DETAILED ANALYSIS OF A NEUROSPORA CROSS

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ACCORDING to a widely accepted view of the mechanism of ascospore formation in *Neurospora crassa*, a single diploid nucleus in the young ascus undergoes meiosis followed by mitotic division of the four resulting nuclei. A spore is formed around each of the eight products, sister nuclei from the mitotic divisions inhabiting adjacent spores. A second mitosis gives binucleate spores. This mechanism leads to the following expectations: that the nuclei within a spore are haploid and genetically alike; that the members of a spore pair are genetically identical and, therefore, that not more than four phenotypes will be found in one ascus; that each ascus represents an independent event.

Occasional exceptions to the first expectation, that is, single-ascospore cultures which were obviously heterocaryotic (PITTENGER 1954) have been thought to result from irregular meioses and, hence, to occur in abnormal asci. There are indications, however, that certain types of heterocaryons, difficult to recognize, may not be noticed ordinarily and that the frequency of exceptions of this sort is unknown.

It is also difficult to estimate, from existing data, the frequency of spore pairs whose members are not alike phenotypically. In some analyses homogeneity has been taken for granted and only one member of a pair tested (segregation of mating type in 1814 asci, HOULAHAN, BEADLE and CALHOUN 1949) or the spores have been isolated and cultured as pairs. Among completely germinated and completely scored asci about one in 20 shows irregularities in the order of phenotypes. Instead of the order 1,1,2,2,3,3,4,4, one may find, for example, 1,2,2,1 or 1,2,1,2, and so forth. Less frequently five to three segregations have been recorded. Among 1878 asci (HOULAHAN, BEADLE and CALHOUN 1949), of which 814 germinated completely and were completely scored, 11 were recorded as five to three segregations. These irregularities have been attributed to errors in technique, although, in some of the latter cases, this seems a bit unlikely.

A recent observation appears to be contrary to the expectation that the formation of each ascus represents an independent event. By examination of immature perithecia, asci were frequently seen to be formed in pairs which are closely associated and appear to be at exactly the same stage of development. The striking similarity of these immature twin asci suggests the possibility that they may resemble one another in genetic constitution.

The work reported here constitutes an attempt to detect twin phenotypic patterns and also to estimate the frequency of heterocaryotic spores and heterogeneous spore pairs in a cross analyzed for that purpose. Hence, in choosing the

cross described the considerations were clarity and simplicity of classification of its progeny rather than suitability of the parents for enzymatic or biochemical studies. The analyses have been done with particular care so as to eliminate, in so far as possible, errors in technique which might serve to explain away irregularities.

Phenotypes

The cross to be considered is of two unlinked colonial mutants, C 102, *cot*, temperature-sensitive colonial and C 136, *sn*, snowflake (MITCHELL 1958). Snowflake shows close linkage with nutritional mutants assigned to positions near the centromere in linkage group I, whereas *cot* exhibits linkage to *pan-1* in group IV. Two descendants of each mutant from crosses to standard wilds were used. Stock cultures of these strains have been maintained on unsupplemented WESTERGAARD and MITCHELL (1947) medium.

The *sn* × *cot* cross was chosen for the present purpose partly because the segregants can be scored on minimal plates within 20 hours after germination. It is then possible to observe in detail the first hyphae which grow from the spore and to see clearly that each colony scored arises from a single ascospore. The minimal medium used is a modified Fries in which ammonium tartrate and ammonium nitrate are replaced by the corresponding potassium salts (STRAUSS 1951). After 15 hours incubation at 25°C two types, *sn* and *sn*+, can be scored. At this temperature *cot* is not distinguishable from *sn*+ *cot*+ nor *sn cot* from *sn*. When the temperature is shifted to 34°C, elongation of the hyphae of *cot* and *sn cot* stops abruptly and, during the next three hours, short branches form at rather regular intervals along the whole length of the hyphae. When the segregants are again incubated at 25°C for half an hour elongation of the hyphae is resumed and the short branches formed at 34°C elongate rapidly, so that the *cot* character becomes more conspicuous.

