J. Mol. Biol. (1963) **6**, 175–181

Study of Genetic Targets with Labelled Mutagens. The Action of Myleran† on the Resistance to Streptomycin of *Chlamydomonas eugametos*

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(*Received 14 July 1962, and in revised form 28 September 1962*)

Cells of *Chlamydomonas eugametos* Moewus, sex minus, were treated with tritiated myleran and a correlation was established between the mutation frequency (loss of resistance to streptomycin) and the tritium content of the isolated DNA.

It was concluded that the resistance to streptomycin is due to a single DNA locus per cell; this is particularly interesting in view of the fact that these algae cells are haploid.

It has been supposed that the observed mutations were due nearly exclusively to the cross-linking of the complementary strands of the DNA molecule carrying the competent locus. The size of this DNA molecule was provisionally calculated to be 3,600,000 or 2,900,000 a.m.u.;† these results are derived from two separate sets of experiments.

1. Introduction

Myleran reacts *in vivo* with DNA (Verly, Dewandre, Dulcino, Moutschen-Dahmen & Moutschen-Dahmen, 1960; Brookes & Lawley, 1961) and is mutagenic (Fahmy & Fahmy, 1956; Röhrborn, 1959; Wettstein, Gustafsson & Ehrenberg, 1959).

In the present research, myleran labelled with tritium has been used to study the mutagenic action of the drug on the monocellular alga, *Chlamydomonas eugametos* Moewus, sex minus. The strain used is naturally resistant to streptomycin; it is haploid and the DNA per cell was determined to be $1{\cdot}55 \times 10^{-10}$ mg. The algae are cultivated in Knop saline (Dop & Gautié, 1928) in the light; they are completely autotrophic and no mitosis occurs in darkness. When cells kept for some time in the dark are exposed again to the light, they all start to divide about 10 hours later.

2. Experimental

(1) *Myleran labelled* with tritium in positions 2 and 3 of the butanediol moiety was synthesized by reduction of butynediol with tritium hydrogen, followed by esterification with methanesulphonyl chloride (Koch, Verly & Bacq, 1959; Koch, 1959). The specific activity of the tritiated myleran used in the present work is 666 mc/m-mole.

(2) Chlamydomonas *cells*, 2 hr after re-exposure to light, were suspended in a solution of tritiated myleran for 3 hr at room temperature. The concentration of the myleran varied from 10 to 100 mg/l. in the different experiments. The cells were then centrifuged and carefully washed by multiple resuspensions in water followed by centrifugations. They were finally divided into two parts: one for genetic analysis and the other for the isolation of the DNA.

† Myleran is the name given to 1,4-dimethanesulphonyloxybutane. a.m.u. is an abbreviation of atomic mass units.

(3) *Genetic analysis.* Cells in Knop saline were immediately poured onto agar plates containing the necessary mineral nutrients so as to have about 1000 cells per Petri dish. After 3 weeks, each of the clones (about 2 mm diameter) derived from the surviving cells were cut in two: the halves were laid, one on normal agar, the other on agar containing 20 μg of streptomycin/ml. After 3 more weeks, the plates were examined. If x is the number of growing colonies on streptomycin and y the number of growing controls, then $(1-x/y)$ is the fraction of the cells surviving the action of myleran which have become sensitive to streptomycin, i.e. the mutation frequency.

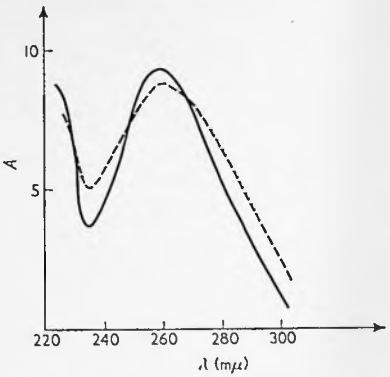

FIG. 1. Ultraviolet spectra of normal and alkylated DNA from *Chlamydomonas* cells. A = extinction per P equivalent; ———— normal; — — — alkylated.

