FORD, E. B. 1964. *Ecological Genetics.* Methuen, London.
GORDON, C. 1935. *Amer. Nat., 69,* 381.
HALDANE, J. B. S. 1930. *Amer. Nat., 64,* 87-90.
HALDANE, J. B. S. 1939. *J. Genet., 37,* 365-374.
KETTLEWELL, H. B. D. 1963. *Proc. 16th Int. Cong. Zool., 2,* 197-198.
PLUNKETT, C. R. 1932. *Proc. 6th Int. Cong. Genet., 2,* 158-160.
RENDEL, J. M. 1962. *The Evolution of Living Organisms.* Ed. G. W. Leeper. Melbourne University Press.
SHEPPARD, P. M. 1961. *Advance. Genet., 10,* 165-216.
WRIGHT, S. 1929*a*. *Amer. Nat., 63,* 274-279.
WRIGHT, S. 1929*b*. *Amer. Nat., 63,* 556-561.
WRIGHT, S. 1934. *Amer. Nat., 68,* 24-53.

AN EFFECT OF FIXATION AND STAINING ON THE REALIZATION OF COLD-INDUCED HETEROCHROMATIN IN *TRILLIUM*

C. J. GRANT

Service de Génétique, Institut National Agronomique, 16 Rue Claude Bernard Paris V—France *

Received 14.viii.65

1. INTRODUCTION

Root tips of *Trillium* species continue to synthesise DNA between 0° and 3° (Boothroyd and Lima de Faria, 1964; Grant, 1964) and replication of the heterochromatin is completed at the same time as that of the euchromatin (Grant, 1965). But studies of the first (X_1) mitosis indicate that the H-segments which are labelled with ^{3}H-thymidine at ordinary temperatures apparently lose this label if they are cooled to 0° after their entry in the G2 phase (Haque, 1963; Grant, 1964).

Haque concluded that cold treatment induced " a true loss of the pre-formed DNA ". This, however, seems unlikely in view of the nature of the DNA replication process and of the lack of recovery during prophase at ordinary temperatures as shown by both colchicine (Dyer, 1964) and auto-radiographic techniques (Grant, 1964; Woodard and Swift, 1964).

Woodard and Swift suggested that the H-segments arise by a process of localised uncoiling and, consequently, that there is not enough tritium label per unit area to activate the silver grains in the photographic emulsion. However, the increase in the length of the H-segments is relatively small (except in *T. undulatum*) and it cannot account for unlabelled H-segments in cells as heavily labelled as that shown in the plate.

Grant (1964) offered two hypotheses. First, it was supposed that the enzymes necessary for DNA-precursor synthesis might be available only during the S period when replication might be impossible for the highly condensed H-segment DNA. Thus DNA precursors might be formed in S and, in some way, become attached to the chromosome or held in the nucleus during G2 to be fully incorporated in the H-segments only when these lost their super-condensation in prophase. However, the failure to label H-segments at prophase (Grant, 1964; Woodard and Swift, 1964) renders this unlikely.

Alternatively it was suggested that although DNA synthesis might be

* Present Address: The Botany Department, The University, Bristol.

completed in S, cold treatment could still affect the secondary processes of chromosome replication, viz. the changes in chromosome structure, histone synthesis, etc. which must be completed before the end of G2 to prepare the chromosome (as opposed to the DNA) for division. Then, differences between eu- and hetero-chromatin might result in H-segment DNA being preferentially attacked during fixation and/or staining.

On the first hypothesis, label would be lost after cold treatment before X_1, but if allowed to become fully incorporated into the chromosome would not be lost from an H-segment cold-treated before X_2. According to the second, label would be lost from heterochromatin in both X_1 and X_2 divisions. This experiment was designed to test these two alternatives and to see if different procedures could affect the labelling.

2. MATERIAL AND METHODS

In this study two plants were used from a clone of *Trillium grandiflorum* of which a photograph of the chromosomes after cold treatment is given in Grant (1965).

Attached roots were labelled in 2 μC/ml. ^{3}H-thymidine (specific activity 5·8 C/mM) for 12 hours at 25°. They were then grown for 6 days at 25° to allow the labelled cells to divide once, to complete a second DNA replication in the absence of label, and to enter the G_2 preceding the X_2 division (at this temperature one mitotic cycle takes about 5 days, for other data see Grant, 1965). At this stage the roots were cooled to 2·5° and after 5 more days they were separated into two groups of 5. One group was fixed overnight in Benda's solution and the other in 1:3 acetic alcohol.

The roots fixed in Benda were stored for 2 days in a mixture of one 50 per cent. glycerol:2 glacial acetic acid:2 70 per cent. alcohol. Before feulgen staining they were washed, bleached and softened for 15 minutes in a mixture of 20 per cent. hydrogen peroxide and saturated ammonium oxalate, and hydrolysed for 13 minutes in 1N HCl at 60°. The roots fixed in acetic alcohol were stored in 70 per cent. alcohol and hydrolysed for 6 minutes before staining.

Squash preparations were made by the " dry-ice " method and covered with Kodak AR 10 stripping film for auto-radiography. Slides were exposed for 8 weeks in deep freeze before being developed and made permanent with euparal. Thus, apart from the fixation and the pre-staining procedures, all treatments were identical.

3. RESULTS AND DISCUSSION

Although this experiment was timed to show labelled X_2 cells in division, in practice the considerable degree of asynchrony between cell populations (Grant, 1965) allows a comparison of three different types of cell: those which were not in S at the time of labelling (67 per cent. of the total mitoses); the fully labelled cells that are in their first (X_1) division since labelling (18 per cent.); and the half-labelled cells in X_2 (15 per cent.). In each group, the percentage of mitoses showing H-segments is the same after a given fixation.

