MITOTIC RECOMBINATION IN PSEUDO-WILD TYPES OF NEUROSPORA[1]

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SOMATIC recombination has been known since the early work of STERN (1936) with Drosophila and more recently has been studied in a variety of fungi (see PRITCHARD 1963 for review). In some cases, most notably *Aspergillus nidulans* (PONTECORVO and ROPER 1953; ROPER and PRITCHARD 1955) and *Ustilago maydis* (HOLLIDAY 1961), it has been possible to demonstrate that recombination may occur by mitotic crossing over in heterozygous diploid cells formed by the rare fusion of different haploid nuclei in heterokaryons. That neither diploid nuclei nor haploid nuclei containing somatic crossovers have yet been isolated directly from forced heterokaryons of Neurospora suggests that diploid nuclei are either extremely rare or are not formed in the vegetative mycelium of this heterothallic organism. However, a single instance of recombination between unlinked markers in a heterokaryon of Neurospora has been reported (WEIJER and DOWDING 1960). In a cross between a wild type and a heterokaryon, asci were recovered from a single perithecium which contained spores with markers from both components of the heterokaryon. They attributed their finding to crossing over between nuclear filaments within the heterokaryon.

Diploid nuclei in a heterokaryon of Neurospora are not a prerequisite to studying somatic crossing over, however, since it has been demonstrated that somatic recombinants can be recovered from pseudo-wild type strains (PITTENGER and COYLE 1963). Pseudo-wild types (PWTs) are phenotypically wild-type cultures which can be isolated from the progeny of crosses between linked mutants. They have been isolated from asci containing four aborted and four adjacent normal-appearing black PWT spores or as single normal-sized ascospores among the random progeny. When PWTs are crossed to standard wild types, half of the progeny are wild and the remainder are the two original mutants. Furthermore, homokaryotic conidia of both original mutant parents can be recovered from PWT cultures, demonstrating that they are heterokaryotic. Behavior of those strains is consistent with the idea that they originate as unstable $n + 1$ nuclei (MITCHELL, PITTENGER, and MITCHELL 1952; PITTENGER 1954), but it is now clear that pseudo-wild types need not all originate by nondisjunction, as once thought, since some have been recovered from eight-spored asci (THRELDKELD 1962; CASE and GILES 1964).

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Although it has been known for some time that PWT strains are heterokaryotic for nuclei containing markers from the two original parental strains (PITTENGER 1954), a more recent analysis using linked markers has shown that such cultures may also contain reciprocal recombinants, which have been interpreted as having arisen by somatic crossing over in the aneuploid nucleus (PITTENGER and COYLE 1963).

In this study, rather than using PWTs recovered as random ascospores, we have extended our original observations by analyzing PWTs recovered from asci with four normal and four aborted spores, i.e., the type of asci expected to contain n + 1 nuclei resulting from primary nondisjunction. Recombinants were found in 13 of the 15 PWTs recovered from such asci. In addition, using strains with uninucleate microconidia for some of the analyses made it possible to screen more effectively for recombinants. Recombinant conidia were isolated from 15 of the 16 microconidial PWTs, which were isolated as random spores, and the recombinants were recovered at an average frequency of about 0.8%. Reciprocals were present in 14 of the 15 cultures. The present analysis confirms the earlier finding that somatic recombination is largely confined to the vicinity of the centromere, although it also occurs in other regions. The recovery of seven PWTs, which contained not only the two parental type nuclei but also three different recombinant types as well, is difficult to reconcile with any hypothesis other than somatic recombination.

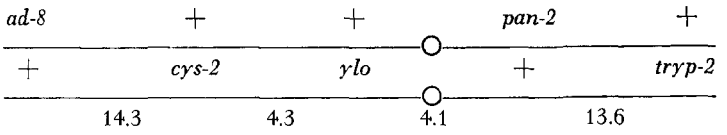
MATERIALS AND METHODS

Pseudo-wild type progeny are easily recovered from crosses of closely linked genes by plating ascospores on minimal medium and isolating the phenotypically wild-type progeny. The PWTs can be readily distinguished from recombinant wild types either by crossing and examining the progeny for mutants or, as is usually done, by plating conidia on various types of supplemented media. Since PWTs are known to be heterokaryotic, they can be readily distinguished from true wild types by plate counts or isolation of homokaryons.

Pseudo-wild types in which somatic crossing over in Neurospora was first detected were among the progeny of a cross of *ad-8 pan-5* × *ylo pan-3 tryp-2* (PITTENGER and COYLE 1963). Analyses of PWTs of this constitution were hampered not only by the presence of the complementing alleles *pan-3* and *pan-5* which could only be distinguished by heterokaryon tests or crosses, but also by the multinucleate nature of the conidia. Here we have omitted the *pan-3* marker from one of the parents and substituted a *cys-2* marker on the opposite arm of the chromosome.

To further facilitate screening procedures for obtaining recombinant-type nuclei from PWTs, two genes which controlled the production of uninucleate conidia were incorporated into two of the strains so heterokaryotic conidia would be eliminated. Unfortunately, such uninucleate strains could not be used exclusively in our analyses because the perithecia from such crosses did not eject many ascospores. Consequently, when the recovery of large numbers of unordered tetrads was an essential feature of an experiment, strains other than those producing microconidia were used.

Strains: The mutant markers in linkage group VI used in crosses to obtain pseudo-wild type progeny are diagrammed below. The relative distances separating the various loci were determined from a small sample of 324 randomly isolated progeny from Cross 2 shown below. Distances separating the genes in general agree with published data from other crosses.



The mutants include *ylo:* yellow (Y30539y, BARRATT, NEWMEYER, PERKINS, and GARNJOBST 1954); *tryp-2:* tryptophan-2 (45302, BARRATT *et al.* 1954); *cys-2:* cysteine-2 (80702, PITTENGER 1954); *ad-8:* adenine-8 (Y152–M7, ISHIKAWA 1960); *pan-2:* pantothenic-2 (B5 allele, CASE and GILES 1958). In addition, the mutants *fl* (fluffy) and pe^m (peach) on linkage group IIR were also used; when present together, these mutants produce uninucleate conidia almost exclusively (BARRATT and GARNJOBST 1949).

