11. G. SERMONTI. — **Genetics of Penicillium chrysogenum. — I. Heterokaryosis in Penicillium chrysogenum.**

Summary. — A simple technique is presented for constituting heterokaryons between mutant strains with complementary nutritional requirements in *Penicillium chrysogenum* Thom. The heterokaryons were obtained from mixed cultures on limiting media.

They develop on such media as tufts with exuberant growth in the zones of contact between colonies of different type. Isolated and transferred to MM they give colonies which show an irregular edge, and a surface colour intermediate between those of the parent colonies.

Plating of the conidia of the heterokaryons on MM does not give rise to growth; on fully supplemented medium the conidia give rise to colonies belonging partly to one and partly to the other parent type. The heterokaryons can be preserved by means of tranfer of vegetative mycelium, and also of single hyphae.

Recovery of the two parent types from a colony derived from a single hypha of a supposed heterokaryon constitutes a proof of its heterokaryotic state.

Heterokaryons may also be detected by their ability to give rise to heterozygous diploids.

Riassunto. — E' presentata una semplice tecnica per la costituzione di eterocarion tra ceppi mutanti con richieste nutrizionali complementari in *Penicillium chrysogenum* Thom. Gli eterocarion si ottengono da culture miste su terreni limitanti. Essi si sviluppano su tali terreni dalle zone di contatto fra colonie di tipo diverso, come ciuffi a crescita rigogliosa. Isolati e trasferiti su MM presentano un aspetto caratteristico per il margine frastagliato delle colonie e un colore della superficie intermedio tra quelli dei ceppi parentali.

Il piastramento dei conidi degli eterocarion su MM non dà luogo a crescita; su terreno completo (CM) i conidi dànno luogo a colonie appartenenti alcune all'uno, le altre all'altro tipo parentale.

Gli eterocarion possono mantenersi mediante trasferimento di micelio vegetativo ed anche di singole ife.

La segregazione di due tipi parentali da una colonia proveniente da una singola ifa di un eterocarion rappresenta la prova della condizione eterocariotica della colonia originaria.

L'eterocariosi di una colonia è altresì rilevabile dalla capacità di questa di produrre eccezionalmente diploidi eterozigoti.

Résumé. — On décrit une technique simple pour la formation d'hétérokaryons parmi les mutants de *Penicillium chrysogenum* Thom. Ayant des besoins nutritifs complémentaires. Les hétérokaryons ont été obtenus à partir de cultures sur milieux partiellement enrichis.

Ils se développent sur de tels milieux comme des touffes avec une croissance exubérante aux zones de contact de colonies de typer différents. Isolés et transplantés sur un milieu minimum (MM) ils donnent des colonies qui affectent un contour irrégulier et dont la couleur de surface est intermédiaire, comparée à celles des colonies-mères.

Les conidies des hétérokaryons en « plating » sur MM ne donnent lieu à aucune croissance; sur un milieu complet, les conidies donnent naissance à des colonies appartenant soit à l'un, soit à l'autre type parental. On peut conserver les hétérokaryons en transférant soit le mycélium végétatif, soit des hyphes isolées.

Le récupération des deux types parentaux à partir d'une colonie dérivée d'une hyphe isolée d'un hétérokaryon supposé, est une preuve de son état hétérokaryotique.

On peut aussi révéler les hétérokaryons par leur faculté d'engendrer des diploïdes hétérozygotes.

Zusammenfassung. — Es wird eine einfache Technik beschrieben zur Herstellung von Heterokaryons zwischen Ernährungsdefekt-Mutanten von *Penicillium chrysogenum*, die sich in ihren Ernährungsbedürfnissen ergänzen. Die Heterokaryons wurden mit Hilfe von Mischkulturen auf einem unvollständigen Medium erhalten.

Sie entwicklen sich auf einem solchen Medium als kräftig wachsende Büschel zwischen den beiden verschiedenen Kolonien. Wenn man Stücke dieser Büschel auf ein Minimalmedium bringt, erhält man Kolonien, die eine unregelmässige Kante besitzen und deren Oberflächenfarbe als Intermediärprodukt der Pigmente der Elternstämme anzusehen ist.

Die Konidien des Heterokaryons wachsen nicht auf Minimalmedium; aus Maximalmedium dagegen bringen sie Kolonien hervor, die zum Teil dem einen und zum Teil dem anderen Elterntyp angehören. Die Heterokaryons können erhalten werden durch dauernde Übertragung des vegetativen Myzeliums und durch Übertragung von Einzelhyphen.

Wenn in einer Kolonie, die durch Übertragung von einer Einzelhyphe eines mutmasslichen Heterokaryons entstanden ist, sich die beiden Elterntypen zeigen, so ist dies der Beweis für das Vorhandensein des Heterokaryons.

Heterokaryons können auch entdeckt werden durch ihre Fähigkeit heterozygote Diplonten zu bilden.

Heterokaryons are strains whose hyphae contain two, or more, different kinds of nuclei. A « balanced heterokaryon » is one whose heterokaryotic condition stays constant on some chosen medium. As stated by PONTECORVO (1947), « the essential feature of heterokaryotic systems is that they are based on mechanisms of segregation and recombination of hereditary particles other than meiosis and karyogamy ».

By means of heterokaryosis tests of allelism and dominance can be carried out without recourse to sexual reproduction. Furthermore a technique for the isolation from balanced heterokaryons of strains carrying in their hyphae diploid heterozygous nuclei was devised by Roper (1952) in *Aspergillus*, and recently applied in *Penicillium chrysogenum* (PONTECORVO & SERMONTI, 1953; 1954).

Simple techniques are in use in *Aspergillus* (PONTECORVO, 1953) and *Neurospora* (RYAN, 1950) for synthesis of balanced heterokaryons between pairs of genetically marked strains. In *Penicillium* attempts to form balanced heterokaryons using the techniques which had proved effective with other species of moulds were unsuccesful, as reported by BONNER (1946) and PONTECORVO & SERMONTI (1954).

Recently PONTECORVO & SERMONTI (1953; 1954) reported the first positive results in synthesizing balanced heterokaryons in *P. chrysogenum*. Since then work in this laboratory has been directed towards the development of suitable techniques for producing balanced heterokaryons in *P. chrysogenum*. This paper deals with the description and practical application of such techniques.

STRAINS AND MEDIA.

The starting strains were three penicillin producing strains of *P. chrysogenum*, X 1612, Wis 176 and 47. 1564 (*).

All the mutant strains used in the course of this work were obtained after ultraviolet treatment. Mutants in colour of conidia were detected by inspection; for the physiological mutants, the « total isolation » method, as described for *Ophiostoma* by Fries (1948), was adopted. (see tab. 1).

(*) Obtained by courtesy of Dr. M. J. Johnson, Department of Biochemistry, University of Wisconsin, Madison, Wis., U.S.A.

TAB. 1. - Mutants (*) of *P. chrysogenum* used in course of study, and strain from which they have been derived.

Single mutants	*Double mutants*	*Triple mutants*
from X 1612 117 cy (**)	from 15 y 35 hy y	from 35 hy y 52 nic hy y
	92 sp y	55 me hy y
from 47. 1564 15 y		
20 w	from 20 w 42 me w	from 42 y sp 65 pr y sp
22 su		
23 ts	from 29 pr 51 thi pr	from 44 py w § 74 nic py w
26 le		
29 pr	from 99 sp 42 y sp	from 46 me w 121 sp me w
99 sp		
		from 51 thi pr 86 y thi pr
		from 92 sp y 63 ts sp y

(*) The strain *20 w* was selected after nitrogen mustard treatment, all the others after ultra-violet rays treatment. The colour mutants were detected by inspection, the nutritional mutants by « total isolation ».
(**) For each strain the figure indicates the collection number; the symbols have the following meanings:

cy	requirement of cysteine or methionine	su	requirement of sulphite, thiosulphate, cysteine or methionine
hi	requirement of histidine		
hy	requirement of hypoxan. or adenine	thi	requirement of thiamine
le	requirement of leucine	ts	requirement of thiosulphate, cysteine or methionine
me	requirement of methionine		
nic	requirement of nicotinamide	w	white conidia
py	requirement of pyridoxine	y	yellow conidia
sp	penicillin suppression		

§ Strain 44 py w (8w) was obtained by the courtesy of Dr. G. Pontecorvo (Glasgow, Scotland).

The media used in the course of this study were:

(a) a minimal medium (MM) (Czapek-Dox, as modified by Clutterbuck et al. 1932): distilled water, 1 litre; $NaNO_3$, 3 g; KCl, 0.5 g; $MgSO_4.7H_2O$, 0.5 g; $FeSO_4.7H_2O$, 0.01 g; KH_2PO_4, 1 g; glucose, 40 g; agar, 20 g; pH 7.

