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# The Effect of Arsenate and Other Inhibitors on Early Events during the Germination of Lettuce Seeds (*Lactuca sativa* L.)

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## ABSTRACT

**The effect of arsenate, arsenite, 2,4-dinitrophenol, and anaerobiosis on early events in seed germination was investigated using both intact and punched seeds of lettuce (*Lactuca sativa* L.). It was found that punching the seed removes penetration barriers to the entrance of inhibitors without an undue loss of germination or light responses. The kinetics of the action of germination inhibitors were established by 2-hour pulse experiments. Arsenate and 2 , 4-dinitrophenol have very different kinetics. The inhibition of germination in punched seeds by arsenate given in conjunction with phosphate compared with the lack of inhibition of arsenate plus phosphate on the growing seedling, suggest a distinct metabolic change in the germinating embryo at some time between the onset of germination and subsequent seedling growth.**

The involvement of phytochrome in the control of many light-mediated processes in plants is well established (4, 7). The primary events of light absorption by phytochrome and its resulting spectral and conformational changes are fairly well documented (12, 19). There is still a considerable lack of information concerning the events between the interaction of phytochrome with light and the final biological event (1).

The Grand Rapids variety of lettuce seeds provides an excellent tool for the investigation of some of the biochemical events occurring between phytochrome induction and seed germination. Of obvious concern is the utilization of the energy stored in the seed in the form of reserve materials. Since an increase in respiration is one of the first observable events that occurs after contact of the seed with water (14), this would seem to be a logical mechanism to investigate. Therefore, we carried out a series of experiments using known inhibitors of phosphorylation in order to elucidate the kinetics of energy utilization very early in seed germination.

Our experiments involved adding metabolic inhibitors to the seeds in "pulses" spaced over 2-hr increments from zero time to hr 12 of germination. Our results show that there are distinct changes in the effects of the inhibitors with increasing time of germination.

## MATERIALS AND METHODS

**Seeds.** Seeds of *Lactuca sativa* L. var. Grand Rapids were used. Seeds were purchased from Buckerfield's Ltd. of Vancouver, Canada and were batch L 185, 1969 harvest. Seeds were stored in glass jars over Drierite ($CaSO_4$) in a freezer at −18 C. These seeds exhibited an average germination of 49% in the dark, 90% after 5 min of red light (660 nm), and 12% after 5 min of far red light (>700 nm).

Seeds were germinated in 6-cm glass Petri dishes on two circles of Whatman No. 1 filter paper and in 1.5 ml of liquid. For all experimental points, triplicate dishes were used, each contained 33 seeds, thus giving a total of 99 seeds for any single point in a particular experiment.

In experiments where punched seeds were used, seeds were punched with a No. 0 insect pin in the midsection through the cotyledons. Punching in this area gave minimal inhibition of germination and only slightly affected the response to red and far red light (Table I).

Seeds were imbibed in the dark in liquid for 1 hr prior to light treatment, at which time either 5 min of red or 5 min of far red light was given.

Germination was determined at the end of 24 hr after light irradiation. The criteria for germination was any visible extrusion of the radical tip beyond the outer seed coat when viewed at 60 magnification under a dissecting microscope. No quantitative consideration was given to the sometimes obvious differences in radicle length at the end of 24 hr.

Seedling growth was determined on seeds germinated as described above and then kept in the dark for 4 to 5 days.

**Light Sources.** The red light source was isolated from four 150W incandescent spot lamps (Sylvania, Cool-Lux, 150 PAR 38/2 SP). Light from these lamps was passed through a filter consisting of 4 cm of 1% $CuCl_2$ in a Plexiglas container and then through a sheet of red plastic filter (Red 590, Percival Refrigeration Co.). This combination gave a spectral distribution with an emission peak at 645 nm and a half-band width of 50 nm.

The far red light source was also isolated from four 150W incandescent lamps as above. In this case, the light was passed through 12 cm of flowing water in a Plexiglas container and then through a sheet of far red plastic filter (FR-700, Percival Refrigeration Co.). This system isolated wavelengths greater than 700 nm.

The irradiation of all seeds was done in boxes constructed of opaque black Plexiglas (30 cm × 30 cm × 15 cm high). These boxes were lined with aluminum foil to increase internal reflection, and the appropriate filter systems were fitted to the top of the box. Incident energies at the bottom center of the boxes were $4.7 \times 10^3$ ergs $cm^{-2}$ $sec^{-1}$ for red light and $4.4 \times 10^4$ ergs $cm^{-2}$ $sec^{-1}$ for far red light. Measurements were made with a YSI radiometer (Yellow Springs Instrument Co., Yellow Springs, Ohio, Model 65).