In addition to *sn*, *cot*, *sn cot* and *sn*+ *cot*+ (designated here as + since only the phenotypes are being considered) it was possible to score two "modified" forms of *cot*, designated as *dcot*, dilute, and *ddcot*, dilute dilute, and one variegated form, designated as *vcot*. Variegated *cot* was not frequent in this cross but it is quite interesting since the simplest interpretation of it is in terms of genetic heterogeneity of single spores. At the time of scoring, *cot* and + colonies show several hyphae, around five or six, as branches of the original short germ tubes. In *vcot* one or two of these hyphae expressed "typical" *cot* throughout their length while the remaining hyphae were typically +. The latter continued elongation at 34°C and branched infrequently in the manner characteristic of +. The *dcot* form appears homogeneous and, like typical *cot*, ceases elongation at 34°C but it differs in that the branches formed are shorter and less frequent. Hyphae of the remaining type, *ddcot* do not elongate at 34°C and form few branches which are very short and appear near the ends. The term, dilute, describes the appearance of the colonies but, in so far as the temperature effect is concerned, the dilute forms may be the more extreme ones. They show less growth, fewer and shorter branches, at 34°C.

A curious attribute of *sn* is the phenotypic shift in the direction of + which it regularly undergoes. The initial growth is colonial in character, that is, the hyphae show excessive branching, but this decreases as growth continues and, in a slant culture, the final growth is almost normal. Colonies from single ascospores or conidia on plates show sectoring but even the less extreme sectors are usually quite distinct from *sn*+ for several days.

At the time of scoring *sn* colonies are small and consist of a single layer or network of hyphae in which individual branches of the original germ tubes can often be followed. Like *cot*, *sn* was variable in expression but there were more *sn* classes, less distinct from one another; therefore no attempt was made to score them separately. The *sn cot* colonies do not enlarge at 34°C, but become more dense, as the *cot* branching is superimposed on the frequent branches already formed. A *sn cot* colony is distinguished from an extreme *sn* by the uniformity in length and spacing of the *cot* branches. It seems likely, however, that *sn vcot* would be scored as *sn* and that *sn ddcot* might also be so scored. However, in completely germinated asci, *sn* and *sn cot* spore pairs appeared quite homogeneous. It will be seen that in only one case was there observed a convincing difference which could not be accounted for in terms of mistakes in recording spore order.

Random spores

Crosses were made on slants of WESTERGAARD and MITCHELL medium by placing inocula from the two parents together on the slant and mixing them. After 17 days or more, samples of spontaneously released ascospores were suspended in about 0.5 ml of sterile water. The suspensions were spread on plates containing the modified Fries medium solidified with four percent agar. After heat treatment at 60°C for ½ hour the plates were incubated, first at 25°C for 15 hours, then at 34°C for three hours and again at 25°C for ½ hour. Incubation at 34°C was sometimes omitted for *sn* × *sn* crosses.

The cross of the two *sn* isolates used here gave mainly *sn* progeny. About 15 to 20 percent of the normal-appearing spores failed to germinate and one or two percent formed colonies which grew too little to be scored after 20 hours. No typical + colonies were observed but it should be mentioned that crosses of these isolates to *sn* segregants from certain other crosses have been observed to give typical + progeny and also slow growing *sn*+ segregants.

Typical + progeny have not been found from *cot* × *cot*, but about one percent behaved as auxotrophs in that they formed short germ tubes which did not continue to grow. About 0.5 percent of the progeny showed a colonial character at 25°C and 2.3 percent among 1350 germinated spores were classified as *dcot* or *ddcot*. Rather large scale tests have been performed incidental to the use of *cot* × *cot* crosses to introduce colonial growth at 31°C in material examined for new mutants. Typical + offspring were not found even though one parent was treated with a mutagen, usually ultraviolet light. For this reason *cot* is regarded as an unusually stable mutant.

The cross *sn A* × *cot a* was somewhat more fertile than either of the "selfs,"

and germination of spontaneously released black spores was usually above 98 percent. Spores were plated repeatedly in order to work out the best conditions for classification. A series of four plates, incubated in the manner described above, gave the following frequencies among 2553 spores: *sn*, 25.1; *sn cot*, 24.4; *cot*, 20.8; +, 29.6, all in percent. The four forms of *cot* were counted together. All plates contained a few segregants which had grown so little that they could not be classified.

The cross *sn a* × *cot A* gave about the same fertility and germination, but scoring was more difficult. The variability of *sn* was greater and "small," unclassifiable individuals more numerous. The following frequencies were obtained from two plates containing 1515 spores; *sn*, 24.3; *sn cot*, 27.3; *cot*, 21.3; +, 27.1 percent.

