BIOCHEMICAL MUTANTS IN THE SMUT FUNGUS USTILAGO MAYDIS

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THE usefulness of growth-factor mutants for genetic studies was demonstrated by BEADLE and TATUM in 1941, using the mold Neurospora. Although a number of other microorganisms have since been employed in studies of a similar nature, there has until recently been no instance of the use of biochemical mutants in sexual fungi other than Ascomycetes. This paper is concerned with the production, characterization and inheritance of requirements for essential metabolites in a parasitic basidiomycete, the smut-fungus of corn, *Ustilago maydis* (DC.) Cda. (*Ustilago zeae, cf.* STEVENSON and JOHNSON 1944).

The smut-fungi, comprising the order Ustilaginales, are characterized by the production of smut-spores or *chlamydospores* which constitute the zygotes and are typically the only diploid cells in the life cycle. During chlamydospore germination, meiosis occurs in a short hypha, the *promycelium*, from which haploid cells, the *sporidia*, are budded off. In the family Ustilaginaceae these products of the reduction divisions are arranged in a linear order that reflects the events of meiosis. The sporidia multiply saprophytically as discrete, yeast-like cells. In certain environmental circumstances monocaryotic, mycelial growth may occur. (Mycelia may replace sporidia completely in some species, as well as in some variants of species that normally produce sporidia.) Under suitable conditions, haploids of compatible mating-types fuse, giving rise to a dicaryotic mycelium which reproduces by conjugate nuclear division, characteristic of the Basidiomycetes. Formation of a dicaryon is generally essential for virulent infection. In *U. maydis*, though not in many other species, infection is evidently initiated by the haploids and the dicaryon is formed within the host (HANNA 1929). The life cycle is completed by production of chlamydospores within which the two nuclei fuse to form a diploid nucleus. In the case of the maize parasite, this occurs in hypertrophied lesions, the smut-galls. References to the work of mycologists who contributed fundamentally to knowledge of life-cycles of the smuts are given by STAKMAN (1913), and the ecology of the group has been reviewed by TAPKE (1948).

Genetic studies on the Ustilaginales have dealt with the inheritance of factors governing sexual compatibility (*e.g.* KNIEP 1929; BAUCH 1930), colony morphology (*e.g.* DICKINSON 1928, 1931), and pathogenicity (*e.g.* HANNA 1929; ALLISON 1937). Mendelian studies have hardly gone beyond the stage of demonstrating that segregation occurs, and that certain phenotypes can be attributed to single or to multiple factors. Aside from data on the relative fre-

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quency of first- and second-division segregation for a few loci (HÜTTIG 1931, 1933; DICKINSON 1928, 1931), which might be used to establish centromere relations, no adequate information on linkage in any smut has been obtained. This absence of linkage data is particularly surprising since the haploid chromosome number is reportedly two for most species studied (including *U. maydis*, KHARBUSH 1928), a circumstance which would be expected to increase the probability of finding linked characters. The extensive literature on inheritance has been reviewed by CHRISTENSEN and RODENHISER (1940).

In the case of *U. maydis* it has been demonstrated that morphological (STAKMAN, KERNKAMP, KING and MARTIN 1943) and compatibility factors are inherited in a mendelian fashion, that the genetic control of compatibility involves multiple loci (HANNA 1929; CHRISTENSEN 1929, 1931), and that segregants showing different rates of occurrence of morphologically variant colony sectors can be obtained (STAKMAN *et al.* 1943). Segregation of factors governing compatibility and morphological characters may occur at either first or second division (HANNA 1929; CHRISTENSEN 1931; STAKMAN *et al.* 1943). Although virulent infection terminating in chlamydospore formation can ordinarily be obtained only when two haploid lines of compatible mating-type are inoculated together (STAKMAN and CHRISTENSEN 1927), exceptional lines occur in which single sporidial isolates, evidently diploid (CHRISTENSEN 1931; CHILTON 1943; GATTANI 1946), can give rise to infection and spore-formation. While fusion between sporidia from compatible lines has been observed *in vitro* (SLEUMER 1931) the life-cycle of *U. maydis* has never been completed outside the host-plant, as has been possible with several other smut species (see CHRISTENSEN and RODENHISER 1940).

Genetic analysis of *U. maydis* has been impeded by several technical difficulties, most serious of which are the lack of stable, easily characterized differences capable of being used as mendelian markers, the necessity of making crosses in the host plant, and the existence of multipolar sexuality, decreasing as it does the freedom with which segregants can be recombined. The use of growth-factor mutants promises to remove the first of these impediments. There is no reason to believe that proper conditions for making crosses *in vitro* cannot be found. The difficulties imposed by multiple compatibility-factors (themselves of intrinsic interest) can be minimized by identifying the loci concerned and establishing tester stocks.

Certain features of *Ustilago maydis* should amply justify the work involved in overcoming these difficulties. The organism is completely auxoautotrophic (SCHOPFER and BLUMER 1938), requiring no exogenous growth-factors. Haplophase sporidia can be handled like yeasts or bacteria, making possible the application of screening procedures and the quantitative treatment of growth, enzyme adaptation, mutation and selection, as well as facilitating physiological studies (*e.g.* FELDMAN 1948). Sexual reproduction increases the ease of studying biosynthesis and gene-action. Single zygotes can easily be isolated in large numbers prior to germination and the four products of meiosis in each case can be obtained either in order or at random. (The life cycle, even with

crosses in the host, is of about three weeks duration.) The fact that this fungus parasitizes *Zea mays*, which is better known genetically than any other plant, should permit a precise analysis of host-parasite relationships impossible where the host is not controlled. Finally, *Ustilago maydis* represents a major group of sexual fungi as yet little explored genetically.

