UPTAKE AND TRANSLOCATION OF 4,6-DINITRO-O-CRESOL (DNOC) IN YOUNG PLANTS OF WINTER RYE (*Secale cereale* L.)

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SUMMARY

The action of the herbicidal compound, 4,6-dinitro-o-cresol (DNOC), on rye roots was studied by analyzing its uptake from solutions and subsequent translocation by roots of intact plants and by isolated roots. The distribution of DNOC in the root cells was investigated by differential centrifugation of tissue homogenates.

DNOC is readily accumulated and spreads throughout the plant by apoplastic movement. Redistribution hardly occurs.

Next to uptake with the transpiration stream there is an intense passive absorption of mainly undissociated molecules. DNOC is partly adsorbed to the walls of xylem vessels and parenchymous cells, partly present in a soluble form. Although adsorption to protein sometimes occurs, DNOC is not metabolized.

Soon after the accumulation, unaltered DNOC is released again into the outer solution by a mechanism sensitive to temperature and oxygen tension. The nature of this mechanism,its possible localization and role in detoxification and resumption of growth are discussed.

1. INTRODUCTION

The herbicidal compound, 4,6-dinitro-o-cresol (DNOC), influences the rates of growth and development of young winter rye plants; after an initial retardation growth is accelerated (BRUINSMA 1962). This influence is exerted primarely on the roots (BRUINSMA 1963; BRUINSMA & SCHUURMAN 1966).

Therefore, the uptake of DNOC by roots, its fate in the root system, and its translocation through the plant were studied in more detail, mainly with young winter rye plants on culture solutions.

2. MATERIAL AND METHODS

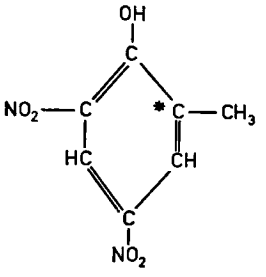
2.1 Material

Seeds of Petkus winter rye were germinated on moist sand in Petri dishes, 18 cm in diameter, in the greenhouse. After 4 to 6 days seedlings were selected and transferred onto an aerated solution of 400 ppm $Ca(NO_3)_2.4H_20$, 100 ppm KNO_3, 100 ppm KH_2PO_4, 100 ppm $MgSO_4.7H_20$, a trace of $FeCl_3$ and trace elements. On this solution, which was refreshed once or twice a week, the plants were grown in the greenhouse for at least one week before being used in the experiments.

2.2 Treatments

Roots of intact plants or isolated root systems were placed in 250 ml glass beakers containing a weighed amount of either the culture solution or a 5 mM citrate-phosphate buffer, each with a known DNOC content. In several experiments radioactive DNOC was used, labelled with ^{14}C in the ring (*Fig. 1*) and with a specific activity of 4μC per mg. The solutions were continuously aerated, also to ensure a homogeneous DNOC concentration.

In the experiments on the distribution of DNOC in the cell, the roots were quickly rinsed 3 times after DNOC uptake, superficially dried and cut into pieces. The root parts were homogenised in a MSE homogeniser or in a mortar without sand, with citrate-phosphate buffer. The homogenate was centrifuged at different speeds in a refrigerated MSE 'High Speed – 17' centrifuge.



4,6-dinitro-o-cresol-2-^{14}C Fig. 1

2.3 Determinations

The uptake of DNOC was measured by periodical determination of DNOC in the outer solution, after a modified method of PARKER (1949). Triplicate samples of 5 ml were saturated with NaCl: Na_2CO_3 = 9:1, and shaken against 5 ml of dried ethyl methyl ketone, the optical density of which was subsequently measured at 422 nm with an Unicam SP 600 spectrophotometer. From the weighed amounts of solution and of roots the uptake was calculated in μg DNOC per g fresh root weight, all data referring to free DNOC, H-form.

In the experiments with ^{14}C-labelled DNOC the uptake and distribution were also determined from G.M. countings of evaporated 0.2 ml samples of solutions, and of dried fractions of the homogenized material, corrected for self absorption after ATEN (1951).

In order to detect adsorption of DNOC on large molecules, root extracts were chromotagraphed on columns of 8 g Sephadex G-25 fine (Pharmacia), 2 cm diameter, and eluted with citrate-phosphate buffer at room temperature; fractions of 4.6 ml were collected with the LKB RadiRac fraction collector. Distribution and redistribution of radioactive DNOC was followed qualitatively by autoradiography of freeze-dried plants after the methods described by CRAFTS & YAMAGUCHI (1964).

