Equivalent inhibition of oxygen uptake was obtained with cyanide and freezing, but no additional depression of respiration was found when cyanide was added to frozen seedlings. It was suggested that freezing abolishes the cyanide-sensitive respiration.

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METABOLISM OF C^{14}-BICARBONATE, P^{32}-PHOSPHATE, OR S^{35}-SULFATE BY LETTUCE SEED DURING GERMINATION [1]

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The time course of germination of lettuce seed has been divided into the following phases: imbibition, activation, mitosis, protrusion of radicle, and growth (5). Although some enzymes have been found in extracts of seeds throughout the course of germination, very little is known of the metabolism of intact seeds during the phases before protrusion. It has been shown that lettuce seeds respire in the earliest phases of germination (5, 9, 13). Presumably, germination-promoting light and chemicals act during the early phases preceding protrusion. We used radioactive bicarbonate, phosphate, and sulfate in the experiments reported here to explore the metabolism of lettuce seeds during the phases of germination preceding radicle protrusion.

Materials and Methods

Seeds of *Lactuca sativa,* var. Grand Rapids, were germinated in covered 5.5-cm Petri dishes, each containing a pad of filter paper moistened with 1 ml of solution containing 100 μc of $NaHC^{14}O_3$ buffered at pH 9 with 0.05 M Tris (hydroxymethyl)aminomethane 0.005 M in Cl^-, or 60 μc of $NaH_2P^{32}O_4$ in distilled water at pH 5.8, or 100 μc $Na_2S^{35}O_4$ in distilled water at pH 5.8. Radioisotopes were purchased from the Oak Ridge National Laboratory. Specific activities of the tracers at the beginning of the experiments were approximately 160 to 1200 mc of C^{14} per gram of carbon and 10,000 to 40,000 mc of P^{32} per gram of phosphorus. The S^{35} was carrier free. All experiments were performed at 21 to 22° C. Light treatments (room lighting or far-red) were given continuously. Unless otherwise designated, seeds were germinated under 20 ft-c of white light. Seeds designated as punctured were pricked through the surface of the seed coats covering the cotyledons with a number 8 needle so that the embryo was not touched and viability was unaffected.

Six to ten seeds were extracted by boiling for several minutes in 3 ml of 85 % ethanol and then in 3 ml of water. Unless otherwise indicated, the alcohol- and water-soluble extracts were combined for analysis by two-dimensional paper chromatograhy as described by Bassham and Calvin (1). When indicated, the following solvents were also used for developing one-dimensional chromatograms: methanol : 80 % formic acid : water (80 : 15 : 5 v/v) ; *tert*-amyl alcohol saturated with water; ethanol : glacial acetic acid (95 : 5 v/v) (3).

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RESULTS

Under the conditions of room temperature and lighting, the seeds used in these experiments entered the protrusion phase around 15 hours after the beginning of imbibition, whether or not they had been punctured. Imbibition of water, as determined by weighing groups of 100 seeds, was complete after the 1st 4 hours in unpunctured seeds (fig 1). This rate of water uptake per intact seed was unaffected by exposure to far-red light in the presence or absence of germination-promoting chemicals (gibberellic acid, kinetin, or thiourea), but was completed within 15 to 30 minutes in punctured seeds (Haber, unpublished).

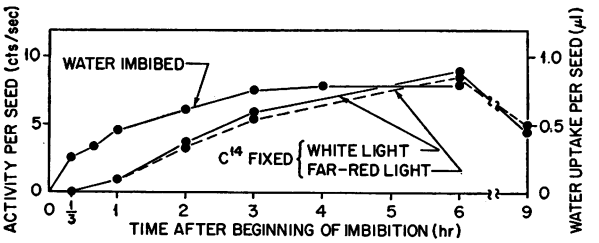

FIG. 1. Fixation of C^{14} from $NaHC^{14}O_3$ and water imbibition. Dry seeds were put into a solution of $NaHC^{14}O_3$ at zero time. Groups of seed were removed and extracted at times indicated.

