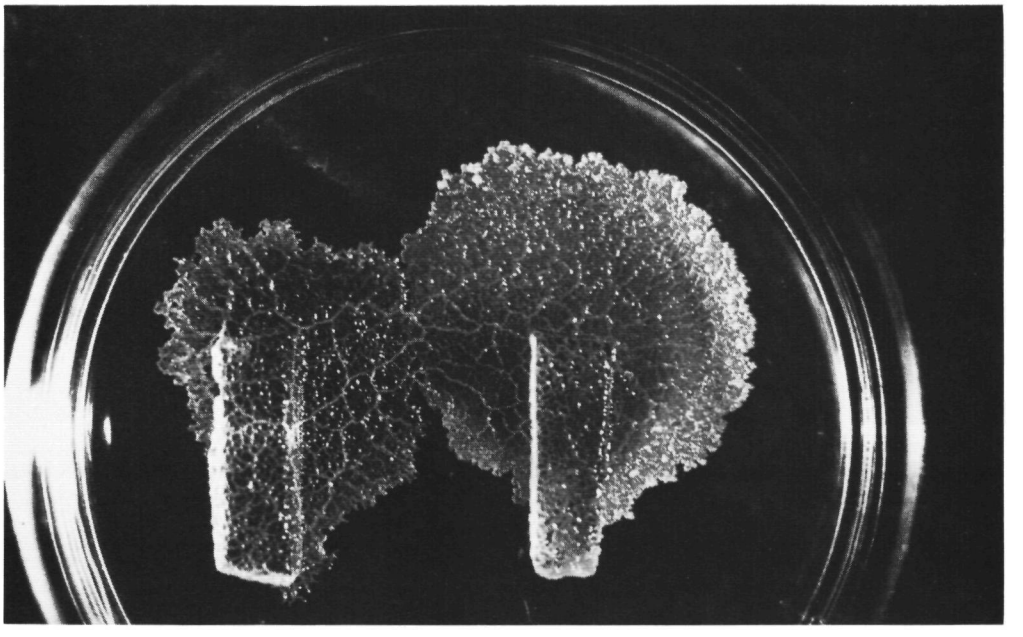

Fig. 4. About 30 min after Fig. 3. The two plasmodia are not fused completely but prominent veins have connected and changed their original position

The results of genetical studies have shown that the fusion of plasmodia is controlled by many loci with a pair of alleles at each locus, and at some loci one allele may be dominant. Studies on the progeny of Wisconsin 1 plasmodia revealed the presence of two fusion loci, now designated *fus A* and *fus B*

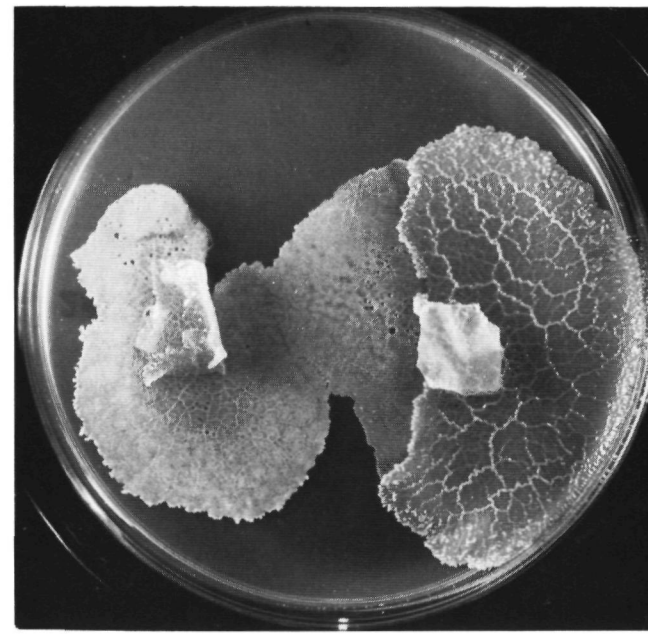

Fig. 5. Two plasmodia of the same strains 17 h after fusion. The plasmodium on the *left* (strain 29) is dead and the surviving plasmodium has just begun to re-invade. (CARLILE 1972)

(COOKE and DEE 1975); the fusion alleles are designated *fus* A_1 and A_2 and *fus* B_1 and B_2 respectively. The two alleles at the *fus A* locus are co-dominant, but B_2 is dominant to B_1. Mating of amoebae from the Colonia and Indiana isolates demonstrated a third fusion locus, *fus C* (ADLER and HOLT 1974). For plasmodial fusion to occur phenotypic identity with respect to fusion loci is required. The presence of 13 fusion loci with dominant and recessive alleles was demonstrated in other strains (COLLINS 1979). JEFFERY and RUSCH (1974) claimed that fusion incompatibility could be bypassed by gentle homogenization of mixtures of microplasmodia. This, presumably by damaging the plasma membrane, allows fusion of plasmodial fragments and regeneration of heterokaryotic plasmodia. However, fusion between two incompatible plasmodia from another strain resulted in a lethal reaction, independent of damage of the membranes by enzymatic treatment prior to fusion (SCHRAUWEN 1979). So far it seems likely that fusion compatibility is dependent upon identity with respect to some aspects of the membrane surface, but biochemical evidence is lacking. Following plasmodial fusion, an incompatibility reaction may occur due to genetical differences between the two plasmodia at certain other loci. ADLER and HOLT (1974) demonstrated that for the Colonia and Indiana strains several alleles are involved with the lethal effects. CARLILE (1976), studying the progeny of strain i × 1029, itself derived from Wis 1 plasmodia, demonstrated three loci, designated *let A*, *let B* and *let C*, with phenotypic differences which led to extensive lethal reactions a few hours after fusion. A dominant (*let A*) and a recessive allele (*let A* or *let a*) occurred at each locus, and lethal reactions

occurred in a plasmodium that was homozygous recessive with respect to a *let* locus and was damaged by a plasmodium having one or two dominant alleles at the *let* locus. Thus a Wisconsin strain carrying *let A let A* will fuse harmlessly with a strain carrying *let A let a*, but both strains can kill a strain carrying *let a/let a*. Physiological experiments were performed with two closely related strains, 15 and 29, both derived from the Wisconsin strain, in which 15 acts as the killer and 29 as the sensitive strain. The events visible with the naked eye and with a light microscope which occur after fusion between both strains have been described in detail (CARLILE 1972) and are demonstrated in Figs. 3, 4 and 5.

Fusion of killer and sensitive strain is an absolute requirement for the lethal reaction to occur in the sensitive strain. Contact only of both plasmodia, intact or not, has no effect at all (SCHRAUWEN 1979).

For the post-fusion somatic incompatibility reaction in *P. polycephalum* two kinds of reactions can be distinguished: a very specific reaction that is directed only at sensitive nuclei, and an unspecific reaction which may be the result of the disorganization in the cell caused by a large number of disrupted sensitive nuclei.

Between the moment of fusion of plasmodia of strains 15 and 29 and the lethal reaction several processes occur in succession. There is an irreversible spreading of the influence of the killer strain throughout the whole plasmodium of the sensitive strain within 1 h. This is followed by the synthesis of special RNA and protein respectively 2.5 and 4 h after fusion; and the series of events ends with the lysis of the fused plasmodium (Table 2). Also the involvement of the DNA of both strains was proved experimentally (SCHRAUWEN 1981).

High resolution autoradiographic studies after fusion of another pair of strains demonstrate step-wise ultrastructural changes (LANE and CARLILE 1979). In the first 1.5 h after fusion there are apparently no changes. After that time a very specific assault is performed on the chromatin structure of the sensitive nuclei, which results in a reduction of size of the nucleolus of the sensitive strain and chromatin condensation. This is followed by disintegration of the nucleolus and the elimination of the sensitive nuclei at 3–5 h after fusion. Mean while the killer nuclei change little in appearance, and the number and size of rough endoplasmic reticulum increases. Unspecific damage to the plasmodium between 4–5 h after fusion is followed by a slow recovery of the surviving part of the plasmodium during the next few days (Table 2). After that time the fused plasmodium behaves as the killer plasmodium. This conversion of a sensitive plasmodium into a killer plasmodium as a result of the events that occur after fusion of plasmodia of both strains can be obtained in the absence of the unspecific lysis reaction (CARLILE 1972). This opportunity facilitates biochemical studies and has been used to follow the disappearance of sensitive nuclei after fusion of plasmodia of strain 15 and 29 by the labelling of DNA. The results showed no change in the amount of sensitive and killer nuclei for at least 8 h and a dramatic decrease in DNA from sensitive nuclei 9–11 h after fusion of plasmodia of strain 15 and 29 in ratio 1:10 (SCHRAUWEN 1981) (Table 2).

Table 2. Changes after fusion of a sensitive and a killer plasmodium (Events are described in detail by LANE and CARLILE 1980, SCHRAUWEN 1979)

Time after fusion	Ultrastructural[a]	Biochemical[b]
0–1 5 h	No visible change	Irreversible outward spreading of the influence of the killer strain
2 2 5 h	Membranes of sensitive and killer nuclei develop small blebs Increase in amount of rough endoplasmatic reticulum	Synthesis of specific DNA
3–4 h		Synthesis of specific proteins
3–5 h	Alterations in sensitive nuclei, starting with a reduction in the size of the nucleolus to disruption Fusion of nuclei, both homologeous (from same strains) and heterologeous (from different strains)	
4–5 h	Unspecific plasmodial damage	
8–11 h		Disappearance of DNA from sensitive nuclei
12–16 h	Increase in size of remaining nuclei (11 µm) Protoplasmic streaming and movement are resumed Far fewer nuclei are present	
2 5 d	The plasmodium behaves as killer and nuclear sizes are normal (3–5 µm)	

[a] From LANE and CARLILE (1979), using strains 1 × A1029 (killer) and 29 02 × 29 19 (sensitive)
[b] From SCHRAUWEN (1979, 1981), using strains 1 × A1015 (killer) and 1 × A1029 (sensitive)

14.3.2 *Didymium iridis*

Extensive work on somatic fusion of the plasmodia *Didymium iridis* has been carried out by O R COLLINS, J CLARK, H and M LING, C D THERRIEN and I.C UPADHYAYA and has been reviewed by CARLILE and GOODAY (1978), and COLLINS (1979) The results of their studies demonstrate that the incompatibility reaction between plasmodia of *D iridis* is controlled by a polygenic system with dominant alleles at many loci The existence of at least 13 loci could be proved Two plasmodia must be phenotypically identical at all these somatic incompatibility loci in order to fuse and to produce a heterokaryon Thus difference of one allele at any of the large number of loci can prevent heterokaryon formation The results of LING and LING (1974) suggest that these imcompatibility loci can be divided into two classes, one that controls membrane fusion and the other involved in the post-fusion reaction The number of fusion (*Fus*)

and post-fusion loci which can be distinguished vary from strain to strain For the strains Pan-1 and Hon-1 seven *Fus* loci have been distinguished (Ling and Clark 1981) The post-fusion incompatibility loci are designated, from their appearance, as clear-zone (*Cz*) loci Ling and Clark (1981) recognized six *Cz* loci Fusion between plasmodia of *D iridis* is only possible if both plasmodia have the same fusion loci, however, this fusion terminates quickly if the two plasmodia differ at any of their clear-zone loci The post-fusion reaction can develop within seconds of fusion This zone of intermixture loses pigment to become a clear zone, undergoes coagulation and generally dies The size of the intermixed zone and the speed of the reaction is determined by the *Cz* genes, each of which has a characteristic strength The question arises whether the *Cz* loci act not only in "cytotoxic" post-fusion reactions but also in the instances of apparent non-fusion The latter reactions would then in fact be very fast cytotoxic reactions following transient fusion Clark (1980a and b) performed many fusion experiments between isolates of a Honduras strain and Panama–Honduras hybrids Thirteen different plasmodial incompatibility phenotypes were prepared from eight haploid myxamoebal clones of the Hon-1 strain The results of the fusion experiments between plasmodia of these strains show that one locus (designed G) is mainly a fusion controller (Clark 1980a) In the model, the presumed competitive survivor of each of these multiple-clone crosses could be predicted by comparing the relative cytotoxities of the potential phenotypes Comparable fusion experiments between plasmodia from Pan-1–Hon-1 hybrids indicated that plasmodial-incompatibility phenotypes control the survival of plasmodia in crosses and demonstrated the existence of different genes which are fusion controllers (Clark 1980b) These results seem to indicate that not all seven fusion loci described by Ling and Ling (1974) are cytotoxic Further studies may clarify the precise function of the *fus* and *Cz* loci to which Ling and Clark (1981) have given a unified nomenclature

Somatic incompatibility studies other than in *P polycephalum* and *D iridis* are few and are described by Carlile and Gooday (1978) and Collins (1979)

14.3.3 Conclusions Concerning Somatic Incompatibility

Intolerance of genetically non-identical tissue is very common in nature This phenomenon is regularly encountered, for instance with grafting in plants and the transplantation of organs in man Unless cells of higher organisms are treated with specific agents, somatic fusion between these cells is very difficult to perform. Somatic fusion experiments between plasmodia of the slime mould may be helpful for a better understanding in the processes which are involved before and after fusion of two different cells So far the results obtained from fusion between plasmodia of *P polycephalum, P cinereum, D iridis* and *Badhamia utricularis* demonstrate that the capacity for plasmodial fusion is controlled by a polygenic system with dominant alleles at many loci Somatic incompatibility in myxomycetes can take two forms, fusion and post-fusion incompatibility

1. Fusion Incompatibility. Fusion between two plasmodia occurs only when they are phenotypically identical for all fusion loci. The kind of interaction processes which distinguish between fusion from non-fusion are not completely understood, but the outer surface of the cell, membrane and slime, plays an important role.

2. Post-Fusion Incompatibility. The results of fusion between two plasmodia phenotypically identical for all fusion loci, but with differences for the post-fusion loci, designated as *let* or *kil* for *P. polycephalum* (CARLILE and GOODAY 1978) and *Cz* for *D. iridis* (LING and LING 1974), is a heterokaryotic plasmodium, which, however, reverts to a single homokaryon within 24 h (COLLINS 1979, CARLILE 1976). In this conversion from a heterokaryotic to a homokaryotic plasmodium one can distinguish two phases, suggested by the results of biochemical experiments and submicroscopical observations (SCHRAUWEN 1979, 1981, LANE and CARLILE 1979).

a) Specific phase – recognition of the alien nuclei. After fusion of a killer and a sensitive plasmodium, a spreading of the killer strain follows throughout the whole plasmodium. In the subsequent reactions, the involvement of the DNA of both plasmodia, RNA and protein synthesis can be demonstrated. The process concludes with the elimination of sensitive nuclei and the acquisition of the properties of the killer plasmodium by the fused plasmodium.
b) Non-specific phase. Beside the processes which lead to the elimination of the sensitive nuclei in the specific phase, there may be the occurrence of damage to the whole or part of the fused plasmodium. This damage, designated the cytotoxic reaction for *D. iridis* and lethal or killer reaction for *P. polycephalum,* may arise within 1 min for the former or after about 5 h for the latter. Fusion experiments with other strains may show different time lags between fusion and damage of the heterokaryon formed. Dead areas did appear in a heterokaryon of fused hybrids of *P. polycephalum* 30 min after fusion (CARLILE personal communication). Both myxomycetes suffer similar damage in the disruption of membranes and organelles followed by the loss of cytoplasm. One difference is that the cytotoxic reaction is strictly local, while the lethal reaction is widespread, sometimes over the whole plasmodium.

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3 THE LETHAL REACTION AS A FUNCTION OF FUSION AND TRANSLATION

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Post-Fusion Incompatibility in *Physarum polycephalum*

The Requirement of de novo Synthesized High Molecular-weight Compounds

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Abstract. In the myxomycete *Physarum polycephalum* the somatic incompatibility between plasmodia of a killer strain and a sensitive strain is expressed as a lethal reaction (LR) in the sensitive strain within 6 h after fusion of both plasmodia.

