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Recombination during blocked chromosome replication in temperature-sensitive strains of *Ustilago maydis*

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SUMMARY

*tsd*1–1 strains of *Ustilago maydis* are blocked in DNA synthesis under restrictive conditions. On recovery from the block, both intergenic and allelic recombination are enhanced, and aneuploids are generated. UV is synergistic in the induction of recombination. Direct assays of allelic recombination have shown that gene conversion occurs during the block in DNA synthesis.

1. INTRODUCTION

We have previously described the isolation and characterization of the temperature-sensitive mutant *tsd*-1 (Unrau & Holliday, 1970). Transfer of growing cells from the permissive (22 °C) to the restrictive temperature (32 °C) is followed by some DNA synthesis for 3–4 h, but thereafter there is very little increase. There is no direct effect on RNA and protein synthesis. Much of the initial increase in DNA at 32 °C can be attributed to the completion of a round of chromosome replication and the proliferation of mitochondria. The mutant can be used to examine the relationship between genetic replication and recombination in suitably marked diploids homozygous for *tsd*-1. More particularly, we wish to know whether mitotic recombination can occur when DNA synthesis is blocked. Although a small amount of DNA synthesis during this period cannot be excluded, we know that replication of whole chromosomes cannot take place.

In several studies of meiosis it has been clearly demonstrated that recombination occurs after rather than during chromosome replication, i.e. by breakage and reunion of chromatids (see Peacock, 1970). In experiments with *tsd*-1 diploids of *Ustilago*, we are able to show that the same is true for mitotic recombination. The inhibition of DNA synthesis at 32 °C stimulates mitotic recombination, and by using the direct biochemical assay for recombination (Holliday, 1971) it can be shown that this occurs during the period of inhibition.

2. MATERIALS AND METHODS

(i) *Strains*

Two temperature-sensitive diploid strains were derived from heterozygous diploids by UV-induced mitotic crossing-over. The strains were therefore isogenic with their respective parental strains except for the chromosome arm distal to the

tsd 1–1 locus. The parental strains were used as controls because *tsd* 1–1 is recessive (Unrau, 1968).

I-1:

$$\frac{nar\ 1\text{–}6}{nar\ 1\text{–}1}\ \frac{tsd\ 1\text{–}1}{+}\ \frac{+}{rec\ 1\text{–}1}\ \frac{+\quad +}{ad\ 1\text{–}1\ me\ 1\text{–}2}\bigcirc\ \frac{+\quad +}{nic\ 1\text{–}1\ inos\ 1\text{–}3}\bigcirc\ \frac{pan\ 1\text{–}1\ a_1}{+\quad a_2}\bigcirc\ \frac{b_2}{b_1}$$

H-54

$$\frac{nar\ 1\text{–}6}{nar\ 1\text{–}1}\ \frac{tsd\ 1\text{–}1}{tsd\ 1\text{–}1}\ \frac{+}{rec\ 1\text{–}1}\ \frac{+\quad +}{ad\ 1\text{–}1\ me\ 1\text{–}2}\bigcirc\ \frac{+\quad +}{nic\ 1\text{–}1\ inos\ 1\text{–}3}\bigcirc\ \frac{pan\ 1\text{–}1\ a_1}{+\quad a_2}\bigcirc\ \frac{b_2}{b_1}$$

M 146:

$$\frac{tsd\ 1\text{–}1}{tsd\ 1\text{–}1}\ \frac{nar\ 1\text{–}12\ nir\ 1\text{–}1}{nar\ 1\text{–}13\ nir\ 1\text{–}1}\ \frac{nic\ 1\text{–}2\ inos\ 1\text{–}3}{+\quad inos\ 1\text{–}3}\bigcirc\ \frac{a_2}{a_1}\bigcirc\ \frac{b_2}{b_1}$$

The nomenclature for *Ustilago maydis* mutants has been altered to correspond to that used in other fungi. The first number represents the locus or gene, and the second the allele isolation number.

Previous designation	New designation	Phenotype
tsd-1	*tsd* 1–1	Temperature-sensitive with regard to DNA synthesis
uvs-1	*rec* 1–1	Radiation-sensitive, recombination deficient
ad-1	*ad* 1–1	Adenine-requiring
me-15	*me* 1–2	Methionine requiring
nic-3	*nic* 1–1	Nicotinic acid requiring
	nic 1–2	Nicotinic acid requiring
inos-3	*inos* 1–3	Inositol requiring
pan-1	*pan* 1–1	Pantothenic acid requiring
nar-6	*nar* 1–1	Require nitrite or ammonium (mutants in structural gene for nitrate reductase)
nar-13	*nar* 1–6	
	nar 1–12	
	nar 1–13	
nir-1	*nir* 1–1	Requires ammonium (lacks nitrite reductase)

a_1, a_2, b_1 and b_2 are mating-type alleles.