3. RESULTS

3.1 Uptake and translocation in intact plants

The course of uptake and the distribution and redistribution patterns of DNOC upon continued plant growth in a DNOC-free medium were followed by placing plants on nutrient solutions containing 2, 4 and 10 ppm DNOC-2-^{14}C during 1, 8 and 24 hrs. The DNOC uptake was measured by determinations in the outer solution. After the treatments the plants were either sampled for autoradiography or further grown on nutrient solutions with non-radioactive DNOC or without DNOC and sampled at a later point of time. Some of the autoradiographs are shown in *plate 1*.

DNOC turned out to be readily taken up by the roots, particularly the root tips being often yellow-orange coloured by the end of the treatment. The xylem vessels, too, became DNOC-coloured and the radioactivity spread throughout the plant within 1 hr. (*plate 1,A*). The label was mainly restricted to the veins, its translocation being typically apoplastic, with the transpiration stream towards the leaves. The uptake of DNOC was enhanced by increased rates of transpiration. There was hardly any disappearance or redistribution of radioactivity upon further growth on a DNOC-free solution, except that the high activity of the root tips diminished. The leaves formed after treatment were practically label-free except for the tips that might already have been present

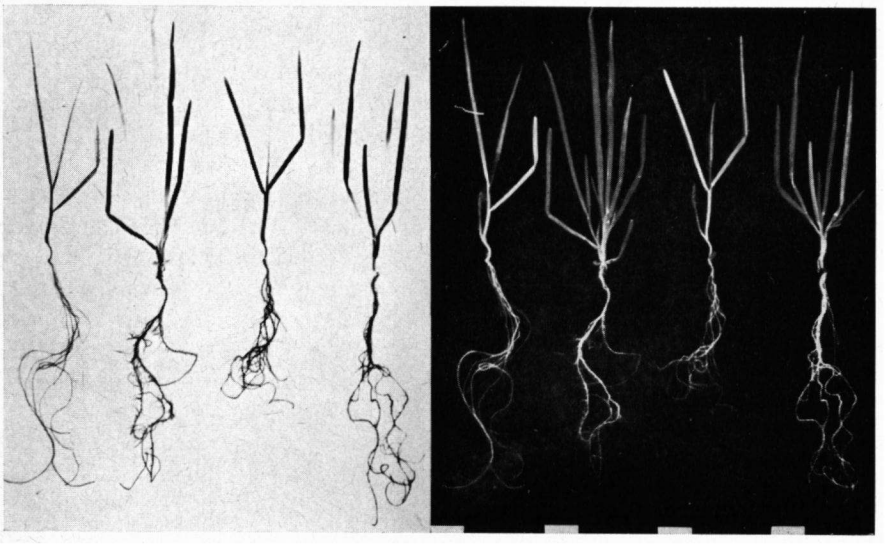


Plate 1.
Mounted freeze-dried plants (right) and their autoradiographs (left).
A: Root uptake of DNOC-2-^{14}C during 1 hr. From left to right; 2 ppm; 2 ppm, next 143 hrs. on DNOC-free nutrient solution; 10 ppm; 10 ppm, next 143 hrs. on DNOC-free nutrient solution.

B: Root uptake of DNOC-2-^{14}C during 8 hrs., next 136 hrs. on nutrient solution. From left to right: 2 ppm, 1.78 μC total activity; 10 ppm, 1.82μC total activity; 10 ppm, 9.16μC total activity; the same, but also with 10 ppm non-radioactive DNOC during the further 136 hrs.

during the treatment (*plate 1,A*). The bases of already existent but not yet full-grown leaves, too, showed little activity. Newly developed roots sometimes did contain some label, probably derived from the nutrient solution to which some radioactivity was released by the treated roots as will be seen below.

Concentrations of 2 and 4 ppm DNOC, which may well be expected to prevail in the soil solution upon a practical herbicidal application, were tolerated, but 10 ppm was definitely toxic at prolonged exposure time. The uptake was relatively lower at 10 ppm (*table 1, plate 1,B*, second plant). This may be connected with an inhibited water uptake: whereas the plants remained fully turgescent at 2 and 4 ppm solutions, a loss of turgescence occurred already upon 1 hr exposure to 10 ppm DNOC. After this treatment the plants were still able to recover (*plate* 1,*A*), but after 8 hrs. of exposure leaf shrinkage occurred and subsequent growth on DNOC-free solutions was very poor (*plate 1,B*).