The lethal reaction was not caused by any of the extra-cellular compounds produced by the myxomycete nor was the result of intra-cellular compounds of the killer strain which were able to diffuse or could be transported into the plasmodium of the sensitive strain.

The lethal reaction could be prevented using inhibitors of RNA or protein synthesis applied within a certain time after fusion. This suggests that the sequence of events leading to the LR involves RNA and protein synthesis. These results are in agreement with genetic experiments which prove that the post-fusion incompatibility reaction is controlled genetically.

Key words: Lethal reaction – *Physarum polycephalum* – Somatic incompatibility.

The plasmodial incompatibility reaction in myxomycetes has been studied extensivily (Carlile, 1976; Clark, 1977; Collins, 1972). Carlile (1976) has demonstrated that the genetic control of incompatibility, after fusion of surface plasmodia resides at three loci in a sensitive strain (no. 29) of *Physarum polycephalum*. However, studies of somatic fusion have shown that a first stage of recognition is associated with the membrane (Jeffery and Rusch, 1974). Then, following fusion of the plasmodia of the sensitive strain (no. 29) and the killer strain (no. 15) of *Physarum polycephalum* the nuclei of the sensitive strain are destroyed in the lethal reaction (LR) (Border and Carlile, 1974). The lethal reaction is visible to the naked eye 6 h after the first contact between plasmodia of the killer and sensitive strains (Carlile, 1972). This time lag suggests that changes in metabolic activities occur eventually leading to the lethal reaction. For this reason we have studied biochemical processes leading to the breakdown of nuclei of the sensitive strain and the LR after fusion.

Since many organisms produce and excrete compounds in nature which are able to kill other organisms and *Physarum polycephalum* is known to produce a number of extra-cellular compounds (Braun and Behrens 1969; Farr et al., 1974; McCornick et al., 1970), the possible involvement of extra-cellular material in the development of the lethal reaction has also been examined.

Non-standard abbreviations DOC = sodium deoxycholate, ME = 2-mercaptoethanol, SDS = sodium dodecyl sulfate, TCAA = 5% trichloroacetic acid in 50% acetone, LR = lethal reaction, CPM = counts per minute, TCA = trichloroacetic acid

Materials and Methods

Media The origin of the somatic incompatibility of sensitive strain (no 29) and killer strain (no 15) of *Physarum polycephalum* and the composition of a semi-defined growth medium (SD as well as SDst agar) are described by Carlile (1972) Incompatibility tests are described by Border and Carlile (1974) Experiments were carried out at 24° C and aseptic procedures were employed throughout

Inhibition of Nucleic Acid and Protein Synthesis Inhibitors were added to SDst agar medium at the final concentration indicated

a) for inhibition of DNA synthesis. hydroxyurea (HU) 50 mM (Fouquet et al , 1975 a), ethidiumbromide (EB) 250 µg/ml (Fouquet et al , 1975b), 5-fluoro-2-deoxyuridine (FUdR) 0,02 mM and uridine (Ur) 0,40 mM (Hildebrandt and Sauer, 1973, Sachsenmaier and Rusch, 1964)

b) for inhibition of RNA synthesis actinomycin D (act D) 250 µg/ml (Fouquet et al , 1975 b), cordycepin (cord) 150 µg/ml (Fouquet et al , 1975 b)

c) for inhibition of protein synthesis cycloheximide (CH) 40 µg/ml (Nations et al , 1974)

Incubation Plasmodia were incubated in the presence of precursor and/or inhibitor with the aid of a nylon sieve (35 × 35 mm) Plasmodium of strain 29 (diameter 10 mm) was placed on a nylon

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sieve (pore 130 × 150 µ) immersed and layered on SDst agar medium The plasmodium grew to a size of approximatly 40 mm² in 24 h

Plasmodium together with support was easily transferred to a medium with inhibitor At the end of the incubation period the treated plasmodium on the nylon support was transferred back to SDst agar medium

Incorporation experiments Synthesis of RNA and protein was followed by measuring the incorporation of the precursors, [^{3}H]-uridine and [^{14}C]-leucine 50 µl of a radioactive solution was pipetted onto the medium below the nylon sieve which became detached from the agar medium completely for a few minutes The amount of activity is indicated for each series of experiments in the legend of the figures At the end of an incorporation experiment, the plasmodium was scraped off the nylon sieve, sonicated in 1 ml acetone for 10 s at position 9 in a Megason Ultrasonic Disintegrator and then mixed with 4 ml ice-cold trichloroacetic acid in acetone (5% ICA in 50% acetone = TCAA) The suspension was centrifuged and the pellet was washed successively with icecold TCAA (two times) and 5 ml acetone The dry residue was dissolved in 1 ml 0 3 N KOH Samples were taken for protein determination (Lowry et al , 1951), with bovine serum albumine as standard, and for measurement of radioactivity The latter samples were mixed with 5 ml aqualuma scintillation liquid and counted in Philips Liquid Scintillation Counter (PW 4540)

Treatment of Plasmodia with Enzymes Plasmodia scraped off the SDst agar medium or a plasmodium on a nylon support were suspended in an enzyme solution during five minutes room temperature At the end of the incubation period the treated plasmodia were either placed on SDst agar medium or brought in direct contact with a treated or untreated plasmodium, on SDst agar medium The final enzyme concentrations used in the incubations were hemicellulase 6 mg/ml pH 5 5, pectinase 5 mg ml in 0 25% EDTA pH 4, phospholipase A_2 1 mg ml pH 6, phospholipase C 25 µg ml pH 6, protease 2 mg ml pH 7, thermolysine 0 2 mg ml pH 7 5

Treatment of Plasmodia with a Detergent or an Organic Solvent Plasmodia scraped of the SDst agar medium were immersed for 10 s in the solution, immediately followed by washing with distilled water After these handlings the plasmodia were either placed on SDst agar medium or brought into contact with another treated or untreated plasmodium, on SDst agar medium *Uv-Irradiation* Plasmodia were irradiated at 1 mJ mm^{-2} with a Gamag-Lamp at 254 nm at a dose rate of 60 µJ mm^{-2} s^{-1} (Sudbery and Grant, 1975)

Radiochemicals [5,6 -^{3}H] Uridine (43 Ci $mmol^{-1}$) and L-[1-^{14}C] Leucine (50 mCi $mmol^{-1}$) were purchased from the Radiochemical Centre, Amersham *Enzymes* Hemicellulase (grade II), pectinase, protease (type VI) and thermolysine (type X) were obtained from Sigma Phoslipase A_2 (pancreas) and phospholipase C (grade II) were purchased from Boehringer, Mannheim

Results

When a plasmodium of strain 15 is brought into contact with a plasmodium of strain 29 both plasmodia fuse. The first symptom of lysis, visible to the naked eye, appears within 6 h. The cytoplasm turns brown and diffuses into the agar by that time.

Requirement for Direct Contact of Active Growing Plasmodia for the Lethal Reaction

It is important to know whether or not the killer strain contains compounds which can cause a LR in the sensitive strain without fusion and /or complete mixing of both cytoplasms.

Table 1. Plasmodium of strain 15 (diameter 1 cm) with underlying medium was brought into direct contact with the surface of the object LR = lethal reaction

Object	Pore size	After ≥ 6 h
medium		growth, no LR
Dialysis membrane layered on medium	2 nm	growth, no LR
Plasmodium of str 29		I R
Dialysis membrane layered on plm of str 29	2 nm	growth, no LR
Millipore filter HAWP layered on plm of str 29	0 45 µm	growth, no LR
Millipore filter SCWP layered on plm of str 29	8 µm	growth, no LR

Experiments were first performed to test the influence of direct contact between the membranes of both strains and of the condition of the membranes on the lethal reaction.

When both plasmodia were separated by filters of various pore size (Table 1) no fusion occurred. The extra-cellular compounds produced by the mold and expected to pass freely through the filter did not cause a lethal reaction and both plasmodia maintained their normal growth (Table 1). This demonstrates that neither extra-cellular material nor compounds which may diffuse or be transported from one plasmodium into the other are able to cause a LR. The plasmodial surfaces of strain 29 and/or strain 15 could possibly be treated in such a way that extra- or intra-cellular material of strain 15 could diffuse freely into plasmodium of strain 29. It would be possible to test the influence of components of plasmodium of strain 15 on the LR. Washings with different types of liquid could affect the membrane in such a way. However, washing the plasmodia of both strains with a buffer solution or tap water did not influence the LR. After the plasmodium of strain 15 was cut with a razor blade into pieces smaller than 1 mm² and the pieces washed in 5 ml buffer (100 mM tricine pH 7.0, 10 mM KCl and 1.5 mM MgAc) or destilled water, the pieces retained the capacity to fuse and to induce lysis. If, however, one of the plasmodia was damaged by centrifugation (Table 2) or treatment with a detergent (Table 2) or an organic solvent (Table 3), the plasmodia did not fuse and products inside the treated plasmodia of strain 15 were unable to penetrate into the plasmodium of strain 29 and to cause a LR. These results show that, with the procedures used, no material from the killer strain is

Table 2. Plasmodium of strain 15 free of medium was given different treatments LR = lethal reaction

Treatment	After treatment placed on top of	
	plm 29	SD(st) medium
1. Washed for 10 min in 5 ml buffer I (300 mM sucrose, 100 mM tricine, 10 mM KCl, 1,5 mM MgAc)	LR	growth
2 Cut into pieces (< 1 mm^2)	LR	growth
3 Cut (as in 2), washed (as in 1), residue tested	LR	growth
4 Residue 3, centrifuged 1 min, 100 × *g*	no LR	growth after > 24 h
5 Submerged in 50 µl buffer I, centrifuged 1 min, 100 × *g*		
supnt	no LR	no growth
residue	no LR	growth after > 24 h
6. Sonicated 10 s in 100 µl buffer I	no LR	no growth
7. Washed 1 or 10 min in distilled water	LR	growth
8 Washed 1 min buffer I in 2% SDS and 5%ME	no LR	no growth
9 Washed 1 min in 1% DOC	no LR	growth after > 24 h
10 Washed 1 min in 25% Bry-35	no LR	slow growth

Table 3. The influence of organic solvents prior to fusion on the LR LR = lethal reaction Plasmodium of strain 15 free of medium submerged for 10 s in an organic solvent The organic solvent was washed away with water

Organic solvent	After handling placed on top of	
	plm 29	SD(st) medium
Acetone	no LR	no growth
Ethylalcohol	no LR	no growth
Chloroform	no LR	no growth
Di-ethylether	no LR	no growth
Dioxane	no LR	no growth
Petroleum ether	a small LR < 1 out of 3 exp.	growth

able to cause a lethal reaction in the sensitive strain. Only plasmodia which have the capacity to fuse will cause a LR. However, fusion only occurs after contact between plasmodia of strain 29 and strain 15 if both plasmodia grow well (Table 2 and 3).

Another way to alter the plasmodium surface is by treatment of the membrane with enzymes. We have tested enzymes which can have an influence on the polysacharide outerlayer and those which could possible interfere with the selective membrane permeability. However, treatment of the plasmodia with an enzyme, described in methods, did not prevent the occurence of the LR, independent of the enzyme used for the pretreatment (Table 4). If a plasmodium of strain 15 was disrupted, by sonication or homogenization and placed on plasmodium of strain 29, either treated enzymatically or not, the result was that no uptake of plasmodium 15 was visible under the microscope and no lethal reaction appeared (Table 4). Evidently it is not possible to damage the membrane surface with enzymes that either free diffusion is possible or that the information for fusion and LR is lost.

Table 4. The effect of enzymatical treatment prior to fusion on the LR. LR = lethal reaction

Enzymes	Placed on medium			sonicated plm of str 15 brought into contact with enzymatically treated plm of str 29
	plm 15 on plm 29	control plm str 29	control plm str 15	
Phospholipase C	LR	growth	growth	no LR
Phospholipase A_2	LR	growth	growth	no LR
Hemicellulase	LR	growth	growth	no LR
Protease	LR	growth	growth	no LR
Hemicellulase (first), Protease (second)	LR	growth	growth	no LR
Pectinase	LR	growth	growth	no LR
Thermolysine	LR	growth	growth	no LR

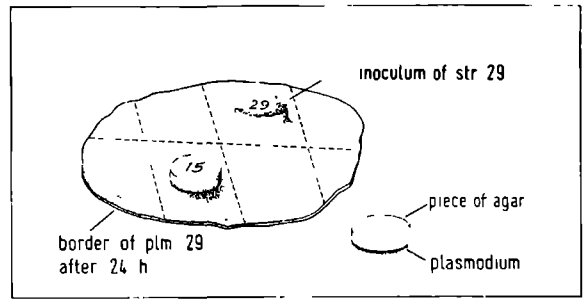


Fig. 1. Experimental design for the study of the LR in *Physarum polycephalum*
About 24 h after inoculation of strain 29 on medium an inoculum of plasmodium of strain 15 is placed upside down on the plasmodium of strain 29 The plasmodium of strain 29 was cut into pieces along the dotted lines. Each plm part was tested individually for the LR on SDst agar

Spreading of the Influence of the Killer Strain

Since there is time lag of 6 h between the moment of fusion and the lethal reaction the question arises whether this time gap will be caused by a slow

outward-spreading of the influence of the killer strain or by slow metabolic processes leading to the lethal reaction. Removal of the plasmodium of strain 15 (Fig. 1) one hour after inoculation did not prevent the occurrence of a lethal reaction. This demonstrates that from this moment on the presence of the inoculum of the killer strain is not essential for the lethal reaction to occur. If one hour after the first contact the fused plasmodium was cut into small pieces of about 8 × 8 mm (Fig. 1) and these small heterokaryons were transferred to individual plates, a lethal reaction appeared in all individual pieces 5 h later. Prior to 1 h after first contact, only plasmodial pieces from around the inoculum of strain 15 developed the lethal reaction. This demonstrates that the sign of the lethal reaction will be spread out from the inoculum of strain 15. These data also show that the lethal reaction is irreversible induced in the whole plasmodium within one hour after contact between killer and sensitive strain.

The Influence of Inhibitors of Nucleic Acid and Protein Synthesis on the Lethal Reaction

The results presented above show a time lag of 5 h between the moment that the whole plasmodium is committed to the lethal reaction and the moment of lysis. In addition mixing of the cytoplasm is required for a lethal reaction. These data suggest metabolic activities leading eventually to a lethal reaction. Because somatic incompatibility is genetically determined for plasmodia of strain 29 and strain 15 (Carlile, 1973), we have tested inhibitors of DNA, RNA and protein synthesis. It is found that inhibitors of RNA and protein synthesis can prevent the lethal reaction, while UV light of 254 nm and inhibitors of DNA synthesis do generally not affect the lethal reaction (Table 5). The period after fusion during which inhibitors of RNA and protein synthesis can be applied and yet remain effective in preventing a lethal reaction varies with the type of inhibitor (Table 5).

Reducing protein synthesis with cycloheximide prevents the lethal reaction if the inhibitor is added up to two hours before the lethal reaction appears. Inhibitting RNA synthesis with cordycepin becomes inefficient in inhibitting the lethal reaction $1\,^1/_2$ h earlier (Table 5). This time gap and the fact that inhibitors of RNA and protein synthesis prevent the lethal reaction suggest that RNA and protein synthesis are involved in this process. The observation that other inhibitors, which inhibit DNA synthesis, generally have no influence support this hypothesis.

