AUTORADIOGRAMS WITH TRITIATED MYLERAN

J. and M. MOUTSCHEN-DAHMEN, W. G. VERLY and G. KOCH

Département de Génétique (Institut de Morphologie Végétale), Laboratoire des Isotopes (Institut Léon Fredericq, Biochimie et Centre belge de l'Energie Nucléaire), University of Liège, Belgium

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THE major importance of alkylating agents in mutagenesis has been stressed for a long time, especially by the Swedish school. These compounds seem to induce specific mutations, although this view is still controversial. It would be desirable to know more about the chemical processes which lead to mutation after the action of alkylating agents. Herriot [5] reported that the *Pneumococcus*-transforming principle, mainly composed of deoxyribonucleic acid, showed great sensitivity toward mustard gas. In *Hemophilus influenzae*, Zamenhof *et al.* [12] inactivated the transforming principle with nitrogen mustard. Although these results strongly suggest a reaction between alkylating agents and nucleic acids, they do not show that the latter compounds are the only sites of action in the cell. Other *in vitro* chemical experiments indicate that primary amino groups as well as sulfhydryl groups can also be alkylated [10]. It would be interesting to know if the alkylating agents react with the nucleoproteins *in vivo* and if they enter the cell nucleus; so far little is known about localization in the cell of the molecules which undergo alkylation.

Myleran was found by von Wettstein, Gustafsson, and Ehrenberg [11] to have a strong mutagenic activity in plants. It was also shown to be mutagenic in *Drosophila* [3]. The quantitative modifications of chromosome breaks and their localization have been followed during the development of root tips in several species of plants [9].

Material and methods.—Myleran was prepared synthetically by Koch *et al.* [6, 7] by catalytic reduction of butyne-2-diol-1,4 with tritiated hydrogen followed by an esterification with methanesulphonyl chloride. The specific activity was 105 mC per millimole.

Ten seeds of *Vicia faba* (var. Åkerböna Weibull) were put into 50 ml of water containing 10 mg of tritiated myleran for a period of three hours. After this treatment, seeds were carefully washed for two hours in distilled water and then germinated in Petri dishes on filter paper soaked with distilled water. A set of control seeds was grown in the same way. Three days later, the growing root tips were cut, fixed in alcohol and acetic acid (3:1) for 2–4 hours and stained with Feulgen.[1] Squash preparations were made according to Conger's technique [2].

Kodak AR10 stripping films were used for making autograms. We also made some autograms by immersion of the slides in a liquid emulsion. After 5 days of exposure, autograms were developed following the usual technique [1, 4].

[1] The HCl treatment must be of short duration, otherwise the radioactivity bound to macromolecules entirely disappears.

We also treated some seeds of *Vicia* with a solution of tritiated thymidine Schwartz (specific activity 1.9 C/m*M*) for two hours. After washing, they were treated with nonlabelled myleran (20 mg/100 ml) for three hours. The seeds were then germinated as indicated above.

Results.—The number of chromosome breaks after treatment with tritiated myleran was found to be of the same order of magnitude as for the nontritiated compound as reported in previous experiments [8, 9].

Since the distribution of disturbed dividing cells was observed to be irregular in meristematic tissues, it could be expected that labelling would show irregularities in its distribution. We found that labelling varies not only from one histogen to another but also within the same histogen.

It became clear that some nuclei showed a high degree of labelling. We selected ten of these labelled nuclei to compare with the background labelling. Table I summarizes the results. The differences are highly significant. It was also observed that

TABLE I. *Counted silver grains in tissue after action of tritiated myleran.*

	Values (counted grains per 20 μ^2)	95 % confidence interval $\left[2\sqrt{\frac{\Sigma\Delta^2}{n(n-1)}}\right]$	Numbers of counted samples
Nuclei	22	±2.0	50
Micronuclei with nucleolus	20	±1.9	10
Pycnotic micronuclei	0.33	±0.03	15
Cytoplasm	0.42	±0.16	50
Nucleoli	22	±1.9	50
Background	0.44	±0.10	50

TABLE II. *Counted silver grains in tissue after uptake of labelled thymidine and treatment with nonlabelled myleran.*

