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Effects of NDA, a New Plant Growth Retardant, on Cell Culture Growth of *Zea mays L.**

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Abstract. A new plant growth retardant, the norbornenodiazetine derivative 5 - (4 - chlorophenyl) - 3,4,5,9,10 - pentaaza - tetracyclo - 5,4,1,$0^{2,6}$,$0^{8,11}$ - dodeca - 3,9 - diene (NDA) was tested for its effects on growth of *Zea mays* suspension cultures. It was shown that NDA could inhibit cell division almost completely at a concentration of 5×10^{-5} M, while 80% of cells could be considered viable. Tracer experiments revealed that NDA inhibited thymidine, uridine, and leucine uptake into cells after 30 min of application. In contrast, amino acid incorporation into proteins was reduced only after one day of treatment and incorporation of precursors into DNA and RNA still later. Since NDA stimulated DNase, RNase, and protease activity in the cells simultaneously, an enhancement of DNA and RNA in cells possibly was prevented. That NDA affected protein synthesis indirectly seemed to be proved by the late point in time of its action on leucine incorporation and by only slight effects on cell free translation. An explanation of these findings could be an alteration in or inhibition of sterol biosynthesis caused by NDA, because it is known that sterols play an important role in controlling permeability of plant membranes as well as in maintaining normal protein synthesis. Thus we tested NDA for its effects on sterol production in maize cells and demonstrated that the composition of the sterol fraction, mainly stigmasterol and β-sitosterol, was clearly changed qualitatively as well as quantitatively.

* Dedicated to Professor Martin Bopp on the occasion of his 60th birthday.

Many synthetic compounds are known to modify plant growth. Among them, plant growth retardants are of special economic importance in horticulture and agriculture, since they reduce the rate of stem elongation by inhibiting subapical meristem activity in responsive plants. The mode of action of most plant growth retardants is generally seen as influencing the gibberellin system of the treated plants, thereby slowing or inhibiting cell division and cell elongation (Dicks 1980). However, further possible explanations, e.g., via inhibition of the sterol biosynthesis (Douglas and Paleg 1981) or via a direct effect of the substances on cell division processes, should be kept in mind.

For the study of the effects of growth retardants at a cellular level, exponentially growing plant cell cultures offer a homogenous system that is similar to plant meristems because cell division is the major cellular event (Sloan and Camper 1981). Recently, we have demonstrated that seedlings and cell cultures of the same plant species show a correlated behavior in response to various plant growth retardants, thus indicating the usefulness of cell cultures for identifying and investigating their mode of action (Grossmann et al. 1982). A new plant growth retardant, the norbornenodiazetine derivative NDA, proved to be the compound with the greatest inhibitory action of cell division growth of all cultures tested and is also active in intact plant of all higher plant species hitherto studied. It can be assumed that it affects a central point in the steering of plant growth (Jung et al. 1980). Thus we investigated the influence of NDA on uptake and incorporation of precursors for DNA, RNA, and protein synthesis in cultured maize cells. Besides effects on membrane permeability and the anabolic pathways of the molecular genetic metabolism, we also studied the influence on the catabolic pathways of these macromolecules as represented by the DNase, RNase, and protease activity in the cells.

The results seen in connection with qualitative and quantitative changes in sterol contents caused by NDA treatment raised the question of a possible involvement of sterol biosynthesis as a direct or indirect requirement for growth in plant cell cultures.

Materials and Methods

Freely suspended callus cells from *Zea mays L.* (cv. Inbred B 73) derived from shoot were cultivated in a Murashige-Skoog medium modified according to Seitz and Richter (1970). The cells, grown in culture for several years, were subcultivated at intervals of 7 days in the exponential growth phase. The culture conditions were as follows: 250 ml Erlenmeyer flasks containing 80 ml of cell suspension were shaken on a rotary shaker at 110 rpm in the dark at 25°C. The plant growth retardant 5 - (4 - chlorophenyl) - 3,4,5,9,10 - pentaaza - tetracyclo - 5,4,1,$0^{2,6}$,$0^{8,11}$ - dodeca - 3,9 - diene (= NDA, = LAB 102 883, = BAS 10600 W) was prepared in acetone and added to the medium as indicated. The cell number was determined in a hematocytometer as previously described (Grossmann et al. 1979). Viability of the suspension cells was examined with fluorescein diacetate (Larkin 1976) and phenosafranin staining (Ferrari et al. 1975).