Green safelights were green fluorescent tubes (General Electric, 20 w, F20T12-G) wrapped with layers of blue, amber, and green plastic sheets according to the method of Klein (11). The spectral emission of all light sources was scanned with an Isco spectroradiometer (Model SRR). However, since the sen-

sitivity of this instrument was set to keep the largest peak of the emission spectrum on scale, when using it to scan the green safelights, there could have been enough energy present at wavelengths recording near zero on the scale to affect the lettuce seeds. Therefore, the green safelights were bioassayed. This was done by imbibing dishes of intact and punched seeds in water for 1 hr, exposing them to 5 min of far red light, and then placing them at working distance (2 m) from the safelights for 1 hr and then in the dark. These were then scored for germination at 24 hr and were compared to similar control dishes not exposed to the green lights. Under these conditions, no reversal of the far red inhibition of germination was noted (Table I).

**Experiments with Nitrogen Gas.** Nitrogen gas from a tank was passed through a gas scrubber (Fisher-Milligan, Fisher Scientific Co.) containing 10 g of pyrogallol in 200 ml of 22 M KOH (3) and then through a similar scrubber filled with distilled water to which a pH indicator (Bromothymol Blue) and 1 drop of sulfuric acid was added. Nitrogen gas thus scrubbed was passed into a black plastic box (16 cm × 16 cm × 16 cm high), and the flow rate was adjusted such that the box was flushed in 2 min.

Water used in making up the pyrogallol was deoxygenated by repeated flushing with nitrogen and evacuation by a vacuum pump. Dishes of seeds used in these experiments were also flooded and aspirated twice with 4-ml aliquots of deoxygenated water to reduce the amount of dissolved oxygen in contact with the seeds.

**Inhibitor Pulse Experiments.** All inhibitors used were buffered to pH 6.5 with tris-malate buffer, 20 mM. In 24- and 48-hr experiments, the inhibitors were left in contact with the germinating seeds. In 2-hr pulse experiments, inhibitors were added to the Petri dishes of seeds in the following regime. The dishes were flooded with 4 ml of the inhibitor, shaken for 1 min, and then the excess inhibitor was aspirated off. Aspiration was facilitated by the use of a plastic tube (7 mm diameter) over which was stretched a piece of fine mesh nylon cloth. At the end of the 2-hr pulse period, the inhibitor was removed by flooding the dish with water and aspirating off the excess water. Flooding was repeated once more, and the seeds were then washed into a new dish containing two new circles of filter paper, and again excess water was aspirated off. Dishes of seeds were stored in a black plastic box until germination was scored. All operations were carried out at room temperature under green safelights.

Table I. *A Comparison of the Photoresponsiveness of Intact and Punched Seeds*

These data are a compilation of data from 13 separate experiments. The mean is the mean of the number of seeds that germinated in a dish of 33 seeds ± the standard error. The total number of dishes of 33 seeds is given by *n*. The data for the green safelights are from a single experiment and therefore the mean values vary somewhat from the other data due to small sample size.

| Treatment | Punched Seeds | | | Intact Seeds | | |
|---|---|---|---|---|---|---|
| | Mean | Germination | *n* | Mean | Germination | *n* |
| | | % | | | % | |
| Red, 5 min | 24.4 ± 1.07 | 73.9 | 30 | 29.81 ± .40 | 90.3 | 36 |
| Far red, 5 min | 12.84 ± .66 | 38.9 | 33 | 4.17 ± .65 | 12.6 | 12 |
| Dark, 65 min | 16.97 ± .95 | 51.4 | 33 | 16.08 ± 1.17 | 48.7 | 12 |
| Far red, 5 min (green 1 hr at 2 m) | 4.67 ± .88 | 14.1 | 3 | 4.67 ± 1.1 | 14.1 | 3 |
| Far red, 5 min (0 hr green) | 11 ± 1.2 | 33.33 | 3 | 4.5 ± .5 | 13.6 | 3 |

Table II. *Effect of Various Drugs on the Germination of Intact and Punched Seeds*

All drugs are buffered in 20 mM tris-malate, pH 6.5. The drugs are left in contact with the seeds for the full 48 hr. All concentrations are 20 mM, unless stated otherwise. Seeds were given 5 min red light after imbibition for 1 hr.

