Effect of 4,6-Dinitro-o-sec-butylphenol on Phosphorus Accumulation and Incorporation in Tomato Leaf Disks

T. Wojtaszek, J. H. Cherry, and G. F. Warren
Horticulture Department, Purdue University, Lafayette, Indiana

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Summary. The accumulation and incorporation of externally applied P\textsuperscript{32} into ATP and the effect of 4,6-dinitro-o-sec-butylphenol (DNBP) on these processes was studied using tomato (Lycopersicon esculentum Mill.) leaf disks.

\textsuperscript{32}P was, in most part, actively accumulated into leaf disks with time and was incorporated into ATP and other organic phosphates. DNBP inhibited both P\textsuperscript{32} accumulation and ATP generation. The amount of inhibition increased with time of incubation.

It is concluded that P\textsuperscript{32} accumulation is related to ATP generation. Even though DNBP greatly inhibits phosphorus accumulation, there is little or no effect on its retention.

DNBP has the ability to uncouple oxidative phosphorylation. Therefore, it is assumed that its inhibitory effect on phosphate accumulation and generation of high-energy phosphorus esters is related to its inhibition of oxidative phosphorylation.

A method is described which appears to be satisfactory to determine the relative amounts of ATP and ADP in leaf disks labeled with P\textsuperscript{32}.

2,4-Dinitrophenol (DNP) and other substituted phenols inhibit metabolic processes coupled to the electron transport system which lead to the formation of high-energy metabolites. The flow of electrons along the respiratory chain results in high-energy intermediates and finally leads to ATP formation (6, 7). It is generally recognized that ion uptake and accumulation is an energy-requiring process (7, 8, 11) which is inhibited by DNP (2, 10).

A compound related to DNP, 4,6-dinitro-o-sec-butylphenol (DNBP) is used as a commercial herbicide. Tomato plants are sensitive to DNBP when applied to foliage (3). Even a low dose at 28\textsuperscript{°} inhibits growth by approximately 30\%. Little is known about the differences in the mode of action of DNP and DNBP. Also very little is known about the effect of dinitrophenols on mineral uptake and distribution of ions in plants (1). It has been generally agreed that the mode of action of all dinitrophenol compounds is the same as that of DNP (4, 13) including some of the chloro-substituted phenols (14). As a rule the toxicity of DNBP is noted to be higher than that of DNP, but on certain plant species, and under certain conditions DNBP is less active than DNP at the same concentrations (4, 5).

Since it has been established that mitochondria are centers of oxidative phosphorylation, most work dealing with the action of DNP has been carried out using mitochondrial preparations. For the studies reported in this paper, leaf disks were used instead of mitochondria because the effects of DNBP on leaf disks should more closely resemble those on intact leaves. Studies on the mechanism of action of this compound were directed toward the effect on uptake, accumulation and incorporation of external P\textsuperscript{32} into ATP by tomato leaf disks. If DNBP acts in the leaf disks by uncoupling oxidative phosphorylation an effect on ATP generation should be noted. On the other hand, DNBP is recognized as a selective herbicide. In agreement with this data it may be speculated that the selectivity of DNBP depends upon the ability of the plants to synthesize ATP by some other mechanism or the ability to store high-energy phosphates. Hackett (6) concluded that practically nothing is known about the storage of energy-rich phosphates in higher plants.

In this initial part of our studies it was necessary to find a satisfactory technique to estimate the relative amount of ATP in leaf tissue. In this paper it will be shown that the application of DNBP to leaf disks inhibits the level of ATP by either uncoupling oxidative phosphorylation or by inhibiting some other energy generating process required for ion uptake.

Materials and Methods

\textbf{Plant Material}. Tomato plants (Lycopersicon esculentum Mill.) used in the following studies were grown in the greenhouse in sterilized soil in plastic pots. Depending on the experiment, the fifth and sixth leaves of 6- to 7-week-old plants or the fourth leaf of 4- to 5-week-old plants were used.
**Determination of Phosphate Accumulated in Leaf Disks during Incubation.** About 100 mg samples of leaf disks, 6 mm in diameter, from 6- to 7-week-old plants, were washed with distilled water and were placed in manometric vessels with 2.5 ml of $10^{-4} \text{M}$ ammonium citrate buffer, pH 6.0, containing KH$_2$P$_{32}$O$_4$ (about 0.02 µc P$_{32}$ per ml). DNBP as the alkanolamine salt (supplied by Dow Chemical Company, Midland, Michigan) was applied to the leaf disks either before, at the same time, or after incubation with P$_{32}$. The samples were incubated in the dark at 28° in an atmosphere of air. The frequency of shaking was 120 cycles per minute. At the termination of the incubation period the incubation solution was removed and the disks washed and incubated with cold phosphate buffer at pH 6.7 in an ice bath for 30 minutes to exchange the adsorbed P$_{32}$. The leaf disks were washed 3 times with water, removed and dried. The amount of P$_{32}$ accumulated in dried leaf disks was determined directly with a gas-flow counter.

