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The genetics of *Ustilago maydis*

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The Ustilaginales, or smut fungi, are an important group of parasitic Basidiomycetes the genetics of which have remained relatively unexplored. An examination of the characteristics of certain species indicates that there are no features which should prevent or hinder genetic analysis; on the contrary they would appear to provide very suitable material for the investigation of problems of fundamental interest.

Standard genetic analysis is possible since a normal sexual cycle occurs. The haploid stage consists typically of uninucleate sporidia which divide by yeast-like budding to form compact colonies on artificial media. Inoculation of haploid strains of opposite mating type into the appropriate host results in the formation of an infective dikaryon within the host tissue. The binucleate cells of the dikaryon eventually round off to form thick-walled brandspores, or chlamydospores, within which the fusion of the nuclei takes place. When the brandspore germinates, meiosis occurs and a tetrad of haploid basidiospores is formed. The members of the tetrad (which is linear in the Ustilaginaceae) can be isolated, and each gives rise to a colony of sporidia. The absence of a fruiting body makes it particularly easy to control the environment of the cells undergoing meiosis.

The chromosomes of several species of smut fungi, including *Ustilago maydis* (Kharbush, 1928), have been examined and there is general agreement that the haploid chromosome number is two (see reviews by Christensen & Rodenhiser, 1940, and Ainsworth & Sampson, 1950).

Many of the smut fungi grow vigorously on a simple medium consisting of carbohydrate, asparagine and mineral salts (Schopfer & Blumer, 1938). The production of mutant strains requiring particular growth factors has already been achieved by Perkins (1949) in *U. maydis*. Such strains provide a large source of genetic markers.

The sporidia are uninucleate, which simplifies the processes involved in the production of mutants and the measurement of mutation rates. The compact growth makes it possible to use replica plating (Lederberg & Lederberg, 1952) and other bacteriological techniques which enable large numbers of colonies to be handled more easily than can be done with filamentous fungi.

Mendelian segregation of a pair of mating-type factors was first demonstrated in the smut fungi by Kniep (1919), and has since been shown in most species examined. Spore and sorus characters have also been shown to be controlled by single loci in several species (see Christensen & Rodenhiser, 1940), and more recently segregation of a single gene affecting pathogenicity has been observed in

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U. avenae (Halisky & Holton, 1956). Claims of linkage between genes have been made by Dickinson (1931) in *U. hordei*; Rodenhiser (1934) in *Sphacelotheca sorghi* and *S. cruenta* hybrids; Fischer (1940) in *Ustilago bullata*, and Holton (1951) in *Tilletia foetida*, but in none of these has adequate quantitative data been presented to substantiate the claim. The most satisfactory genetical data have been collected by Dickinson (1931) and Huttig (1931, 1933). By analysing linear tetrads in *Ustilago hordei*, they obtained the frequencies of first and second division segregation at meiosis for the mating-type locus. Some of their data suggest that the centromere and this locus do not segregate at random, which indicates that the centromere distance is less than the theoretical maximum of 33·3 units.

Since there were few indications from the available genetical data as to which smut fungus would be most convenient for further genetical study, the choice of species was determined by certain technical considerations. *Ustilago maydis* (DC.) Corda (syn., *Ustilago zeae* Beckm. Unger) was selected primarily because the life cycle can be completed in a few days (Stevens, Melhus, Semenink & Tiffany, 1946). In addition its host, *Zea mays*, has been more fully investigated genetically than any other flowering plant, which opens up possibilities in the investigation of the genetics of the host parasite relationship. Another characteristic unusual among smut fungi which was considered to be useful, is that the sporidia or basidiospores of opposite mating type do not fuse on artificial media. This removes many difficulties which might otherwise be encountered in the classification of progeny.

Numerous genetic investigations with *Ustilago maydis* have been mainly concerned with the isolation and description of mutants and with heterothallism. Apart from Perkins' (1949) work on the production of biochemical mutants, large numbers of mutations affecting the morphology or colour of sporidial colonies have been examined (see Stakman, 1936; Christensen & Rodenhiser, 1940; Holton, 1953). A 2:2 segregation in tetrads for factors affecting colony appearance or mutability has occasionally been obtained (e.g. Hanna, 1929; Stakman, Kernkamp, Martin & King, 1943*a*; Stakman, Kernkamp, Thomas & Martin, 1943*b*); but none of the characters which have been studied showed a consistent segregation, and it has generally been concluded that their inheritance is controlled by two or more loci.

From extensive early investigations of heterothallism it was believed that *U. maydis*, unlike the majority of smut fungi, had a complex mating system involving more than one locus. This work has been critically reviewed by Whitehouse (1951). More recent studies by Rowell & De Vay (1954) and Rowell (1955*a*) showed that the production of brandspores in the host is controlled by alleles at two loci. Homothallic or solopathogenic strains, which appear as a result of irregular brandspore germination in certain crosses, have been frequently encountered (see Christensen & Rodenhiser, 1940; Holton, 1953). Induced segregation of mating-type factors from one such strain has been reported by Rowell (1955*b*).

The very slow progress in the genetics of *U. maydis* and other smut fungi can be attributed to the absence of easily distinguishable characters controlled by single

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genes, and to the lack of techniques allowing the handling and scoring of large numbers of progeny. As Perkins (1949) has pointed out, the first difficulty can be removed by using biochemical mutations as genetic markers. The present paper is largely concerned with an attempt to remove the second difficulty.

MATERIALS AND METHODS

(a) *Strains*

Brandspores of *U. maydis*, which had been collected near St Paul, Minnesota, U.S.A., were kindly supplied by Dr J. J. Christensen. Of three basidiospores isolated from the promycelium of a germinating brandspore, two were interfertile. Later three basidiospores were taken from a second brandspore, and again two were found to be interfertile, but neither was fertile with either of the original isolates. These four strains are the four basic mating types: a_1b_1, a_1b_2, a_2b_1 and a_2b_2. All subsequent strains were derived from their sexual or asexual progeny. In addition strains derived from brandspores collected near Saint-Benoît-sur-Loire, Dept. Loiret, France, were used in investigations of the mating system.

(b) *Media*

Minimal medium: glucose, 10 g.; salt solution, 250 ml.; distilled water to 1 litre. pH 5·8 after sterilization.

Salt solution: ammonium nitrate, 24 g.; potassium dihydrogen phosphate, 16 g.; sodium sulphate, 4 g.; potassium chloride, 8 g.; magnesium sulphate ($7H_2O$), 2 g.; calcium chloride, 1 g.; trace element solution, 8 ml.; distilled water to 2 litres.

Trace element solution (modified from Ryan, Beadle & Tatum, 1943): boric acid, 30 mg.; manganous chloride, 70 mg.; zinc chloride, 200 mg.; sodium molybdate, 20 mg.; ferric chloride, 50 mg.; copper sulphate, 200 mg.; distilled water to 500 ml.

All compounds were of Analytical Reagent standard.

Complete medium: glucose 10 g.; hydrolysed casein, 10 ml.; hydrolysed nucleic acid, 5 ml.; vitamin solution, 10 ml.; 'Difco' yeast extract, 1 g.; salt solution, 250 ml.; distilled water to 1 litre. pH 5·8 after sterilization.

Casein hydrolysate (according to the method of McIlwain & Hughes, 1944): 200 g. casein acid hydrolysed to produce 1 litre of hydrolysate, sterilized by Seitz filtration.

Nucleic acid hydrolysate (according to the method of Pontecorvo, Roper, Hemmons, Macdonald & Bufton, 1953): 2 g. yeast nucleic acid and 2 g. thymus nucleic acid produced 40 ml. of a mixture of acid and alkali hydrolysates.

Vitamin solution (Beadle & Tatum, 1945): thiamin, 0·1 g.; riboflavin, 0·05 g.; pyridoxin, 0·05 g.; calcium pantothenate, 0·2 g.; *p*-amino benzoic acid, 0·05 g.; nicotinic acid, 0·2 g.; choline chloride, 0·2 g.; inositol, 0·4 g.; folic acid, 0·05 mg.; distilled water to 1 litre.

Supplemented minimal medium: individual growth factors were added to minimal medium as required; amino acids 100 mg.; purines and pyrimidines 10 mg., and vitamins 1 mg. per litre.

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For solid minimal or supplemented minimal medium, 1·5% 'Difco' agar was added. For solid complete medium, 1·5% or 2·0% unwashed agar was added.

(c) *Incubation and storage of cultures*

All cultures were incubated at 30°C. Stock cultures were maintained on 3% complete agar slants (Perkins, 1949), stored at 3°C. and sub-cultured every two months.

(d) *Plating and counting*

Sporidia grown on complete medium were fairly easily dispersed by shaking in water to form a uniform suspension of single cells, whereas those grown on minimal medium disperse much less readily. Sporidia remain viable in distilled water for several days, and it has been found unnecessary to use saline for diluting and plating suspensions. Estimations of the concentration of sporidia or brand-spores were made with a Neubauer haemocytometer. Standard microbiological techniques were used for diluting cells and for spreading or suspending them in plates of agar.