Multiple-marked strains involving these mutants were prepared for the following three crosses from which PWTs were obtained:

1. *ad-8 pan-2* 180–1*a* × *cys-2 ylo tryp-2* 44*A*
2. *ad-8 pan-2* 18*A* × *cys-2 ylo tryp-2* 28*a*
3. pe^m *fl ad-8 pan-2* 423-9*A* × pe^m *fl cys-2 ylo tryp-2* 43–2*a*.

Media and supplements: Vogel's medium (VOGEL 1956), with appropriate supplements, was employed to maintain cultures and determine growth requirements. Crosses were carried out on the medium of WESTERGAARD and MITCHELL (1947). This medium was also employed for conidial platings when supplemented with 1% sorbose and .01% glucose. Media were supplemented as necessary with adenine or adenine sulfate (symbolized A), DL tryptophan (T) and DL methionine (M) at concentrations of 200 μg/ml, and with DL calcium pantothenate (P) at 5 μg/ml. The more soluble adenine sulfate was used in place of adenine in the more highly buffered Vogel's, but not with Westergaard's medium. Methionine was used as a supplement for *cys-2* (STADLER and TOWE 1963). Minimal medium is abbreviated "Min".

Analysis of PWTs collected from 4:4 asci: With one of the strains as the female parent, crosses were made in petri dishes on Westergaard's medium supplemented with the necessary growth factors and 1% sucrose. Crosses were maintained at 25°C in the dark until the perithecia started to eject ascospores. Unordered tetrads were collected by the technique of STRICKLAND (1960) which takes advantage of the spontaneous ejection of groups of eight ascospores from individual asci. Groups of eight spores were collected on a 4% agar surface placed a few millimeters away from the ostioles of the perithecia. Only groups of eight ascospores containing four normal black spores and four small hyaline aborted spores were selected for further study. The 4:4 asci containing PWTs were assumed to have originated by primary nondisjunction of the VIth linkage group. The four black and four aborted spores were assumed to have been adjacent in the ascus and to be n + 1 and n − 1, respectively, except where specific exceptions are noted. The black spores were individually transferred to AMPT slants. The four aborted spores, none of which was ever observed to germinate, were placed together in a single tube. Spores were allowed to ripen for at least 2 weeks before being activated by heat shock in groups of ten asci for further analyses. Asci containing the desired PWTs were selected for further study.

PWTs isolated from 4:4 asci were analyzed for two parental types as well as somatic recombinants. Original cultures, i.e. mycelia which developed from the isolated ascospores without intervening subculturing, were used almost exclusively for analyses. Random samples of conidia for plating were prepared by adding a few milliliters of sterile water directly to the culture tubes of the PWT cultures. Aliquots of conidial suspensions from each culture were overplated on Min, AM, AT, MP, PT, AP, MT, and AMPT media.

Analyses of the PWT cultures differed in regard to the number of colonies isolated for subsequent testing and the media from which they were isolated, but subsequent procedure for testing isolates was the same for all PWTs. Conidial-derived colonies were transferred onto AMPT slants from nonparental types of doubly supplemented media that could support growth of some of the possible recombinants as well as heterokaryotic conidia containing *ad pan* and *cys ylo tryp* nuclei. Homokaryotic and heterokaryotic auxotrophic isolates were distinguished from the prototrophic heterokaryons by their inability to grow on minimal medium. Prototrophs were

discarded and auxotrophs were provisionally classified by testing in tubes of APT, APM, AMT, and PMT media to distinguish all possible combinations of markers.

Recombinant isolates were plated on combinations of media which permitted distinguishing homokaryons from heterokaryons as well as verifying their nutritional requirements. When colony counts indicated that an isolate was heterokaryotic, the genotype of the heterokaryon was determined by isolating as many colonies as necessary to recover the homokaryotic components. The genotypes of such homokaryons were again confirmed by plating and by growth on differential media. Not all isolates were plated, but when more than three of any type were recovered from one PWT culture, two to five representative isolates from the culture were plated. The mating types were determined for 153 isolates.

Analyses of microconidial pseudo-wild types: Sixteen microconidial PWTs isolated as random spores were analyzed in much the same manner as the PWTs from 4:4 asci. The only difference was that all conidial-derived colonies were transferred to minimal rather than APMT slants to eliminate further testing of colonies originating from heterokaryotic hyphal fragments or multinucleate conidia.

Isolates incapable of growing on minimal medium were then supplemented with APMT by adding a few drops of concentrated APMT medium. The concentrated medium was injected between the cotton plugs and the side of the culture tube with a 23-gauge needle on a Cornwall pipette. Contamination was avoided by flaming the needle frequently and raising the plugs slightly to expose a sterile entrance.

After the auxotrophic isolates had grown, their requirements were determined by differential growth in tubes of APT, APM, AMT, and PMT media. All isolates having a single nutritional requirement were plated on appropriate media to determine if any were heterokaryotic.

RESULTS

Analysis of pseudo-wild types from 4:4 asci: 99 asci containing four normal and four aborted spores were collected as unordered tetrads from Crosses 1 and 2. Approximately 60% of the normal-appearing spores germinated. PWTs were present in ten of the 4:4 asci (Table 1). Four of these asci contained non-PWTs as well as PWTs. Four asci (Nos. 5, 13, 14, and 81) were selected for an analysis of recombination. The remaining 4:4 asci with four viable spores, only some of which were PWTs, are discussed later.

