Genet. Res., Camb. (1980), **35**, *pp.* 291–307
With 9 text-figures
Printed in Great Britain

Repair of double-strand breaks and lethal damage in DNA of *Ustilago maydis*

By S. LEAPER,* M. A. RESNICK† and R. HOLLIDAY
National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA

(*Received* 17 *January* 1980)

SUMMARY

The size of nuclear DNA from wild-type *Ustilago maydis* was determined to be approximately $6{\cdot}09 \pm 0{\cdot}3 \times 10^8$ daltons from neutral sucrose gradient sedimentation analysis. Following exposure to ionizing radiation the nuclear DNA size was reduced due to the production of double-strand breaks in the DNA. These breaks were repaired when the irradiated cells were incubated in medium for at least one hour after irradiation. The repair was seen as a shift in the DNA profile from a low molecular weight region where the control DNA sedimented. Inhibition of protein synthesis by cycloheximide prevented this type of repair. Blocking protein synthesis also decreased the survival of irradiated wild-type cells but not radiation-sensitive mutants. Protein synthesis was necessary within the first one and a half hours after irradiation for the survival of wild-type cells to be unaffected. The results provide additional evidence for an inducible repair process in *U. maydis*.

1. INTRODUCTION

Double-strand breaks (DSB) in DNA are among the most lethally damaging effects of exposure to ionizing radiation. They have been implicated as lethal events in bacteriophage (Friefelder, 1965) in a transformation system (Randolph & Setlow, 1972), and in the *rad* 52 mutant of *Saccharomyces cerevisiae* (Resnick & Martin, 1976) where a one-to-one relationship between DSB and lethal events has been demonstrated. Repair of DSB in *Escherichia coli* was initially not thought to occur (Kaplan, 1966; Bonura *et al.* 1975), but a recent report has shown that DSB are repaired in this organism and that the repair requires a functional *rec* A gene and the presence of a duplicate genome (Krasin & Hutchinson, 1977). Repair of DSB has been demonstrated in a number of both prokaryotic and eukaryotic systems including *Micrococcus radiodurans* (Burrell, Feldschreiber & Dean, 1971; Kitayama & Matsuyama, 1971), *Bacillus subtilis* (Hariharan & Hutchinson, 1973), yeast (Resnick & Martin, 1976) and mammalian cells (Corry & Cole, 1973;

* Present address: Central Public Health Laboratory, Colindale Avenue, London, NW9 5HT.
† Present address: Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709, U.S.A.

0016–628723/0/288–0000 $01.00

Lange, 1974; Lehmann & Stevens, 1977). A mechanism for the repair of DSB in DNA has been proposed by Resnick (1976) which relies on a recombination intermediate, heteroduplex DNA, and repair enzymes. The model predicts that non-reciprocal recombination (gene conversion) as well as reciprocal recombination (crossing over) could occur during repair of DSB. A prerequisite for repair is the presence in cells of either sister chromatids or homologous chromosomes (Resnick, 1978*b*).

Ustilago maydis is particularly well suited to studies on DNA repair as it is a very radiation-resistant organism (Holliday, 1965, 1971; Holliday *et al.* 1976) and presumably possesses efficient repair mechanisms. Most previous studies have concentrated on the repair of UV damage to DNA, yet *U. maydis* is particularly resistant to ionizing radiation (Holliday, 1971). Holliday (1971, 1975) has presented evidence for an inducible repair mechanism operating in wild-type *U. maydis*. If an inducible repair mechanism is operating in the repair of lethal damage caused by ionizing radiation it should be possible to block it with cycloheximide, a potent inhibitor of protein synthesis (Kerridge, 1958). We have demonstrated the repair of DSB and present further evidence that this is an inducible process, depending upon protein synthesis.

2. METHODS

(i) *Strains*

Most of the strains were derived from stock cultures in the laboratory. The growth requirements of these strains are: *ad* 1, adenine; *me* 1, methionine; *pan* 1, pantothenate; *inos* 1, inositol; *nir* 1, inability to use nitrite as sole source of nitrogen; *nar* 1, inability to use nitrate as sole source of nitrogen. *rec* 1 and *rec* 2 are UV and ionizing radiation sensitive, recombination deficient mutants (Holliday, 1967; Holliday *et al.* 1976). *uvs* 3 is a UV sensitive mutant (Holliday, 1965*b*; Unrau, 1975) deficient in excision repair. a_1, a_2 and b_1, b_2 are mating type alleles.

Stock no.	Genotype
127–15	*ad*1–1 *nir*1–1 a_2b_1
341	*ad*1–1 *me*1–2 *nar*1–6 *rec*1–1 a_2b_2
594	*pan*1–1 *nar*1–1 *rec*2–1 a_1b_2
204	*ad*1–1 *me*1–1 *inos*1–5 *uvs*3–1 a_2b_1
701	*inos*1–5 *uvs*3–1 *rec*2–1 a_2b_2
260	*pan*1–1 *nar*1–1 *rec*1–1 *rec*2–1 *uvs*3–1 a_1b_2
702	*pan*1–1 *nic*1–1 *nar*1–1 *γs*30 a_1b_1

(ii) *Media*

Complete medium (CM), synthetic media and genetical methods were in general as given in Holliday (1961*a*, *b* and 1974).

(iii) *Growth*

The incubation temperature was 32 °C. Liquid cultures were grown in flasks with continuous mechanical shaking. To obtain stationary phase cultures, cells

were incubated for 48 h from an inoculum of about 10^5 cells/ml. Wild-type strains in complete growth medium reached a stationary phase concentration of 2×10^8 cells/ml. Log phase cultures were harvested at 1×10^6 to 1×10^7/ml. Cell concentrations were determined with a Coulter Counter Model A. Viable counts were made by plating after suitable dilution onto CM.

(iv) *Irradiation*

UV irradiation was from a Hanovia low-pressure germicidal lamp delivering 5·6 J/m²/s at a distance of 15 cm, and 2 J/m²/s at 30 cm. Cells were irradiated after spreading on agar plates. Precautions were taken to prevent photoreactivation.

γ Irradiation was from a Gammabeam 650 ^{60}Co source delivering 70 krad/min at the centre of the source, and 14 krad/min 30 cm from the centre. Cells were irradiated at 10^7/ml in a beaker with stirring at the centre of the source, or on agar plates 30 cm from the centre. Stationary phase cells were used unless otherwise stated.