MATERIALS AND METHODS

Biological Materials

Chlamydospores from a single smut gall obtained in September 1947 from maize growing at Irvington-on-Hudson, New York, were germinated on minimal agar, the sporidia suspended and plated, and stocks made from single colonies picked from plates of suitable dilution. Isolates 4, 5, and 6 were used in subsequent work. DR. E. C. STAKMAN kindly furnished his *U. maydis* stocks 10A4 and 17D4, which were used in preliminary tests of compatibility. An inbred line of maize known to be highly susceptible to smut infection was used for crosses.

Media

The following medium (GRAY and TATUM 1944, modified by addition of trace elements according to RYAN, BEADLE and TATUM 1943) is adequate for growth of non-mutant strains, and will be referred to as minimal medium: NH_4Cl, 5 gms; NH_4NO_3, 1 gm; Na_2SO_4, 2 gms; $MgSO_4 \cdot 7H_2O$, 0.1 gm; $CaCl_2$, trace; K_2HPO_4, 3 gms; KH_2PO_4, 1 gm; Glucose, 5 gms; trace element solution, 1 cc; H_2O distilled, 1,000 cc. The trace element solution is composed of: Boric acid, 114 mg; $FeCl_3 \cdot 6H_2O$, 192 mg; $ZnCl_2$, 840 mg; $MnCl_2 \cdot 4H_2O$, 288 mg; Na_2MoO_4, 84 mg; $CuSO_4$, 750 mg; H_2O, 2 liters. The pH after autoclaving is 6.8.

For solid medium 1.5 percent agar was added. In any experiment where traces of growth-factors might interfere, agar washed fifteen times in distilled water and three times in 95 percent alcohol was used. Minimal medium was supplemented with 1 percent yeast extract (Difco), 1 percent casamino acids (Difco) (acid hydrolysate of casein), 1 percent nucleic acid (Schwarz) plus 2 percent nucleic acid hydrolysate (10 gms yeast nucleic acid autoclaved 20 minutes at 15 lbs. pressure in 50 cc normal NaOH and neutralized), or with individual growth factors as required. Difco "A.C. Broth" (containing, per liter, proteose-peptone, 20 gms; beef extract, 3 gms; yeast extract, 3 gms; malt extract, 3 gms; and dextrose, 5 gms) was in some cases used as complete medium in place of supplemented minimal.

Culture methods

Stocks were maintained on 3 percent agar slants (mortality increases on more moist medium), stored at 3°C, and transferred monthly. Material was grown routinely at 30–32°C. Growth in deep liquid requires aeration; for this purpose a "roller-tube" apparatus of the type used for tissue-culture work (PARKER 1944) was found most convenient. 16×150 mm Pyrex test-tubes

containing 5 cc of liquid medium were placed in fixed position in a drum inclined with axis at an angle of 15° to the horizontal, and rotated at ten revolutions per minute in an incubator maintained at constant temperature. The continuous stirring results in growth comparable to that obtained with vigorous shaking or bubbling. Use of the roller-tubes was suggested by DR. R. BALLENTINE.

Dilutions for plating were made in 0.9 percent NaCl. All plates were prelayered with a small amount of agar to prevent spreading growth at the glass-agar interface.

Turbidometric measurement of growth

A Klett-Summerson photoelectric colorimeter with No. 59 filter and an adapter to take 16×150 mm test-tubes was calibrated against number of cells, using stock 5. Density readings are directly proportional to total cell number over a range from 0 to 250 densitometer units (0 to about 10^7 cells per cc), and then fall off to introduce a 15 percent error at a reading of 360, and progressively greater errors with further increase in density. Since absolute values were not required, growth is expressed in densitometer units.

Ultraviolet irradiation

Freshly grown sporidia suspended in liquid 2 mm deep in 9-cm Petri dishes were exposed to a General Electric 15-watt Germicidal lamp 51 cm distant. Samples of 1 cc, taken at intervals from 0 to 60 minutes after the beginning of the treatment, were diluted and plated in minimal medium.

Detection of mutants

The procedure described by LEDERBERG and TATUM (1946) was used to recover growth-factor deficient mutants from among the survivors of irradiation. This method, applicable to any organism forming discrete colonies in agar medium, allows one to select from a large population any mutant individuals that require an exogenous supply of some water-soluble substance. The detection technique consists of plating the population in minimal agar, which supports development of growth-factor independent colonies but not of mutants devoid of the ability to synthesize some essential metabolite. The latter appear as colonies only after the diffusion of growth-factors from a newly added surface layer of supplemented agar has enabled them to multiply. By varying the composition of the supplement one can select classes of mutants, or mutants with specific requirements.

One cc portions of treated sporidia in various dilutions were pipetted into Petri dishes and thoroughly mixed with liquid minimal agar. After solidification, a thin layer of agar was poured on top to prevent surface growth. The positions of all colonies visible at 48 and 56 hours were marked on the bottom dish with glass-marking ink, and another thin agar layer added containing triple-strength Difco A. C. Broth. Colonies appearing subsequently were picked and transferred to stock tubes of complete medium and tested for growth-factor deficiencies.

Characterization of mutants

The most widely used method for determining requirements of biochemical mutants has been that of inoculating the unknown into a series of tubes containing liquid or solid test media of various compositions. In the present study, two more satisfactory identification procedures were used which involved, in one instance, the spotting of unknown isolates on the surface of agar test-media in Petri dishes, and in the second, the spotting of test substances on a dense minimal-agar plating of an unknown isolate (auxanography). These methods can be employed both for the identification of newly isolated mutants and for the characterization of progeny from crosses.