Germination of sporidia was complete on minimal and complete medium. On complete medium the colonies derived from single sporidia were visible to the naked eye after incubation for 24 hours and about 2·5 mm. in diameter after 72 hours. Growth was slightly slower on minimal medium, and the colonies, instead of being round and smooth as they were on complete medium, tended to be convoluted and irregular in outline.

(e) *Crosses*

A modification of the method of inoculation used by Stevens *et al.* (1946) has been used. Sporidia from colonies growing on complete medium were added to a tube of sterile water and shaken until a suspension of about 10^7 sporidia per ml. was obtained. Suspensions were then mixed in the desired combinations before inoculation. The susceptible variety of maize 'Golden Bantam' was used throughout (Stakman & Christensen, 1927). Before germinating the seeds, they were dusted with a mixture of a mercury seed dressing and tetrachlorobenzoquinone. This was the only treatment which effectively prevented the growth of troublesome laboratory fungal contaminants on the seedlings. The seeds were germinated on damp filter paper at 30°C. After 72 hours, about 1 mm. of the tip of the coleoptile of each seedling was removed. Using a hypodermic syringe of 1 ml. capacity the mixed sporidial suspensions were inoculated just above the first node, until a droplet of suspension emerged from the open end of the coleoptile. The seedlings were sown in sand supplemented with a mineral salt solution of the following composition: potassium nitrate, 0·02%; potassium monohydrogen phosphate, 0·002%; magnesium sulphate, 0·002%, and ferric phosphate, 0·0005%. This solution was obtained by diluting one of 100 times these concentrations. For routine mating tests 4–6 seedlings were inoculated; for making crosses 10–16 seedlings were inoculated. The seedlings were grown in an illuminated incubator

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at 28°C., in which humidity was over 90%. Continuous illumination was provided by two 40-watt daylight fluorescent tubes.

Galls appeared on leaves or stems occasionally as soon as 3 days, but almost always 5 days after inoculation. Brandspores were usually formed between 8 and 10 days after inoculation. Normally 70–80% of the seedlings developed galls, but brandspores were not always produced in all of these. Galls containing spores were ground in a small mortar with a few drops of 1% or 1·5% copper sulphate solution. More solution was added and the mixture filtered through cotton wool to remove debris and clumps of brandspores. The suspension was left at room temperature for 18 hours (Christensen & Stakman, 1926) to kill any contaminants or vegetative cells, and was then centrifuged. The supernatant was decanted and the brandspores suspended in sterile distilled water. The fact that the spores do not germinate in the absence of nutrients, unlike those of many other smut fungi, makes this procedure possible. Germination of brandspores was not higher than 5% on complete medium, and less than 1% on minimal medium. This low germination proved to be useful in certain techniques of genetic analysis. Where brandspores were not required immediately, it was found most convenient to keep them by drying galls or pieces of gall tissue and storing them at room temperature.

Other methods are described in the text where relevant.

THE PRODUCTION AND IDENTIFICATION OF BIOCHEMICAL MUTANTS

Since the sporidia are uninucleate and haploid they are particularly suitable for treatment with mutagenic agents. All the mutations were induced in the strain a_1b_1 with ultra-violet light. A sterile 13-ml. quartz tube was filled with 11 ml. of a sterile suspension of freshly grown sporidia. The tube was corked and rotated about its long axis in a horizontal position, at a distance of 18 cm. from a low-pressure mercury discharge lamp. The lamp was supplied by Thermal Syndicate Ltd. and emitted 87% of its energy at 2500–2600 Å. The dose at this distance was 10,000–12,000 ergs/cm^2/sec.

Since only a relatively small proportion of the survivors of irradiation would be expected to carry suitable mutations, it was necessary to use selective techniques in order to isolate adequate numbers of mutants. Perkins (1949) isolated a number of biochemical mutants of *U. maydis* following ultra-violet irradiation, by the delayed enrichment technique of Lederberg & Tatum (1946). This method would appear to be ideally suited to *Ustilago* since sporidia form very small compact colonies when embedded in agar. After irradiation, about 400 viable sporidia were suspended in each plate of minimal agar medium. After two or more days the colonies were marked and a layer of complete medium added to each plate. A high proportion of the colonies which subsequently develop should have been auxotrophs. In practice ultra-violet light treatment appeared to delay considerably the germination of some of the sporidia so that the proportion of these colonies which were in fact auxotrophs was very low. This difficulty has also been encountered with *Aerobacter aerogenes* (Devi, Pontecorvo & Higginbottom, 1951) and

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Aspergillus nidulans (Pontecorvo *et al.*, 1953). In a number of experiments in which less than 10% of the sporidia survived irradiation, the time of supplementing the minimal plates with complete medium was varied considerably; nevertheless, only one auxotroph was recovered from a sample of over 200 colonies which developed after supplementation. The method was discontinued in favour of that of indirect selection by means of the technique of replica plating (Lederberg & Lederberg, 1952).

After irradiation about 200 surviving sporidia were spread on each of a series of plates of 2% complete agar. After incubation for 3 days most of the colonies were between 1 and 3 mm. in diameter. Each plate was replicated to minimal medium with a piece of sterile velvet spread over a circular piece of cork and held in position with a loop of wire. The minimal plates were incubated 2 days, and rare auxotrophs were then detected by placing the minimal plate over the complete and searching for gaps in the replica pattern. Unmatched colonies were isolated and retested on minimal. Fig. 1, Plate I, shows a master plate and its replica.

In a number of experiments with fresh sporidia in which the dose of irradiation was varied, the proportion of auxotrophs amongst the survivors remained constant at about 0·1%. The results from four experiments are shown in the first part of Table 1. This result is in agreement with data from other fungi where linear relationships between the ultra-violet light dose and the proportion of mutants induced have often not been observed with very high doses (see review by Pomper & Atwood, 1955). In a final experiment in which sporidia which had been stored at 3°C. for several weeks were irradiated, the proportion of auxotrophs in the survivors rose to 0·277% (see Table 1). This is a significantly higher figure than

Table 1. *The yield of biochemical mutants obtained under different experimental conditions*

Period of U.V. irradiation (min.)	Survivors (%)	Colonies examined	Stable mutants	
			No.	%
Freshly grown sporidia:				
1·25	7	8400	6	0·071
1·50	0·8	3500	5	0·143
3·00	0·04	4000	3	0·075
4·50	0·008	11400	9	0·079
		Total 27300	23	0·084
After storing sporidia at 3°C.:				
2·75	0·05	18400	51	0·277

that obtained with fresh sporidia ($2 \times 2\ \chi_1^2 = 25{\cdot}2, P < 0{\cdot}001$). Nevertheless, even this yield of mutants is considerably lower than that obtained following ultra-violet treatment in other fungi. For instance, in *Aspergillus* 0·6% of the survivors were auxotrophs (Pontecorvo *et al.*, 1953) and in *Neurospora* 0·72% were auxotrophs (Beadle & Tatum, 1945).

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Of 100 stable auxotrophs which were obtained, the basic biochemical requirements. of 94 were identified by a method which has been described elsewhere (Holliday, 1956). The six which were not identified may have had requirements which were not anticipated, or they may have been inhibited by the particular combinations or concentrations of growth factors in the test media. Some groups of mutants were further classified by testing which of certain possible precursors in the biosynthesis of their requirement would also support growth. These tests were carried out by means of the auxanographic method (Lederberg, 1946). The final classification of the auxotrophs is given in Table 2.

Comparison of the types of mutants identified with those obtained in *Penicillium* (Bonner, 1946), *Ophiostoma* (Fries, 1947), *Neurospora* (Tatum, Barrat, Fries & Bonner, 1950) and *Aspergillus* (Pontecorvo *et al.*, 1953) shows two notable differences. Firstly no lysine requiring mutants have been recovered in *Ustilago maydis* although these are one of the commonest types in the other four fungi. Secondly these fungi produced a smaller proportion of nicotinic acid requiring

Table 2. *The types of auxotrophs obtained following U.V. irradiation of prototrophic sporidia*

Requirement	Genetic symbol*	Number of mutants
Nucleic acid components:		
adenine/hypoxanthine	*ad*	17
cytidine/uridine	*cyt*	6
Vitamins:		
nicotinic acid	*nic*	3
nicotinic acid/anthranilic acid	*nic*	1
nicotinic acid/anthranilic acid/indole	*nic*	11
thiamin	*thia*	7
choline/dimethylethanolamine/monomethylethanolamine	*cho*	5
pyridoxin	*pdx*	2
pantothenic acid	*pan*	2
inositol	*inos*	2
p-amino benzoic acid	*paba*	2
Amino acids:		
arginine	*arg*	7
arginine/ornithine	*arg*	5
methionine	*meth*	2
methionine/homocysteine	*meth*	5
methionine/homocysteine/cysteine/cystathionine	*meth*	7
leucine	*leu*	4
histidine	*hist*	1
serine/glycine	*ser*	1
Others:		
reduced sulphur	*rs*	4
unidentified		6
	Total	100

* Mutants were numbered in the order of identification and are referred to as *ad-1*, *ad-2*, . . . etc.