| Treatment | 24 Hr Germination | | 48 Hr Germination | |
|---|---|---|---|---|
| | Intact | Punched | Intact | Punched |
| | % | | | |
| $H_2O$ | 90.9 ± 0 | 92.9 ± 1.0 | 96.0 ± 2.7 | 98.0 ± 1.0 |
| Buffer | 91.9 ± 2.7 | 88.9 ± 4.0 | 97.0 ± 1.8 | 99.0 ± 1.0 |
| $Na_2HPO_4$ | 91.9 ± 2.7 | 69.7 ± 6.3 | 98.0 ± 1.0 | 92.9 ± 2.7 |
| $Na_2HPO_4$, 10 mM | 93.9 ± 3.5 | 84.8 ± 9.3 | 98.0 ± 2.0 | 98.0 ± 1.0 |
| $Na_2HAsO_4$ | 86.7 ± 1.0 | 0.0 | 93.9 ± 1.7 | 0.0 |
| $Na_2HAsO_4$, 0.5 mM | | 49.5 ± 8.3 | | 53.5 ± 4.4 |
| $Na_2HPO_4$, 1 mM + $Na_2HAsO_4$, 0.5 mM | 86.86 ± 2.0 | 31.31 ± 4.0 | 93.9 ± 3.0 | 33.3 ± 3.0 |
| $NaAsO_2$ | 0.0 | 0.0 | 0.0 | 0.0 |
| 2,4-Dinitrophenol | 0.0 | 0.0 | 0.0 | 0.0 |
| $MnCl_2$ | 92.9 ± 2.0 | 67.7 ± 2.7 | 97.0 ± 1.8 | 92.9 ± 2.7 |
| $ZnCl_2$ | 92.9 ± 4.4 | 74.7 ± 6.1 | 100 ± 0.0 | 91.9 ± 2.7 |
| $NaHSO_4$ | 90.9 ± 1.8 | 77.8 ± 6.6 | 98.0 ± 2.0 | 98.0 ± 2.0 |
| NaCl | 97.0 ± 1.8 | 77.8 ± 4.0 | 99.0 ± 1.0 | 94.9 ± 1.0 |

## RESULTS

**Intact and Punched Seeds.** Ikuma and Thimann (10) found that disruption of the integrity of the seed coat and endosperm resulted in full germination in either darkness or light. In our experiments, disrupting the seed coat by punching with a pin results in some loss of light response but clearly not a total loss (Table I). The data in Table I show that, in red light, punched seeds germinate about 80% as well as intact seeds. Punched seeds have about a 28% loss of far red inhibition of germination, and the values for germination in the dark are nearly equal. The primary effect of punching therefore is mainly a reduction of the far red inhibition of germination. Punching the seeds had no effect upon the subsequent growth of the seedling, as they grew as well as intact controls, the punch marks through the two cotyledons being quite evident.

**Effect of Inhibitors on Intact and Punched Seeds.** Experiments were carried out using intact and punched seeds in which various metabolic inhibitors were left in contact with the seeds for periods of 24 and 48 hr (Table II). For these experiments, it was essential that punched seeds be used. The intact seed evidently acts as a selective barrier to some substances; punching the seed removes this barrier. This is particularly evident in the case of arsenate and arsenite. The germination of intact seeds is affected only slightly by 20 mM arsenate, whereas punched seeds are completely inhibited at this concentration. The arsenate concentration must be reduced to 0.5 mM in order to achieve 50% inhibition of punched seeds. Arsenite at 20 mM gives complete inhibition of intact seeds as does also 2,4-dinitrophenol.

The specificity of the arsenate inhibition was checked by comparing it with 20 mM sulphate, phosphate, and sodium chloride. At the end of 24 hr, there was only slight inhibition of germination in punched seeds due to sulphate and sodium chloride; there was a much greater inhibition with 20 mM phosphate.