(b) a complete medium (CM), (modification of the medium used by Pontecorvo 1953): water, 1 litre; « Costantino » hydrolysate of casein, 1.5 g; « Costantino » autolysed yeast 1 g; « Costantino » peptone, 1.5 g; corn steep liquor, 10 g; hydrolysed yeast nucleic acid, (**) 3 ml; solutione of B-complex vitamins (***), 1 ml; glucose, 20 g; agar, 20 g; pH 6.

(**) 2 g nucleic acid are dissolved in 15 ml N NaOH and 2 g nucleic acid in 15 ml N HCl; the two solutions are heated at 100°C for 20 minutes, mixed, brought to pH 6 and filtered while warm. The volume is brought to 40 ml with water.
(***) Riboflavin, 10 mg; nicotinamide, 10 mg; para-aminobenzoic acid, 1 mg; pyridoxin HCl, 5 mg; aneurin HCl, 5 mg; biotin, 0.025 mg; calcium pantothenate, 20 mg; choline HCl, 20 mg; inositol, 40 mg; water 20 ml. To be kept in the dark.

GENERAL CRITERIA FOR THE FORMATION OF BALANCED HETEROKARYONS.

The best known type of balanced heterokaryon is that formed between two strains with complementary requirements of growth factors. Let us take as an example a heterokaryon formed between a strain that requires proline for its growth (and is able to synthesize leucine like the wild type strain) and a strain that requires leucine (and synthesizes proline). The two isolated strains do not grow on MM when proline and leucine are absent. When a heterokaryon is formed between the two strains it appears as a prototroph, that is, capable of developing on MM. The heterokaryon contains in its hyphae nuclei of both types (see below) and is able to synthesize proline and leucine. On MM the heterokaryon is thus superior relatively to its parents, and this selective advantage is to be correlated with the stability displayed in this medium by the heterokaryon. Transferred to a medium which contains leucine and proline the heterokaryon segregates into sectors which slow the characteristics of the components.

The ideal medium for the preservation of a heterokaryon between two complementary auxotrophic strains is one totally lacking in the growth factors required by the parents. On the other hand, on such a medium the formation of heterokaryons cannot take place, for this would require at least a beginning of development of the original strains, in order that the nuclei of the latter may coalesce in the same cytoplasm.

The conditions suitable for the growth and preservation of heterokaryons do not favour their formation. Thus the problem is to find the culture conditions which would lead to synthesis and establishment of heterokaryons.

Such a difficulty is negligible with *Neurospora crassa*, where the simple mixed inoculation of conidia from two complementary auxotrophic strains on MM leads to the stabilization of the heterokaryons (RYAN, 1950).

Synthesis and selection of heterokaryons in *Aspergillus nidulans* involve a more complicated procedure (PONTECORVO, 1953). The process which gives most constant results in this species consists of two phases: a first phase for the formation, and a second for the emergence and stabilization of the heterokaryon. The two complementary auxotrophic strains are inoculated in a liquid medium containing the required growth factors (CM), and allowed to grow together so as to permit the formation of heterokaryotic hyphae. The mycelium thus developed is removed after 24 hours, washed and spread out on an agarized minimal medium.

This operates selectively for the heterokaryotic hyphae formed, which succeed in finding their way through the parental mycelium.

In *Penicillium chrysogenum* the formation and selection of the heterokaryons presents the greatest difficulties. The method used in *Aspergillus nidulans* was found to give uncertain results, giving the impression that the heterokaryon produced was perhaps a chance occurrence (Pontecorvo & Sermonti, 1954).

It is noteworthy that the slower the rate of growth of a species, the greater are the difficulties encountered in heterokaryon production.

Neurospora crassa grows on agar at a rate of 4 mm per hour (advancing at the front of the colony); *Aspergillus nidulans* at a rate of 0.25 mm per hour; *Penicillium chrysogenum* 47.1564 at a rate of 0.05 mm per hour. Evidently a heterokaryon which develops on agar very slowly finds the growth medium rich in products of its metabolism; on such a medium the heterokaryon no longer has a marked selective advantage over its parental auxotrophic strains.

During two attempts at synthesis of heterokaryons in *Penicillium chrysogenum* (Pontecorvo & Sermonti, 1954), the unexpected appearance of several tufts of mycelium was observed — which were later shown to be heterokaryons — in the zone of contact between complementary auxotrophic colonies growing on CM. The emergence of heterokaryotic mycelium on CM was attributed to the fact that, in the above mentioned instances, the auxotrophic strains showed stunted growth on CM, allowing the heterokaryon on this medium to gain advantage over the parents and to emerge. In one case the heterokaryon was formed between two auxotrophic dwarf strains; in the other, both strains (and one in particular) failed to grow normally on CM because they were specifically inhibited by substances present in the medium.

The work reported in this paper originates from these observations and aims at defining a culture condition in which similar heterokaryotic tufts might appear in the zone of contact between complementary auxotrophic colonies of any type.

Limiting media.

A medium for the production and selection of heterokaryons should — according to the considerations outlined above — show conditions which, while limiting the growth of the auxotrophic strains, would have no inhibitory action on the heterokaryons that might be formed from them.

The first experiments were carried out on agarized minimal media (MM) to which the substances required by the two auxotrophic strains were supplied in amounts less than the minimal quantities needed for optimum growth of the strains under experiment. About ten days after spreading a mixed suspension of conidia of the two complementary auxotrophic strains on the surface of a similar medium, there regularly appeared tufts of heterokaryotic mycelium in the zone of contact between colonies of different type (fig. 1).

Such media will be termed in the course of this study *limiting media*; and it will be stated (where not obvious) for which auxotrophic strains the medium in question is limiting, or which substances were added to MM in reduced concentrations relative to the minimal requirement of one or more given strains. The *limiting concentration* of a substance relative to a given strain is that concentration in agar MM which was shown to be suitable (or possibly was the best) for obtaining synthesis of heterokaryons between that strain and other strains. By *optimal concentration* is meant any concentration (generally the minimal one) which allowed a given strain requiring the substance in question the maximum growth and sporulation.

In the course of all the experiments to be reported, Petri dishes of various sizes were used (8, 10 or 12 cm diameter) always containing 20 ml of agarized medium. The 20 ml were measured before autoclaving the media, and distributed in 50 ml test-tubes plugged with cotton-wool.

The two concentrations, optimal and limiting, of each substance with reference to determined strains, were established by adding to 20 ml of melted agar MM different quantities of some solutions, at known concentrations, of these substances. On solidified agar in Petri dishes a suspension of conidia of the strain being tested was spread (0.1 ml containing about 100 conidia) and the dishes were then placed at 24° C. After a week the various dishes concerned were brought under observation. If above a certain concentration of the substance added, growth and sporulation were constant, this concentration was considered optimal. The limiting concentration of a substance was established as that which allowed stunted growth, on which in general sporulation was hardly apparent. The fixed concentration was then checked in successive heterokaryon synthesis experiments. The response of the various strains at different concentrations did not always have the same pattern with the decrease of concentration of the required substance: some strains appeared in smaller but richly sporing colonies; others lost the ability to form conidia before the size of their colonies diminished; still

others showed a decrease in the number of colonies per dish, and a slight alteration in their appearance. These latter strains (e.g. 46 me w) were not very suitable for synthesis of heterokaryons.

In cases where the optimal and limiting concentrations could not be detected in the series of concentrations tried, the experiments were repeated, varying the range of concentration.

The optimal and limiting concentrations of some substances with reference to determined strains are listed in table 2.

TAB. 2. — Optimal and limiting concentrations in agar MM of some substances with reference to strains requiring them for growth.

Substance	*Mol. weight*	*Stock Solution (SS)*		*Optimal Concentration*		*Limiting Concentration*			*Test strains* (**)
		mg/ml	*mM per l*	*mM per l*	*ml of SS to be added to 20 ml of agar MM*	*mM per l*	*Diluted Stock Solutions (ss)*	*ml of ss to be added to 20 ml of agar MM*	
adenine HCl	171	5	29.2	0.58	0.4	0.007	SS. 10^{-2}	0.5	*33 hy y*
dl-methionine	149	10	67.1	0.34	0.1	0.067	SS. 10^{-1}	0.2	*55 me hy y*
l-leucine	131	10	76.3	1.14	0.3	0.076	SS. 10^{-1}	0.2	*26 le*
l-proline	115	5	43.5	0.65	0.3	0.087	SS. 10^{-1}	0.4	*29 pr*
thiamine HCl	337	0.25	0.74	—	0.1 *	0.000037	SS. 10^{-2}	0.1	*51 thi pr*
nicotinamide	122	1	8.2	—	0.1 *	0.0082	SS. 10^{-1}	0.2	*24 nic py w*
pyridoxine HCl	205	0.15	0.73	—	0.1 *	0.000037	SS. 10^{-2}	0.1	*74 nic py w*
histidine	191	5	26.2	0.65	0.5	0.026	SS. 10^{-1}	0.2	*114 hi*

(*) In excess

(**) When the test strain has two requirements, the substance not to be tested was added to the agar in the optimal concentration.

