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A search for temperature-sensitive mutants of *Ustilago maydis* blocked in DNA synthesis

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SUMMARY

Over 400 temperature-sensitive mutants of *Ustilago maydis* have been tested for DNA synthesis at the restrictive temperature of 32 °C by measuring ^{14}C adenine incorporation into DNA and RNA. Five mutants were defective in DNA synthesis but none was completely blocked. One mutant, *tsd*-1, which is unlinked to the others, forms long uninucleate filaments at 32 °C which die exponentially after 4 h temperature treatment. The phenotype is comparable to that of thymine-starved bacteria, but it is possible that rather than being specifically defective in DNA synthesis the mutant is blocked in nuclear division.

1. INTRODUCTION

Although fungi provide excellent experimental material for genetic studies on recombination, they have not so far been successfully used to study related cell processes such as genetic replication and repair, an understanding of which may well be necessary before the mechanism of recombination can be fully explained. This lack of progress can be attributed very largely to the difficulty of specifically labelling DNA with radioactive tracers, owing to the absence of known DNA precursors. Thymine or thymidine mutants have never been isolated in any fungus, and neither substance is specifically incorporated into the DNA of wild-type cells of several species (Chakraborty & Loring, 1960; La Cour, Martin & Holliday, 1960; Fink & Fink, 1962; Grivell & Jackson, 1968; D. H. Williamson, personal communication). Nor are specific inhibitors of DNA synthesis known, since those which have been used—such as mitomycin C or 5-fluorodeoxyuridine (FUDR)—do not prevent residual synthesis or recovery from a period of inhibition (Williamson & Scopes, 1962; Esposito & Holliday, 1964).

One possible approach is to isolate conditional lethal temperature-sensitive mutants which are able to synthesize DNA at the permissive temperature whilst being incapable of doing so at the restrictive temperature. Temperature-sensitive indispensable (*tsi*) mutants were first isolated in micro-organisms by Horowitz & Leupold (1951), and they were subsequently used extensively in phage genetics. In principle, it should be possible to isolate such a mutant in any gene which specifies a protein. Mutants specifically blocked in DNA synthesis have been

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isolated in *Escherichia coli* (Bonhoeffer, 1966; Hirota *et al.* 1968) and *Bacillus subtilis* (Mendelsohn & Gross, 1967). The aim of the present study was to isolate such mutants in *Ustilago maydis*. By using temperature-sensitive mutants blocked in DNA synthesis it should be possible to gain information about the relationship of genetic recombination to replication; to determine, for instance, whether or not recombination can occur in the absence of DNA synthesis (see Holliday, 1968). The mutants which have been isolated (*tsd* mutants) are strongly affected in DNA synthesis at the restrictive temperature, although in no case is there a complete block in synthesis. During the course of the work, similar mutants were reported in yeast by Hartwell (1967).

2. METHODS

(i) *Strains*

All mutants were derived from a haploid wild type of mating type a_2b_1 or from an adenine auxotroph, *ad*-1, of the same mating type. To obtain mutant strains of opposite mating type they were crossed with *pan*-1 *nar*-13 a_1b_2, which requires pantothenic acid and nitrite or ammonium salts, and the appropriate progeny were detected amongst the random products of meiosis (see Holliday, 1961). Crosses between mutant strains were analysed by the same method. Mutant *tsd*-1 was also incorporated into a heterozygous diploid, from which strains homozygous for *tsd*-1 and *ad*-1 were derived. The genotype and use of this diploid in recombination studies will be described in a subsequent paper.

(ii) *Media and growth conditions*

Minimal medium (NM) was the same as previously described (Holliday, 1961) except that the nitrogen source was 0·3 % KNO_3. Complete medium (CM) had the same additions to NM as previously described, but a semi-enriched version (SCM) lacking hydrolysed nucleic acid was used for testing mutants.

Previously, *U. maydis* was incubated routinely at 30 °C. In these experiments 22 °C was chosen as the permissive and 32 °C as the restrictive temperature.

Cell concentrations were determined with a Model A Coulter particle counter. Survival after heat or U.V. treatment was determined by embedding cells in molten CM. Other microbiological or genetical techniques were as previously described, modified to take account of the temperature sensitivity of the mutant strains.

(iii) *Cytology*

Nuclei were stained with Giemsa, according to the method of Williamson (1965). They could also be detected by phase contrast microscopy. Cell volumes were determined with the Coulter counter.