Segregation in asci

Asci were dissected on the minimum plates; the spores were placed at suitable distances apart in rows and all unused material was removed. In order to ensure that whole asci were being examined two checks were made. A group of asci still attached to the cluster was chosen and watched while a small drop of water was placed on the cluster. The asci float in water and an intact ascus floats as a unit. The water was allowed to evaporate and the ascus to be dissected was stretched with a glass needle placed between the first and second spores. All the spores of an intact ascus move in response to this pull and then move back in the direction of their former positions when the ascus is released.

The two more frequent types of asci from *sn A* × *cot a* after about 17 days incubation were those with eight black spores or with eight colorless spores. Asci in which no spores had formed were also numerous. One hundred and fifty-five asci containing black spores were dissected. Most of these had eight spores but a few had only six or seven. Only the 109 eight-spored asci from which all spores germinated will be considered here, except to say that the more frequent reason for failure to germinate may have been immaturity. This is suggested by the observation that samples of spontaneously released black spores regularly gave almost perfect germination.

Tabulations of the asci, in Tables 1 and 2, show the order in which the spores were isolated as well as the phenotypes of the segregants. The asci in Table 1 contained only the four phenotypes, *sn*, *sn cot*, *cot* and + and were regular except for the last four which show irregularities only in the order of phenotypes. Table 2 shows all other asci with heterogeneous spore pairs and also six with homogeneous pairs but which contained one of the "atypical" forms of *cot*.

In the first eight irregular asci the heterogeneous spore pair consists of + and one of the three *cot* types, *cot*, *vcot* or *dcot*. These + segregants were indistinguishable from + individuals in regular asci. The heterogeneous pair in the next 11 asci consists of *cot* with one of its "modified" forms. It is considered unlikely that the heterogeneity here is due to chance environmental or physiological differences not reflecting differences in genetic constitution of the hyphae. Envi-

TABLE 1

Regular segregations from sn A × cot a

1*	2	3	4	5	6	7	8	Number of asci
sn	*sn*	*sn*	*sn*	*cot*	*cot*	*cot*	*cot*	9
cot	*cot*	*cot*	*cot*	*sn*	*sn*	*sn*	*sn*	7
sn cot	*sn cot*	*sn cot*	*sn cot*	+	+	+	+	9
+	+	+	+	*sn cot*	*sn cot*	*sn cot*	*sn cot*	16
sn cot	*sn cot*	*sn*	*sn*	*cot*	*cot*	+	+	4
+	+	*cot*	*cot*	*sn*	*sn*	*sn cot*	*sn cot*	7
sn cot	*sn cot*	*sn*	*sn*	+	+	*cot*	*cot*	6
+	+	*cot*	*cot*	*sn cot*	*sn cot*	*sn*	*sn*	3
sn	*sn*	*sn cot*	*sn cot*	*cot*	*cot*	+	+	5
cot	*cot*	+	+	*sn*	*sn*	*sn cot*	*sn cot*	6
sn	*sn*	*sn cot*	*sn cot*	+	+	*cot*	*cot*	2
cot	*cot*	+	+	*sn cot*	*sn cot*	*sn*	*sn*	6
+	+	+	*sn cot*	+	*sn cot*	*sn cot*	*sn cot*	1
sn	*sn cot*	*sn*	*sn cot*	+	+	*cot*	*cot*	1
sn cot	*sn*	*sn cot*	*sn*	+	+	*cot*	*cot*	1
cot	+	*cot*	+	*sn*	*sn*	*sn cot*	*sn cot*	1
								—
								84

* Each line shows the order of spores from 1 to 8 in an ascus sac.

ronmental effects seem an unlikely explanation because, in each case, the two segregants were growing a few mm apart on the same plate. Chance physiological differences would not account for the pattern that is followed among the *sn*+ segregants. It will be seen that no ascus contains more than two of the *cot* forms and, when there are two, one of them is always "typical" *cot*. No effort was made at the time of scoring to fit the asci into this pattern. It was not noticed until after the analysis was completed.

It seems possible, however, that, in some cases at least, the members of the heterogeneous spore pairs were originally alike genetically but both were heterocaryotic. Chance factors affecting multiplication of the components of the heterocaryon might then account for the difference in expression in the two segregants. This seems particularly applicable to those pairs having *vcot* as one member, since this type appears as a heterogeneous colony.