(1) *Spotting of inocula.* Since *U. maydis* is characterized by localized growth on the surface of agar, it was possible to use, instead of tubes, a series of Petri dishes prepared with test media, and to test up to twenty different isolates on a single plate by inoculating them at marked points. Suspected mutants, obtained from those colonies that appeared after layering with complete medium, were grown on complete slants, suspended, and washed three times in 10 cc portions of saline. A small loopful of each washed culture was inoculated on a series of four plates containing, respectively, minimal agar, minimal+yeast extract, minimal+casamino acids, and minimal+nucleic acid. Those isolates growing only on yeast extract were further tested on plates containing individual B-vitamins (concentrations as used by BEADLE and TATUM 1945); those responding to casamino acids, on individual amino acids (0.2 mg/cc); those showing growth on nucleic acid or both yeast extract and nucleic acid, on individual purines and pyrimidines (1.0 mg/cc or saturated solution).

(2) *Spotting of test-substances (auxanography).* A yet more satisfactory method for characterizing mutants consists of plating dense suspensions of individual isolates in minimal medium and spotting each plate with a series of test-substances. The procedure used with Ustilago is shown in figure 1. A small loopful of sporidia from a freshly-grown (12–24 hr.) slant is suspended in 1 cc sterile saline and pipetted or poured into a prelayered plate to which minimal washed-agar medium is added, thoroughly mixed and allowed to solidify. The test-substances, which need not be sterile, can be spotted on as solid or liquid. With amino acids and nucleic acid components a small particle has generally been used; with vitamins, a loopful of concentrated stock solution. Best results are obtained if plates are incubated 12 hours before spotting. Positive responses are apparent in 24 hours, and are illustrated in figures 3 and 4.

Crosses

Dense suspensions of sporidia from actively growing liquid roller-tube cultures, or from 24-hour slants, were mixed in the desired combinations and inoculated with a hypodermic syringe into maize plants two weeks or more of age, each plant being inoculated at several points at and above the apical bud. Advanced infection, manifested by extensive chlorosis and gall formation, was obtained only at temperatures averaging well above 25°C. Galls appeared after ten days at the earliest, and chlamydospores were obtainable several

days later. Chlamydospores from fresh galls germinate readily, as do those stored for several months at 3°C.

Recovery of products by random isolation

Chlamydospores were suspended in a 1 percent solution of $CuSO_4 \cdot 5H_2O$ for at least 12 hours (CHRISTENSEN and STAKMAN 1926) in order to destroy contaminants or vegetative Ustilago cells, and were then diluted and plated in complete agar. At 32°C chlamydospore germination occurs and promycelia are

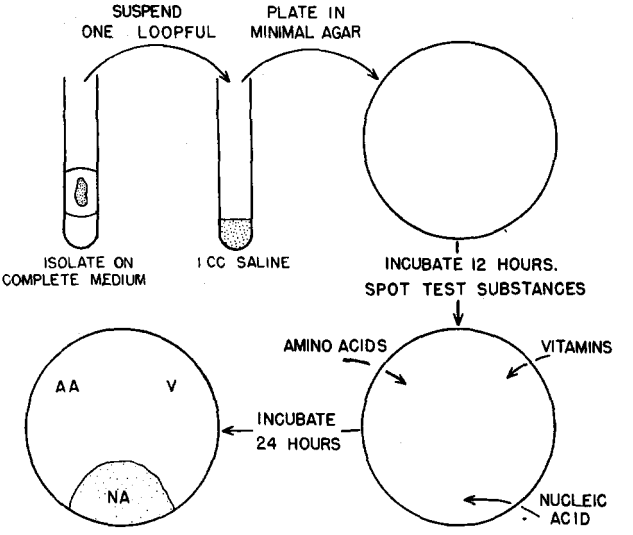

FIGURE 1.—The auxanographic method applied to identification of biochemical mutants in Ustilago. A mutant requiring some component of nucleic acid is illustrated, and specific characterization would be accomplished by spotting a second test-plate with individual purines and pyrimidines.

visible in from 24 to 36 hours. When colonies of sporidia arising from the meiotic products of a chlamydospore (*single chlamydospore colonies*) appeared, whole colonies were cut out individually from plates on which they were widely spaced, and a homogeneous suspension of each was diluted and plated in minimal agar for screening. After non-mutant colonies of unisporidial origin had appeared in these plates, and been marked, the various mutant types were induced to grow either by layering with properly supplemented agar, or by spotting the required substances directly on the agar surface. In the case of crosses involving several loci, segregants can be brought up type by type or all together, as desired. Mutant colonies were transferred to stock tubes and tested auxanographically.

EXPERIMENTAL RESULTS

Description of mutants

Twenty-one isolates unable to grow on minimal medium were obtained from among the sporidia surviving 95 to 99.99 percent killing by ultraviolet irradia-

tion. The specific requirements of fifteen of these mutants were determined by spotting washed inocula of each on the surface of a series of agar plates that contained diagnostic supplements, or by the auxanographic method. (Six mutant isolates have not been fully characterized.) Requirements were rechecked auxanographically, and in most cases by demonstrating a quantitative relation between growth and concentration of the required substance. Mutants were designated by the number of the wild-type sporidial isolate from which they were derived, followed by a specific mutant number; *e.g.*, 5–91 is an isoleucine-requiring mutant obtained following irradiation of sporidia from wild-type stock number 5, which was one of the haploid progeny of chlamydospores obtained from nature. Identified mutants are described in table 1.

All mutants are morphologically and culturally similar to wild-type when grown on optimal medium, with the exception of one yeast-extract requiring isolate which grows as a mycelium and produces no sporidia. All mutants have remained stable through from 4 to 11 monthly transfers on supplemented medium. Ten have been recovered as progeny from crosses (five in two successive generations), demonstrating that they remain stable during several weeks' growth within the host plant. No cases of reversion to growth-factor independence have been observed, except those instances of adaptations in liquid culture noted in table 1.