(iv) *Mutation induction*

Log.-phase cultures growing at 22 °C in liquid NM (or NM plus adenine) were chilled to 15 °C. A fresh solution of 1 mg/ml. *N*-methyl-*N*-nitro-*N*-nitrosoguanidine

(nitrosoguanidine) was added to give a final concentration of 100 μg/ml. Mutagen treatment was ended by a hundredfold dilution and the cells spread on CM at about 200 survivors per plate. Plates were incubated at 22 °C until the colonies were suitable for replica plating to NM (or NM plus adenine). The replicas were incubated at 32 °C. Colonies producing non-growing replicas were picked to CM grid plates and the colonies retested and classified into major groups by replicating to NM and CM at 22° and 32 °C.

(v) *Screening* tsi *mutants*

Williamson (1965) used tritiated adenine to follow the course of DNA replication in *Saccharomyces cerevisiae* by means of autoradiography. The same approach was used here in developing a rapid method for measuring the relative incorporation of labelled adenine into RNA and DNA. Mutant cells were grown to stationary phase in SCM at 22 °C, diluted into radioactive SCM supplemented with ^{14}C adenine (specific activity 1 μc per 0·1 μM; 0·74 μc per ml). The strains were incubated at 32 °C for 8 h with vigorous shaking and then harvested on to 2·1 cm Whatman GF/A glass fibre disks by vacuum filtration; all extractions and washes were made on the disks to eliminate losses in centrifugation. The residual radioactivity could be counted directly on the disks (Davies & Cocking, 1966).

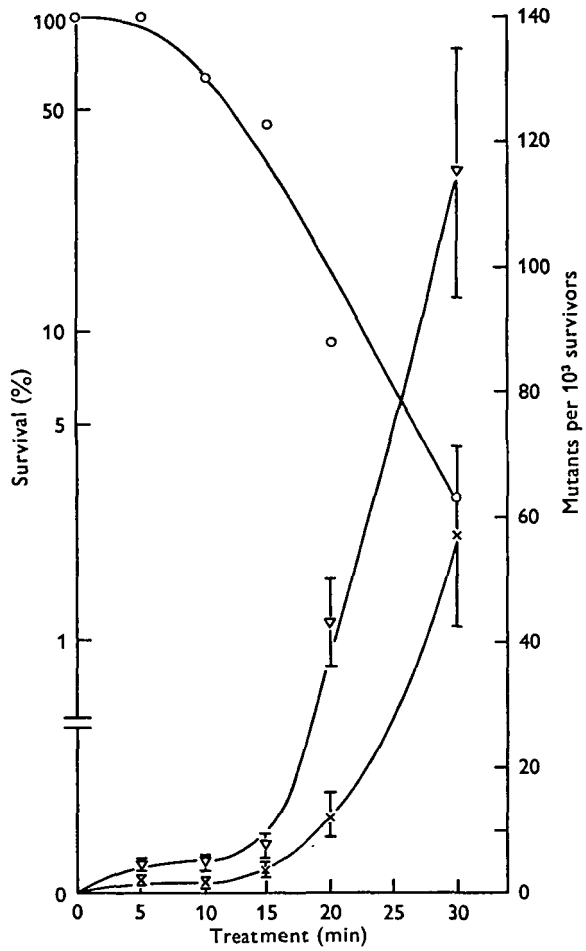
Acid-soluble material was removed by extraction for 2 h in ice-cold 0·2 N-PCA followed by a rinse with 96 % ethanol. RNA was removed by enzymic extraction in 2 ml RNAase solution (Sigma type III-A), at 250 μg per ml in 0·02 M phosphate buffer, pH 7·6. After 2 h digestion at 32 °C 0·2 ml of the resulting extract was dried on to a GF/A disk to determine RNA-bound activity. Any residual RNA was removed by a further 2 h extraction in 2 ml 1 N-NaOH at 32 °C. The samples were then chilled ice-cold and acidified with 0·2 ml 11 N-PCA to precipitate nascent DNA, and finally rinsed in 96 % ethanol. The disks containing DNA-bound and RNAase extracted activity were then dried over an infra-red lamp and counted in 0·2 ml toluene-based scintillation fluid. After correcting for quench by the channels ratio method the resulting CPM values were used to compute the DNA/RNA ratios for each mutant.

Mutants classified as deficient in DNA synthesis at 32 °C were further tested by rapidly shifting log.-phase populations grown at 22° to 32 °C and sampling in triplicate between 1 and 2×10^8 cells at 3 h intervals. Gross chemical synthesis of DNA, RNA and protein were examined using a modified Ogur & Rosen (1951) technique. Relative DNA increase was measured using Burton's (1956) modified diphenylanine reaction. RNA increase was measured using the orcinal reaction after Hurlbert *et al.* (1954). Protein increase was estimated using the Folin–Ciocalteus reaction according to Lowry *et al.* (1951).