Inhibition of protein synthesis by cycloheximide concentrations of 20 µg/ml and lower gave variable results in preventing the lethal reaction. The same unsatisfactory results were obtained for treatment with cordycepin at 100 µg/ml. The incorporation of [^{14}C]-leucine was inhibited by 98% with 40 µg/ml and by 95% with 5 µg/ml cycloheximide when the plasmodium was preincubated for 15 min with the inhibitor (Fig. 2). These results show that inhibition of protein synthesis is almost complete 15 min after incubation with the inhibitor (Fig. 2). Inhibiting of protein synthesis of 96% is not sufficient to prevent the lethal reaction (Fig. 2). The incorporation of [^{3}H]-uridine was inhibited by 96% when a macroplasmodium was treated with 150 µg/ml cordycepin 15 min prior to the addition of the isotope, [^{3}H]-uridine was used instead of uracil to follow RNA synthetic activity because, like in microplasmodia (Birch and Turnock, 1976), uridine is taken up ten times faster than uracil.

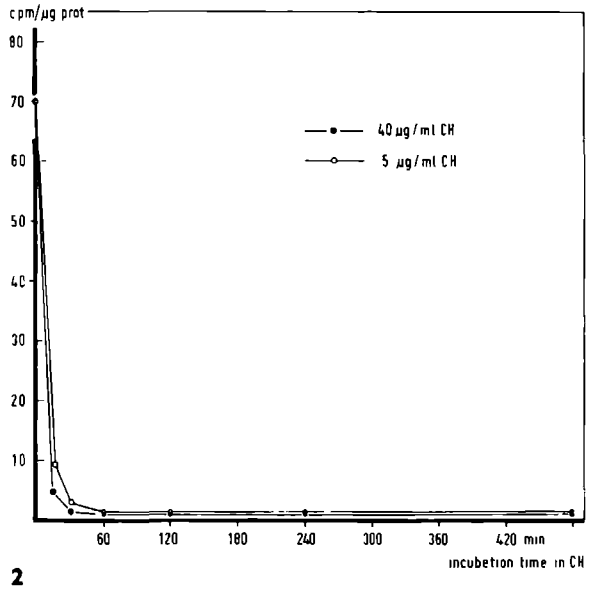
Table 5. The influence on the LR of inhibitors for DNA, RNA and protein syntheses after fusion of plasmodia of strain 29 and strain 15
LR = lethal reaction

Inhibits the synthesis of	DNA				RNA		prot
Time after fusion at which the incubation started	HU	EB	FUdR + Ur	$UV_{254\,nm}$	Act D	Cord	CH
$^1/_4$ h	+		(+)		−		
$^1/_2$ h	+	+	(+)	(+)		−	
$^3/_4$ h				(+)			
1 h	+	+	+	(+)	−	−	−
$1\,^1/_2$ h	+	+	+	+	−		−
2 h	+	+	+	+	(+)	−	−
$2\,^1/_2$ h		+	+			−	
$2\,^3/_4$ h						+	
3 h		+	+	+	+	+	−
$3\,^1/_2$ h		+				+	−
$3\,^3/_4$ h							−
4 h							+
$4\,^1/_4$ h							+

+ = a normal lethal reaction
(+) = irregularly results or delayed lethal reaction
− = no lethal reaction even after an incubation period of only 2 h

The Incorporation

The activity of [^{3}H]-uridine or [^{14}C]-leucine uptake into the plasmodium of strain 29 after fusion with strain 15 remained at the same level for 5 h (Fig. 3). In the overall synthesis of these high molecular compounds no moment of significantly increased activity could be demonstrated. At the moment the lytic reaction started, both RNA and protein synthesis had decreased and the rate of incorporation fell to nearly zero at 9 h after the first contact between strain 29 and strain 15. However, the uptake of [^{3}H]- uridine and [^{14}C]-leucine started to



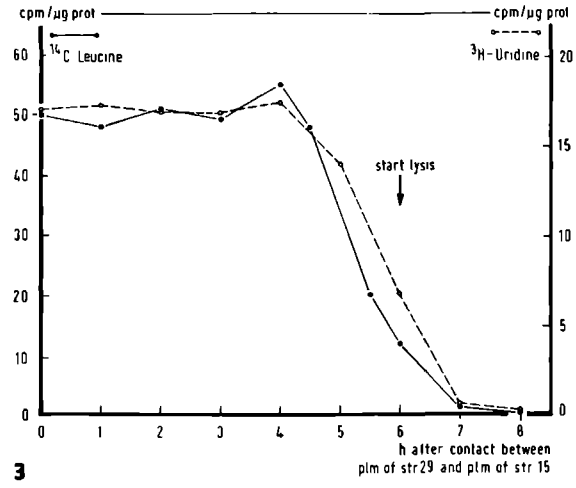


Fig. 2. The incorporation of [^{14}C]-Leucine in plasmodium after pretreatment with cycloheximide Plasmodia were continuously incubated in cycloheximide Fifteen minutes prior to the moment of extraction 1 25 µCi L-[1-^{14}C] Leucine (53 mCi/mmol) was supplied below the nylon support on the medium Radioactivity exceeded 1200 cpm in all vials

Fig. 3. The incorporation of [^{14}C]-Leucine or [^{3}H]-Uridine in fused plasmodia of strain 29 and strain 15
Fifteen minutes prior to the extraction 1 25 µCi L-[1-^{14}C] Leucine (53 mCi mmol) or 5 0 µCi [5,6-^{3}H] Uridine (43 Ci, mmol) was pipetted below the nylon support on the medium Radioactivity exceeded 1500 cpm in all vials

decrease 1/2 h after plasma streaming began to slow down and an hour before any lytic reaction was visible. The time gap between this decrease and the visible symptoms of the lethal reaction seemed to be at least 30 min.

Discussion

Requirement of Fusion for the Lethal Reaction

Heterogenic incompatibility in all groups of Eukaryotes has been described extensively by Esser and Blaich (1973). Carlile (1972) has described the somatic incompatibility reaction in *Physarum polycephalum* after fusion of surface plasmodia of a killer strain (no. 15) and a sensitive strain (no. 29). The genetic conditions for fusion and the lethal reaction between surface plasmodia of a myxomycete have also been described (Carlile and Gooday, 1978; Carlile, 1976; Clark, 1977; collins, 1972).

Substances localized at the plasmodium surface control the somatic fusion in *Physarum polycephalum* (Jeffery and Rusch, 1974) as is the case for other mechanisms (Capdeville et al., 1978). However, in the current experiments treatment of plasmodia of strain 29 and/or strain 15 of *Physarum polycephalum* with enzymes or liquids of different type did not influence the fusion and the somatic incompatibility reaction as long as both plasmodia were able to grow well (Table 2–4). If the membrane surfaces of plasmodia of strain 29 and strain 15 have any influence on the somatic incompatibility reaction, the described treatments were not able to affect this reaction. Rapid repair of the membrane (Wohlfarth-Bottermann, 1964) could have had an influence on the results of these experiments. The results in Table 1–4 demonstrate that fusion of killer and sensitive strain is an absolute requirement for the lethal reaction, to occur in the sensitive strain, and that contact only of both plasmodia, intact or not, has no influence at all. Comparable results were obtained in *Neurospora crassa* (Williams and Wilson, 1968) and in *Podospora anserina* (Esser, 1959; Bernet, 1965).

The Influence of Inhibitors of Nucleic Acid and Protein Synthesis on the Lethal Reaction

By inhibition of RNA synthesis (Fouquet, 1975b) or protein synthesis (Nations et al., 1974; Haugli and Dove, 1972) in *Physarum polycephalum* with inhibitors different concentrations of inhibitors has been applied. Our results show that the lethal reaction is prevented completely in the sensitive strain after fusion with the

killer strain following inhibition of RNA synthesis with cordycepin at 150 µg ml or inhibition of protein synthesis with cycloheximide at 40 µg ml. These experiments and the data from Figs. 2 and 3 suggest that either minor concentrations of newly synthesized RNA's and proteins can develop a lethal reaction or that just the synthesis of products leading to the lethal reaction, probably RNA and protein, are inhibited by cordycepin only at 150 µg/ml and cycloheximide at 40 µg/ml. An indication of the latter hypothesis are the results of Nations et al. (1974), who have found specifically labelled proteins in *Physarum polycephalum* after treatment with 50 µg/ml cycloheximide and an inhibition of 88% of the total protein synthesis. The involvement with the lethal reaction of RNA and protein synthesis is sustained by the striking increase of rough endoplasmic reticulum after fusion of incompatible plasmodia (Lane and Carlile, (1979). A close association does exist between synthesis of proteins during the incompatibility reaction in *Petunia hybrida* (van der Donk, 1975) and *Podospora anserina* (Esser, 1959; Bégueret and Bernet, 1973). It has been demonstrated for *Podospora anserina* that the synthesis of proteolytic enzymes is diminished by cycloheximide, while actinomycin D had no inhibition effect (Boucherie et al., 1976; Boucherie and Bernet, 1977). The latter inhibitor stops the lethal reaction in *Physarum polycephalum* (Table 5), which suggests that the incompatibility systems in *Podospora anserina* and *Physarum polycephalum* are not the same.

In *Physarum polycephalum* DNA synthesis can be effected by inhibtiors (Fouquet et al., 1975a; Hildebrandt and Sauer, 1973) or UV-irradiation (Sudbery and Grant, 1975). Their influence on the lethal reaction show that DNA synthesis is probably not involved in the lethal reaction (Table 5). However, these results do not rule out a role for DNA in the lethal reaction.

The Time Scale

From the moment of contact between plasmodia of strain 15 and strain 29 up to the moment of destruction of the sensitive plasmodium, one can distinguish several time markers in the results Figs. 1 and 3 and Table 5. On the basis of the results presented in this study and those of other investigators, a time scale of processes leading to the lethal reaction can be proposed (Fig. 4): 1. Recognition at the plasmodial suface (Carlile, 1976; Clark, 1977; Collins, 1972; Jeffery and Rusch, 1974). 2. Fussion of both strains (Table 1–4) within a few minutes after contact (Carlile, 1973; Collins, 1972). 3. Spreading of the influence of the killer strain throughout the whole plasmodium of the sensitive strain within 1 h (Fig. 1). 4. The latest moment at which

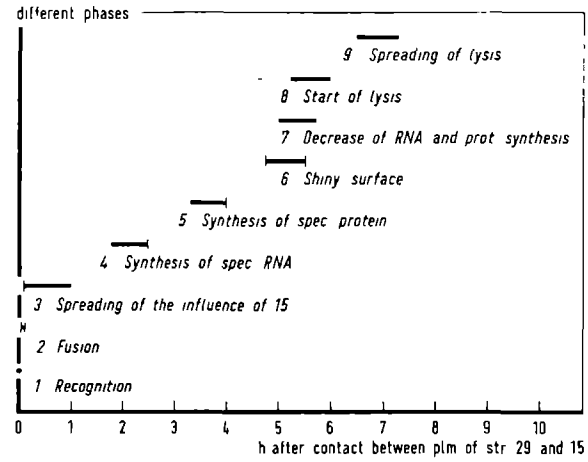


Fig. 4. Time scale of different events after contact of plasmodia of strain 29 and strain 15 of *Physarum polycephalum*
The abbreviated text is described in more detail in the discussion

inhibitors of RNA synthesis can still prevent the lethal reaction at $2^1/_2$ h after the first contact between both strains (Table 5). This suggest synthesis of special RNA till this moment. 5. The latest moment at which inhibitors of protein synthesis can still prevent the lethal reaction at 4 h after the first contact between the plasmodia of both strains (Table 5). This suggests synthesis of special protein till this moment. 6. Slowing of plasma streaming and a plasmodial surface that becomes more shiny at about 5 h after contact. Protein synthesis can still go on (Fig. 3) as is described also by Bernstam (1974). 7. A decrease in RNA and protein synthesis half an hour before lysis is perceptible (Fig. 3). 8. Appearance of the symptoms of lysis, first in the surrounding of the inoculation place of strain 15, within 6 h after contact between both plasmodia (Carlile, 1972). 9. Rapid spreading of the lysed area followed by desintegration of nuclei (Border and Carlile, 1974) and a complete drop of RNA and protein synthesis (Fig. 3).

This time scale is indicative of a series of processes taking place during the somatic incompatibility reaction between plasmodia of strain 15 and strain 19 of *Physarum polycephalum*, which reject the sensitive strain.

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4 DNA AS AN INTERMEDIATE AND TARGET OF THE INCOMPATIBLE PROCESSES

Post-Fusion Incompatibility in *Physarum polycephalum*

The Involvement of DNA

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Abstract. The lethal reaction following fusion of plasmodia of a sensitive and a killer strain of *Physarum polycephalum* could be prevented by the incorporation of 5-bromo-2′-deoxyuridine in both strains. This result suggests the involvement of transcription in the lethal reaction. Although under appropriate conditions fusion of the strains is not followed by a lethal reaction the fused plasmodium will behave as the killer strain after subculturing.

At different times after fusion, DNA was isolated from a plasmodium in which visible lethal reaction was prevented and separated on CsCl gradients. Killer DNA remained intact but sensitive DNA was broken down, mainly from 8–11 h after fusion.

Key words: *Physarum polycephalum* – Lethal reaction – Incompatibility

After fusion of plasmodia of strain 29 and strain 15 of *Physarum polycephalum* heterokaryon forms (Carlile 1972) which is unstable as a consequence of a genetically controlled lethal reaction (Carlile 1976). Similar heterogenic incompatibilities have been described in other myxomycetes and in filamentous fungi (Collins 1972; Clark 1980; Delettre et al. 1978; Ling and Upadhyaya 1974) and in higher eukaryotes (Esser and Blaich 1973).

The lethal reaction is dependent on de novo synthesis of RNA and protein in the interacting plasmodia, whereas DNA synthesis is not involved (Schrauwen 1979).

Modification of the genetic information will affect RNA and protein synthesis and might therefore affect the incompatibility reaction. Incorporation of the base analogue 5-bromo-2′-deoxyuridine (BrUdR) sensitizes DNA to light of 313 nm (Ben-Hur and Elkind 1972; Krasin and Hutchinson 1978) and produces DNA of heavier density (Funderud et al. 1978). So, incorporation of BrUdR makes it possible to study the influence of "modified" DNA on the lethal reaction after fusion of plasmodia of strain 15 and 29 in ratio 1:3 and the kinetics of the breakdown of sensitive nuclei after fusion of plasmodia in ratio 1:10.

Materials and Methods

Growth of Physarum Cultures *Physarum polycephalum* sensitive strain (no 29) and killer strain (no. 15) were cultured at 26 C on SD liquid medium as described by Carlile (1972). Macroplasmodial cultures with synchronous S-phase were made by sloping a flask with exponential growing microplasmodia at 45 After 10 min of settling, 0 35 ml of the sedimented microplasmodia was transferred with a wide-bore tip pipette (diam 3 mm) onto a filter paper (diam. 7 cm). Plasmodial fusion took place in closed petri dishes for 30 min at room temperature in the dark, after which cultures were incubated at 26 C on SD liquid medium. Under these conditions, the third S-phase started 20 h after the onset of incubation and was essentially complete 2 h later

Irradiation Experiments. Macroplasmodia of strain 29 or strain 15 (diam. 15 mm) were placed on a nylon sieve (Schrauwen 1979) and incubated at 26 C either in the presence of 160 µM BrUdR on SDst agar medium (Funderud et al. 1978) or on SDst agar medium (Carlile 1972). After 24 h, a well-grown plasmodium was transferred on the nylon support to SDst agar medium and incubated at 26 C for 2 h

Plasmodia were irradiated at 40 mJ cm $^{-2}$ at 313 nm. The light from a Philips high pressure lamp (90 W, arc length 25 cm) was filtered through a 3 mm UG5 glass-filter (Schott, Jena) and 10 mm K_2CrO_4 solution (135 mg·l $^{-1}$) (Rauen 1956)

For fusion experiments, plasmodia of strains 15 and 29 were brought into contact by the sandwich method (Chin et al. 1972) at a ratio of approximately 1 3.