The inhibition of germination in punched seeds by 0.5 mM arsenate was not reversed when given in conjunction with 1 mM phosphate. However, the effects of arsenate and phosphate on seedling growth are more obvious. While 0.5 mM arsenate does not affect the germination of intact seeds it does affect sub-

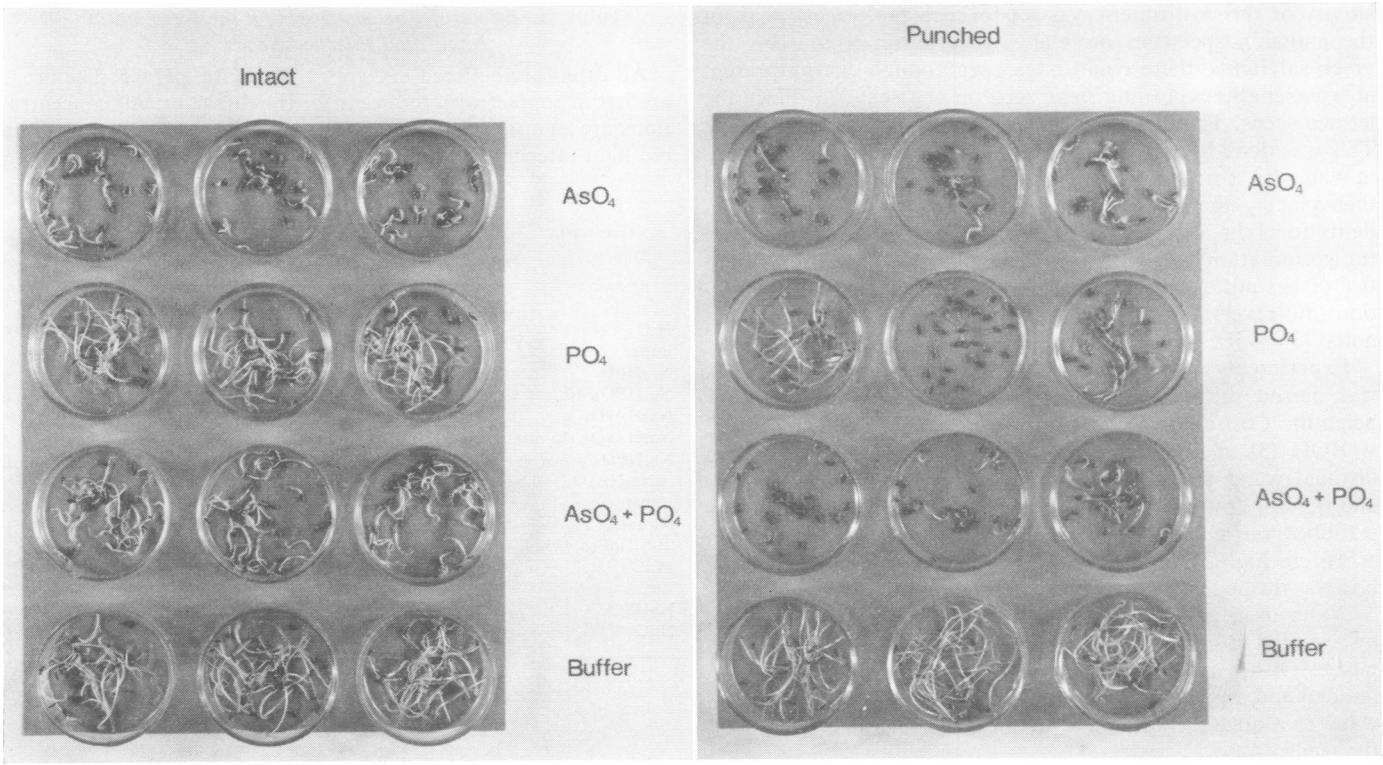


FIG. 1. Effect of 0.5 mM arsenate and 1 mM phosphate on the germination and seedling growth of intact and punched seeds. All solutions were in 20 mM tris-malate buffer, pH 6.5. Photographs were taken 115 hr postimbibition, filter papers were removed to increase photographic contrast.

sequent seedling growth of both intact and punched seeds (Fig. 1). Intact seeds left in contact with 0.5 mM arsenate show about 94% germination at the end of 48 hr but little or no subsequent seedling growth after 115 hr. There is a reversal of this arsenate inhibition of seedling growth when arsenate is given in the presence of phosphate. This could be explained on the basis that arsenate is acting as a competitive inhibitor of both oxidative and substrate phosphorylation, phosphate thus acting competitively to reverse the inhibition. Other explanations are also possible, but the data show that the intact germinating seed excludes substances such as arsenate and phosphate, but the growing seedling does not.

