# DNA UNWINDING IN ALKALI APPLIED TO THE STUDY OF DNA REPLICATION IN MAMMALIAN CELLS

Björn RYDBERG

*Department of Physical Biology, Gustaf Werner Institute, University of Uppsala, Slottsgränd 3, S-752 20 Uppsala, Sweden, and Research Institute of National Defence, Department 4, S-104 50 Stockholm, Sweden*

Received 13 February 1975

## 1. Introduction

DNA unwinding in alkali has recently been used as a basis for very sensitive methods for the detection of radiation-induced lesions in DNA of mammalian cells [1–3]. After the cells have been lysed, at pH $\gtrsim$ 12, gradual separation of the two strands in DNA occur at a rate that seems to be proportional to the number of DNA strand breaks. The fraction of the DNA transformed to single strands after a given time of alkali treatment (1 min to 1 hr) can be tested by neutralization, immediate fragmentation of the partly unwound molecules with a brief ultrasound treatment, and subsequent estimation of the relative amount of single-stranded and double-stranded DNA molecules by hydroxylapatite chromatography [1,3]. According to our interpretation [3], the unwinding of DNA is initiated at breaks in the DNA molecules and proceeds from there in both directions, with rates governed by frictional forces opposing the rotation of the separated single strands.

Similarly, the replication forks in mammalian cells, with accompanying nicks between 'Okazaki' segments [4,5], can be expected to serve as starting points for unwinding in alkali. Accordingly, in a pulse-chase experiment with [$^3$H]TdR, the distances by which the replication forks have moved in relation to the pulse-labelled regions, after a given time of DNA replication in unlabelled medium, might be estimated from the time needed for subsequent DNA unwinding in alkali to reach the labelled regions and transform them to single strands.

The results of experiments reported here support the above considerations, provided termination and sealing of the replications are taken into account, in accordance with current models for DNA replication in mammalian cells [6,7]. The proposed method seems to be useful for the study of normal or abnormal DNA replication. The effect of hydroxyurea on DNA replication is studied as an example.

## 2. Materials and methods

### 2.1. *Cell culture and labelling*

A primary cell line of Chinese hamster lung fibroblasts [8] was cultured in Leighton tubes under conditions described elsewhere [3]. Pulse labelling was performed by the addition of 0.5 $\mu$Ci/ml [$^3$H]TdR (5 Ci/mmol), incubation for further 3 or 5 min at 37°C, washing the cells twice and incubating again with prewarmed fresh non-radioactive medium, in some experiments containing $10^{-5}$ M TdR. The postlabel incubation was stopped by trypsination (2 min at 37°C) and cooling of the cell suspension to 0°C.

### 2.2. *Alkali treatment*

The freshly made alkaline solutions were the same as previously used [3], see figure legends. The alkali treatment was started by transferring 50 $\mu$l of the cell suspension (about $5 \cdot 10^4$ cells) to a test tube and rapidly adding 1 ml of the alkaline solution. The DNA strand separation was then allowed to take place in the dark at 20°C. The treatment was terminated by rapid addition of 1 ml 0.034 M HCl and immediate sonication of the resulting neutral solution for 15 sec (Branson sonifier B-12, microtip,



*North-Holland Publishing Company – Amsterdam*

level 2). After addition of 0.1 ml 5% sodium dodecyl sulfate (SDS) the samples were stored at −20°C.

2.3. *Hydroxylapatite chromatography*

The single-stranded and double-stranded DNA were separated, after thawing, on 0.5 ml columns (diameter 10 mm) of hydroxylapatite (Bio-Rad: Bio-Gel HTP), held at 60°C. The columns, packed with the dry powder, were washed with 1.5 ml 0.5 M and 1.5 ml 0.01 M potassium phosphate ($K\text{-}PO_4$) buffer, pH 6.9, before the samples were applied. Most low molecular weight products were washed out with 1.5 ml 0.01 M $K\text{-}PO_4$ buffer, after which the single-stranded DNA was eluted with 3 ml 0.125 M $K\text{-}PO_4$ buffer and the double-stranded DNA with 1.5 ml 0.25 M $K\text{-}PO_4$ buffer, pH 6.8–6.9. The efficiency of the separation was as reported earlier [3]. With a flow rate of 0.75 ml/min, up to 12 simultaneous separations were completed in about 20 min. The $^3H$ activity in the eluates was then determined by liquid scintillation counting.

