Biological effect and the toxicity mechanisms of some dinitrophenyl ethers

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Abstract

It is assumed that dinitrophenols hinder the proton translocation through the mitochondrial inner membrane, inhibiting thus the oxidative phosphorylation. However, the toxicity mechanism of these uncouplers is still unclear, because the corresponding dinitrophenyl ethers retain the uncoupling behaviors. Therefore, we have investigated the biological effect of 2,4-dinitrophenetole (DNF), dinitroanisole (DNAN), and 3-(2,4-dinitrophenoxy)-propane-1,2-diol (DNG) in wheat experiments as compared to that of classical dinitrophenols. The biological effect of all these dinitrophenyl derivatives was effective even at very low concentration. Part of their toxicity seems to be related to the solubility of the investigated compounds. Contrary to the current theories, the toxicity induced by the investigated dinitrophenyl ethers was not related to the corresponding dinitrophenols obtained by hydrolysis. Besides, both the dinitrophenylether and the other uncoupling agents are characterized by a significant absorbance in IR at about 6000 cm⁻¹, corresponding to an energy quantum similar to that of ATP molecule. Consequently, a hypothetically alternative mechanism of toxicity based on spectral measurements was introduced.

Keywords: dinitrophenyl ethers; dinitrophenetole; dinitroanisole; FT-IR; toxicity mechanism; wheat germination.

Introduction

Dinitrophenols and their derivatives have multiple biological effects, being used in agriculture as insecticides, fungicides, herbicides and acaricides or in medicine and biology as metabolic inhibitors [1,2]. It was also assumed that dinitrophenols (DNP) hinder the proton translocation through the mitochondrial inner membrane and therefore oxidative phosphorylation is inhibited (ATP is no longer formed and the cells deprive of essential energy supply) [3-5]. Their toxic action, chronic or acute one, on human being is manifested during manufacturing, conditioning, transportation, storing and usage of these products or by manipulating and consumption of treated products [6,7]. It was shown that 2,4-dinitrophenol and related compounds produce a prolonged choleretic and a marked pyrexia in dogs [8]. However, the effect was associated with nitro groups in positions 2 and 4 of the benzene ring, and a free or potential free hydroxyl group. Our previous results showed that free hydroxyl group might be less important than the absorption of electromagnetic radiation around 6000 cm⁻¹ [9,10]. We hypothesized that the biological effect of dinitrophenols can be associated with the energy transfer from the respiratory chain to ATP-synthase and not with proton translocation as the chemiosmotic hypothesis claims [3-5]. Therefore, the present study was undertaken in order to investigate more fully the effects of some dinitrophenyl ethers, such as 2,4-dinitrophenetole, 2,4-dinitroanisole, and 3-

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(2,4-dinitrophenoxy)-propane-1,2-diol on wheat germination. Because germination experiments are easy, cheap, fast and spectacular, they can reveal if these compounds act as ethers or phenols [11-16]. The possible mechanism of toxicity of these chemicals and pesticides are discussed in the light of Eugen Macovschi’s biostuctural theory [17,18] as well as the chemiosmotic theory by Peter Mitchell [4,5].

Materials and Methods

Equipment. The dinitroderivatives were quantified using a UV-Vis Libra S35PC spectrophotometer from Biochrom Ltd (Cambridge, England). The infrared spectra were taken on a Jasco FT/IR660 Plus Fourier spectrometer in the range from 0 to 15000 cm⁻¹.

Chemical reagents. The reagents used were of analytic purity (Merck, Sigma, chimney) and the solution and the water slurries were prepared using bidistilled water. Several dinitrophenol ethers such as 2,4-DNF, 2,4-DNAN, and DNG were synthesized, starting from dinitrochlorobenzene, with variants of the methods described in literature [19]. The separation and purification of the compounds obtained were carried out using thin layer chromatography on silica gel (Kieselgel 60F₂₅₄, Merck) and on silica gel column. The melting points and the elemental composition were also determined.