Tracer Experiments

In experiments for studying DNA, RNA, and protein synthesis, both the uptake of [methyl-^{3}H] thymidine (44 Ci/mmol), [5-^{3}H] uridine (28.3 Ci/mmol), and L-[4,5-^{3}H] leucine (24.9 Ci/mmol), and their incorporations into DNA, RNA, and protein, respectively, were determined. Cell suspension (0.5 ml) and radiolabeled precursor (2 μCi/ml) were incubated in sterile test tubes on a rotary shaker at 400 rpm in the dark at 25°C. Immediately after incubation, the cells were washed 3 times with 5 ml of medium and recovered by centrifugation (4°C). The cells were lysed with 0.2 ml of 2% sodium dodecylsulfate followed by 2 ml of 10% trichloroacetic acid (TCA). After precipitation of TCA insoluble material overnight (4°C) and centrifugation, a sample of 50 μl of the supernatant was placed on a glass fiber disc (Whatman GF/C), dried, and counted in toluene-PPO-POPOP scintillation fluid (TCA soluble radioactivity). The TCA insoluble material was filtered onto filter discs, washed 5 times with 5% TCA and once with 96% ethanol, dried, and counted (TCA insoluble radioactivity). The sum of TCA soluble and insoluble radioactivity was taken for total uptake, while TCA insoluble activity represented the incorporation of the isotope.

In vitro Translation

Wheat germ cell-free system was purchased from Bethesda Research Lab (Gaithersburg, Maryland, USA). The reaction assay based on a 30-μl volume contained: Wheat germ extract (27 A_{260} units/ml), 27 mM HEPES, 2.37 mM Mg-acetate, 63.3 mM K-acetate, 33.3 mM KCl, 1.67 mM β-mercaptoethanol, 1.2 mM ATP, 0.1 mM GTP, 5.5 mM creatine phosphate, 0.2 mg/ml creatine kinase, 80 μM spermidine phosphate, 19 amino acids at 50 μM each, 0.15 μg rabbit globin mRNA, and 4 μCi L-[4,5-^{3}H] leucine (130 Ci/mmol). NDA was added in acetone solution (0.5% final concentration of acetone in the incubation mixture).

The assay mixture was incubated for 60 min at 25°C, and the reaction was stopped by chilling. An aliquot (10 μl) of each mixture was spotted onto filter discs, which were then transferred into cold 10% TCA containing 0.1 mM leucine for 10 min. The filters were washed with 5% TCA at room temperature, boiled for 10 min in 5% TCA, and washed again in 5% TCA and ethanol at room temperature. The filters were dried and counted for radioactivity. The value of the endogenous protein synthesis was subtracted.

Determination of DNase, RNase and Protease Activity

Four ml of cell suspension was filtered onto filter discs, washed, and homogenized with mortar and pestle under liquid nitrogen. After the addition of 25 mM Tris · HCl (pH 7.5), the enzyme activities were measured spectrophotometrically as described previously (Grossmann and Jung 1982).

Sterol Extraction and Analysis

Total sterols were extracted and isolated by the methods described by Stedman and Rusaniwski (1959), Grunwald (1970), and Buchenauer and Röhner (1981). The cell material (ca. 40 g fresh weight) was extracted with 250 ml acetone in a Soxhlet apparatus for 24 h. After the solvent was evaporated from the extract at 35°C, the residue was redissolved in 25 ml of 95% ethanol containing 0.13 ml sulfuric acid. Sterol glycosides were then cleaved under 12 h of reflux. Thereafter, 15 ml of 10% KOH in 25% ethanol was added, and the extract was again refluxed to hydrolyze sterol esters. After neutralization with HCl, the sample was extracted 3 times with 30 ml of n-hexane. The combined n-hexane phases were washed twice with 5 ml of 90% methanol and then dried. The residue was solved in 20 ml of boiling ethanol, and the sterols were precipitated for 12 h after the addition of 10 ml of hot 2% digitonin solution in 80% ethanol and 5 ml of boiling water. The precipitates were washed 3 times with 80% ethanol and 3 times with ether (10 ml each). Cleavage of the sterol-digitonide complexes was achieved by heating the samples for 2 h in a 70°C water bath after the addition of 2 ml of dimethylsulfoxide. Thereafter, sterols were extracted with 40 ml of ether, while digitonin precipitates were removed by centrifugation. Ether was removed *in vacuo*.