The DNOC uptake depended both on the transpiration rate and on the pH of the outer solution. This was shown in experiments with non-radioactive DNOC in solutions of different pH values upon which plants were placed under different transpiration conditions: in darkness or in diffuse day light (20.10^3 ergs. $sec^{-1}.cm^{-2}$) with or without an air stream from a fan. The net DNOC uptake of these plants, presented in *fig. 2*, was always higher than could be accounted for by a passive trailing along with the transpiration stream, particularly so at low transpiration rates and at low pH values. Undissociated

Table 1. Net rates of DNOC intake with intact plants over a period of 24 hrs.

initial concentration	rate of intake, in g DNOC per hr and per plant		
	0–2 hrs	2–8 hrs	8–24 hrs
2 ppm	4.85	2.12	1.31
10 ppm	12.0	9.5	—0.20

DNOC molecules must have been taken up by the root system by another way than via the water absorption.

Other phenomena, too, pointed to an exchange of DNOC irrespective of the transpiration. Generally, the DNOC concentration in the outer solution at first decreased but subsequently increased again. Initial accumulation of DNOC must, therefore, be followed by a release that even may about equal the uptake via transpiration (*table 1*). This agrees with the drop in radioactivity of the initially higly labelled root tips, after exposure to DNOC-^{14}C, during further growth on DNOC-free solutions. Afterwards these solutions showed a slight but significant radioactivity.

Whereas the radioactivity in the leaves apparently is immobilized in a form that is neither translocated nor dissimilated to an appreciable extent any more, the fate of DNOC in the roots might be more interesting for the reasons mentioned above. Attention was therefore focussed on the uptake by root systems excised from their shoots, to avoid interference from transpiration.

3.2 Uptake by isolated root systems

The dissociation degree of DNOC at the pH values 5.6, 4.6 and 3.6 is 96, 80 and 23 per cent respectively.

The effect of the degree of dissociation of DNOC molecules on their uptake by isolated root systems was studied using 5 mM citrate-phosphate buffer

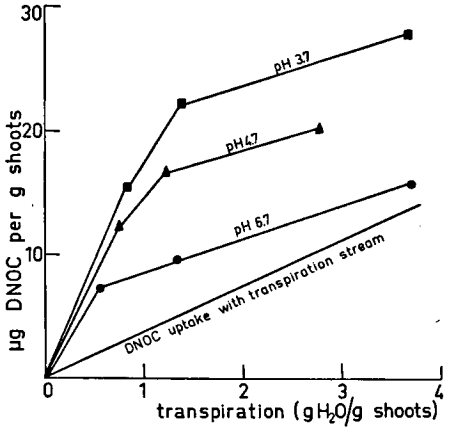


Fig. 2. Uptake of DNOC by plants during 1 hr. exposure of the roots to buffered solutions of 3.7 ppm DNOC under different transpiration conditions.

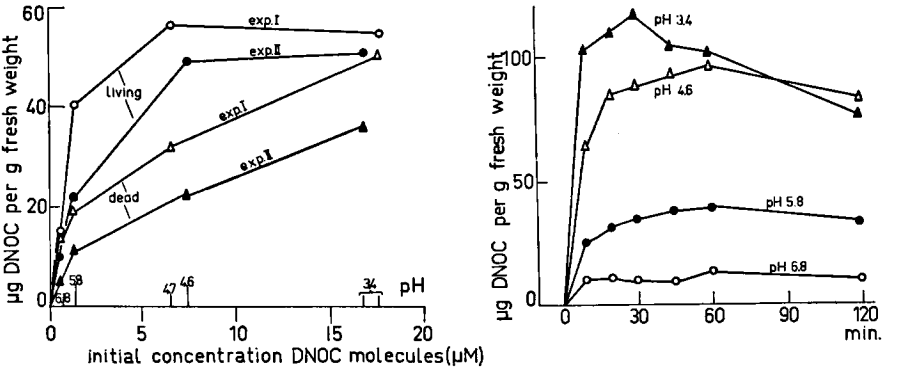


Fig. 3. Uptake of DNOC from 3.8 ppm solutions, buffered at different pH, by living and dead isolated root systems, during 2 hrs.