DISTRIBUTION OF C^{14}: Detectable C^{14} was fixed from $NaHC^{14}O_3$ into soluble compounds by intact seeds within the 1st hour of imbibition (fig 1). Extracts from seeds, taken at various times from 90 minutes after the beginning of imbibition until after radicle protrusion, were chromatographed. A radioautograph of the chromatograph of seeds after 90 minutes is reproduced in figure 2. Analysis of extracts after 3 hours imbibition showed that about one third of the total fixed C^{14} appeared in malic acid, and 12 to 15 % in each of the following: glutamine, aspartic acid, citric acid, or glutamic acid. Other labeled compounds were asparagine, serine, glycine, succinic acid, fumaric acid, glyceric acid, and alanine. The over-all qualitative distribution of C^{14} among soluble compounds was similar during all phases of germination, even after protrusion of the radicle. The data suggest that the C^{14} was fixed primarily by carboxylations into organic acids and that the tricarboxylic acid cycle and transamination mechanisms functioned during the earliest phases of germination. The lag in C^{14} fixation as compared with water imbibition does not necessarily imply a lag in C^{12} fixation, but may be attributed to failure of sufficient C^{14} to enter the seed during the 1st 20 minutes of imbibition. Neither the time course of total fixed C^{14} (fig 1) nor the distribution of C^{14} among soluble compounds per seed appeared to be altered by continuous exposure during the 1st 12 hours to far-red light, even though seeds germinated in this

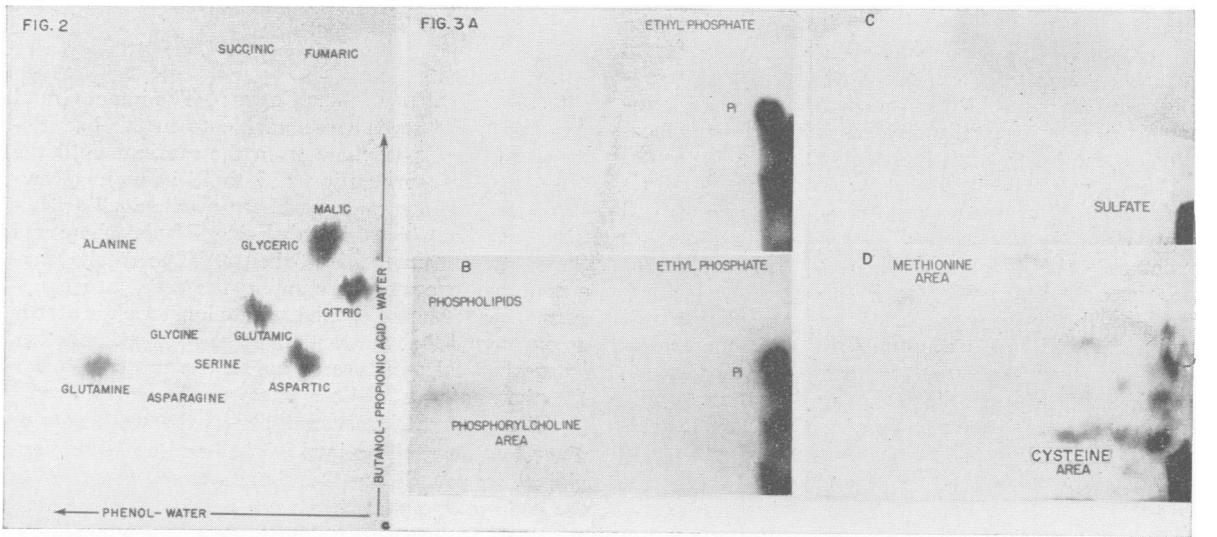

FIG. 2. Radioautogram of chromatograph of extracts from seeds after 90 minutes in $NaHC^{14}O_3$.

FIG. 3. Radioautographs from extracts of seeds that had been in solutions containing tracers for 12 hours. The chromatograms from which the films were made were developed from right to left with phenol : water, and from bottom to top with butanol : propionic acid : water. 3 A. unpunctured seed; with P^{32}-orthophosphate; 3 B. punctured seed; with P^{32}-orthophosphate; 3 C. unpunctured seed; with S^{35}-sulfate; 3 D. punctured seed; with S^{35}-sulfate. The chromatograms of 3 A and 3 B were folded parallel to the direction of phenol movement so as to expose both sides of the film. Consequently, the position of the phospholipid spot in 3 B is not indicative of its actual R_f in butanol : propionic acid : water.

way gave 3 % germination after 24 hours under far-red light compared with 85 % germination under white light.

