Effect of Chemicals on Mitosis Studied in *Tradescantia* Cells *in vivo*. III. 2-Amino-1,3-diazazulene, a chromosome doubling agent^{1,2}

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Received July 27, 1953

In this report the writer is giving the results of his investigations upon the action of amino-diazazulene, which recently was synthesized by Prof. Nozoe and his coworkers (1952)³. As the investigations were carried on according to the same material and methods which were described in the writer's two recent papers, they are not repeated here (see Wada 1952 a, b).

The yellowish pulverous crystal of amino-diazazulene is soluble in water of more than 20 times of its own weight. In an agar plate containing this compound, changes of the mitotic spindle were observed in the cells of pro-metaphase, metaphase and anaphase. Corresponding to the state of chromosome distribution in the atractosome, the spindle body, there appeared either a large nucleus with doubled chromosome number or a binucleate cell. In more diluted concentrations, such tetraploid or binucleate cells were also induced, but they were provided with incomplete or remnant-like cell walls.

Observations

0.5 mg per cc. In the illustrations, a step by step description is given to explain the processes of spindle disintegration and the formation of nuclei with doubled chromosome number or of binucleate cells (Figs. 1-6).

11:20⁴. July 11, 1953. Cells were embedded in an agar plate con-

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The present studies were carried out as part of the investigation of "Chemical and Biological Studies on Troponoid Compounds" represented by Prof. Tetsuo Nozoe, and financed partly by the Grant in Aid for Scientific Research from the Ministry of Education of Japan as well as the Zaidanhoin Wada-Kunkokai.

³ Amino-diazazulene used in the present studies was provided through the kind favour of Prof. Nozoe of the Tohoku University (Sendai, Japan), for which the writer wishes to express his deep gratitude both to Prof. Nozoe and to his coworkers, Messrs. Seto and Kitahara.

⁴ 11:20 means 11 o'clock 20 minutes; the words "o'clock" and "minute" are in the following figure also omitted.

taining 0.5 mg/cc of this tropolone compound; "a" cell in Fig. 1 was in mid-anaphase and "b" cell in early prophase at the beginning of the experiment.

Figs. 1-6. Chromosome doubling of anaphase nucleus in cell "a" and reversion of prophase nucleus in cell "b" treated with a 0.5 mg/cc solution of amino-diazazulene. 1, "a" cell in mid-anaphase and "b" cell in early prophase. Photo. 11:26. 2, beginning of disintegration of anaphase spindle and of mid-prophase nucleus. 3, spindle-shaped metabolic nucleus with doubled chromosome number and reverting prophase nucleus. 4, cells on the second day of the experiment. 5, cells on the third day. 6, cells on the fourth day. The hair with "b" cell has coagulated and died. Detailed description in the text.

11:26. Changes were scarcely observed in the mid-anaphase nucleus. The phase boundary of the atractoplasm and the outline of each chromosome were still maintained (Fig. 1).

11:58. The state of the prophase nucleus seemed to go on further and has just entered into the beginning of the mid-prophase. In the mid-anaphase nucleus, the movement of anaphase chromosomes was found to be suppressed entirely and the contour of each chromosome became rapidly indistinct. Moreover, the spindly body itself was decreasing in volume.

These changes of the anaphase mitotic figure gave the impression that the atractoplasm, one of the basic components in the field of chromosome movement in the anaphase, was going on to leave from the spindle cavity. The state of the chromosomes in this anaphase seemed to have become altered into the state of chromosomes as in the mid-telophase. In this mitotic stage, the disintegration of chromosomes into chromonema-structure in the metabolic nucleus, the resting nucleus, would then commence. Concerning the chromosome structure alterations, similar processes were found also in the prophase nucleus, but the disintegration of chromosomes proceeded more slowly (Fig. 2).