Sterol Analysis

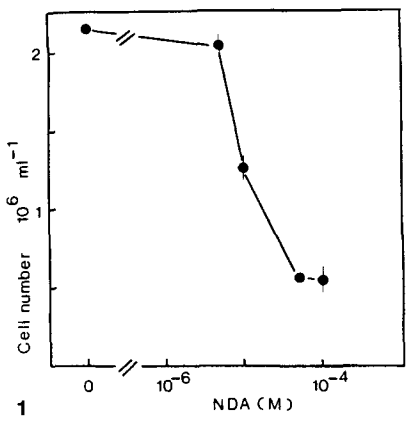
An aliquot of the final ether extract (usually 10%) was dissolved in 50 µl of ethyl acetate of which 2 µl was analysed by gas-liquid chromatography (Packard 430 instrument equipped with an FID). Separations were carried out using 2% OV 101 on Chromosorb W/AW-DMCS (80–100 mesh) in a glass column (180 cm in length; 4 mm i.d.) with nitrogen as the carrier gas. The column temperature was kept at 260°C.

Results

In a previous study, we have shown that NDA could inhibit cell culture growth (Grossmann et al. 1982).

In Fig. 1 the influence of different concentrations of NDA on cell division of cultured maize cells is shown. While a concentration of 5×10^{-5} M NDA inhibits the increase in cell number nearly completely, 5×10^{-6} M NDA has only slight effect on growth.

Approximately 30 min after addition of 10^{-4} M NDA to the cell culture, the total uptake of precursors for DNA, RNA, and protein synthesis was significantly reduced (Fig. 2). Since the incorporation processes were not affected over a period of 100 min, a direct or indirect effect of NDA on membrane permeability is likely. In the pulse experiments shown in Fig. 3, the influence of NDA (10^{-4} M) was studied over a period of 5 days. After various times of treatment, aliquots were removed and cultivated in test tubes for 4 h in the presence of radioactive precursors for DNA, RNA, and protein synthesis,



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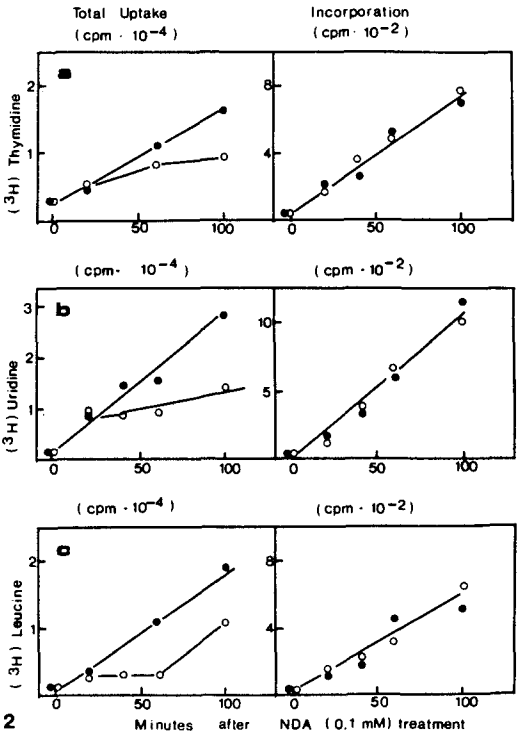

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Fig. 1 and 2. Growth of the maize cell suspension culture under the influence of different concentrations of NDA. Cell suspension (0.8 ml) was incubated for 8 days. The vertical bars represent standard deviation when larger than the size of the point in the graph. Each point is the average of 3 replicates. **Fig. 2.** Time course of the effect of NDA on [methyl-^{3}H] thymidine, [5-^{3}H] uridine and L-[4,5-^{3}H] leucine uptake and incorporation into exponentially growing maize cells. 0.5 ml of cell suspension was incubated with 10^{-4} M NDA (○—○) and without NDA (●—●). The uptake and incorporation rates of the isotopes were referred to 10^6 cells · ml^{-1}. Each point is the average of 3 replicates. Individual standard errors are not presented, since the relative standard deviation of the mean was less than 10%.