The results of the analysis are summarized in Table 2. Homokaryotic conidia of the two parental types, i.e., *ad pan* and *cys ylo tryp*, were recovered from

TABLE 1

The 4:4 asci from which PWT spores were obtained

Cross	Ascus number	Number of viable ascospores	Genotypes of ascospores
2	5	4	4 PWT
2	10	3	3 PWT
2	12	2	2 PWT
1	13	4	3 PWT; 1 *cys ylo tryp*
2	14	4	4 PWT
1	76	3	3 PWT
2	79	4	2 PWT; 2 *cys ylo tryp*
2	81	4	4 PWT
2	98	4	1 PWT; 2 *ad pan*; 1 *cys ylo tryp*
2	117	4	3 PWT; 1 *ad ylo tryp*

TABLE 2

Results of plating analyses and types of recombinants recovered from macroconidial pseudo-wild types isolated from four 4:4 asci

PWT No.	Average number of colonies per plate* Media Min	AM	AT	MP	PT	AP	MT	APMT	Total no. of colonies tested	Number and phenotypes of recombinant isolates†
5.1	47	**48**	47	42	**56**	67	84	107	312	1 *cys ylo*; 2 *pan*
5.1‡	80	..	**59**	**90**	..	..	..	178	300	3 *pan*; 1 *ad tryp*; 2 *cys ylo pan*
5.2	46	**53**	**42**	**50**	**49**	69	71	96	569	4 *ad*; 1 *cys ylo*; 1 *pan*; 3 *tryp*
5.3	35	**40**	**36**	**42**	**40**	91	48	91	464	0
5.3‡	14	..	**10**	**14**	..	..	..	131	125	5 *ad*; 1 *ad tryp*
5.4	33	**33**	32	33	**41**	61	63	71	206	1 *pan*
5.4‡	73	..	**59**	**64**	..	..	..	280	350	0
13.1	0.33	..	0	0.66	..	0.33	364	278	4	0
13.2	23	..	**21**	**26**	..	33	402	368	400	2 *tryp*
13.3	117	..	**71**	**119**	..	135	751§	753§	400	0
13.4	39	..	**29**	**42**	..	nc‖	57	nc‖	400	2 *pan*
14.1	227	**194**	**245**	**217**	**234**	396	421	574	400	1 *ad*; 3 *pan*; 2 *tryp*
14.2	105	**120**	**149**	**104**	**105**	266§	145	311	400	29 *ad*; 1 *pan*; 2 *tryp*; 8 *ad tryp*; 2 *cys ylo pan*
14.3	23	**27**	**26**	**26**	**19**	207	32	256	400	3 *ad*; 5 *pan*; 1 *tryp*#; 3 *cys ylo pan*
14.4	30	**41**	**51**	**312**	**278**	458	33	538	400	27 *ad*; 158 *pan***; 4 *ad tryp*; 1 *cys ylo pan*
81.1	38	**41**	**39**	**42**	**39**	337	55	314	400	2 *pan*; 1 *cys ylo pan*
81.2	117	**118**	**79**	**121**	**99**	225	272	402	400	0
81.3	83	**79**	**63**	**89**	**86**	400	98	388	400	4 *ad*; 2 *cys ylo*; 7 *pan*; 1 *cys ylo pan*
81.4	90	**105**	**75**	**89**	**95**	354	133	303	400	1 *ad*; 2 *pan*

* Averages in table calculated from two plates each of AP, MT and AMPT media and at least three plates each of Min, AM, AT, MP and PT media. (See MATERIALS and METHODS for abbreviations.) Numbers in bold face indicate type of medium from which colonies were isolated and tested for recombinants. Total colonies tested is sometimes larger than average number per plate, since all colonies from three or more plates often were tested.

† The isolates having a single requirement were heterokaryons of the genotypes shown in Figure 1; two exceptions are indicated below.

‡ Indicates subculture of original.

§ Only one plate was counted.

‖ Plates too crowded to count; plates had at least 1000 colonies.

Heterokaryon between *cys ylo tryp* and *ad pan tryp*.

** *pan* homokaryons or heterokaryons between *pan* and *ad pan*

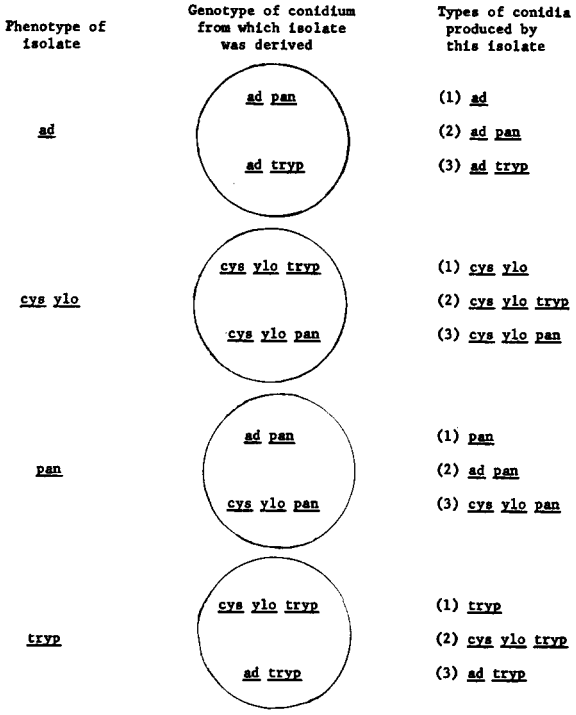


FIGURE 1.—The phenotypes and genotypes of auxotrophic heterokaryons isolated from PWTs containing the parental nuclei, *ad pan* and *cys ylo tryp*, and the recombinant nuclei *cys ylo pan* and *ad tryp*. Only conidia containing two nuclei are represented although the number of nuclei varies, averaging between two and three per conidium.

every PWT analyzed, although this is not specifically mentioned in the table. Recombinants were found in 13 of the 16 cultures analyzed and all of the tested recombinant isolates were the same mating type as the PWT culture from which they were recovered. Table 2 shows that a high proportion of the nonparental-type isolates had single growth requirements for either adenine, pantothenate, methionine, or tryptophan. On subsequent testing, most of them (Figure 1) proved to be auxotrophic heterokaryons between one of the parental types and a recombinant. With only two exceptions, the recombinant types were either *ad tryp* or *cys ylo pan*, which represent the reciprocal products of an exchange in the region enclosing the centromere between *ylo* and *pan*. This was the only region involved in the recombination previously observed in random PWTs (PITTENGER and COYLE 1963). There were two notable exceptions. Many *pan* isolates recovered from PWT 14.4 were characterized as either *pan* homokaryons or heterokaryons between *pan* and *ad pan*. Such *pan* homokaryons were recombinants between *ad* and *cys* but the reciprocal *ad cys ylo tryp* recombinant was not found among the isolates tested from PWT 14.4. An exceptional *tryp* isolate from PWT 14.3 was found to be a heterokaryon between *cys ylo tryp* and *ad pan tryp*, the latter being a rare recombinant between *pan* and *tryp*.