COLLECTION OF HETEROKARYONS ON LIMITING MEDIA.

The procedure adopted for synthesizing a heterokaryon from two given strains was as follows. A series of Petri dishes were prepared with 20 ml of MM, to which were added the requirements of the two strains in limiting concentrations. These concentrations were previously fixed as stated above, using separately the strains to be associated in the heterokaryons, as test strains. In general optimal and limiting concentrations of a substance appeared to be similar in different strains having the same nutritional requirement.

It was advisable, when a strain was used for the first time in heterokaryon synthesis, to repeat the experiments using two or three concentrations of the substance (or substances) required by that strain, nearest to that considered in the preliminary experiments as the most

suitable. This precaution, initially adopted for synthesis of all heterokaryons, helped to demonstrate that the operating range of concentrations which permit heterokaryon formation is generally wide. This range was from nearly three times to near one third the limiting concentration of the particular required substance.

From each of the two strains to be associated in the heterokaryon, a suspension of conidia was obtained, and was brought to a density of approx. 100 conidia in 0.1 ml. On a certain number of agar dishes (4 were generally sufficient when the limiting concentrations were well established) 0.1 ml of conidial suspension of both types was spread. On some dishes, which were used as controls, each of the two types of conidia (0.1 ml of suspension) was plated separately. The drops were spread with a glass rod over the whole surface of the agar, taking care that the different suspensions were well mixed with one another.

It was advisable to have thick pourings of agar in the dishes, in order to permit maximum growth of the prototrophic colonies, so as to avoid non-specific growth limitation of both the heterokaryon and the parental strains as a result of insufficient quantities of culture medium.

The dishes were placed at 24° C and kept at this temperature for 9-10 days, during which time small, slightly raised and poorly sporulating colonies developed. Some of these joined each other by contact during growth; others remained isolated. By comparing the type of growth thus obtained with the appearance of one or the other colony type in the controls, it was often possible to recognize the two types in the mixed cultures.

In the zone of contact between colonies of different types something resembling a small tuft of cotton-wool could be observed, distinguishable from the rest of the culture, in its initial stages of growth by its whiteness, in later stages by the rich sporulation which it presented (figs. 1, 2, 8).

A small hyphal tuft from one of the areas of excess growth was transferred by means of a needle to fresh agar MM. After some days some of the transferred tufts had started growing, leading to the formation of characteristic colonies: the balanced heterokaryons.

It should be noted that the heterokaryotic zones that developed in mixed culture did not expand on agar, but were composed exclusively (or almost so) of aerial mycelium which developed vertically. Some heterokaryons of *P. chrysogenum* obtained on limiting media in this laboratory are listed in tab. 3. Beside each is indicated the test which permitted its designation as a heterokaryon.

TAB. 3. — Balanced heterokaryons in *P. chrysogenum* synthesized on limiting media. Tests (1) of heterokaryosis.

Balanced heterokaryons (2)	*Media on which obtained MM supplemented by* (3)	*Typical growth* (*)	*Recovery of components*	*Recovery of components after isolation of single hyphae*	*Formation of heterozygous diploid*
57 nic hy y + 74 nic py w	ad nic py	+	—	(—)	—
23 ts + 22 su	me	+	+	—	—
55 me hy y + 22 su	ad me	+	+	—	—
55 me hy y + 74 nic py w	me nic py	+	+	—	—
29 pr + 26 le	le pr	+	+	—	+
63 ts sp y + 46 me w	me	+	+	+	+
63 ts sp y + 65 pr y sp	me pr	+	—	—	+
51 thi pr + 63 ts sp y	me pr thi	+	+	+	+
86 y thi pr + 117 cy	me pr thi	(—)	+	(—)	+
51 thi pr + 55 me hy y	me hy thi pr	+	—	+	—

(1) +: positive test; —: test not made; (—) no positive test.

(2) Heterokaryons are designated by the symbols of the components joined by the sign +

(3) The substances, indicated by the symbols of the corresponding requirements (see tab. 1), were added to MM in the limiting concentrations reported in tab. 2.

(*) as described in the text.

CHARACTERISTICS OF THE HETEROKARYONS.

All the heterokaryons between complementary auxotrophic strains grew on MM, and were selected for this property.

Their rate of growth, measured as the daily increase of diameter of the colony on MM agar, was approximately the same as that of the common ancestor of the two parental strains. The heterokaryons were distinguished from all other types of colony by the characteristic appearance of the edge during growth. This was composed, for a depth of 1-2 mm, of mycelium submerged beneath the agar, which was seen microscopically to be composed of scattered, differently protruding hyphae. In the homokaryotic colonies the marginal hyphae are dense, compact, in line with one another, covered with aerial mycelium. Macroscopically the edge of the heterokaryon appeared irregular (fig. 3), but its most evident characteristic was its transparency to indirect light (fig. 4). In the heterokaryons which had among their parents a strain derived from X 1612, the edge of the colony appeared almost regular, but retained its characteristic transparency.

Sporulation was markedly later in the heterokaryons than in the wild type. The colour of the sporing surface always appeared interme-

diate between the conidial colours of the two parents, as observed by PONTECORVO & SERMONTI (1954). In different heterokaryons selected from the same mixed culture or derived from transfer of hyphae from the same heterokaryotic colony, the colour of the sporing surface was variable, showing different gradations between the colour of one parent and of the other.

Heterokaryons between one strain with white conidia and one with yellow always had a more or less pale yellow colour. Those between yellow and green strains appeared yellowish-green in colour. Two heterokaryons between two green strains and one between two yellow strains gave respectively a dark green and a bright yellow colour. These observations are tabulated in tab. 4.

TAB. 4. — Colour of the sporing surface of some balanced heterokaryons of *P. chrysogenum*

Balanced heterokaryons	*Conidial colour of parents*	*Colour of the sporing surface of the heterokaryons*
57 nic hy y + 74 nic py w	yellow + white	yellowish
63 ts sp y + 46 me w	yellow + white	yellowish-white
63 ts sp w + 65 pr y sp	yellow + yellow	yellow
51 thi pr + 63 ts sp y	green + yellow	greenish-yellow
86 y thi pr + 117 cy	yellow + green	yellowish-green
23 ts + 22 su	green + green	green
29 pr + 23 le	green + green	green

After a period of about ten days following transfer, the heterokaryon ceased to grow, and the edge of the colony although keeping its irregularity lost its transparency and became covered with aerial mycelium.

Transfer of the heterokaryons was effected by picking up from the edge of the developing colony a small fragment of agar containing the growing hyphae, and transferring it to MM. The heterokaryon reproduced regularly, provided that the colony from which the transfer was attempted was not allowed to grow so old that the vegetative mycelium died.

The transfer of the conidia of the heterokaryon on to MM did not produce growth. Transferred on to CM, conidia germinated and gave

rise to colonies of which some belonged to one, the remainder to the other parent type. Segregation was immediately evident when the heterokaryon had been synthesized between two strains with differently coloured conidia. On MM to which the substance (or substances) required by one parental strain were added, only that type whose requirement was satisfied developed. Counting the colonies that developed on differentially supplemented media, or recording the number of colonies of either colour that developed on complete medium, made it possible to estimate the ratio between the frequencies of the two parental types in the conidia of the heterokaryon. Some of these ratios are recorded in tab. 5.

TAB. 5. — Recovery of the component types from some balanced heterokaryons in *P. chrysogenum*.

Balanced heterokaryons		*Media on which plated. MM supplemented by*		*Number of colonies of the component types obtained*		*Percentage of the c mponent types*	
a	*b*	1	2	a	b	a	b
51 thi pr +	63 ts sp y	pr thi	me	29	131	*18*	*82*
86 y thi pr +	117 cy	pr thi	me	7	43	*14*	*86*
26 le +	pr	le	pr	164	200	*45*	*55*
63 ts sp y +	121 sp me w	cy	me **	22	180 **	*10*	*90*
65 pr y sp +	121 sp me w	pr	me	46	366	*11*	*89*

* The substances, indicated in table 3, were added in the optimal concentrations as reported in table 2.

** Both strains grow on MM supplemented by methionine. The number of b colonies was calculated as the difference of the colony numbers on (1) and (2) (202-22 = 180)

TESTS FOR HETEROKARYOSIS.

A heterokaryotic strain is one which has in its continuous cytoplasm two or more genetically different types of nuclei intermingled.

Proof of heterokaryosis is therefore reached by mechanically isolating a single hypha of the supposed heterokaryotic colony and then recovering from the mycelium produced by it the two clones combined in the heterokaryon.