Since seeds require oxygen to germinate, oxidative phosphate metabolism may be involved in these early events (5). Phosphate is normally stored in seeds as phytin (inositol hexaphosphate) and is made available during seed germination by enzymatic cleavage by the enzyme phytase (13). Peers (16) reported that wheat phytase is completely inhibited *in vitro* by 10 mM $Zn^{2+}$ or $Mn^{2+}$. We therefore did experiments using intact and punched seeds in 20 mM zinc chloride and manganese chloride (Table II). The results using intact seeds show no effect of these ions on germination. With punched seeds, there is some inhibition of germination at 24 hr, but germination is equal to that of intact seeds by 48 hr. Seedling growth subsequent to germination is completely inhibited.

**Effect of Inhibitors in Pulse Experiments.** In order to try and identify the point in time at which various metabolic reactions occur, 2-hr pulse experiments were done. Inhibitors were added to punched seeds at 2-hr time intervals and then washed out. Concentrations of inhibitors were chosen that gave about 50% inhibition of germination, so that any subtle changes in germination response could be detected. Since the inhibitor is in contact with the germinating seed for only 2 hr, much higher concentrations were necessary to achieve 50% inhibition than those concentrations that gave 50% inhibition in 24- to 48-hr experiments.

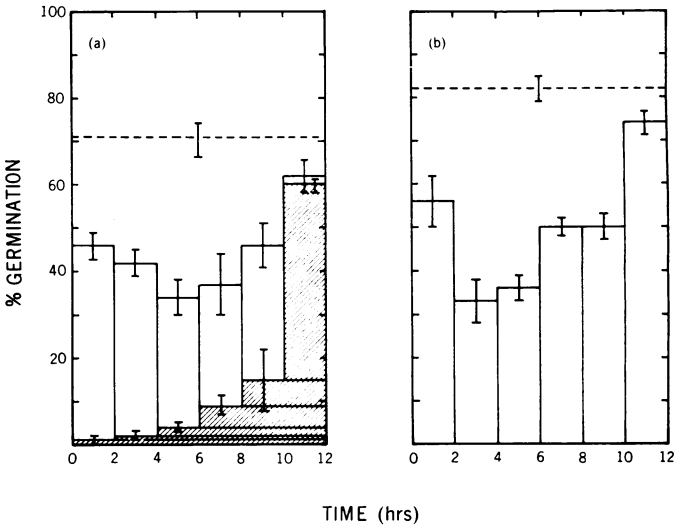


FIG. 2. Time course of the effect of 10 mM arsenate (a) and 2 mM arsenite (b) on the germination of punched seeds. The drugs were added in 2-hr pulses in 20 mM tris-malate buffer, pH 6.5 and then washed out at the end of the 2-hr period. The cross hatched values represent points in which the drug was left in contact with the seeds until hr 12 and then washed out. The dotted line represents buffer controls. Germination was scored at 24 hr. The data are the mean of four separate experiments representing a total of 12 dishes of 33 seeds for each time point. Range marks indicate standard error.

The results of these experiments using arsenate are shown in Figure 2a. In the 0- to 2-hr time period, arsenate is somewhat

inhibitory at the 10 mM concentration. The arsenate inhibition increases up to about the 4- to 6-hr time period, at which time it decreases until hr 12, when it is near the control level. Some experiments were carried out from hr 12 to hr 24, but there is no significant inhibition by 10 mM arsenate during these time periods.

In order to determine if the inhibitor is actually removed by washing at the end of the 2-hr pulse, experiments were done in which the inhibitor was not washed out at the end of the 2-hr period. These seeds were then washed at hr 12, and germination was scored at hr 24. These data are shown in Figure 2a as the cross hatched bars. The difference between the two sets of data represent some combination of the degree to which the drug can be washed out and the reversibility of the inhibition.

Since arsenite was so much more effective in inhibiting germination in the 24- to 48-hr experiments, its effects were compared with arsenate in a 2-hr pulse experiment. The metabolic effects of arsenate and arsenite are quite different. Arsenate uncouples both oxidative and substrate level phosphorylation (21), while arsenite is reported to be an inhibitor of SH groups, especially adjacent SH groups on a protein (22). Because of these different modes of action, we predicted different effects of the two drugs in our 2-hr pulse experiments. The results for arsenite are shown in Figure 2b. The arsenite data look much like the arsenate, there being less sensitivity at 0 to 2 hr and 10 to 12 hr than at times in between.