In both labelled and half-labelled hetero-chromatic mitoses, the large terminal H-segments of the B chromosomes were scored for the presence or absence of label. In only 14 of the 200 labelled heterochromatic cells examined, was there appreciable label over these segments. Since there was no difference in this respect between X_1 and X_2 divisions (7 cells in each showing labelled H-segments) the loss of pre-formed but unincorporated DNA precursor is ruled out.

Tables 1 and 2 show that there are considerable differences due to the fixation and staining procedures in that after Benda *more* mitoses are heterochromatic and *fewer* cells are labelled. In confirmation of these results, Wakonig-Vaartaja and Read (1965) have shown that fixation in Benda with subsequent feulgen staining reduces the area (and by implication the volume) of *Allium cepa* chromosomes in squash preparations by between 10 and 20 per cent. as compared with stained or unstained acetic alcohol fixed material. Also Taylor and McMaster (1954) showed that 4 minutes' excess hydrolysis before staining could release up to 85 per cent. of P^{32}-labelled DNA from *Lilium* chromosomes after formic or acetic fixatives.

TABLE 1

The percentage of mitoses showing heterochromatin after different fixations. 300 mitoses were scored for each fixation

Fixation	Unlabelled mitoses	Labelled mitoses	½ labelled mitoses
Benda . . .	80	79	78
Acetic alcohol . .	51	44	46

TABLE 2

The percentage of cells showing label after different fixations

Fixation	Mitosis	Interphase
Benda . . .	26	19
Acetic alcohol . .	44	37

To explain these results in *Trillium* it is suggested that cold-treatment does interfere with the completion of chromosome replication and that this effect is more marked in the H-segments. Thus the extra hydrolysis and bleaching required after Benda fixation result firstly, in the solution of more histone or other protective material, especially in the cold-sensitised H-segments; and secondly, in the solution of more DNA, reducing labelling in general and especially in the less well-protected H-segments. This would explain the observed labelling pattern and also, if chromosomal coiling is due largely to changes in the histone/protein structure, the variations in the amount of despiralisation noted by different authors (see Dyer, 1964).

This reasoning has additional merit in that it can explain the apparently contradictory results of LaCour, Deeley and Chayen (1956) on the one hand and those of Heyes and Shaw (1958) on the other. The former found that cold teatment reduced the photometrically determinable DNA content of the cell in species both with and without heterochromatin; while the latter were unable to confirm this by chemical analysis. The fixatives used (osmic or acetic) would also explain the difference between LaCour (1960), and Woodard and Swift (1964) who respectively, could and could not demonstrate a cold-induced DNA loss in *Trillium*. Further, it is no longer necessary

to imagine or to try to understand the consequences of a true DNA loss in the living material.

In conclusion, the suggested lack of protection of the H-segment DNA agrees well with the localisation in heterochromatin of those aberrations induced by many chemical mutagens, but not those induced by ionising radiations which would be unselective in their primary effects.

4. SUMMARY

The effect is described of two fixation and staining procedures on the realisation of cold-induced H-segments in the first and second divisions after labelling *Trillium* roots with tritiated thymidine.

It is suggested that cold treatment, while not directly affecting DNA synthesis, impedes the secondary processes of chromosome replication more in hetero- than in eu-chromatin. This results in selective loss of DNA from the H-segments during pre-staining procedures although there is no loss in the living plant.

Acknowledgments.—I should like to thank Dr H. Heslot for his helpful criticism of the manuscript.

This investigation was supported by Euratom, the Commissariat à l'Energie Atomique and the Institut National de la Recherche Agronomique (Contrat No. 009-62-10 BIAF).

5. REFERENCES

BOOTHROYD, E. R., AND LIMA DE FARIA, A. 1964. DNA synthesis and differential reactivity in the chromosomes of *Trillium* at low temperature. *Hereditas*, *52*, 122-126.

DYER, A. F. 1964. Heterochromatin in American and Japanese species of *Trillium*. II. The behaviour of the H-segments. *Cytologia*, *29*, 171-190.

GRANT, C. J. 1964. Induced chromosome breakage and the chromosome cycle. D.Phil. Thesis, Oxford University.

GRANT, C. J. 1965. Chromosome aberrations and the mitotic cycle in *Trillium* root tips after X-irradiation. *Mutation Research*, *2*, 247-262.

HAQUE, A. 1963. Differential labelling of *Trillium* chromosomes by H^3-thymidine at low temperature. *Heredity*, *18*, 129-133.

HEYES, J. K., AND SHAW, G. W. 1958. Chemical determination of deoxyribonucleic acid in root tips before and after cold treatment. *Nature*, *181*, 1337-1338.

LACOUR, L. F. 1960. The reaction of heterochromatic segments of chromosomes to the feulgen stain. *John Innes Institute, Annual Report*, *51*, 38-39.

LACOUR, L. F., DEELEY, E. M., AND CHAYEN, J. 1956. Variations in the amount of feulgen stain in nuclei of plants grown at different temperatures. *Nature*, *177*, 272-273.

TAYLOR, J. H., AND MCMASTER, R. D. 1954. Autoradiographic and microphotometric studies of desoxyribose nucleic acid during microgametogenesis in *Lilium longiflorum*. *Chromosoma*, *6*, 489-521.

WAKONIG-VAARTAJA, R., AND READ, J. 1965. Measurement of *Allium cepa* chromosomes. *Experimental Cell Research*, *38*, 264-271.

WOODARD, J., AND SWIFT, H. 1964. The DNA content of cold-treated chromosomes. *Experimental Cell Research*, *34*, 131-137.

Plate

Heavily labelled heterochromatic cell showing absence of label over some of the H-segments. Note the terminal segment of the short arm of a B chromosome in the centre of the plate.