Incorporation Experiments DNA of the killer strain was radioactively labelled by growing a synchronous macroplasmodium for 24 h at 26 C in the presence of 0 5 µCi [^{14}C]-thymidine ml^{-1} of SD liquid medium DNA of the sensitive strain was labelled by growing the macroplasmodium during the third S-phase in SD liquid medium containing 10 µCi [^{3}H]-thymidine, 2 1 µCi 5-bromo-2′-deoxy [1′,2′-^{3}H]-uridine, 50 µg BrUdR per ml. At the end of the incorporation-period, the labelled plasmodium was washed for 2 min with SD liquid medium (20 ml). A radioactive labelled plasmodium of strain 15 (about 2 cm^2) was brought into contact with a radioactive and density-labelled plasmodium of strain 29 (about 20 cm^2), layered on SDst agar medium and cultured At different times, approximately equal parts of one fused plasmodium were taken to make direct comparisons of the recovered dpm possible. About 1 h of fusion is needed for full mixing of the killer and sensitive strain (Schrauwen 1979), so the first sampling was at 1^1 $_2$ h after fusion

Other samples from labelled plasmodia of the two strains were cultured separately on SDst agar medium as controls

Isolation of Nuclei and DNA At different times a part of the fused labelled macroplasmodium was scraped from the agar layer and mixed with a non-labelled plasmodium (diam. 6 cm) of the same age in 25 ml isolation medium (250 mM sucrose, 5 mM $MgCl_2$, 10 mM Tris-HCl pH 7.1, 0 1 % Triton X-100, 0 5 mM phenylmethylsulfonylfluoride (PMSF) (Mohberg and Rusch, 1971) At the same time controls were made by extracting parts of nonfused plasmodia from both strains Homogenization was carried out by hand (6 strokes) in a loose-fitting glass, glass Potter-homogenizer The homogenate was filtered through two layers of milk filter (Schel a id Wanka 1973). The milk filters were washed with 50 ml isolation medium. Nuclei were collected from the 75 ml filtrate by centrifugation at 1,000 × *g* for 15 min over an underlay of 25 ml isolation medium containing 1 M sucrose The supernatant was decanted and the pellet was resuspended in 3 ml buffer (150 mM NaCl, 15 mM Na-citrate pH 7.0, 5 mM Na-EDTA) and treated with 100 µg RNase (preheated at

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Non-Standard Abbreviations BrUdR, 5-bromo-2′-deoxyuridine; EDTA, ethylenediamine tetra acetic acid, PMSF, phenylmethylsulfonylfluoride, dpm, disintegrations per minute, ThdR, thymidine

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70 C for 30 min) at 37 C for 30 min Then 100 µg proteinase K (preincubated at 37 C, 30 min) was added and the incubation was continued at 37 C for 1 h DNA was precipitated from the solution with 7.5 ml 96 % alcohol (−20 C) containing 1 % K-acetate, kept at −20 C for 30 min and collected by centrifugation at 5,000 × g for 15 min The pellet was washed with 70 % alcohol and resuspended with 2 7 ml 0 1 × buffer. The solution was clarified by centrifugation (5,000 × g, 10 min) and 2.4 ml was mixed with 100 µg calf thymus DNA and 2 0 g CsCl The clear solution was carefully layered over 3 6 ml CsCl solution (refractive index 1.405) in 0.1 × buffer and centrifuged in a Beckman 50Ti rotor for 60 h at 34,000 rpm and 15 C All extraction conditions were performed at 0–4 C unless specified otherwise. The gradients were fractioned and aliquots were used for measuring absorption at 260 nm, refractive index and radioactivity Samples were mixed with 0 4 ml water and 5 ml scintillation liquid (Packard Emulsifier Scintillator 299) and counted in a Philips liquid scintillation counter (PW 4540)

Table 1. The influence on the lethal reaction of 5-bromo-2′-deoxyuridine incorporation and irradiation at 313 nm prior to fusion of plasmodia of strains 15 and 29

Plasmodia treated with BrUdR	Plasmodia irradiated			
	29	15	both	neither
Neither	+	+	+	+
29	(±)	(±)	(±)	(±)
15	(−)	(−)	(−)	(±)
Both			−	−

\+ = lethal reaction, 100 % of the fused plasmodium was destroyed
(±) = small lethal reaction, to 20 % of the fused plasmodium
(−) = small lytic spots, after retardation of plasma streaming
− = no visible lethal reaction

Results

The Influence of Modified DNA on the Lethal Reaction. An inoculum of either strain 29 or strain 15 on SDst agar medium with 160 µM BrUdR grew out as well as on SDst agar medium in 24 h. Irradiation of plasmodia at 313 nm in the presence of 160 µM BrUdR or immediately following a BrUdR treatment of 24 h resulted in the subsequent death of the plasmodia. Since incubation for at least 1 h on SDst agar medium overcame this phenomenon, irradiation of BrUdR-treated plasmodia was started 2 h after transfer to SDst agar media.

After irradiation, the protoplasmic streaming in the irradiated plasmodia decreased or stopped, but recovered in less than 10 min. Fusion experiments were started at that time. Table 1 shows that fusion of control plasmodia of strain 29 and strain 15 untreated with BrUdR resulted in a lysis of the total fused plasmodium, a complete lethal reaction. Irradiation of plasmodia untreated with BrUdR did not affect this lethal reaction. About 6 h after fusion of BrUdR-treated and untreated plasmodia, the lytic activity varied from small lytic spots to a complete lethal reaction. The severity of the reaction was a little different depending on the kind of a plasmodium that had been treated, sensitive or killer (Table 1). BrUdR treatment of either sensitive or killer plasmodium caused a weak hydrolytic reaction with very small lytic spots. The lethal reaction was completely absent after fusion of killer and sensitive plasmodia when both plasmodia had been treated with BrUdR. Irradiation of these plasmodia did not change the results.

After 26 h had elapsed following fusion of BrUdR-treated and untreated plasmodia, some of the plasmodia, in which no lethal reaction was observed, were found to have died. The dead plasmodium looked as if a lethal reaction had taken place. After 48 h had elapsed any fused plasmodium that survived behaved as strain 15, and the features of the sensitive strain had disappeared completely.

Disappearance of Sensitive Nuclei after Fusion. The relative density of DNA of sensitive plasmodium was increased by labelling with BrUdR during one S-phase. The denser DNA could be separated from marker calf thymus DNA on a CsCl gradient (Fig. 1). Labelling of DNA of a plasmodium with BrUdR and [^{3}H]-thymidine during one S-phase followed by culturing of the sensitive plasmodium for 26 h on SDst agar medium did not change this sensitive DNA. The radioactive profile, the amount of radioactivity and the density of these DNA on the CsCl gradient was the same as in Fig. 1 for a

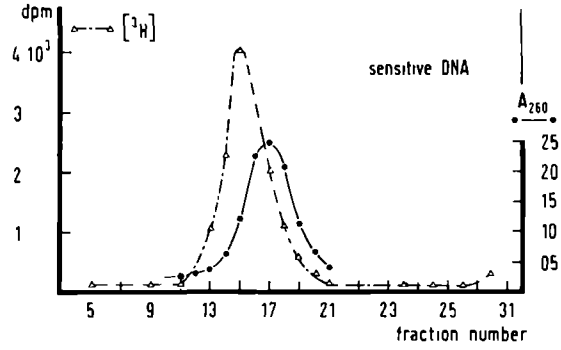

Fig. 1. CsCl gradient analysis of DNA from sensitive plasmodium. DNA was labelled with BrUdR and [6-^{3}H]-thymidine (23 Ci mmol^{-1}) during one S-phase At the end of the incubation period DNA was isolated, centrifuged in CsCl and counted for radioactivity (△-- △) Calf thymus DNA (standard) was estimated by absorbance at 260 nm (●— ●)

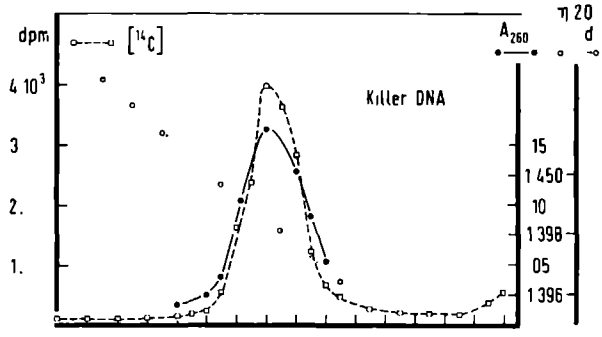

Fig. 2. CsCl gradient analysis of DNA from killer plasmodium. DNA was labelled with [2-^{14}C]-thymidine (56 mCi mmol^{-1}) during at least two S-phases (□----□). Calf thymus DNA (standard) was estimated by absorbance at 260 nm (●— ●). The refractive index of the CsCl gradient was represented by (○····○)

comparable size of plasmodium, not incubated for that 26 h. The radioactive profile of DNA isolated from the killer plasmodium, grown on non-BrUdR SD liquid medium, was coincident with the UV-profile of calf thymus DNA in the CsCl gradient (Fig. 2).

In the CsCl gradient the ratio of radioactivity (^{3}H/^{14}C) of DNA isolated from a plasmodium after fusion (ratio 1 : 10) of

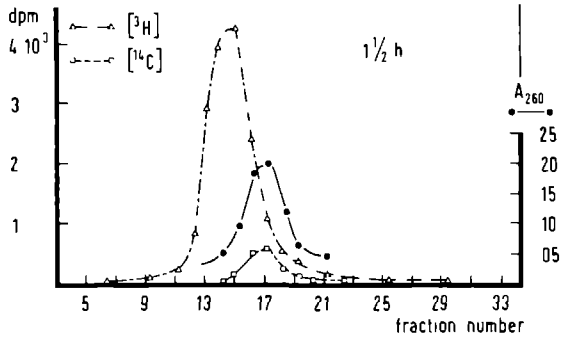

Fig. 3. CsCl gradient analysis of DNA from a plasmodium 1½ h after fusion of sensitive plasmodium (treated as in Fig. 1) and killer plasmodium (treated as in Fig. 2) The ratio of plasmodial fusion of killer : sensitive = 1 10. Radioactivity was determined as in Fig. 1 (△---△) sensitive DNA, (□---□) killer DNA, (●——●) calf thymus DNA

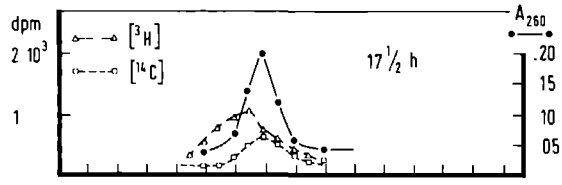

Fig. 4. CsCl gradient analysis of DNA from the same plasmodium as in Fig. 3 but 17½ h after fusion Symbols as in Fig 3

[¹⁴C]-labelled killer plasmodium, and BrUdR and [³H]-labelled sensitive plasmodium is about 10 at 1.5 h after fusion (Fig. 3). For a comparable size of the same plasmodium this ratio has dropped to about 2 at 17½ h after fusion. The amount of [³H]-label has been decreased while the [¹⁴C]-label kept constant (Fig. 4).

The ratios of radioactivity of sensitive and killer DNA in CsCl gradients of different experiments are represented in Fig. 5. The ratio of sensitive to killer DNA is stable for 8 h in the fused plasmodium. Between 8 and 11 h after fusion it drops rapidly whereas after 11 h a slow continuous decrease takes place.

Discussion

The results of Table 1 demonstrate that the expression of intact DNA of strain 29 as well as strain 15 is a requirement for the lethal reaction to occur. "Modification" of killer DNA or sensitive DNA with BrUdR is not sufficient to stop the lethal reaction completely in a fused plasmodium. Irradiation of one of the plasmodia having "modified" DNA did not change the results, even though irradiation of DNA at 313 nm after incorporation of BrUdR causes damage of DNA (Ben-Hur and Elkind 1972; Krasin and Hutchinson 1978).

Concerning the inhibition of enzyme synthesis by BrUdR in other systems (Scrive et al. 1977) the results of Table 1 suggest that the lethal reaction after fusion of strain 29 and strain 15 is caused by enzymes which can be synthesized by either strain. This hypothesis is sustained by the involvement of common hydrolytic enzymes in the incompatibility reaction in *Podospora* (Delettre et al. 1978).

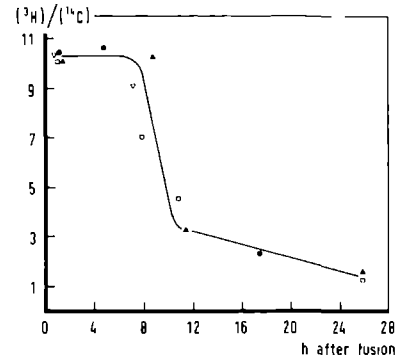

Fig. 5. The time course of disappearance of sensitive DNA in a plasmodium after fusion of killer and sensitive plasmodia (ratio 1·10) as indicated by the ratio between [³H] (sensitive) and [¹⁴C] (killer) labelling Different symbols represent different experiments

The results shown in Table 1 and those previously published (Schrauwen 1979) make clear that DNA is involved in the lethal reaction.

Though the dramatic lytic reaction was not visible in a fused plasmodium after modification of DNA of both strains, this fused plasmodium behaved as strain 15 within 48 h. This suggests that disappearance of sensitive nuclei has occurred in this plasmodium. The reversion of the influence of BrUdR can take place in two S-phases completely (Felenbok et al. 1974). From that point the now-intact DNA could be involved in the elimination process of the sensitive nuclei in the heterokaryon. Ultrastructural studies by Lane and Carlile (1979) on fused plasmodia of two strains differing at the let A incompatibility locus showed damage to sensitive nuclei 3–4 h after plasmodial fusion. In contrast, the DNA of the fused plasmodia of strains 15 and 29, which differ at a different locus, remains intact for at least 8 h after plasmodial fusion (Fig. 5). This difference in timing may be related to either differences in the incompatibility loci or differences in the metabolic rates of the strains involved.

After that time sensitive DNA is broken down in a short period whereas no change in killer DNA is observed (Figs. 3 and 4). The great loss of sensitive DNA and the non-loss of killer DNA indicate that the disruption must be a very specific effect. That the only visible change which takes place 8–11 h after fusion of plasmodia (ratio 1:10) of strain 15 and 29 is retardation of the growth, also suggests a specific effect. Concerning the post-fusion incompatibility reaction in *Ph. polycephalum* one can distinguish two different processes:

1. A series of reactions which lead to the destruction of the whole or a large part of the fused plasmodium. Results in this paper and earlier (Schrauwen 1979) demonstrate the involvement of DNA and the synthesis of RNA and protein.

The process develops only under conditions of good nutrition and after fusion of killer and sensitive plasmodia in an appropriate ratio (Carlile 1972).

2. A reaction which occurs under other conditions and causes elimination of sensitive DNA only. Experiments are in progress to describe these two processes in more detail.

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5 MEMBRANES AS A SITE OF INCOMPATIBLE REACTIONS

Post–Fusion Incompatibility in *Physarum Polycephalum*

Involvement of membranes

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Abstract Fusion of sensitive and killer plasmodia of *Physarum polycephalum* caused a change in membrane composition which did not occur after fusion of two sensitive plasmodia. This change, which was mainly the result of an increase in the synthesis of phosphatidylethanolamine, began one and a half hours after fusion. In the processes which underlie the lethal reaction, destroying the fused plasmodia of sensitive and killer strains, energy seems involved. Free radicals had no apparent role in these processes.

