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Utilization of Labelled 'Myleran' for the Determination of the Size of DNA Molecules in Cells

THE mutagenic action of the electrophilic alkylating agents has been attributed to many different causes. For Alexander and Stacey[1], the alkylation of the DNA phosphates could be sufficient to induce mutations; but these could also be the consequence of the hydrolysis of the very unstable triester phosphate formed, with rupture of the macromolecule followed by large deletions. Lawley and Brookes[2] have postulated that the 7-alkylation of guanine makes this base couple with thymine instead of cytosine and so is responsible for transitions. But the alkylation of the guanine and adenine residues can also be followed by the loss of the alkylated base[3]; this depurination might act in two ways: the hole left gives the opportunity for transitions, transversions or nucleotide deletions when the DNA replicates, or the DNA sugar-phosphate 'backbone' can break with loss of a part of the chain. All these chemical mechanisms damage only one helix of the DNA molecule exposed to the mono- or bi-functional alkylating drug and the mutation ought to appear only in one half of the genomes of the following generations.

Bridge formation between the two helices by a bifunctional alkylating agent would prevent the DNA replication and lead to the deletion of the cross-linked macromolecule which will have no descent in any genome of the following generations[4]. Besides this qualitative difference, mutation following bridge formation must be quantitatively the most important for bifunctional alkylating agents because the target for the drug is then the whole DNA molecule carrying the competent gene instead of a few sensitive triplets inside this gene.

In a previous publication[4], we tried to evaluate the size of a DNA molecule carrying a known genetic locus by the use of a bifunctional alkylating reagent labelled in its alkyl moiety. Cells of *Chlamydomonas eugametos* Moevus, sex minus, haploid and spontaneously resistant to streptomycin, were treated with 'Myleran' (1,4-dimethanesulphonyloxybutane) labelled with tritium in the butanediol moiety and a correlation was found between the frequency of cells becoming sensitive to streptomycin and the radioactivity of the isolated DNA. Starting from the hypothesis mentioned earlier that, when the mutation reaches all the cells issued from the treated one, the chemical damage must be a cross-linking between the

complementary helices of the DNA molecule, and, taking into account the fraction of alkyl groups attached on the DNA which is utilized for cross-linkings, it must be possible to calculate the size of the DNA molecule carrying the competent locus. In the case of *Chlamydomonas eugametos* and the resistance to streptomycin, we tentatively evaluated for the DNA molecule specifically concerned a size of about 3,000,000 A.M.U.; this figure was only provisional in view of the several rather arbitrary corrections introduced in the arithmetics and that must be checked.

The reliability of the labelled 'Myleran' method for the estimation of the weight of an *in situ* DNA molecule biologically characterized depends on two things: (*a*) the stability of the alkyl groups bound to the DNA; (*b*) the knowledge of the percentage of bound alkyl groups forming bridges between the complementary strands of the macromolecule. The examination of these two problems was the purpose of the work reported hereafter.

Calf thymus DNA and 'Myleran' labelled with tritium in positions 2 and 3 of the butanediol moiety (20 mc./m.mole) were used throughout these experiments which were carried out at 22° C. They were dissolved separately before being put together; the final concentration of 'Myleran' was always 0·1 mg/ml. The radioactivity determinations were performed by liquid scintillation counting after suitable preparation of the sample.

(*A*) In a first set of experiments, the DNA was left for 3 h in contact with the *T*-'Myleran' before precipitation (addition of solid sodium chloride to bring the concentration to 1 M, then of an equal volume of 95 per cent ethanol). The fibres were washed three times with absolute ethanol and afterwards with anhydrous acetone until this solvent did not extract any more radioactivity. At this stage the alkylated DNA radioactivity was 9,100 d.p.m. mg.

This alkylated DNA was redissolved in water and

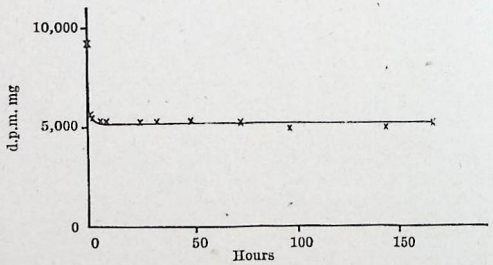

Fig. 1

DNA reprecipitated from aliquots at various time intervals. Fig. 1 shows that there was a sudden drop of the DNA radioactivity followed by a plateau.

To understand the meaning of the initial drop, the supernatant was percolated through an 'Amberlite *IR*-120(*H*)' column: 80 per cent of the activity came with the effluent and the water washings, while 20 per cent were retained and could be eluted with N ammonium hydroxide. The 20 per cent are thus alkylated bases. The 80 per cent (2,800 d.p.m. mg DNA) are due either to the hydrolysis of alkyl phosphates[5], or to a contamination by unreacted 'Myleran'. It is noteworthy that, when the DNA is precipitated from the solution immediately after it has been put in contact with the *T*-'Myleran', the activity bound to the DNA is precisely 2,800 d.p.m. mg.

Of the total of 9,100 d.p.m., we see that only 700 d.p.m. was lost as hydrolysed alkylated bases. Of course, there could also be some depurination during the 3 h contact between DNA and *T*-'Myleran': but the large radioactivity of the unreacted 'Myleran' prevents the detection of alkylated bases in the supernatant after precipitation of the DNA.