Experiments were conducted using other known inhibitors of oxidative phosphorylation (2,4-dinitrophenol and anaerobiosis) and arsenate in conjunction with nitrate. Nitrate was chosen because it has been shown to promote seed germination (8); it is a member of the same atomic group as phosphorus and arsenic, and arsenite may act as a specific anion competitor, depressing nitrite oxidation by some organisms (2). The results of these experiments are shown in Figures 3 and 4.

Both 2,4-dinitrophenol and nitrogen gas show a maximal effect on germination in the 0- to 2-hr time period with a diminishing effect as germination continues (Fig. 3). The experiments with arsenate in the presence of nitrate and arsenite in the presence of nitrite were only done from 4 to 8 hr after imbibition, the time period of maximal sensitivity to the drugs (Fig. 4).

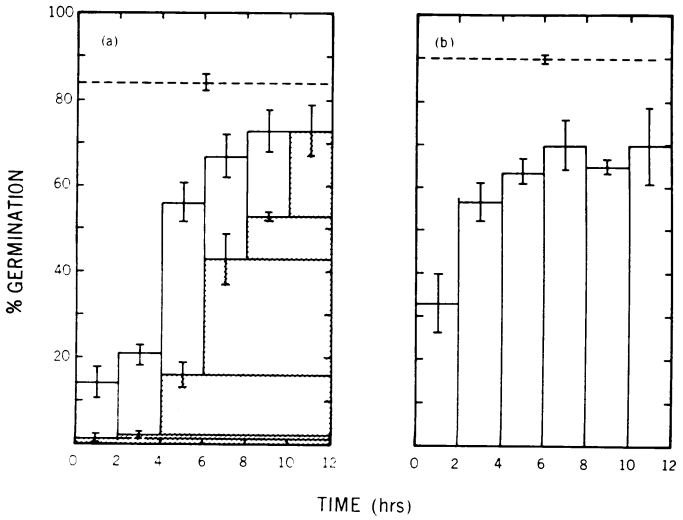


FIG. 3. Time course of the effect of 0.5 mM 2,4-dinitrophenol (a) and nitrogen gas (b) on the germination of punched seeds. 2,4-dinitrophenol was added in 2-hr pulses in 20 mM tris-malate buffer, pH 6.5, and then washed out at the end of the 2-hr period. The cross hatched values represent data in which the drug was left in contact with the seeds until hr 12 and then washed out. The dotted line represents buffer controls. Germination was scored at 24 hr. Range marks indicate standard error.

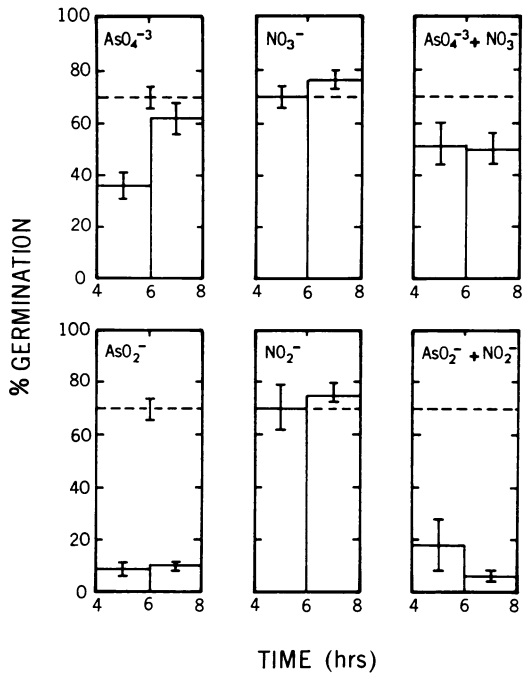


FIG. 4. Effect of 10 mM arsenate, 10 mM nitrate, 5 mM arsenite, and 10 mM nitrite on punched seeds during the period of 4 to 8 hr postimbibition. The drugs were added in 2-hr pulses in 20 mM tris-malate buffer, pH 6.5, and then washed out at the end of the 2-hr period. The dotted line represents buffer controls. Germination was scored at 24 hr. The data are the mean of three separate experiments representing a total of nine dishes of 33 seeds for each time point. Range marks indicate standard error.

## DISCUSSION

It is evident that with the Grand Rapids variety of lettuce seeds, germination should be distinguished as two separate events: (*a*) the breaking of seed dormancy by imbibition of water and extrusion of the radicle, (*b*) subsequent seedling growth. Haber and Luippold (6) have shown that cell division and cell elongation are distinct and separate events in Grand Rapids seeds. Ikuma and Thimann (9) have described the germination process as consisting of four separate phases culminating when the radicle breaks through the surrounding seed coat. Other work shows that respiration increases markedly at about 14 to 18 hr postimbibition, the time of the onset of seedling growth (17).

