STUDIES OF REST PERIOD. II. NITROGEN AND PHOSPHORUS CHANGES IN EMBRYONIC ORGANS OF AFTER-RIPENING CHERRY SEED \textsuperscript{1,2}

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Information on changes which occur during after-ripening is essential to an understanding of the mechanism of the rest period. In the first paper of this series \textsuperscript{7} we reported that during after-ripening growth occurs in the embryonic axis of the cherry seed. This growth is accompanied by translocation of materials from cotyledons to axis, an increase in respiration rate of the axis, and an increase in the efficiency with which the respiratory enzyme system is utilized. These data suggest that the respiratory rise might be due to an increased supply of available phosphate or phosphate acceptors. The present work was performed to test this hypothesis. The observations are divided into two parts: A, measurement of changes in total nitrogen and phosphorus in the embryonic axis during after-ripening, and B, fractionation of total phosphate and measurement of the changes in quantities of these fractions during after-ripening. The results show that total phosphate concentration in the axis increases during after-ripening. This increase is not reflected uniformly in all fractions measured.

MATERIALS AND METHODS

Seeds of the sour cherry, \textit{Prunus cerasus} L. var. Montmorency, were obtained and prepared by the methods previously described \textsuperscript{7}. The measurements of translocation of total nitrogen and total phosphorus were actually part of the experiment previously described; the sampling procedures and cell counts upon which figures 1 and 2 are based are detailed in that paper.

Total nitrogen was determined by titration following digestion according to the method of Boell and Shen \textsuperscript{3} and recovery by diffusion according to a micromodification of the Conway method \textsuperscript{4}. Diffusion was carried out in paraffined vessels 13 mm in diameter and 13 mm deep in which 18 \textmu l aliquots of the 6 \textpercnt acid digest and 18 \textmu l of 8 \textpercnt NaOH were mixed with a magnetic flea \textsuperscript{5}. A drop containing 18 \textmu l 0.04 \textpercnt H\textsubscript{2}SO\textsubscript{4} with bromoresol green indicator \textsuperscript{3} was suspended from a glass plate covered with "Parafilm" and sealed in place with vaseline. The drop was prevented from shifting during manipulation by a circular depression formed in the parafilm with a dull No. 1 cork borer. After diffusion at room temperature for 70 minutes, the receiving acid was transferred with rinsing to micro tubes and titrated with 0.01 \textpercnt NaOH delivered from a horizontal microburette and mixed with a magnetic flea \textsuperscript{5}. This modification of the Conway method is sensitive to 0.05 \textmu g nitrogen per diffusion vessel.

Total phosphorus of leaf primordia was determined by the method of Bernhardt and Wreathe \textsuperscript{2}. To facilitate pH adjustment, the digestion method was modified by evaporating the nitric-perchloric acid digestion mixture (N-PCA, 2.5 volumes nitric acid plus 1 volume 70 \textpercnt perchloric acid) to a dry residue at 130°C. This residue was dissolved and converted to orthophosphate by heating with 1 \textpercnt HCl; the pH was then adjusted to about 2.4 with NaOH. The un-reduced phosphomolybdate yellow was intensified by adding acetone and measured at 430 nm in a special microcuvette in the Beckman DU spectrophotometer. This method is sensitive to 0.025 \textmu g phosphorus.

To determine both total nitrogen and total phosphorus in aliquots from a digest of an individual embryonic axis, a sealed tube digestion was adapted from Lowry \textsuperscript{6}. A minimum quantity of 12 \textpercnt H\textsubscript{2}SO\textsubscript{4} was used for digestion at approximately 500°C in sealed ampules. After digestion the ampules were opened under an air pressure of approximately 30 psi which was reduced slowly to prevent frothing. Aliquots of the solution were then analyzed for total nitrogen and total phosphorus as described above.

Following the fractionation procedure outlined below, phosphorus was analyzed by the method of Schaffer et al \textsuperscript{8}.

The experiments on fractionation of total phosphorus were performed with seeds from the 1958 crop. In Delaware cherries are normally picked at

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a stage in which the fruits do not readily separate from the stems; seeds from such fruits were used for all except the 1958 experiments. In 1958 the fruits were allowed to ripen on the trees to a stage which corresponded with that normally used for commercial canning and freezing of sour cherries. After air-drying, the dry stones were stored in sealed containers at 5° C until used.

Because of the small quantities of phosphorus in some of the fractions, three samples of five seeds each were analyzed at each time interval. The axis and leaf primordia were analyzed as a unit rather than separately as reported in the previous paper (7). Each sample of five axis-leaf primordia units was carried through the entire fractionation procedure separately. The data were then calculated on a unit basis to permit direct comparison with previous data.