DISTRIBUTION OF P^{32}: Intact seeds were germinated in $NaH_2P^{32}O_4$ and extracted at various times through radicle protrusion (15 to 18 hours after beginning imbibition) and into the seedling growth phase. At any time prior to radicle protrusion, the only labeled compounds detected were orthophosphate and ethanol-1-phosphate (fig 3 A). The P^{32}-ethyl phosphate could be accounted for by formation of ethyl phosphate from orthophosphate and ethanol as an artifact produced during the extraction procedure, since it could be detected after administration of P^{32}-phosphate to extracts of lettuce seeds not previously in contact with the phosphate tracer. Runeckles (14) and Loewenberg, Tolbert, and Haber (unpublished) observed similarly the formation of ethyl phosphate after extraction of various plant tissues with ethanol. Extracts from unpunctured seeds before protrusion were also chromatographed with methanol : formic acid : water. No esterification of P^{32}-phosphate was detected on chromatograms developed with this solvent, which we have found to separate orthophosphate from other phosphorus compounds (sugar phosphates, pyrophosphate) that chromatograph near the orthophosphate region in the double solvent system routinely used (Haber and Tolbert, unpublished). Thus, before appearance of the radicle, no P^{32}-orthophosphate that could be detected with our chromatographic procedures was esterified by intact seeds. At the time of radicle protrusion, unpunctured seeds attained the capacity to esterify detectable P^{32}-orthophosphate from the external solution, and during the subsequent seedling growth phase, activity was detected in many compounds characteristically labeled when P^{32}-orthophosphate is administered to intact plants.

In contrast, when punctured seeds were used, esterification of P^{32}-orthophosphate was detected within 3 hours after the beginning of imbibition, even though punctured seeds did not germinate any sooner than intact control seeds. Of the P^{32}-phosphate esterified, considerable activity corresponded to phosphorylcholine (12) and phospholipids (2). Radioautographs of chromatograms of extracts from intact and punctured seeds after 12 hours in a solution containing P^{32}-phosphate are shown in figure 3 A, B. The presence of P^{32}-orthophosphate on the chromatograms can probably be accounted for by adsorption of the external solution on surfaces external to the embryo, since the seeds had not been washed so as to quantitatively remove adsorbed P^{32}-phosphate. The distribution of tracer in extracts of punctured seeds before radicle protrusion was similar to that obtained from unpunctured seeds after protrusion.

Far-red light exposure in the presence or absence of gibberellic acid, kinetin, or thiourea did not seem to alter either the inability of the seeds to metabolize the tracer or the distribution of P^{32} in extracts of punctured seeds before protrusion.

DISTRIBUTION OF S^{35}: Intact seeds that were germinated with $Na_2S^{35}O_4$ and extracted any time prior to radicle protrusion yielded no labeled organic compounds (fig 3 C). Around the time of radicle protrusion, unpunctured seeds attained the capacity to metabolize S^{35}-sulfate from the external medium. These results are parallel with those reported above with intact seeds and phosphate tracer.

When punctured seeds were used, S^{35}-sulfate was metabolized within 3 hours after beginning of imbibition, suggesting a further parallel between results with sulfate and phosphate tracers. The distribution of S^{35} in extracts of punctured seeds before radicle protrusion was similar to that in unpunctured seeds soon after radicle protrusion. S^{35} was detected in numerous spots, 2 of which corresponded to cysteine and methionine. Radioautographs of chromatograms of extracts from intact and punctured seed after 12 hours in a solution containing radioactive sulfate are shown in figure 3 C, D. The presence of radioactive sulfate on the chromatograms can probably be accounted for by adsorption of the external solution on surfaces external to the embryo.

During the seedling growth phase (followed up to 60 hours after beginning imbibition), the fraction of S^{35} appearing as methionine increased so that methionine had more activity than any other organic compound. The S^{35}-methionine from chromatograms of extracts from such seedlings was identified as methionine by elution and cochromatography with *tert*-amyl alcohol : water and with ethanol : glacial acetic acid.