**Determination of ATP Formed in Leaf Disks during Incubation.** The same method of incubation was used as described above with the exception that the amount of P$_{32}$ in the incubation medium was 0.5 µc per ml. Samples of leaf disks from 4- to 5-week-old plants weighing about 250 mg were incubated for different time periods (depending on the experiment) and were homogenized with 1.0 ml of 0.2 M HClO$_4$. The homogenate was centrifuged at 2500 × g for 10 minutes to remove cell debris. The supernatant fraction was neutralized to pH 7.0 with KOH. The insoluble KClO$_4$ was removed by centrifuging at 3500 × g for 10 minutes. All centrifugations were done in a refrigerated centrifuge at 4°. The clear supernatant solution was transferred into 4 ml beakers, adjusted to pH 5.0 and evaporated to dryness at room temperature using a fan. Additional tests using labeled ATP showed that during a 45- to 60-minute drying period the amount of ATP did not change significantly. Following the drying step the samples were redissolved in 0.25 ml of water and spotted with a capillary tube on Whatman No. 1 chromatography paper. The paper chromatograms were developed by ascending chromatography at 25° with the following solvent system (9): 0.1 M sodium phosphate, pH 6.8: (NH$_4$)$_2$SO$_4$: n-propanol (100: 60: 2, v/v/w/v). The position of ADP and ATP on the chromatograms of the extract was determined by scanning for radioactivity and their $R_f$ values compared to those of the known compounds which were located under ultraviolet light. The position of the ATP spot was checked further by adding ATP-C$^{14}$ to an unlabeled extract of tomato tissue incubated under the same conditions. A third verification of the ATP spot was obtained by eluting the area of the chromatogram containing the suspected ATP and rechromatographing with known ATP in another solvent system (9). This solvent was composed of isobutyric acid: concentrated NH$_4$OH: H$_2$O (66: 1: 33, v/v/v). In all cases the position of the unknown corresponded to the known ATP.

**Results**

**Accumulation of Phosphate in Leaf Disks.** Leaf disks rapidly accumulated P$_{32}$ (fig 1) over a 3-hour period; the amount accumulated increased with time. Since ion uptake is an energy-requiring process, DNBP should reduce P$_{32}$ accumulation by disrupting oxidative phosphorylation or some part of it. Figure 2 shows that a marked inhibition of P$_{32}$ uptake occurs when leaf disks are incubated with DNBP. The lowest concentration of DNBP (1.04 × $10^{-4}$ M) for 3 hours of incubation inhibited P$_{32}$ accumulation by 20% while the highest concentration (8.35 × $10^{-6}$ M) for the same incubation period resulted in about 90% inhibition. Also in figure 2 it can be seen that the time to reach 50% inhibition (T$_{50}$) decreased with increasing DNBP concentration.
Concomitantly with the estimation of P\textsuperscript{32} accumulation measurements on the rate of respiration show that at concentrations of 2.08 × 10\textsuperscript{-6} M to 4.17 × 10\textsuperscript{-5} M DNBP, O\textsubscript{2} uptake is stimulated (fig 3). Greater stimulation was observed at lower rates of DNBP.

![Graph](image)

**Fig. 3.** The effect of DNBP on the respiration rate of tomato leaf disks. Leaf disks and conditions of incubation are described in figure 1. The data represent an average of 2 experiments in duplicates. Control values (as µl O\textsubscript{2}/g fr wt) for 30, 60, 90, 120 and 150 minutes of incubation were 77, 160, 233, 307 and 360, respectively.

The next experiment was carried out to test the effect of DNBP on P\textsuperscript{32} accumulation when applied before incubation with P\textsuperscript{32} (see illustration at the bottom of the graphs in fig 4). For this purpose leaf disks were incubated for 120 minutes without (treatment 1) and with (treatments 2 and 3) 2.08 × 10\textsuperscript{-5} M DNBP. Afterwards, another 120-minute period of incubation was made with P\textsuperscript{32}. The results of this experiment demonstrate that DNBP when applied before incubation with P\textsuperscript{32} gave about 65% inhibition of P\textsuperscript{32} accumulation (fig 4, column 2). Little difference was observed between samples treated with DNBP only during the first period of incubation (column 2) and the samples which were treated during both the first and the second period (column 3).