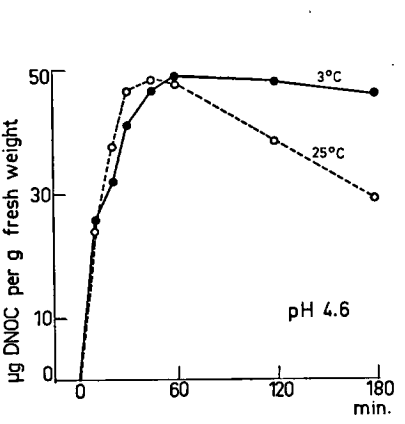
Fig. 4. Course of the DNOC uptake by isolated root systems from 4.4 ppm solutions buffered at different pH.

solutions at different pH values containing a fixed amount of DNOC. From various experiments, the results of two of which are shown in *fig. 3*, the absorption of DNOC was found to depend on the concentration of associated molecules, especially below about 5.10^{-6}M. The curves are no true saturation curves because their shapes vary with the time of exposure, as can be concluded from the course of uptake with time in *fig. 4*. The affinity of associated molecules to living roots was higher than to roots previously killed by being dipped in hot water (*fig. 3*), but this need not involve some active transport mechanism.

On the contrary, although the initial rate of uptake was very high, it was affected neither by anaerobiosis nor by different temperatures as is shown for the latter factor in *fig. 5*. This independence of temperature and oxygen deficiency of the DNOC uptake was also found with isolated roots of such plants as bean and melon that are otherwise very sensitive to these conditions. Effects of inhibitors of respiration were not studied because DNOC is an effective uncoupler of oxidative phosphorylation (KERR & WAIN 1964).

The rapid absorption of DNOC during the first minutes of exposure soon slowed down and generally became even negative after 30 to 60 minutes (*figs.4* and *5*). The concentration of the outer solution, after initial dilution to sometimes one third of the original values, increased again due to the egress of unaltered DNOC. In contrast to the preceeding DNOC uptake, this release was increased both upon aeration and at higher temperatures (*fig. 5*), so that it might be looked upon as an active process. It was also observed with root systems of bean and melon, and occurred, too, with intact plants if the DNOC uptake with the water intake was nullified by preventing transpiration (*fig. 6*). The excretion of DNOC may, therefore, also occur in the field.

The maximum amount of DNOC per g fresh weight of roots varied in the different experiments. The younger the roots the greater the proportion of root



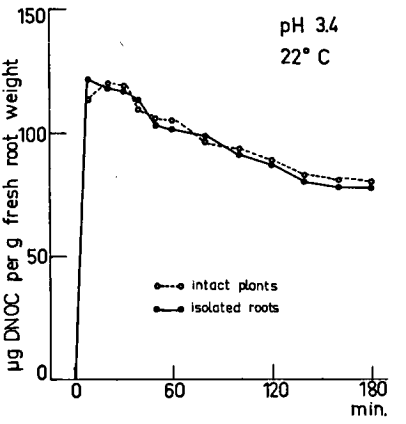


Fig. 5. Course of the DNOC uptake by isolated root systems at different temperatures.
Fig. 6. Course of the DNOC uptake by isolated root systems and intact plants. Transpiration was prevented by enveloping the shoots in black polyethylene film.

tips that most intensively accumulated DNOC. Also the subsequent release of DNOC was observed best with young root systems (the roots, grown in well-aerated solutions, had no root hairs). Moreover, the observation that living roots absorbed DNOC better than dead ones (*fig. 3*), led to the supposition that it might be protein to which the DNOC was reversibly adsorbed. It was therefore analysed with which parts of the root cells the absorbed DNOC was associated.

3.3 Distribution over cell constituents

The first partition was to homogenise the root tissue, to precipitate the cell fragments at 30,000 g and to determine the DNOC in the supernatant; the difference with the total amount of absorbed DNOC was considered to be precipitated DNOC, adsorbed to the cell particles. Results of such an experiment, performed with different samples of isolated roots that were washed after 30 minutes of DNOC uptake for 0, 30 and 60 minutes prior to their homogenation, are presented in *fig. 7*. Immediately after uptake, most of the absorbed DNOC could not be recovered from the supernatant and was assumed, therefore, to be adsorbed to the precipitated cell particles. Upon washing of roots, however, the amounts of DNOC, determined in the combined washing solutions, surpassed the amount of initially soluble DNOC and, hence, must have been removed from the cell particles.