Keywords: incompatibility – lethal reaction – phospholipids – *Physarum polycephalum* - PC, phosphatidylcholine - PE, phosphatidylethanolamine - - PI, phosphatidylinositol - PS, phosphatidylserine

Introduction

In the myxomycete *Physarum*polycephalum, the somatic incompatibility between plasmodia of a killer strain and those of a sensitive strain is expressed as a lethal reaction (LR) in the sensitive strain (Carlile, 1972). The damage caused by the lethal reaction is a result of a series of processes which take place after fusion of the plasmodia of both strains. These reactions involve the synthesis of high molecular weight compounds (Schrauwen, 1979, 1984b) and an increase in the size and frequency of the cytoplasmic vesicles of the endoplasmic reticulum (Lane and Carlile, 1979; Schrauwen, 1984a). In *Didymium iridis* (Upadhyaya and Ling, 1976), *Neurospora crassa* (Williams and Wilson, 1966), and other fungi (Esser and Blaich, 1973; Lane, 1981), the membranes are either the primary target during the incompatibility reaction or a membranous barrier is formed. In other systems where cells are attacked by a lytic reaction, such as that of the complement system of red blood cells (Lachman, 1983) the mechanism seems to reside in a disturbance of the lipid bilayer, whose structure and function is strongly dependent on the phospholipid composition (van Zwaal, 1975; Rothman and Lenard, 1977). These data lead to the view that membranes may be involved in the lethal reaction after fusion of sensitive and killer plasmodia.
Therefore the present study was undertaken to follow up the quantitative and qualitative changes in phospholipids after plasmodial fusion. In addition, a preliminary examination has been made into the possible role of changes in energy level and radical formation after fusion of plasmodia of this myxomycete.

Materials and Methods

Culture of plasmodia

Strains 137 and 149 of *P.polycephalum* were maintained and somatic incompatibility tests were performed as described by Carlile (1972), except that in the medium Bacto–Soytone replaced Bacto–peptone (Turnock and Dee, 1981). The sensitive strain (137) was originally derived by the mating of the amoebae strains IC 137 and LU 219, the killer strain (149) by mating of the amoebae strains IC 149 and LU 219, as described by

Dee (1962). Both plasmodial strains were a kind gift of Dr M.J.Carlile.

Phospholipid labeling

For labeling experiments a macroplasmodium of 1x1 cm supported by a nylon sieve(Schrauwen, 1979) was incubated on 2mm agar medium B (=agar medium containing one-eihgth of the original KH2PO4 concentration of the standard SDmedium (Carlile, 1972)) and mixed with 6.66 MBq/ml [32P]-orthophospate (Amersham). After an incubation of one hour the plasmodium was either analysed immediately or further incubated on unlabelled agar medium.

Lipid analysis

After incubation, the treated plasmodium was scraped off the agar medium, the wet weight was estimated and phospholipids were extracted as described by Comes and Kleinig (1973), and Schacht (1981). This resulted in a chloroform phase containing the phospholipids and a methanol–water phase. An aliquot of the chloroform phase was counted for total radioactivity and the rest was analysed for phospholipid composition by thin-layer chromatography on Silicagel 60 (Merck) with chloroform-methanol-acetic acid-water = 50–28–4–8 as the solvent. Individual radioactive spots were visualised on Kodak XAR–5 film and counted for radioactivity. The lipids were identified by co-chromatography with known standards.

An aliquot of the original methanol-water fraction was counted for radioactivity. Samples were counted in a Philips Liquid Scintillation Counter (PW 4540). The minimum number of counts measured per sample was 1000 and efficiencies were determined by the external standard ratio method.

Heat exchange

Heat production of a plasmodium was measured continuously with a LKB Bio Activity Monitor type 2277 of agar medium (diameter 8 mm) half covered with plasmodium. The oxygen consumption was measured by Warburg manometry and energy production calculated on the assumption, that all oxygen taken up was used in the combustion of glucose, and converted to energy (Tilberg, 1971; 6 mol O2 = 1 mol glucose = 28.16 10*5 J).

Results and Discussion

Incorporation rate of [32P] into phospholipids

The phospholipid composition of the membranes and their synthesis or turnover, was estimated in macroplasmodia of *P.polycephalum* by measuring the incorporation of the precursor [32P] orthophosphate.

Table 1.Uptake and incorporation of phosphate into phospholipid in the plasmodium of *P.polycephalum*. Results are expressed as pmol exogenously supplied phosphate per mg plasmodium (wet weight).

Fraction	Treatment			
	pulse	1 h	1 h	1 h
	chase	-	1 h	6 h
CHCl3		1.85±0.2	4.27±0.08	9.44±0.2
MeOH-H2O		328±14.4	65.7±0.4	34.9±2.0
Ratio:MeOH-H2O/CHCl3		178	15	3.7

CHCl3	Chloroform fraction=phospholipid fraction
MeOH-H2O	Methanol-water fraction
Pulse	Plasmodium was incubated on agar medium B with [32P] phosphate (specific activity 0.41 pmol/ml).
Chase	Plasmodium was transferred to agar medium after the pulse. The medium was refreshed twice during 6 h.

The amount of [32P] incorporated into phospholipid depends on the specific activity of [P32] in the medium and the incorporation period. The results in Table 1 indicate that the amount of phosphate incorporated into the phospholipid fraction increased not only during the pulse but also during the chase period. This increase is almost linear in time over the whole period. In contrast, the amount of radioactive phosphate in the methanol-water fraction dropped a factor 5 during the first hour of the chase period (Table 1). After a chase of 6 h, during which time the agar medium was refreshed twice, the label in the phospholipids had increased 6 fold while the polar labelled phosphate content in the plasmodium had dropped 90% (Table 1).

This suggests that the phospholipids, once synthesized, are very stable and do not turn over to any extent.

The incorporation rate of [32P] into the phospholipid fraction was constant for non-fused plasmodia (Table 1) and after fusion of two plasmodia of a sensitive strain (Table 2). The incorporation rate was the same for fused plasmodia of both sensitive and killer strains until the moment plasma streaming stopped. This shows that the rate of total phospholipid biosynthesis is not influenced by the type of plasmodia fusion - either sensitive and sensitive or sensitive and killer. Immediately after lysis, the uptake of label into the phospholipid fraction was lower by a factor of 10, while uptake into the nonphospholipid fraction was increased (Table 2).

Table 2.Uptake and incorporation of exogenous phosphate in one hour into the plasmodium of *P polycephalum* after fusion of two strains. Results are expressed as pmol per mg plasmodium (wet weight).

Combination of strains	Feature	Time of incubation after fusion (h)			
		¾ - 1 ¾	2 - 3	3 ¼ -4 ¼	5 - 6
137+137	CHCl3	1.93	1.64	1.76	1.76
	MeOH-H2O	308	336	353	353
	pl.str.	++	++	++	++
137+149	CHCl3	1.93	1.76	1.79	0.16
	MeOH-H2O	246	268	268	657
	pl.str.	++	++	++→+/−	−

137 sensitive strain
149 killer strain
++ normal plasma streaming (pl.str.)
+ little plasma streaming
− no plasma streaming

See legends of Table 1 for other abbreviations.

Table 3.Distribution of [32P] phosphate over individual phospholipids after thin layer chromotography. Extracts were prepared from plasmodia at different intervals after fusion of sensitive and sensitive strain.

Phospholipids	Time of incubation after fusion (h)			
	3/4 - 1 3/4	2 - 3	3 1/4 - 4 1/4	5 - 6
Phosphatidylethanolamine	39±2	39±2	39.8±0.4	42±2
Phosphatidylserine	10.0	8.5±1	7.9±0.1	11±2
Phosphatidylcholine	31±0.2	31±0.3	32±0.5	33±5
Phosphatidylinositol	11.5±1	13±2	12.3±0.3	10±2

The data are means and S.D. of three samples of one fused plasmodium. The numbers are percentages of the total radioactive phosphate present in one lane after chromatographic separation.

Fig 1 Thin-layer chromatogram of phospholipids separated after labeling for one hour with [32P]–orthophosphate Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS)

After lysis, a part of the plasmodial content diffused visibly into the agar medium causing a decrease of about 50% in the wet weight of the plasmodium Since all cpms are related to the measured wet weight, the values for uptake and label in phospholipid fraction of the lysed plasmodium are probably too high

Changes in phospholipid composition

The distribution of [P32] in the phospholipids is comparable to that found in *Dictyostelium discoideum* (Davidorf and Korn, 1963) and *P polycephalum* (Comes and Kleinig, 1973), except that phosphatidylserine was not found by Comes and Kleinig (1973) (Fig 1)
Table 3 shows that during a 1 hour incubation the proportions of [32P] incorporated into the four main phospholipids remained constant after fusion of two sensitive plasmodia irrespective of the measuring time However, the distribution of [32P] over the main phospholipids in plasmodia of *P polycephalum* changed after fusion of sensitive and killer plasmodia (Fig 2) Synthesis of phosphatidylinositol and phosphatidylcholine remained constant whereas that of phosphatidylethanolamine increased by 30% until 4 h after fusion Incorporation of phosphate into phosphatidylserine decreased to the original value of the sensitive plasmodium after attaining a maximum at 1 h after fusion (Fig 2) This change in lipid distribution suggests that after fusion of sensitive and killer plasmodia the membrane composition changes This may be the result of synthesis of a new type of membrane as well as an increase of phosphatidylethanolamine at one side of the bilayer of an existing membrane Asymmetric distribution of phospholipids has been demonstrated for inner and outer leaflets of membranes of red blood cells (v Zwaal et al, 1975), influenza virus (Rothman et al, 1976, Rothman and Lenard, 1977) and in mitochondria of plants (Cheesbrough and Moore, 1980) The inner leaflet of these membranes is enriched by one or two phospholipids in comparison to the outer leaflet These data and the results of Fig 2 suggest that after fusion of sensitive and killer plasmodia there is

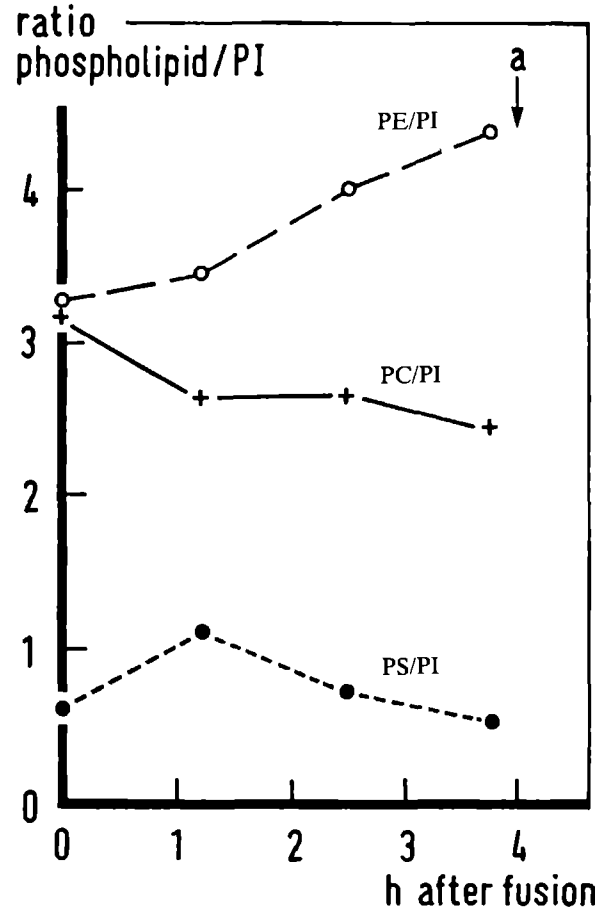

Fig 2 Ratios of individual phospholipids to phosphatidylinositol (PI) after fusion of sensitive and killer plasmodium of *Physarum polycephalum* The plasmodia(1x1cm2) were incubated with [32P] phosphate for 1h at different times in the period between fusion and lysis

a cessation of plasmastreaming at 4 h after fusion

either a different rate of synthesis of phospholipids for the inner and outer leaflets, or that much more irregular or special membranes are developed (Rothman and Lenard, 1977) Both hypotheses are in agreement with the observation of ultrastructural changes after fusion of sensitive and killer plasmodia in *P polycephalum* (Lane and Carline, 1979) and formation of thick membrane

barriers spread over the cytoplasm of *Didymium iridis* (Upadhyaya and Ling, 1976)

Radical reactions

The destruction of the entire, or at least large portion of the plasmodium 5–6 h after the fusion of sensitive and killer plasmodia is a result of a disruption of at least the plasma membrane since some of the cytoplasmic content diffuses into the agar layer Lysis of cells, as in several animal cell types (Lachman, 1983), is caused by hydroxyradicals whose first target seem to be the nuclei (Mello Filho et al, 1984) Since nuclei of killer and sensitive plasmodia of *P polycephalum* are involved in the lysis process (Schrauwen, 1981), an attempt was made to influence a possible formation of radicals by using chelating agents Growth of either sensitive, killer or fused plasmodia on SDagar medium in the presence of ethylenediaminetetraacetate (EDTA) (0 5–20 mM), ethyleneglycol–bis(2–aminoethylether)tetraacetic acid (EGTA) (0 5–15 mM) or phenantroline (0–100uM) had no visible influence Since none of these compounds could prevent or delay the lethal reaction after fusion of sensitive and killer plasmodia, neither hydroxyradicals nor divalent cations (Lachmann, 1983) seem to be involved in the reaction chain

Energy exchange

Fig 3 shows that the available energy supplied by the combustion of glucose has a constant value for plasmodia, fused or not fused, as long as plasma streaming is present In the fused plasmodium of sensitive and killer strains, the oxygen uptake declined at the moment the plasma streaming stopped and decreased to 10% when 30% of the plasmodium had been lysed

The values for heat production increased continuously in killer, sensitive and homologous fused plasmodia during the measuring time (Fig 3) The plasmodium derived after fusion of sensitive and killer strains produced an increasing amount of heat at a rate between sensitive and killer plasmodia until two hours after fusion Ater that time the rate of heat production began to decline and decreased to zero at the moment plasma streaming stopped This change at two hours could be caused by an alteration in the membrane composition or by a cessation in growth which, until that time, was small but observable

The ratio of 10 for respiration over heat production is quite large but of the same magnitude as for *Neurospora crassa* (Lambowitz and Slayman, 1971)

Heat production in plants and microorganisms (Lambers, 1982, Laties, 1982, Ainsworth et al, 1980) has often been related to the CN-insensitive respiration (Meuse, 1975, Day et al, 1980) for which membrane integrity (Laties, 1978) is essential

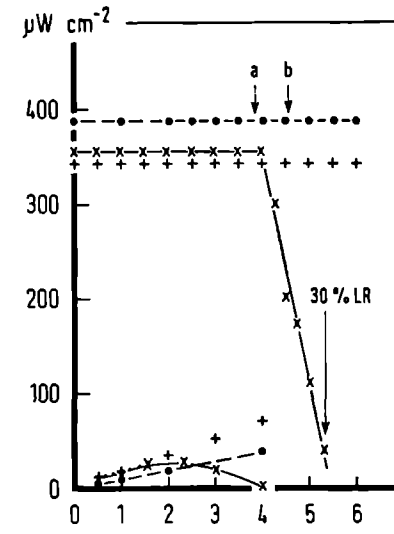

Fig 3 Respiration and heat production in fused and non-fused plasmodia of *Physarum polycephalum*
sensitive plasmodia and two fused sensitive plasmodia
killer plasmodia and two fused killer plasmodia
fused killer and sensitive plasmodia
a cessation of the plasmastreaming at 4 h after fusion
b start of visible lethal reaction (I R) which spread over 30% of the entire plasmodium of *Physarum polycephalum*

Conclusion

A prerequisite for the occurence of the lethal reaction is fusion of plasmodia of sensitive and killer strains (Schrauwen, 1979, 1984a) In the early period after fusion of two plasmodia, synthetic capacity for RNA, protein (Schrauwen, 1979) and phospholipids, and the respiration rate, did not change All these metabolic parameters declined only at the moment that plasma streaming, observed under the microscope, stopped This suggests that the processes which lead to the cessation of plasma streaming and the lethal reaction are not caused by changes in the overall rates of these biosynthetic pathways The initial signal leading to the lethal reaction is given at the moment of fusion by the interaction between plasma membranes of both strains (Jeffery and Rusch, 1974) Since organelles, such as endoplasmic reticulum and vesicles form a continuously changing

interconnection between the plasma membrane and the nuclear envelope ((Whatley and Whatley, 1984) a clue to understanding the lethal reaction may be found in the total membrane system. This hypothesis is supported by changes in the membrane composition as evidened by (1) changes in phospholipid composition and heat production (this article) (2) the involvement of DNA of both plasmodia (Schrauwen, 1981) (3) use of the electronmicroscope (Lane and Carlile, 1979). The participation of RNA and protein synthesis in the processes (Schrauwen, 1979, 1984b) which lead to the lethal reaction supports this view. The connection between the changed membrane composition and the lethal reaction might be that membranes around vesicles in the cytoplasm become weak or develop defects in inner membrane sites (Lachman, 1983), comparable to those found *Coriolus versicolor* (Rayner and Todd, 1979).