Twin asci

The twin asci were seen in preparations of immature asci fixed in ethanol-acetic acid and stained lightly with aceto-carmine or aceto-lacmoid. Examinations were made with phase contrast which makes it possible to see structural details not visible in ordinary light. The twins are attached to the ascus cluster

TABLE 2

Irregular segregations from sn A × cot a

1*	2	3	4	5	6	7	8	Number of asci
sn cot	*sn cot*	*sn*	*sn*	+	+	+	*cot*	1
sn	*sn*	*sn cot*	*sn cot*	*cot*	+	+	+	1
sn cot	*sn cot*	*sn*	*sn*	+	*cot*	+	+	1
sn	*sn*	*sn cot*	*sn cot*	+	+	*vcot*†	+	1
+	+	+	*vcot*	*sn*	*sn*	*sn cot*	*sn cot*	1
sn	*sn*	*sn*	*sn*	*dcot*‡	*dcot*	*dcot*	+	1
sn	*sn*	*sn cot*	*sn cot*	*dcot*	+	+	+	1
sn cot	*sn cot*	*sn*	*sn*	+	+	*dcot*	+	1
sn	*sn*	*sn cot*	*cot*	*sn cot*	*vcot*	+	+	1
sn	*sn*	*sn*	*sn*	*cot*	*dcot*	*cot*	*cot*	1
cot	*cot*	*cot*	*dcot*	*sn*	*sn*	*sn*	*sn*	1
dcot	*dcot*	*cot*	*dcot*	*sn*	*sn*	*sn*	*sn*	1
+	+	+	+	*sn dcot*	*sn cot*	*sn cot*	*sn cot*	1
sn	*sn*	*sn cot*	*sn cot*	+	+	*dcot*	*cot*	2
+	+	*cot*	*dcot*	*sn*	*sn*	*sn cot*	*sn cot*	1
cot	*dcot*	+	+	*sn*	*sn*	*sn cot*	*sn cot*	1
cot	*cot*	*ddcot*	*cot*	*sn*	*sn*	*sn*	*sn*	1
sn	*sn*	*sn cot*	*sn cot*	*cot*	*ddcot*	+	+	1
sn	*sn*	*sn*	*sn*	*dcot*	*dcot*	*cot*	*cot*	2
cot	*cot*	*dcot*	*dcot*	*sn*	*sn*	*sn*	*sn*	1
sn	*sn*	*sn cot*	*sn cot*	+	+	*dcot*	*dcot*	1
dcot	*dcot*	+	+	*sn*	*sn*	*sn cot*	*sn cot*	1
sn cot	*sn cot*	*sn*	*sn*	*ddcot*	*ddcot*	+	+	1
								—
								25

* Each line shows the order of spores from 1 to 8 in an ascus sac.
† *vcot* = variegated *cot*.
‡ *dcot* and *ddcot* = modified forms of *cot*.

at adjacent points and appear almost identical in such details as size, shape, degenerate areas in the cytoplasm, the appearance of the apical pore, the number and appearance of nuclei or the number, shape, size and degree of maturity or degeneration of spores (Figure 1). Some details regarding association of the twins during development will be omitted here. It appears that the actual process of ascus formation is an intricate one and it is hoped that after further study this process can be described more completely than would be possible now.

In order to be sure that adjacent mature asci are twins it would be necessary to see details which are not visible with the magnification used for dissection. With the equipment available, all that could be done was to dissect adjacent asci whenever possible and to record their positions. One pattern of twin phenotypes which is suggested by the associations thus observed is set forth in Table 1, the