Treatment solutions of dinitrophenyl ethers and dinitrophenols with the concentrations in the range from 10⁻⁴ M to 10⁻² M were prepared. Each time, a control with redistilled water was carried out.

Biological material. The wheat samples (Triticum aestivum), Henika variety, were taken from the Agricultural Research Station in Suceava.

Procedure. The germination parameters were measured according to ISTA recommendations (Seed Science and Technology, 1993), however we worked also with lots of 50 seeds which were laid to germinate on filter paper, in Petri dishes, in three repetitions. The treatment lasted for an hour; afterwards the seeds were laid as uniformly as possible in Petri dishes, on double filter paper, together with the treatment solution. The seeds with a visible root were considered germinated. The seeds were supplemented daily with 5 ml of redistilled water. The germinated, abnormal and dead seeds as well as the resulting plantlets were counted. The plantlets were cut off at the level of the seeds 7 days after, measured and weighed (height, H, in cm and mass, m, in grams). The percent of germinated seeds were reported 3 days later (energy of germination, EG) and 7 days later (the germination rate, GR), respectively. A seed with visible coleorhizae was considered germinated. Young wheat plantlets were harvested from their seeds, measured (height H, expressed as cm) and mass (M, expressed as grams).

Statistics. The data were validated using the Tukey test [20].

Results and Discussion

During the 1-week treatment, both 10⁻² M dinitroderivatives completely inhibited wheat seed germination (Figure 1, treatment 2 and 4). Germination partly recovered after rinsing before being introduced in Petri dishes (Figure 1, treatment 3 and 5). Because DNAN, DNG, and other dinitrophenyl ethers are less toxic than the corresponding dinitrophenols at very low concentrations, but as noxious as them at 10⁻² M concentration, we hypothesized that the solubility might play an important role in dinitrophenol toxicity (Table 1). Thus, a significant difference was also calculated by Tukey test between the control and the sample treated with...
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3-(2,4-dinitrophenoxy)propane-1,2-diol, with a concentration of $10^{-3}$ M. At this concentration, 2,4-dinitrophenol proved to be significantly more noxious than DNG was.

![Figure 1](Figure 1. Effect of 2,4-dinitrophenol (DNP) and 2,4-dinitroanisole (DNAN) on wheat germination. The seeds were treated with dinitrophenyl derivatives for 1 hr, then they were rinsed with redistilled water to release the toxic compound or put directly on filter paper in Petri dishes. 1) Control, H$_2$O; 2) DNP, $10^{-2}$ M; 3) rinsed DNP, $10^{-2}$ M; 4) DNAN, $10^{-2}$ M; 5) rinsed DNAN, $10^{-2}$ M)

It was assumed that, in order to manifest its action, dinitrophenetole hydrolyzes to afford the toxic dinitrophenol [8]. The two dinitrophenyl ethers (DNAN and DNF) in Table 2 behaved almost similarly, although DNAN had a rather more powerful effect on wheat germination than DNF had (Figure 2). We expected that DNF to be more toxic, being hydrolyzed to 2,4-DNP, as previously showed [8]. However, the presence of depleted ether was not confirmed after thin layer chromatography separation and specific color reaction (in the presence of iron chloride for 2,4-DNP identification).

![Figure 2](Figure 2. The inhibitory effect of 2,4-dinitrophenetol (DNF), 2,4-dinitrophenol (DNP),

<table>
<thead>
<tr>
<th>Treatment *)</th>
<th>G.R. **)</th>
<th>Number of plantlets in a lot (N)</th>
<th>Total height of the plantlets in a lot (H, cm)</th>
<th>Plantlets size (S, cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNG, $1x10^{-4}$ M</td>
<td>94%</td>
<td>44</td>
<td>$572.2\pm5.4$</td>
<td>$13.0\pm0.4$</td>
</tr>
<tr>
<td>DNG, $5x10^{-4}$ M</td>
<td>96%</td>
<td>44</td>
<td>$575.7\pm7.7$</td>
<td>$13.1\pm0.3$</td>
</tr>
<tr>
<td>DNG, $1x10^{-3}$ M</td>
<td>94%</td>
<td>41</td>
<td>$480.7\pm8.4$</td>
<td>$11.7\pm0.7$</td>
</tr>
<tr>
<td>Blank, H$_2$O</td>
<td>93%</td>
<td>41</td>
<td>$550.1\pm23.1$</td>
<td>$13.3\pm0.3$</td>
</tr>
<tr>
<td>D (Tukey test)</td>
<td>3</td>
<td>4</td>
<td>76.8</td>
<td>0.9</td>
</tr>
</tbody>
</table>