It was difficult to make an unequivocal conclusion about the nature of asco-

spore 13.1. The poor growth of this yellow culture on liquid minimal medium resembled that of a leaky *cys ylo tryp* strain. Except for the four prototrophic colonies found on a total of 18 plates, the culture appeared to be a *cys ylo tryp* homokaryon. One of the four prototrophs was a wild-type contaminant of mating type *a*. The other three were heterokaryons between *ad pan* and *cys ylo tryp* and were mating type *A*, which corresponded to the mating type of ascospore 13.1. Since the ratios of *ad pan* to *cys ylo tryp* conidia in all of the PWTs of Ascus 13 exhibited a greater deviation from a value of 1 than was found in 11 of the other 12 PWTs, it appeared possible that 13.1 was a PWT with the *ad pan* component present at a very low frequency. If the failure to recover *ad pan* homokaryons from ascospore 13.1 were due only to the low viability of the *ad pan* component, the viability difference might be evident in the three heterokaryotic isolates. Instead, plating those heterokaryons revealed that the *ad pan* nuclei were the more frequent type in two of the three cultures. How a *cys ylo tryp* isolate might arise in a 4:4 ascus with three PWTs is considered in the discussion.

Recovery of reciprocal recombinants together with noncrossover parental types from PWTs with a known 4:4 origin is consistent with the hypothesis that mitotic crossing over occurs in disomic nuclei which had arisen by nondisjunction. The significance of the failure to recover reciprocal types from five of the 13 cultures that yielded recombinants could not be known without more extensive analysis.

Analysis of random microconidial pseudo-wild types: The search for recombinant types in macroconidial PWTs was encumbered by the large fraction of heterokaryotic conidia capable of growing on minimal and supplemented media. Analyzing microconidial PWTs recovered as random spores from Cross 3 proved highly efficient for detecting recombinants. Since less than 0.2% of the conidia from microconidial PWTs were prototrophic, it was technically feasible to recover recombinants occurring at a much lower frequency than the macroconidial strains permitted.

Plating data and number of recombinants recovered from the microconidial PWTs are presented in Table 3. No attempt was made to recover recombinants with triple growth requirements since any medium supporting the growth of such recombinants would also contain an excessively large number of one of the parental types. The reciprocals of such recombinants, i.e., those with a single requirement, could be recovered from two types of doubly supplemented media. The possibility that any culture contained a large number of recombinant conidia that required three supplements was eliminated to some extent by a later experiment in which colony counts on AMPT medium roughly equalled the totals from AP and MT media. Conidia were not routinely plated on AMPT medium since work with macroconidial strains showed this medium to be inhibitory.

Recombinant types were recovered from all but one of the 16 pseudo-wild types. Fourteen of the cultures contained both *ad tryp* and the reciprocal *cys pan* isolates. Unforunately, in these pe^{m} *fl* PWT strains the *ylo* marker is not expressed, so it was not possible to localize the exchange between *ylo* and *pan* as with the macroconidial PWTs. However, the majority of exchanges were between *cys* and *pan*, a region roughly eight units long.

Five PWTs were found to contain a third type of recombinant, either *cys* or

TABLE 3

Results of plating analyses and types of recombinants recovered from microconidial pseudo-wild types isolated as random spores

PWT No.	Average number of colonies per plate* Media: Min.	AM	AT	MP	PT	AP	MT	Number and phenotypes of recombinant isolates
26	10	8 **17**	60 **20**	14 **16**	8 **15**	1180	6410	2 *cys*; 2 *tryp*; 16 *ad tryp*; 6 *cys pan*
27	5	4 **9**	2 **4**	29 **20**	2 **3**	1880	3080	3 *cys*; 1 *cys*†; 1 *pan*; 1 *tryp*; 12 *cys pan*; 3 *ad tryp*
28	0	0 **0**	0 **0**	0 **0**	0 **0**	60	3390	0
30	12	11 **9**	87 **20**	27 **25**	8 **9**	7380	3480	3 *cys*; 2 *pan*†; 2 *tryp*; 16 *ad tryp*; 13 *cys pan*
31	1	2 **4**	19 **20**	5 **6**	4 **9**	590	4470	5 *tryp*; 15 *ad tryp*; 1 *cys pan*
32	30	42 **48**	700 **30**	221 **17**	25 **27**	20360	2610	9 *ad*; 1 *cys*; 6 *cys*†; 3 *pan*; 25 *ad tryp*; 14 *cys pan*
33	13	14 **17**	437 **10**	34 **34**	13 **17**	16660	1880	3 *ad*; 2 *cys*; 9 *ad tryp*; 21 *cys pan*
35	18	25 **30**	40 **44**	51 **57**	16 **17**	7080	7600	4 *cys*; 2 *tryp*; 14 *ad tryp*; 26 *cys pan*
36	31	54 **27**	51 **54**	1740 **20**	30 **23**	5700	6390	7 *cys*; 1 *cys*†; 4 *pan*; 2 *tryp*; 24 *ad tryp*; 19 *cys pan*
37	8	9 **18**	19 **21**	132 **14**	6 **6**	1950	5760	2 *cys*; 1 *cys*†; 1 *tryp*; 10 *ad tryp*; 12 *cys pan*
40	16	18 **19**	20 **25**	45 **44**	21 **22**	2050	3590	3 *tryp*; 2 *ad tryp*; 14 *cys pan*
43	1	2 **4**	1 **2**	19 **23**	0 **0**	230	2410	5 *cys*; 2 *ad tryp*; 4 *cys pan*
44	2	2 **5**	6 **11**	1 **1**	0 **0**	180	4290	1 *tryp*; 7 *ad tryp*
45	6	9 **13**	46 **20**	63 **20**	4 **10**	2420	6600	1 *tryp*; 14 *ad tryp*; 18 *cys pan*
47	4	6 **11**	8 **9**	38 **20**	4 **7**	7420	390	3 *pan*; 4 *ad tryp*; 15 *cys pan*
49	28	25 **25**	30 **30**	33 **31**	24 **25**	2650	3100	1 *cys*; 2 *tryp*; 8 *ad tryp*; 9 *cys pan*