A number of mutants were grown in roller-tube culture (at $32° \pm 1°C$) and the course of growth on media supplemented with graded concentrations of the required metabolite was followed turbidometrically. Response curves are given in figure 2.

Under conditions where liquid growth exceeded 2×10^7 cells per cc, a rapid decrease in turbidity commenced immediately after maximum concentration was reached, and might deplete the densest populations by one-eighth within 12 hours. For this reason, points on response curves that indicate turbidities of 300 or more are in error by being too low, since readings were taken at intervals of as great as 12 hours, and it is unlikely that the true maximum was recorded. In special cases measurements were rendered inaccurate by pigment-formation or by clotting, but no such difficulty was encountered for the majority of mutants.

The Ustilago mutants so far identified appear to fall into the same categories as biochemical mutants reported in other microorganisms. They can by no means be considered a representative sample of all growth-factor requiring variants, since the type of mutant recovered is a function of the selective screening methods used.

Crosses involving biochemical mutants

Those mutants derived from wild-type isolates 5 and 6 proved to be compatible in crosses with mutant 4–24 and with STAKMAN'S wild-type stock 10A4. Crosses from which first-generation mutant progeny were recovered, either as parental or recombination types, are indicated in table 1. All mutant stocks used in crosses were derived from the same smut gall, and are genetically

TABLE 1

Description of mutants. Under Range of Response is given the highest metabolite concentration supporting negligible growth and the lowest concentration supporting optimal growth. Stability is expressed as the ratio of number of cultures in which adaptation was observed to the total number of cultures in which adaptation could have been detected (tubes containing suboptimal supplements).

ISOLATE NUMBER	REQUIREMENT	RANGE OF RESPONSE	STABILITY IN LIQUID CULTURE	RECOVERED IN PROGENY OF CROSS WITH	REMARKS
5-55	arginine	3×10^{-4}–10^{-1} mg/cc L-arginine	0/28	—	No response to citrulline
5-91	isoleucine	3×10^{-3}–3×10^{-1} mg/cc DL-isoleucine	0/34	4–24 (and in f_2)	Valine without effect
4-24	methionine	10^{-3}–3×10^{-2} mg/cc DL-methionine	1/34	5-4B, 5-25, 5-55 5-76, 5-91, 5-92 5-95, 5-132, 6-A (and in f_2)	No response to cystine, cysteine
5-40	Reduced sulfur		0/13	—	Responds to K_2S, $Na_2S_2O_3$, S-containing amino acids.
5-78	Reduced sulfur		0/6	—	Responds to K_2S, $Na_2S_2O_3$, S-containing amino acids
5-25	adenine or hypoxanthine		18/20	4-24	Order of adaptation is that of decreasing adenine concentration Slight response to guanine
5-95	adenine or hypoxanthine	3×10^{-4}–10^{-1} mg/cc adenine sulfate	0/42	4-24, 10A4	Slight response to guanine
5-132	adenine or hypoxanthine			4 24 (and in f_2)	Slight response to guanine
6 A	adenine or hypoxanthine		4/37	4-24 (and in f_2)	Slight response to guanine
5-92	uracil		6/6	4-24	Order of adaptation is that of decreasing uracil concentration
5-4B	thiamin	$<10^{-4}$–10^{-2} μg/cc thiamin-HCl	0/27	4-24 (and in f_2)	
5-112	thiamin				
5-66	nicotinic acid		10/12		
5-76	nicotinic acid	1.5×10^{-2}–1.5×10^{-1} μg/cc nicotinic acid	0/46	4/24	
4-102	nicotinic acid				