(v) *Preparation of protoplasts*

A method was devised by Duell, Inoue & Utter (1964) whereby the contents of yeast cells could be gently extracted using glusulase from the gut of the snail *Helix pomatia*. Radioactively labelled log phase *U. maydis* cells were harvested by centrifugation and 10^7 cells were resuspended in 1 ml 10 mM EDTA, pH 7·6, 1 % 2-mercaptoethanol. After 10 min at 32 °C the cells were washed twice in distilled water and resuspended to the same titre in 20 % sorbose plus 5 % glusulase (Endo Laboratories). The suspension was incubated at 32 °C for 60 min with occasional shaking. In order to release the protoplasts from the partially degraded cell wall the suspension was gently centrifuged and resuspended in 0·2 ml 1·0 M sodium chloride, 20 mM EDTA pH 7·0.

(vi) *Radioactive labelling of DNA*

Cells were grown from a titre of 1×10^5/ml to 2×10^6/ml in minimal medium supplemented with ammonium nitrate, hydrolysed casein, vitamins (at concentrations found in CM) and 5 μCi/ml [^{3}H]adenine (Radiochemicals, Amersham) to obtain log phase cells with the maximum label. The labelled cells were washed, resuspended to the same titre in medium without label and left on ice for 11 h. The temperature was then raised to 32 °C and the cells allowed a further generation doubling.

(vii) *Sucrose gradient sedimentation*

Linear neutral gradients, 15–30 % sucrose in 1·0 M-sodium chloride, 20 mM-EDTA pH 7·0, were prepared in cellulose nitrate tubes. 0·2 ml lysing solution (5 % sarkosyl, 3 % sodium deoxycholate, 5 % sodium dodecylsulphate, 20 mM-EDTA, 10 mM-tris, pH 8·0, Blamire *et al.* 1972) was layered on top of the gradients. The protoplast sample was layered onto the lysing layer and gently stirred, then left at room temperature for 45 min. Freshly deproteinized T4 phage (10 min at 65 °C in 1 % sarkosyl) which had previously been labelled as described by Kutter

& Wiberg (1968) was layered on top. Within 10 min of layering the phage, centrifugation was begun at 18 °C using a Beckman SW 50.1 rotor in a Beckman L5-50 ultracentrifuge. Except where noted (speed $= \omega) \times$ (time $= t$) $= 1450$ krpm^2 h and the speed of centrifugation was less than 10 krpm to avoid speed dependence artifacts as reported by Zimm (1974).

The gradients were fractionated from the bottom by puncturing the centrifuge tube with a 1 mm diameter needle. A steady flow of air was pumped onto the top of the gradient with a peristaltic pump, and equal volumes of liquid were collected on Whatman 3MM chromatography paper strips. The filter strips were immersed in 1 N sodium hydroxide and incubated at 37 °C for $2\frac{1}{2}$ h, then placed in 5% trichloroacetic acid at 4 °C for 30 min, followed by alcohol washes of 15 min and then 5 min. The filter strips were dried and the radioactivity determined in toluene-based scintillation fluid (12·5 g 2,5-diphenyloxazole (PPO) and 0·5 g 1,4-bis2-(4-methyl-5-phenyloxazole) benzene (dimethyl POPOP) dissolved in 2·5 l. toluene).

(viii) *Molecular-weight determination*

The molecular weight of DNA in any fraction i was determined according to Burgi & Hershey (1963) from the relationship $(d_i/d_T) = (M_i/M_T)^x$ where d_i is the distance from the top (less two fractions) to fraction i and d_T is the distance to the average position of $T4$. The d_T was determined from 16 gradients in this study and found to be 26·57% from the top of the gradient for $\omega^2 t = 1450$. The values M_i and M_T are respectively the molecular weight of *U. maydis* DNA in fraction i and the $T4$ DNA which is assumed to be $1{\cdot}2 \times 10^8$ daltons (Leighton & Rubenstein, 1969; Friefelder, 1970). The exponent $x = 0{\cdot}409$, as determined by Resnick & Martin (1976). The number average molecular weight Mn over fractions y–z was calculated as

$$\mathrm{Mn} = \sum_{y}^{z} \mathrm{Ci} \Big/ \sum_{y}^{z} \left(\mathrm{Ci}/\mathrm{Mi}\right)$$

Where Ci represents the counts in the i fraction. The corresponding weight average molecular weight, Mw, is equal to

$$\sum_{y}^{z} \mathrm{CiMi} \Big/ \sum_{y}^{z} \mathrm{Ci}$$

(ix) *Caesium chloride gradient sedimentation*

To determine the density of the DNA 10^8–10^9 cells were protoplasted as described and resuspended finally in 0·5 ml 1·0 M sodium chloride, 20 mM-EDTA pH 7·0. 0·5 ml lysing solution was added and gently mixed in the bottom of a polyallomer centrifuge tube. After 60 min at room temperature 5 ml 0·1 M sodium chloride, 20 mM EDTA, pH 7·0 and 7·4 g caesium chloride (Harshaw) were added. The solution was centrifuged at 18 °C using a Beckman 50 Ti rotor in a Beckman L5–50 ultracentrifuge for 60 h at 40 krpm. Fractions were collected in Eppendorf tubes, in the same manner as for sucrose gradients, and 100 μl spotted onto filters for treatment as described. The density distribution within the gradient was determined using a refractometer.

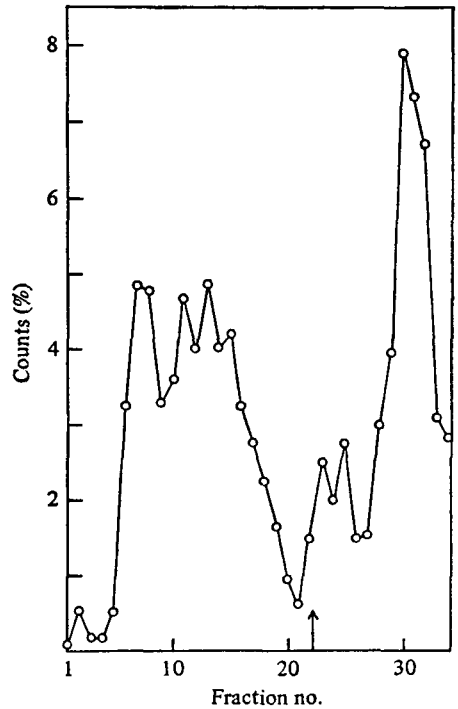

Fig. 1. Neutral sucrose gradient sedimentation of unirradiated *U. maydis* DNA obtained from log phase cells. The sedimentation is from right to left ($\omega^2 t$ = 1680 krpm² h). The arrow indicates the position of T4 DNA.

