SUPPRESSION OF MITOSIS AND MACROMOLECULE SYNTHESIS IN ONION ROOTS BY HEAVY WATER

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Exposure of microorganisms to high concentrations of D_2O slows or stops growth, but the inhibition is often overcome after a time; in many instances, serial subculture leads to successful adaptation (Crespi, Conrad, Uphaus, and Katz, 1960; Crespi, Marmur, and Katz, 1962). Cell division, among the many component processes of population growth, seems generally to be the most sensitive process. Shifts in the patterns of nucleic acid synthesis are sometimes seen (Flaumenhaft, Conrad, and Katz, 1960), but there is thus far scant evidence that DNA synthesis is especially sensitive.

The situation in animal cells is different. Here, too, cell division is the first process to suffer a blockade (Gross and Spindel, 1960 *a*), but, in contrast to microorganisms, animal cells cease dividing very soon after immersion in D_2O-rich media. In sea urchin eggs, which are among the most thoroughly studied cell types, the effect on mitosis is virtually instantaneous (Gross and Spindel, 1960 *b*; Marsland and Zimmerman, 1963). There is no doubt that the speed of this effect depends upon the extremely short equilibration time between medium and intracellular water,—probably a few seconds (Tucker and Inoué, 1963),—and that it is related to the presence of a large and complex mitotic apparatus, whose mechanical properties are changed by deuteration (see, for review, Gross, Spindel, and Cousineau, 1964). But there is also a profound effect upon DNA synthesis. In sea urchin eggs, high concentrations of deuterium in the medium cause a rapid and reversible block of DNA replication (Gross and Harding, 1961). RNA synthesis in fertilized eggs is also inhibited, although to a smaller extent than DNA synthesis, while protein synthesis is even less affected (Cousineau, 1963). In fact, treatment of *unfertilized* eggs with D_2O evokes protein synthesis which is ordinarily blocked, and the return of such treated eggs to normal sea water brings about parthenogenetic activation and prolonged mitotic activity (Gross, Spindel, and Cousineau, 1963). Over the intervals in which animal cells can survive in media containing 80 per cent or more D_2O, there appears to be no adaptive recovery.

These facts lead to the suggestion that in higher

cells the organization of the genetic material into large and complex chromosomes, which are in turn moved about by a large and complex organelle, confers a special sensitivity to D_2O; that the rapid and complete suppression of DNA synthesis may reflect not so much an inhibition of some elementary enzymatic step in replication but the slowing of some critical process in which supramolecular units of the chromosome must move or function as a whole.

It seemed potentially useful, from this point of view, to determine what effect D_2O might have on mitosis and DNA synthesis (and incidentally on the synthesis of RNA and protein) in plant roots, whose cells also have large chromosomes but in addition a mitotic apparatus and a cytokinetic mechanism differing in some important features from those in animal cells.

Roots of *Allium cepa* L. were grown in Bonner's medium (Bonner, 1943). Deuterium-enriched media were prepared by dissolving the salts directly in 99.9 per cent D_2O, redistilled from alkaline permanganate, plus ordinary water, mixed in the desired ratios. Bulbs were set over beakers containing the test media, and the roots immersed intact. Sample roots were removed at intervals for determination of mitotic indices, which were obtained from squash preparations stained with acetic orcein.

The results are shown in Fig. 1. Confirming the observations of Stein and Forrester (1963) on roots of *Pisum* and *Zea*, we found that the mitotic indices fell precipitously, reaching zero at 12 hours (*i.e.* in less than one intermitotic time). Thereafter, all cells remained arrested in interphase. No obvious alterations in the appearance of stained chromosomes were detected (at least at the light microscope level) during the time that the mitotic index was declining, but the arrested interphase nuclei had enlarged and very heavily stained nucleoli.

This pattern differs from the one seen in animal cells, to the extent, at least, that the time required for mitotic inhibition is much longer in the plant. While, in addition, all stages of mitosis seem equally sensitive in the animal cells studied, the root meristem cells complete an ongoing mitosis before becoming arrested. This difference may reflect a much slower equilibration of external water with cell water, for which there is some indirect evidence (see below), rather than a real difference in the sensitivity of the mitotic apparatus to high concentrations of D_2O. The interphase arrest is not, however, inconsistent with a rapid block to DNA synthesis, as occurs in animal cells. The results of experiments with tritiated thymidine indicate that such a block does occur.