DISCUSSION

PERMEABILITY OF SEED COATS: The intact Grand Rapids lettuce seeds were unable to metabolize P^{32}-phosphate or S^{35}-sulphate from the medium until the time of radicle protrusion (15 to 18 hours). However, seeds whose coats had been mechanically punctured were capable of metabolizing P^{32}-phosphate and S^{35}-sulfate within the 1st 3 hours. The results suggest that the coats surrounding the embryo are impermeable to phosphate and sulfate ions, even in tracer amounts, and that the coats must be ruptured, whether by mechanical puncture or by radicle protrusion, for the phosphate and sulfate tracers to be metabolized.

The apparent impermeability of the seed coats to phosphate and sulfate ions might be alternatively explained by postulating that these ions did penetrate to the embryo, but that they were not metabolized until the time of protrusion. Metabolic changes have been shown to occur within the seeds around the time of protrusion (6), and our data could thus be interpreted to mean that, at the time of radicle protrusion, metabolic processes were activated that in turn resulted in the metabolism of the labeled phosphate and sulfate. According to this latter alternative, we must similarly postulate that the observed metabolism of phosphate and sulfate tracers by pricked seeds before radicle protrusion was occasioned by a more vigorous phosphate

and sulfate metabolism somehow caused by the mechanical puncture. Mechanically punctured seeds, however, did not germinate any sooner than unpunctured controls. This would suggest that the failure of unpunctured seeds to utilize P^{32}-phosphate and S^{35}-sulfate is indeed caused by failure of the tracers to reach active metabolic sites within the interior of the seeds. This suggestion is supported by experiments cited by Evenari (5), which showed that dinitrophenol stimulated oxygen uptake of Grand Rapids lettuce seeds during the earliest phases of germination. We have confirmed this effect of dinitrophenol by studies using the same experimental conditions and the same batch of seeds used in the tracer studies here reported. 5×10^{-4} M 2,4-dinitrophenol, which completely prevented germination of these seeds, increased the average oxygen uptake per 100 intact seeds from 34.1 to 39.1 microliters ($t = 2.70$; d.f. $= 6$; $p = 0.035$) between 4 and 5 hours after the beginning of imbibition. Stimulation of oxygen uptake by dinitrophenol is generally regarded to be a consequence of the greater availability of phosphate acceptors caused by uncoupling of phosphorylation from oxidation (11). Thus it follows that the seeds probably effected phosphorylations during the earliest phases of germination. Failure to esterify P^{32}-phosphate under conditions in which the seeds nevertheless effect phosphorylations strongly implies failure of the P^{32} to reach the active sites for phosphorylation. That seed coverings may check the intake of salts (KNO_3, NaCl) is also suggested by investigations (reviewed by Crocker and Barton, (4)) performed before the availability of tracers.

The seeds fixed C^{14} within 1 hour from the beginning of imbibition in a buffered $NaHC^{14}O_3$ medium. Since the coats surrounding the embryo were impermeable to phosphate and sulfate ions, it is also possible that bicarbonate ion did not penetrate the seed coats. There is an equilibrium between $C^{14}O_2$ and $NaHC^{14}O_3$ in solution. Moreover, since germinating seeds excrete acid into the medium (4), $HC^{14}O_3^-$ could have been converted to $C^{14}O_2$ at the surface of the seeds. Thus the C^{14} may have reached the embryo in the form of $C^{14}O_2$ rather than as bicarbonate ion. This suggestion is corroborated by the fact that during a 24-hour period the radioactivity of C^{14} in the medium dropped to 2 % of initial activity, although the pH did not fall and the C^{14} fixed by the seeds was probably negligible. As a result, the total fixed C^{14} per seed decreased after 6 hours of imbibition, owing to a loss of C^{14} from the medium as well as from the seeds by respiration (fig 1). The observed fixation of C^{14} by the seeds cannot be explained by microbial contamination on the surface of the seeds, since the intact seeds did not metabolize P^{32}-phosphate or S^{35}-sulfate.

METABOLIC IMPLICATIONS: Poljakoff-Mayber and Evenari (13) have shown that a particulate fraction from Grand Rapids lettuce seedlings is capable of oxidizing a number of substrates of the tricarboxylic acid cycle. However, particulate fractions extracted from untreated seeds before radicle protrusion, or even 1 day after protrusion, showed negligible capacity to oxidize these substrates. Results of our work imply the operation of the tricarboxylic acid cycle as early as 90 minutes after the beginning of imbibition. The pattern of fixation was similar to typical $C^{14}O_2$ dark-fixation patterns (fig 2).