Such proof was obtained in some of the heterokaryons produced in *P. chrysogenum* by means of the following procedure: a portion of the margin of the colony which was to be tested was picked up with

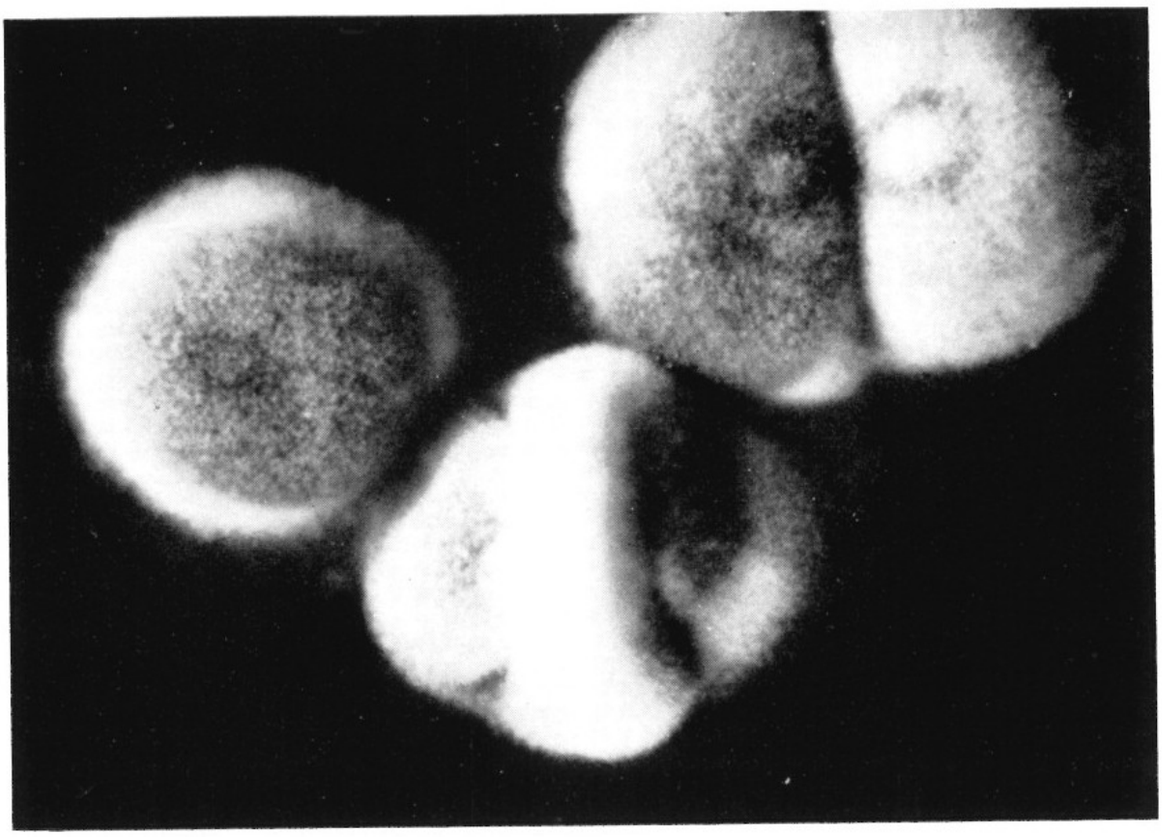

Fig. 1. - Colonies of strain 57 nic_1 hy y and 74 nic_2 py w growing on limiting medium, ten days old. A tuft of better growing mycelium (heterokaryotic) is evident between two colonies. The one on its left is white (74), the one on its right is yellow (57), the other three colonies are yellow ($\times$ 6,5).

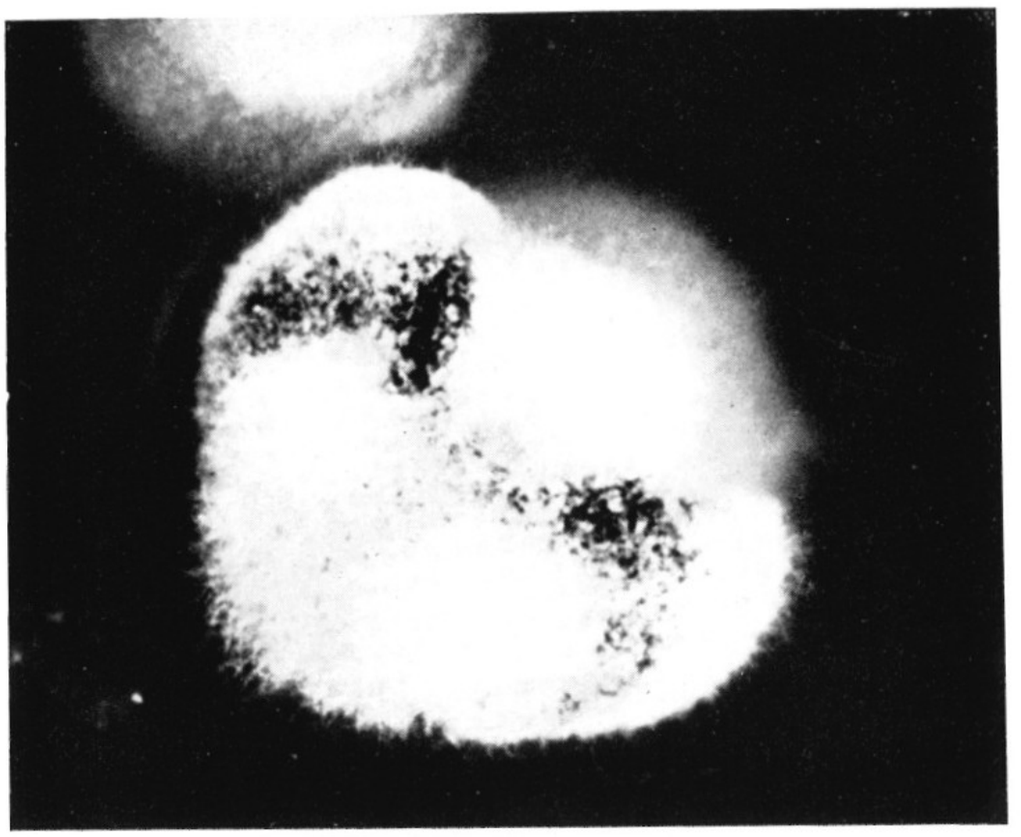

Fig. 2. - Tufts of heavily sporing mycelium arising in the zone of contact between colonies of strain 22 le (left) and 29 pr (right), growing on limiting medium. Ten days old (× 10).

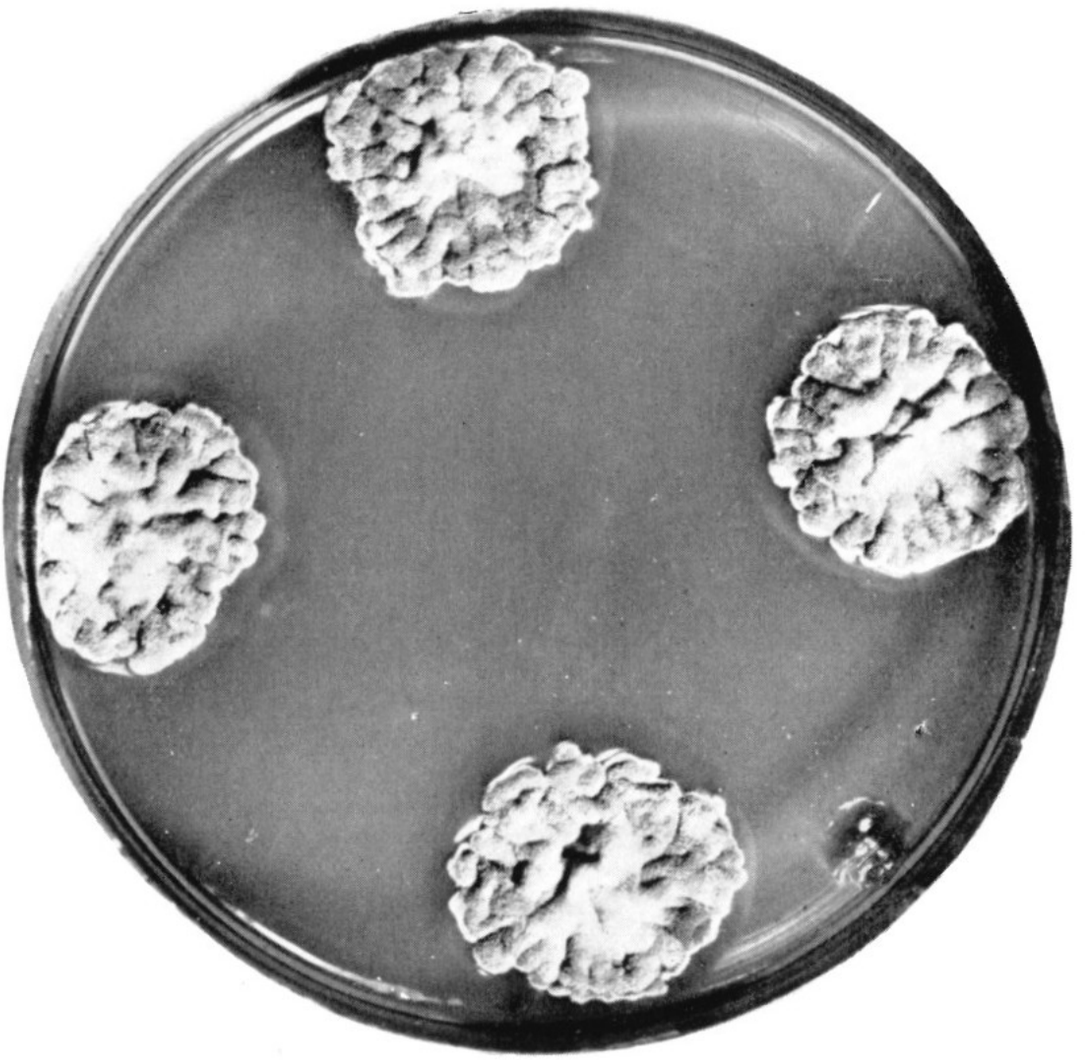

Fig. 3. - Colonies of heterokaryon 26 le + 29 pr isolated in MM (illuminated from above).