3. RESULTS

(i) *Neutral sucrose gradients and the molecular weight of DNA*

The DNA from *U. maydis* protoplasts when analysed by neutral sucrose gradient technique was distributed over a wide range of large molecular weights (Fig. 1). The larger peak (fractions 3–20) was found to be nuclear DNA after rebanding these fractions in neutral caesium chloride gradients, where the DNA had a density of 1·72 g/ml. The peak containing fractions 21–25 was found to be mitochondrial DNA after rebanding in neutral caesium chloride gradients, where the DNA had a density of 1·70 g/ml. These density values correspond well with the values for *U. maydis* DNA determined by Banks (1973). The methods of protoplasting, lysis and centrifugation therefore allowed both nuclear and mitochondrial DNA to be obtained.

An estimation of nuclear DNA size can be determined if it is assumed that the nuclear DNA peak is distinct from the mitochondrial DNA. (The counts in the top fractions of the gradient are variable due presumably to a small amount of RNA remaining undigested.) The Mn of unirradiated *U. maydis* nuclear DNA was $6{\cdot}09 \pm 0{\cdot}3 \times 10^8$ daltons. The nuclear DNA from *U. maydis* ran further in neutral sucrose gradients than yeast DNA, run in parallel gradients under identical conditions. (The Mn of nuclear DNA from yeast is $3{\cdot}0 \times 10^8$ daltons based on the

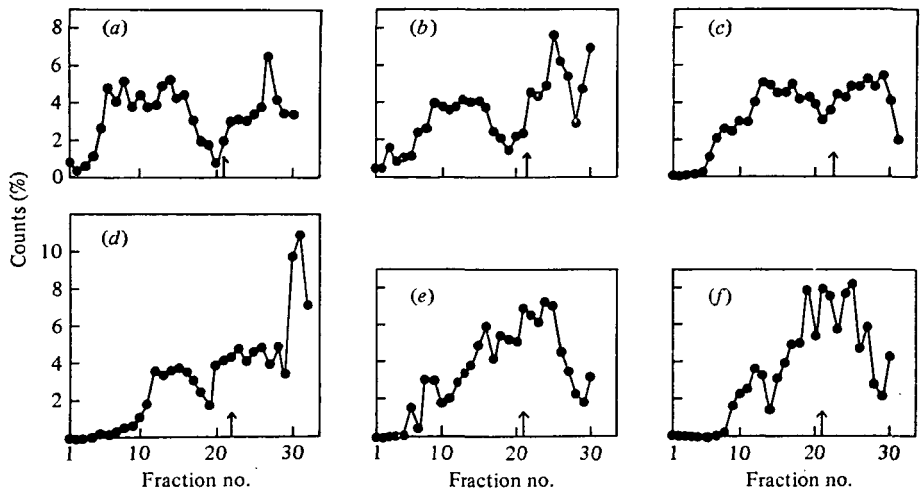

Fig. 2. The effect on wild-type *U. maydis* DNA sedimentation profiles of increasing doses of ionizing radiation. Doses used were (*a*) 0 krad, (*b*) 25 krad, (*c*) 50 krad, (*d*) 75 krad, (*e*) 100 krad and (*f*) 125 krad. The sedimentation is from right to left. The arrows indicate the position of T4 DNA in each gradient.

calculations of Resnick & Martin (1976), or $5{\cdot}6 \times 10^8$ daltons as calculated by Petes & Fangman (1972).)

(ii) *The induction of double strand breaks with ionizing radiation*

The exposure of wild-type cells to ionizing radiation reduced the size of the DNA presumably due to the production of DSB (Fig. 2). As the dose was increased there was a successive shift of the nuclear DNA profile towards the top of the gradient. The pattern of sedimentation was similar if the cells were irradiated with or without oxygen bubbled into the sample, or if the cells were irradiated prior to or after being converted to protoplasts.

The percentage of counts at the top of the gradients varied and the nuclear DNA peak merged with these counts after irradiation. Errors arise in molecular weight estimation when the distribution of DNA is non-random, as discussed by Ehmann & Lett (1973), also counts associated with small molecular weight DNA can alter considerably the value of the Mn. An estimate of the size of the total DNA from irradiated cells (Fig. 2) was made using the Mw relationship, from the distribution of radioactive counts over the whole of the gradient (Table 1). The Mn clearly decreased with doses of irradiation up to 75 krad; above this dose it was not possible to clearly distinguish changes in Mw. The high Mw in the 100 krad sample may be due to a higher percentage of counts in the high-molecular-weight region in this sample than in the 75 krad sample. If we assume that the data are compatible with a continuous reduction in Mw, we can estimate the efficiency of DSB production by ionizing radiation. From Ehmann & Lett (1973) a random distribution of DNA would be expected to have Mn = Mw/2. By using this relationship as an estimate of Mn and plotting 1/Mn versus dose, we calculate

Table 1. *Estimates for the average molecular weight (Mw) of irradiated* U. maydis *DNA*

Dose (krad)	$Mw \times 10^8$ (daltons)	$1/Mn \times 10^8$ (daltons)
0	$6{\cdot}06 \pm 0{\cdot}006$	0·33
25	$4{\cdot}52 \pm 0{\cdot}01$	0·44
50	$3{\cdot}42 \pm 0{\cdot}006$	0·58
75	$2{\cdot}24 \pm 0{\cdot}006$	0·90
100	$3{\cdot}15 \pm 0{\cdot}008$	0·64
125	$2{\cdot}14 \pm 0{\cdot}008$	0·94

that the efficiency is approximately $0{\cdot}55 \times 10^{-10}$ DSB per dalton-krad. This falls within the range (0·4–2·7) reported by others for bacteria, yeast and mammalian cells (see Resnick & Martin, 1976; Lehmann & Stevens, 1977).

(iii) *Repair of double-strand breaks*

To examine the possibility of repair of double-strand breaks, irradiated cells were reincubated in growth medium for various times, before preparing protoplasts and centrifuged in neutral sucrose gradients. Results are shown in Fig. 3 for post-irradiation incubation times of 1 and 4 h.