12:10. The chromonematization of the mid-anaphase chromosomes advanced remarkably and left no contour of the chromosomes. This new metabolic nucleus which was apparently a nucleus with doubled chromosome number, still showed a spindle-shaped outline and a loose arrangement of chromonemata (Fig. 3). During these metamorphoses of the anaphase chromosome into one metabolic nucleus, neither a phragmoplast nor degenerated phragmoplast substances were observed in or out of this mitotic figure.

The mechanism observed in the disintegration of the atractosome under the action of amino-diazazulene revealed an essential difference from that of chromosome-doubling induced by the colchicine-treatment or by the ammonia-chloroform mixed vapour. The explanation of this subject will be discussed in a later chapter.

8:15. July 12. The second day of the experiment. In the "a" cell, the chromonema-structure and the state of nucleoil showed hardly any differences from the so-called "resting nucleus", though the outline of this nucleus still appeared oval. The nucleus in the "b" cell also reverted into the structure of the resting nucleus, which still appeared in a state of minor swelling; the chromonemata were found to have a fine granular appearance in the optical section (Fig. 4).

Figs. 5 and 6 showed the state of the cells on the following days. On the third day of the experiment, the nucleus with doubled chromosome number had a spherical shape and from it a strong cytoplasmic strand stretched through the cell sap and active protoplasmic streamings were observed in the cytoplasm (Fig. 5). On the fourth day of the experiment,

the hair containing the "a" cell was found in a senescent state, but it continued the protoplasmic streaming. The hair with the "b" cell, however, had coagulated already and died. On the fifth day of the experiment, 24 hairs out of 40 survived, but on the seventh day all hairs except 2 had coagulated and died. When amino-diazazulene affected cells in the late anaphase or in the early telophase, in general a binucleate cell occurred.

At the beginning of the experiment, the nucleus in Fig. 7a was in late anaphase and the nucleus in Fig. 7b in the very beginning of prophase. Forty-five minutes after the treatment, two chromosome groups in each spindle pole already showed the appearance of the chromonema structure of daughter nuclei, which would be observed in the nuclei in the late telophase in normal mitosis. During this disintegration of chromosomes into chromonemata, no development of phragmoplast nor of cell-plate

Figs. 7-10. Binucleate cell formation and prophase nucleus reversion treated with a 0.5 mg/cc solution of amino-diazazulene. The cells embedded at 11:20, July 11, 1953. **7a**, late anaphase and **7b**, beginning of prophase. Photo. 11:23. **8a**, daughter nuclei formation without cytokinesis and **8b**, spiral stage. Photo. 12:05. **9**, photo, on the second day. The daughter nuclei in the binucleate cell show still a minor swelling. **10**, photo, on the third day. In both cells rich cytoplasm and active protoplasmic streamings were observed.

formation was observed in and out of the mitotic figure (Fig. 8a). The chromosome structure of the nucleus in Fig. 7b became altered a little further and the cell was just in the spiral stage (Fig. 8b). The cells in Fig. 9 were photographed on the second day and those in Fig. 10 on the third day of the experiment. In Fig. 10, the terminal cell contained two daughter nuclei without cell wall and the second cell one metabolic nucleus.

Both cells were found in a healthy state and showed active protoplasmic streamings.

From this varied behaviour of the mitotic cells, it is concluded that the action of amino-diazazulene on the prophase nucleus seemed to be indirect and was not so destructive as it is found to be on the mitotic spindle. The specific action of this compound primarily induced the disintegration of the atractoplasm in meta- and anaphase and secondarily the suppression of the atractosome development in late prophase or in prometaphase. In other words, the polypeptide chains in the mitotic spindle seem impossible to keep themselves in an unfolded state under the action of amino-diazazulene.

In the experiments treated with 0.5 mg/cc of amino-diazazulene 116 young staminal hairs of *Tradescantia* were investigated. Among them 28 mitotic cells were found at the beginning of the experiments: 16 prophase, 3 prometaphase, 6 anaphase and 3 telophase cells. On the second day of the experiment 24 nuclei were observed to have reverted into the metabolic nuclear structure, and among them, at least 5 nuclei or possibly 8 were considered to be those with the doubled chromosome number. Other cells developed as binucleate cells; one of them was induced from a late anaphase cell and the others from telophase cells. In this concentration of the compound, a number of cells were coagulated but the majority of them were able to survive until the third day of the experiment, but on the fifth day most cells had coagulated and died.