* Averages in tables calculated from only two plates each of the nonparental types of plating media. Plate counts on AP and MT multiplied by 10 since plating suspension for these media were diluted 10-fold prior to plating. Numbers in bold face indicate number of colonies isolated and tested for recombinants.

† Homokaryons, see text.

pan homokaryons, which were detected by plating all the isolates having a single requirement. The reciprocal types, which would have had three nutritional requirements, were not recovered since no colonies were isolated from media with more than two supplements. The small fraction of heterokaryotic conidia or hyphal fragments in a microconidial culture presented little opportunity for heterokaryotic combinations like that of the *tryp* isolate from macroconidial PWT 14.3, which was a heterokaryon between *ad pan tryp* and *cys ylo tryp.*

Estimates of the frequency of recombinants in pseudo-wild type cultures: Although the frequency of recombinants was not an original objective, a reasonable approximation of the frequency of recombinant conidia in microconidial and macroconidial pseudo-wild type cultures might be estimated from data presented in Tables 2 and 3. Since only a sample of the available recombinants was isolated and identified, the frequency of recombinants in the population was adjusted for the fraction of isolates tested. In calculating frequencies of certain recombinants, it was also necessary to consider that some types could be recovered from two types of media. For example, adenine-requiring isolates, either homokaryotic or heterokaryotic, could be recovered from either AT or AM medium and so the frequency of such isolates was divided by two to compare them with

TABLE 4

Estimated numbers of recombinant and parental types of conidia in microconidial PWT cultures as calculated from data in Table 3

	Phenotypes of the isolates								
	Recombinant types								
	Homokaryons		Heterokaryons*				Parental types		Frequency of recombinant conidia ($\times 10^{-3}$)
PWT No.	*ad tryp*	*cys pan*	*ad*	*tryp*	*cys*	*pan*	*ad pan*	*cys tryp*	
26	48	5	0	3	1	0	1180	6406	7.4
27	2	17	0	1	2,1†	1	1879	3077	4.7
28	0	0	0	0	0	0	60	3390	0.0
30	70	14	0	4	2	1†	7379	3474	8.3
31	14	1	0	2	0	0	590	4468	3.4
32	582	182	15	0	1,8†	1	20359	2601	33.3
33	339	21	1	0	2	0	16660	1878	19.2
35	13	23	0	1	2	0	7080	7597	2.6
36	23	1653	0	1	7,1†	45	5655	6381	125.7
37	9	113	0	1	1,1†	0	1950	5757	15.7
40	2	14	0	1	0	0	2050	3589	3.0
43	2	3	0	0	2	0	230	2408	2.6
44	4	0	0	1	0	0	180	4289	0.8
45	32	57	0	2	0	0	2420	6598	9.9
47	3	28	0	0	0	1	7419	390	4.1
49	8	10	0	1	1	0	2650	3098	3.3
							Average = 244/16 = 15.2		
							Average omitting PWT 36 = 118.3/15 = 7.9		

* See Figure 1 for genotypes.
† Homokaryons.

TABLE 5

Estimated numbers of recombinant and parental types of conidia in PWT cultures from 4:4 asci as calculated from data in Table 2

	Phenotypes of the isolates							
	Recombinant types						Parental types	
	Homokaryons		Heterokaryons*					
PWT No.	*ad tryp*	*cys ylo pan*	*ad*	*tryp*	*cys ylo*	*pan*	*ad pan* and *cys ylo tryp*	Frequency of recombinant conidia ($\times 10^{-3}$)
5.1	0.4	1.2	0.0	0.0	0.3	2.4	279	15.5
5.2	0.0	0.0	0.7	0.5	0.2	0.2	92	16.3
5.3	0.2	0.0	0.9	0.0	0.0	0.0	236	4.7
5.4	0.0	0.0	0.0	0.0	0.0	0.4	371	1.0
13.2	0.0	0.0	0.0	0.2	0.0	0.0	412	0.5
13.3	0.0	0.0	0.0	0.0	0.0	0.0	771	0.0
13.4	0.0	0.0	0.0	0.0	0.0	0.4	not counted	. .
14.1	0.0	0.0	1.0	2.4	0.0	3.5	583	11.6
14.2	11.9	2.1	19.2	1.5	0.0	0.5	285	109.8
14.3	0.0	0.8	0.4	0.1†	0.0	0.6	215	8.6
14.4	2.0	3.1	5.8	0.0	0.0	232.7‡	222	523.2
81.1	0.0	0.4	0.0	0.0	0.0	0.4	354	2.3
81.2	0.0	0.0	0.0	0.0	0.0	0.0	380	0.0
81.3	0.0	0.9	1.3	0.0	0.9	3.1	410	14.9
81.4	0.0	0.0	0.4	0.0	0.0	0.9	396	3.3
							Average = 711.7/14 = 50.8	
							Average omitting PWTs 14.2 and 14.4 = 78.7/12 = 6.6	

* See Figure 1 for genotypes.
† Heterokaryon between *ad pan tryp* and *cys ylo tryp*.
‡ *pan* homokaryons or heterokaryons between *pan* and *ad pan*.

parental types. Other factors undoubtedly affected the results to some extent. Throughout the experiments there was some indication that some isolates, but not others, tend to be leaky on certain media, and certain supplements appear to be inhibitory in some cases but not in others. To what extent such phenomena distorted the ratios reported is not known.