Following a dose of 100 krad the nuclear DNA profile was shifted towards the low molecular weight region of the gradient. After 1 h post-irradiation incubation an increase in DNA counts on the high molecular weight side of the nuclear peak was seen, although the major part of the DNA profile corresponded to that for radiation only. The shape of the profile was different from that of the control or after irradiation alone. After 4 h post-irradiation incubation there was a shift in the nuclear DNA profile back towards the profile of unirradiated DNA, indicating substantial rejoining of breaks.

(iv) *The effect of cycloheximide on the repair of double-strand breaks*

The involvement of a repair system requiring protein synthesis was studied by the addition of cycloheximide (CH) to the growth medium during the post-irradiation incubation. CH was added at a final concentration of 100 μg/ml and the effect on the rejoining of DSB was compared with irradiated cells incubated in the absence of the drug (Fig. 4). Repair of DSB induced by a dose of 100 krad was observed after 1 h post-irradiation incubation, but in the presence of CH there was no repair. The nuclear DNA profile was at a similar position in the gradient as the irradiated nuclear DNA profile. This was observed in several experiments. Similar results were obtained for $4\frac{1}{2}$ h post-irradiation incubation. Repair of DSB in *U. maydis* therefore requires protein synthesis. Somewhat similar results were obtained by Resnick & Martin (1976) using yeast and Kitayama & Matsuyama (1971) using *M. radiodurans*. These results would be consistent with an inducible repair system, but another possibility is that CH has an indirect effect by blocking DNA synthesis.

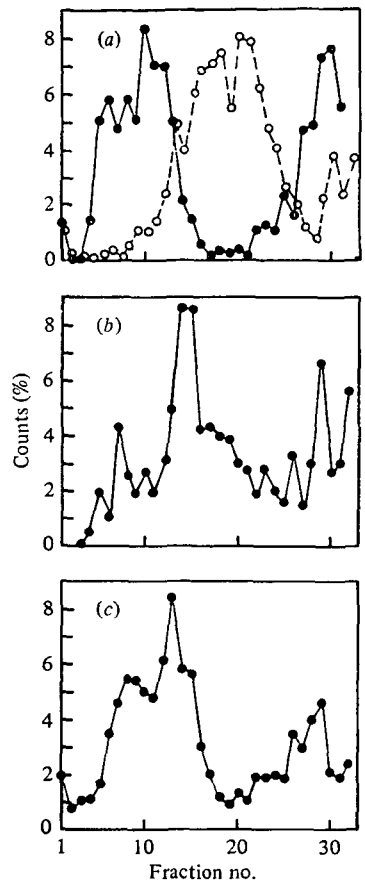

Fig. 3. The effect on the sedimentation profile of wild-type *U. maydis* DNA of incubating the cells in medium after irradiation with a dose of 100 krad. (*a*) DNA from control cells (●—●) and DNA from cells that have been irradiated with 100 krad (○- -○). (*b*) DNA from cells that have been irradiated with 100 krad and allowed 1 h post-irradiation incubation. (*c*) DNA from cells that have been irradiated with 100 krad and allowed 4 h post-irradiation incubation. Sedimentation is from right to left ($\omega^2 t$ = 1750 krpm² h).

(v) *Evidence for an inducible repair system affecting survival*

Cells of *U. maydis* are very resistant to ionizing radiation, but there are marked differences between log phase and stationary phase haploid cells. This is shown in Fig. 5(*a*). Approximately 50% of wild-type log phase cells were extremely sensitive, based on the rapid decrease in survival up to a dose of 50 krad. The remaining cells were resistant up to a dose of 120 krad, after which the survival decreased exponentially. In stationary phase cultures which are mainly in G2, only a small proportion of the cells were very sensitive, the remainder being resistant up to a dose of approximately 350 krad. The survival then decreased exponentially. The importance of repair processes is demonstrated by the isolation of very radiation sensitive mutants (Holliday, 1965; Holliday *et al.* 1976). The sur-

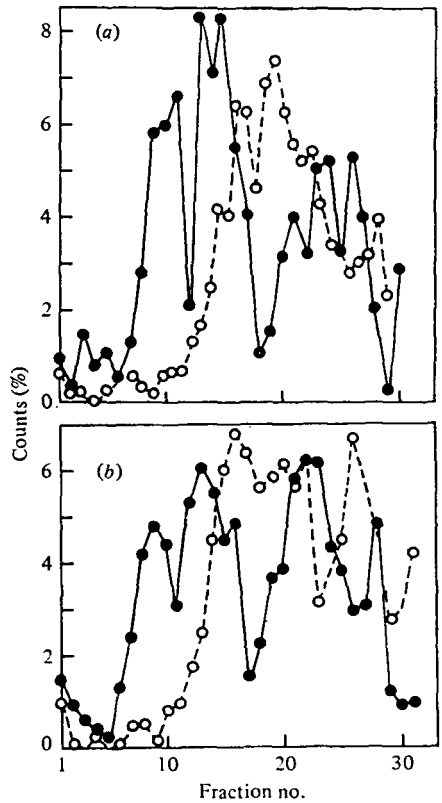

Fig. 4. The effect on the sedimentation profile of wild-type *U. maydis* DNA of incubating irradiated cells in the presence of 100 μg/ml cycloheximide. (*a*) DNA from control cells, ●—●; DNA from cells exposed to 100 krad ○– –○ (*b*) DNA from irradiated cells incubated for one hour in the presence, ○– –○, or absence, ●—●, of cycloheximide. Sedimentation is from right to left.

vival of several of these after treatment with γ-rays is compared to wild-type in Figs. 5(*a*) and (*b*). Table 2 shows the doses of both UV and ionizing radiation which are required to reduce the survival of wild-type and radiation-sensitive strains to 37 %. The *γs*30 strain is a newly isolated mutant, which complements with *rec*1, *rec*2 and *uvs*3.

The effects of CH on the survival of irradiated cells was studied in the following way. Cells were grown to stationary phase in CM, washed and resuspended in CM at a titre of 3×10^7/ml. The culture was irradiated as a stirred suspension, using a dose that reduced the survival to approximately 50 % (for wild-type cells a dose of 350 krad was used; for *rec*1 and *rec*2 cells, 5 krad, and for *uvs*3 cells, 15 krad). The cells were then diluted ten-fold into CM or CM plus 5 μg/ml CH and incubated at 32 °C. After various times samples were removed, appropriately diluted to remove the protein synthesis inhibitor, and plated on CM for survival.