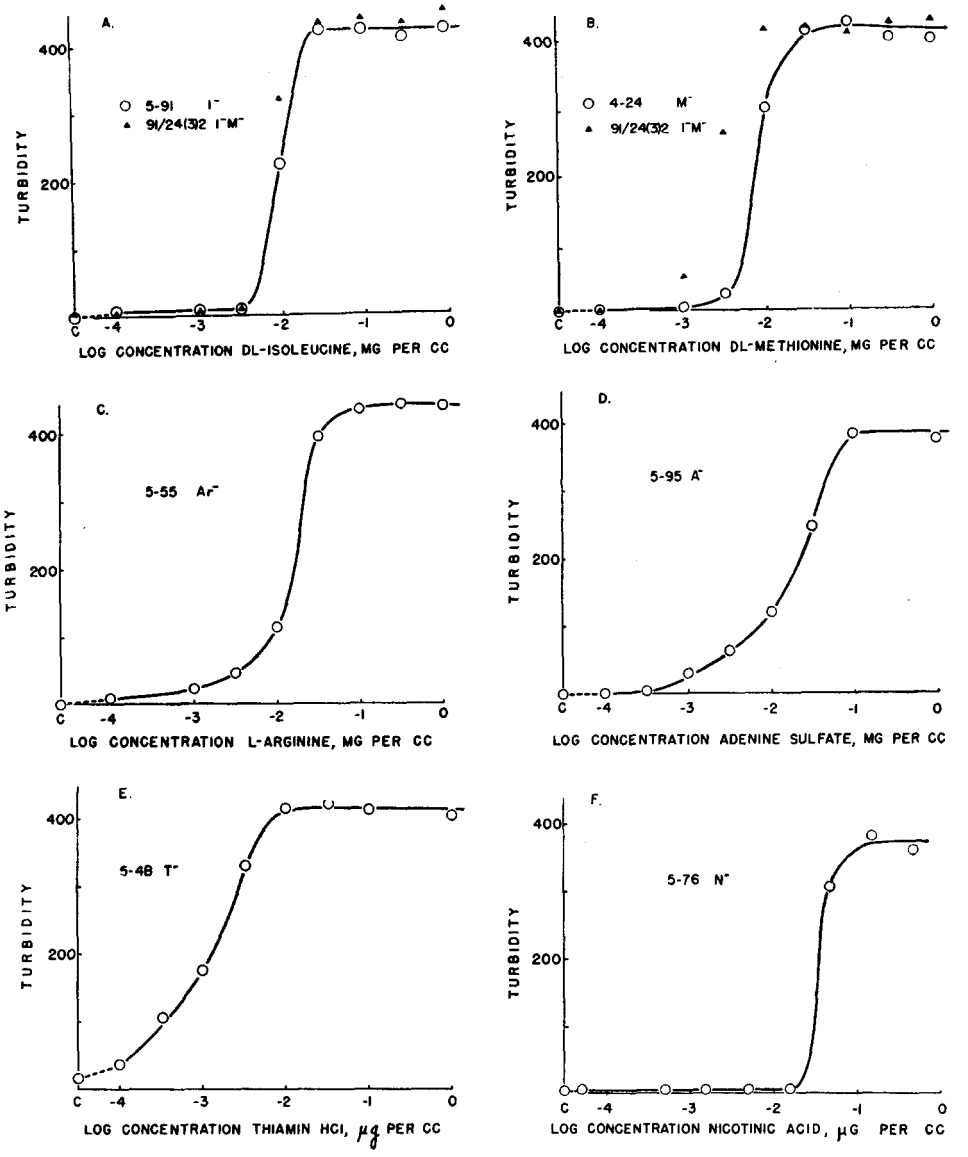

FIGURE 2.—The growth of seven mutant isolates on different concentrations of required substances. Ordinate: turbidity in densitometer units. Abcissa: log concentration of supplement. (C on the abcissa indicates unsupplemented control.) Each point represents the mean turbidity for four tubes at maximum growth or at 230–250 hours after inoculation (except for E where two tubes were used at each concentration).

A. Circles: 5–91 (isoleucineless); triangles: 91/24(3)2 (isoleucineless-methionineless) on minimal medium +0.1 mg/cc DL-methionine. B. Circles: 4–24 (methionineless); triangles: 91/24(3)2 (isoleucineless-methionineless) on minimal medium +0.1 mg/cc DL-isoleucine. C. 5–55 (argininеless). D. 5–95 (adenineless). E. 5–4B (thiaminless). F. 5–76 (nicotinicless).

homogeneous to this extent. (The mutant progeny from 5–95×10A4 were not used in further crosses.)

All four possible types (two parental and two recombinant) were recovered from among the haploid progeny of crosses of 4–24×5–4B, 5–25, 5–76, 5–91, 5–132 and 6A. A number of different double mutants were thus available for use in further crosses. The f_1 isolates were designated by the specific mutant numbers of the parents, separated by a bar, followed by the chlamydospore number, in parentheses, and the f_1 isolate number; *e.g.*, 91/24(3)2 is isolate 2 from a plating of sporidia originating from chlamydospore 3 of the cross 5–91 (I^-M^+)×4–24 (I^+M^-). An isolate bearing this number would be expected to be one of the four types I^+M^+, I^+M^-, I^-M^+, or I^-M^-. This specific isolate proved to be the double mutant.

Characterization of isolates from crosses was accomplished auxanographically, it being possible by this method to determine by use of a single plate which of a number of possible combination-types an unknown segregant represents.

All progeny were obtained from single chlamydospore colonies, by methods described earlier. The type of segregation for individual zygotes could thus be inferred from the kinds of sporidia making up each colony. The three possible types of segregation—$Ab+aB$ (parental ditype), $AB+ab$ (non-parental ditype), and $AB+Ab+aB+ab$ (tetratype) (BURGEFF'S terminology, 1929)—were found in one or another dihybrid cross, and are illustrated in figures 3 and 4. The number of tetrads analyzed from individual crosses is not yet sufficient to permit a reliable estimate of the relative frequencies of different segregation types.

Double mutants from among the f_1 isolates were paired in crosses with one another, and galls obtained from a number of combinations. Progeny isolated from single chlamydospores of the crosses 4B/24(7)6 (thiaminless-methionineless)×132/24(2)1 (adenineless-methionineless), and 91/24(3)2 (isoleucineless-methionineless)×6A/24(1)1 (adenineless-methionineless) included all four expected types of individual segregants; tetratype segregations were again encountered while examining the progeny of single-chlamydospores for the presence of triple mutants.

The two triple-mutant and seven double-mutant combinations obtained are indistinguishable from wild-type in the appearance of their growth on complete medium.

DISCUSSION

Auxanography. With Ustilago, the most satisfactory method for detecting and identifying growth-factor requirements has been to observe the response of an isolate to test substances spotted on the surface of a culture densely plated in solid minimal medium. This method is quicker, easier, and less ambiguous than the alternate procedure of testing each culture by inoculating the organism into a series of tubes or plates which contain different supplements. It allows a comparison of responses to different compounds under uniform conditions, distinguishes a specific response from back-mutation, gives a com-