Roots growing in D_2O-Bonner's solution were labeled with H^3-thymidine (1.920 c/mmole and 20 μc/ml, New England Nuclear Corp., Boston) by exposure to isotope-containing medium for 30 minutes. The D_2O content was varied from 50 to 95 per cent. For the experiments to be reported in detail, the growth and labeling media had more

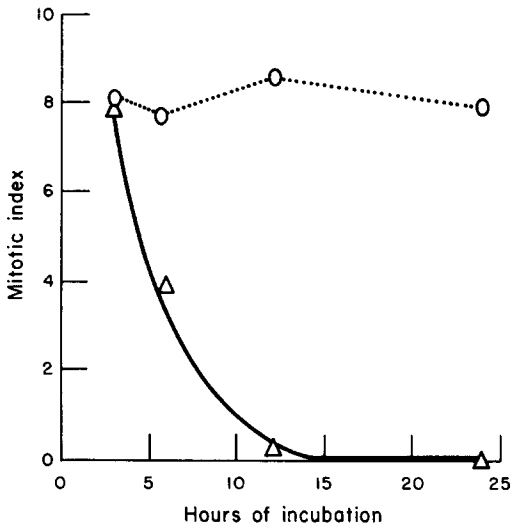


FIGURE 1 Variation of mitotic index with time after immersion of *Allium* roots in 95 per cent D_2O-Bonner's medium. Circles and dotted line are for mitotic indices obtained from control roots, otherwise treated identically with those placed in D_2O, but growing normally in H_2O-Bonner's. Mitotic index counts from acetic orcein squash preparations. Triangles and continuous line are for deuterated roots. Cells in mitosis disappear after 12 hours.

than 90 per cent of their water as D_2O. Some experiments were done in which roots were transferred from 90 per cent D_2O medium to a 50 per cent medium for labeling, but this practice was discontinued when we discovered that significant recovery from the deuterium effect could take place during the short period of exposure to a solution of reduced heavy water content. In all experiments, controls growing in normal H_2O medium were labeled in an H_2O-thymidine-H^3 solution of the same composition as its D_2O counterpart. Labeling was done at various intervals after first exposure to D_2O. At the end of a labeling

period, the roots were fixed in acetic alcohol (3:1) and embedded in paraffin. Sections 3 microns thick were cut and attached to slides, which were then treated for 1 hour in 2 per cent perchloric acid and for 18 hours in running tap water. This removed radioactivity not incorporated into acid-insoluble polymers. The washed slides were dipped in Kodak NTB2 Nuclear Track Emulsion (diluted 1:1 with water), dried, and stored in the dark for photographic exposure of the emulsion. Seven days to 1 month were required for the various precursors used in these studies. The backgrounds were negligible.

TABLE I

Grain Counts and Labeling of Nuclei after Exposure of Allium Roots to H^3-Thymidine for 30 Minutes

Time in D_2O medium	Grain count/nucleus	Per cent of nuclei labeled
hrs.	*mean* ± SD	*mean* ± SD
0	139 ± 20*	49 ± 5*
1.5	133 ± 14	49 ± 3
3	16 ± 5	18 ± 8
6	0	0
12	0	0

* These values are invariant for control roots, growing in normal H_2O medium, at all times during the course of the experiment, and under the conditions used