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mutants than did *Ustilago*. On the other hand, the high frequency of adenine, arginine and methionine requirers which has been encountered is quite typical.

The methionine mutants in so far as they were tested appeared to have the same requirements as those found in *Neurospora* (see review by Wagner & Mitchell, 1955). None of the mutants which grew on arginine or ornithine responded to citrulline, proline or glutamic acid. These are similar to arginine mutants found in *Aspergillus* (Pontecorvo, *et al.* 1953) and differ from those in *Penicillium* (Bonner, 1946) and *Neurospora* (Srb & Horowitz, 1944). The nicotinic acid mutants had unexpected requirements. None of them, with one possible exception, showed any response to tryptophan, although most of them grew vigorously on anthranilic acid. These responses suggest that the nicotinic acid synthesis is not the same as that found in *Neurospora* (see Wagner & Mitchell, 1955) and that the pathway direct from anthranilic acid by-passing tryptophan, which appears to be an inefficient alternative method of nicotinic acid synthesis in *Aspergillus* (Pontecorvo, 1949), is possibly the main if not the only route in *Ustilago*. A full biochemical examination of this group of mutants might well yield interesting results.

The mutant *leu-1*, which has been used extensively as a genetic marker, was found to have unusual responses. It grew only slowly on leucine, but at the normal rate if glycine, serine, glutamic acid or aspartic acid was added to the medium. Methionine inhibited response to leucine, but this inhibition could be removed by any of the other four amino acids. Mutants of this type have not previously been reported.

HETEROTHALLISM AND PATHOGENICITY

Rowell & De Vay (1954) and Rowell (1955*a*) presented evidence for a compatibility system in *U. maydis* which is controlled by a pair of alleles at an *a* locus and multiple alleles at a second *b* locus. This mating system is not only exceptional among smut fungi, but is also unknown in any other group of outbreeding organisms. Moreover, since it has been pointed out by Whitehouse (1951) that anomalous mating behaviour previously reported in *U. maydis* and other smut fungi can be explained on the basis of recessive factors for weak pathogenicity or fertility, it seemed a necessary preliminary to genetic studies to verify the conclusions reached by Rowell & De Vay.

Whereas some workers have been able to obtain fusion of sporidia of opposite mating type on artificial media (Bauch, 1932; Sleumer, 1932; Bowman, 1946), others have been unable to accomplish this (Stakman & Christensen, 1927; Seyfert, 1927; Hanna, 1929). Rowell (1955 *a*) was able to obtain fusion only on a medium containing maize seedling extract. With the strains used in these experiments fusion of sporidia has not been found to be a reliable method for detecting mating type, and the production of galls in the host has been used as the criterion of compatibility. The results from mating-type tests were consistent with the view that the mating system is controlled by two loci. These are designated *a* and *b* but the particular alleles have not been identified with those in Rowell & De Vay's material. The four original basidiospore isolates consisted of two compatible pairs

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(a_1b_1, a_2b_2, and a_1b_2, a_2b_1) and no other combinations were fertile. Two sporidial isolates were obtained from French brandspores. These were tested with the four American strains and both were fertile only with a_2b_2 and a_2b_1; their mating type was therefore a_1b_3. From one of the fertile crosses between a_1b_3 and a_2b_2 brandspores were germinated. Thirteen sporidial isolates were taken at random and these were tested with the American strains. The results are shown in Table 3. The two loci had segregated independently, six of the thirteen isolates being recombinants. It was confirmed that the a_1b_3 and a_2b_3 progeny were not interfertile. In addition, it was found that when progeny from various crosses amongst mutant strains were tested routinely for mating type with the four original isolates, galls were always produced with only one of the four. In all, twenty-seven progeny were tested in this way.

Table 3. *The compatibility reaction of thirteen isolates from a cross between the French strain,* a_1b_3, *and the American strain,* a_2b_2

(Progeny:)		American strains (from 1st brandspore)		American strains (from 2nd brandspore)	
Genotypes	Frequency	a_1b_1	a_2b_2	a_1b_2	a_2b_1
a_1b_3	2	—	+	—	+
a_2b_2	5	+	—	—	—
a_1b_2	3	—	—	—	+
a_2b_3	3	+	—	+	—

+ indicates that galls were produced on the host.

It is not possible to explain these results on the basis of a single pair of mating-type alleles with independent segregation of factors for weak pathogenicity or fertility. Nevertheless, Rowell (1955*a*) has shown that the fusion of sporidia is controlled only the *a* locus; therefore, since it is impossible at present to distinguish pathogenicity from fertility, it cannot be concluded whether the *b* locus is in fact concerned with sexual compatibility or whether it merely determines the pathogenicity of the dikaryon.

In *U. maydis* it is not possible to determine by a simple complementation test on minimal medium whether biochemical mutants with the same requirement are allelic since heterokaryons or dikaryons will not grow outside the host. A test based on the pathogenicity reaction, however, appeared to be an effective though less convenient alternative.

It was never found that pathogenicity was impaired if only one parent carried a mutation at a particular locus. This was true even if each parent carried several mutations. A similar result has been reported by Buxton (1956) with *Fusarium oxysporum f. pisi*. On the other hand, when strains of opposite mating type carrying the same biochemical mutation were inoculated, pathogenicity was reduced or prevented. On selfing *ad-1*, *me-1* or *leu-1* no galls were produced in the host in repeated tests. When *ad-1* was inoculated with *ad-5* or *ad-10* of opposite mating

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type the same result was obtained. This indicates that these three adenine mutants are allelic, a conclusion which was supported by the close similarity of their appearance on complete medium. On selfing *inos-2* or inoculating it with *inos-1*, and on selfing *nic-10* or inoculating it with *nic-9*, small galls were produced in rather a mall proportion of seedlings. These pairs of genes also appear to be allelic.

With a heterozygous dikaryon the wild-type allele compensates for the deficiency of its partner and an exogenous supply of the relevant growth factor from the host is not required. On the other hand, when both nuclei are deficient at the same locus, the dikaryon is dependent on such a supply, and if this is inadequate pathogenicity is restricted. If this is so, it should be possible to restore pathogenicity by supplementing the host artificially with the relevant growth factor. Attempts to do this included methods similar to those devised for use with *Venturia inaequalis* by Kline, Boone & Keitt (1958). With *ad-1* these were completely unsuccessful, either because adenine was not entering the host in sufficient quantities, or because there is some direct relationship between pathogenicity and adenine synthesis within the mycelium. With *inos-2* and *nic-10* supplementation with a solution of 0·1 mg. per ml. of the relevant vitamin had a definite effect. In both crosses larger galls containing brandspores were produced, but normal pathogenicity was not completely restored. Supplementation was not attempted with *me-1* and *leu-1*.

Similar results have been obtained with other pathogenic fungi. Boone, Kline & Keitt (1958) found that mutants requiring nicotinic acid or inositol were pathogenic, whereas those requiring amino acids or adenine were non-pathogenic. Kline *et al.* (1958) were able to restore the pathogenicity of the former by supplementing the host, but this was not true of adenine requirers. Buxton (1956) found that strains of *Fusarium* requiring amino acids had reduced pathogenicity which could be increased by supplementation. On the other hand, Perkins (1949), using different methods of inoculation from those described here, found that one of his methionine requiring strains of *Ustilago maydis* was completely fertile when selfed.

METHODS OF GENETIC ANALYSIS AND EXPERIMENTAL RESULTS

(a) *Random isolation of the products of meiosis*

The basidiospores produced on the promycelium behave as if they were vegetative sporidia. As soon as they are formed they begin to bud off sporidia which continue to divide. The basidiospores are not formed simultaneously, so that two or more sporidia may have been produced from one basidiospore before the next is formed. For these reasons there is no possibility of obtaining a sample of basidiospores except by techniques of micro-manipulation. However, if cells of different genotypes divide at roughly the same rate, and the number of sporidia and basidiospores is high compared with the number of promycelia and ungerminated brandspores, a rapid method for the analysis of random products of meiosis is possible. To get a random sample of sporidia it is necessary to avoid harvesting them too late in the development of the brandspore colony, when different growth-rates might be exaggerated, or too early, when too many promycelia and ungerminated

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brandspores might be included. The procedure used was to spread several thousand brandspores on a plate of complete medium. When the largest brandspore colonies were just visible (after about 36 hours' germination), the agar was cut into strips which were transferred to a 250-ml. flask containing about 100 ml. of sterile distilled water, which was shaken until the colonies had become dispersed. The suspension was filtered through cotton wool and then between 100 and 200 sporidia were spread on each plate of complete medium. After 3 days' incubation the plates were replicated to appropriate test media. By using test media each lacking one of the requirements segregating in the cross, all the genotypes could be identified. The isolated colonies on the complete plates were numbered, and then scored by examination of the replicas. This technique was used not only for the purpose of genetic analysis but also to obtain particular progeny which were required for further crosses.