The results of such analyses are presented in Table 4 for the microconidial strains and in Table 5 for the macroconidial strains only to give some general estimate of frequencies of recombinants in the population. The frequencies vary widely as expected when considering that their proportion in a population depends not only on frequency of recombination, but also on the rate and mechanism of haploidization as well as relative survival of n and n + 1 nuclei in a culture. Nevertheless, if one eliminates two macroconidial cultures that contribute nearly 90% of the total recombinants in this group, and also eliminates one microconidial culture that contributes over 50% of the recombinants among that group, then the average frequency of recombinant conidia in the two groups is approximately the same. In the microconidial group, the average frequency of recombinant conidia was 7.9×10^{-3} and in the macroconidial group, 6.6×10^{-3}.

Absence of recombinants in reconstructed heterokaryons between ad pan *and* cys tryp *strains:* The work described thus far has not excluded the possibility that

somatic recombination occurred in heterozygous diploid cells produced by fusion of different haploid nuclei in a PWT culture. Four different heterokaryons between *ad pan* and *cys tryp* strains, two of which were between uninucleate strains, were prepared in flasks of minimal medium and conidia were examined for recombinant types. No recombinant isolate was recovered among 1200 isolates from macroconidial heterokaryons, although sample sizes were comparable to those in which recombinants had been recovered in PWTs and the procedures for selecting recombinants were identical with the methods employed in analyses of PWT cultures. The combined data from microconidial heterokaryons indicate that recombinant types at a frequency of approximately 1.9×10^{-5} nuclei could have been detected if present. Plating data and the number of colonies tested are shown in Table 6.

The failure to recover any recombinants from heterokaryons suggests that somatic recombinants from PWT cultures were not produced via the parasexual cycle, but these experiments cannot be considered evidence against the occurrence of a parasexual cycle in Neurospora. Sample sizes were much too small to recover recombinants that might arise at the frequency characteristic of the parasexual cycle in Aspergillus. Considering the data from Aspergillus (PONTECORVO 1954), mitotic crossing over in diploid cells resulting in homozygosis of markers on either arm of linkage group VI would be expected to occur in 10^{-10} nuclei of a heterokaryon.

DISCUSSION

The majority of the pseudo-wild type strains studied in this investigation, regardless of whether isolated as random ascospores or from asci containing four normal and four aborted spores, were similar to one another in that they contained at least four different nuclear types, two parental types and two recombinants, the last being crossovers in the vicinity of the centromere and present at an average frequency of less than 1%. The evidence is consistent with the assumptions that the PWTs were aneuploid in origin, that somatic crossing over between homologues in the vicinity of the centromere produced the reciprocal recombinant types, and that haploidization of the partial diploids occurred by either nondisjunction of sister chromatids or lagging of chromosomes.

Although these experiments were designed to obtain evidence for the presence of recombinants in PWTs, a knowledge of the genetic constitution of these cultures is obviously important to the correct interpretation of the results. The PWTs that were recovered from 4:4 asci can best be explained as aneuploids originating by primary nondisjunction of the VIth linkage group. Since PWT cultures are known to be heterokaryotic, a further assumption is that the aneuploids, presumably $n + 1$ nuclei, are unstable and undergo haploidization to form the observed heterokaryons. Such an explanation is much more consistent with the behavior of a variety of PWTs than the assumption that they originate as heterokaryotic ascospores (MITCHELL, PITTENGER and MITCHELL, 1952; PITTENGER 1954, 1958). Furthermore, the assumption of heterokaryotic origin of PWTs makes it necessary to postulate a parasexual cycle involving crossing

TABLE 6

Results of analyses of heterokaryons between ad pan *and* cys tryp *strains for presence of somatic recombinants*

Heterokaryon	Average number of colonies per plate* Media AM		AT		MP		PT		Min	AP	MT	Number of recombinants isolated	Estimated frequency of recombinants
ad pan 15a and *cys ylo tryp 28a*	..	..	69	**300**	54	**300**	..	..	70	175	226	0	$< 6.2 \times 10^{-4}$
ad pan 18A and *cys ylo tryp 281A*	..	..	53	**300**	53	**300**	..	..	56	122	92	0	$< 1.1 \times 10^{-3}$
pe^{m} fl ad pan and *pe^{m} fl cys tryp* from *microconidial PWT 36*	0.5	**1**	0	**0**	0.5	**1**	0	**0**	2	4240	4360	0	$< 5.8 \times 10^{-5}$
pe^{m} fl ad pan and *pe^{m} fl cys tryp* from microconidial PWT 49	3	**6**	3	**6**	6	**11**	6	**11**	5	12700	4800	0	$< 2.9 \times 10^{-5}$

* Average number of colonies calculated from at least two plates. Counts from microconidial cultures on AP and MT media were obtained from higher dilutions than those on other media and adjusted accordingly. Numbers in bold face were the number of colonies isolated and tested for recombinants.

over in diploid nuclei to account for the reciprocal recombinants in these cultures. Reconstruction experiments with heterokaryons demonstrated that if such a cycle is present in Neurospora, it cannot account for the proportions of recombinants recovered in PWTs.

That some PWTs have been recovered from complete asci (CASE and GILES 1964) demonstrates that not all PWTs originate by nondisjunction. That significant observation suggests why PWTs may be recovered in fairly high proportions from some crosses that appear to form too few 4:4 asci to account for the large number of PWT progeny. Although the mechanism responsible for PWTs in complete asci is not understood, the recovery of a recombinant-type nucleus in one of the PWTs studied by CASE and GILES (1964) suggests a similarity between PWTs from complete asci and those from 4:4 asci.