2.4. *Miscellaneous methods*

Estimation of 'acid-soluble' radioactivity was made by addition of 0.1 mg/ml of carrier DNA to the samples, precipitation in 0.2 M perchloric acid at 4°C for 20 min, and centrifugation at about 2000 *g* for 20 min. The radioactivity remaining in the supernatant was regarded as the acid-soluble fraction.

Separations of thymidine mono-, di- and tri-phosphates on ECTEOLA cellulose columns were performed as described in [9].

Ultracentrifugation of DNA in alkaline sucrose gradients was made essentially as described in [10], and evaluation of the data was made according to [11].

## 3. Results

3.1. *Influence of labelled low molecular weight products*

Various tests of the experimental methods have been made earlier [3]. An additional point to consider here is the labelled low molecular weight products present in the cells at short times after pulselabelling with [$^3$H]TdR, including [$^3$H]TdR, dTMP, dTDP and dTTP. When these substances (unlabelled) were tested on the hydroxylapatite columns, under the conditions described above, 98% or more of TdR and dTMP were washed out with the 0.01 M $K\text{-}PO_4$ buffer, but 25% of dTDP and 70% of dTTP were retained, and were instead eluted with the 0.125 M $K\text{-}PO_4$ buffer.

When cells were pulse-labelled for 3 min (no chase period) and then alkali-treated, the acid-soluble radioactivity eluted with the 0.125 M buffer constituted about 30% of the total radioactivity eluted, and behaved as [$^3$H]dTDP and [$^3$H]dTTP when tested by ECTEOLA column chromatography. Chasing the cells with medium containing $10^{-5}$ M TdR reduced the acid-soluble fraction to 5% of the total radioactivity after 15 min and to less than 1% after 2 hr. The data presented below are *not* corrected for the presence of labelled low molecular weight products in the single-stranded DNA fractions.

3.2. *Kinetics for unwinding of pulse-labelled DNA*

Cells were pulse-labelled for 3 min and then incubated in non-radioactive medium, containing $10^{-5}$ M TdR, for further 15 or 30 min. For calibration, a culture was also incubated for 3 hr, following the pulse, and then given 2 krads by exposure to $^{60}Co$ gamma radiation at 0°C. This will induce about one single strand break per 100 $\mu$m ($2 \cdot 10^8$ daltons) of DNA, as estimated from ultracentrifugational data, presumably with a random distribution of breaks along the DNA molecules. The result of subsequent alkali treatments for 1 to 60 min are shown in fig.1. The zero time treatments were performed by lysing cells in 0.25% SDS in already neutralized solutions for 1 hr before sonication for 15 sec.

The scales in fig.1 are chosen in such a way that unwinding of DNA from randomly distributed breaks will result in a straight line, according to the model [3]. Applying this model to the irradiated cells, the top scale in fig.1 can be calculated, showing the distances by which the strand separation has proceeded on each side of the breaks for the alkali treatment times, respectively. Labelled regions having the closest nick in excess of the indicated distances will then remain double-stranded after alkali treatment, and the curves in fig.1 will show the distribution of distances between the labelled regions and the closest nick (provided the model is correct).

The component that seems to be single-stranded without alkali treatment in fig.1 is, at least partly, an artefact due to [$^3$H]dTDP and [$^3$H]dTTP (see