Table 4. *The analysis of random sporidia. Data from 2-point crosses*

Cross	Genotypes		Frequency	Recombination (%)
1. *thia-2*, a_2b_2/*me-1*, a_1b_1	*thia*	+	42	49·0
	+	*me*	57	
	thia	*me*	42	
	+	+	53	
2. *thia-2*, a_2b_2/*ad-3*, a_1b_1	*thia*	+	35	46·7
	+	*ad*	29	
	thia	*ad*	23	
	+	+	33	
3. *thia-2*, a_2b_2/*ad-1*, a_1b_1	*thia*	+	33	53·3
	+	*ad*	37	
	thia	*ad*	36	
	+	+	44	
4. *me-1*, a_2b_2/*nic-3*, a_1b_1	*me*	+	54	50·4
	+	*nic*	70	
	me	*nic*	52	
	+	+	74	
5. *me-1*, a_2b_2/*cho-4*, a_1b_1	*me*	+	21*	51·5
	+	*cho*	42	
	me	*cho*	32	
	+	+	35	
6. *me-1*, a_2b_2/*arg-1*, a_1b_1	*me*	+	31	55·0
	+	*arg*	23	
	me	*arg*	22*	
	+	+	44	
7. *me-1*, a_2b_2/*pan-1*, a_1b_1	*me*	+	36	46·5
	+	*pan*	41	
	me	*pan*	23*	
	+	+	44	

* Indicates a probability of less than 0·05 that complementary genotypes do not deviate significantly from equality.

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Table 5. *The analysis of random sporidia. Data from 3-point crosses*

Cross	Genotypes			Frequency	Recombination (%)
8. *nic-3 me-1*, a_2b_2/*thia-1*, a_1b_1	*nic*	*me*	+	40	
	+	+	*thia*	32	*nic-3* and *me-1*: 46·5
	nic	*me*	*thia*	28*	
	+	+	+	76	*nic-3* and *thia-1*: 55·6
	nic	+	*thia*	39	
	+	*me*	+	40	*me-1* and *thia-1*: 54·1
	nic	+	+	48*	
	+	*me*	*thia*	26	
9. *nic-3 me-1*, a_2b_2/*ad-1*, a_1b_1	*nic*	*me*	+	53	
	+	+	*ad*	40	*me-1* and *ad-1*: 20·6
	nic	+	*ad*	70*	
	+	*me*	+	37	*me-1* and *nic-3*: 50·4
	nic	*me*	*ad*	12	
	+	+	+	20	*nic-3* and *ad-1*: 55·2
	+	*me*	*ad*	1*	
	nic	+	+	19	

* Indicates a probability of less than 0·05 that complementary genotypes do not deviate significantly from equality.

Since the pioneer work of Beadle & Tatum (1941) with *Neurospora*, it has been shown in numerous micro-organisms that the inheritance of biochemical deficiencies resulting from mutations follows the pattern expected for single gene differences. It was not considered necessary, therefore, to demonstrate a 1:1 segregation for each mutant before it was used as a genetic marker. The data from seven 2-point crosses and two 3-point crosses involving nine mutants are shown in Tables 4 and 5. Crosses 1–8 indicate that linkage is not present between any of the markers tested, but a clear deviation from random segregation is present in cross 9. This shows that *ad-1* and *me-1* are linked, but that neither are linked with *nic-3*. Crosses 4 and 8 confirm that *me-1* and *nic-3* are unlinked.

These results indicate that normal segregation is occurring but that significant deviations from expected ratios do occur. Complementary genotypes are not always equal, and there is a general deficiency of progeny carrying mutant genes, particularly *me-1*, *arg-1* and *thia-1* and *2*. These deviations are presumably due either to different growth rates, or to difference in the germination of the basidiospores of the various genotypes. They are not large enough to obscure disturbed ratios due to linkage between genes. By combining the data from crosses in coupling and repulsion carried out under identical conditions, allowance could be made for differences in the germination and rate of growth of each genotype, and accurate linkage values would be obtainable (Mather, 1951).

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Unfortunately the results obtained in later crosses were much less satisfactory. In almost all cases an excess of wild-type sporidia and a deficiency of certain classes of mutant sporidia were found. It was always impossible to determine whether or not linkage was present. Results from tetrad analysis indicate that these unsatisfactory results are almost certainly due to the poor germination of the basidiospores of certain genotypes, rather than their slower rates of division. No evidence is available as to why germination was reduced in these crosses, and after several unsuccessful attempts to obtain reasonable data the method was abandoned in favour of tetrad analysis.

In addition to the results in Tables 4 and 5, some data on linkage were also obtained from routine tests for mating type. It was noticed that when *pan-1* was segregating, progeny carrying this marker or its wild-type allele were always parental with respect to the mating-type locus *a*. In all, eighteen such progeny were tested for mating type and none showed recombination between *a* and *pan-1*. This indicates that the loci are closely linked, with a probability of 0·05 that recombination would exceed 17·6 %.

(*b*) *Analysis of whole tetrads*

The germinating brandspore produces a compact colony which should consist of four haploid clones derived from the products of meiosis. These brandspore colonies will be referred to as whole tetrads. Perkins (1949) has suggested that some of the information which is obtained by the isolation of the individual members of a tetrad could also be gained by the examination of whole tetrads. The method depends on the ease of identification of prototrophic progeny by the use of a selective minimal medium. It is therefore applicable only to the study of biochemical markers. When two genes are segregating at meiosis, only three types of unordered tetrad can be produced: parental ditype (PD), recombinant ditype (RD), and tetratype (T). In the repulsion cross $a+/+b$, the PD tetrads ($a+$ $a+$ $+b$ $+b$) will not grow on minimal medium whereas the RD tetrads (ab ab $++$ $++$) and the T tetrads ($a+$ $+b$ ab $++$) will do so. In the coupling cross $ab/++$, only the RD tetrads do not contain prototrophic sporidia. By measuring the proportion of tetrads in each cross which will not grow on minimal medium, the frequencies of the two ditype tetrads are known and the recombination is given by:

$$r = \mathrm{RD} + \tfrac{1}{2}\mathrm{T},$$

or, since $\mathrm{T} = 1 - \mathrm{RD} - \mathrm{PD}$,

$$r = \tfrac{1}{2}(1 + \mathrm{RD} - \mathrm{PD}).$$

In Perkins' technique, each brandspore colony was tested for the presence of prototrophic sporidia by suspending all the sporidia from a single colony in a plate of minimal medium. This method is expensive in plates, and other techniques of whole tetrad analysis which are more convenient and less laborious have been devised.

About 2000 brandspores were spread on plates of complete medium of which approximately 100 germinated and developed into colonies. In the earlier crosses

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these were allowed to grow to a maximum diameter of about 4 mm. and were then replicated to minimal medium. A random sample of isolated colonies over 1 mm. in diameter was marked and their replicas were then examined. The same technique has been developed independently for use with *Chlamydomonas* (Levine & Ebersold, 1958). In later crosses the colonies were allowed to grow to a maximum diameter of 2 mm., and colonies over 0.25 mm. were picked off the plate with a needle and transferred to small tubes or plates of minimal medium. The sporidia tended to stick together on slightly acid medium (pH 5·8) and whole colonies could be transferred very easily, whereas on neutral medium the sporidia tended to separate and the colonies were removed much less readily.

There are of course several possible sources of error in measuring the frequency of PD or RD tetrads by these methods:

(1) In the galls of the host the brandspores are formed in groups. Most of these are broken down by grinding up the gall and most of the rest removed by filtration. A few are plated on complete medium and it is therefore possible for a single colony to be derived from the germination of more than one spore. But with the germination frequencies of 5% or lower which have been consistently observed, it has been calculated that the chance of this happening is negligible.

(2) It is possible that during the growth of the brandspore colonies mutant genes might revert to wild-type, with the result that colonies would be incorrectly scored. A colony 2·0 mm. in diameter consists of about $2{\cdot}5 \times 10^6$ sporidia and in certain crosses is liable to contain some revertant cells. However, it is very easy to distinguish between the rapid growth on minimal medium of a colony containing many thousands of prototrophic sporidia, and the much slower growth of one containing a few revertant sporidia which form sectors or patches growing out from a mass of auxotrophic cells. Colonies had sometimes been tested in tubes of liquid minimal rather than on agar medium, but this procedure was discarded since it was occasionally difficult to judge whether reversion had occurred. In the replication of colonies, only a few thousand sporidia were transferred to minimal, so that the chance of carrying over revertant cells was extremely slight.

(3) In the majority of smut fungi it is usual for sporidia of opposite mating type to fuse on artificial media. In *U. maydis* this occurs much less readily. Several attempts were made to synthesize balanced heterokaryons or dikaryons by streaking mixtures of different auxotrophs of the same or opposite mating type on minimal medium (Beadle & Coonradt, 1944; Pontecorvo, 1946). None was successful, so it seems certain that prototrophic growth on minimal medium must always be haploid.