The similarity of distribution of C^{14} among compounds in seeds under white light or far-red light suggests that the effects of photomorphogenic radiation on CO_2 fixation are either nonexistent or too subtle for detection by the methods utilized. Similar conclusions were reached by Kunitake et al (10) with respect to CO_2 dark fixation during photoperiodic induction in leaves of Kalanchoë.

Generally, the operation of the tricarboxylic acid cycle is linked to the production of high-energy phosphate compounds, which in turn mediate the phosphorylation of many other compounds. The detection of P^{32} in phosphorylcholine and phospholipids within 3 hours after the beginning of imbibition by punctured seeds in a solution containing P^{32}-orthophosphate suggests that these synthetic processes are linked very early in the germination process. These results do not support the hypothesis that the block of germination effected by far-red light can be identified with an incapacity of the seeds to effect phosphorylations, since the seeds are still photosensitive much later than the time at which esterification of P^{32}-phosphate was first detected in punctured seeds (8). It is not now feasible to defend this last suggestion by the more direct experiment of comparing phosphate esterification in seeds germinated under white light with seeds under far-red light, since puncturing the seeds (necessary for the tracer to reach the embryo) also releases them from far-red light control, as previously described by Evenari and Neumann (7).

Sulfur was detected in sulfur-containing amino acids from punctured seeds after only 3 hours in a solution containing S^{35}-sulfate. Thus the mechanisms by which energy is coupled to the endergonic processes of sulfate reduction and phosphate esterification operate during the earliest phases of germination.

SUMMARY

Grand Rapids lettuce seeds were germinated in solutions containing C^{14}-bicarbonate, P^{32}-phosphate, or S^{35}-sulfate. Extracts from seeds at various times during the course of germination were analyzed by paper chromatography.

C^{14} was detected in soluble compounds as early as 1 hour after the beginning of imbibition. The data suggested that C^{14} was fixed by carboxylations into organic acids and that the tricarboxylic acid cycle and transamination mechanisms functioned during the earliest phases of germination. No difference in the extent or pattern of C^{14} fixation was noted between seeds germinating under white light or under far-red light.

Neither the P^{32}-phosphate nor the S^{35}-sulfate were metabolized by intact seeds until after radicle protrusion (15 to 18 hours after beginning of imbibition). Punctured seeds, however, metabolized the radioactive phosphate or sulfate within 3 hours after the beginning of imbibition. We concluded that the coats surrounding the embryo are impermeable to phosphate and sulfate ions. Most of the organic P^{32} extracted, whether from punctured seeds before radicle protrusion or from unpunctured seeds after protrusion, was found in phosphorylcholine and phospholipids.

S^{35} was detected in cysteine, methionine, and numerous unidentified compounds, both from punctured seeds before radicle protrusion and from unpunctured seeds after radicle protrusion.

Thus within 3 hours after the beginning of imbibition, punctured seeds could esterify phosphate and reduce sulfate to the level of sulfhydryl.

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MECHANISMS OF ACTION OF POLYMYXIN B ON CHLORELLA AND SCENEDESMUS[1, 2, 3]

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The investigations of the mechanism of action of an inhibitor of cell growth or development often yield fundamental information about the metabolism of the species under study. The available data on the biochemical constituents of the organisms are commonly insufficient to permit interpretation of experimental results obtained when the inhibitor is introduced. The examination of the role of polymyxin B, an antibiotic which shows strikingly different effects on related species, is no exception. Evidence has been accumulating which indicates that polymyxin B acts to disorganize the cell wall which in turn causes disruption of the osmotic equilibrium of the cell (1, 8, 9, 10). However, other evidence (2, 11, 17) suggests that damage from surface action does not completely explain polymyxin inhibition. Osmotic disruption seems to be but one of several possible effects. Warren et al (17), studying the sensitivity of polymyxin-treated cells to lysozyme, observed that no correlation could be demonstrated between the sensitivity of the organisms to the antibiotic and the lytic response following the addition of the enzyme. Galloway and Krauss (2) showed that galactose can protect against the inhibition of respiration by polymyxin B in 2 species of bacteria.

The present study was undertaken to further examine the role of polymyxin B, especially with regard to the protective effect of galactose on susceptible algae, as well as the mechanism of resistance in normally resistant algae. Such an investigation has

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