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6 CHANGES IN PROPERTIES OF THE HETEROKARYON

Post-Fusion Incompatibility in *Physarum polycephalum*

Changes in Protein Pattern and Fusion Properties of a Heterokaryon

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Abstract The polypeptide patterns of two different strains of *Physarum polycephalum,* sensitive and killer, showed only minor differences on two - dimensional electropherograms. After heterologous fusion of the sensitive and killer plasmodia newly formed proteins could be demonstrated which were not detectable in homologous fused plasmodia. The sensitization of the heterokaryon plasmodium for the lethal reaction occured after the aformentioned protein synthesis. Microinjection of plasmodial extracts into plasmodia did not cause an effect comparable to a lethal reaction. Residues or diffusable products from lysed plasmodia had no harmful influence on sensitive or killer plasmodia.

Key words: incompatibility - lethal reaction - *Physarum polycephalum* -protein synthesis

Non–standard abbreviations: SDS= sodium dodecyl sulfate

Introduction

Plasmodia from identical strains of the myxomycete *Physarum polycephalum* normally fuse when they come into contact -a process known as homologous fusion. Carlile and Dee (1967) observed a lethal reaction in heterokaryons formed after fusion of two different strains - heterologous fusion. This lethal reaction, referred to as a post–fusion incompatible reaction, has a genetical basis (Carlile , 1972, 1976) and is characterized by lysis of either the entire or a large part, of the plasmodium (Carlile, 1976; Schrauwen, 1979b).

Changes in electronmicroscopical structures (Lane and Carlile, 1979; Border and Carlile, 1974) and in membrane composition (Schrauwen, 1984b) have been shown to begin two hours after fusion of an incompatible pair of strains. The dependence of the lethal reaction on DNA of both plasmodia (Schrauwen, 1981b) and protein synthesis in *P.polycephalum* (Schrauwen, 1979a), as well as the association of proteolytic activity with protoplasmic incompatibility, found in *Podospora anserina* (Labarere and Bernet, 1979), lead to an attempt to characterise the proteins involved. Changes in overall protein composition may be a part of the lethal reaction.

If proteolytic enzymes similar to those found in *Podospora anserina* (Labarere and Bernet, 1979) are responsible for the lytic reaction in the heterokaryon of *P.polycephalum,* microinjection of these substances may cause the same type of reaction in a non–fused plasmodium.

Materials and Methods

Culture of P.polycephalum

Macroplasmodia of sensitive (137) and killer (149) strains were maintained as described previously by Schrauwen (1984b). To induce fusion, two plasmodia were brought into contact by the sandwich method (Schrauwen,1979b). Plasmodia, which had to be transferred, were either in direct contact with the medium or separated from it only by a nylon sieve. Plasma streaming and the development of the lethal reaction were examined microscopically.

Labelling of proteins

For labelling experiments a piece of macroplasmodium (1x1cm), supported by a nylon sieve (Schrauwen, 1979a), was incubated on 2 mm of agar medium C (= agar medium containing one-eighth of the original Bacto–Soytone concentration of the standard SD agar medium (Carlile, 1972) and mixed with 6.0 MBq [35S]–methionine (Amersham) /ml).

Gel electrophoresis

The method of O'Farrell (1975) was followed for two–dimensional electrophoresis: a 3:2 mixture of pH 3–10 and pH 5–8 Ampholines (LKB) with a 5% gel in the isolectric focussing dimension and 12% acrylamide gel in the second dimension. One dimensional gel electrophoresis was performed according to Laemmli (1970) with 12% polyacrylamide gels. Unlabelled proteins were visualised in the gel by silver staining (Eschenbruch and Burk, 1982). Labelled proteins were detected by fluorography on Kodak XAR–5, after treatment of the gels with 2,5–diphenyloxazole and exposure at -70°C (Bonner and Larsky, 1974). Extracts for 2–dimensional electrophoresis were prepared according to Turnock et al (1981). Radioactivity of labelled proteins were measured in a Philips Liquid Scintillation Counter (PW 4540) from the alcohol precipitable residue of the protein extract.

Fractionation of plasmodial organelles

Approximately 1 g plasmodium was scraped of the medium from which both nuclei (Mohberg and Rusch, 1971; Hardman and Gillespie, 1980) and the mitochondrial fraction (Kuroiwa et al, 1976; Holmes and Stewart, 1979) were isolated following procedures described earlier. The residual supernatant after pelleting nuclei and mitochondria was mixed with buffer I (250 mM sucrose, 10 mM KCl, 5 mM MgCl2, 10 mM Tris–HCl, pH 7.2) to a final volume of 36 ml and layered over 5 ml 1 M sucrose in buffer I. A colorless microsomal pellet was derived after 16 hours of centrifugation (100000 g, Beckman SW 27). The surface of the microsomal pellet was quickly rinsed with distilled water (0°C). The residual 100000 g supernatant was mixed with alcohol (-20°C) to a final concentration of 70% (v/v), allowed to settle at -20°C for 30 min and centrifuged (3000 g, 10 min). The pellet was washed with 70% alcohol, than acetone and subsequently dried in a stream of nitrogen. The pellets of nuclei, mitochondria, microsomes and 100000 g sup were taken up with sample buffer (2% sodiumdodecylsulfate (SDS), 6 M urea, 0.1 mM EDTA, 10 mM Tris–HCl pH 7.2, 5% 2–mercaptoethanol), (Laemmli, 1970) 300–500 ul/g plasmodium (wet weight).

Microinjection

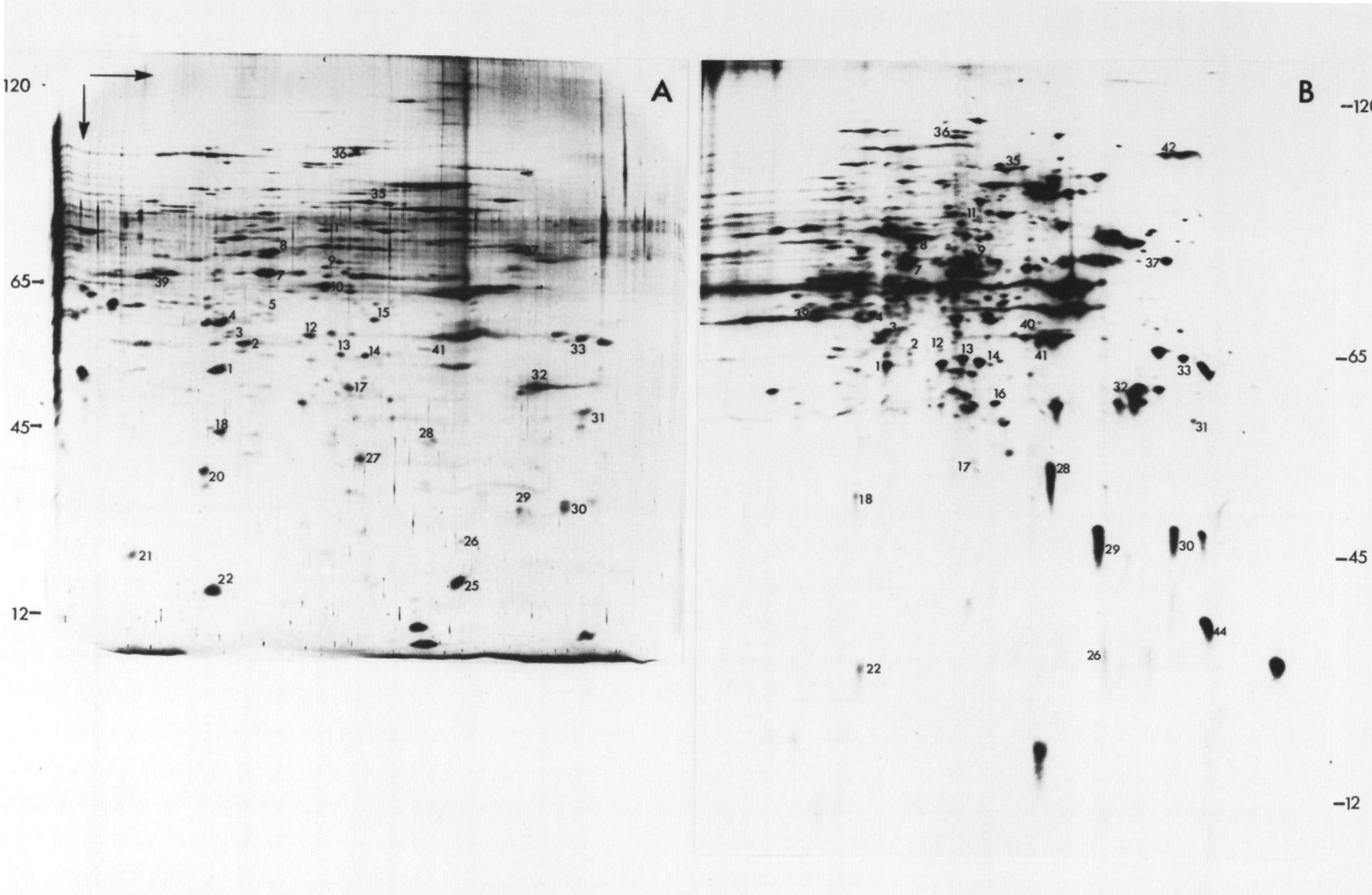
A glassmicrocapillair prepared as described by Graessmann and Graessmann (1976) was connected with a microsyringe and fixed in a micromanipulator (Leitz). Wells (diameter 10 mm) covered for 80% with plasmodium were cut at the border of thriving plasmodium growing on SDagar medium. Solutions were injected into a vein of the tiny plasmodium according to Ueda (1978). Plasmodium with agar medium were kept at 25°C and syringe with injection liquid at 4°C continuously. Plasmodial extracts for microinjection were prepared by homogenising 500 ul buffer II (50 mM Tricine pH 7.2, 5 mM MgCl2, 0°C) and 500 mg plasmodium (wet weight). The homogenate was sonicated (10 s,Megason Ultrasonic Desintegrator), centrifuged (10000 g, 30 min, 0°C), and filtered (Millipore SCWP). The clear extract was used immediately or stored at -70°C.

Results

Changes in synthesis of polypeptides

Two dimensional electrophoresis of plasmodial proteins from *P.polycephalum* resulted in a spotty pattern. A few hundred individual proteins could be detected equally well after silver staining of proteins (Fig 1A) as by fluorigraphy of [35S]-methionine labelled proteins (Fig 1B). The spots were spread over the entire electropherogramme, but by far the most proteins were located in the pH region of 4.7–8.6 and molecular weight

Fig 1:Polypeptide pattern after two–dimensional electrophoresis of an extract from a killer plasmodium. Isoelectric focussing was in the horizontal dimension and SDS in the vertical. Molecular weight markers are shown x 10*3. The numbers are arbritrary and belong mostly to a group of proteins with a specific shape. A: Proteins were stained with the silver method. The amount of protein loaded on the top of an isoelectric focussing gel was 40 ug. B: Fluorogram after two dimensional electrophoresis of proteins, from killer plasmodium, labelled with [35S] - methionine during one hour. At each isoeletrical focussing gel about 100000 precipitable (alcohol 70% v/v) cpm was loaded.


A
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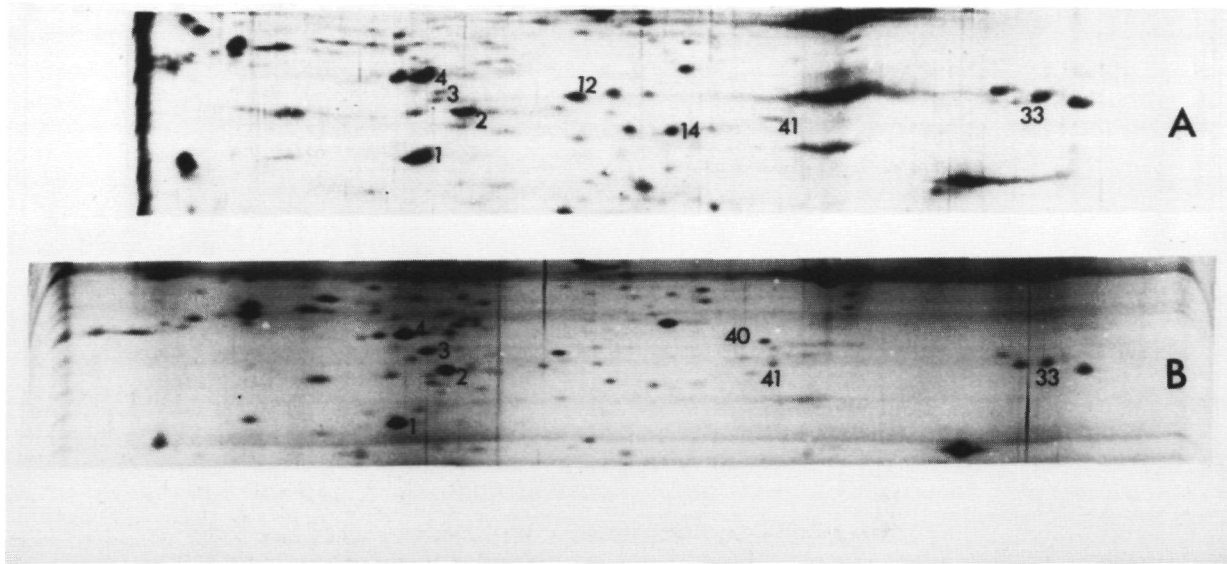

Fig 2:Details from the central area of the two-dimensional gel. Killer plasmodium (A),sensitive plasmodium (B). Conditions as in Fig 1A.