	Values (counted grains per 20 μ^2)	95 % confidence interval $\left[2\sqrt{\frac{\Sigma\Delta^2}{n(n-1)}}\right]$	Numbers of counted samples
Nuclei	17	±1.7	50
Micronuclei with nucleolus	16	±1.0	10
Pycnotic micronuclei	14	±1.1	15
Cytoplasm	1.4	±0.8	50
Nucleoli	2.3	±0.6	50
Background	1.6	±0.4	50

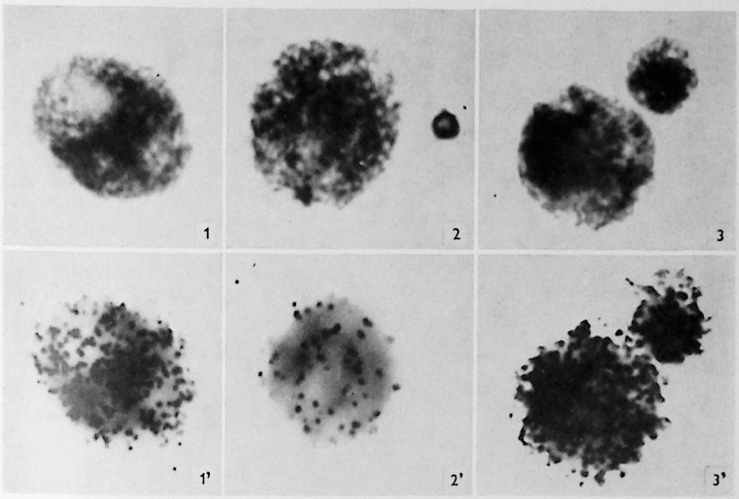

Fig. 1.—Normal nucleus of the root tip of *Vicia faba* which had grown in myleran-^{3}H for 3 hrs. ×2500.

Fig. 1′.—Autoradiogram of the same nucleus at the silver grains level. ×2500.

Fig. 2 and 2′.—Nucleus and micronucleus without nucleolus, photographed at both levels. ×2500.

Fig. 3 and 3′.—Nucleus and micronucleus with nucleolus, photographed at both levels. ×2500.

nucleoli contained statistically the same amount of radioactivity as the rest of the nuclei. Moreover, Table I does not show any significant difference between cytoplasmic and background labelling. Figs. 1 and 1′ show an example of labelling; Fig. 1 has been photographed at the level of the specimen and Fig. 1′ at the level of the silver grains of the emulsion.

It must be pointed out that in these experiments tissues were fixed a long time after treatment allowing for the completion of about two mitotic cycles. On the other hand, fixation leaves only the tritium which is attached to macromolecules; the tritiated myleran used was labelled in the tetramethylene moiety; labelling presumably indicates the position of macromolecules which have been alkylated by myleran.

As a consequence of chromosome breakage, we observed that some micronuclei showed a high degree of labelling whereas some do not. Figs. 2 and 2′ show photographs taken respectively at the nucleus and at the silver grain level. In Fig. 2, a small micronucleus is visible, but one sees in Fig. 2′ no developed silver grain above. On the other hand, in Figs. 3 and 3′, both nucleus and micronucleus are labelled. Differences in the distance between micronucleus and film could explain these findings. In order to test this possibility, we performed the following experiment. Some seeds were treated with labelled thymidine and, after washing, with nonlabelled

myleran (see Material and methods). Table II summarizes the results of this experiment.

As only nucleolar micronuclei were labelled, we conclude that the activity of micronuclei can be correlated with their qualitative chromatin content. Nucleoli may have something to do with such activity but the possible roles of the other micronuclear components cannot be disregarded. At least, the existence of nonlabelled micronuclei evidently demonstrates that some chromosome parts do not react with the alkylating agent. Whether these are heterochromatic, euchromatic or both cannot yet be deduced from the present data. If some heterochromatic centers were proved to take more myleran, these results would confirm previous findings [8]. By this assumption, the localization of the substance would be the site where the chromosomes break.

In summary, after treatment with tritiated myleran and some described histological manipulations, tritium was found to be localized in the nuclei of root tips of *Vicia faba*, while significant cytoplasmic retention was not yet detected. From examination of micronuclei, it could be stated that some chromosomic components do not show any radioactivity. Evidently, the reaction sites of myleran are not uniformly distributed in nuclei. A correlation might therefore exist between these findings and the observed mutagenic activity of the compound.

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