(ii) *Media*

As in Unrau & Holliday (1970); minimal and complete media for strain M 146 were supplemented with inositol at all stages of growth and testing.

(iii) *Growth conditions*

tsd 1–1 homozygotes were grown in liquid NM supplemented with 0·3% ammonium sulphate at 22 °C and heat-treated at 32 °C, the restrictive temperature, in one of two ways. Cells were either plated on pre-warmed plates and incubated at 32 °C followed by recovery at 22 °C, or the whole log-phase culture was shifted to 32 °C in a water-bath and cells were then plated on 22 °C plates for subsequent recovery. Combined heat and UV treatments were made by irradiating the heat-

treated cells after plating. Survival and intergenic recombination was scored on CM plates and allelic recombination was scored on washed heat-treated cells plated on NM.

(iv) *UV irradiation*

Cells were irradiated on plates using a Hanovia germicidal UV lamp delivering between 45 and 51 ergs/mm^2/sec at a distance of 15 cm. Doses were timed with a shutter and precautions were taken to prevent photoreactivation.

(v) *Scoring recombinants*

Mitotic recombination was scored using the standard techniques developed for *U. maydis*. Treated cells were plated on CM, replica plated on to ammonium supplemented minimal medium, and non-growing replicas were picked on to CM master plates. When *rec* 1–1 was segregating, the replicas were irradiated with 15 sec UV, as the *rec* 1–1 phenotype includes extreme sensitivity to UV (Holliday, 1967). The master plates were replicated to a single subtraction series to identify the precise phenotypes of the segregants, with an irradiated CM plate to score *rec* 1–1. A proportion of all survivors after heat-treatment were small slow-growing colonies which were not scored for mitotic recombination by replica plating. A few such small colonies were picked, streaked and subsequent clones were examined for phenotypic segregation by replica plating.

Allelic recombination was scored in two ways. In strain I-1 and its derivative, H-54, thrice-washed cells were plated on NM after the relevant heat-treatments and irradiated if necessary. Convertants which arose after 6 days at 22 °C were scored by direct counting. Populations of up to 10^7 cells per plate could be scored this way. All experiments were carried out on log-phase cells, concentrated during washing by centrifugation, and as all washings and heat-treatments were carried out in NM, no change of cell state to stationary phase would be expected, nor was any observed. In strain M 146 allelic recombination was measured directly by examining the production of nitrite from nitrate by functional nitrate reductase, formed as a result of recombination in the nitrate reductase structural gene during heat-treatment. Using 50 ml aliquots of supernatant evaporated to 5 ml before the addition of reagents, it proved possible to measure a recombination rate of 10^{-5} at the limits of resolution of the method. Cells were removed from the supernatant by centrifugation and filtration, but a considerable amount of scatter was still observed. Reagent concentrations and reaction conditions were as in Holliday (1971). NO_2 concentrations were read on a Zeiss spectrophotometer at 540 nM.

3. RESULTS

Strains I-1 and H-54 showed no detectable difference in spontaneous allelic recombination at 22 °C. Incubating strain I-1 at 32 °C caused no detectable change in spontaneous rates of either allelic or intergenic mitotic recombination. Therefore any differences observed in the largely isogenic H-54 strain represent the effects on recombination of the homozygous *tsd* 1–1 locus. The spontaneous intergenic