As Haber and Luippold (6) point out and our work supports, there are very different metabolic processes occurring between germination and subsequent seedling growth. Since $Zn^{2+}$ and $Mn^{2+}$ totally inhibit seedling growth, these ions must penetrate the seedling and affect processes sensitive to these ions. In intact germinating seeds, $Zn^{2+}$ and $Mn^{2+}$ are either excluded from the seed, as arsenate is, or then penetrate the seed but have no effect upon germination. Since the punched seeds also germinate in the presence of $Zn^{2+}$ and $Mn^{2+}$, we conclude that either germination is not sensitive to these ions, or they do not reach the germinating embryo. This question is difficult to resolve from the present data, however, since arsenite inhibits both intact and punched seeds and arsenate inhibits only punched seeds, it seems that exclusion by the germinating embryo is not the mechanism.

It is clear from our work and that of others (9) that oxidative processes are involved very early in seed germination.

Pradet *et al.* (18) have shown that ATP levels increase sharply from the beginning of imbibition up to about hr 4, at which time the ATP concentration increases much more slowly. They also show that transferring seeds imbibed for 6 hr from air to nitrogen reduces the ATP level to near zero in about 6 min with a concomitant increase in AMP. Our pulse experiments indicate that germination is most sensitive to 2,4-dinitrophenol during the first 4 hr postimbibition (Fig. 3a). This is also the most sensitive time period to anaerobiosis (Fig. 3b). Since Pradet's data shows that ATP is depleted in a matter of minutes upon transfer of imbibed seeds from air to nitrogen, our 2-hr nitrogen pulse should have completely depleted the system of ATP. The effect of anaerobiosis diminishes with time, becoming minimal at about hr 6; this suggests that ATP is relatively more important to events previous to hr 6.

The lack of inhibition of germination by $Zn^{2+}$ and $Mn^{2+}$, from our data, seems to indicate that phytase activity is probably not involved during early events of germination, most likely it is not involved until the onset of seedling growth. This idea is supported by Mayer's (13) data that at 12 hr postimbibition the hydrolysis of phytin is insignificant nor has the level of phytase appreciably increased by this time. There is some problem in the interpretation of Peers' (16) data. His assay was for orthophosphate cleaved from sodium phytinate by his enzyme preparation. However $Zn^{2+}$ and $Mn^{2+}$ give insoluble precipitates of orthophosphate and would thus give an apparent *in vitro* enzyme activity of zero.

Paulson and Srivastava (15), in an electron microscope study of dry *L. sativa* seeds, report that while the mitochondria have fairly well defined outer membranes, their internal structure is somewhat irregular. In a further study, using imbibed seeds, these two authors report that the first detectable ultrastructural changes occur in the embryo at about 12 hr postimbibition. During the period of 12 to 24 hr, mitochondria become more well defined and the number of cristae increase (20). Is it then possible that up to hr 12 postimbibition mitochondrial phosphorylation is low or nonexistent? In our experiments, the inhibitory effects of arsenate are readily reversed in the growing seedling by the addition of phosphate but no such reversal occurs during germination of intact or punched seeds. Since arsenate and phosphate are accessible to the embryo of the punched seed, this suggests that oxidative phosphorylation is of little importance at this period of seed germination. This idea was also considered by Poljakoff-Mayber and Evenari (17), since they could detect no mitochondrial function regarding citric-acid cycle activity until 24 to 48 hr in subcellular fractions of Grand Rapids seeds. The point is that arsenate probably affects some other system, possibly nitrate reduction. In our experiments, there is an indication of a reversal of arsenate inhibition by nitrate. There is no indication that nitrite reverses arsenite inhibition. This suggests that the conversion of arsenate to arsenite by the seed and arsenite being a competitive ion for nitrite is improbable.

## CONCLUSION

In intact seeds there is a distinct penetration barrier to the entrance of materials into the seed. This barrier can be overcome by punching the seed coat resulting in only slight loss of germination or response to red and far-red light. There is a distinct change in the response to inhibitors from germination to subsequent seedling growth. Punched seeds can be used in pulse experiments to establish the time course of biochemical events during seed germination. During the first 12 hr of seed germination, oxidative processes are involved.

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