It is improbable, however, that upon prolonged washing all the bound DNOC should be set free again and should be eluted from the roots. The autoradiography experiments showed already that 143 hrs. after 1 hr. of DNOC-^{14}C

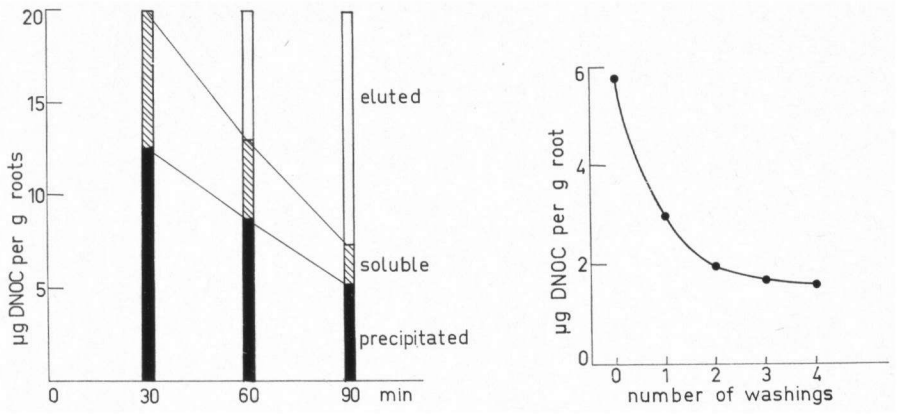


Fig. 7. Elution from and distribution in the cells of DNOC during 1 hr. of washing of isolated roots subsequent to 30 min. of uptake from a 4 ppm solution at pH 4.5. Samples of 3 g roots, washed with portions of 150 ml buffer, pH 4.5, refreshed every 10 min. DNOC determined in all solutions and in the 30,000 g supernatants.

Fig. 8. Elution of DNOC, added to homogenate, from the precipitate upon repeated washings. 10 ml Homogenate in buffer, pH 4.5, containing 4 ppm DNOC, was precipitated at 30,000 g and the pellet resuspended in portions of 10 ml buffer, pH 4.5, and reprecipitated four times.

intake the roots still contain much of the initial labelling. Also DNOC, added to a root homogenate and precipitated at 30,000 g, could not be completely removed from the precipitate after repeated resuspending of the pellet (*fig. 8*).

It was analysed whether the DNOC that could not be detected in the supernatant was indeed quantitatively adsorbed to cell fragments or was perhaps partly metabolized. The pink colour of the water phase, which showed up at the determination of DNOC in the cell extracts, reminded of the intensievty coloured 4-nitro-6-amino-o-cresol that can be obtained by reduction of DNOC with sodium dithionite and is a metabolite in the rabbit (SMITH *et al.*, 1953). This substance was found to be more hydrophilic than DNOC and to absorb in aqueous solution at 460 nm. However, the maximum absorption of the pink water phase was between 420 and 430 nm and was also found with blank DNOC determinations in DNOC-free root extracts. The differential spectra of water phases from root extracts with and without DNOC did not agree with that of any known degradation product. As is shown in *table 2*, no enzymatic breakdown of DNOC could be detected either in root homogenates or in their 30,000 g supernatants. The DNOC concentration remained constant even after 24 hrs. of incubation provided that no bacterial contamination occurred.

What incidentally was found, indeed, was a sharp drop in the DNOC concentration of a homogenate or a supernatant during the first few minutes upon the addition of DNOC, before the stable level was obtained. This was due to adsorption to protein as will be seen below.

Table 2. Absence of enzymatic breakdown of DNOC in a root homogenate and in its supernatant. Roots were homogenized in buffer, pH 4.5, part of which was centrifuged at 30,000 g. At 0 min, DNOC was added to homogenate and supernatant to a final concentration of 4.9 ppm. Temp. 19°C.

	μg DNOC per g root weight	
	0 min.	30 min.
homogenate	30.3	30.3
supernatant	30.2	30.2

It was concluded that DNOC was not metabolically degraded to any appreciable extent.