Holding unirradiated cells in CM plus CH did not alter the survival of the cells, whereas the survival of irradiated wild-type cells was decreased upon CH incu-

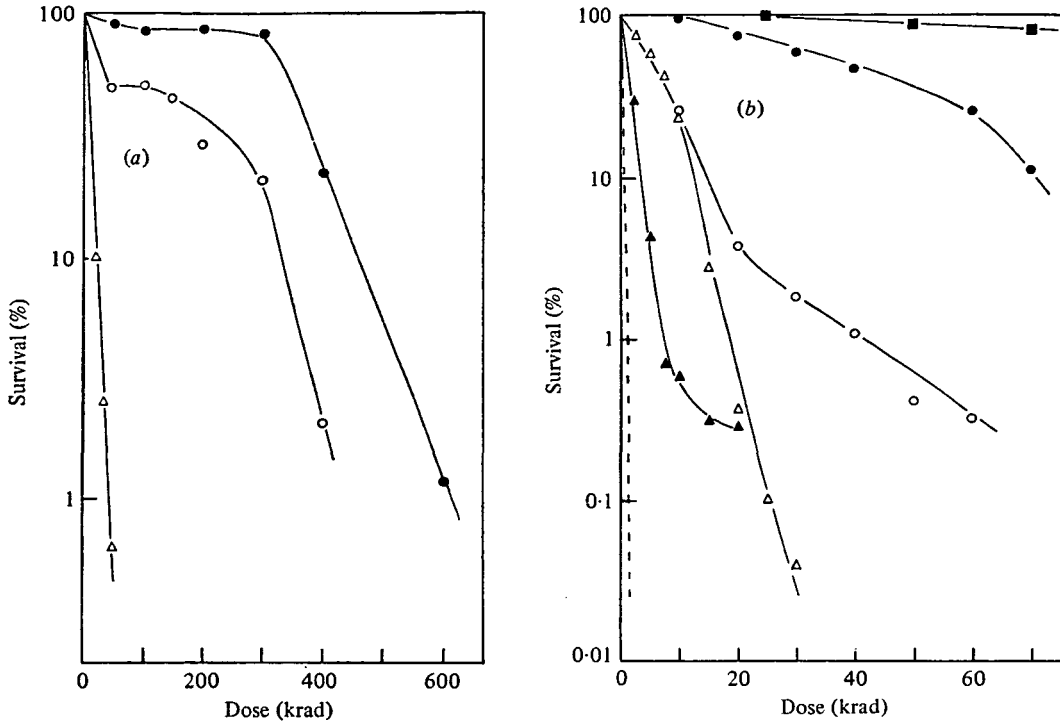

Fig. 5. The effect of ionizing radiation on the survival of *U. maydis* cells: (*a*) ○—○, log phase wild-type; ●—●, stationary phase wild-type; △—△, stationary phase *γs*30. (*b*) Stationary phase cells from the following strains: ■—■, wild-type; △—△, *rec*1; ○—○, *rec*2; ●—●, *uvs*3; ▲—▲, *uvs*3 *rec*2. The survival of a *rec*1 *rec*2 *uvs*3 strain is indicated by a dashed line.

Table 2. *Radiation doses which give 37 % survival to stationary phase cells*

Strain	UV (J/m²)	γ(krad)
Wild-type	342	360
*rec*1	5	8
*rec*2	42	8
*uvs*3	57	50
*rec*1 *rec*2 *uvs*3	1	0·25
*rec*2 *uvs*3	3	2
*γs*30	14	8

bation (Fig. 6). The survival of wild-type cells was reduced 50-fold after 7 h incubation; holding in CM alone did not affect the survival. The effects were similar for both log and stationary phase cells. The survivals of both unirradiated and irradiated *rec*1 and *rec*2 cells was unaffected by 24 h incubation in CM plus CH. Protein synthesis therefore appeared to be required for the repair of ionizing radiation damage in wild-type cells, but not in the radiation sensitive mutants. This suggests that these mutants may be blocked in an inducible repair system.

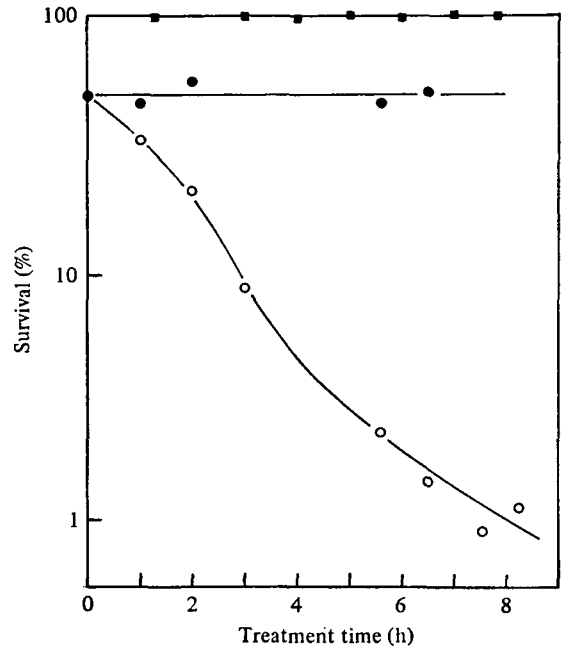

Fig. 6. The effect on the survival of holding wild-type *U. maydis* cells in medium containing 5 μg/ml cycloheximide for varying times: ■—■, unirradiated cells in medium plus cycloheximide; ○—○, irradiated (100 krad) cells in medium plus cycloheximide; ●—● irradiated (100 krad) cells in medium.

The effect on the survival curves of holding cells in CH was investigated further. Cultures, grown and irradiated with various doses as described above, were incubated for 7 h in CH at 32 °C then plated for survival or diluted in water and plated for survival within 1 h. Wild-type cells that were held in CM plus CH for 7 h after irradiation were more sensitive at all the doses given than the cells that were plated immediately (Fig. 7). There was only a slight shoulder to the survival curve at the lowest doses used. At higher doses the curves were almost parallel. *rec*1 and *rec*2 cells showed an increased survival upon holding in CM for 7 h (Table 3) but there was no difference in the survival of *uvs*3 cells.