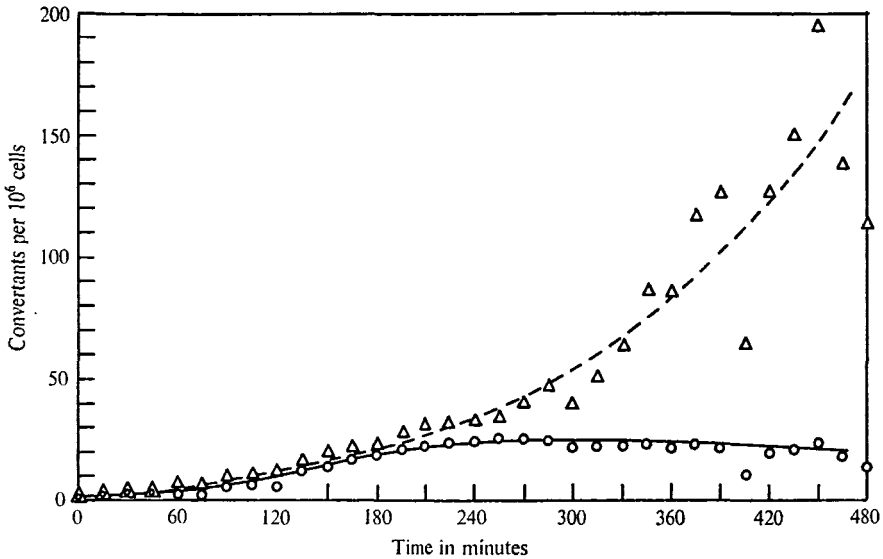

Fig. 1. Allelic recombinants arising after heat-treatment of strain H-54, on a per survivor (△- - - -△) and an absolute (○——○) basis. Convertants were scored after incubation on NM plates at 32 °C for the times shown, and then at 22 °C until convertants had grown into colonies.

recombination rate of strain I-1 was 0·1 %, i.e. within the range of strains studied previously (e.g. Holliday, 1964, 1967).

We observed no spontaneous intergenic recombination in strain H-54 grown and plated at 22 °C. The summed control of 26,770 colonies excludes a recombination rate of greater than 0·02 % (5 % upper fiducial limit) and in this respect strain H-54 clearly differs from the parental strain.

Incubating H-54 at 32 °C was found to influence both intergenic and allelic recombination. Fig. 1 shows allelic recombination in a population plated on NM at 32 °C for varying intervals of time. Survival was measured on a subset of cells plated on CM and convertants per survivor calculated accordingly. There is an increase in the absolute numbers of recombinants until after 270 min of heat-treatment, while on a per-survivor basis the increase is made much more dramatic. Intergenic recombination can only be measured by replica plating in *Ustilago* so fewer heat-treatment times were observed, but in Table 1 we show the increase in recombination after heat-treatment in two aggregated experiments and the phenotypes observed. Compared with the absence of recombinants at 22 °C, a very significant increase in crossing-over was observed for H-54, with the rate increasing to above the normal spontaneous mitotic crossing-over rate of the heterozygous parental strain I-1.

The effects of UV on allelic and intergenic recombination was examined in several experiments and both the parental and *tsd* 1–1 strains were normal at 22 °C. Fig. 2 compares allelic recombination induced in strains I-1 and H-54 and shows UV survival as well; the results are similar to those observed previously

Table 1. *Mitotic crossing-over induced in strain H-54 by heat-treatment at 32 °C*

Time at 32 °C (min)	Survival (%)	Colonies scored	Segregants	Recombination (%)
0	100·0	26770	0	0·02*
120	96·5	3200	1	0·03
240	70·0	3820	7	0·18
360	47·0	4220	14	0·33
480	16·5	8010	23	0·29

Phenotypes segregating:

ad	*me*	*ad me*	*nic*	*in*	*nic in*	*pan*	*uvs*	*doubles*
4	0	4	11	0	15	3	6	2†‡

* 5% upper fiducial limit.
† *ad me, uvs*
‡ *pan, uvs.*

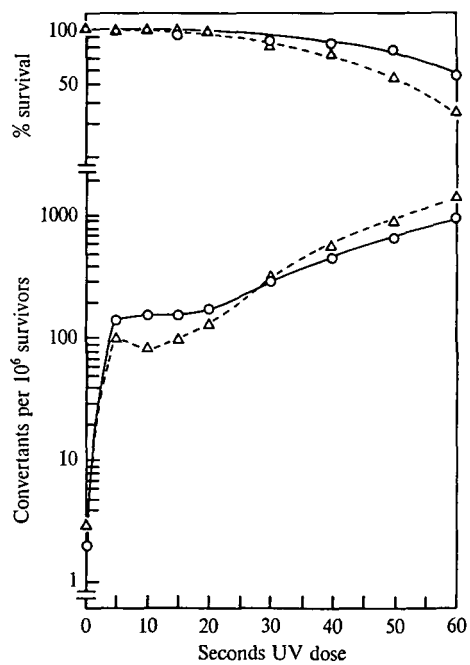

Fig. 2. UV-induced allelic recombination and lethality in a wild-type (○——○) strain I-1 and in a temperature-sensitive (△- - -△) strain, H-54, grown, irradiated and plated at 22 °C.

(e.g. Holliday 1967). In Table 2 the phenotypes and frequencies of UV-induced intergenic recombination is shown for both strains.