respectively (uptake and incorporation of the isotopes run linear during this period). Determination of cell number indicated that a complete inhibition of cell division growth was brought about by NDA treatment. The percentage of viable cells was reduced by ca. 20% (Fig. 3-a, 3-b). A decrease in total uptake of [methyl-^{3}H] thymidine, a precursor for DNA synthesis, was accompanied by reduced incorporation rates during 4 days of NDA treatment and resulted in an approximately constant ratio between uptake and incorporation (Fig. 3c). An explanation of these reduced rates of incorporation could be seen in an influence of the impaired uptake on the specific radioactivity of the internal precursor pool. Table 1 shows that differential rates of labeled thymidine and uridine uptake, as caused by an increase of the external concentration, had pronounced effects on the specific radioactivity of the endogenous thymidine and uridine pools. In contrast, the uptake of leucine has relatively little influence on the specific radioactivity of the internal leucine pool used for protein synthesis. This is a result that is in accordance with Lado et al. (1977). If we look at this in detail, we see that when the external concentrations of labeled thymidine and uridine were increased from 10^{-5} M to 10^{-4} M, the uptake and the incorporation increased more or less in parallel. However, an increase of

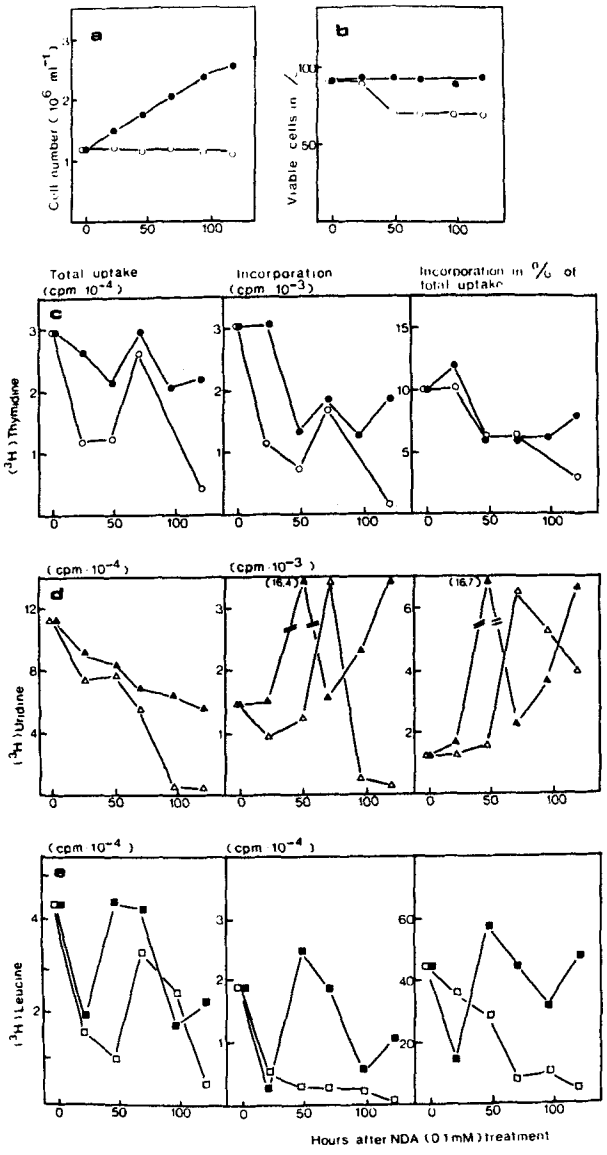

Fig. 3. Investigation of the growth of exponentially dividing maize cells after NDA treatment. Cells were cultivated and treated with NDA in Erlenmeyer flasks (○, △, □ with 10^{-4} M NDA; ●, ▲, ■ without NDA). At various times after treatment, aliquots (0.5 ml) were removed and incubated for 4 h in the presence of labeled precursors. Additional aliquots were removed for determination of cell number and percentage of viable cells. The uptake and incorporation rates of the isotopes were referred to 10^6 viable cells · ml^{-1}. Each point is the average of 2 replicates. Individual standard errors are not presented, since the relative standard deviation of the mean was less than 10%.

Table 1. Effect of various concentrations of [methyl-^{3}H]thymidine, [5-^{3}H]uridine, and L-[4,5-^{3}H]leucine on total uptake and incorporation into maize cells.

		Total uptake (cpm ± S.E.)	Incorporation (cpm ± S.E.)
Thymidine	10^{-4} M	7615 ± 1348	772 ± 17
	10^{-5} M	1161 ± 120	131 ± 10
Uridine	10^{-4} M	21531 ± 4028	3474 ± 1453
	10^{-5} M	2998 ± 329	445 ± 162
Leucine	10^{-3} M	9205 ± 406	208 ± 70
	10^{-4} M	1420 ± 182	117 ± 32

S.E. = standard error of mean.
Exponentially dividing cells (0.5 ml) were incubated for 4 h in the presence of labeled compounds.

the external concentration of leucine from 10^{-4} M to 10^{-3} M caused the uptake of the amino acid to increase 7-fold, while its incorporation rate merely doubled (Table 1).