Reciprocality of recombinants: Because reciprocal recombinants have been recovered from the majority of PWTs analyzed, it has been inferred that somatic recombination in Neurospora occurs by the classical mechanism involving reciprocal exchange of chromatids. However, critical evidence for reciprocality, such as twin spots in Drosophila (STERN 1936) or mosaic colonies, corresponding to twin spots in heterozygous diploids of *Saccharomyces cerevisiae* (JAMES 1955), *Ustilago maydis* (HOLLIDAY 1961) or Aspergillus (KÄFER 1961), were not obtained in these studies.

Failure to recover reciprocal types in some PWTs as well as differences in frequencies of reciprocal types within individual cultures might be used as an argument against crossing over in PWTs being a reciprocal event. However, considering the small number of isolates examined and the average frequency of recombinants of less than 1%, occasional failure to recover both products of crossing over is not surprising. Failure to recover the missing type is explained by the low frequency with which the reciprocal types were found.

Differences in the frequencies of reciprocal types, as well as of parental types, would be expected if the mechanism of haploidization were either by nondisjunction or lagging of chromosomes. Consequently, neither observation is inconsistent with crossing over in n + 1 nuclei being reciprocal. In view of evidence from mitotic crossing over in other organisms, together with PONTECORVO's observation (1958) of no apparent difference in the basic mechanism between mitotic and meiotic crossing over, no other recombination models are needed to explain the data from PWTs.

The mechanism of haploidization: Several mechanisms might be responsible for haploidization of the n + 1 nuclei of the PWTs. Either nondisjunction of sister chromatids or chromosome lagging, as postulated by KÄFER (1961) to account for the results in Aspergillus, seems to satisfactorily explain our results.

There is independent evidence for nondisjunction of chromatids in Neurospora. PITTENGER (1954) recovered ten randomly isolated PWTs from a cross of the linked mutants *a al-2 nic-1* × *A aur lys-3*. The PWTs were homoallelic for the mating type locus on IL, but heteroallelic for the other four markers located on IR. They could have arisen as the result of meiotic crossing over followed by nondisjunction of chromatids in the second meiotic division. The 6:2 ascus con-

taining two PWTs (MITCHELL, PITTENGER and MITCHELL 1952) also is consistent with nondisjunction of sister chromatids.

Although it seems less likely, haploidization and crossing over both might take place during a reductional-type division such as occurs at the first meiotic division. Pairing of homologues in n + 1 nuclei might be stimulated at mitosis by an extra chromosome. There could be a strong tendency for pairing of homologues in *Neurospora crassa*, as there would be no selection for a mechanism to prevent mitotic synapsis in an organism whose life cycle includes only one diploid nucleus that immediately undergoes meiosis. If haploidization by such a mechanism occurred in the first postmeiotic division, one would obtain two PWT spores and two genetically different haploid sister spores. However, the genotypes of haploid spores recovered in 4:4 asci containing PWTs (Table 1) do not support the idea of pairing of homologues during mitosis immediately preceding haploidization. The simplest explanation for such ascus types appears to be that primary nondisjunction in the first meiotic division resulted in the 4:4 spore arrangement, but nondisjunction of chromatids in one of the following nuclear divisions resulted in haploidization of some of the PWT nuclei. In some cases, one spore already would be haploid but its sister spore would be n + 2, if nondisjunction rather than chromosome lagging took place. The data from Ascus 13 seem to favor chromatid nondisjunction which would result in an n + 2 nucleus, since there was a great excess of *ad pan* conidia in PWT 13.4. An excess of *ad pan* nuclei might be expected following haploidization of the n + 2 spore (*ad pan/ad pan/cys ylo tryp*) if it were a sister spore of the haploid *cys ylo tryp* ascospore.

The recovery of one *ad ylo tryp* recombinant spore, together with three PWTs from Ascus 117, is even more significant and has been interpreted to result from mitotic exchange prior to, and presumably independent of, chromatid nondisjunction in a later division, as shown in Figure 2. In such a case, one of the PWT spores in this ascus would be expected to contain the reciprocal crossover of the *ad ylo tryp*, i.e., a *cys pan* nucleus. In fact, when we examined the three PWTs, one (No. 117.1) contained nuclei of the genotype *cys pan*. In addition to recovering *cys pan* nuclei from the presumed n + 2 PWT (*ad pan/cys pan/cys ylo tryp*), we also recovered additional reciprocal recombinants of the genotypes *cys tryp* and *cys ylo pan*. Such reciprocals could have resulted from recombination in the n + 2 nucleus in the region of the centromere between chromosomes of the genotypes *cys pan* and *cys ylo tryp*.

Furthermore, one can also explain the other exceptional asci in Table 1 employing the model of chromatid nondisjunction. For example, Ascus 13 can be explained by chromatid nondisjunction of the *cys ylo tryp* chromosome in the first postmeiotic division, and Ascus 79 by chromatid nondisjunction of the *ad pan* chromosome in meiosis II. Ascus 98 could have resulted from chromatid nondisjunction of the *cys ylo tryp* chromosome in meiosis II and the *ad pan* chromosome in the first postmeiotic division.