Protein synthesis was required for up to 1½ h immediately after irradiation to repair ionizing radiation-induced damage (Fig. 8). The wild-type cells were grown and irradiated with a dose of 350 krad (14·3 % survival in this experiment) as previously described. After irradiation the cells were diluted tenfold into CM and incubated at 32 °C. At timed intervals a sample was removed and added to CH, at a final concentration of 5 μg/ml, and further incubated for 7 h at 32 °C. The sample was appropriately diluted after this incubation and plated on CM to determine survival. If the inhibitor was added after 1–2 h, the survival was not greatly affected.

The effect of splitting the dose on wild-type cells was investigated in the following way. After various doses, cells were removed from the suspension and

https://doi.org/10.1017/S0016672300014154 Published online by Cambridge University Press

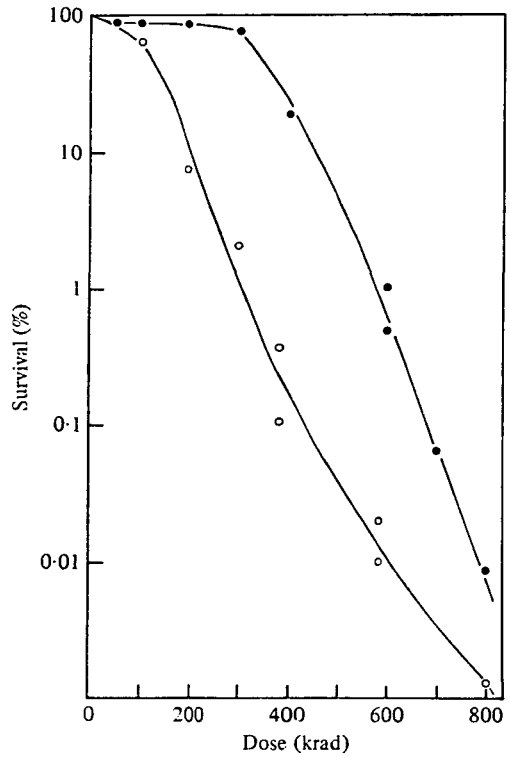

Fig. 7. The effect on the survival curve of holding wild-type *U. maydis* cells in medium plus 5 μg/ml cycloheximide for 7 h after irradiation: ○—○, cells incubated for 7 h; ●—●, cells plated directly after irradiation.

Table 3. *Effect of 7 h post-irradiation incubation on the dose of ionizing radiation which gives 37 % survival to stationary phase cells*

Strain	Dose (krad) giving 37 % survival	
	−CH	+CH
Wild-type	365	135
*rec*1	7	15
*rec*2	8	11
*uvs*3	32	32

plated for survival. Following a cumulative dose of 350 krad one sample was incubated at 32 °C in CM for 2 h before continuation of the irradiation up to a dose of 800 krad. Incubating wild-type cells at 32 °C for 2 h after a dose of 350 krad affected the survival of cells when irradiated with further doses (Fig. 9). The survival at each subsequent dose was higher in the incubated cells versus the unincubated cells. These results are consistent with an inducible repair mechanism.

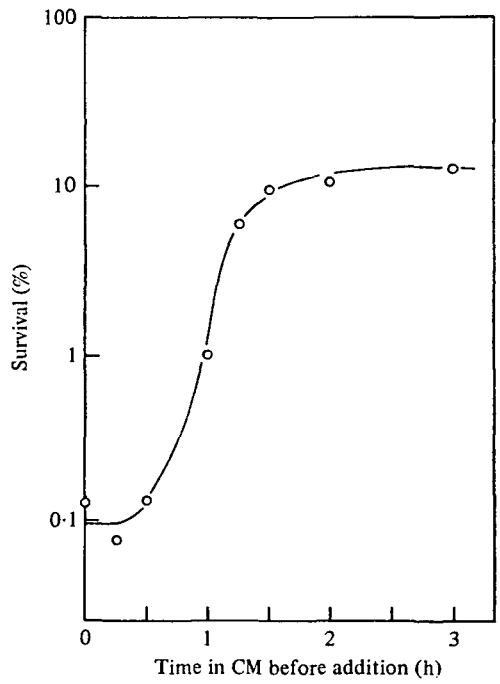

Fig. 8. The effect on the survival of incubation of irradiated (350 krad) wild-type cells prior to the addition of 5 μg/ml cycloheximide for 7 h.

4. DISCUSSION

In order to study the repair of radiation-induced DNA strand breakages in *U. maydis* it was necessary to develop a method whereby large molecular weight DNA could be obtained from the cells. This has been done using a method that allows the formation of protoplasts which can be gently lysed in the top of neutral sucrose gradients to avoid mechanical shearing of the DNA. Using this method the DNA profiles obtained from unirradiated wild-type cells show that both nuclear and mitochondrial DNA can be obtained from log phase cells of *U. maydis* (Fig. 1). (Stationary phase cells could not be converted to protoplasts). The mitochondrial DNA is well separated from the nuclear DNA, but in DNA profiles from irradiated cells it could not be distinguished. The number average molecular weight of unirradiated *U. maydis* nuclear DNA was estimated to be $6{\cdot}09 \pm 0{\cdot}3 \times 10^8$ daltons from neutral sucrose gradients containing T4 DNA as a standard.

Exposure of wild-type *U. maydis* cells to ionizing radiation, over the dose range 25–125 krad, results in a reduction in the molecular weight of the DNA (Table 1). A plot of the reciprocal of the number average molecular weight (1/Mn) versus dose indicates that DSB are produced in a linear fashion suggesting that a single event is required for DSB production, similar to the observations in yeast (Resnick & Martin, 1976). The efficiency of DSB production was estimated to be $0{\cdot}55 \times 10^{-10}$ dalton-krad. This value falls within the wide range (0·4–2·7) reported for bacteria,