0.3 mg per cc. In an agar plate containing 0.3 mg/cc of amino-diazazulene, the disintegration of the atractoplasm was observed in the metaphase and anaphase, but the suppression of the cell-plate formation was not so complete as was found in the treatment of 0.5 mg/cc of this compound.

The cell in Fig. 11 was in the spiral stage at the beginning of the treatment. After 4 hours the cell reached the prometaphase. The nuclear cavity, however, seemed to be in a less turgescient state than that in the normal mitotic cell. The contour of each chromosome appeared to be vague and the longitudinal splitting was hardly observed. In 3 hours after this prometaphase, the chromosomes reverted into chromonema-structure without dividing into two daughter chromosome groups. However, the contour of this chromosome mass was rather vague and had a shallow depression and a thin remnant-like cell-plate lying at its middle part, as if the chromosome mass was making preparation to divide itself later into two groups, although no spindle apparatus had been organized. In this state of the chromosome mass, it was difficult to determine, whether the chromosome mass would become one nucleus with the doubled chromosome number or two daughter nuclei (Fig. 12). On the second day of the experiment, it became apparent that the cell contained one large nucleus with the doubled chromosome number. Near

the one side of this nucleus a thin and short remnant-like cell wall appeared (Fig. 13).

The above mentioned varied behaviour of treated cells revealed that the action of amino-diazazulene upon the attractoplasm in a 0.3 mg/cc solution was not so destructive as in a 0.5 mg/cc solution. Various grades of disintegration of the attractoplasm occurred in this concentration. The cell in Fig. 14 was in early prophase, when embedded in the amino-diazazulene agar plate. Five hours after the treatment the cell had reached just the polar cap stage. On the second day, however, the cell contained 2 daughter nuclei remarkably unequal in size, which were separated with an incomplete cell wall (Fig. 15).

Figs. 11-15. Various types of mitotic abnormality treated with a 0.3 mg/cc solution of amino-diazazulene. **11**, prometaphase. Photo. 15:14. Owing to the disintegration of the attractosome, the chromosome in metaphase became vague. This hair was embedded at 10:10, July 17. **12**, metaphase nucleus reverted into a chromonema-structure of metabolic nucleus. An arrow indicates a shallow depression on the nucleus. Photo. 18:14. **13**, a nucleus with doubled chromosome number. An arrow indicates the remnant-like cell wall. Photo. on the second day. **14**, polar cap stage. The cell was in early prophase when embedded at 10:10, July 17; the cell affected little by the compound. Photo. 15:31. **15**, same cell photographed on the second day. Two daughter nuclei remarkably unequal in size, separated with short incomplete cell wall indicated by an arrow.

Among 50 hairs treated with 0.3 mg/cc solution of amino-diazazulene, 20 mitotic cells were observed. The occurrence of *de novo* mitosis was completely suppressed in this concentration of the compound.

0.1 mg per cc, 0.05 mg per cc and 0.005 mg per cc. In 71 staminal hair cells treated with 0.1 mg/cc solution of amino-diazazulene, 26 mitotic cells were found. 9 among 15 prophase cells at the beginning of the experiment reverted into the metabolic nuclei and the rest finished the

division, but very seldom did abnormal mitoses occur. (Figs. 16-17). Mitotic cells in metaphase, anaphase and telophase carried on their mitoses in the normal way, but no *de novo* mitosis occurred in this concentration.

In a 0.05 mg/cc solution of amino-diazazulene, 13 mitotic cells were found among 33 staminal hairs. These mitotic cells, except three, finished their divisions in the normal way. Two cases, however, developed into binucleate cells, one of which was in the spiral stage at the beginning of the experiment and the other was found in a *de novo* mitosis. The third cell, which was at first in very early prophase, reverted into metabolic nucleus. Thus it is concluded that in a lower concentration, the reaction of amino-diazazulene concerning the occurrence of mitotic abnormalities seemed partly to be affected by inner conditions of the cell.