*) Average of three independent values.

**) Germination rate.

Table 1. The biological effect of 3-(2,4-dinitrophenoxy)propane-1,2-diol (DNG) on wheat germination and the growth of the resulted plantlets.

Indeed, all the experiments carried out with dinitrophenyl ethers in our laboratory showed that they act without hydrolysis. It was assumed that 2,4-DNP dissociates releasing protons, which penetrate the mitochondrion, replacing to one translocated from the mitochondrion into the cytosol, preventing thus the formation of ATP [3-5]. Thus, the energy that the other enzymes in the chain use to accumulate protons in the intermembrane space is recuperated. This energy is necessary for the ADP phosphorylation reaction with the mineral phosphate, in the presence of Mg ions, the reaction being endothermic and requires more than 31 kJ/mol. Dinitrophenols disrupt the H⁺ gradient reducing ATP synthesis. However, dinitrophenols are uncoupling agents due to their free hydroxyl group. Nevertheless, DNAN, DNF, DNG and other uncoupling agents have no free –OH groups to determine an H⁺ gradient through the biological membranes. Therefore, we searched for an alternative mechanism of biological activity for all dinitrophenyl derivatives. The biostructural theory by Eugen Macovschi [17,18] considers that dinitrophenols break down the biostructure similar to electric stimulation. However, this theory did not explain the molecular mechanism of energy release from the cell due to the dinitrophenols. On the other hand, DNP activity is associated with a pH change, as the chemiosmotic hypothesis considers. Therefore, the toxic effect of the investigated substances has to be explained by another mechanism and cannot be attributed solely to the proton translocation through membranes. We explained that the pH modification could be a secondary phenomenon associated with electron transition from the ground state to the excited one.

Table 2. The inhibitory effect of 2,4-dinitrophenol (DNP), 2,4-dinitrophenetol (DNF), 2,4-dinitroanisole (DNAN) on wheat germination and the growth of the resulted plantlets.

<table>
<thead>
<tr>
<th>Treatment 1)</th>
<th>G.R. 3)</th>
<th>N 4)</th>
<th>H 5) (cm)</th>
<th>S 6) (cm)</th>
<th>SM 7) (mg)</th>
<th>sm 7) (mg)</th>
<th>RM 8) (mg)</th>
<th>rm 8) (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, H₂O</td>
<td>83%</td>
<td>30</td>
<td>109.3±17.1</td>
<td>3.6±0.3</td>
<td>436±75</td>
<td>14.4±5.6</td>
<td>838±114</td>
<td>27.5±1.3</td>
</tr>
<tr>
<td>DNP, 3x10⁻³ M</td>
<td>0%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DNF, 3x10⁻³ M</td>
<td>78%</td>
<td>26</td>
<td>59.5±9.6</td>
<td>2.3±0.1</td>
<td>138±44</td>
<td>5.7±0.9</td>
<td>484±82</td>
<td>18.7±1.0</td>
</tr>
<tr>
<td>DNA, 3x10⁻³ M</td>
<td>85%</td>
<td>31</td>
<td>42.8±0.8</td>
<td>1.30±0.2</td>
<td>200±47</td>
<td>6.6±0.9</td>
<td>330±34</td>
<td>12.6±0.8</td>
</tr>
<tr>
<td>D (Tukey test)</td>
<td>8%</td>
<td>14</td>
<td>77.7</td>
<td>0.5</td>
<td>52</td>
<td>0.9</td>
<td>74</td>
<td>3.2</td>
</tr>
</tbody>
</table>