3. RESULTS

(i) *Isolation of mutants*

Over 600 mutants were isolated in the wild-type strain and 400 in the *ad*-1 strain. The major classes of mutant which were identified are given in Table 1, together



Text-fig. 1. Survival and mutation induction in log.-phase *ad* − 1 cells treated with NG. Bars indicate standard errors of all mutants (▽—▽) and of t.s.i. mutants (×—×). (○—○) Survival.

Table 1. *Types of mutant obtained after nitrosoguanidine treatment of the wild-type strain*

Growth response				Mutants	
Complete medium		Minimal medium			
22 °C	32 °C	22 °C	32 °C	Number*	%
+	+	+	+	–	– (wild type)
+	+	–	–	179	35·0 (auxotrophs)
+	–	–	–	96	18·8 (complex)
+	+	+	–	77	15·1 (t.s. auxotrophs)
+	–	+	–	159	31·1 (t.s. indispensable)
			Total	511	100·0

* Totals for four separate mutation runs.

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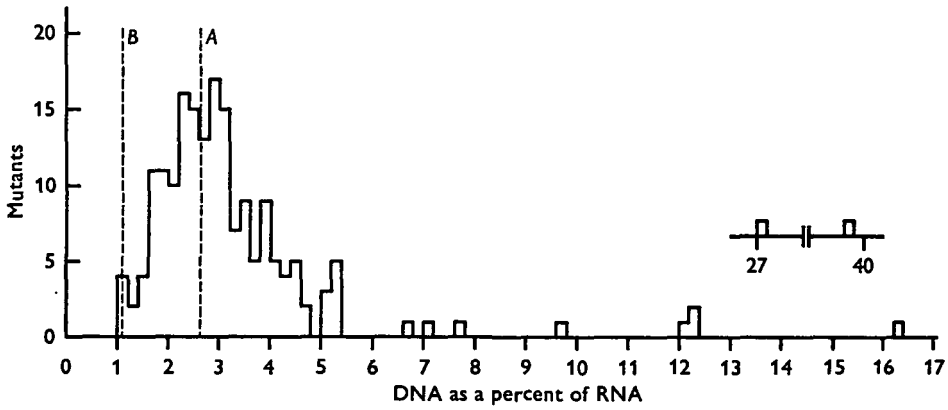
with combined totals from four independent experiments with the wild type. Auxotrophic and temperature-sensitive indispensable (*tsi*) mutants were the commonest, and temperature-sensitive auxotrophs the least common. There was a surprisingly high frequency of mutants which grew only on complete medium at low temperature. Some of these could be double mutants, as there is a report that NG acts on



Text-fig. 2. DNA/RNA incorporation ratios of 183 t.s.i. mutants derived from wild-type cells; line *A* represents the mean wild-type control ratio and line *B* represents the mean ratio of five trials of *tsd*-1.

Table 2. *DNA/RNA incorporation ratios at 32 °C of temperature-sensitive indispensable mutants tentatively classified as being blocked in DNA synthesis*

Mutant no.	Locus	CPM × 10^{-3} DNA	CPM × 10^{-3} RNA	DNA % of RNA
ts-115	*tsd*-1*	3·8	313·4	1·21
ts-84	—	2·7	227·4	1·19
ts-207	—	4·8	411·2	1·18
ts-220	—	7·0	451·0	1·41
ts-92 ad-1	—	0·4	608·0	0·06
ts-197 ad-1	—	3·4	535·0	0·64
Wild-type control†	—	21·2	808·0	2·63

* *tsd*-1 is not allelic to any other DNA⁻ts mutant yet isolated.

† This DNA/RNA ratio represents the sum of several trials of wild-type cells at 32 °C.