(4) *Isolation of DNA.* All operations were performed at 0°C. About 2×10^{10} centrifuged cells of *Chlamydomonas* were dissociated in 10 ml. of a solution containing 0·075 M-NaCl and 0·025 M-EDTA, disodium salt (Zubay & Doty, 1959), then centrifuged; the supernatant fluid was discarded. This treatment was repeated 3 times. The residue was dispersed in 10 ml. of 0·14 M-NaCl, then centrifuged; this operation was performed twice. To dissociate the DNA proteins of the pellet, the latter was suspended in 10 ml. of 0·14 M-NaCl to which 1·1 ml. of detergent had been added (5 g of dodecylsulphate in 100 ml. of 45% ethanol (Kay & Dounce, 1953)) and stirred for 3 hr. The concentration of NaCl was brought to 1·0 M by the addition of solid salt and stirring was continued for 10 more minutes. After centrifugation, the addition of an equal volume of 95% ethanol to the supernatant solution precipitated the DNA which was removed by centrifugation 3 hr later, washed 3 times with ethanol, 3 times with acetone and then dried. For further purification, the dried DNA was redissolved, treated with detergent and reprecipitated 4 more times. After desiccation under vacuum over phosphoric anhydride, the yield is about 3 mg of DNA. The u.v. spectrum of one of these preparations in 0·1 M-acetic acid solution (pH 3) is shown in Fig. 1 together with the spectrum of DNA from *Chlamydomonas* cells not treated with myleran; the two spectra are slightly different.

(5) *Radioactivity of the isolated DNA.* About 1 mg was dissolved in 0·5 ml. of a 1·0 M solution of hyamine hydroxide (di-isobutylcresoxyethoxyethyldimethylbenzyl ammonium hydroxide) in methanol for 40 hr at 60°C in the dark. The solution was diluted with methanol and toluene and a scintillating solution (10 g of diphenyloxazole, 250 mg of di[phenyloxazolyl]-benzene and 100 g of naphthalene/l. of *p*-dioxane) added. When the chemiluminescence had subsided, the tritium assay was performed in a Packard Tri-Carb spectrometer. A tritiated naphthylacetamide standard, calibrated with respect to the N.B.S. tritiated water, was used and the results are expressed in disintegrations/min/mg of DNA (see Table 1).

(6) *Corrections of the DNA-specific radioactivity.* The values found experimentally are corrected for water content (15%) and loss of alkylated bases (25%).

(a) *Hydration water.* The base ratio of DNA can be estimated, after denaturation, from a comparison of the u.v. absorbancy at 260 and 280 mμ (Fredericq, Oth & Fontaine, 1961); by this method, the mean molecular weight of a nucleotide residue (in the sodium form) was calculated to be 331 for *Chlamydomonas* DNA. The u.v. spectrum of the denatured DNA being equal to the sum of the u.v. spectra of the constitutive nucleotides, it was easy to calculate what the absorbancy at 260 mμ of 1 mg of denatured DNA, having the right base composition, should be. This theoretical value was higher than the experimental one, showing that *Chlamydomonas* DNA dried in vacuum in the presence of phosphoric anhydride still contains 15% of an additional component. As the same result is obtained with highly purified calf thymus DNA, the additional component is most likely hydration water.

TABLE 1

DNA-specific radioactivities from Chlamydomonas *cells after treatment with tritiated myleran (first set of experiments)*

	Alkylated DNA-specific activities		
[^{3}H]Myleran mg/litre	*d*/min/mg	Corrected *d*/min/mg	Molecules of myleran/10^6 nucleotides
20	22,800	35,800	8·1
	26,300	41,300	9·3
50	41,700	65,400	14·7
	39,000	61,400	13·8
100	85,000	134,000	30·1
	82,600	130,000	29·4

(b) *Loss of alkylated bases.* Brookes & Lawley (1960, 1961) have shown that, *in vitro* and *in vivo*, the electrophilic alkylating agents react mostly with the N_7 of guanine residues of DNA. This reaction is followed by a slow hydrolysis of the alkylated guanine (see also Bautz & Freeze, 1960).