We earlier reported a synergistic killing when heat-treatment is followed by UV (Unrau & Holliday, 1970). A similar synergism was observed when we measured mitotic recombination. Fig. 3 shows allelic recombination of a log-phase population

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Table 2. *UV-induced mitotic recombinants in strains heterozygous and homozygous for tsd 1–1*

Strain and treatment	Dose (ergs/mm²)	Colonies scored	Survival (%)	Segregants	%
I-1 control	0	5200	100	5	0·10
I-1+UV	3000	11060	55	60	0·54
H-54 control	0	26770	100	0	0·02*
H-54+UV	3000	5870	60	18	0·30

	Phenotypes segregating								
ad	*me*	*ad me*	*nic*	*in*	*nic in*	*pan*	*uvs*†	*ts*	*doubles*
7	4	2	8	0	17	3	12	12	0
2	4	2	2	0	5	0	3	—	0

* 5 % upper fiducial limit.

† The *rec* 1–1 phenotype includes extreme UV sensitivity and is scored by irradiating a CM replica.

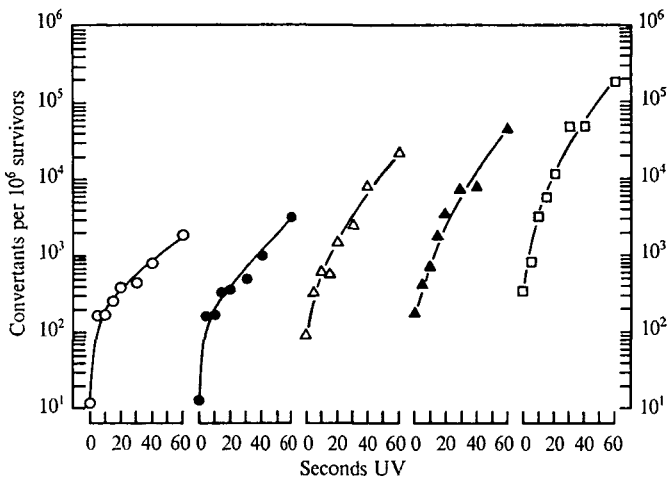

Fig. 3. The combined effects of heat-treatment and UV irradiation on the induction of mitotic allelic recombination in strain H-54. Times at 32 °C before irradiation and subsequent incubation at 22 °C are: ○——○, 0 h; ●——●, 2 h; △——△, 4 h; ▲——▲, 6 h; □——□, 8 h. Survival was as in Unrau & Holliday (1970), fig. 6A.

washed 3 times, incubated at 32 °C in NM, plated on NM and irradiated with the doses shown. The experiment was repeated twice with similar results. Survival was measured on a subset plated on CM and irradiated similarly. There is a rise in the numbers of convertants for heat-treated cells until between 240 and 360 min, when cell killing begins to outweigh this effect. Mitotic crossing-over was also affected synergistically and in Table 3 the phenotypes and frequencies of segregants are given.

Among the survivors of 480 min heat-treatment of strain H-54 we noticed a large proportion of minute slow-growing colonies. These were not scored for intergenic recombination directly, but were cloned to examine single cell isolates for

Table 3. *Mitotic segregants induced in strain H-54 both heat-treated and UV-irradiated*

Time at 32 °C (min)	UV dose (ergs/mm²)	Survival (%)	Colonies scored	Segregants	%
0	3000	65	1970	6	0·30
240	3000	8	2190	11	0·55
480	1500	3·3	1400	32	2·30
480	3000	1·2	1063	19	1·80

	Phenotypes segregating								
Time at 32 °C (min)	*ad*	*me*	*ad me*	*nic*	*in*	*nic in*	*pan*	*uvs*	*doubles*
0	0	2	2	1	0	1	0	0	0
240	0	0	0	1	0	7	2	0	1*
480	1	1	11	6	0	12	4	10	3†‡§

* *me, uvs.*
† *pan, uvs.*
‡ *pan, uvs.*
§ *nic in, ad me.*

Table 4. *Putative aneuploids after heat-treatment of H-54 for 480 min at 32 °C*

Colonies examined		
No.	No. segregating	Segregants
28 (variable)	12	7 *nic in*; 1 *nic in pan* 1 *ad me nic in*; 2 *ad me*; 1 *pan*
24 (homogeneous)	3	2 *nic in*; 1 *ad me**

* This exceptional colony segregated only auxotrophic cells.

phenotypic requirements. As can be seen from Table 4, two types of initial minutes were present, giving rise to either variable or homogeneous single cell isolates. Fifteen out of 52 minute colonies segregated phenotypic requirements, and of these, 14 out of 15 segregated both phenotypic requirements and wild-type cells from the same initial isolate. The results of this limited survey suggest that whole chromosome segregants, probably aneuploid multisomics, are generated on recovery from heat-treatment, resulting in a high frequency of linked marker segregation. Holliday (1961) had previously observed UV-induced aneuploidy in *U. maydis* characterized by absence of breakdown to haploids. As not all chromosomes are marked in strain H-54, a proportion of aneuploids segregating only wild types would be expected.