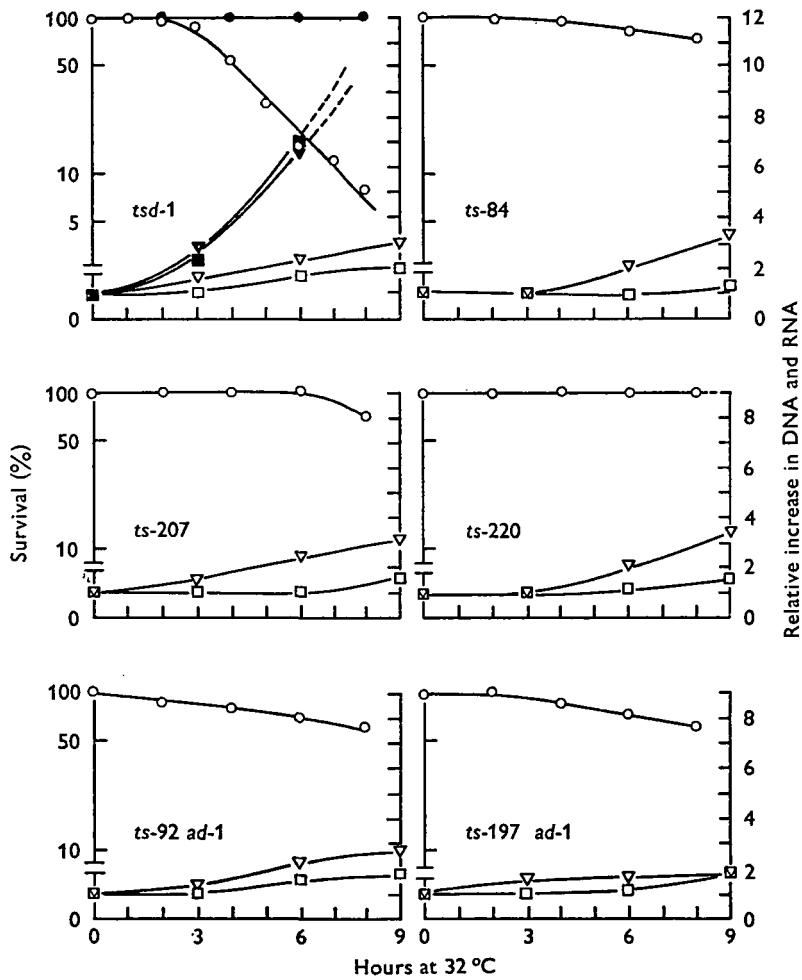
the replication point of DNA and is therefore likely to induce more than one mutation in the same cell (Cerdá-Almeda, Hanawalt & Guerola, 1968); alternatively they might be mutants defective both in the synthesis and uptake of small metabolites.

Text-fig. 1 shows the result of an experiment with *ad*-1 in which the time of treatment with NG was varied. In this case only the temperature-sensitive and auxotrophic mutations were scored. Both survival and mutation induction follow a 'multi-hit' curve, which suggests that at high doses the repair system becomes saturated. The highest mutation frequency which was recorded was 11 % per

survivor, which is a considerable improvement on the best yield after UV treatment, which is about 1 % (R. Holliday, unpublished data).

(ii) *Screening temperature-sensitive mutants*

The incorporation of ^{14}C adenine into DNA and RNA was determined for each *tsi* mutant. The overall results for a batch of 183 mutants isolated in the wild-type strain are shown in Text-fig. 2. In a number of experiments with non-mutant cells the ratio of incorporation into DNA to that into RNA was 2·5 %. Mutants with a ratio of less than 1·5 % were selected for retesting. It can be seen from Text-fig. 2



Text-fig. 3. Survival, RNA and DNA synthesis of mutants putatively blocked in DNA synthesis at 32 °C. Triplicate samples from log.-phase cultures switched to 32 °C were extracted to determine RNA biosynthesis. Relative increase in DNA (□—□) and RNA (▽—▽) are indicated in the lower vertical scale on the left and the vertical scale on the right; % survival (○—○) is shown in the upper vertical scale on the left. A wild-type control (solid symbols) is shown on the graph for *tsd*-1 (see also Text-fig. 5).

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that some mutants appear to be defective in RNA synthesis (*tsr* mutants); these were not examined further. In all, over 400 *tsi* mutants were screened by this technique.

Table 2 shows those mutants which were tentatively classified as blocked in DNA synthesis on the basis of their DNA/RNA incorporation ratios at 32 °C. It is clear that such mutants differ widely from wild type in incorporation ratios, even though substantial incorporation occurs. In Text-fig. 3 the amount of DNA and RNA synthesis of log.-phase cultures switched to 32 °C was determined chemically. Any discrepancy between these results and the isotopic labelling experiments may reflect the differences between log.-phase and stationary-phase cultures but this point was not investigated. The four mutants derived from the wild type all seemed to be defective in DNA synthesis whilst showing considerable increase in RNA, but only one of the two derived from *ad*-1 fell into this category. The other appeared to be initially misclassified as it was equally deficient in RNA and DNA. Protein synthesis was also determined (see Unrau, 1968); although in all cases this increased, it was not always in parallel with RNA synthesis. In mutants *tsd*-1 and *ts*-84 the increase was significantly less than that of RNA, whereas in mutant *ts*-207 it was significantly more. It is not surprising that all the mutants showed subnormal RNA and protein synthesis in comparison to the control, since none of the mutants were actively dividing at the restrictive temperature.

Since it was thought that mutants blocked in DNA synthesis might be so unbalanced in growth as to be unable to survive at 32 °C, their viability during an 8 h incubation period was also examined. These survival curves are also shown in Text-fig. 3. After a lag period *tsd*-1 cells did become inviable (see also Text-fig. 6*A*), but the other mutants were less affected or unaffected by this treatment.