(4) In a mixture of two auxotrophs on minimal medium each can secrete the growth factor the other lacks and allow mutual feeding or syntrophism to occur. Pronounced syntrophism leading to vigorous growth on minimal medium was never observed except after a lag of a week or more, whereas the prototrophic growth of brandspore colonies was scored after 2–3 days.

(5) Homothallic or solopathogenic strains have been reported in *U. maydis.* They appear to arise as a result of abnormal brandspore germination and are

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probably diploid. They appeared frequently in certain crosses (e.g. Christensen, 1931; Chilton, 1943), but Schmitt (1940) encountered only three such strains in over 4000 he examined. In numerous routine tests for mating type no solopathogenic strains have been found, but the possibility of obtaining rare brandspore colonies containing prototrophic diploid or aneuploid sporidia cannot be ignored.

(6) There is a wide scatter in the time of germination of brandspores after they have been plated on complete medium which results in great variation in size of the colonies. Variation could also be due to the different growth rates of different types of colony, and since it is not possible to score a completely random sample of colonies, this could result in biased data. In a number of crosses tetrads were scored both by replication (colonies 1–4 mm.) and by transfer of whole colonies (0·25–2 mm.), and in none of these was there a significant difference in the results obtained by the two methods (crosses 13, 14 and 21 in Table 6, and crosses 59 and 60, and 37, 39 and 40 in Table 7. The numbering of crosses in Tables 6 and 7 does not indicate the order in which they were carried out.) However, in a later cross a strong correlation between colony size and genotype was observed when scoring was by replication, although this correlation was absent if the other method was used (Holliday, 1959). It was considered that in certain crosses the replicator could fail to pick up prototrophic sporidia from the smaller colonies, or that a greater growth rate of prototrophs in comparison with certain mutant genotypes became evident only in large colonies.

None of the above six possible sources of inaccuracy in the method of whole tetrad analysis was thought likely to lead to other than slight errors in quantitative results, provided that the tetrads were scored by transfer of whole colonies. This method of scoring was therefore adopted in all later crosses.

Unfortunately two other sources of error were discovered during the course of the work. Some haploid sporidia or mycelial fragments from gall tissue survived the 1% copper sulphate treatment recommended by Christensen & Stakman (1926), and formed colonies indistinguishable from brandspore colonies. The scoring of such colonies led to the detection of differences between the PD and RD tetrad frequencies which could not have been due to linkage (Holliday, 1959). This error was prevented by using a 1·5% copper sulphate solution, which had no adverse effect on the viability of the brandspores. It was also discovered that in certain crosses there was incomplete basidiospore germination. This possibility had been ignored, since the basidiospores are indistinguishable morphologically from vegetative sporidia, whose germination is always complete on nutrient media. In addition the only reports of failure of germination of basidiospores in *U. maydis* and other smut fungi were in strains in which the germination of the brandspores was abnormal, being in association with lysis of sporidia and/or the production of solopathogenic lines (e.g. Christensen, 1931; Chilton, 1943; Fischer, 1940; Holton, 1951). Such abnormalities had not been observed in the strains used in the present investigations. The effect of failure of basidiospore germination on the detection and measurement of linkage by analysis of whole tetrads will be discussed below.

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In a routine search for linkage by analysis of whole tetrads information is gained by making only the repulsion or the coupling cross. If the repulsion cross gives a PD tetrad value of greater than 50% then the genes are 25 or less units of recombination apart. With values between 50% and the theoretical minimum of 16·67%, the genes may be loosely linked or unlinked. In the coupling cross RD tetrad values of less than 16·67% indicate linkage, whereas higher values indicate

Table 6. *The estimation of parental ditype (PD) tetrad frequencies by the examination of whole tetrads from repulsion crosses. (In each cross the first parent was of mating type* a_2b_2 *and the second* a_1b_1)

	Cross	Brandspore treatment and scoring method	Auxotrophic colonies / Total colonies	% Auxotrophic colonies (PD tetrads)
2.	*thia-2/ad-3*	CR	6/29	20·7
3.	*thia-2/ad-1*	CR	31/86	36·0
10.	*thia-2/nic-3*	CT	12/41	29·3
5.	*me-1/cho-4*	CR	350/1069	32·7
6.	*me-1/arg-1*	CR	197/460	42·8
11.	*me-1/ad-3*	CR	99/567	17·5
12.	*me-1/paba-1*	CR	144/558	25·8
13.	*me-1/nic-5*	CR	53/233*	24·0
		CT	10/30	
14.	*nic-1/arg-5*	CR	47/194*	22·1
		CT	17/95	
15.	*me-1/ser-1*	CT	0/13	0·0
16.	*me-1/ad-4*	CT	53/150	35·3
17.	*me-1/pdx-2*	CT	10/34	29·4
18.	*nic-3/paba-1*	CT	12/59	20·3
19.	*nic-3/pdx-2*	UT	18/74	24·3
20.	*nic-3/nic-2*	CT	69/274	25·2
21.	*nic-3/nic-1*	CR	58/189*	30·7
		CT	49/159	
22.	*nic-3/nic-12*	UT	18/61	29·5
23.	*nic-10/pdx-2*	UT	25/102	24·5
24.	*nic-10/nic-2*	UT	35/95	36·8
25.	*nic-10/nic-7*	UT	27/103	26·2
26.	*nic-10/nic-13*	UT	30/102	29·4
27.	*nic-10/nic-14*	UT	13/38	34·2
28.	*ad-1/nic-1*	UT	11/84	13·1
29.	*ad-1/ad-3*	CR	12/58	20·6
30.	*ad-1/ad-6*	CT	25/96	26·0
31.	*ad-1/ad-7*	CT	0/4	0·0
32.	*ad-1/ad-4*	UT	25/116	21·5
33.	*inos-2/nic-1*	UT	42/111	37·8
34.	*inos-2/nic-13*	UT	5/13	38·5
35.	*inos-2/pdx-2*	UT	8/23	34·8
36.	*leu-1/pdx-2*	UT	32/115	27·8

C indicates that the brandspores were treated with 1% copper sulphate and may have been contaminated with vegetative cells.

U indicates that the spores were treated with 1·5% copper sulphate and were therefore not contaminated in this way.

R indicates that brandspore colonies were scored by replication.

T indicates that they were scored by transfer of whole colonies.

* indicates that the CR and CT data are not significantly different.

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that the genes are on different chromosomes and that neither is further than 33·3 units from its centromere (Whitehouse, 1949; Papazian, 1952).

The results of a series of repulsion crosses between various markers chosen more or less at random are shown in Table 6. Where only small numbers of whole tetrads were classified this was due to a poor harvest of brandspores from that particular cross. As expected, the proportion of mutant colonies in any of these colonies was never significantly lower than 16·67%; nor was it ever higher than 50%, so that none of these pairs of genes could have been closely linked. In crosses 2, 3, 5, 6 and 10 evidence of the absence of linkage had already been obtained from analysis of random sporidia. Where 1% copper sulphate had been used to purify the brandspores, some of the auxotrophic colonies may have been derived from parental cells. The real proportion of whole tetrads which are PD would then be lower than that given in Table 6. If the germination of the prototrophic basidiospores was not complete the same effect would be produced. Therefore neither of these possibilities alters the conclusion that none of these markers are closely linked.

Since the occurrence of close linkage seemed to be uncommon, several crosses were made both in coupling and repulsion phase in order to distinguish between loose linkage (PD > RD) and absence of linkage (PD = RD), and in the hope of obtaining tetratype frequencies which might be used in the calculation of centromere distances (Whitehouse, 1950). Seven loci were examined in detail: *ad-1*, *me-1* (for which evidence of linkage had already been obtained), *leu-1*, *pan-1*, *inos-2*, *nic-3* and *nic-10*. In some cases only the repulsion or the coupling cross was made. The data are presented in Table 7. If it is accepted that differences between the observed PD and RD tetrad frequencies indicate linkage, it is possible to allocate each of these markers to one of two linkage groups. The first contains four loci with the probable order: *me-1*, *ad-1*, *leu-1*, *pan-1*; and the second the other three loci in the order *nic-3*, *inos-2*, *nic-10*. There is no evidence of linkage between any two markers on different groups, except *me-1* and *nic-3*, where the significant excess of observed PD over RD tetrads can be explained by the survival of a small proportion of parental cells in the sample of brandspores. These two markers had been shown to be unlinked in three different crosses by analysis of random sporidia.