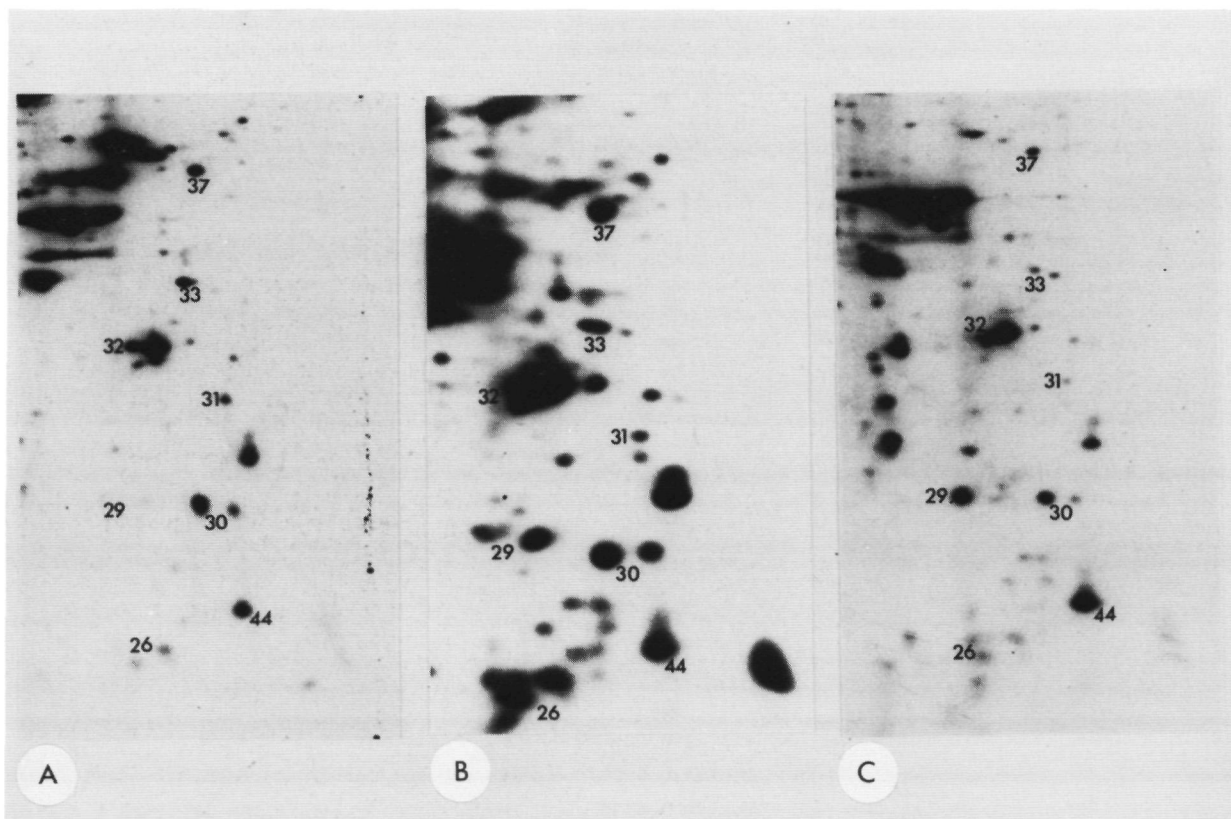

Fig 3:Details from fluorograms of labelled proteins from plasmodia after fusion of sensitive and killer strains. Discs cut from the fused plasmodium were labelled with [35S]–methionine for one hour. A,from 1/2–1 1/2; B,from 2–3; C,from 3 1/2–4 1/2 hours after fusion. Cessation of plasma streaming at 4 1/2 after fusion.

45–100K, as described earlier (Lafler et al, 1981; Turnock et al, 1981). Protein patterns of sensitive and killer strain are almost identical. Differences could be distinguished only around spotnumbers 3,33,40 and 41 (Fig 2).

To distinguish newly formed proteins after fusion from the bulk, polypeptides were labelled for a period of one hour. Small pieces cut from a plasmodium derived from the fusion of two plasmodia or from a plain strain were incubated with [35S]–methionine. Differences in the protein patterns of labelled polypeptides could be demonstrated only after fusion between sensitive and killer plasmodia. Fig 3 shows a change in the

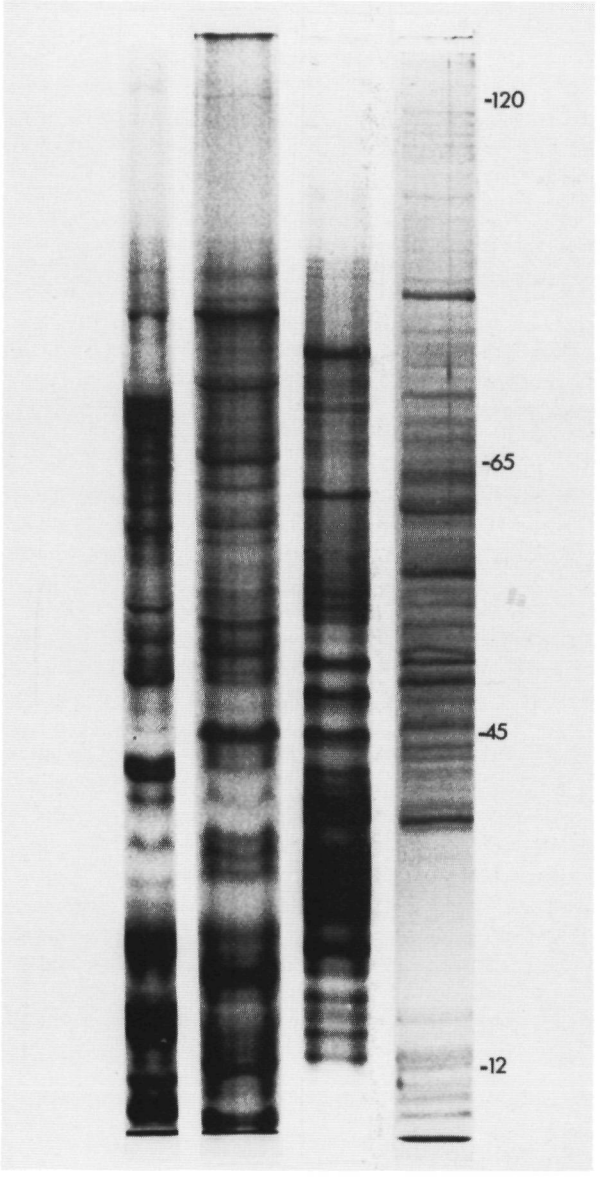

Fig 4:SDS acrylamide gel electrophoresis from fractionated sensitive plasmodium,loaded 10 ug protein on the top. From left to right nuclei, mitochondria, microsomen and 100000 g supernatant. Proteins were stained with the silver method.

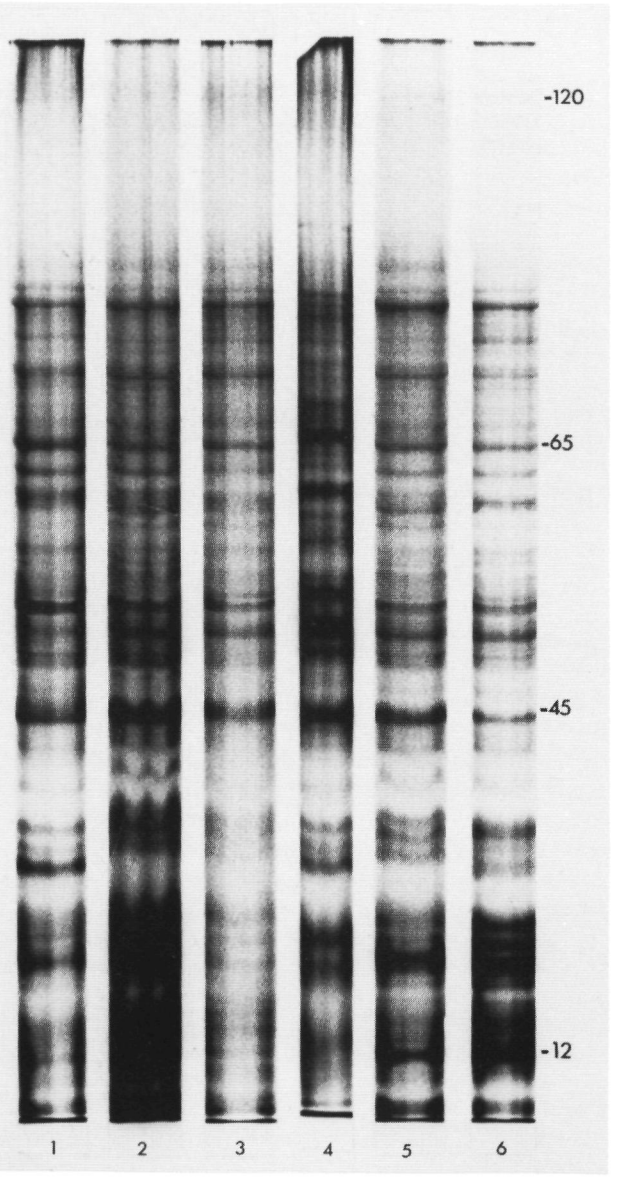

Fig 5:SDS acrylamide gel electrophoresis of mitochondrial fraction from fused plasmodia of sensitive and killer strains. Lane 1,2 and 3 resp. 1,2 and 3 1/2 hours after fusion. Lane 5, sensitive plasmodium; lane 6, killer plasmodium, lane 4, from plasmodial residues 1/2 hour after lysis of the fused plasmodium. Cessation of plasma streaming at 4 hours after fusion, decrease of plasma streaming at 3 1/2 hours.

fluorographic pattern of proteins synthesised in a plasmodium of sensitive and killer strain between respectivily 1 hour (A), 2 1/2 hours (B) after fusion and the cessation (C) of plasma streaming. No changes could be observed in the protein pattern, fluorographic or silver stained, between 1 and 4 hours after fusion of sensitive and sensitive or killer and killer plasmodia.

Changes in the protein composition of plasmodial fractions

A non-fused plasmodium was first separated into nuclear, mitochondrial, microsomal and supernatant fractions and than the proteins of these fractions were separated on SDS acrylamide gel and visualised by silver staining. Fig 4 shows several differences in the protein patterns of these fractions.

The polypeptide pattern of the mitochondrial fractions from a plasmodium derived after fusion of sensitive and killer strains changes from two hours after fusion. Fig 5 shows a qualitative change at 2 and 3 1/2 hours after fusion (lane 2 and 3) for polypeptides with molecular weight between 40 and 60 Kdalton. Changes occurred in almost all protein patterns of fractions from lysed plasmodia by 6 hours after fusion of sensitive and killer plasmodia. For the mitochondrial fraction this change is shown in lane 4, Fig 5. However, no changes were observed in other plasmodial fractions from fused sensitive and killer plasmodia. Protein patterns of plasmodial fractions from homologous fused plasmodia never demonstrated any visible change. The molecular weights of both the newly formed proteins demonstrated in Fig 3 and the proteins from the mitochondrial fraction, the latter undergoing changes between 1 and 5 hours after fusion, are of the same magnitude,about 50 Kdaltons.

Microinjection of plasmodial extracts

Injection of solutions into a plasmodium can be performed and the distribution of the injected substances can be monitored. Within one hour of injecting [14C]-Leucine into a plasmodium, it was almost evenly distributed.

Treatment of plasmodia, sensitive or killer, by injection of plasmodial extracts resulted in an immediate temporary and local expansion of the plasmodium as well as a temporary cessation of the plasma streaming. Within an 1/2 hour after injection, the plasmodium shape and streaming appeared to have returned to normal. Injuries were concentrated mostly around the point of injection. Crude extracts plugged the microcapillary tip. Therefore,the most complex plasmodial extract which could be injected was the extract after homogenisation, centrifugation (10000 g) and filtration. However, none of the tested plasmodial extracts caused a visible process comparable to the lethal reaction. The maximum effect that could be demonstrated was an irreparble damage around the place of injection and was more related to the amount of injected material than to the type. The tested plasmodial extracts were from plasmodia of sensitive and killer strain as well from fused plasmodia at different moments after fusion, even from lysed residues. Plasmodial extracts present in the capillaries led to spontaneous formation of blebs, starting at the tip if the capillaries were kept at room temperature. The rate of bleb formation was temperature dependent, which suggests that the extracts has retained some physiological activity.

Repeatable-fusion and refusion of plasmodia

Small pieces of plasmodia (10x5 mm) were cut from a large plasmodium and brought into contact with another plasmodium or agar medium. Transport of such tiny plasmodia to another homologous plasmodium resulted in complete mixing of the plasmodia (Table 1). Making contact of such tiny plasmodia, either sensitive or killer, with a fused plasmodium from sensitive and killer strain resulted in complete mixing as long as plasma streaming was present in the heterokaryon (Table 1). In the newly contacted plasmodium the lethal reaction appeared, as in the larger earlier fused plasmodium, with a short delay of time. Contact of a tiny plasmodium with a fused plasmodium of sensitive and killer strain in which the plasma streaming had been stopped caused no mixing and no lethal reaction in the attached tiny plasmodium (Table 1). The lethal reaction developed normally in the earlier fused large plasmodium in which the plasma streaming had stopped (Table 1). No clear phenomenaes and surely no cessation of plasma streaming or lethal reaction appeared in a tiny plasmodium after making contact with either a lysed plasmodium or the agar medium of which a lysed plasmodium had been scraped off and into which several compounds had diffused visibly (Table 1).

Discussion

The patterns of the electropherogrammes (Fig 1 A and B) show a great number of polypeptides present in plasmodia of *P.polycephalum*, qualitatively reliable for the used detection methods, silver staining and radioactive labeling. The failure to demonstrate any change in the protein pattern between 1 and 4 hours after fusion of

Table 1.The influence on tiny plasmodia at mixing, plasma streaming and lethal reaction by larger earlier fused plasmodia.

Plasmodium type		Tiny plasmodium			
large fused	tiny	moment of contact with large fused plasmodium, hours after fusion of large plasmodia.	mixing with large	plasma streaming	lethal reaction
s + k	s	‹ 4 h	+	++→−	+
s + k	k	‹ 4 h	+	++→−	+
s + k	s	› 4 h	−	++	−
s + k	k	› 4 h	−	++	−
s + s	s	0 - 24 h	+	++	−
k + k	k	0 - 24 h	+	++	−

In the large fused plasmodium of sensitive and killer strain, plasma streaming decreases at 4 h and stopped at 5 h after fusion. In this large plasmodium lysis started at 5 h after fusion. In the tiny plasmodium there is a delay of cessation of plasma streaming and lethal reaction. This delay is greater if large plasmodia were fused longer before the moment of contact with the tiny plasmodia.

two identical plasmodia,sensitive or killer, suggests that fusion of plasmodia of *P.polycephalum* needs no special protein synthesis. After fusion of plasmodia of sensitive an killer strains, the synthesis of proteins not present originally, occurred between 1 1/2 and 5 hours after fusion (Fig 3). Since protein synthesis decreases at the moment that the plasma streaming declines (Schrauwen, 1979a), between 4 and 5 hours after fusion of sensitive and killer plasmodia, measurements could be taken until a visible change in plasma streaming. Since transcription (Schrauwen, 1981a) and translation (Schrauwen, 1984a) seem to be involved in the processes, leading to a lethal reaction after fusion of sensitive and killer plasmodia, newly formed proteins may play a role in this series of reactions. Cyclohexemide inhibits protein synthesis for at least 99% and overcomes the lethal reaction in a plasmodium if the inhibitor is added before and up to 4 hours after fusion of sensitive and killer plasmodia (Schrauwen,1979a). Since all new proteins are formed between 1 and 4 hours after fusion of sensitive and killer plasmodia (Fig 3) it is questionable if these new proteins are direct responsible for the lethal reaction. It is more likely that this newly formed proteins are a part of the change in the membrane composition which starts at 1 1/2 hour after fusion (Schrauwen, 1984b) ore are a part of the series of reactions that ends in the lethal reaction.

Though plasmodia of *P.polycephalum* can be influenced by the injection of different compounds (Ueda and Olenhusen, 1978; Stockhem et al, 1978) as well by incubating plasmodia with plasmodial extracts (Loidl and Grobner, 1982), neither an injected plasmodial extract nor the residues from a lysed plasmodium, membranes as well as diffusable components, have the power to influence a plasmodium harmfully. Cessation of the plasma streaming in the entire plasmodium followed by a lethal reaction, a typicaly phenomenon after fusion of sensitive and killer plasmodium, could not be demonstrated with residues from a lysed plasmodium or after injection of plasmodial extracts . The absence of any influence on the lethal reaction of these components from fused or non–fused plasmodia and the slow spreading of the lysis process over the entire plasmodium suggests that the hypothetical lysis compounds are firmly bound to plasmodial vesicles. This idea correlates with the hypothesis that the development of the lethal reaction may be caused by disjunction of vesiclemembranes in the cytoplasm after fusion of sensitive and killer plasmodium (Schrauwen, 1984b).

Fusion of tiny plasmodia, sensitive or killer, with a fused plasmodium of sensitive and killer strain between 1 and 4 hours after the first contact of the large plasmodia, causes with some delay, a lethal reaction in the tiny plasmodium. This delay suggests that before the lysis compounds can be active a change in the tiny plasmodium has to occur. This is perhaps a developmental prerequisite or a kind of preparation of the plasmodium to make the

plasmodium sensitive for the lethal reaction (Lane and Carlile, 1979; Schrauwen, 1984b). This may explain why no visible reactions could be demonstrated after injection of compounds from a lysed plasmodium into another plasmodium.
The occurence of the lysis reaction in the later fused tiny plasmodia, sensitive as well as killer, suggests that both plasmodia are targets and participate in their own lysis in some active way as in mammalian cells (Lachmann, 1983).