The time of allelic recombination was determined in strain M 146 by incubation at 32 °C and measurement of the accumulation of nitrite in the supernatant. We observed considerable variability as the numbers of convertants was low and evaporation of 50 ml of supernatant to 5 ml introduced further error. However,

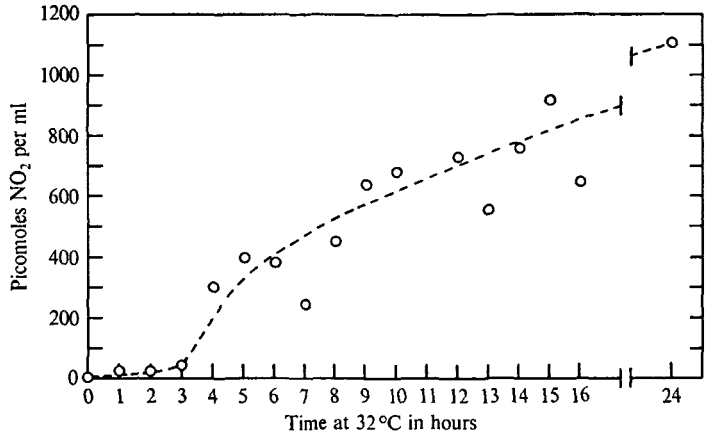

Fig. 4. Production of NO_2 by recombinant and active nitrate reductase in strain M 146, during incubation at 32 °C in nitrate minimal medium. Per cell amounts were calculated on the basis of no cell increase from the initial inoculum.

Fig. 4 shows the increase in NO_2 per cell when corrected for the increase observed in control populations grown at 22 °C. Holliday (1971) observed little NO_2 increase when a non-temperature-sensitive strain was grown at 32 °C and the observed increase therefore reflects an increase in recombination within the *nar* 1–1 locus at 32 °C. A number of experiments gave the same result, and by 24 h the amount of NO_2 per cell was 2–3 times higher than in the control, even though by 24 h less than 0·1 % of *tsd* 1–1 cells would have survived (Unrau, 1968).

From this result we conclude that the allelic and intergenic recombination measured in standard ways (Figs. 1, 3) reflects the effect of a transient block in DNA synthesis at 32 °C.

4. DISCUSSION

The main findings of our studies with *tsd*-1 diploids are that a temperature-induced block in DNA replication stimulates recombination, and this recombination actually occurs during the period of inhibition at 32 °C rather than during recovery at 22 °C. We are able to demonstrate this by detecting nitrate reductase produced by recombinant genes during the period when DNA synthesis is blocked. A small amount of DNA synthesis or turnover during or subsequent to recombination cannot be excluded, but it is clear that chromosome replication *per se* is unnecessary for mitotic recombination. This recombination must therefore occur by breakage and reunion of chromosomes. It is also known that the cells which recombine contain the 4C amount of DNA, therefore the recombination we are observing occurs at the post replicative four-strand stage. Thus, our observations on mitotic recombination are comparable with several studies of meiosis which show that recombination occurs by breakage and reunion after the premeiotic S period (Taylor, 1965; Henderson, 1966; Rossen & Westergaard, 1966; Chiang & Sueoka, 1967; Peacock, 1970). They also agree with classical genetical studies with *Aspergillus nidulans* (Pontecorvo, 1958), which make it clear that mitotic re-

combination occurs at the four-strand stage. Somewhat different results have recently been obtained with heteroallelic diploids of yeast by Wildenberg (1970). By pedigree analysis, she obtained strong evidence that X-ray-induced gene conversion and reciprocal exchange most frequently occurs at the two-strand pre-replicative stage. However, this observation in itself also shows that mitotic recombination does not occur during replication, and therefore to this extent is in agreement with ours.