(iii) *The properties of* tsd-1

This strain when transferred from the permissive to the restrictive temperature forms very long filaments which die exponentially after a lag of about 4 h. Since thymine starvation of bacteria induces the same phenotype (Cohen & Barner, 1954), it was thought that *tsd*-1 might be the most interesting to examine in more detail, and it was also used in recombination studies (Unrau, 1968; P. Unrau & R. Holliday, in preparation).

Almost half the cells of log.-phase populations of *tsd*-1 growing at 22 °C possess buds, and of these 45 % are binucleate. On shifting to 32 °C, it appears that only

Table 3. *Distribution of buds and nuclei in a* tsd-1 *culture after incubation at* 32 °C

(Based on observations of at least 400 cells per sample.)

	Hours at 32 °C		
	0	2	4
% budded cells	49·5	81·8	99·7
% binucleate cells	22·0*	3·1	0

* All binucleate cells were budded.

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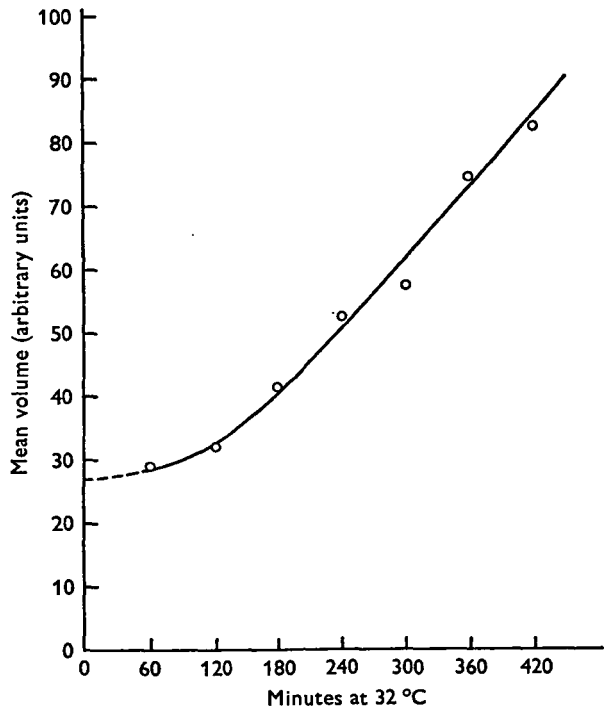
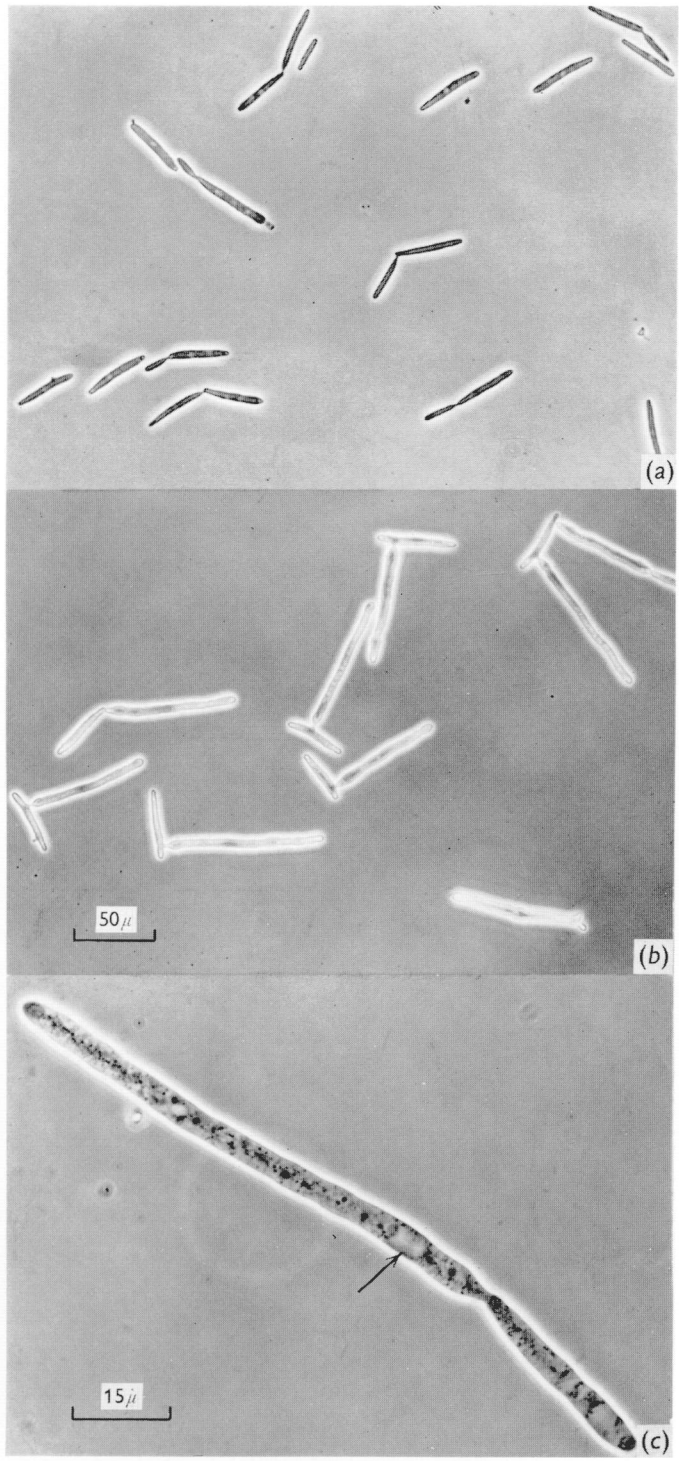
the binucleate cells divide to give a consistently observed overall increase in cell numbers of 20–30 %. During incubation at 32 °C all the cells eventually produce buds, but all remain uninucleate. These results are summarized in Table 3. The parent cell does not increase in size, but the bud elongates. Coulter counter measurements show that the total cell volume increases linearly with time (see Text-fig. 4).