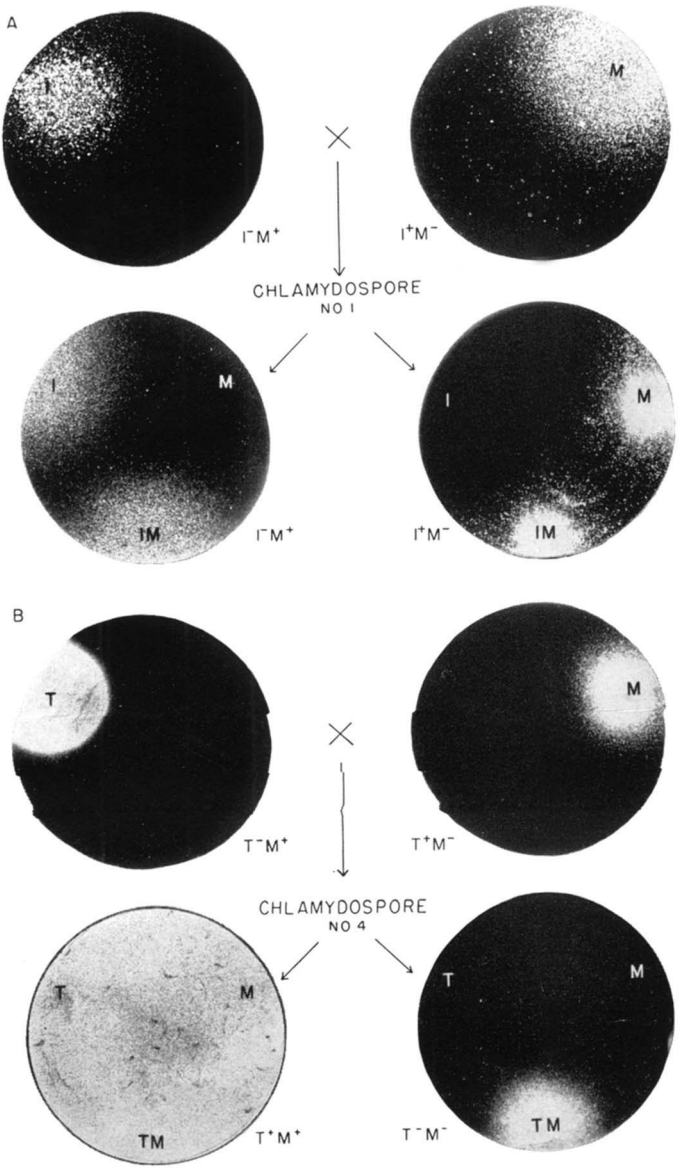

FIGURE 3.—Auxanograms of parents and segregants of single chlamydospores from two crosses.

A. Segregation producing parental types only. Parents: 5–91 (isoleucineless) and 4–24 (methionineless).

B. Segregation producing non-parental types only. Parents: 5–4B (thiaminless) and 4–24 (methionineless). The spots at which test-substances were placed on the plate are indicated by letters on the photographs. I = DL-isoleucine, M = DL-methionine, T = thiamin hydrochloride.

plete concentration-gradient for each substance, permits testing substances of low solubility, and allows testing for single and multiple requirements on the same plate. It can be used to reveal inhibition as well as stimulation of growth.

The surface-spotting of test substances was proposed by BEIJERINCK (1889),

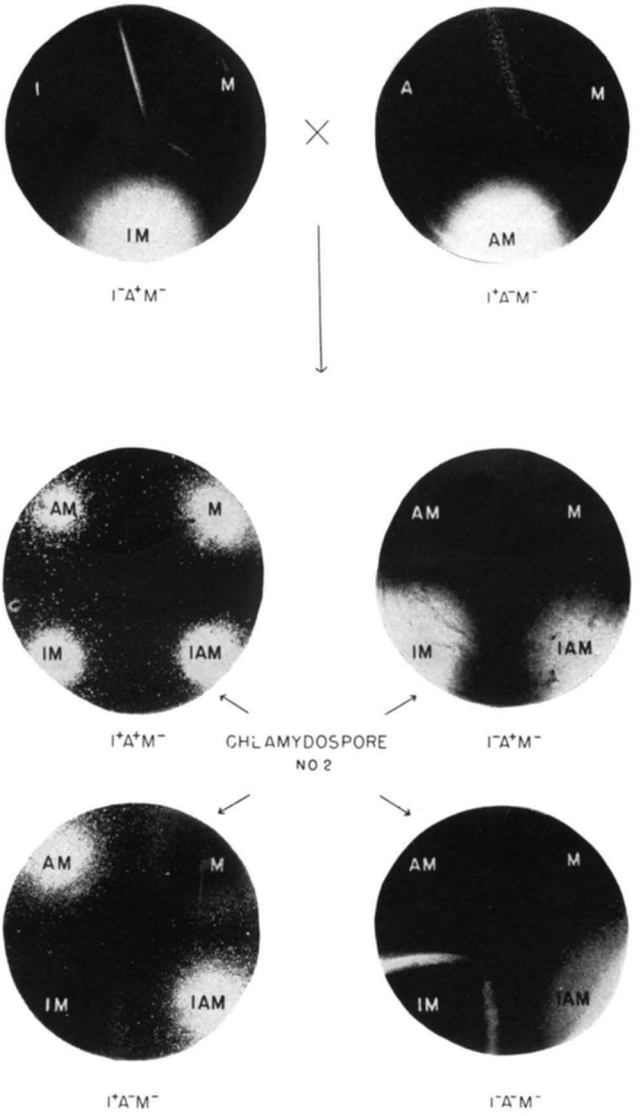

FIGURE 4.—Auxanograms of parents and segregants of a single chlamydospore from a cross of 91/24(3)2 (isoleucineless-methionineless)×6A/24(1)1 (adenineless-methionineless). Segregation produced all four possible types. A = adenine sulfate, I = DL-isoleucine, M = DL-methionine.

who termed the technique *auxanography* and applied it to determine the effect of various substances on luminescence and on the production of acids and pigments. In modified form it has recently been brought into prominence by its use in studies on the effect of antibiotics. Auxanography has been used extensively to test the ability of microorganisms to utilize various carbohydrates and nitrogen sources (*e.g.*, KLUYVER and CUSTERS 1939) and has been adapted to investigating the production of antibiotic-destroying enzymes (WATERWORTH 1948), the effect of inorganic ions (*e.g.*, DUFRÉNOY 1948), and

the effect of substances stimulating sexual-spore formation (HAWKER 1948). Several workers have reported the use of auxanography in studying growth-factor requirements (LUTERAAN and DENIS 1946; LEDERBERG 1946; PONTECORVO 1947; WILLIAMS and PRICE 1948; BACHARACH and CUTHBERTSON 1948; B. DAVIS 1948). At COLUMBIA UNIVERSITY, DR. K. C. ATWOOD has successfully applied auxanographic methods to the characterization of Neurospora mutants, using plated conidia. The manifold applications of BEIJERINCK'S method have been reviewed by DUFRÉNOY (1947).