To investigate this process when myleran was the alkylating agent, a solution of calf thymus DNA was mixed with a solution of tritiated myleran (final concentration 100 mg/l.) and the mixture left for 3 hr at room temperature. Solid NaCl was added to make the concentration 1·0 M, then an equal volume of 95% ethanol. The DNA fibres were washed 3 times with ethanol and 3 times with acetone (acetone is a very good solvent for the myleran). To free the DNA completely from unreacted myleran, the fibres were dissolved, precipitated and washed 3 more times. The alkylated DNA was finally dissolved in water and portions were taken at different times from 1 to 170 hr. The DNA was precipitated from these portions and analysed for tritium content. The loss of radioactivity reached 30% at the 50th hr and did not seem to increase afterwards.

3. Results and Discussion

Sager (1960) has shown that there are two types of *Chlamydomonas reinhardi* resistant to streptomycin: *sr*-100 has a chromosomic determinant and *sr*-500, which is far less frequent, a cytoplasmic one. Mating with *ss*+ has shown that the natural *gametos* strain *sr*−, used in the present work, belongs to the first category. The streptomycin-sensitive mutant *ss*− obtained by the action of the myleran was isolated and mated with *sr*+; half of the products of the zygotes was sensitive and

half resistant to 20 μg of streptomycin per ml. proving a Mendelian inheritance of the character. It can thus be supposed that the resistance to streptomycin of a *Chlamydomonas eugametos* strain depends on the integrity of portions of DNA molecules and that these functional units are inactivated when they have reacted with the myleran.

Two sets of experiments have been carried out. Figs. 2 and 3 give the mutation frequencies as a function of the corrected DNA-specific activities in linear coordinates.

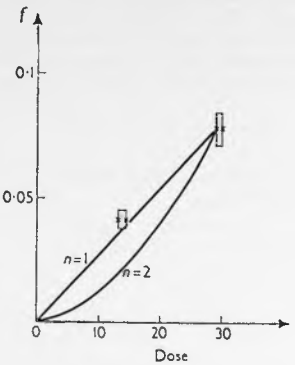

Fig. 2

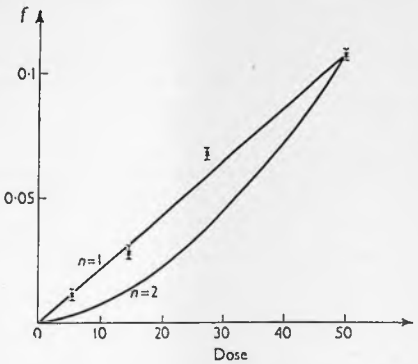

Fig. 3

FIGS. 2 and 3. Mutation frequencies f (loss of resistance to streptomycin) *versus* dose in *Chlamydomonas* cells treated with tritiated myleran. The dose is expressed as mean numbers of myleran molecules per 10^6 nucleotides; they are equivalent to DNA-specific radioactivities (see Table 1).

X shows experimental results (with standard deviations on the biological assays; the error on the radioactivity determination is negligible). Curves are theoretical for $f_{n=1}$ and $f_{n=2}$. Two separate experiments have been performed for each concentration of tritiated myleran in the first set (Fig. 2) and one only in the second set (Fig. 3).

The corrected DNA-specific radioactivities can be converted from disintegrations per minute per mg into the mean number, D, of myleran molecules attached to 10^6 nucleotides.