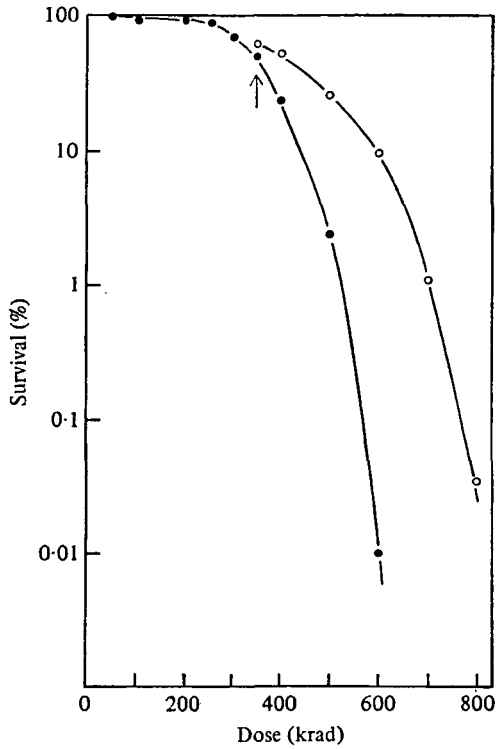

Fig. 9. The effect on survival of holding wild-type cells for 2 h after a dose of 350 krad (↑), before exposure to higher doses of ionizing radiation (○—○) compared to the survival of wild-type cells which were not incubated.

yeast and mammalian cells (see Resnick & Martin, 1976; Lehmann & Stevens, 1977). The wide range is thought to reflect either differences in the state of the DNA in the various systems or differences in techniques.

Rejoining of DSB has been observed in a variety of organisms and has now been observed in *U. maydis* by the reappearance of large molecular weight DNA in neutral sucrose gradients, when irradiated cells are allowed at least one hour post-irradiation incubation in growth medium (Fig. 3). The repair of ionizing radiation induced DSB requires protein synthesis since the addition of CH prevented repair (Fig. 4). Incubation in the presence of CH also affects the survival of wild-type cells (Figs. 6, 7). The survival was decreased if the irradiated cells were held for 1–8 h in medium plus CH. After 7 h the survival was reduced by 50-fold. Holding in medium alone does not alter the survival of irradiated cells. Since DSB repair is prevented in the presence of CH it is possible that the absence of repair of this type of DNA damage is responsible for the decrease in survival.

Holliday (1975) showed that protein synthesis was required within two hours after UV irradiation for full survival and production of recombinants. Experiments carried out with ionizing radiation show that wild-type cells require protein synthesis within approximately 1½ h after irradiation for survival to be unaffected (Fig. 8). The kinetics of the induction of repair of these cells is similar to the appear-

ance of the enzyme nitrate reductase, in a wild-type strain of *U. maydis* after the addition of inducing nitrate medium (Resnick & Holliday, 1971). However, results using CH must be interpreted with caution since a block in protein synthesis will also prevent initiation of DNA synthesis and this could also have effects on survival. It is also possible that a repair enzyme with a short half life exists and this rapidly disappears in the presence of CH. Nevertheless, all the observations support the hypothesis that an inducible repair mechanism is present in wild-type *U. maydis* cells. The split-dose experiments (Fig. 9) provide additional evidence that radiation damage in the cell induces a repair system. The increased survival seen with cells incubated for two hours after an initial dose of 350 krad before exposure to higher doses, would be explained by a repair mechanism that is induced during the period of incubation and is capable of repairing some of the subsequent damage that is inflicted by further exposure of ionizing radiation.

Recombination repair is thought to play a major part in the repair of DNA damaged by radiation, as the frequency of allelic recombination is greatly increased after irradiation and mutants which are deficient in recombination are radiation-sensitive (Holliday, 1967, 1971; Holliday *et al.* 1976; Resnick, 1978*a*). If recombination repair is induced by irradiation of the cells, then it is of interest to look at the effects of blocking protein synthesis on some of the radiation-sensitive mutants that are also known to be recombination-deficient. Irradiation of *rec*1 and *rec*2 cells with a dose of ionizing radiation that reduces the survival to 50 %, and subsequent holding in medium plus CH does not significantly alter their survival over an 8 h period. Protein synthesis is therefore not required in the repair of ionizing radiation-damaged recombination-deficient strains; this would be consistent with their being blocked in an inducible repair pathway. Irradiated *uvs*3 cells show no difference in survival if they are held for 7 h in CH medium. It might have been expected that *uvs*3 cells would behave similarly to wild-type cells with respect to their survival after holding in CH as they are proficient in recombination (Holliday, 1967) and deficient in excision repair (Unrau, 1975). However, Unrau (1975) has argued that the excision repair and recombination repair pathways act sequentially and are in some way co-ordinated. It may be that a block in excision repair also has effects on the inducibility of a recombination repair pathway. Preliminary experiments (results not reported) using a diploid strain homozygous for *uvs*3 and heteroallelic for *nar*1, indicate that UV-induced recombination is blocked by the addition of CH within approximately three hours after irradiation. Viability closely parallels the induction of recombinants. These results are similar to those obtained by Holliday (1975) using wild-type cells heteroallelic for *nar*1.

The repair of ionizing radiation damage would appear to involve recombination as the *rec*1, *rec*2, *rec*2 *uvs*3 and *rec*1 *rec*2 *uvs*3 strains are extremely sensitive (Fig. 5). *rec*1, *rec*2 and *uvs*3 are known to be involved in different repair pathways, as each of the double mutants are more sensitive to UV than any single mutant and the triple mutant is even more sensitive than the double mutants (Holliday *et al.* 1976). The mutant *γs*30 has similar characteristics to *rec*1, and is found to com-

plement with *rec*1, *rec*2 and *uvs*3 which shows that it is a mutant at another locus either in the *rec*1 pathway or in a new pathway. *rec*2 cells are more sensitive than *rec*1 cells above a dose of 10 krad. The mutations are in different pathways, but may share a common recombination pathway to a certain point, accounting for their identical survival up to 10 krad. Excision repair would also appear to be involved in the repair of ionizing radiation damage as the *uvs*3 mutant is significantly sensitive. These results provide a basis for further investigations of the molecular basis for the repair of DSB in *U. maydis*.

REFERENCES

BANKS, G. R. (1973). Mitochondrial DNA synthesis in permeable cells. *Nature New Biology* **245**, 196–199.

BLAMIRE, J., CRYER, D. R., FINKELSTEIN, B. & MARMUR, J. (1972). Sedimentation properties of yeast nuclear and mitochondrial DNA. *Journal of Molecular Biology* **67**, 11–24.

BONURA, T., TOWN, C. D., SMITH, K. C. & KAPLAN, H. S. (1975). The influence of oxygen on the yield of DNA double-strand breaks in X-irradiated *Escherichia coli* K12. *Radiation Research* **63**, 567–577.

BURGI, E. & HERSHEY, A. D. (1963). Sedimentation rate as a measure of molecular weight of DNA. *Biophysics Journal* **3**, 309–321.