In the case of [^{3}H] uridine, too, reduced rates of total uptake were approximately correlated with reduced rates of isotope incorporation after NDA treatment (Fig. 3-d). However, there was a reduced maximum incorporation rate 1 day later than that of the control, and a steep decrease incorporation rate at the end of the culture period, demonstrating some quantitative and qualitative changes in RNA synthesis. To sum up, NDA had a pronounced influence on thymidine and uridine uptake, while the reduced incorporation rates could be mainly explained by changes in the specific radioactivity of the internal precursor pools. In contrast to these findings, after 1 day of treatment NDA affected the incorporation of [^{3}H] leucine in protein much more than its uptake (Fig. 3-e). Furthermore, differential rates of labeled leucine uptake had relatively slight effects on the specific radioactivity of the internal leucine pool (Table 1). Thus it could be concluded that NDA influenced both leucine uptake and protein synthesis.

Since NDA inhibited cell division almost completely while DNA and RNA synthesis were only slightly reduced, its effects on the activities of enzymes degrading DNA and RNA were tested. In an experiment shown in Fig. 4, it was demonstrated that NDA treatment resulted in an enhancement of DNase, RNase, and protease activity.

Another experiment was related to the effect of NDA on protein synthesis. To ascertain directly whether NDA inhibited amino acid incorporation, the effect of NDA on protein synthesis was measured in a cell-free translation system (Table 2). Concentrations that inhibited cell division and leucine incorporation in the intact cells exerted only slight effects on the cell-free system, indicating an indirect influence of NDA on protein synthesis of the cells. In 1974, Douglas and Paleg postulated that inhibition of sterol biosynthesis in

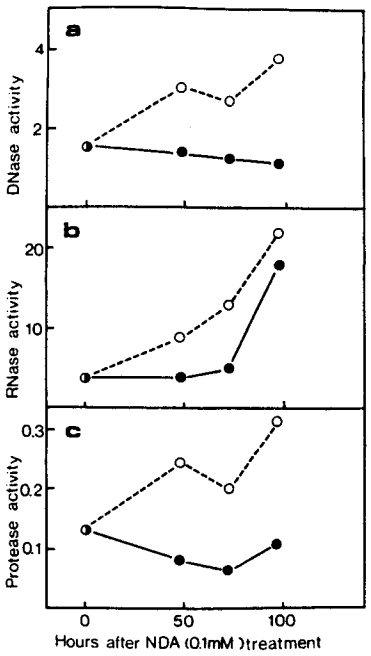

Fig. 4. Influence of NDA on DNase, RNase, and protease activity of exponentially dividing maize cells. Cells were cultivated in Erlenmeyer flasks with 10^{-4} NDA (○—○) and without NDA (●—●). At various times after treatment aliquots (4 ml) were taken and analyzed for enzyme activity. The enzyme activities were referred to 10^6 cells · ml^{-1} and are expressed as A_{260} (nucleases) or nmol leucine (protease) values over the zerotime control. Each point is the average of 2 replicates. Individual standard errors are not presented, since the relative standard deviation of the mean was less than 10%.

Table 2. The effect of NDA on in vitro amino acid incorporation into polypeptides.

		NDA	
	Control	0.06 mM	0.1 mM
cpm ± S.E.	135 379 ±8 802	128 022 ±6 939	118 810 ±6 691
% of control	100	95	88

S.E. = standard error of mean.

plants could account for at least part of the growth reduction caused by certain growth retardants. Later the same authors demonstrated that growth retardants such as AMO-1618 caused qualitative and quantitative changes in the sterols of tobacco plants. Growth retardation in these plants could be overcome by the exogenous application of stigmasterol (Douglas and Paleg 1981). An alteration in or inhibition of sterol biosynthesis has been shown to affect membrane permeability (van Deenen et al. 1972), and a role in protein synthesis seems likely (Hradec et al. 1974). Thus NDA was tested for its effects on sterol production in maize cells. Cells from the exponential phase were diluted with medium to which 10^{-4} NDA was added. After 8 days (exponential phase of controls) and 15 days (stationary phase of controls), the cells were harvested and the sterols were extracted and analyzed. Stigmasterol and β-sitosterol could be identified as the major sterols. As shown in Table 3, the composition of these sterols from cells grown in the presence of NDA was profoundly changed qualitatively and quantitatively. After 8 days, the total sterol content

Table 3. The effects of NDA (10^{-4} M) on sterol contents of maize suspension cells after various times of treatment.