The specific activity of the tritiated myleran is 666 mc/m-mole, i.e.

$$\frac{666 \times 2{\cdot}22 \times 10^9}{6{\cdot}06 \times 10^{20}}\ d/\text{min per molecule} = A.$$

If R is the radioactivity in d/min/mg of the isolated DNA, the activity per 10^6 nucleotides (mean molecular weight of 1 residue = 331) is

$$\frac{R \times 331 \times 10^6}{6{\cdot}06 \times 10^{20}}\ d/\text{min} = B$$

$$D = B/A$$

10^6 nucleotides is conveniently used as a unit in which to express the volumes (or masses), V, of the functional units (or targets) on which the resistance to streptomycin depends. If an alkylation of the DNA by the myleran is considered as one hit on the targets, then D has the dimensions of a dose; it is the mean number of hits per unit volume.

If there are n targets of volume V per cell of *Chlamydomonas eugametos* and if each target is destroyed by a single hit, the mutation frequency f for a dose D is

$$f = (1 - e^{-VD})^n. \quad (1)$$

In the experiments reported, the mutation frequency barely ever exceeded 10%. For low values of f, equation (1) can be reduced to

$$f = (VD)^n. \quad (2)$$

When there is only 1 target per cell, equation (2) becomes

$$f_{n=1} = VD, \quad (3)$$

which means that, for low values, $f_{n=1}$ is nearly proportional to D.

In Figs. 2 and 3, f is represented *versus* D in linear coordinates. The experimental results are represented by crosses (with standard deviations). The theoretical functions $f_{n=1}$ and $f_{n=2}$ have been fitted to pass through the experimental value corresponding to the highest concentration of myleran used. It is easy to see that, in both instances, the experimental crosses are essentially on a straight line as expected for a function of the type $f_{n=1}$. This means that *it suffices to attach a single molecule of myleran to a single specific target to change the sensitivity towards streptomycin of a* Chlamydomonas *cell*. This result is particularly interesting in view of the fact that this cell is haploid; there is only one target controlling the resistance to streptomycin per genome. The target may of course be a composite one. From our results we cannot say whether it has a unique localization within one chromosome.

The volume of the target is easily deduced from equation (3) using the experimental result with the lowest error (in this part of the discussion, only the first set of experiments represented in Fig. 2 will be dealt with). A dose of 30 myleran molecules per 10^6 nucleotides gives 8% mutation.

$$V = 0{\cdot}08/30 = 0{\cdot}0027 \times 10^6 \text{ nucleotides.}$$

The 'apparent' volume of the target is 2700 nucleotides or about 900,000 a.m.u.

Significance of the 'apparent' target

Spotts & Stanier (1961) have recently shown that resistance to, sensitivity to and dependence on streptomycin as well as the organization of the ribosomes depend on the same genetic locus in *E. coli*, and have suggested that resistance, sensitivity and dependence are allelic forms of the same gene leading to different structures of the ribosomes. In the present discussion, this hypothesis will be extrapolated to *Chlamydomonas*. In addition, we shall assume that resistance to streptomycin of the *Chlamydomonas* cells depends on the formation of a *special ribosomal protein* and attention will be directed towards the genetic locus controlling its production.

According to Brookes & Lawley (1961), the bifunctional alkylating agents, such as myleran, react with native DNA in two different ways:

(a) in 75% of the cases (the experiment has been performed with calf thymus DNA), the drug reacts with only one guanine, the other sulphonic ester function being eventually hydrolysed;

(b) in the other 25%, it reacts with two guanines linking them together. In most instances, these guanines belong to the complementary helices of the same DNA molecule

If the DNA molecule is cross-linked before the increase of the cellular DNA content preceding the morphological mitosis, this will probably prevent the reproduction of this particular molecule, which will be lost for the daughter cells. This argument supposes that the hydrolysis of alkylated guanines before premitotic DNA synthesis does not have an important mutagenic role. This seems likely in our experimental conditions. The replication of DNA starts about 1 hour after the end of the treatment with the myleran, while the loss of alkylated bases is a slow process. This point of view is analogous to that of Lawley & Brookes (1961) who suppose that it is not the hydrolysis of the guanine but the alteration of the acidity of the NH in the 3 position by the N_7 alkylation which is responsible for mutations following damage to a single DNA chain.