That the DNOC not to be recovered from the supernatant was bound, indeed, to the precipitate was further affirmed in experiments with DNOC-^{14}C, in which the amounts of radioactivity in precipitates was calculated after corrections for the radioactivity owing to the amount of solution in the wet pellet and for the self absorption of the dried pellet material. Balances were drawn up for the recovery of radioactivity, both upon administration of DNOC to isolated roots that were next homogenized, and after addition of DNOC to already homogenized root tissue. The homogenates were centrifuged at 4°C at 600, 3600 and 30,000 g to isolate precipitates rich in cell wall materials, mitochondria, and ribosomes respectively. Fractions of the ultimate supernatant were chromatographed over Sephadex columns to detect DNOC adsorbed to large molecules.

The results turned out to be not exactly reproducable. Sometimes part of the DNOC in the supernatant was adsorbed to protein. In one experiment, 25% of all the DNOC absorbed by the roots could be precipitated with $(NH_4)_2SO_4$ from the final supernatant. On the Sephadex column, a distinct zone was observed in the protein fractions, moving ahead of the main zone of unaltered DNOC. In most cases, however, both the DNOC and the radioactivity were confined to the zone of small molecules, as can be seen in *fig. 9*. The small amount of radioactivity in the protein fractions 4 to 10, together less than 5% of the total activity, is not quite significant.

As was expected from *fig. 7*, varying proportions of the DNOC, absorbed by the roots or added to the homogenate, could be recovered from the supernatants. The remainder was present in the precipitates, as followed from the determinations of the radioactivity of the pellets. Generally, most or all of the residual DNOC was traced back in the first precipitate, adsorbed to cell wall fragments. The specific activity of this 600 g precipitate was always much higher than that of the corresponding supernatant. In one experiment, for example, 20 ml of homogenate contained 134.10^3 cpm, the first supernatant 65.10^3 cpm, the first precipitate 76.10^3 cpm, with a specific activity of 178 cpm per μg dry weight; the further precipitates showed hardly any net radioactivity.

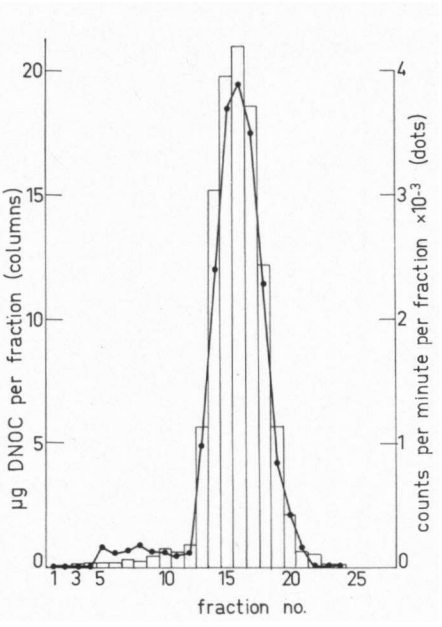


Fig. 9. Determinations of DNOC and radioactivity in fractions of supernatant eluted from a Sephadex column.

The distribution of DNOC over the cell constituents was not substantially influenced by the way of administration of the compound. Both after absorption by intact roots and upon addition to root homogenates, the vast majority of the residual DNOC was adsorbed to the cell wall fragments that, of course, formed the heaviest precipitate.

4. DISCUSSION

In the present investigation the attention was focussed on the uptake of DNOC by the root system. The foliar absorption of DNOC was studied already by FOGG (1948), who established that DNOC may enter the leaf by liquid and gaseous diffusion and that this way of translocation occurs within the leaf, too, but to a limited extent and towards the leaf margin only.

Our results on the uptake of DNOC-^{14}C by roots of intact plants agree with these findings. The radioactivity, absorbed by the roots, readily spreads throughout the plant, but by apoplastic movement only, that is through the water system in the xylem and the cell walls. Part of the label is irreversibly adsorbed to these structures, there is no redistribution to newly formed organs.

Apart from some adsorption to protein, no conversion of DNOC could be detected either in intact or in homogenized tissues. This enzymatic inertia allows for the tentative conclusion that the radioactivity traced back in plant organs and cell constituents is likely to be unaltered DNOC.

Next to uptake with the transpiration stream, DNOC is strongly accumulated by the passive absorption of mainly undissociated molecules. Apart from the

high absorption rate, this has frequently been found, for instance with the molecules of the auxin-type of herbicide, 2,4-D (2,4-dichlorophenoxyacetic acid) (YAMAGUCHI 1965).