The observation that a block in DNA synthesis stimulates recombination is in line with many others in both fungi and bacteria. For instance, Joshi & Siddiqi (1968), using methods very similar to ours, showed that allelic recombination proceeded at enhanced rates when DNA synthesis was blocked in a temperature-sensitive mutant of *Escherichia coli*. Gallant & Spotswood (1965) inhibited DNA synthesis by thymine starvation and also observed a stimulation of recombination in partial diploids of the same organism. Conditional mutants of bacteriophage T 4 which are defective in DNA synthesis also recombine at higher than normal frequency (Bernstein, 1967). In fungi a variety of agents which affect DNA synthesis have been found to increase the frequency of mitotic recombination. Apart from UV light and ionizing radiation, mitomycin C and 5-fluorodeoxyuridine do so (Holliday, 1964; Esposito & Holliday, 1964), as well as a variety of nitrosamides and other mutagens (Zimmermann, Schwaier & Van Laer, 1966). Guerola, Ingraham & Cerdá-Olmedo (1970) have recently shown that the initial effect of the nitrosamide *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine is to cause a complete block in DNA synthesis. It is possible that when DNA synthesis is blocked, by whatever mechanism, genetic damage results, perhaps as a result of a small amount of abortive or defective synthesis, or the failure to repair spontaneous lesions. A proportion of this damage might then be repaired by a mechanism which involves recombination between sister or homologous chromosomes (Howard-Flanders, 1968; Witkin, 1969). This interpretation of the stimulation of recombination in *tsd*-1 diploids is supported by several observations. Exposure to 32 °C for more than 4 h results in a exponential loss in viability. Cells which are held at this temperature become progressively more sensitive to the lethal effects of UV light, as would be expected if the repair mechanisms are already partially saturated and cannot deal efficiently with additional damage. Furthermore, if an excision repair mechanism is unable to cope with both temperature and radiation-induced damage, then a larger than normal proportion of lesions may be repaired by the presumed recombination pathway of repair. This would account for the observed synergism in the recombination effect of the two treatments. Several features of the *tsd*-1 phenotype resemble that of thymine-starved cells of bacteria (see Unrau & Holliday, 1970). Friefelder (1969) has suggested that thymineless death could be attributed to a defect in polynucleotide ligase function caused by an excessive level of dATP. It is possible that *tsd*-1 may have altered or defective ligase, leading to the accumulation of single strand nicks or Okazaki fragments. Studies are in progress to see whether such nicks or fragments accumulate at 32 °C.

Alternatively, the phenotype of *tsd*-1 can be explained on the basis of a block in

mitotic division (see Unrau & Holliday, 1970), which in turn prevents genetic replication. Hartwell (1971) suggests that this mutant is analogous to those he has studied in yeast which are blocked in cell division. The cells held several hours at 32 °C increase their volume several times but contain only one nucleus. They are not dissimilar in appearance to the filamentous promycelium from the germinating brandspore in which a single diploid nucleus undergoes meiosis. It is possible that the altered DNA/protein ratio or an accumulation of precursors of DNA synthesis induce an abortive meiotic-like state, together with the synthesis of recombination enzymes which are normally absent from the mitotic cell. Evidence has been presented elsewhere for the induction of a recombination repair mechanism in Ustilago after irradiation (Holliday, 1971; Holliday & Resnick, 1970; and in preparation). It may be that the genetic block in *tsd*-1 has the same effect, but in this case the recombination which subsequently occurs need not necessarily be playing a role in repairing damage in DNA. The production of large numbers of slow-growing aneuploids amongst the survivors of heat-treatment, and their characteristics, perhaps support the argument that a meiotic-like condition has been induced. Unlike aneuploids arising after irradiation (Holliday, 1961*b*, and unpublished observations), those from *tsd*-1 diploids segregate both unmarked and marked homologous chromosomes; moreover, more than one homologous pair may segregate (see Table 4). The exact genetic status of these aneuploids has not been determined, but they are likely to be trisomics. In addition, there is a strong indication that in cells which have undergone mitotic crossing-over, there is a greater chance that multiple exchanges will occur than is the case with radiation-induced recombination.

The two alternative explanations for the stimulation of recombination in *tsd*-1 diploids which have been suggested are not mutually exclusive. It is possible that damage to DNA in the heat-treated cells in some way induces a recombination repair mechanism, as is believed to occur after irradiation, and to this extent at least the cells are in a meiotic-like state. Experiments are in progress to attempt to determine whether the recombining *tsd*-1 cells contain a protein or proteins absent from mitotic cells at 22 °C, but present in the promycelium where meiosis takes place.

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