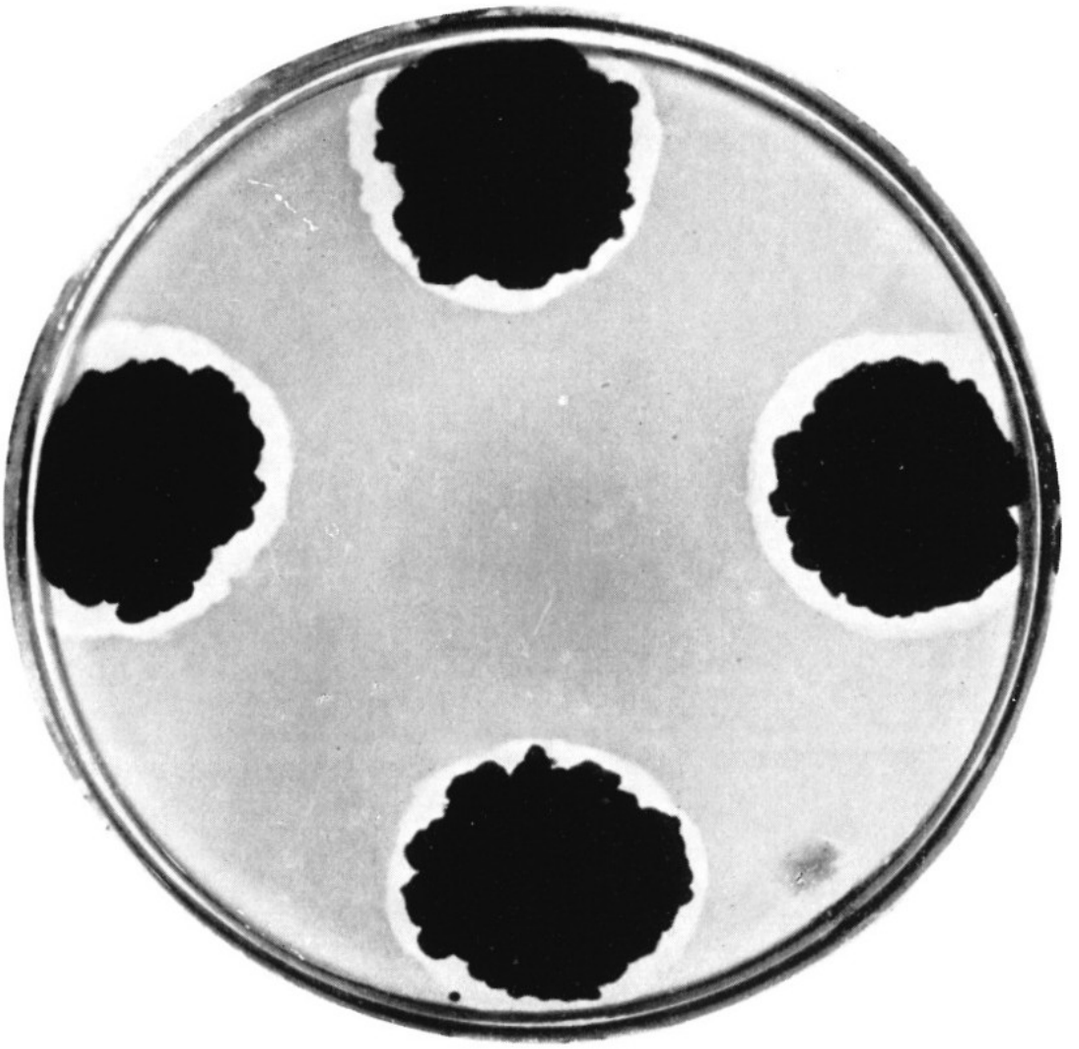

Fig. 4. - Colonies of heterokaryon 26 le + 29 pr isolated in MM (illuminated from below). Notice the wide margin of mycelium growing inside the agar.

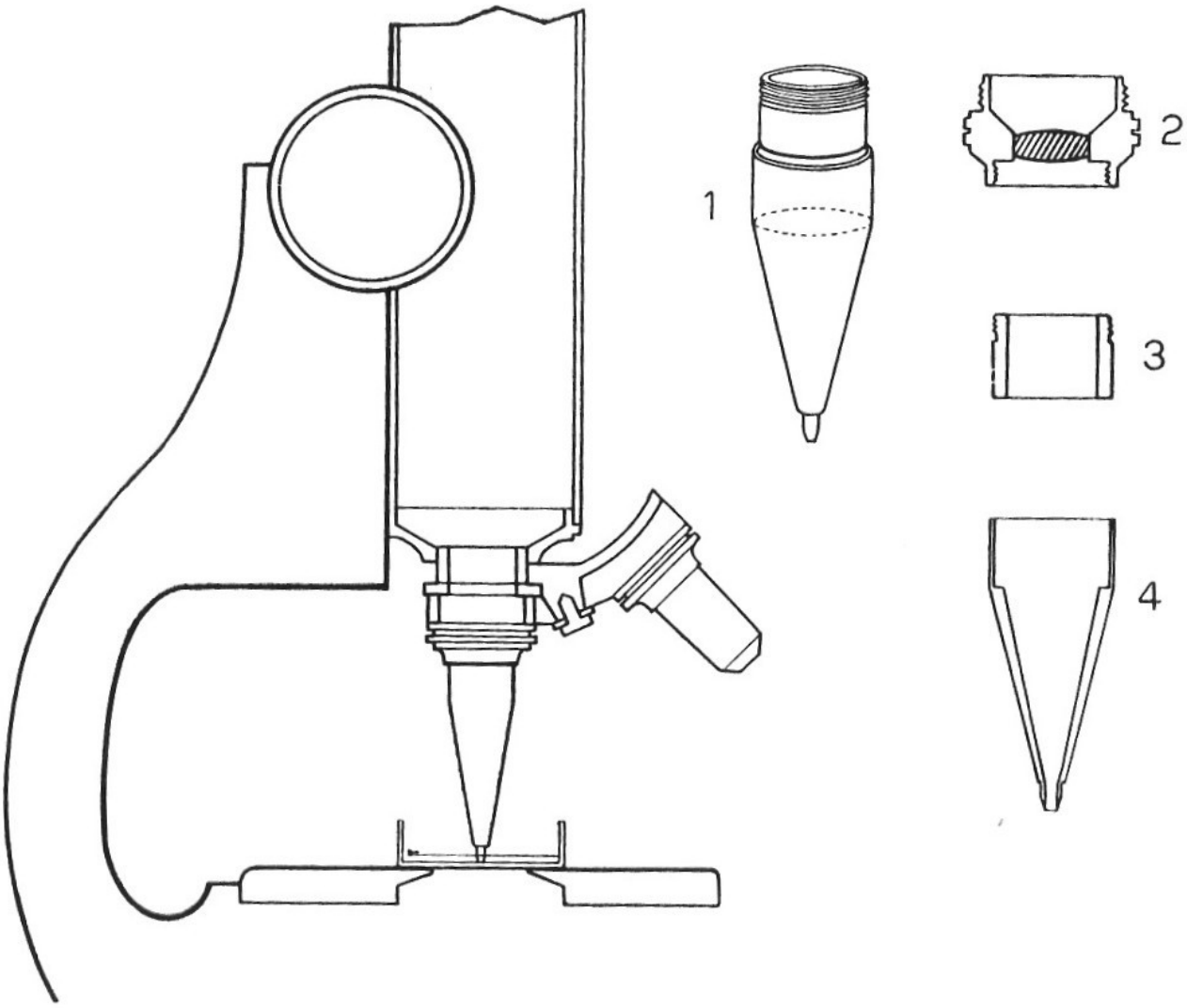

Fig. 5. - Stainless steel circular cutter for isolation of single hyphae applied to an objective of the microscope instead of the frontal lens. (1) view of the complete cutter removed from the objective; (2) section of the objective, from which the frontal lens and its support were unscrewed; (3) sleeve connecting the objective with the cutter proper; (4) conical cutter, the end of which is a small cylinder with a sharp edge cutting into the agar. The conical cutter can be rapidly exchanged during work.