Figs. 16-17. Various types of mitotic abnormality of cells treated with a 0.1 mg/cc solution of amino-diazazulene. **16**, polar cap stage. The cell was in mid-prophase, when embedded at 11:45, July 17; the chromosomes were affected a little by the compound, and became thick and short. Photo. 15:31. **17**, oval shaped metabolic nucleus with doubled chromosome number. Photo. on the second day.

Figs. 18-19. Various types of mitotic abnormality of cells treated with a 0.05 mg/cc solution of amino-diazazulene. **18**, binucleate cell. Photo. on the second day. The cell was in spiral stage, when embedded at 11:45, July 14. **19**, binucleate cell. Photo. on the second day. The cell was in resting stage, when embedded at 11:45, July 14.

In a 0.005 mg/cc solution of amino-diazazulene, 21 mitotic cells were observed among 56 staminal hairs. 6 mitoses occurred *de novo* from resting cells and other 14 cells finished their mitoses normally; only one cell in a very early prophase reverted into a resting cell. These results indicated that any toxic action on the mitotic cells was hardly found in this low concentration, so the mitosis occurred *de novo* and cells continued growth and metabolism in the normal way and could survive for weeks.

The frequency of normal and abnormal mitoses in each mitotic phase, when treated with various concentrations of amino-diazazulene, is summarized in Table 1.

Table 1. The frequency of normal and abnormal mitosis in each mitotic phase, when treated with various concentrations of amino-diazazulene. Figures in the table indicate following types of cells: □ cells finished mitosis normally. ▨ prophase cells whose nuclei reverted into resting one. ■ cells with doubled chromosome number. ▲ binucleate cells. Number put in bracket in the column of mitotic cell numbers are those of staminal hairs observed.

Stage Conc.	0.5 mg/cc	0.3 mg/cc	0.1 mg/cc	0.05 mg/cc	0.005 mg/cc
<i>de novo Mitosis</i>				□▲	□□□□□ □
<i>Prophase</i>	▨▨▨▨▨ ▨▨▨▨▨ ▨▨▨▨▨ ▨	□□□□□ ▨▨▨■▲	□□□□□ □▨▨▨▨ ▨▨▨▨▨	▨□□□□ ▲	▨□□□
<i>Prometaphase and Metaphase</i>	■ ■ ■	■	■ □ □ □		□
<i>Anaphase</i>	■ ■ ■ ■ ■ ▲	▲▲	□ □ □ □	□ □	□ □ □ □
<i>Telophase</i>	▲▲□	▲ □ □ □ □ □ □	□ □ □	□ □ □	□ □ □ □ □ □
<i>Mitotic Cells</i>	28 (116)	20 (50)	26 (71)	13 (33)	21 (56)

Results and discussion

1. *Comparison between colchicine and 2-amino-1,3-diazazulene with reference to the mechanism of chromosome doubling.* As already reported by the writer (1940, 1949), within a certain range of concentrations, colchicine selectively attacks the atractoplasm and induces a disturbance of the orientation of the unfolded polypeptide chains. In fact, by colchicine treatment, the birefringence of the mitotic spindle is extinguished (Inoué 1952). Under the colchicine action, a very fluid hyaline plasm with innumerable fine granules in rapid Brownian movement would accumulate near the one large chromosome grouping. This plasm mass, due to degenerated phragmoplast substances, has no potential to form the cell-plate. The cell thus fails in cytokinesis. The other important character

of colchicine is that, even in such concentrations as to destroy the atractosome as well as phragmoplast mitosis can occur *de novo*.

Contrary to the colchicine action, amino-diazazulene, whose concentrations are high enough to destroy the atractosome, no longer induces the occurrence of *de novo* mitoses. Moreover, cells in prophase fail to carry out their further development and revert into resting nuclei. In short, it is concluded that colchicine acts exclusively on the atractoplasm, but amino-diazazulene acts not only on the atractoplasm but also on the nucleoproteins in the prophase nucleus.