It seems that the biological consequences of these different chemical damages must be as follows.

(a) When the myleran reacts with a single guanine, the target must be the sum of the hot spots of the competent genetic locus; these hot spots, which probably contain no more than a few tens of nucleotides, presumably correspond to the amino acids essential for the biological activity of the protein which confers resistance to streptomycin upon the ribosomes.

(b) When the cross-linking of the complementary DNA strands is the chemical damage, the target must be the whole DNA molecule carrying the competent locus because the position of the alkyl bridge is without influence on the final loss for the daughter cells. This target contains thousands of nucleotides.

Although damage of type (a) is three times more frequent than that of type (b), the corresponding target is likely to be hundreds of times smaller, and its biological consequence is negligible compared with that following cross-linking reactions. Moreover, the screening procedure used in this work enables the detection of the mutation only if all the descendants from a myleran-treated cell are mutated to sensitivity. This cannot happen if only one DNA chain is altered by the mutagen. It is concluded that the mutations recorded by the assay can only be due to damage of type (b).

Because two different phenomena are simultaneously dealt with, the 'apparent' target of 900,000 a.m.u. has no reality. As only about 1 hit out of 4 is efficient, i.e. leads to cross-linking, the size of the DNA molecule carrying the competent locus can be estimated by multiplying the mass of the 'apparent' target by 4; it is thus 3,600,000 a.m.u. (the second set of experiments yields a value of 2,900,000 a.m.u.). That the mechanism of the mutation depends on such a large deletion is confirmed by the fact that no spontaneous back-mutation from sensitivity to resistance has yet been observed with *Chlamydomonas eugametos* mutated by the action of myleran.

These conclusions call for a few remarks.

(1) The mutagenic effect of tritiated myleran is exclusively due to the chemical reactivity of the drug; it is exactly the same as that of non-radioactive myleran.

(2) The conditions were strictly standardized only within one particular set of experiments, so that the results of both cannot be pooled together; they are significantly different perhaps because the loss of alkylated bases was not the same at the time of determination of the DNA radioactivity.

(3) The final value of 3,600,000 (or 2,900,000) a.m.u. for the mass of the DNA molecule carrying the locus responsible for the formation of streptomycin-resistant

ribosomes is liable to revision because it depends on three assumptions that ought to be checked further.

(a) The loss of alkylated guanine after reaction of the tritiated myleran with DNA, during the biological experiment of 3 hr and the isolation manipulations, was estimated to be 25%. This estimation is very approximate and we have already mentioned that it may be different in the two sets of experiments. In the study with pure DNA *in vitro*, where a maximum loss of 30% was found, the hydrolysis occurring during the 3 hr reaction period and the purification of the alkylated DNA is ignored.

(b) Brookes & Lawley (1961) have shown, with calf thymus DNA, that 25% of the reacted bifunctional alkylating agent can be isolated as a diguanyl derivative after acid hydrolysis of the DNA and that this bridge formation happens more easily with native DNA than with a denatured DNA. But it is still to be determined in what percentage of the cases the bridge links together the complementary strands. If only a fraction leads to this consequence, it is obvious that the size of the target must be increased accordingly.

(c) The calculation also supposes that the guanine content of the DNA molecule carrying the competent locus is the same as the mean value measured on the total *Chlamydomonas* DNA and that the probability of cross-linking complementary strands is the same as for calf thymus DNA.

(4) It must finally be recalled that the value of about 3,000,000 a.m.u. (eventually corrected as indicated above) must perhaps be divided between several DNA molecules. But, for our discussion, this represents a single genetic target in the sense that a single cross-linking anywhere in these competent molecules is sufficient to yield the mutation.

Associé du Fonds National Belge de la Recherche Scientifique (W.G.V.). Boursière (A.D.) et Chercheur agréé (M.M.D.) de l'Institut Interuniversitaire des Sciences Nucléaires.

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