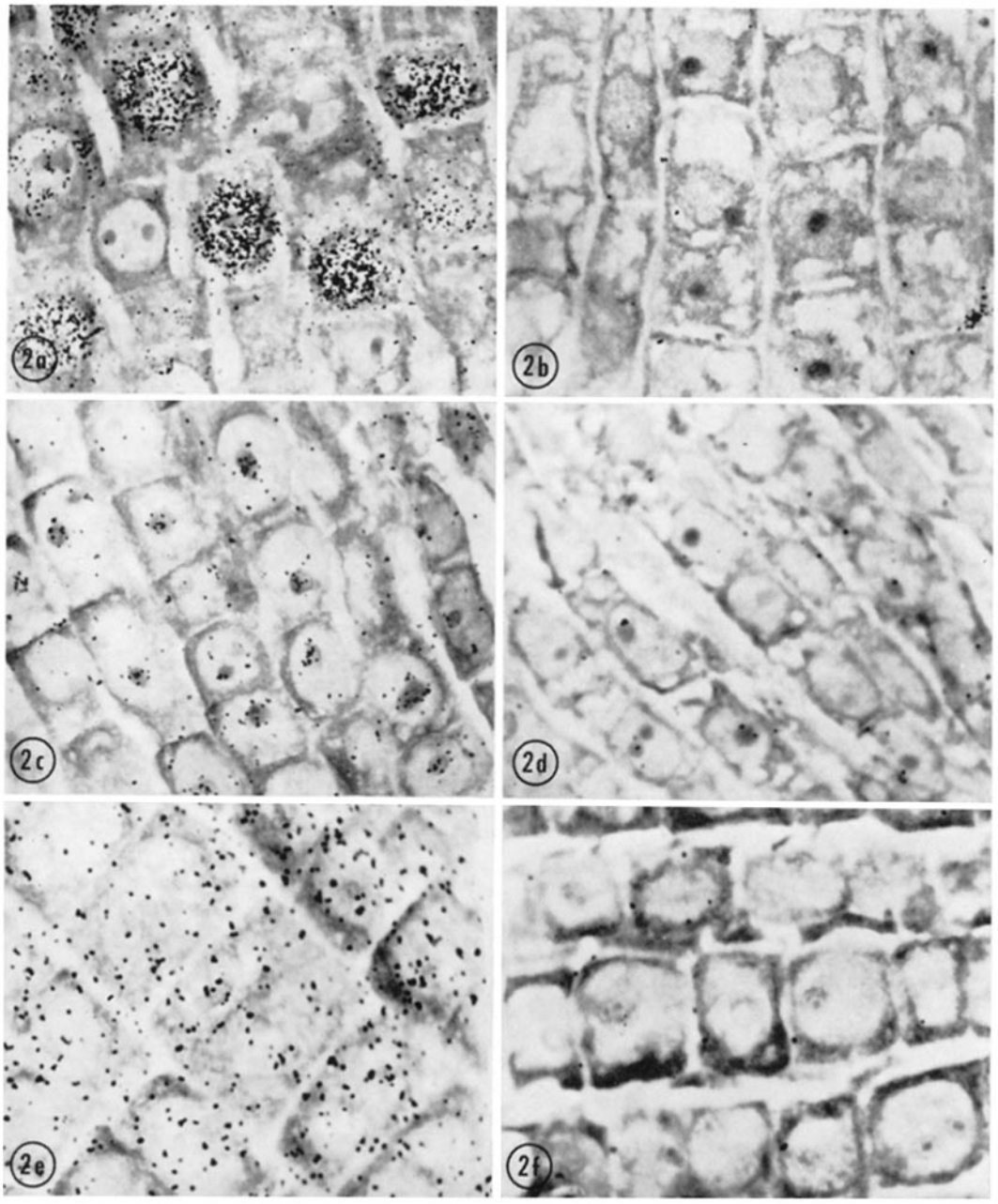
Table I shows that by 6 hours after first exposure to D_2O the synthesis of DNA, as represented by thymidine incorporation, had ceased. The mitotic index (Fig. 1) had by this time fallen to half the control value. The effect is not, as is the case in sea urchin eggs, immediate: at 1½ hours after immersion in deuterium, the roots labeled approximately the same fraction of their nuclei, and at about the same rate, as did control roots in normal water. After an additional 90 minutes, however, the grain counts per nucleus and the labeled fraction of all nuclei had fallen to 12 and 37 per cent of control values, respectively. Roots exposed to labeled thymidine after 12 and 24 hours in D_2O showed no incorporation into DNA. The inhibition is thus essentially complete by 6 hours, and no recovery is seen during the subsequent 18 hours. The possibility of some later adaptive recovery, as has been seen in other plant species (Stein and Forrester, 1963), is not ruled out. Figs. 2 *a* and 2 *b* show autoradiograms of normal and deuterated roots, exposed to H^3-thymidine after 24 hours in their respective media.