2. *Data supporting the kinetochore hypothesis with reference to mitosis mechanism.* Concerning the kinetochore hypothesis, the writer (1950) gave his opinion that the spindle body submicroscopically consists of two basic components; one is the interfibrillar fluid and the other the unfolded polypeptide chains lying parallel to each other, but converging to the spindle poles. There are then, at least two possibilities to be considered in the destruction of the spindle body. The one possibility can be attained by disturbing the parallel orientation of unfolded polypeptide chains in the atractoplasm and the other either by preventing the unfolding of the polypeptide chains in the prometaphase or by refolding the polypeptide chains in the atractoplasm.

The first category is demonstrated by action of colchicine on the mitotic spindle in *Tradescantia* cells *in vivo* and the action of ammonium-chloroform mixed vapour may also belong to this action type (Wada 1939, 1940, 1949). The second category is illustrated, as mentioned above, by the action of amino-diazazulene. Within a certain range of concentrations of amino-diazazulene, the suppression of prophase nuclei entering into metaphase may be caused by the prevention of the unfolding of nucleoproteins in the karyolymph and also the chromosome doubling due to the disintegration of the atractoplasm by the precocious refolding of polypeptide chains in the atractosome. Except for the action of amino-diazazulene on the nucleoproteins or polypeptide chains, this compound scarcely acts on other mitotic elements, such as development of chromosomes or longitudinal splitting of chromosomes.

Further confirmation of these specific characters of amino-diazazulene, the writer believes, will be demonstrated biochemically in the future; it should also be examined whether the folding and unfolding of polypeptide chains may be induced by the treatment with amino-diazazulene *in vitro*.

Summarizing all above data it can be said, that amino-diazazulene is one of the c-mitotic substances most recently synthesized in the laboratory. The general character of tropolone compounds, which act selectively on nuclear substances (Wada 1952a), was confirmed also for amino-diazazulene. This tropolone compound acts especially upon nucleoproteins and seems to prevent any unfolding of polypeptide chains, and it suppresses the

development of nucleoproteins in the karyolymph into atractoplasm and also disintegrates the atractoplasm in meta- and anaphases. The chromosome doubling is thus induced. Treatment with this compound, however, is not applicable for inducing a polyploid individual, because this amino-diazazulene suppresses not only the anaphase movement of chromosomes, but also prevents the development of prophase nuclei and the occurrence of *de novo* mitosis. In other words, this tropolone compound can stop either the development of meta- and anaphase nuclei or all of the cells which will enter into mitoses.

Therefore this tropolone compound is a typical mitotic poison and in a certain range of concentrations, no mitosis can occur *de novo*, though no toxic effect can be found in resting nuclei. This peculiar character of amino-diazazulene seems to have high potency as a chemical, which may be available as an anti-tumor substance; because it may prevent any active proliferation of malignant tissues by mitosis, but is hardly injurious to any somatic cells in the resting state.

Summary

By means of the agar plate method, the action of 2-amino-1,3-diazazulene on mitotic cells was investigated in the *Tradescantia* test *in vivo*.

In a 0.5 mg per cc solution of this tropolone compound, disintegration of atractoplasm occurs. Therefore, nuclei with doubled chromosome number are induced from mitotic cells in metaphase and mid-anaphase, and binucleate cells from those in late anaphase and early telophase. In this concentration, nuclei in prophase cells revert into resting stage and the occurrence of *de novo* mitosis is entirely suppressed.

In lower concentrations (0.3, 0.1 and 0.05 mg per cc), various grades of abnormality appear corresponding to the stage of mitosis and to concentrations of the compound in the medium.

From the standpoint of the submicroscopic molecular structure of mitotic elements, differences in the disintegration mechanism of atractoplasm caused by colchicine and amino-diazazulene respectively and data supporting the kinetochore hypothesis with respect to the mitosis mechanism are discussed.

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