		Sterol content (μg/g fresh weight)	
		after 8 days	after 15 days
control	Stigmasterol	39.7	22.5
	β-sitosterol	169.1	169.4
NDA treated	Stigmasterol	85.7	37.2
	β-sitosterol	116.3	45.9

Exponentially dividing cells were incubated in Erlenmeyer flasks in the presence of NDA. Sterol contents of the cells were analyzed after various times of treatment.

of the treated cells was similar to that of the control cells, while there were qualitative changes in the ratio of β-sitosterol to stigmasterol. The stigmasterol content strongly increased, whereas the β-sitosterol content decreased. After 15 days of treatment, the total content was reduced by 50%, while the ratio of β-sitosterol to stigmasterol remained almost constant. Further, still unknown sterols were found in the treated cells.

Discussion

Recently we have demonstrated the usefulness of cell suspension cultures for identifying growth retardants and for investigating their mode of action (Grossmann et al. 1982). In this study we reported that the norbornenodiazetine derivative NDA, a very potent growth retardant with a broad spectrum of activity (Jung et al. 1980, Rademacher and Jung 1981), could inhibit cell-division growth of cell-suspension cultures almost completely at a concentration of 10^{-4} M and induced red pigmentation of the cells. Staining methods revealed that a high percentage of cells could be considered viable and substantiated the evidence that NDA inhibits the cell division process directly or indirectly while the cells remain metabolically active. Using a cell-free enzyme system from immature pumpkin endosperm, it was found that NDA inhibits the formation of gibberellins by blocking the oxidative steps leading from *ent*-kaurene to *ent*-kaurenoic acid (Hildebrandt 1982, Graebe 1982). Since the endogenous gibberellins could play a functional role in cell culture growth (Weiler 1981), this finding could explain the inhibitory action of NDA. However, since both the sterol and gibberellin biosynthesis pathway involves cyclization and oxidation steps that are very similar to each other (Siegel 1981), further possible explanations, e.g., inhibition of the sterol biosynthesis (Douglas and Paleg 1981) or a direct effect on cell division processes, should be kept in mind.

In order to elucidate the influence of NDA on the molecular genetic metabolism of maize suspension cells derived from shoot, tracer experiments for studying precursor uptake and incorporation in DNA, RNA, and protein were carried out. While the total uptake of these compounds was reduced as soon as approximately 30 min after NDA (10^{-4} M) treatment, DNA, RNA, and protein synthesis were not affected at this stage. In pulse experiments over 5

days of NDA treatment, a decrease in total uptake of label was accompanied by reduced rates of incorporation revealing an inhibitory effect of NDA on protein synthesis, while the reduced rates of incorporation in DNA and RNA can be mainly explained by changes in the specific radioactivity of the internal pools used for the synthesis. It can be assumed that any increases of DNA and RNA content during the inhibition of cell division are reduced by the stimulated action of DNase and RNase.

Nevertheless, a higher level of polyploid cells can also be expected as has been shown for chloromequatchloride on cell growth and ploidy level of *Phaseolus vulgaris* plants (Essigmann-Capesius et al. 1981). To sum up, that NDA limited membrane permeability and affected protein synthesis indirectly was proven by the reduced rates of leucine incorporation, which were detected after only 1 day of treatment and by data concerning the action of NDA on protein synthesis in vitro. An explanation of these findings could be an alteration in the sterol composition or an inhibition of sterol biosynthesis caused by NDA, because it is known that sterols play a multifaceted role in all organisms. They are not only of outstanding importance in controlling permeability and maintaining the integrity and function of plant membranes but also, and even more important, they are thought to be involved in the functioning of protein synthesis (Hradec et al. 1974, Douglas and Paleg 1974, 1981).

Thus we tested NDA for its effects on sterol production in maize suspension cells and demonstrated that the composition of the sterol fraction, mainly stigmasterol and β-sitosterol, was clearly changed qualitatively and quantitatively.

Considering the results presented, it can be hypothesized that NDA inhibits cell division growth by affecting membrane permeability and indirectly the protein synthesis. However, additional experiments are necessary to determine to what extent NDA acts in cell cultures by altering sterol biosynthesis and/or by lowering the production of gibberellin as the primary mode of action.

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