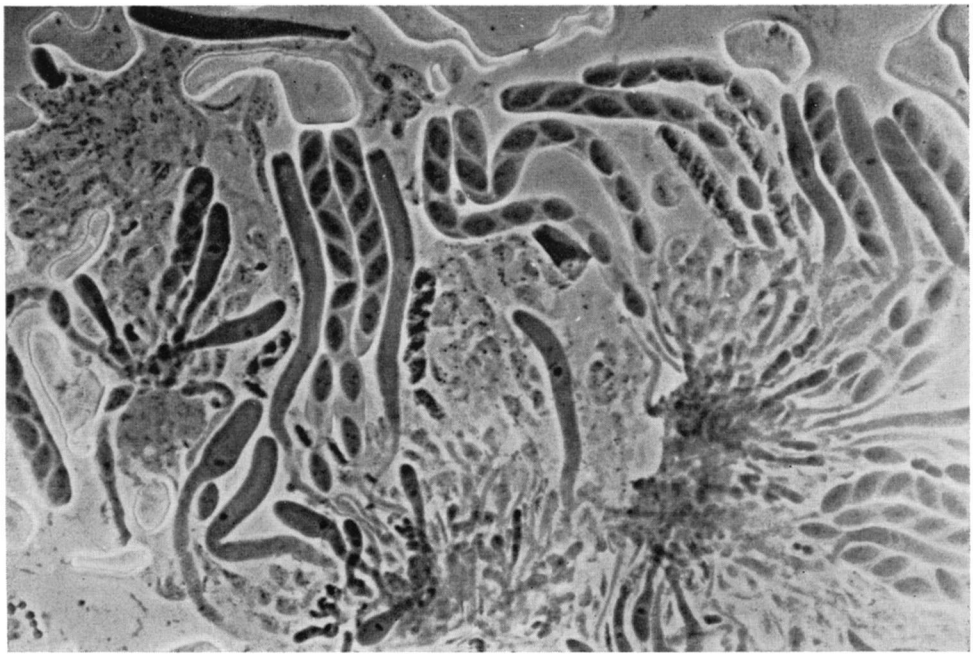

FIGURE 1.—Immature or degenerate twin asci from a typical cross.

first and second ascus types (the two parental-ditype arrangements) being twins, the third and fourth, and so on. This pattern seems more likely than one which makes the twins identical in both arrangement and phenotype. It is an interesting possibility, partly because it would, presumably, fit all crosses as well as would the identical pattern, and partly because of the suggested relationship between corresponding spore pairs in twin asci. There is, however, no assurance at present that the same pattern will be found in all crosses, nor is it certain that all asci are formed in pairs. Indeed, one recognizable type, seen more frequently in certain other crosses than in this one, appears not to have a twin.

One other point might be mentioned in favor of the suggested pattern, namely that it would, in part, account for odd coincidences sometimes found. Data from a cross of the linked mutants, *pan-1* and *pdx* (HOULAHAN, BEADLE and CALHOUN 1949) will serve as an illustration. Twenty-one among 49 asci were recombinant and, in terms of crossovers, 17 of these can be accounted for as singles between *pdx* and *pan*. Of the four requiring double crossovers, two were as follows:

pan	*pan*	*pdx*	*pdx*	+	+	*pdx pan*	*pdx pan*
−	*pdx*	*pan*	*pan*	−	*pdx pan*	+	+

Seven perithecia were analyzed and these two asci, the only representatives of this type among 49, were obtained from the same perithecium. Thus it is possible that they were twins.

DISCUSSION

In earlier analyses only 11 of 814 asci were recorded as five to three segregations whereas, in the present study 19 among 109 were found to contain heterogeneous spore pairs. One may well ask whether this difference is due to differences in the strains, in the methods, or in the attitude of the observer. It is the author's opinion that all three factors may have played a part. Certainly it appears that some phenotypes segregate more irregularly than others, at least in some crosses. This is indicated by the fact that all irregularities here (disregarding irregularities in order only) involve *cot*, while segregation of *sn* was recorded as regular in each ascus. As for methods, the ones used here and the properties of *cot* make it possible to detect slight differences in phenotype which would have been unnoticed or ignored in the earlier work. If, for example, *cot* were a nutritional mutant, scored by the earlier method of establishing single-spore cultures on complete and sub-culturing on minimal, such differences as those between *cot*, *dcot* and *ddcot* would have been unnoticed or attributed to environmental effects. Thus ten of the heterogeneous spore pairs would not have been recorded. The attitude of the observer, at the time of the earlier work, may well have been influenced by the conviction that the two members of a spore pair were genetically identical. During the present study a more neutral point of view has been entertained.

Because of recent interest in six to two segregations in Neurospora (MITCHELL 1956; CASE and GILES 1958) it may be worth while to emphasize the fact that no unambiguous six to two segregations were recorded here. It appears, however, as was suggested above, that the distinction between six to two and five to three segregation may be an artificial one. Two asci (the fourth and fifth in Table 2) would most likely have been scored as six to two segregations if *cot* were a nutritional mutant. The heterogeneous pair consists of + and *vcot* and, even by the method used here, such a variegated form of a nutritional mutant would have been scored as +. The mutant hyphal branches would merely have stopped growing very early and the presence of one or two short branches would have been disregarded since this is commonly seen in + colonies. Nor does it seem assured that subsequent tests would reveal the heterogeneous nature of such variegated segregants, even if the initial growth occurred on supplemented medium. It is well known that growth of many nutritional mutants is slower than that of +, even on supplemented medium. The mutant component might, therefore, have become so outnumbered by + as to make its recovery from an outcross unlikely.