However, in certain crosses between linked markers unexpected results were obtained. In crosses 45, 48 and 57 the RD values when considered in relation to PD values are higher than would be expected for linked markers. Papazian (1952) has given formulae for the expected frequencies of PD and RD tetrads for linked genes in the absence of chromatid or chiasma interference. These are:

$$\mathrm{RD} = \tfrac{1}{2} - \tfrac{1}{3}(1 - e^{-3m/2}) - \mathrm{e}^{-m/2},$$
$$\mathrm{PD} = \tfrac{1}{2} - \tfrac{1}{3}(1 - e^{-3m/2}) + \mathrm{e}^{-m/2},$$

where m is the mean number of cross-overs. These have been modified by Whitehouse (personal communication) to the more amenable form:

$$\mathrm{RD} = \tfrac{1}{3}(1 - 2r)^{3/2} + r - \tfrac{1}{3},$$
$$\mathrm{PD} = \tfrac{1}{3}(1 - 2e)^{3/2} - r + \tfrac{2}{3},$$

where r is the recombination frequency.

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Table 7. *The estimation of parental ditype (PD) and/or recombinant ditype (RD) tetrad frequencies by the analysis of whole tetrads from repulsion and/or coupling crosses among the seven loci:* ad-1, me-1, leu-1, pan-1, nic-3, nic-10 *and* inos-2.

	Crosses	Brandspore treatment and scoring method	Auxotrophic colonies / Total colonies	% auxotrophic colonies PD	% auxotrophic colonies RD	Recombination %	Expected PD and RD (%)
	Within linkage group I:						
37.	*me-1*, a_2b_2/*ad-1*, a_1b_1	CR	88/224*				
		CT	63/139	41·0			42·2
38.	" " " "	UT	50/127				
39.	*me-1 ad-1*, a_2b_1/a_1b_2	CR	10/223*			*31·8*	
40.	" " " "	CT	6/130		4·6		5·8
41.	" " " "	UT	6/126				
42.	*ad-1*, a_2b_2/*leu-1*, a_1b_1	UT	84/128§	65·6			
43.	" " " "	UT	57/66	86·4			36·8–65·1
44.	*ad-1*, a_1b_1/*leu-1*, a_2b_2	UT	98/183	53·6		*18·8–35·4*	
45.	*ad-1 leu-1*, a_1b_2/a_2b_1	UT	31/129		24·0		2·7–7·2
46.	*me-1*, a_2b_2/*leu-1*, a_1b_1	UT	49/160*	31·9			26·8
47.	*me-1*, a_1b_1/*leu-1*, a_2b_2	UT	43/128			*42·0*	
48.	*me-1 leu-1*, a_1b_1/a_2b_2	UT	24/151		15·9		10·8
49.	*leu-1*, a_2b_2/*pan-1*, a_1b_1	UT	48/191	25·1		*40·9*	28·4
50.	*leu-1 pan-1*, a_1b_2/a_2b_1	UT	13/192		6·8		10·1
51.	*me-1*, a_2b_2/*pan-1*, a_1b_1	UT	9/104	8·7			16·7
52.	*me-1 pan-1*, a_1b_1/a_2b_2	UT	14/104		13·5	*52·4*†	16·7
53.	*ad-1*, a_2b_2/*pan-1*, a_1b_1	UT	12/107	11·2		*ca.50*	
	Within linkage group II:						
54.	*inos-2*, a_2b_2/*nic-10*, a_1b_1	UT	110/264	41·6		*29·9*	45·4
55.	*inos-2 nic-10*, a_1b_2/a_2b_1	UT	3/231		1·3		5·1
56.	*inos-2*, a_2b_2/*nic-3*, a_1b_1	UT	59/105	56·2		*28·0*	48·5
57.	*inos-2 nic-3*, a_1b_1/a_2b_2	UT	13/107		12·1		4·4
58.	*nic-10*, a_2b_2/*nic-3*, a_1b_1	UT	23/101	22·8		*ca.45*	
	Between unlinked markers:						
59.	*me-1*, a_2b_2/*nic-3*, a_1b_1	CR	62/277*	24·2		*45·2*	
		CT	69/264				
60.	*me-1 nic-3*, a_2b_2/a_1b_1	CR	87/497*		17·2		
		CT	42/255				
61.	*leu-1*, a_2b_2/*inos-2*, a_1b_1	UT	13/89	14·6			
62.	*leu-1 inos-2*, a_1b_2/a_2b_1	UT	31/134		23·1	*45·8*†	
63.	*inos-2*, a_2b_2/*pan-1*, a_1b_1	UT	7/40	17·5			
64.	*inos-2 pan-1*, a_1b_2/a_2b_1	UT	30/104		28·8	*55·7*†	
65.	*inos-2*, a_2b_2/*me-1*, a_1b_1	UT	35/106	33·0			
66.	*inos-2 me-1*, a_2b_2/a_1b_1	UT	28/109		25·7	*46·3*†	
67.	*leu-1*, a_2b_2/*nic-10*, a_1b_1	UT	46/125	36·8			
68.	*leu-1 nic-10*, a_2b_2/a_1b_1	UT	55/133		41·3	*52·3*†	
69.	*ad-1*, a_2b_2/*inos-2*, a_1b_1	CT	8/55	14·5		*ca·50*	
70.	*nic-10*, a_2b_2/*me-1*, a_1b_1	UT	10/56	17·9		"	
71.	*nic-10*, a_2b_2/*pan-1*, a_1b_1	UT	20/103	19·4		"	
72.	*nic-10*, a_1b_1/*ad-1*, a_2b_2	UT	8/60	13·3		"	
73.	*nic-3 pan-1*, a_1b_1/a_2b_2	CT	29/121		24·0	"	
74.	*nic-3 leu-1*, a_1b_2/a_2b_1	CT	36/157		22·9	"	

C, U, R and T: see footnote to Table 6.
* Indicates that the data from different sources are not significantly different.
† Indicates that the observed RD and PD values are not significantly different.
§ The data from crosses 42, 43 and 44 are heterogeneous ($\chi^2_2 = 23{\cdot}3$; $P < 0{\cdot}001$).

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The expected values for PD and RD tetrads (see Table 7) have been calculated by using the recombination frequency obtained from the observed PD and RD values. The justification for doing this is discussed below. With *ad-1* and *me-1*, *leu-1* and *pan-1*, and *nic-10* and *inos-2* the observed are not higher than the expected values, whereas this is not so for *ad-1* and *leu-1*, *me-1* and *leu-1*, and *nic-3* and *inos-2*. In addition three separate crosses between *ad-1* and *leu-1* gave results which were statistically heterogenous.

These anomalous results can be explained if the germination of the basidiospores is not complete in certain crosses, and also if degree of failure of germination can be affected by an unknown environmental factor. The germination of basidiospores was tested by crossing mutants *ad-1* or *leu-1* to wild type and examining brandspore colonies. With complete germination of prototrophic basidiospores all these colonies should have grown on minimal medium. A small proportion did not do so, and it could be calculated that the basidiospore germination in three crosses of this type varied from 60% to 90% (Holliday, 1959).

In the crosses with *nic-3* and *inos-2*, and *leu-1* and *me-1*, it can be estimated (Holliday, 1959) that the germination is between 80% and 90% in both coupling and repulsion crosses; and with the unlinked markers the observed PD and RD frequencies are not significantly different (with the exception of *me-1* and *nic-3*, where the difference can be attributed to survival of parental cells). The evidence therefore suggests that germination tends to be the same in repulsion and coupling crosses. If this is so it is possible to calculate the effect low germination will have on the detection of linkage. If, for instance, germination was as low as 50% and the recombination calculated from the observed PD and RD values (as has been done in Table 7), then for real recombination values of 10, 20, 30, 40 and 50%, the observed values would be 20·5, 27·5, 35·0, 42·5 and 50%. With 90% germination the observed values would be 10·4, 20·4, 30·2, 40·1 and 50%. Linkage is therefore still detectable and even measurable with some accuracy under these circumstances. Unfortunately it is impossible to obtain tetratype values for unlinked markers, since there is no means of determining whether high ditype values are due to poor basidiospore germination, or to linkage of the markers with their centromeres. Without this information the position of the centromeres cannot be identified, and it is not possible to be certain that the two linkage groups really correspond to separate chromosomes.