In order to determine whether DNBP enhances leakage of accumulated P\textsuperscript{32} labeled phosphates, leaf disks were preincubated with P\textsuperscript{32} for 2 hours followed by incubation in DNBP for 2 hours (treatment 3, fig 5). Treatments 1 and 2 of this experiment were controls (no DNBP). The data presented in figure 5 indicate that during the second period of incubation, either with or without DNBP, there was a small loss of P\textsuperscript{32}. However, this loss or leakage was only slightly larger when the tissue was incubated with DNBP (treatment 3). Therefore, DNBP must inhibit P\textsuperscript{32} accumulation but apparently does not affect retention of P\textsuperscript{32} phosphates (fig 4, columns 2 and 3; fig 5, columns 4 to 6).

**Incorporation of Phosphate into ATP** The data represented in figure 6 show a typical separation of the P\textsuperscript{32} labeled compounds accumulated during 2 hours (6B) and 3 hours (6C) of incubation, as well as the effect of DNBP on the incorporation of P\textsuperscript{32} into those substances (6D and 6E). Most of the P\textsuperscript{32} was located in 2 major areas having R\textsubscript{f} values of 0.93 (peak I) and 0.68 (peak II). Additional experiments indicate that peak I is P\textsubscript{1} and at least some sugar phosphates, Addition of P\textsuperscript{32} (as KH\textsubscript{2}P\textsuperscript{32}O\textsubscript{4} or fructose-C\textsuperscript{14} 6-phosphate after incubation and before homogenizing gave peaks with R\textsubscript{f} values between 0.93 and 0.96. Since peak III (R\textsubscript{f} 0.37) was identified as ATP and peak IV (R\textsubscript{f} 0.30) as ADP it may be assumed that peak II contains other esters, possibly phytic acid or sugar phosphates. Several attempts by the authors to positively identify peak II were unsuccessful. Roux and Lesanis (12) showed that the amount of organic phosphates in tomato plants, as percent of total phosphate, ranges from 38 to 53%. They also found that 29 to 37% of the organic phosphate is composed of phytin plus esters.

From figure 6D and 6E it is evident that DNBP caused a great inhibition of P\textsuperscript{32} incorporation into peaks II, III and IV as compared to nontreated tissue. The only activity present was located in the area which corresponds to P\textsubscript{1}.
Fig. 6. The effect of DNBP on the formation of ATP and other $\text{P}^{32}$ labeled phosphates in tomato leaf disks. Six hundred mg samples of leaf disks from 4- to 5-week-old plants were incubated in Warburg vessels with 2.5 ml ammonium citrate buffer at pH 6.0, containing 0.5 $\mu$C $\text{P}^{32}$ for experiment presented in 6B and 6D (2 hrs) and 6C and 6E (3 hrs). 6B and 6C are untreated samples and 6D and 6E were treated with $1.04 \times 10^{-6}$ M DNBP. The acid soluble phosphates were chromatographed on paper (see Methods). The data presented are profiles of the radioactivity obtained by scanning paper strips with a windowless autoscan.
Discussion

The results indicate that most of the P\(^{32}\) in the leaf disks is accumulated by an active process. Results of supplementary experiments showed that leaf disks incubated in the dark under anaerobic conditions (5% CO\(_2\) and 95% N\(_2\)) accumulated very low amounts of P\(^{32}\) and none was incorporated into organic phosphates. Leaf disks accumulated approximately the same amount of P\(^{32}\) aerobically in the presence of DNBP as tissue incubated in CO\(_2\)-N\(_2\) without DNBP. Under the first of these conditions, metabolic processes were blocked by lack of O\(_2\), while in the second, P\(^{32}\) incorporation was impaired because of the uncoupling effect of DNBP. This compound, like DNP, has the ability to uncouple oxidative phosphorylation. It stimulates O\(_2\) uptake and greatly inhibits phosphate accumulation in leaf disks at 2.08 \(\times\) 10\(^{-5}\) M.

Since ion uptake is related to oxidative phosphorylation (11, 12) DNBP would be expected to inhibit both of these processes. In the work reported in this paper it was found that the inhibition of phosphorus accumulation by DNBP can not be reversed by removing the herbicide from the incubation medium, washing the tissue, and subsequently incubating without DNBP but with P\(^{32}\) (treatment 2 in fig 4). The results presented in figure 4 show no marked difference between samples treated with DNBP prior to incubation with P\(^{32}\) (treatment 2) and the samples which were treated both before and at the time of incubation with P\(^{32}\) (treatment 3). The demonstration that DNBP applied before incubation with P\(^{32}\) resulted in inhibition of P\(^{32}\) accumulation, although the tissue was washed, suggests that this herbicide lowers the capacity of the tissue to absorb ions.

Nonetheless, some P\(^{32}\) was absorbed both under anaerobic conditions and in the presence of DNBP. This amount could be accumulated passively by diffusion and exchange (11). Even at a concentration of 8.35 \(\times\) 10\(^{-5}\) M DNBP some P\(^{32}\) was accumulated by leaf disks.

Literature Cited