Testing the segregants of irregular asci by outcrossing seems less to the point here than in the case of six to two segregation. The finding that a pair of cot^+ segregants, from a six cot^+ to two *cot* ascus, behaved in outcrosses as suppressed *cot* could be regarded as an explanation of the irregular segregation. In an ascus such as the first one in Table 2, however, the more interesting question is not why the seventh segregant (assuming the spore order to have been recorded cor-

rectly) is wild, but why it is phenotypically different from the eight. The latter question would not, of course, be answered by the finding that number seven was suppressed *cot*, which, moreover, does not seem very likely. When the same phenotypic difference, that between + and *cot*, is observed in a regular ascus it is assumed, without further tests, to represent a genetic difference. In the absence of prior convictions, it therefore seems reasonable to assume a genetic difference between the seventh and eighth segregants in the irregular ascus, particularly when it is remembered that the genetic identity of members of a spore pair has not been demonstrated. They have been assumed to be regularly alike genetically, partly because they so often appear to be phenotypically alike. Hence it was decided to forego the outcrosses and to pursue instead, as a continuation of this study, such considerations as the possible correlation suggested here between five to three segregation of phenotypes and the ability to segregate "at the second division".

The phenotypic shift towards + regularly exhibited by *sn* cultures is of interest because this sort of "adaptation" is characteristic of many single-ascospore strains of Neurospora. Certain growth responses, for example, have been described as "adaptive" when the growth rate is slow initially but becomes more rapid as the culture ages. A possible interpretation of this behavior can be made in terms of genetic components which give different expressions and which are capable of independent multiplication. If neither alone could produce a viable mycelium, then, together they might constitute an obligate heterocaryon whose phenotype could shift within certain limits in response to culture conditions. Deficient components of heterocaryons were suggested earlier to account for the behavior of certain pseudo wilds (MITCHELL, PITTENGER and MITCHELL 1952). In the present case it seems premature to attempt to devise an explanation of the regular appearance of different and independently multiplying components from single spores of normal eight-spored asci. The fact that *vcot* spores, obviously heterogeneous, were obtained from such asci, suggests, however, that it may be useful to keep such a possibility in mind.

It may be seen that of 19 asci in which the "modified" forms, *dcot* and *ddcot*, appeared, 13 were irregular, whereas only six among 90 not containing these forms showed the same sort of irregularity. This indicates a correlation between irregular segregation and "modification". If *cot* is regarded as a depressor of growth at 34°C, then *dcot* becomes an enhanced form of *cot* and *ddcot* an enhanced *dcot* or, perhaps, a doubly enhanced *cot*. This suggests the possibility that the enhancer is *cot* itself, *dcot* then being *cot cot* and *ddcot*, *cot cot cot*. Possibly, on some basis, the frequencies of the three types among the sn^+ segregants from asci would then be as expected. The parent type, *cot*, is about eight times as frequent as *dcot* which is about six times as frequent as *ddcot*. The same basis might also provide reason to expect a correlation between "modification" and the occurrence of heterogeneous spore pairs.

SUMMARY

A cross of two unlinked colonial mutants has been carefully analyzed, with particular attention given to segregations in asci. The four phenotypes expected, two parental and two recombinant, and also one variegated and two "modified" forms of one parent, could be classified as very young colonies which could be seen to have originated from single ascospores germinated on minimal plates.

All completely germinated asci (109) showed four to four segregation of one of the mutants. Nineteen of these asci contained heterogeneous spore pairs which could not be accounted for in terms of mistaken spore order. The heterogeneous pairs consisted of the second mutant and wild, or one of its atypical forms and wild, or the typical with an atypical form. Thus six asci contained five phenotypes instead of the expected maximum of four. Three of the asci contained one spore each which gave rise to a heterogeneous colony, consisting of the typical mutant form and wild. Curiously, the mutant implicated in the irregularities is one which, in other tests, has shown exceptional stability. The regularly segregating mutant, on the other hand, has been observed to give nonmutant offspring from crosses to certain descendants of itself. No detailed interpretation of the irregularities is offered. The possible prevalence of genetically heterogeneous spores is considered.

Observations of immature perithecia have shown that asci develop as closely associated pairs, or twins. The twins are so nearly identical with respect to structural details as to suggest that they may be identical in phenotype. Twin phenotypes, suggested by the ascus analyses, are discussed.

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