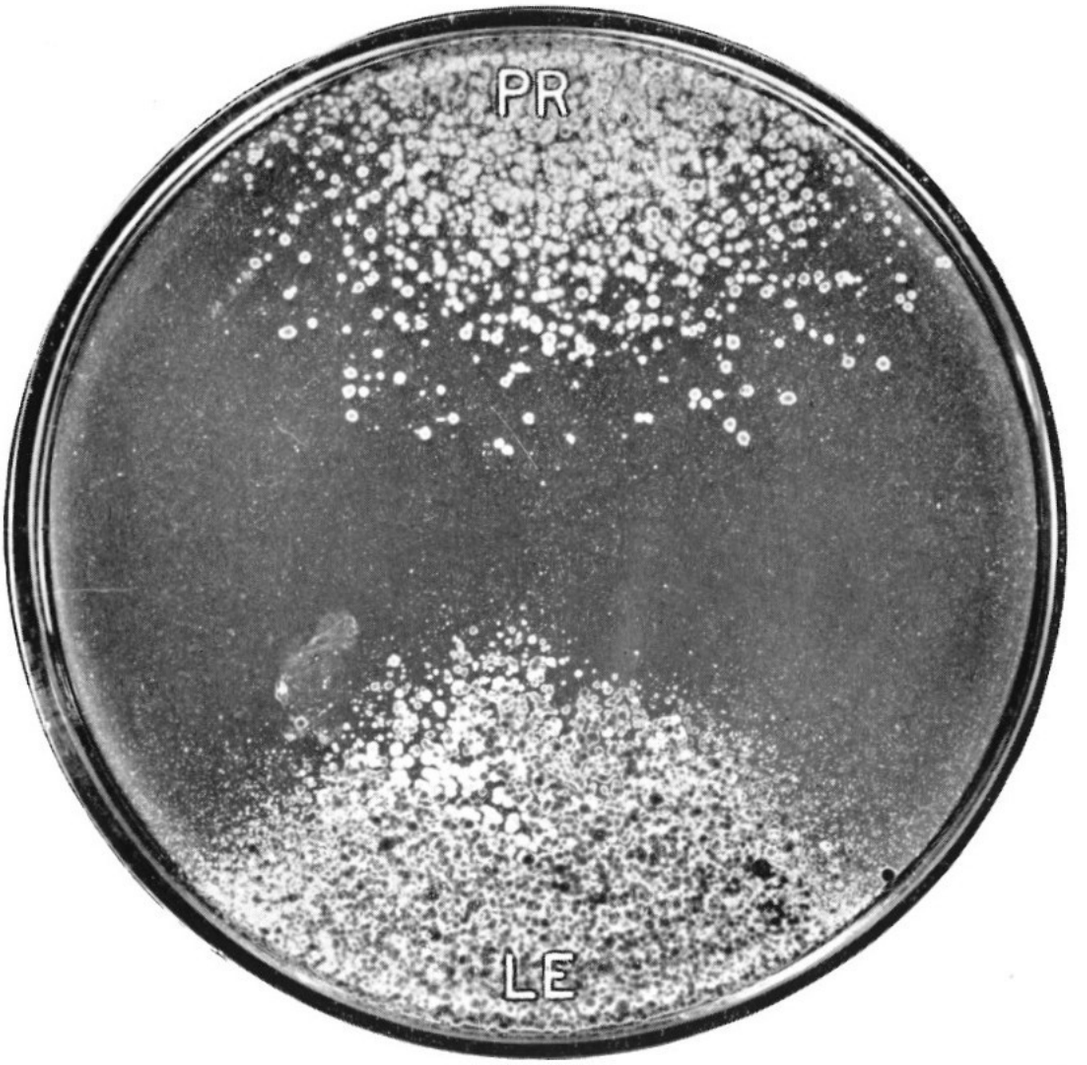

Fig. 6. - Auxanographic test of heterokaryosis. Conidia from heterokaryon 26 le + 29 pr are embedded in agar MM. Some crystals of leucine (below) and of proline (above) are stabbed into the agar at opposite sides of the dish. Growth of the segregated component strains occurs in the areas where the substances diffuse.

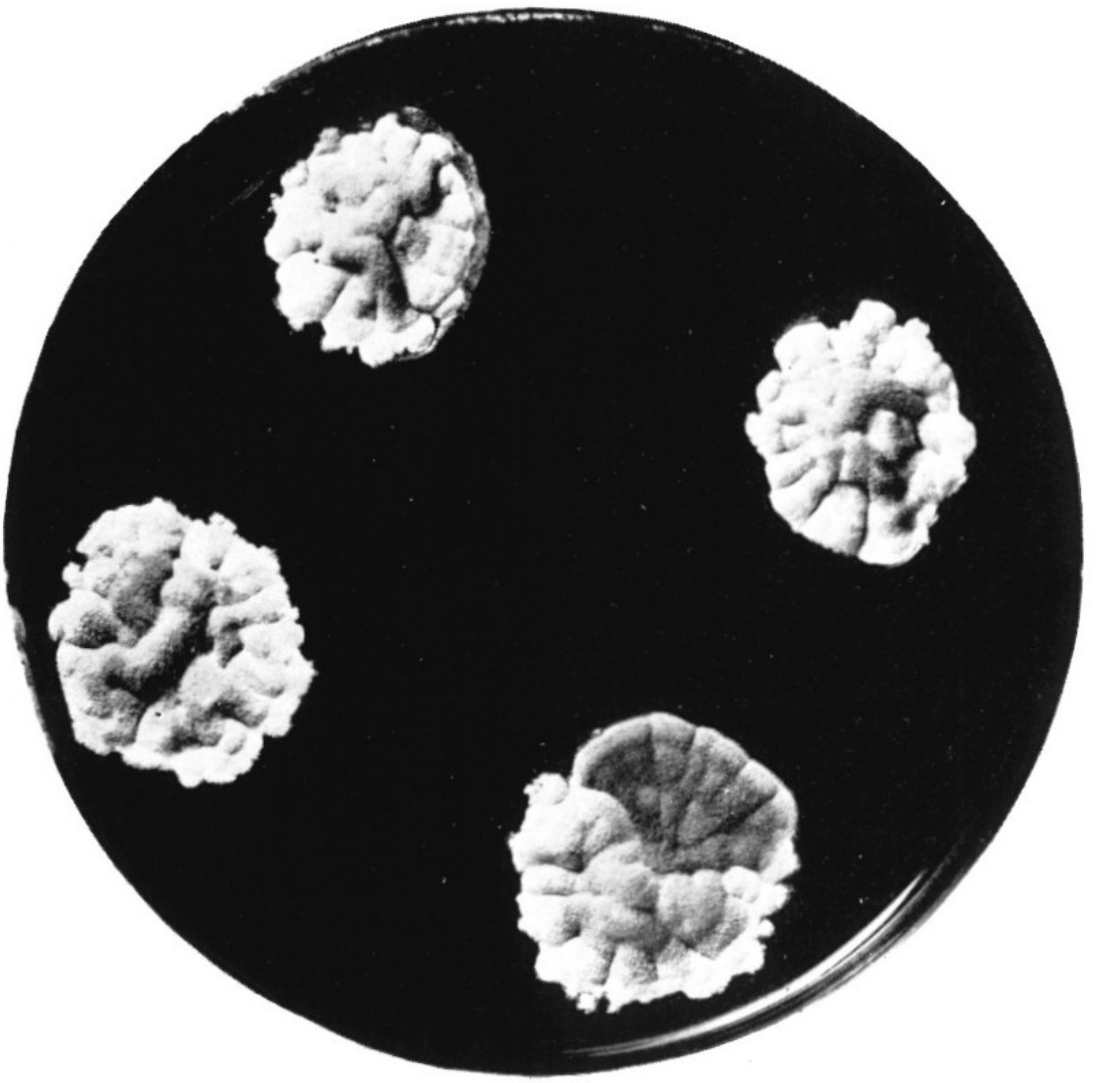

Fig. 7. - Colonies of heterokaryon 65 pr $y_3 sp_2$ + 124 hy y_2 sp_1 isolated on MM. From one of them a heterozygous diploid, with green spores and regular growth, sectored out.