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Loidl P, Grobner P (1982) Acceleration of mitosis induced by mitotic stimulators of *Physarum polycephalum.* Exp Cell Res 137:469–472

Mohberg J, Rusch HP (1971) Isolation and DNA content of nuclei of *Physarum polycephalum* Exp Cell Res 66:305–316

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7 GENERAL CONCLUSION

The tolerance of cells from *Physarum polycephalum* is regulated genetically. Haploid amoebae form zygotes - diploid plasmodia - in response to their composition of alleles regulating mating and fusion Two plasmodia fuse if their strains are identical or if their fusion *(fus)* loci match each other. A post–fusion incompatibility reaction follows fusion of plasmodia of two strains which have a different composition of *let* alleles The plasmodium which has the major dominant *let* alleles of the two interacting strains are called killer and the other sensitive. A series of reactions is initiated in the fused heterokaryon which causes the destruction of a part or of the entire fused plasmodium (Chapter 3) The involvement of transcription (Chapter 4) and translation (Chapter 3) in these processes can be demonstrated The results of Chapter 5 and 6 show the participation of qualitative changes in proteins and phospholipids The destruction of the heterokaryon plasmodium, called lysis or the lethal reaction, is not only directed toward the sensitive plasmodium (Chapter 3 and 6) A severe lysis reaction will destroy both sensitive and killer plasmodia Fusion of killer and sensitive plasmodia not followed by a lethal reaction, or by a weak one, leads to the formation of a plasmodium with killer properties within 48 hours (Chapter 3) The disappearance of sensitive DNA can be demonstrated in this heterokaryon during the period of change (Chapter 4). Though several metabolic processes were shown to be the result of the interactions between sensitive and killer plasmodia in the fused heterokaryon, none of these compounds could be demonstrated by direct in vitro or in vivo tests (Chapter 6)

From the results described in this thesis it may concluded that after fusion of sensitive and killer plasmodia two types of reactions are initiated. Type 1, a very specific one, that is directed only toward the sensitive strain, and perhaps mainly at their nuclei as a target (Chapter 4) A second type (Scheme) of reaction results in the lysis reaction, in which both strains participates as targets as well as attackers (Chapters 3,4 and 6) Another conclusion is that in both type of reactions a series of products may have to be produced before the end–product is effective (Chapters 3,4,5 and 6)

This conclusion is sustained by· (1) the length of time between the moment of conact and the rejection reaction in respect to the presence of the killer strain over the entire heterokaryon within one hour (Scheme) (2) the different time periods in which the demonstrated metabolic compounds are active or formed (Scheme) (3) the failure to demonstrate the different components involved with both types of reactions

Scheme about the interactions in a heterokaryotic plasmodium of *Physarum polycephalum*.

Type 2	Moment of contact between sensitive and killer plasmodium	0 h
	Interaction between nuclei of both plasmodia presumble by membranes	0 h→
	Spreading of killer properties over the entire plasmodium	0-1 h
	Change of the membrane composition	1½ h
	Visible change of the surface of the fused plasmodium	~2h
	Qualitative change of protein pattern	~2 h
	Latest moment at which the lethal reaction can be stopped by inhibiting RNA synthesis	2 ½ h
	After this moment the inhibition of protein synthesis cannot prevent the lethal reaction anymore.	3¾ h
	Surface of the heterokaryon gets shiny	4 h
	Decrease of the rate of RNA and protein synthesis	4 h
	Plasma streaming until this moment very fast starts to decrease	4 h
	Fusion with plasmodia of the original strains is not longer possible	4 h
	Lysis of the plasmodium starts, first at place of contact between the two plasmodia	4 - 5 h
	A major part of the plasmodium has beeen destructed	6 h
Type 1	A specific attack at the sensitive nuclei	8 h→

8 SAMENVATTING

Samenvatting

Onverenigbaarheid van plasmodia van verschillende stammen van de slijmzwam *Physarum polycephalum* wordt genetisch gereguleerd.
De vorming van diploide plasmodia uit haploide amoebae is afhankelijk van de allel samenstelling voor zowel paring als fusie van de gameten.
Fusie van twee plasmodia kan tot stand komen indien de twee stammen identiek zijn of bij volledige overeenstemming van de fusi loci. Wordt er een plasmodium gevormd uit twee niet identieke stammen dan resulteert dat meestal in een post–fusie incopatibiliteits reaktie. Deze laatste reaktie wordt gecontroleerd door de samenstelling van de aanwezige *let* (lethaal) allelen van beide plasmodia. Iedere stam beschikt over een set *let* allelen samengesteld uit "dominante" en "recessieve" genen. De stam met de meeste dominante *let* allelen wordt "killer" genoemd en de ander "sensitief".
Na fusie van sensitief en killer plasmodium ontwikkelen er zich een serie reakties, waardoor een deel of soms zelfs het gehele gevormde plasmodium vernietigd wordt. De betrokkenheid van transkriptie en translatie kon worden aangetoond bij de processen die voorafgaan aan de lethale reaktie. Tussen het moment van kontakt van de twee plasmodia en het optreden van de eerste beschadigingen in het heterokaryon konden kwalitatieve veranderingen van eiwitten en membranen worden aangetoond. Al deze makromoleculaire veranderingen verlopen gefaseerd (schema).
De lytisch aandoende reaktie waarbij de heterokaryon beschadigd wordt lijkt niet alleen gericht te zijn op het sensitieve plasmodium. Vrijwel altijd wordt een belangrijk deel en soms zelfs het gehele killer plasmodium vernietigd.
Fusie van plasmodia van twee stammen leidt soms tot geen zichtbare afbraak van de heterokaryon, maar heeft wel tot gevolg dat het gevormde plasmodium binnen 48 uur de eigenschappen van de killer verkrijgt. Tijdens deze omzetting kon de verdwijning van sensitief DNA uit de heterokaryon worden aangetoond.
Uit de resultaten van de hoofstukken 3,4,5 en 6 blijkt, dat meerdere metabolische processen optreden na fusie van sensitief en killer plasmodia,die niet voorkomen na fusie van homologe plasmodia. Toch kon geen enkele component worden geisoleerd of aangetoond, die verantwoordelijk was voor de lethale reaktie of voor de afbraak van sensitief DNA, in vitro noch in vivo.
Uit de resultaten die in deze thesis beschreven zijn, kan men concluderen dat na fusie van sensitief en killer plasmodia mogelijk twee type reakties ontstaan. Type 1, dat alleen gericht is op de sensitieve stam en vermoedelijk voornamelijk op de sensitieve kernen. Een tweede type waarin beide stammen als aanvaller en slachtoffer optreden,die eindigt in een vernietiging van beide stammen.
Beide reaktie typen bestaan vermoedelijk uit stapsgewijze processen (schema), die zich geheel of voor een groot deel afspelen aan de membranen. De conclusie van het stapsgewijze verlopen van de processen kan men trekken uit het gefaseerd optreden van de aangetoonde veranderingen. Dit geldt zowel voor de fusie eigenschappen van de heterokaryon als voor de makromoleculaire veranderingen. De betrokkenheid van de membranen wordt gesteund door een verandering in zijn samenstelling en de afwezigheid van aantoonbare verbindingen in het cytosol, die beschadigingen aan plasmodia kunnen oproepen.

Dankwoord

In dit proefschrift wil ik oprecht zeggen: **BEDANKT.**
Aan alle medewerkers van de hele botanie, dus inclusief de tuin.
Voor de vrijheid die mij gegeven werd eigen ideeen te kunnen aanpakken en uitwerken.
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Voor de treffende warme belangstelling, die ik heb ondervonden in de afgelopen jaren bij een aantal persoonlijke zaken.
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Aan de medewerkers van alle technische diensten.

Onderzoek doen of onderwijs geven zonder hulpmiddelen is voor mensen van deze tijd bijna onmogelijk. Gelukkig bezit de faculteit een uitgebreide technische staf die vakbekwaam vrijwel iedere vraag helpt oplossen, zelfs vanuit zeer sumiere aanwijzingen. Opvallend was hierbij in de afgelopen decennia, de bereidheid om het gevraagde snel en zo goed mogelijk te leveren. Zelfs als de leveringsdatum "gisteren" was,werd het probleem opgewekt en energiek aangepakt. De resultaten van het groot aantal keren, dat ik medewerking kreeg zijn terug te vinden , zowel in dit proefschrift als in eerder verschenen publikaties. Rechtstrecks via illustraties,foto's of beschreven apparatuur. Misschien niet direct herkenbaar maar zeker een belangrijk onderdeel vormend waren het voorkomen van "rampen", door het direct herstellen van een storing of het geven van advies over een veelheid van zaken, die nooit beschreven worden en heel gewoon lijken.
Aan de vele studenten met wie ik mocht discussieren en samenwerken. Die bereid waren mij te aanhoren bij kennis overdracht, die mij stimuleerden en jong hielden tot in mijn nieren.

Hierbij wil ik vermelden, dat een gedeelte van de inhoud van dit proefschrift is verwerkt met UNIX/TM software (nroff en troff) en afgedrukt op een linotron fotozetter 202 van de Faculteit Wiskunde en Natuurwetenschappen.

Curriculum vitae

De schrijver van dit proefschrift werd geboren te Breda, waar ook de middelbare school en het Analysten diploma, chemische richting, werd behaald. Na het vervullen van de militaire dienstplicht trad hij op 1 juni 1958 in dienst van de Faculteit der Wiskunde en Natuurwetenschappen. Vanaf die datum nam hij deel aan het onderzoek naar de incompatibiliteit bij planten op het Botanisch Laboratorium onder leiding van Professor Dr H.F.Linskens. Na een aanvullende studie in wiskunde werd in 1961 begonnen aan een avondopleiding M.O.-A Scheikunde en Natuurkunde in Utrecht,waarvoor in 1965 het examen met goed gevolg werd afgelegd. Deze opleiding werd gevolgd door een part-time studie Scheikunde in Nijmegen, waarvan het doctoraal examen werd behaald in januari 1972. In 1972 en 1973 was hij voor een jaar verbonden aan State University of New York te Syracuse, waar werd deelgenomen aan onderzoek bij dormante zaden. Speciale aandacht kreeg hierbij de aktivering van organellen bij het doorbreken van de rusttoestand. Vanaf 1974 heeft hij aan de Katholieke Universiteit deelgenomen aan pre- en post-candidaats onderwijs, waaronder de begeleiding van de cursus Plantenfysiologie in het tweede jaar en Research cursus in het derde jaar van het curriculum Biologie. In de laatste tien jaar is hij achtereenvolgens secretaris geweest van de werkgroep Bevruchtingsonderzoek bij Planten en de werkgemeenschap Genetisch-Fysiologisch en Bevruchtings onderzoek bij Planten in BION verband. Gedurende deze tijd werden vele recenties geschreven voor vakbladen en werden voordrachten gegeven in Dronten, Eerbeek,Wageningen, Lille, Seefeld, Leicester. In 1975 is een begin gemaakt met het onderzoek zoals beschreven in dit proefschift. In october 1963 ben ik getrouwd met Lia van Maurik, waarna wij twee kinderen kregen Saskia en Bob.

STELLINGEN

I

De vorming respectievelijk vermeerdering van bepaalde celorganellen in zaden van *Fraxinus americana* treedt pas op na het doorbreken van de dormante toestand
Schrauwen JAM, Sondheimer E (1975) Z Pflanzenphysiol 75,67-77

II

De hoge concentratie van het aminozuur proline in pollen, zoals deze in sommige plantensoorten voorkomt, kan alléén voordat de kieming plaats vindt een fysiologische betekenis hebben
Linskens HF, Schrauwen JAM (1969) Acta Bot Neerl 18,605-641
Linskens HF, Britikov EA, Schrauwen JAM (1970) Acta Bot Neerl 19,515-520
Zhang HQ, Croes AF, Linskens HF (1982) Planta 154,199-203
Zhang HQ,thesis (1983)

III

Allergenen kunnen direct worden aangetoond Isolatie en karakterisering van deze stoffen zijn daarom belangrijke voorwaarden voor ontwikkeling van methoden ter bestrijding van allergene aandoeningen
Linskens HF, Schrauwen JAM (1978) Schriftenreihe der Allergopharma Joachim Gauzer KG 9,53-58

IV

De verschillen in samenstelling van suikers en aminozuren in nectar van plantenfamilies zijn aanwijzingen voor een specifieke voedselafhankelijkheid van de bezoekers
Gottsberger G, Schrauwen JAM, Linskens HF (1984) Plant Syst Evol 145,55-77

V

De eiwitten die een rol spelen bij de kieming van pollenkorrels en bij de groei van de pollenbuis zijn verschillend
Linskens HF, Schrauwen JAM, Konings RNH (1970) Planta 90,153-162
Herpen MMA van (1984) Acta Bot Neerl 33,195-203

VI

Er is een overeenkomst in de ultrastructuur van pollenbuizen bij lelies, die in vitro zijn gegroeid (in een medium met 10% sucrose en 0 03% $Ca(NO_3)_2$) , en pollenbuizen die in vivo zijn gevormd na een incompatibele bestuiving
Miki-Hirosige H, Nakamura S, Yamada Y (1984) Internat Symp on "Sexual reproduction in seed plants, ferns and mosses', Wageningen p 24

VII

De resultaten van het onderzoek naar de gedetailleerde chemische samenstelling van binnen- en buitenmembranen van chloroplastenveloppen ondersteunen de endosymbiose theorie over het ontstaan van de chloroplast
Block MA, Dorne AJ, Joyard J, Douce R (1983) J Biol Chem 258,13281-13286

De conclusie van Rauch et al (1984), dat de door hen beschreven monoclonale antilichamen gericht zijn tegen de phosphodiester band van polynucleotiden en phospholipiden, is onjuist

Rauch J, Tannenbaum H, Stollar BD, Schwartz RS (1984) Eur J Immunol 14,529-534

IX

Analyse van chromosomen met behulp van de flowcytometrie methode kan een belangrijke bijdrage leveren aan de karakterisering van de chromosoomsamenstelling van plantaardige cellen

Lebo RV (1982) Cytometry 3,145-154

X

Het model van Aelen et al (1983) en van der Velden et al (1984) omtrent de aanhechting van eukaryotische replikatie 'origins' aan de kernmatrix is aannemelijk

Aelen JMA, Opstelten RJG, Wanka F (1983) Nucleic Acids Res 11,1181-1195

Velden HMW van der, Willigen G van, Wetzels RHW, Wanka F (1984) FEBS Lett 171,13-16

XI

Een dietiste wordt vaak ten onrechte gezien als iemand die opgeleid is om zich bezig te houden met de slanke lijn

XII

Als plantenfysiologen hun resultaten willen vertalen in een bijdrage aan de oplossing van het wereldvoedsel probleem, moet meer aandacht besteed worden aan de plantaardige producten, die in de "derde" wereld geconsumeerd worden

XIII

In de intramurale gezondheidszorg maken velen zich ongerust over de voorgenomen arbeidstijdverkorting, die een nadelige invloed kan hebben op de patient.
Een andere taakverdeling binnen dit instituut kan onrust en nadelen wegnemen

XIV

Eenmaal per jaar
is Sinterklaas daar
Om dit feit vandaag te memoreren
wil ik de volgende stelling offreren

De verkorting van studietijd in het w.o.
en de reorganisatie van het h b o.
heeft gevolgen voor beiden
waaronder één zal lijden
het is de afgestudeerde van het wetenschappelijk onderwijs
die op de arbeidsmarkt betaalt de hoogste prijs