The absorbed DNOC could largely be traced back in two forms: free in solution or adsorbed to cell wall materials. Part of the adsorbed DNOC can be set free again as the concentration of soluble DNOC drops because of release into the outer solution, the remainder is irreversibly bound. In contrast to 2,4-D, the washing-resistent fraction of which is assumed to be metabolically fixed (YAMAGUCHI 1965), DNOC is immobilized mainly to inactive cell structures.

The protein-bound DNOC, that was sometimes met with, might originate from the protein-rich root tips that intensively accumulate DNOC initially, but later on release much of it again. Root-tip accumulation itself is not specific for DNOC, it was also found for the herbicide, dalapon (2,2-dichloropropionic acid) (PRASAD & BLACKMAN 1965). With graminaceous plants, particularly, binding to protein appears to be a detoxification mechanism, as was shown for 2,4-D by ZEMSKAJA & RAKITIN (1964) and RAKITIN *et al.* (1966). This protein adsorption might also play a role with the excretion of DNOC, which was the highest, the larger the proportion of root tips was.

Egress of previously absorbed toxic substances by roots has been demonstrated by Blackman and co-workers (BLACKMAN 1961; SAUNDERS *et al.* 1965 a and b, 1966; VENIS & BLACKMAN 1966). They found a temperature-sensitive release of those auxin-type substances for which the particular plant species were sensitive, relatively harmless compounds being continuously accumulated. As in our experiments, the auxins were released without being metabolically changed, but some active excretion mechanism has been suggested. The supposition that the egress of auxins might result from desorganisation owing to the sectioning of tissue, does not hold for DNOC because isolated root systems and intact plants show identical patterns of uptake and release. Whereas the auxin excretion starts only after about 12 hours, a net DNOC release shows up within half an hour already. Although this release, in contrast to the uptake, is sensitive to temperature and oxygen tension, it is doubtful whether active excretion is involved because DNOC is an even more effective uncoupler of oxidative phosphorylation than DNP (2,4-dinitrophenol) (KERR & WAIN 1964). Such processes as the uptake by roots of phosphate (LOUGHMAN & SCOTT RUSSELL 1957), chloride (BUTLER 1953) and sodium (PEARSON 1962), that are inhibited by DNP, are also reduced by DNOC: RIEPMA (1958) reported a 50% inhibition of the overall salt intake at 2 ppm DNOC. The phloem transport, too, is inhibited by DNP (HAREL & REINHOLD 1966) and root growth is known to be arrested shortly upon a DNOC application (BRUINSMA & SCHUURMAN 1966). For these reasons, excretion of DNOC at the cost of metabolic energy is not likely to occur. However, its dependence on temperature and aeration might suggest an active excretion of DNOC from more outward plasmatic parts driven by energy from more inner parts where DNOC did not yet penetrate in sufficient amounts to uncouple phosphorylation completely.

For the original problem, the elucidation of the growth stimulating action of DNOC on root growth after its initial retardation, the apparent inertia of DNOC is not very promising. But although DNOC is neither metabolized nor significantly accumulated at active cell particles, its high concentration in the root cells may considerably affect the cell metabolism. The only known way up to now is the inhibition of oxidative phosphorylation and by that, of all energy-requiring processes in the root. The concentration of the outer solution in most experiments, 4 ppm, must about equal that of the soil solution after a practical DNOC application that initially inhibits root growth. The higher dose of 10 ppm turned out to be definitely toxic, as was also found by RIEPMA (1958). LYNDSAY & HARTLEY (1963, 1966) reported doses above 10 ppm to be lethal to young cucumber and pea plants. They described a collapse of the root tissue that prevented water uptake and allowed a passive entry of DNOC into the aerial parts.

How the growth retardation at sublethal doses changes into subsequent acceleration remains obscure. The change might be connected with the release of DNOC into the outer solution by the root tips that are also activated at the resumption of growth.

ACKNOWLEDGEMENTS

The DNOC-2-^{14}C was a generous gift from Dr. M. Czech, Farbwerke Hoechst A.G., Germany.

The author gratefully acknowledges the co-operation of his collegue, Dr. H. C. M. de Stigter, with the autoradiographic techniques, and the skilful technical assistence of Mr. J. Swart and Mr. R. A. Vermeulen.

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