To prepare the phosphorus fractions, the freshly dissected material was quickly frozen in dry ice in a mortar made from thick-walled pyrex capillary tubing (35 × 2 mm ID). The frozen material was powdered by pounding with a cold stainless steel pestle and hammer (9). The frozen tissue was rubbed into suspension in 50 µlizers 10 % perchloric acid (PCA) with a pyrex rod. After centrifuging at room temperature for 1 minute at 1,000 × G the supernatant was transferred to a 6 × 45 mm micro tube. The precipitate was washed twice with 50 µlitzer aliquots of 5 % PCA and the washings were added to the supernatant. The residue was dried, digested, and analyzed to provide a measurement of acid-insoluble phosphorus. To the combined supernatant approximately one milligram of specially purified Norite A carbon was added, the solution was stirred, and the carbon was removed by centrifugation. This carbon precipitate was then washed three times with 100 µlizers of glass distilled water and the supernatants were combined. The total solution was then divided into two equal aliquots, one of which was analyzed directly for inorganic orthophosphate and the other dried, digested with N-PCA mixture, and analyzed to give the acid soluble non-nucleotide phosphorus residue.

The nucleotides adsorbed on the charcoal were hydrolyzed in 75 µlizers 1.0 N HCl at 100° C for 12 minutes; the time for hydrolyzing high-energy phosphorus on carbon was found to be slightly longer than in solution. The carbon was washed once with 100 µlizers water and the combined supernatants analyzed to give high-energy nucleotide phosphorus. The remaining adsorbed nucleotides containing the low-energy nucleotide phosphorus were determined by complete digestion on the carbon in 25 µlizers N-PCA, followed by analysis in the presence of partially-digested carbon. Since the analysis required octanol extraction, this carbon did not interfere, but required special purification to remove contaminating phosphates.

This fractionation procedure produced five fractions, which, according to literature descriptions (2, 5, 9, 11) should have included the following types of compounds: High-energy nucleotide: adenosine di- and tri-phosphate. Low-energy nucleotide: adenosine monophosphate. Inorganic: inorganic orthophosphate. Acid soluble residue: chiefly pentose and hexose phosphates. Acid insoluble residue: chiefly nucleic acids and phosphoproteins.

RESULTS

The data on total nitrogen (fig 1a, 1c) show an increase with after-ripening time at 5° C, but not at 25° C. Total phosphorus shows similar trends (fig 2a, 2c). These increases represent translocation of nitrogen and phosphorus from the reserves in the cotyledons to the growing cells of the axis. They are correlated with the breaking of rest at least to the degree that after-ripening at 5° C breaks rest. After-ripening at 25° C does not break rest but acts as a control to show changes which occur during moist storage.

Using the data on cell division previously published (7), the data (figs 1a, 1c, 2a, 2c) were recalculated to

![Fig. 1](https://www.plantphysiol.org/content/45/2/971.f1)

Fig. 1. The effect of after-ripening time and temperature on the total nitrogen content of cherry embryonic leaf primordia and axes. The vertical lines represent ± the standard error of the mean.
show the changes in quantity of nitrogen and phosphorus per cell (figs 1b, 1d, 2b, 2d). In the case of total nitrogen, the rate of translocation almost exactly parallels the rate of cell division, resulting in a constant amount of nitrogen per cell. However, the amount of phosphorus per cell increases with after-ripening at 5°C. Since this increase occurs in cells whose average nitrogen content remains constant during cell multiplication, this may be interpreted as a real increase in total phosphorus, i.e., phosphorus concentration per unit protoplasm.

Between 12 and 16 weeks after-ripening at 25°C there is a sharp increase in phosphorus per leaf primordium cell (fig 2b). This is the result of a relatively small increase in total phosphorus per leaf primordium (fig 2a) coupled with a relatively stable cell number. It was noted that at this time a number of seeds from this particular seed crop were actually capable of limited growth; this abnormally high concentration of total phosphorus may reflect that growth potentiality.

In the previous paper (7) data were presented to show that after-ripening at 5°C results in increased efficiency in utilization of the respiratory enzymes present. Such increased efficiency might be the result of increased availability of phosphorus or phosphate acceptors in the respiring cells. Since the data (fig 2) show such an increase in phosphorus concentration, it seemed desirable to determine whether this increase represents a general increase in the level of phosphate compounds, or whether it is the result of an increase in a particular class of compounds. Therefore, data were obtained in an experiment performed as nearly as possible under conditions identical to those of the experiment described in the previous paper and illustrated in figures 1 and 2 above.

The data in figures 3a to e show the comparison of phosphorus fractions from seeds after-ripened at 5°C and at 25°C. In the nucleotide high- and low-energy phosphorus fractions, as well as the acid-insoluble residue, the data are much as would be expected. These fractions increase with after-ripening at 5°C but not at 25°C; the quantities are higher in material after-ripened at 5°C than at 25°C. However, the fractions containing inorganic phosphorus (fig 3d) and acid-soluble phosphorus (fig 3e) are distinctly different. The general increase with after-ripening time is not apparent. More important, the quantities are higher, in some cases very much higher, in material from seeds after-ripened at 25°C than at 5°C. This difference is completely reversed from that of any quantity previously related to after-ripening.