It was observed, and not unexpectedly, that root elongation continued in D_2O, although at steadily decreasing rates, for 6 to 12 hours. But by the 12th hour after transfer to heavy water, growth in length had always stopped. This is, of course, related to the cessation of mitosis in the meristem, also after 12 hours, and is consistent with the much earlier failure of new cells to enter into the S period of interphase.

Thus, under conditions in which the intracellular concentration of D_2O builds up slowly, as seems to be the case in plant roots (*i.e.* as deduced from the 1½ to 3 hour lag in the response), mitotic inhibition is not indifferent to stage, as in animal cells, but results from interphase block during the period of DNA synthesis and possibly at the end of G_1.

The effect is reversible: after 24 hours in heavy water, roots replaced in normal medium resume elongation, mitosis, and macromolecule synthesis at normal rates in considerably less than an additional 24 hours. We have seen no compelling evidence of induced division synchrony. Macromolecule synthesis recovers with particularly impressive speed. Even transfer from D_2O (95 per cent) to D_2O (50 per cent) allows significant recovery within 30 minutes, as can be shown when

FIGURE 2 Autoradiograms from sections of roots labeled with tritiated thymidine, uridine, and leucine. Conditions of exposure to labeled precursors are given in the text. Fig. 2 *a*, control (H_2O) root, thymidine. Fig. 2 *b*, root in deuterium for 24 hours, thymidine. Fig. 2 *c*, control, uridine. Fig. 2 *d*, deuterated, after 24 hours, uridine. Fig. 2 *e*, control, leucine. Fig. 2 *f*, deuterated, after 24 hours, leucine. Silver grains over the sections labeled with thymidine, uridine, and leucine detect radioactive DNA, RNA, and protein, respectively. Note localizations: thymidine; nuclear and non-nucleolar. Uridine; mainly nucleolar. Leucine; general. After 24 hours in D_2O, incorporation of thymidine into DNA and leucine into protein has ceased; incorporation of uridine into RNA has been reduced by approximately 90 per cent. × 1000.



the 50 per cent D_2O medium contains tritiated thymidine or uridine. Roots which show no incorporation of thymidine into DNA, after 6 or more hours in D_2O, always permit the entry of numbers of cells into DNA synthesis during a 30 minute exposure to 50 per cent D_2O. It is difficult to explain in a satisfactory way why the initial D_2O effect requires 1½ to 3 hours for its appearance, whereas reversal of the same effect is considerably faster. The initial delay in appearance of the block and the quick recovery in 50 per cent D_2O are, however, quite reproducible, so that an explanation will have to be sought.

Knowing that DNA synthesis, mitosis, and elongation were all brought to a halt after 24 hours in D_2O, and that the system remained nevertheless fully recoverable, we thought it of some interest to determine whether RNA and protein synthesis were stopped at this time. Again, the motivation was in part the possibility of an instructive comparison with the situation in the sea urchin egg, where protein synthesis survives either enucleation (Denny and Tyler, 1964), or the suppression of RNA synthesis by Actinomycin or deuterium (Gross, Spindel, and Cousineau, 1963), at least for many intermitotic times.

Roots kept in D_2O-Bonner's or in control medium for 24 hours were exposed for 30 minutes to H^3-uridine (25 μc/ml, New England Nuclear Corp.) or for 1 hour to DL-leucine-4,5-H^3 (30 μc/ml, New England Nuclear Corp.). Further treatment of the roots in preparation for autoradiography was as described above. Autoradiograms from control and deuterated cells exposed to uridine are shown in Figs. 2 *c* and 2 *d*, while autoradiograms of leucine-labeled cells are shown in Figs. 2 *e* and 2 *f*. The uridine incorporation, during the 30 minute pulse, was intranuclear and mainly nucleolar. Leucine incorporation was everywhere in the cells, with a very slight preponderance of cytoplasmic over nuclear grains. Treatment of the uridine-labeled sections with ribonuclease removed essentially all of the incorporated radioactivity.