1) Average of three independent values; 2) Germination rate; 3) Number of plantlets in a lot; 4) Total height of the plantlets in a lot; 5) Plantlets size; 6) Plantlets mass in a lot; 7) Average mass of plantlets; 8) Roots mass in a lot; 9) Average mass of rootlets

In the electron transport chain, NADH + H⁺ containing 52.52 kcal/mol is used to produce the three moles of ATP (17.51 kcal for each mol of ATP), whereas FADH₂ with only 35.94 kcal/mol, will produce two moles of ATP. The hydrolysis of phosphoenolpyruvate, which is involved in ATP formation under anaerobic conditions, is accompanied by a standard free-energy change of 14.77 kcal/mol. Therefore, if the ATP formation is a radiation-associated process, then an energy quantum corresponding to 14.8-17.5 kcal/mol would be necessary for an ATP mole, which corresponds to an infrared radiation from 5169 cm⁻¹ to 6111 cm⁻¹.

Both dinitrophenols and dinitrophenyl ethers absorb mostly in the near infrared, while compounds without uncoupling activity e.g. 2,4-dinitrobenzoic acid or 4-nitrophenol had no such optical properties (Figure 3). The spectra of uncoupling agents, such as dinitrophenols and dinitrophenyl ethers displayed a high absorption in the wavenumber region around 6000 cm⁻¹. Therefore, the toxicity mechanism of dinitrophenyl ethers has to be discussed in direct...
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2,4-Dinitrophenol, 2,4-dinitronaphthol, 4,6-dinitro-o-cresol, 2,4-dinitrophenetole, 2,4-dinitroanisole, and to a lesser extent picric acid, produced an increase in bile flow and a rise in body temperature in the anaesthetized dog [8]. Nevertheless, dinitroresorcinol, 2,4-dinitrobenzoic acid or nitrophenols had no such uncoupling activity. Now, we are able to explain that by the following hypothesis: cytochrom c oxidase or other excited compounds in the respiratory chain transfer directly energy to ATP-synthase. The conformation of ATP-synthase protein molecules changes accordingly and releases a bound ATP molecule. Dinitrophenols and other uncoupling agents absorb the radiation with an energy of about 14-17 kcal/mole and dissipate it as heat effect. Dinitroresorcinol or dinitrobenzoic acid, but dinitrophenyl ethers, do not absorb this energy, and therefore are not uncoupling agents.

**Figure 3.** FT-IR spectra of some dinitrophenyl derivatives with uncoupling activity (left) versus those of phenylalanine and dinitrophenyl derivatives without uncoupling activity (right). The transmittance of uncoupling agents decreased drastically above 5000-6000 cm$^{-1}$. 

Thus, all of them have a significant absorbance at about 6000 cm$^{-1}$ in IR, corresponding to $\Delta G$ of ATP formation.
Overall, these findings provide support for further investigation of the toxicity mechanisms of dinitrophenols and related compounds.

Conclusion

Wheat seed germination experiments revealed their sensitivity to low concentrations of dinitrophenols and dinitrophenyl ethers. Both dinitrophenols and dinitrophenyl ethers inhibit wheat germination, acting most probably as uncoupling agents. The solubility seems to play an important role in dinitrophenol toxicity. Contrary to the findings in the literature, we found that dinitrophenetole and dinitroanisole act as ethers, without hydrolysis to the corresponding dinitrophenol. The mechanism of toxicity is probably related to acido-basic properties of dinitrophenyl derivatives, with their solubility and spectral properties. The strong absorption of these compounds at about 6000 cm\(^{-1}\) in the near infrared, at energies approximately equal to those for the formation of the ATP molecule suggest a direct radiation-based energy transfer. Besides, the excitation of organic molecules is connected with changes in their acidity and, therefore, a proton pump cannot explain ATP production. Further investigation with new theoretical instruments should be undertaken to clarify these obscure aspects of dinitrophenols biological activity.

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References

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