(*c*) *The isolation of the individual products of meiosis*

Most of the previous workers who have studied the genetics of *Ustilago* spp. isolated individual basidiospores from the promycelium in a linear order. In *U. maydis* Kernkamp & Petty (1941) demonstrated that there was immense variation in the development of the promycelium and the arrangement of basidiospores. Normal germination with the production of a four-celled promycelium and a linear arrangement of basidiospores occurred regularly only in certain spore populations. With the strains used in the present work it has been found that normal germination is uncommon. Frequently more than one promycelium

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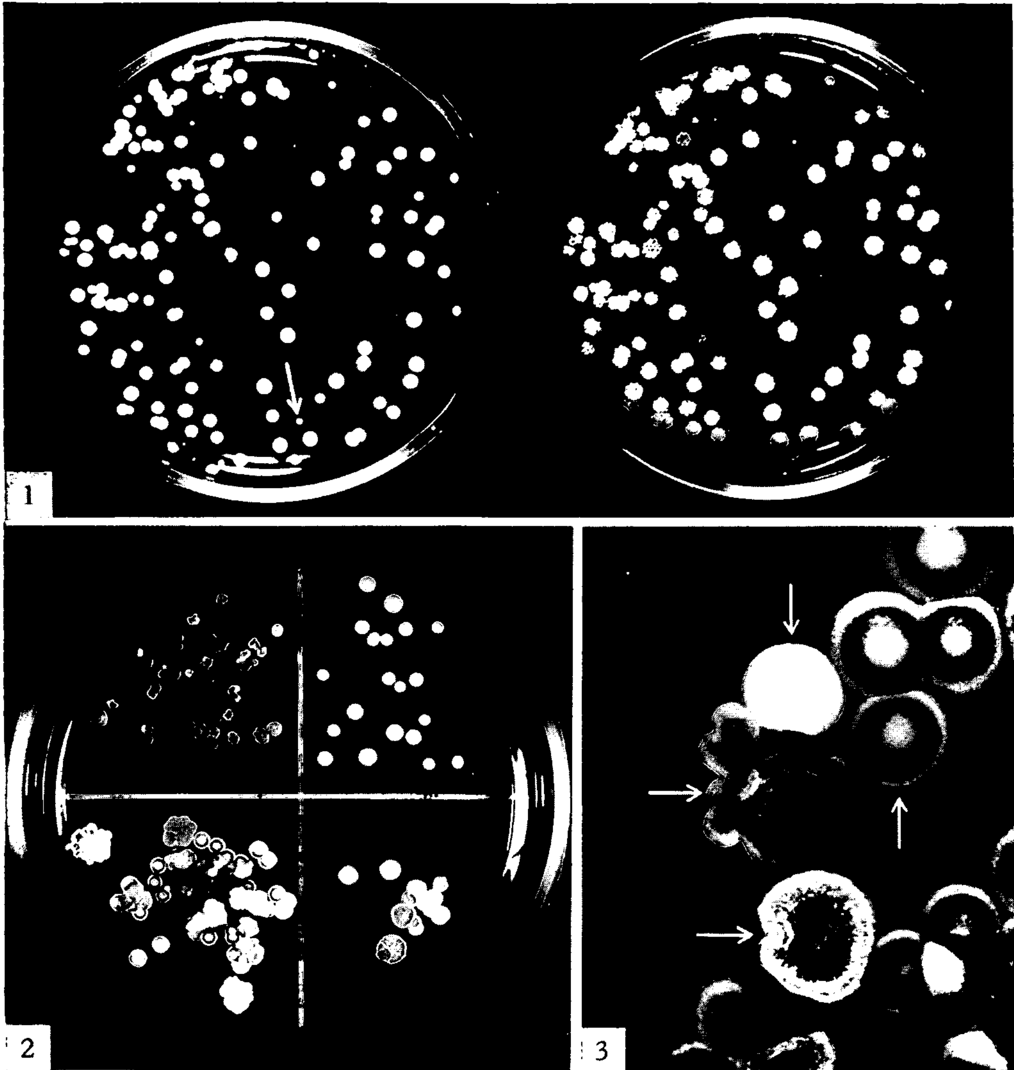

Fig. 1. The isolation of biochemical mutants by replica plating. One of the colonies on the master plate of complete medium (left) is auxotrophic since it does not grown on the replica plate of minimal medium.

Fig. 2. Tetrad analysis. A plate of complete medium on which four young colonies each derived from a single brandspore have been spread.

Fig. 3. Part of a spread of a single brandspore colony. The four distinct types of colony have different biochemical requirements. Each is derived from one of the members of a tetratype tetrad.

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appeared, or the proximal basidiospore had produced numerous sporidia before the terminal basidiospore had been formed. It was therefore difficult to identify by visual examination the individual members not only of ordered tetrads, but also those of unordered tetrads. Even if this difficulty was not present, the small size of the basidiospores makes micro-manipulation necessary for their direct isolation, and the slowness of this technique considerably outweighs its other advantages. Another method of isolation has therefore been devised.

Brandspores were plated on complete medium. Plates were examined after about 36 hours and colonies with between about 10 and 50 cells, which could be seen to have been derived from a single brandspore, were removed on small blocks of agar. Plates of complete medium were marked into four equal sectors, and about 0·01 ml. of sterile water was pipetted onto the centre of each. A single brandspore colony was placed in each of the drops of water. The block of agar was squashed and the sporidia spread over the surface of the agar within the sector with an instrument consisting of a triangular piece of copper sheet of $\frac{1}{2}$-in. side, soldered to an inoculating needle. The sporidia from each brandspore colony separated very easily and on incubation a uniform distribution of colonies was obtained (Fig. 2, Plate I).

The genotypes of the various types of colony were at first identified by replicating the plates to appropriate test media until it was noticed that within sectors colonies of different biochemical genotypes (as determined by replica tests) were always of different appearance and colonies with the same requirements were of the same appearance. The differences were in colour, margin or shape, surface topography or size of colonies. Four colonies from one sector with different biochemical requirements are shown in Fig. 3, Plate I. It was therefore possible to determine merely by inspection how many genotypes were present in a sector. Only those with three or four genotypes were examined further. One of each type of colony in these sectors was inoculated to plates of complete medium; between twenty-five and fifty were accommodated on each plate, and their requirements identified by replication to test media. A somewhat similar method of isolating the products of meiosis has been developed independently by Levine & Ebersold (1958) for use with *Chlamydomonas*.

The disadvantage of this method of tetrad analysis is that if germination of basidiospores is not complete, it is impossible to distinguish between ditype tetrads and tetrads in which only two of the products of meiosis have been recovered. It is therefore necessary to make multi-point crosses between loosely linked or unlinked markers in which the great majority of the tetrads would be tetratype. Since with *Ustilago* most of the labour lies in the isolation of the products of meiosis and not in the identification of the genotypes of the individual progeny, it is a considerable advantage to incorporate as many markers as possible into a cross in order to increase the yield of information per tetrad.

A cross between *ad-1 me-1* $a_2 b_1$ and *leu-1 pan-1* $a_1 b_2$ was selected for detailed analysis. It was hoped to confirm the linkages between these markers and to obtain information on chromatid interference. The linkages between the markers

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were sufficiently loose to make the probability of obtaining ditype tetrads very low.

The germination of the basidiospores (as determined by the numbers of genotypes per brandspore colony) varied from 40 to 50% with different crops of brandspores. This meant that only 10–30% of the brandspore colonies yielded three or four genotypes. In those that yielded three, it could be deduced that the genotype which had not been recovered was more often than not one with three or four requirements. The overall low germination rate may therefore have been due to the fact that so many markers were segregating in the cross. With one crop of basidiospores attempts were made to increase germination by altering environmental conditions. These included: (1) reducing the glucose content of the medium to 0·5% or increasing it to 2·0%; (2) adjusting the pH to 7·0; (3) adding 1 ml. of maize extract per plate (10 g. of maize seedling tissue was ground up in a mortar, 75 ml. distilled water was added and the mixture sterilized by Seitz filtration); (4) using malt agar medium, and (5) incubating the brandspores at 25°C. None of these conditions significantly stimulated basidiospore germination.

A total of 124 tetrads were completely analysed. In 24 of these all four genotypes were recovered, and these showed normal 2:2 segregation for each marker. In 100 three genotypes were recovered and none of these were incompatible with normal segregation for each marker, i.e. for each locus the mutant gene was present in either one or two, but never in none or all three of the genotypes identified. The recombination values were obtained from the frequency of each of the three tetrad types for each pair of markers. These data are given in Table 8. The order of the genes is the same as that deduced from whole tetrad analysis, but the evidence for linkage between *leu-1* and *pan-1* is less conclusive, particularly if only those tetrads in which all four genotypes were recovered are considered. That the proportion

Table 8. *Complete analysis of tetrads. The frequency of each class of tetrad for each pair of loci in the cross:* me-1 ad-1, a_2b_1 / leu-1 pan-1, a_1b_2. *The figures in brackets give the frequency of tetrads in which all four products of meiosis were recovered*

Loci		PD	RD	T	Recombination (%)
me-1, ad-1		49 (11)	1 (0)	74 (13)	30·6
	%	39·5	0·8	51·7	
ad-1, leu-1		74 (18)	0 (0)	50 (6)	20·2
	%	59·7	0	40·3	
me-1, leu-1		22 (5)	5 (0)	97 (19)	43·1
	%	17·7	4·0	78·2	
pan-1, leu-1		28 (4)	13 (4)	83 (16)	44·0
	%	22·6	10·5	66·9	
pan-1, ad-1		21 (1)	20 (3)	83 (20)	49·6
	%	16·9	16·1	66·9	
pan-1, me-1		17 (2)	23 (5)	84 (17)	52·4
	%	13·7	18·5	67·7	

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of undetected ditype tetrads was small is shown by the very close agreement to the expected 1:1:4 ratio for PD, RD and T tetrads for the markers *ad-1*, *pan-1* and *me-1*, *pan-1* which show independent segregation. A surprisingly high proportion of the tetrads were tetratype with respect to the markers *me-1* and *leu-1*. This indicates that positive interference of the type described by Barrat, Newmeyer, Perkins & Garnjobst (1954) may be operating in this region of the chromosome. The coincidence value for cross-overs in this region is in fact 0·52; but since it is not possible to allow for the undetected ditype tetrads this figure can only be regarded as approximate.