BURRELL, A. D., FELDSCHREIBER, P. & DEAN, C. J. (1971). DNA membrane association and the repair of double breaks in X-irradiated *Micrococcus radiodurans*. *Biochemica et Biophysica Acta* **247**, 38–53.

CORRY, P. M. & COLE, A. (1973). Double strand rejoining in mammalian DNA. *Nature New Biology* **245**, 100–101.

DUELL, E. A., INOUE, S. & UTTER, M. F. (1964). Isolation and properties of intact mitochondria from sphaeroplasts of yeast. *Journal of Bacteriology* **88**, 1762–1773.

EHMANN, U. K. & LETT, J. T. (1973). Review and evaluation of molecular weight calculations from the sedimentation profiles of irradiated DNA. *Radiation Research* **54**, 152–162.

FRIEFELDER, D. (1965). Mechanism of inactivation of coliphage T7 by X-rays. *Proceedings of the National Academy of Sciences of the U.S.A.* **54**, 128–134.

FRIEFELDER, D. (1970). Molecular weights of coliphages and coliphage DNA. IV. Molecular weights of DNA from bacteriophages T4, T5 and T7 and the general problem of determination of M. *Journal of Molecular Biology* **54**, 567–577.

HARIHARAN, P.V. & HUTCHINSON, F. (1973). Neutral sucrose gradient sedimentation of very large DNA from *Bacillus subtilis*. II. Double-strand breaks formed by γ-ray irradiation of cells. *Journal of Molecular Biology* **75**, 479–494.

HOLLIDAY, R. (1961*a*). The genetics of *Ustilago maydis*. *Genetical Research, Cambridge* **2**, 204–230.

HOLLIDAY, R. (1961*b*). Induced mitotic crossing-over in *Ustilago maydis*. *Genetical Research, Cambridge* **2**, 231–248.

HOLLIDAY, R. (1965). Radiation sensitive mutants of *Ustilago maydis*. *Mutation Research* **2**, 557–559.

HOLLIDAY, R. (1967). Altered recombination frequencies in radiation sensitive strains of *Ustilago*. *Mutation Research* **4**, 275–288.

HOLLIDAY, R. (1971). Biochemical measure of the time and frequency of radiation induced allelic recombination in *Ustilago*. *Nature New Biology* **232**, 233–236.

HOLLIDAY, R. (1974). *Ustilago maydis*. In *Handbook of Genetics*, vol. 1 (ed. R. C. King), pp. 575–595. New York: Plenum Press.

HOLLIDAY, R. (1975). Further evidence for an inducible recombination repair system in *Ustilago maydis*. *Mutation Research* **29**, 149–153.

HOLLIDAY, R., HALLIWELL, R. E., EVANS, M. W. & ROWELL, V. (1976). Genetic characterization of *rec*-1, a mutant of *Ustilago maydis* defective in repair and recombination. *Genetical Research, Cambridge* **27**, 413–453.

KAPLAN, H. S. (1966). DNA-strand scisson and loss of viability after X-irradiation of normal and sensitized bacterial cells. *Proceedings of the National Academy of Sciences of the U.S.A.* **55**, 1442–1446.

KERRIDGE, D. (1958). The effect of actidione and other antifungal agents on nucleic acid and protein synthesis in *Saccharomyces carlbergensis*. *Journal of General Microbiology* **19**, 497–506.

KITAYAMA, S. & MATSUYAMA, A. (1971). Double-strand scissons in DNA of γ-irradiated *Micrococcus radiodurans* and their repair during post-irradiation incubation. *Agricultural & Biological Chemistry* **35**, 644–652.

KRASIN, F. & HUTCHINSON, F. (1977). Repair of DNA double-strand breaks in *Escherichia coli* which require *rec*A function and the presence of a duplicate genome. *Journal of Molecular Biology* **116**, 81–98.

KUTTER, E. M. & WIBERG, J. S. (1968). Degradation of cytosine-containing bacterial and bacteriophage DNA after infection of *Escherichia coli* B with bacteriophage T4 D wild-type and with mutants defective in genes 46, 47 and 56. *Journal of Molecular Biology* **38**, 395–411.

LANGE, C. S. (1974). The organization and repair of mammalian DNA. *FEBS Letters* **44**, 153–156.

LEHMANN, A. R. & STEVENS, S. (1977). The production and repair of double strand breaks in cells from normal humans and from patients with ataxia telangiectasia. *Biochemica et Biophysica Acta* **474**, 49–60.

LEIGHTON, B. & RUBENSTEIN, I. (1969). Calibration of molecular weightscales for DNA. *Journal of Molecular Biology* **46**, 313–328.

PETES, T. D. & FANGMAN, W. L. (1972). Sedimentation properties of yeast chromosomal DNA. *Proceedings of the National Academy of Sciences of the U.S.A.* **69**, 1188–1191.

RANDOLPH, M. L. & SETLOW, U. K. (1972). Mechanism of inactivation of transforming deoxyribonucleic acid by X-rays. *Journal of Bacteriology* **106**, 221–226.

RESNICK, M. A. (1976). The repair of double-strand breaks in DNA: a model involving recombination. *Journal of Theoretical Biology* **59**, 97–106.

RESNICK, M. A. (1978*a*). The induction of molecular and genetic recombination in eukaryotic cells. *Advances in Radiation Biology* (In the Press.)

RESNICK, M. A. (1978*b*). The importance of DNA double-strand break repair in yeast. In *DNA Repair Mechamisms* (ed. P. C. Hanawalt, E. C. Friedberg & C. F. Fox), New York: Academic Press. (In the Press.)

RESNICK, M. A. & HOLLIDAY, R. (1971). Genetic repair and the synthesis of nitrate reductase in *Ustilago maydis* after UV irradiation. *Molecular and General Genetics* **111**, 171–184.

RESNICK, M. A. & MARTIN, P. (1976). The repair of double-strand breaks in the nuclear DNA of *Saccharomyces cerevisiae* and its genetic control. *Molecular and General Genetics* **143**, 119–129.

UNRAU, P. (1975). The excision of pyrimidine dimers from the DNA of mutant and wild-type strains of *Ustilago*. *Mutation Research* **29**, 53–65.

ZIMM, B. H. (1974). Anomalies in sedimentation. IV. Decrease in sedimentation coefficients of chains at high fields. *Biophysical Chemistry* **1**, 279–291.