Most important from our point of view is the fact that, after the initial drop, the specific activity of the alkylated DNA is completely stable, around 5,000 d/min mg in our experiment. At this stage, the DNA can be dissolved and precipitated many times in succession without change of the specific activity: this proves that the precipitation procedure does not differentiate between alkylated and non-alkylated DNA.

This alkylated DNA of stable specific activity was hydrolysed for 30 min in 98 per cent formic acid at 175° C[6] and the hydrolysate evaporated to dryness under vacuum. The residue was dissolved in N hydrochloric acid and filtered. After substitution of water for N hydrochloric acid, the solution was passed through a column of 'Amberlite *IR*-120(*H*)' and the ion-exchanger washed with water; the effluent contained 880 d.p.m. (per mg hydrolysed DNA) which was not bound to compounds absorbing at 260 mμ. The column was afterwards eluted with N ammonium hydroxide which carried away the weak bases and 3,500 d.p.m. per mg DNA. This shows that most of the radioactivity firmly bound to the DNA is in alkyl groups attached on the bases of the macromolecule. The base fraction was submitted to a two-dimensional paper chromatography (methanol/ethanol/conc. hydrochloric acid/water (50 : 25 : 6 : 19, by vol.) and *n*-butanol/conc. ammonium hydroxide/water (86 : 1 : 13, by vol.)): the ultra-violet showed the fluorescent spots of the four bases plus two faint extra ones at the sites given by Brookes and Lawley[7] for the 7-alkyl derivative of guanine ($R_F = 0{\cdot}48$ and $0{\cdot}15$) and the corresponding di-guanyl compound ($R_F = 0{\cdot}26$ and $0{\cdot}00$).

Radioactivity was found only at the sites of these two latter spots: respectively 2,950 and 250 d.p.m. (per mg hydrolysed DNA). The conclusion is that the stable radioactivity fixed on DNA after reaction with the *T*-'Myleran' is mostly in alkylated guanines with about 8 per cent forming bridges between two neighbouring guanine residues.

(*B*) In a second set of experiments, the DNA was left continuously in the presence of the *T*-'Myleran' in a 0·1 M phosphate buffer solution of *p*H 6·6. The DNA was precipitated from aliquots at various time-intervals and the fibres carefully washed with anhydrous acetone before the radioactivity determination. The solid line of Fig. 2 shows a continuous but non-linear increase of the radioactivity bound to the DNA. (The radioactivity found at time 0 was taken as a contamination by unreacted 'Myleran' and all the results were corrected accordingly.) In the reaction kinetics, the DNA can be considered in excess since, at the highest point of the curve, there is only one alkyl group per 3,000 nucleotides. On the other hand, the half-life of the 'Myleran', at the same temperature and in the same buffer, was shown to be 132 h, which means that the k_1 constant of the hydrolysis of the di-ester to the mono-ester has a value of $8{\cdot}75 \times 10^{-5}$ min^{-1}. Taking into account only the hydrolysis of the 'Myleran' (and assuming no participation of the mono-ester), the radioactivity (d.p.m.) bound per mg DNA ought to be:

$$x = \frac{k_2}{k_1}(1 - e^{-k_1 t})$$

where $k_2 = 19{\cdot}2$ min^{-1} is read from the initial slope of the solid curve drawn on Fig. 2. The theoretical curve of the 'x' values is depicted by a dashed line on the same graph: it is above the experimental one. The difference might be due, at least in part, to the existence of labile pools of tritium bound to the DNA.

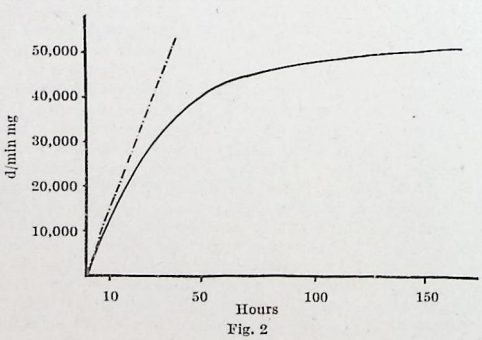

Fig. 2

The foregoing results indicate that, after treatment of the DNA with tritiated myleran, several pools of bound tritium are formed: a very labile, which might be alkyl phosphates but the existence of which has not been proved unambiguously (it could be a contamination by unreacted 'Myleran'); a medium labile one of alkyl bases; and, quantitatively most important of all, a stable pool also of alkyl bases. The stably bound tritium can be used as an index of the magnitude of the alkylation undergone by the DNA.

The analysis of the stably bound radioactivity has shown that it is mostly in alkyl groups attached to guanine residues with about 8 per cent linking together two guanines. We have no proof that these two guanines belong, in every instance, to the DNA complementary strands. Further work must be done to have the exact ratio between the stably bound alkyl groups and the bridges between the DNA helices. Even without this knowledge, relative molecular weights of *in situ* DNA molecules could be estimated by the 'Myleran' method; the ratio is, however, expected to be influenced by the base composition of the DNA's.

This technique of *in situ* evaluation of the weight of a DNA molecule functionally characterized by the use of a bifunctional alkylating agent has also some bearing on the elaboration of a reasonable model for non-bacterial cell chromosomes. The underlying hypothesis can be borne out only if the chromosomes of such cells are composed of independent DNA molecules glued together by more fragile linkers. On the other hand, the method actually provides a means to detect genetic loci carried by the same DNA molecule and to inquire whether each DNA molecule is an integrated operational unit[6].

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