Five tetrads in which three of the genotypes were recovered were also analysed for mating type. Four of them were parental ditype with respect to *pan-1* and the *a* locus, the other was tetratype. The evidence from random sporidia for the close linkage of these loci was therefore confirmed.

From unordered tetrads it is possible to determine the strand relationship of cross-overs in adjacent intervals. Only the adjacent intervals between *me-1* and *ad-1*, and *ad-1* and *leu-1* will be considered, since too high a proportion of undetected multiple exchanges would be expected in the other interval. When three genes are segregating, there are twelve possible types of unordered tetrad. Using Whitehouse's (1956) classification, the frequencies and cross-over derivation of each class are shown in Table 9.

Table 9. *The derivation and frequency of the twelve possible classes of unordered tetrad amongst 124 tetrads from the cross in which the three linked markers* me-1, ad-1 *and* leu-1 *were segregating. These markers are represented in the Table by A, B and C respectively*

Class	AB	BC	AC	Cross-overs (simplest derivation)	Frequency
1	PD	PD	PD	none	18
2	PD	RD	RD	4st in BC	0
3	RD	RD	PD	(4st in AB):(4st in BC)	0
4	RD	PD	RD	4st in AB	0
5	PD	T	T	1 in BC	31
6	RD	T	T	(4st in AB):(1 in BC)	1
7	T	T	PD	(1 in AB) 2st (1 in BC)	3
8	T	T	RD	(1 in AB) 4st (1 in BC)	5
9	T	PD	T	1 in AB	56
10	T	RD	T	(1 in AB):(4st in BC)	0
11 & 12	T	T	T	(1 in AB) 3st (1 in BC)	8+2

2st, 3st and 4st indicate two-strand, three-strand and four-strand relationships.
: indicates that the strand relationship between adjacent cross-overs may be of any kind.

Classes 7, 8, 11 and 12 give information concerning chromatid interference. As Whitehouse (1956) has pointed out, classes 6 and 10 give a measure of the frequency of triple cross-overs within the region. Only one tetrad of this type was observed, therefore there are likely to be few, if any, undetected two- or three-strand double exchanges within each interval. The observed frequency of two-, three- and four-strand relationships are 5, 10 and 3, which give a close fit to the expected 1:2:1 ratio for these types of double cross-overs in the absence of chromatid interference.

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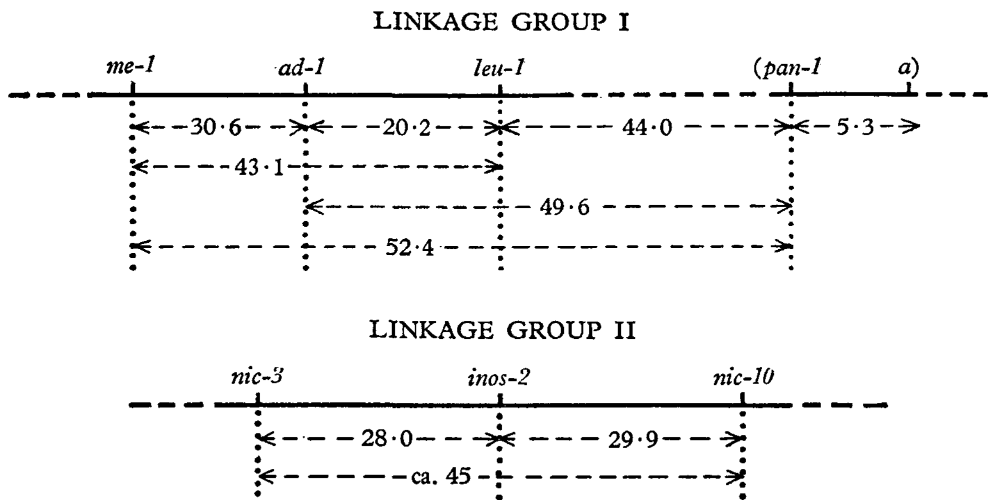
DISCUSSION

Much of the genetic experimentation has been concerned with the development of techniques suitable for carrying out genetic analysis with biochemically deficient strains. The aim throughout was to invent methods which would allow extensive linkage data to be collected at the expense of accuracy, rather than the converse. Each of the methods which has been investigated has been subject to certain sources of error, yet there were no contradictions between results derived from different analyses, nor were there inconsistencies in the linkage data used to determine the linear order of genes on the chromosome. The combined data from different sources were used to construct the chromosome maps shown in Text-



Text-fig. 1. Chromosome maps of *U. maydis*. Recombination values for linkage group II were obtained from analysis of whole tetrads; for *a* and *pan-1* (the order of which is uncertain) from random sporidia and complete tetrad analysis, and for *me-1* to *pan-1* from complete tetrad analysis.

fig. 1. Since the centromeres have not been identified it is not yet possible to be certain that the two linkage groups correspond to the two chromosomes which have been observed cytologically. In a total of fifty-four crosses made largely at random between different pairs of biochemical mutants, linkage has been detected relatively rarely. Moreover, the linkages which have been detected, with one exception, have been found between markers widely spaced on the chromosome. The evidence suggests that the linkage groups may be very long.

Most of the difficulties which have been encountered in the three methods of analysis which have been investigated can be attributed to poor germination of the basidiospores. There is little information concerning the factors which control the rate of germination. In crosses between certain strains germination of the basidiospores was always very high. In other crosses germination was somewhat lower but remained constant when crosses were repeated. Germination

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therefore appeared to depend on the genetic constitution of the brandspore. Yet in a third group inexplicable variation in basidiospore germination occurred in different crosses between the same parents. In these, environmental influence must have been interacting with genetic factors in determining the level of germination. There are no indications as to whether the environmental effect is derived from the medium on which the brandspores were germinated or was due to the host environment in which the brandspore developed. Conditions for improving the germination have scarcely been explored. The fact that in certain crosses germination rates are particularly susceptible to environmental influence; that low germination of basidiospores in smut fungi has rarely been previously recorded, and that in certain early crosses in which random sporidia were analysed, germination must have been uniformly high for various genotypes, strongly suggest that the correct conditions for high germination could be found.

The compact growth of sporidial colonies is an immense advantage, since the technique of replica plating can be used for the very rapid identification of the requirements of large samples of progeny. Another feature which has turned out to be invaluable is the failure of sporidia to form heterokaryons or dikaryons on artificial media. Had this not been so it is doubtful whether any of the techniques which have been used would have been possible. With high germination of the basidiospores the methods of genetic analysis which have been developed could be used to accumulate data very rapidly. In particular the method of complete analysis of tetrads would benefit, and would enable information relevant to an understanding of crossing-over to be accumulated on a larger scale than has been possible previously in organisms in which tetrads must be dissected manually or with the aid of a micro-manipulator.

There is evidence that positive chiasma interference occurs between the loosely linked markers *me-1* and *leu-1*. If so it is improbable that the centromere lies within this interval. In a sample of eighteen double cross-overs between these markers which were detected by tetrad analysis, no indication of chromatid interference was obtained. These data therefore provide additional support for the growing body of evidence from organisms in which tetrad analysis is possible, that such interference is absent within chromosome arms (see review by Perkins, 1955; Strickland, 1958 (see Whitehouse, 1958*a*); Levine & Ebersold, 1958; Whitehouse, unpublished). On the other hand conflicting evidence has been obtained when double crossing-over across the centromere has been examined (see Whitehouse, 1958*b*).

SUMMARY

1. Many of the Ustilaginales, or smut fungi, appear to have the qualities necessary for the application of modern techniques of microbial genetics. *Ustilago maydis* is considered the most suitable species.

2. Investigations of the mating system confirm reports that the production of diploid brandspores in the host is controlled by alleles at two loci.

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3. Genetic markers were obtained by inducing mutations in a wild-type strain with ultra-violet light. Of 100 biochemical mutants which were isolated, the growth requirements of 94 were identified. Thirty of these were used in genetic tests.

4. The compact growth of colonies on artificial media allowed new techniques to be developed by means of which large samples of progeny could be isolated and identified easily. The analysis of brandspore colonies consisting of the products of single meiotic divisions is the quickest method for detecting linkage, but its accurate measurement appears to be achieved by examining the individual members of tetrads.

5. Linkage was detected relatively rarely, but eight markers, including the *a* mating-type locus, were assigned to one or other of two linkage groups. Although recombination values were not always determined accurately owing to irregular basidiospore germination, the auxotrophic markers in each group could be mapped in a linear order. Since no indication of other linkage groups was obtained, the genetic evidence is so far consistent with cytological reports that the basic haploid chromosome number is two in the smut fungi.

6. Three linked markers were used to investigate chromatid interference by tetrad analysis. None was detected in a total of eighteen double exchanges.

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