In retrospect, more could have been learned from a detailed analysis of 4:4 asci with fewer than four PWTs. The original cultures were subcultured several times and data concerning frequency of the nuclei in such PWTs would, conse-

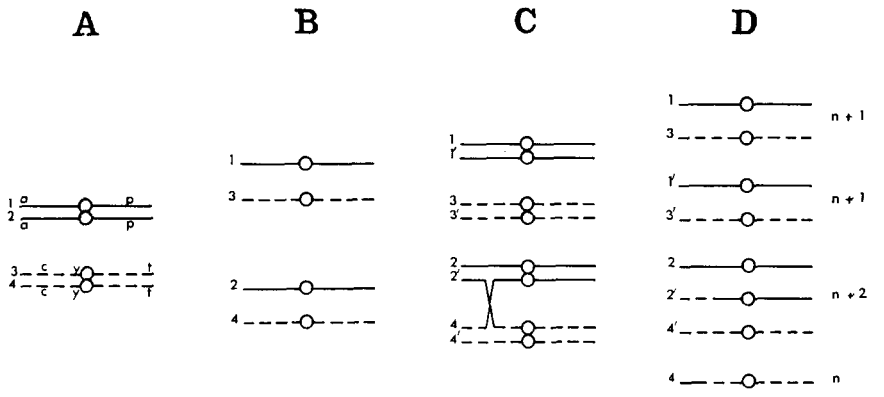


FIGURE 2.—A model employing mitotic crossing over and nondisjunction of chromatids to explain the formation of Ascus 117 containing three PWTs and one haploid recombinant ascospore. The diagram shows only the constitution of linkage group VI (a = *ad*, p = *pan*, c = *cys*, y = *ylo*, and t = *tryp*) during various stages of division leading to the formation of the four viable spores in a 4:4 ascus. (A) An n + 1 nucleus resulting from nondisjunction in meiosis I; the other division product is n − 1 but is not shown. (B) Two heterozygous n + 1 nuclei after meiosis II. (C) Chromosomes after replication at interphase following meiosis II showing a mitotic crossover between *cys* and *ylo*. (D) Two n + 1 ascospores and one n + 2 (*ad pan/cys ylo tryp/cys pan*) and one haploid recombinant (*ad ylo tryp*) ascospores following chromatid nondisjunction in the first postmeiotic division.

quently, have less meaning. Nevertheless, the recovery of reciprocal products of crossing over that took place in one nucleus from two separate ascospores, one haploid (117.2) and one aneuploid (117.1), is of particular significance. That this exchange was rare, being between *cys* and *ylo*, makes it much less likely that the reciprocal products in two separate ascospores could have had some other origin.

Frequency of haploidization: Earlier studies (PITTENGER 1958, and unpublished) indicated that haploidization of a significant proportion of PWT cultures occurred as early as the first nuclear division within the ascospore. In populations of ascospores in which the frequency of PWTs and recombinant wild types had been determined, the number of viable nuclei per ascospore was effectively reduced from two to one by X-irradiation. Among viable progeny, the reduction in frequency of PWT spores was significantly greater than in recombinant wild-type spores, suggesting that many binucleate PWT spores already had undergone haploidization.

At the same time, no direct evidence for the persistence of disomic nuclei has been obtained from analyses involving conidial isolates or crosses of PWTs, but an estimate of their maximum frequency based on number of prototrophic conidia produced by microconidial PWTs is approximately 0.13%. That is obviously an overestimate since microconidial PWTs produce multinucleate conidia, as evidenced by the isolation of auxotrophic heterokaryons. Some were undoubtedly due to hyphal fragments, but multinucleate conidia have been observed in such cultures (PITTENGER, unpublished). That the number of prototrophic colonies

was extremely low in reconstructed heterokaryons between microconidial strains (Table 6) could be interpreted to mean that the relatively more frequent prototrophic colonies from microconidial PWTs could be due to some n + 1 nuclei in these cultures. That has not been experimentally verified, however.

If KÄFER's (1960) findings with Aspergillus are relevant to Neurospora, the great excess of haploid nuclei found in PWTs is more indicative of inability of disomic nuclei to compete with the haploid segregants than it is of the frequency at which haploids are produced. In any event, a prerequisite for recombinant types occurring at the low frequencies found is the persistence of some disomic nuclei, at least until sufficient divisions of either disomic nuclei or their haploid segregants have occurred to produce the observed ratios of parental to recombinant types.

If the 4:4 asci containing four viable spores (Table 1) can be assumed to have originated by primary nondisjunction and the haploid spores assumed to have resulted by nondisjunction of chromatids, then such asci represent an estimate of the frequency of haploidization. With such assumptions, data in Table 1 indicate that three normal divisions of disomic nuclei took place in Asci 5, 14, and 81, to produce the four observed PWTs. In Asci 13 and 117 there were two normal divisions and one nondisjunctional; Ascus 79, one normal division and one nondisjunctional, while in Ascus 98 nondisjunctions occurred during both of the divisions of the disomic nuclei. Thus, nondisjunction occurred during five of the 19 mitoses of disomic nuclei.

This small sample indicates that the chance of haploidization during mitosis of a disomic nucleus is approximately 25%. If this rate of haploidization is of the right order of magnitude, it must be assumed that the rates of nuclear division of n and n + 1 nuclei are very different, in order to account for the fact that PWT cultures contain an extremely high proportion of n nuclei.

SUMMARY

Somatic recombinants and parental type nuclei were recovered from the conidia of 28 of 31 pseudo-wild type cultures isolated from a cross of *ad-8 pan-2* × *cys-2 ylo tryp-2*. Reciprocal types of recombinants were found in 22 of the cultures. Seven pseudo-wild types contained a fifth type of nucleus. In general the region of exchange was in the vicinity of the centromere. In macroconidial pseudo-wild types, the exchanges were localized in the *ylo-pan* interval which includes the centromere and is approximately 4 map units long.—The average frequency of recombinant conidia was estimated to be 7.3×10^{-3}, excluding data from three cultures that had unusually high frequencies of recombinants.—Pseudo-wild types isolated from asci containing four pseudo-wild types and four aborted spores (4:4 asci) were presumed to have originated from disomic (n + 1) nuclei that arose by primary nondisjunction during the first meiotic division. The origin of 16 pseudo-wild types isolated as random spores was not determined, but the presence of more than two types of nuclei in 15 of the cultures indicated that they also originated from nuclei heterozygous for markers on linkage group

VI.—Some of the 4:4 asci contained haploid spores together with pseudo-wild type spores. The haploid spores were interpreted to be products of haploidization of the n + 1 nuclei during one of the divisions prior to spore formation.

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