Growth-factor requirements and parasitism. SCHOPFER and BLUMER (1938) reported that of ten Ustilago species they studied, seven were auxoautotrophic, while the rest required only thiamin or one of its components. This is in surprising contrast to the very exacting requirements of other plant-parasites such as the rust fungi, and raises the question of the relation of growth-factor deficiencies to the parasitic habit.

The fact that combinations of Ustilago mutants, homozygous for an induced growth-factor requirement, are unimpaired in their infective ability suggests that ability to synthesize essential metabolites is probably unrelated to the process of parasitism, but that the selective advantage of growth-factor independence (which must exist to have prevented the accumulation of spontaneously arising deficiency mutations in nature) accrues during some part of the life-cycle that involves saprophytic growth outside the host.

Biochemical mutants as genetic tools. In most discussions of the usefulness of gene controlled growth-factor requirements, the primary emphasis has been placed on their value in furthering knowledge of gene action and biosynthesis. Their importance in this regard has been demonstrated repeatedly, and so successfully that other lines of investigation in which mutants of this type are useful may have been overlooked. Factors governing metabolite requirements can be equally important for biology as tools in the formal genetic study of organisms previously neglected because of technical difficulties. Biochemical mutants are eminently suitable for use as unambiguous genetic markers. They possess a second advantage—the ability to undergo selective screening—in common with but one other category of mutants, those conferring resistance to environmental agents. To illustrate what can be accomplished by employing biochemical mutants as markers, it is only necessary to mention the work of TATUM and LEDERBERG (1947) and LEDERBERG (1947) on sexual recombination in bacteria.

Since BEADLE and TATUM reported the first biochemical mutants in *Neurospora crassa* and *N. sitophila* in 1941, growth-factor mutations have been obtained in *Escherichia coli* (ROEPKE, LIBBY and SMALL 1944; GRAY and TATUM 1944; ANDERSON 1944), *Acetobacter melanogenum*[2] (GRAY and TATUM 1944) *Clostridium septicum* (RYAN, SCHNEIDER and BALLENTINE 1946), *Bacillus subtilis* (BURKHOLDER and GILES 1947), *Bacterium aerogenes* (DEVI, PONTECORVO

[2] Cases where neither the test of sexual recombination nor statistical analysis of the pattern of variation (LURIA and DELBRUCK 1943) have been used to indicate the genic basis of requirement differences are included in this listing because of their resemblance to instances where one or both of these criteria have been applied.

and HIGGENBOTTOM 1947), *Achromobacter fischeri* (MCELROY and FARGHALY 1947), *Azotobacter agilis* (KARLSSON and BARKER 1948), *Absidia glauca* (GILES 1946), *Penicillium notatum* (BONNER 1946; PONTECORVO 1947), *Aspergillus terreus* (RAPER, COGHILL and HOLLAENDER 1945), *Aspergillus nidulans* (HOCKENHULL 1948; PONTECORVO 1948), *Ophiostoma multiannulatum* (FRIES 1945), *Saccharomyces cerevisiae* (REAUME and TATUM 1949; S. POMPER, unpublished), *Coprinus fimetarius* (L. FRIES 1948), and *Ustilago maydis* (this paper). Naturally occurring differences in growth-factor requirements of yeasts have been used by LINDEGREN and LINDEGREN (1947).

No attempt will be made here to deal with the cases where differences in ability to utilize specific energy sources (*e.g.*, WINGE and LAUSTSEN 1939; AUDUREAU 1942; LINDEGREN and LINDEGREN 1946; MONOD 1947; LEWIS 1948, WINGE and ROBERTS 1948; LEDERBERG 1948; EPHRUSSI *et al.* 1949) have been investigated, except to point out that they, like inherited differences in the utilization of nitrogen and sulfur sources (HOROWITZ *et al.* 1945; RAPER *et al.* 1945; FRIES 1946; LAMPEN *et al.* 1947), resemble growth factor mutants, and can be equally useful to the geneticist. Heritable differences of yet another type, affecting chlorophyll synthesis (GRANICK 1948) and photosynthesis (E. DAVIS 1948) have been obtained in Chlorella. The relation of growth-factor mutations to luminescence has been studied by MCELROY and FARGHALY (1947).

Although by far the greatest effort in biochemical genetic studies has been concentrated on *Neurospora crassa* (reviewed by BEADLE 1945), there has already been a considerable extension of similar investigations to other forms. However, the only sexual fungi, aside from Ascomycetes, to which these methods have been applied are Absidia (in which case spore germination could not be obtained), Coprinus, and Ustilago. It seems that the potentialities of biochemical mutants for genetic studies, especially in sexual organisms, have hardly begun to be realized.

SUMMARY

Stable biochemical mutants have been obtained and characterized in the auxoautotrophic smut fungus *Ustilago maydis*, after ultraviolet irradiation. Fifteen isolates requiring specific B-vitamins, amino acids, nucleic acid components, or a source of reduced sulfur, are described.

An auxanographic method has been used for the ready identification and characterization of biochemical mutants.

All possible recombinations of mutant factors were recovered from crosses involving two and three loci at a time. All types of segregations were obtained from single zygotes heterozygous at two loci.