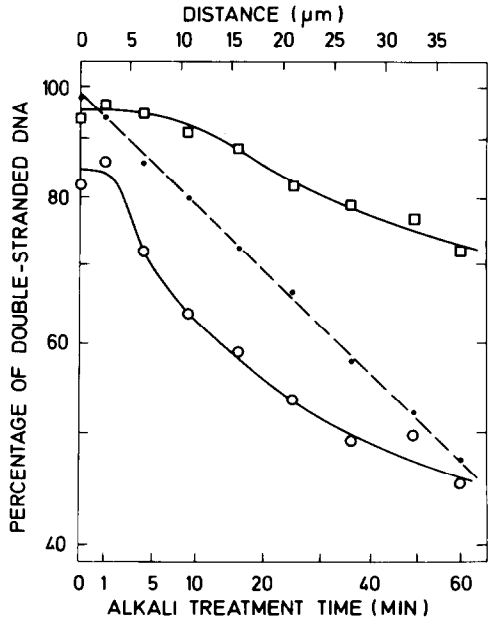


Fig.1. Kinetics of strand separation of pulse-labelled DNA. *Ordinate*: Percentage of labelled DNA that remained double-stranded after alkali treatment in 0.03 M NaOH, 0.01 M $Na_2HPO_4$ at 20°C (logarithmic scale). *Abscissa*: Alkali treatment time, plotted proportionally to $(time)^{0.66}$. Postlabel incubation: 15 min (○——○), 30 min (□——□), 3 hr followed by 2 krads $^{60}Co$ gamma radiation (●- — -●). Explanation of the top scale is given in the text.

above). 'Okazaki' fragments, if present, may also contribute to this component because these often appear single-stranded after isolation [12,13].

The results suggest that there is a wide, non-random distribution of distances between labelled regions and the closest nick (or alkali-labile bond). This is in accordance with previous autoradiographic investigations [6,14], where a wide distribution of synthesis rates for individual growing points was found. The mean value for the synthesis rate at the replication forks, in mammalian cells, has generally been found to be in the range of 0.5 to 1 μm/min [2,6,11,14]. This corresponds well to the steepest portions of the curves in fig.1, that is, at the distance of 7.5–15 μm for the cells chased for 15 min and at the distance of 15–30 μm for the cells chased for 30 min. A large fraction of the labelled regions, however, seem to be located at distances in excess of that expected for the replication forks. This may result from ligase activity in connection with coalescing or terminating replicons.

### 3.3. *Effect of hydroxyurea on DNA replication*

Cells were pulse-labelled for 5 min and then

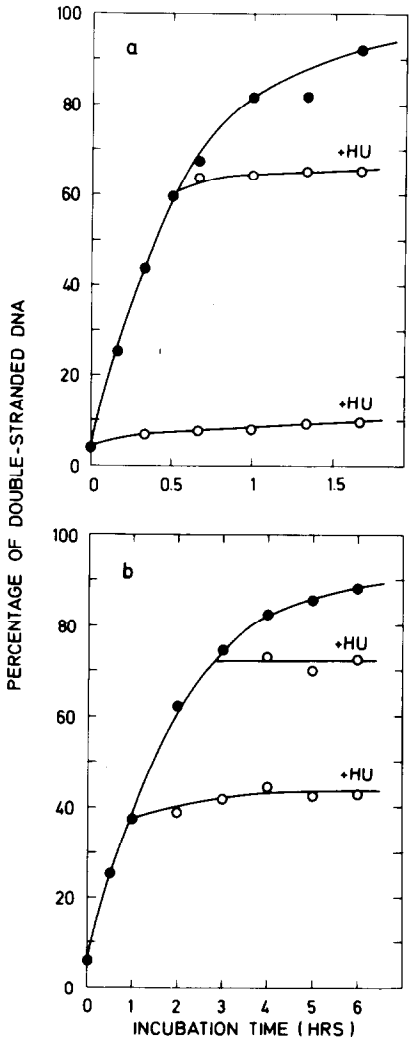


Fig.2. Strand separation of pulse-labelled DNA after various chase periods. *Ordinate*: Percentage of labelled DNA that remained double-stranded after 30 min alkali treatment at 20°C in (a) 0.03 M NaOH, 0.01 M $Na_2HPO_4$ or (b) 0.03 M NaOH, 0.01 M $Na_2HPO_4$, 0.9 M NaCl. *Abscissa*: Incubation time at 37°C in non-radioactive medium after pulse-labelling with [$^3$H]TdR (●——●). To some cultures hydroxyurea (HU) was added at respectively 0, 0.5, 1 or 3 hr after the pulse and the incubation was continued (○——○).

allowed to continue DNA replication in non-radioactive medium. To some cultures 2 mM hydroxyurea (HU), an inhibitor of DNA replication [15], was added at 0, 0.5, 1 or 3 hr after the pulse-labelling. Following the post-label incubation (10 min–6 hr), the cells were trypsinized and alkali-treated for a fixed time (30 min) under the same conditions as above (fig.2a), and also in an alkaline solution [3] where the unwinding is about 10 times faster (fig.2b). As can be seen for the untreated controls, an increasing fraction of the labelled regions remained double-stranded after alkali treatment as the incubation time was prolonged, probably reflecting the increasing mean distance between label and replication forks (or other nicks and alkali-labile bonds). In terms of the model discussed above, unwinding has proceeded about 25 $\mu$m ($5 \cdot 10^7$ daltons) on each side of the breaks in fig.2a, and about 250 $\mu$m ($5 \cdot 10^8$ daltons) in fig.2b. Since the length of most replicons probably is less than 250 $\mu$m [6,7], the effect seen in fig.2b probably reflects mainly the termination of the replicons.