Text-fig. 4. Increase in volume of stationary-phase *tsd*-1 cells diluted into fresh CM at 32 °C. Cell volumes were determined by Coulter counter and cell numbers did not rise appreciably during this experiment.

Both Giemsa staining and phase contrast microscopy demonstrate that the nucleus passes into the bud and usually comes to rest just above the neck. The appearance of the cells after incubation at 32 °C is shown in Plate 1. Elongation of the cells does not continue indefinitely, as the cells begin to lyse at the tip after about 6 h, and almost all are lysed after 18 h ($< 0{\cdot}01$ %) survivors.

These observations make it easier to interpret the measurements of DNA and RNA synthesis at 32 °C. A detailed experiment is shown in Text-fig. 5. In the control population, cell numbers, RNA and DNA show a parallel exponential increase. In the *tsd*-1 population RNA synthesis begins at a similar rate to the control, but the rate slows down after 3 h and after 6 h the total RNA may even decrease, presumably owing to cell lysis. A slow continual rise in DNA occurs, reaching in several experiments 1·6–1·8 times the initial amount. From what is known of the division cycle in *U. maydis* (Holliday, 1965) it can be calculated that if log.-phase populations all move into the G_2 period (i.e. they all have the 2C



Phase contrast photographs of diploid *tsd*-1 cells grown at 22 °C (*a*), and after 6 h incubation at 32 °C (*b*) showing elongated bud cells. A single cell after 8 h incubation at 32 °C (*c*) shows the single nucleus (arrowed), with nucleolus, at the proximal end of the bud.

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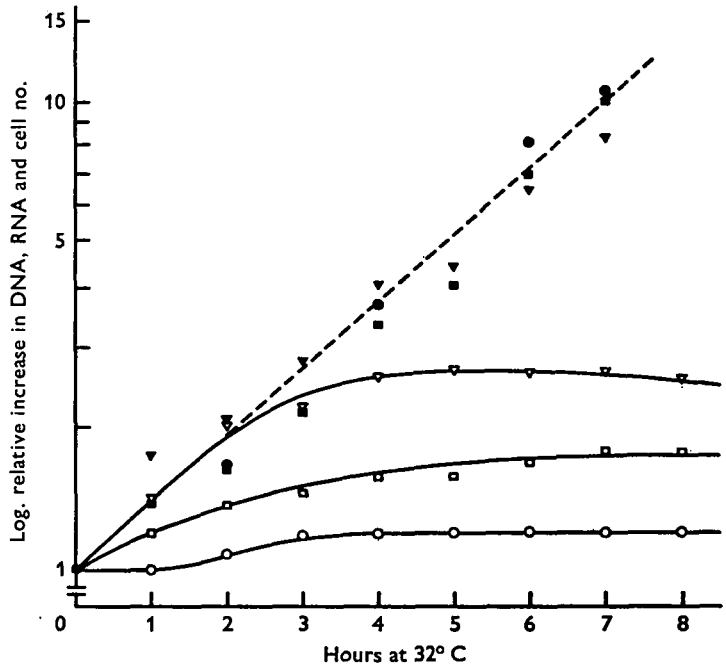
amount of DNA) the total DNA content will increase by only 25–40 %. This does fully account for the increase which is observed with *tsd*-1 populations. However, we now know that at least 10 % of the total DNA is mitochondrial (D. H. Williamson, unpublished data) and if this replicates normally at 32 °C it could account for the additional 30–40 % increase in DNA.