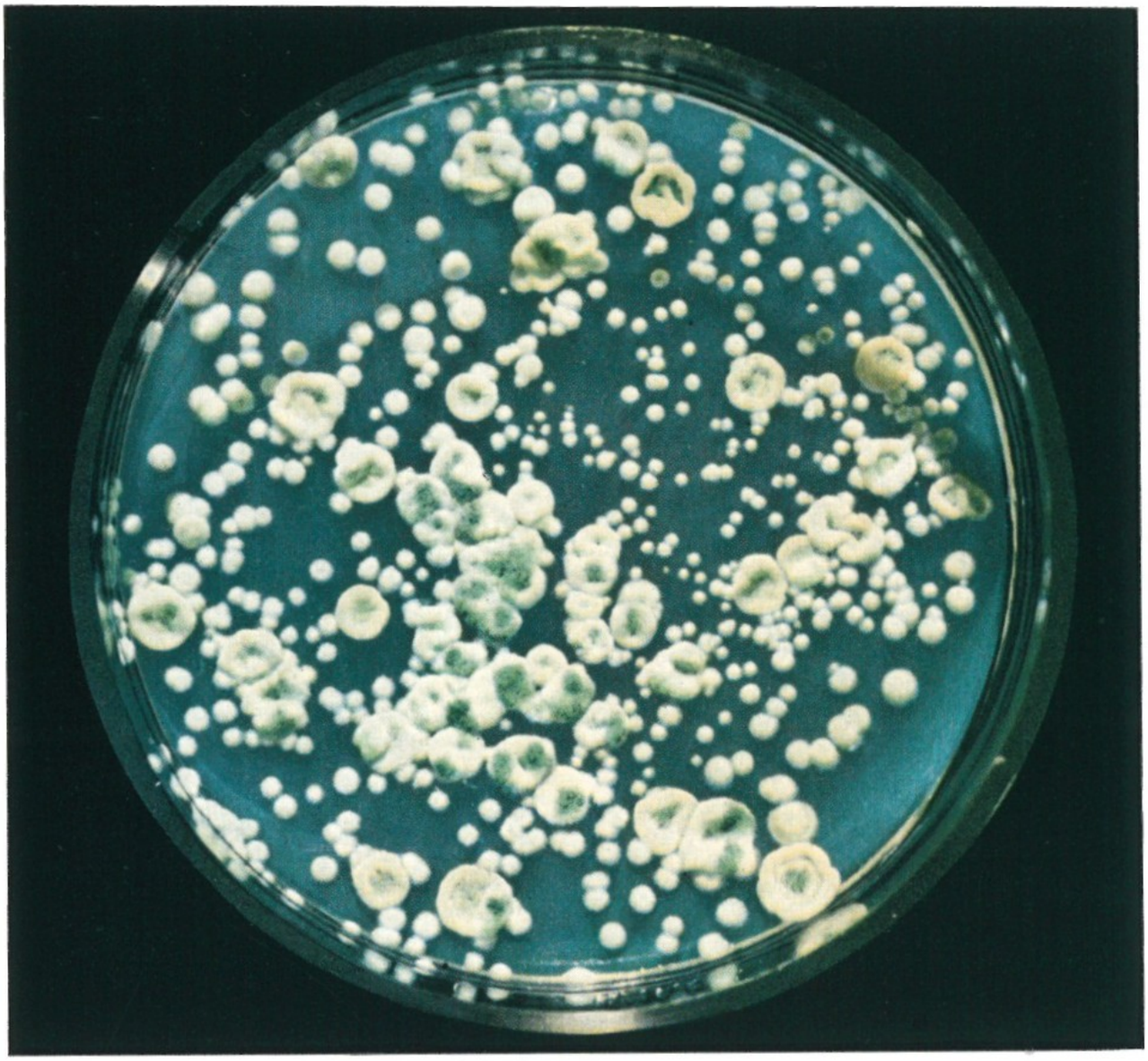

Fig. 8. - Green sporing tufts of heterokaryotic mycelium arising from a mixed culture on limiting medium of strains 26 le (small whitish colonies) and 29 pr (yellowish ring-shaped colonies).

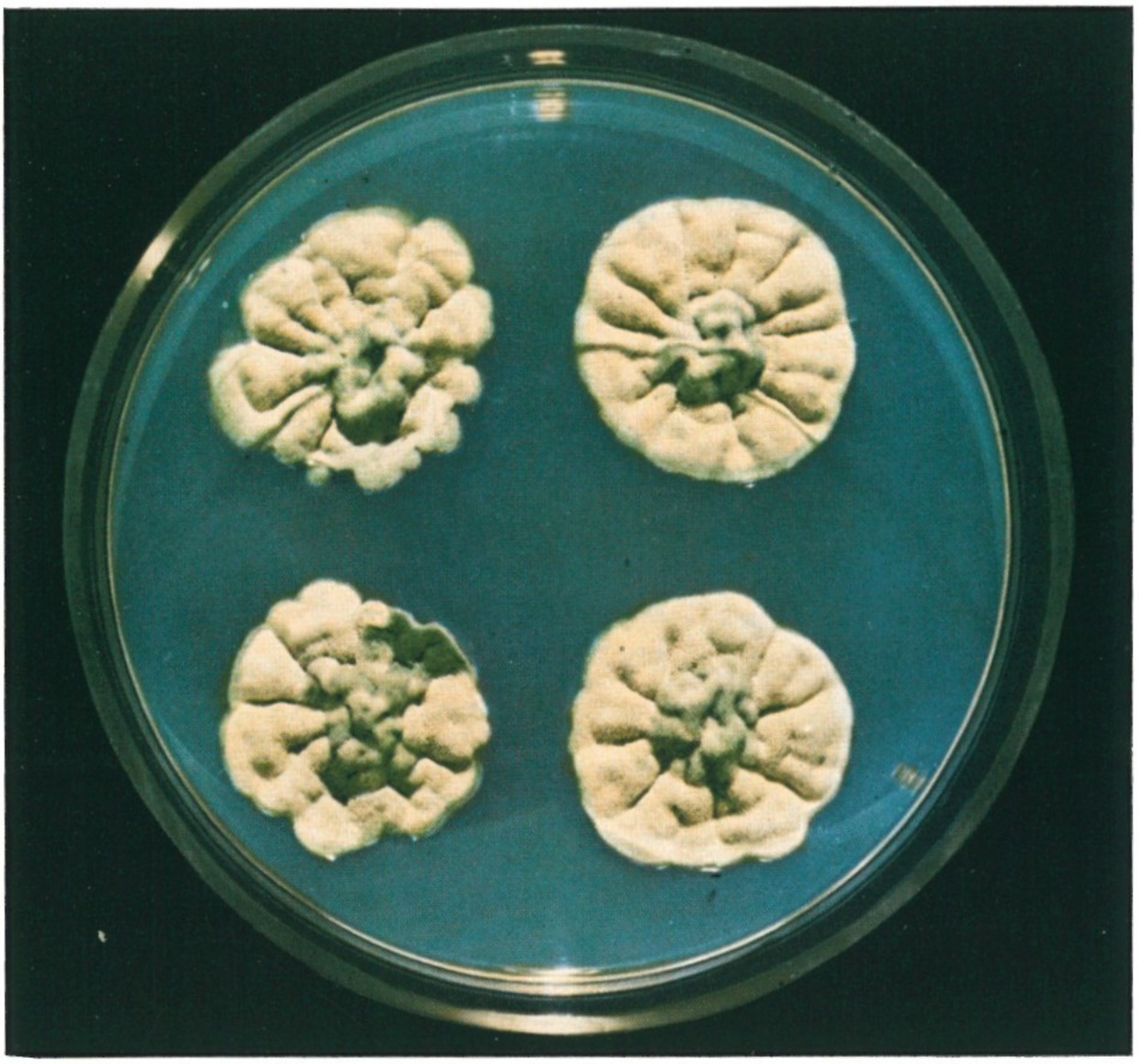

Fig. 9. - Colonies of heterokaryon 63 ts sp y + 51 thi pr, isolated on MM. From one of them a dark green sporing heterozygous diploid is sectoring out.

a needle together with the agar in which it was embedded, taking care to avoid contact between the needle and the conidia of the colony. The patch of mycelium was transferred to the bottom of a sterile test-tube in a few drops of water, and thoroughly broken up with a small glass rod. With a thin Pasteur pipette the suspension was then repeatedly sucked up and forced down to the bottom of the test-tube, in order to separate the hyphae as much as possible.

Meanwhile some Petri dishes were prepared, with as flat a base as possible, on which a very thin layer of agar MM (about 2 mm thick) was poured.

A drop of the hyphal suspension was placed on a slide, put under the microscope and explored in order to evaluate the degree of separation of the hyphae, and their number. If the hyphae were well separated and there were about a hundred of them in one drop, a drop of the suspension was placed in each of the dishes of MM prepared as specified above.

The drop placed on the agar was spread with a glass rod over the entire surface. It was found advisable to carry out the same plating in some dishes of CM, which acted as a control of the number of living hyphal fragments. The dishes were placed at 24° C and kept there until the following day.