It is evident that hydroxyurea is inhibiting the process during incubation that results in a changed response to alkali treatment (fig.2). The simplest interpretations are that the replication forks stop movement, and the ends (or other nicks) remain open, or that sealing of gaps or nicks is inhibited, in which case short DNA fragments may still be synthesized [16].

The presence of [$^3$H]dTDP and [$^3$H]dTTP in the single-stranded DNA fractions have affected the results in fig.2 to a small extent. If the percentage of double-stranded DNA at zero time in fig.2b, for example, is corrected for the presence of 30% labelled low molecular weight products (see above), the value will change from 7% to 10%. After chasing, the relative corrections will be even smaller.

## 4. Discussion

Study of DNA chain elongation at the replication forks in mammalian cells can also be made using autoradiography [6,14] or ultracentrifugation [11,17,18]. In comparison, the outstanding advantage of the method presented here is its simplicity and the speed by which the measurements can be made. Further, the use of FUdR is also avoided.

Other procedures, based on DNA unwinding, have been applied to newly replicated DNA. In one study [2], partly unwound DNA molecules were allowed to rewind after alkali treatment, and separation of single-stranded and double-stranded DNA molecules was made with isopycnic ultracentrifugation. The interpretation of the results is different, because of the rewinding, and experimental variability was reported to be a problem. Another seemingly related procedure [19], based on alkaline elution of DNA from lysed cells absorbed to cellulose triacetate filters, seems to be very simple, experimentally, but the underlying mechanisms are not clear.

## Acknowledgements

The skillful technical assistance of Mrs K. Uisk is gratefully acknowledged. The work has been financially supported by grants from the Swedish Atomic Research Council and the Swedish Cancer Society.

## References

[1] Ahnström, G. and Erixon, K. (1973) Int. J. Radiat. Biol. 23, 285–289.
[2] Jolley, G. M. and Ormerod, M. G. (1974) Biochim. Biophys. Acta 353, 200–214.
[3] Rydberg, B. (1975) Radiat. Res. 61, 274–287.
[4] Sakabe, K. and Okazaki, R. (1966) Biochim. Biophys. Acta 129, 651–654.
[5] Schandl, E. K. and Taylor, J. H. (1969) Biochem. Biophys. Res. Commun. 34, 291–300.
[6] Huberman, J. A. and Riggs, A. D. (1968) J. Mol. Biol. 32, 327–341.
[7] Lehmann, A. R. and Ormerod, M. G. (1972) Biochim. Biophys. Acta 272, 191–201.
[8] Sturelid, S. (1971) Hereditas 68, 255–276.
[9] Johanson, K. J. (1972) Scand. J. Gastroent. 7, 477–482.
[10] Elkind, M. M. (1971) Biophys. J. 11, 502–520.
[11] Lehmann, A. R. and Ormerod, M. G. (1970) Biochim. Biophys. Acta 204, 128–143.
[12] Painter, R. B. and Schaefer, A. (1969) Nature 221, 1215–1217.
[13] Wanka, F. (1973) Biochem. Biophys. Res. Commun. 54, 1410–1417.
[14] Cairns, J. (1966) J. Mol. Biol. 15, 372–373.
[15] Pfeiffer, S. E. and Tolmach, L. J. (1967) Cancer Res. 27, 124–129.

[16] Magnusson, G. (1973) J. Virology 12, 600–615.
[17] Goldstein, N. O. and Rutman, R. J. (1973) Nature New Biol. 244, 267–269.
[18] Gautschi, J. R. (1974) J. Mol. Biol. 84, 223–229.
[19] Kohn, K. W. and Grimek-Ewig, R. A. (1973) Cancer Res. 33, 1849–1853.