Text-fig. 5. Comparison of DNA and RNA synthesis and cell number increase in log.-phase cultures of wild type and *tsd*-1 at 32 °C. DNA (□—□); RNA (▽—▽) and cell number (○—○); wild type in solid symbols.

Apart from chemical determinations of DNA and RNA synthesis, a number of ^{14}C adenine uptake experiments were done with haploid or diploid strains of *tsd*-1. These consistently show a lower ratio of uptake into the DNA and RNA fractions than the control (1·02–1·47 %, with a mean of 1·1 %, compared with 2·53 % for the control. See *A* and *B* in Text-fig. 2). In addition, it has been shown that the synthesis of the inducible enzyme nitrate reductase in *tsd*-1 at 32 °C is comparable to that in the wild type. This shows that *m*RNA and protein synthesis can occur under the restrictive conditions.

If *tsd*-1 cells die at 32 °C owing to unbalanced growth, then it should be possible to prevent their death by blocking RNA or protein synthesis. This was shown by treating cells with the lowest fungistatic concentration of cycloheximide, a well-known inhibitor of protein synthesis in eukaryotic organisms (Siegel & Sisler, 1964; Ennis & Lubin, 1964) and by withholding adenine from an adenine requiring derivative of *tsd*-1. These results are shown in Table 4.

tsd-1 cells are no more sensitive to the lethal effects of UV light than wild type,

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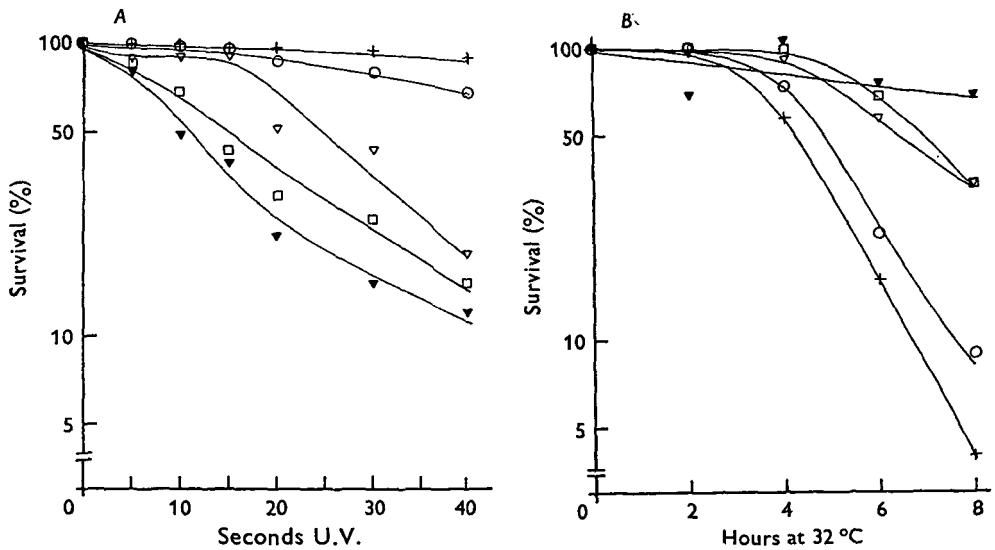
but when they are incubated at 32 °C they progressively become more sensitive. On the other hand, when they are irradiated at 22 °C and then incubated at 32 °C the survivors of irradiation are not killed so readily by the high temperature treatment. These results are shown in Text-fig. 6.

Table 4. *Survival of* tsd-1 *and* tsd-1 ad-1 *cells at* 32 °C *in minimal medium containing* 7 μg/ml *cycloheximide* (*CH*) *or lacking adenine*

	% survival			
	tsd-1		*tsd*-1 *ad*-1	
Treatment (h)	−CH	+CH	+adenine	−adenine
0	100	100	100	100
8	26	80	13	86



Text-fig. 6. *A*, Effect of prior incubation at 32 °C on U.V. sensitivity of *tsd*-1 cells: (+——+), −0 h at 32 °C; 100 % initial survival; (○——○), −2 h at 32 °C, 98 % initial survival; (▽——▽), −4 h at 32 °C, 58 % initial survival; (□——□), −6 h at 32 °C, 19 % initial survival; (▼——▼), −8 h at 32 °C, 5 % initial survival. Cells were incubated at 22 °C after irradiation. *B*, Effect of prior U.V. irradiation on *tsd*-1 cells subsequently incubated at 32 °C. (+——+), −No U.V.; (○——○), −5 sec. U.V. 100 % survival; (▽——▽), −30 sec. U.V., 98 % survival; (□——□) −60 sec. U.V., 85% survival; (▼——▼), −90 sec. U.V., 45 % survival. Cells were incubated for the indicated times at 32 °C after irradiation and then returned to 22 °C. The UV source was a standard germicidal lamp emitting 47 ergs/mm²/sec. at the target distance of 15 cms.