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APPENDIX

Tetrad analysis without knowledge of linearity

Although exhaustive treatment has been given to tetrad analysis where the linear order of segregants is known (MATHER and BEALE 1942; WHITEHOUSE 1942),[3] the more general situation where complete single tetrads are subjected to analysis without consideration of linearity has never received detailed consideration. As genetic studies are increasingly extended to organisms in which the isolation of meiotic products in order is difficult or impossible (Phycomycetes, yeasts, Basidiomycetes, Algae, Bryophytes), mapping procedures will be required that make full use of the information obtainable from single, non-linear tetrads.

In any organism where all four products of a single meiosis can be obtained, it is possible, given any two linked factors, to determine the frequency of recombination between the two, corrected for double-crossovers, by an analysis involving no knowledge of the linear arrangement of segregants. It is further possible, given three unlinked loci, to determine the frequency of crossing over between each locus and its centromere.

A zygote heterozygous at two loci can segregate in any one of three possible ways (table 2). Various chromosomal events and the segregation types to which they give rise are given in table 3.

TABLE 2

Segregation types from a dihybrid cross

TYPE	DESCRIPTION	CONSTITUTION OF TETRAD	
		FOR CROSS $Ab \times aB$	FOR CROSS $AB \times ab$
I	Parental Ditype	$Ab+Ab+aB+aB$	$AB+AB+ab+ab$
II	Non-Parental Ditype	$AB+AB+ab+ab$	$Ab+Ab+aB+aB$
III	Tetratype	$AB+Ab+aB+ab$	$AB+Ab+aB+ab$

For the case where A and B are linked, the frequency of four-strand double-crossovers is that of type II segregations, which give rise only to non-parental types (ignoring multiple crossovers of more than two). Assuming no chromatid interference, the fraction of segregations with no crossovers is given by the frequency of type I minus the frequency of type II segregations,

$$\text{f(non-crossover tetrads)} = \text{f(I)} - \text{f(II)}. \qquad 1$$

Also

$$\text{f(single-crossover tetrads)} = \text{f(III)} - 2\text{f(II)} \qquad 2$$

and

[3] MATHER, K., and G. H. BEALE, 1942 The calculation and precision of linkage values from tetrad analysis. J. Genetics **43**: 1–30; WHITEHOUSE, H. L. K., 1942 Crossing-over in Neurospora. New Phytologist **41**: 23–62.

$$\text{f(double-crossover tetrads)} = 4\,\text{f(II)}. \qquad 3$$

$$\text{Recombination frequency} = \frac{\text{f(singles)} + 2\text{f(doubles)}}{\text{f(non-crossovers)} + \text{f(singles)} + \text{f(doubles)}}$$

$$= \text{f(III)} + 6\text{f(II)}. \qquad 4$$

If frequencies be expressed as decimal fractions,

$$\text{map-distance} = [\text{f(III)} + 6\text{f(II)}] \times 50. \qquad 5$$

This holds regardless of the position of the centromere with respect to the two loci.

For the case where A and B are independent, type III segregations will occur with a frequency equal to $(x-xy)+(y-xy)+1/2xy$, that is,

$$\text{f(III)} = x + y - 3/2xy, \qquad 6$$

where x is the fraction of segregations with crossing-over between A and its centromere, and y the corresponding fraction for B and its centromere (assuming no double-crossovers). By testing both A and B against a third locus independent of both, three simultaneous equations can be obtained and solved for the three unknown recombination-frequencies, each of which is equal to twice the conventional map-distance from locus to centromere.

TABLE 3

The relation of segregation type to crossing-over

TWO LOCI, LINKED		TWO LOCI, INDEPENDENT	
CROSSING-OVER	SEGREGATION TYPE	CROSSING-OVER BETWEEN	SEGREGATION TYPE
No crossing-over	I	Neither locus and centromere	1-I:1-II
Single-crossover	III	One locus and centromere	III
Double-crossovers	1-I : 2-III : 1-II (2-strand:3-strand:4-strand)	Both loci and centromeres	1-I:2-III:1-II

Application to Ustilago maydis. In the corn-smut each colony in a plating of chlamydospores (the zygotes) contains all the products of a single meiosis. Using growth-factor mutants, an auxanogram of the pooled progeny of $Ab+Ab+aB+aB$ segregations will show growth only at the spots where the plate has been supplemented, whereas segregations giving $AB+AB+ab+ab$, or $AB+Ab+aB+ab$, will grow diffusely throughout the plate owing to the presence of growth-factor independent wild-type cells in the population.

For the cross $Ab \times aB$, the frequency of single-chlamydospore cultures giving non-diffuse auxanograms is that of type-I (parental) segregations, while for

$AB \times ab$, non-diffuse plates will represent non-parental segregations (type-II). The frequency of type-III segregations can be obtained by subtraction. An equal frequency of I's and II's indicates that A and B are in separate chromosomes or are separated by at least 50 map-units if on the same chromosome. In cases where I's are more frequent that II's, linkage is indicated, and equation (5) gives the map-distance corrected for occurrence of double-crossovers.

Compared with alternate methods of mapping in Ustilago, this method, whereby a complete tetrad is characterized in a single operation, should be highly efficient. Microdissection is necessary to isolate products in order from the promycelium, and to the difficulty of separating cells whose dimensions are about 10μ is added the problem of irregular formation of promycelia encountered very commonly in some crosses (KERNKAMP and PETTY 1940).[4] Isolation of individual haploid segregants will be necessary for the study of loci incapable of auxanographic characterization, but this method yields no information with respect to biochemically mutant loci that cannot be obtained from pooled tetrads. Such sources of error as selective elimination of the prototrophic haploid type, or early mutation during growth of the mixed colony, must be guarded against.

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