About 10 per cent of the control incorporation of uridine into RNA survived after 24 hours in deuterium. Mean grain counts for this series were 28 ± 7.4 in control nuclei and 2.7 ± 1.3 in deuterated cells. The latter is a significant incorporation, because, on the basis of grains per unit area of section, it is well above background, and because, in addition, these grains were mainly nucleolar, as in the controls. RNA synthesis is, therefore, on the limited basis of this observation, considerably less sensitive to deuteration than is DNA synthesis, which is brought to undetectably low levels within 6 hours. Such a differential sensitivity is seen also in animal cells (Cousineau, 1963).

Protein synthesis, as represented by leucine incorporation, suffers an essentially complete block within the 24 hour period, as shown in Figs. 2 *e* and 2 *f*. This is significantly different from the situation in sea urchin eggs, and it may possibly reflect differences in the immediacy of genetic control over the manufacture of protein in these two kinds of developing system. In the animal embryo, deuteration of fertilized eggs slows but does not stop protein synthesis (Cousineau, 1963), and in that system the bulk of protein synthesis occurs on templates of unusual stability (Gross, Malkin, and Moyer, 1964).

Plant cells, therefore, share at least one important characteristic with animal cells, with regard to their response to a deuterium-rich environment: a blockade of DNA synthesis. It justifies continued study of the possibility that the essential difference between these cells and most microorganisms, whose DNA synthesis is not so dramatically affected, lies in the organization of the DNA into chromosomes. Some evidence is available which favors the idea that the deuterium effect may be expressed through a cooperative effect on multiply hydrogen-bonded structures at the level of organelles rather than single macromolecules (Gross, Spindel, and Cousineau, 1964). The most important differences between the higher plant and animal cells seem to arise from a significantly slower equilibration of cell and external water, and may also depend upon the consequent departures from osmotic equilibrium which were observed and explained in the early experiments of Brooks (1937).

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REFERENCES

BONNER, J., 1943, *Bull. Torrey Bot. Club*, **70**, 184.

BROOKS, S. C., 1937, *Science*, **86**, 497.

COUSINEAU, G. H., 1963, Brown University, PH.D. Thesis.

CRESPI, H. L., CONRAD, S. M., UPHAUS, R. A., and KATZ, J. J., 1960, *Ann. New York Acad. Sc.*, **84**, 648.

CRESPI, H. L., MARMUR, J., and KATZ, J. J., 1962, *J. Am. Chem. Soc.*, **84**, 3489.

DENNY, P. C., and TYLER, A., 1964, *Biochem. and Biophysic. Research Commun.*, **14**, 245.

FLAUMENHAFT, E., CONRAD, S. M., and KATZ, J. J., 1960, *Ann. New York Acad. Sc.*, **84**, 634.

GROSS, P. R., and HARDING, C. V., 1961, *Science*, **133**, 1131.

GROSS, P. R., MALKIN, L. I., and MOYER, W. A., 1964, *Proc. Nat. Acad. Sc.*, **51**, 407.

GROSS, P. R., and SPINDEL, W., 1960 *a*, *Ann. New York Acad. Sc.*, **84**, 745.

GROSS, P. R., and SPINDEL, W., 1960 *b*, *Ann. New York Acad. Sc.*, **90**, 500.

GROSS, P. R., SPINDEL, W., and COUSINEAU, G. H., 1963, *Biochem. and Biophysic. Research Commun.*, **13**, 405.

GROSS, P. R., SPINDEL, W., and COUSINEAU, G. H., 1964, *Pure and Appl. Chem.*, **8**, 483.

MARSLAND, D., and ZIMMERMAN, A. M., 1963, *Exp. Cell Research*, **30**, 23.

STEIN, O. L., and FORRESTER, G. M., 1963, *Planta*, **60**, 349.

TUCKER, R. W., and INOUÉ, S., 1963, *Biol. Bull.*, **125**, 395.