In crosses *tsd*-1 showed normal segregation for a nuclear mutation. Selfed crosses were invariably sterile, even at low temperatures. *tsd*-1 was crossed with the other *tsd* mutants and in each case at least 25 % wild-type progeny were obtained, showing that the loci were unlinked to *tsd*-1.

4. DISCUSSION

These results show that after nitrosoguanidine treatment high yields of temperature-sensitive mutants can be recovered in *U. maydis*. These mutants could not be screened directly for a block in DNA synthesis at the restrictive temperature, since no specific precursor of DNA is known for this or any other fungus. Instead a method was developed for labelling DNA with ^{14}C adenine, based on the procedure used by Williamson (1965) for removing RNA from yeast cells labelled with ^{3}H adenine. This made it possible to screen over 400 temperature-sensitive mutants; and amongst these five were found which appeared to be defective in DNA synthesis whilst being much less affected in RNA and protein synthesis. During the course of the work, similar mutants were reported in yeast by Hartwell (1967). Neither his nor those described here showed an absolute block in DNA synthesis, and this seems to be true of many such mutants in bacteria (Bonhoeffer, 1966; J. D. Gross, personal communication). In fungi one might expect that mutants blocked in the synthesis of precursors would show residual synthesis, as there may well be substantial intracellular pools of such precursors. A complete block might be expected with defective DNA polymerase (or any enzyme directly involved in DNA replication); such a conditional mutant has so far been reported only in phage T4 (Speyer, 1965).

Mutant *tsd*-1, when incubated at 32 °C, shares an impressive number of characteristics with cells of *E. coli* starved of thymine. These form long filaments which die exponentially after a lag period (Cohen & Barner 1954; Barner & Cohen, 1957) and inhibition of protein or RNA synthesis prevents or reduces thymineless death. Again, the longer the cells are deprived of thymine the more sensitive they become to UV light (Gallant & Suskind, 1961). Finally, thymine starvation is recombinagenic in merodiploids (Gallant & Spottswood, 1965). It will be shown in a subsequent publication that incubation at 32 °C induces recombination in diploids homozygous for *tsd*-1. It was previously demonstrated in *U. maydis* that the inhibitors of DNA synthesis, mitomycin and FUDR, are recombinagenic (Holliday, 1964; Esposito & Holliday, 1964).

Pauling & Hanawalt (1965) have suggested that thymineless death may result from a failure to repair single strand gaps or breaks which are formed during the transcription of the genetic material. It is possible that the DNA which is synthesized at 32 °C in *tsd*-1 is abnormal, and contains lesions which are continually being repaired. The cells then begin to die as the repair system becomes saturated. This would explain the increasing sensitivity to UV during the treatment. There is evidence that DNA is synthesized in short pieces which are later joined up into long strands (Okazaki, Okazaki, Sakabe & Sugimoto, 1968; Sugimoto, Okazaki & Okazaki, 1968). If *tsd*-1 lacked this ability, the DNA which it synthesized at high temperature would contain many single strand nicks. UV light is a well-known inhibitor of DNA synthesis. UV treatment prior to heat treatment would prevent the synthesis of abnormal DNA and therefore protect the cells from this genetic damage.

On the other hand, it may well be that *tsd*-1 is not in fact directly blocked in DNA synthesis at all, but that the effect on DNA is secondary to a block in nuclear division. It is quite clear that when a random population of cells is transferred to the restrictive temperature, it is transformed into a uniform population of uninucleate cells, all of which are probably blocked in the G2 period of division. This is what would be expected if there was a temperature-sensitive step in nuclear division in this mutant, perhaps a deficiency in the intranuclear spindle apparatus which has now been demonstrated in fungi (Robinow & Marak, 1964; Heath & Greenwood, 1968). This would presumably result in the cessation of DNA synthesis and cause unbalanced growth. It would not account for the gradual slow increase in DNA over several hours incubation, as the cells come into their uniform state fairly quickly. The additional DNA could be attributed to a proliferation of mitochondria in the growing cells.

The potential value of conditional lethal mutants for investigations of cell structure and function is not open to question. It is evident that the limiting factor is not the isolation of such mutants, but their complete characterization. In the present study a number of mutants with altered capacity for DNA synthesis have been isolated, and one has been examined in some detail; but to fully identify the specific defect in this or the other mutants would demand a much more thorough physiological or biochemical investigation than that attempted here.

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