Isolation of the single hyphae previously separated was carried out with the micro-manipulator shown and described in fig. 5. The hollow cone was sterilized in a flame and allowed to cool in a sterile Petri dish. The operation was carried out with the lid removed, there being a negligible risk of contamination from the air, in view of the minute fraction of agar surface which was to be removed. The open dish was placed on the microscope table and the microscope, with the cone inserted in the objective, was focussed on the surface of the agar. The field was then explored with a magnification of approx. 40 times. Some of the hyphae appeared as shadows, others sparkled on the surface due to refraction; among the latter, those with the most extensive branching were selected, but only if they had clearly marked continuity of the central trunk and its branches. The microscope tube was then lowered by moving the macrometric screws, until the cutting end of the cone reached the bottom of the dish. If the agar was too thick, it was necessary to avoid that the end of the cone sank too deeply into the agar, causing the patch of agar to occlude the aperture of the cone. The patch cut out by the action of the micro-manipulator remained in place after lifting the tube of the microscope, and the isolated hypha

could be observed at its centre. The operation was repeated on other hyphae.

The patches of agar thus obtained were then transferred with a lancet on to dishes of agar MM of normal thickness. Here some of the transferred hyphae gave rise during the next few days to development of colonies. When the new colonies sporulated the conidia could be washed away and plated for recovery of the original clones.

In all cases in which isolation of single hyphae from heterokaryotic colonies was attempted, frequency of positive results was low (tab. 6).

Tab. 6. — Isolation of single hyphae and recovery of component strains from balanced heterokaryons of *P. chrysogenum*.

Balanced heterokaryons	*Age of the HK in days*	*Number of isolated hyphae*	*New HK formed*	*Recovery of component types **
51 thi pr + 63 ts spy	9	15	2	+
117 cy + 86 y thi pr	8	14	0	—
63 ts sp y + 46 me w	7	12	3	+
51 thi pr + 55 me hy y	7	22	1	+

* from the individual colonies derived after isolation of single hyphae. A positive result is marked +

The recovery of colonies of the two parental types from the mycelium derived from the isolation of single hyphae constitutes a critical test of heterokaryosis. In routine work, satisfactory evidence of the heterokaryosis could also be obtained by recovery of the parental types from heterokaryotic colonies not arising from single hyphae.

In such cases heterokaryosis could not be distinguished critically from syntrophism, a condition in which the hyphae of two complementary auxotrophs, mixed together, grow on MM without combining to form heterokaryons. Syntrophic growth on agar in *P. chrysogenum* gave rise to minute colonies, which had no margin growing inside the agar, and could not be confused with heterokaryotic colonies.

The recovery of the parental strains from the conidia of a heterokaryon was carried out in two ways: — (a) By plating of a certain number of conidia on different media which permitted differentiation of the two types of parental colonies: in this case it was possible to calculate the relative frequency of the two types (see tab. 5). (b) With

an auxanographic test of conidia of the heterokaryon. A dense suspension of conidia washed away from the heterokaryon was included in agar MM in a Petri dish. The substance or substances required by one parent were added in the form of a few crystals at a point in the agar near the edge of the dish. At the opposite extremity of the same diameter the substance required by the other parent was added. If a halo of growth formed after a few days, surrounding the two substances added, the presence of the two types of conidia was considered proved (fig. 6). (The test was obviously not significant if one of the two added substances satisfied the requirement of both the original strains).

Since in *P. chrysogenum* the hyphal cells contain several nuclei, while the conidia are uninucleated (Tonolo & Urbani, 1952), heterokaryotic growth would be possible from hyphae, but not from individual conidia. In species with bi- or multinucleate conidia heterokaryotic growth occurs also from individual conidia (Prout, Huebschman, Levene & Ryan, 1953). Therefore, proof of heterokaryosis could be obtained in *P. chrysogenum* when heterokaryotic growth occurs from hyphae but not from conidia of the colony being tested. When the heterokaryon is synthesized between auxotrophic strains, no growth whatever on MM could occur from conidia. Indeed, the experimentally established absence of growth on MM from conidia of such a heterokaryon confirms the cytological observations of Tonolo & Urbani (1952).

A rapid screening test for distinguishing a heterokaryon from a back-mutant or a diploid consisted in gently passing a loop containing a drop of sterile water over the surface of the colony being tested, in order to pick out only the conidia, then streaking the loop across agar MM: under these conditions only the heterokaryon did not reproduce.

To distinguish the heterokaryon from one of the parent strains (in limiting media) it was enough to transfer a patch of agar from the edge of the colony in question on to MM. Only the heterokaryon reproduced in this state. The latter tests are based on the assumption, referred to above, that heterokaryotic growth in *P. chrysogenum* is possible from the hyphae (polynucleated) but not from individual conidia (mononucleated).

A final proof of heterokaryosis of a kind different from those already described, since it does not presuppose cytological observations or knowledge, is that based on the capacity of heterokaryons to lead on to the formation of heterozygous diploids.

A heterozygous diploid is a strain which phenotypically resembles

the common ancestor of the parents of the heterokaryon, and which reproduces by single conidia. It contains in its single nuclei the dominant and recessive genes of the two parents of the heterokaryon from which the strain derives (Pontecorvo & Sermonti, 1954; Sermonti, 1954).

Diploids occasionally appeared after millions of conidia of a heterokaryon between complementary auxotrophic strains had been plated on MM (see tab. 7). As has been stated, the conidia of such a heterokaryon

Tab. 7. — Heterozygous diploids obtained from some balanced heterokaryons in *P. chrysogenum*

a) Detection of heterozygous diploids from conidia of balanced heterokaryons plated on MM

Balanced heterokaryons	*Plated conidia total number*	*Heterozygous diploids obtained*	*Ratio plated conidia : obtained diploids*
55 me hy y + 66 ad cy w	9.750.000	139	70.144 : 1
26 le + 29 pr	10.240.000	5	2.048.000 : 1
63 ts sp y + 65 pr y sp	21.000.000	ca 1.400	15.000 : 1
51 thi pr + 63 ts sp y	6.000.000	6	1.000.000 : 1
86 y thi pr + 117 cy	2.740.000	ca 800	3.400 : 1

b) Balanced heterokaryons on which sectors of heterozygous diploids have appeared.

63 ts sp y	+ 65 pr y sp
46 me w	+ 63 ts sp y
51 thi pr	+ 63 ts sp y

do not give growth on MM. Heterozygous diploids, when exceptionally formed, develop from single conidia, giving on MM rare colonies with the characteristics of the common ancestor of the parental strains.

A heterokaryon between colour mutant strains has a sporing surface whose colour is intermediate between the parent conidial colours. A heterozygous diploid between the same strains has, however, a sporing surface like that of the common ancestor of the component strains. If, for instance, a parent is yellow spored and the other is white, and both derive from a green strain, the heterokaryon they form is yellow-

ish, the corresponding heterozygous diploid is green. In similar conditions a heterozygous diploid may be detected as a green sector, occasionally emerging from the pale heterokaryon (figs. 7, 9).

The formation of heterozygous diploids involves karyogamy between different nuclei, present in the same cytoplasm, and therefore heterokaryosis.

Conclusions.

By means of the technique described in this paper, synthesis of balanced heterokaryons between auxotrophic strains in *P. chrysogenum* becomes (except for the somewhat tedious preliminaries for establishing the limiting concentration of the requirements) a very easy process.

The technique is suitable also for establishing heterokaryons between two strains both differing from the wild type in a sole nutritional requirement. This makes the preparation of the strains to be associated in a heterokaryon as rapid as in *Neurospora* and *Aspergillus*.

After a little practice with the method heterokaryons may be isolated with the greatest ease. In mixed cultures on limiting media, heterokaryons were successfully isolated from the zone of contact between colonies of different type, also in places where the tufts of better growing mycelium were hardly detectable.

Beside the ability of a strain to segregate in two component types, its ability to give heterozygous diploids is additional evidence for heterokaryosis. The detection of heterozygous disploids is, in fact, a very simple way for ascertaining the heterokaryotic state of a colony. It is therefore a particularly convenient test in genetic studies in *P. chrysogenum*, for which the synthesis of a balanced heterokaryon, as the only possible experimental way to diploidism, represents a necessary preliminary.

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REFERENCES

Bonner D. (1946). Amer. J. Bot., 33: 788.
Clutterbuck P. W., Lovell R. and Raistrick H. (1932). Biochem. J. 26: 1907.
Fries N. (1948). Physiol. Plantarum, 1: 330.
Pontecorvo G. (1947). Cold Spr. Harb. Symp. Quant. Biol., 11: 193.
Pontecorvo G. (1949). J. gen. Microbiol., 3: 122.
Pontecorvo G. (1953. Advances in Genetics, 5: 141.
Pontecorvo G. and Sermonti G. (1953). Nature, 172: 126.
Pontecorvo G. and Sermonti G. (1954). J. gen. Microbiol., in press.
Prout T., Huebschman C., Levene H. and Ryan F. J. (1953). Genetics, 38: 518.
Roper J. A. (1952). Experientia, 8: 14.
Ryan F. J. (1950). Meth. Med. Res., 3: 51.
Sermonti G. (1954). Rend. Ist. Sup. Sanità, *17*, English Edition, pag. 231.
Tonolo A. and Urbani E. (1952). Bull. World Hlth Org., 6: 177.