In figure 3, the values of the ordinates vary with the different fractions. In the non-after-ripened axis, the bulk of the total phosphorus appears in the acid-soluble and insoluble fractions (55% and 27%), while the nucleotide and inorganic fractions are relatively small. After-ripening at 5°C results in a decrease in the percentage of acid-soluble fraction and some increase in the relative amount of the low-energy nucleotide fraction. Otherwise, the relationship between the fractions remains approximately constant. However, with 25°C after-ripening, the acid-soluble residue remains the largest fraction, and the inorganic phosphate fraction increases in amount at the expense of the acid-insoluble fraction.

Because of the time-consuming nature of the analyses, it was not possible to perform all of the experiments with seeds from the same crop. The experiments in the previous paper (7) and in figures 1 and 2 of this paper were performed with seeds of the 1957 crop; experiments on phosphate fractionation utilized seeds from the 1958 crop. By totaling the amounts of phosphorus in the various fractions, it is possible to directly compare the 1957 and 1958 crops. This comparison is shown in figure 4. The shape of the curve is similar in the two cases, but the magnitude of total phosphorus is very different, being almost twice as high in the 1957 as in 1958 crops. Some of the data obtained suggest that the rates of after-ripening and growth were slightly different between the two crops, but in the absence of more direct and careful comparison, it is difficult to determine whether or not significant differences did exist.
DISCUSSION

The total amount of material in the embryonic axis of the cherry seed is almost insignificant in comparison with the total material in the storage organs. For example, the dry weight and total phosphorus of the axis plus leaf primordia is only about 0.7% of the total in the seed. Because of the errors inherent in analytical methods, it is not possible to balance the gains in the growing organs against losses in the storage organs. Therefore, the gains in the embryonic axis have been assumed to be the result of translocation from the cotyledonary reserves. Although these gains are expressed on a per cell basis, this does not imply that the cells in the embryonic growing regions are structurally or physiologically equivalent. The obvious specialization of cells within the growing organs has not been included in the present study.

The observation that a close correlation exists between nitrogen translocation and cell division suggests that the growth occurring during after-ripening is primarily the result of cell division rather than cell enlargement. There is one obstacle to the interpretation of these data, however. In the seed, storage is not restricted to the storage organs. A certain amount of storage also occurs within the potentially growing cells of the embryonic axis. This can be seen in a gross way by noting the change from an...
The present data go beyond the relation of total phosphate concentration to respiration and the breaking of rest. The accumulation of phosphate compounds in embryonic organs after-ripened at 5°C shows a pattern consistent with the movement of inorganic phosphate into the metabolic system through such intermediate compounds as sugar phosphates and nucleotides into more stable protoplasmic components such as nucleic acids, i.e., a normal synthetic system is operative. In seeds held moist at 25°C and not progressing toward the growing condition, the data suggest a breakdown of the more stable types of compounds and the accumulation of inorganic phosphate. The normal pathways of metabolism and synthesis are not functioning for growth.

In the absence of actual measurements on phosphate turnover, the above considerations remain suggestive and speculative. They might, however, be interpreted as suggesting that the rest period is associated with a metabolic block in the phosphorylation system between the initial phosphorylation of respiratory intermediates and the synthesis of end products. However, they might equally well be interpreted as being the result of a metabolic block elsewhere in the synthetic system which merely is reflected in the accumulation of metabolic intermediates.

It should be emphasized that there is a further problem of interpreting these results in relation to the rest period. It has been shown that in the cherry embryonic growing organs the rest period does not constitute an absolute block to growth; slow growth in the form of cell divisions and accumulation of metabolic constituents does occur even in the earliest stages of after-ripening. It is possible that the changes noted here are associated primarily with this slow growth and only secondarily with the breaking of rest.

The difference between the two years' cherry crops in content of total phosphate raises a question as to the normal differences of distribution of elements and compounds within the seed, and how these may be related to environmental conditions and to physiological behavior of the seed. Von Abrams and Hand (10) have presented data for Rosa indicating a relationship between pre-harvest environment and dormancy. The present data suggest this type of phenomenon.

**SUMMARY**

During after-ripening at 5°C, nitrogen and phosphate are translocated from the storage to the potentially growing organs of the cherry seed. The rate of translocation of nitrogen is equal to the rate of cell division, so the nitrogen concentration per cell remains constant. However, the rate of translocation of phosphorus is in excess of cell division and the concentration of phosphorus in the cells increases. Fractionation experiments suggest that the translocated phosphorus moves through normal synthetic pathways into all phosphate compounds in the cells. However, in seeds held moist at 25°C and not pro-

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**FIG. 4.** Comparison of the total phosphorus in cherry embryonic organs of the 1957 and 1958 crops.

Opaque to a translucent condition as the embryonic organs begin active growth. Microscopic examination shows the disappearance of starch grains and fat droplets at this time; these reserves are apparently the first used during active growth. Gross chemical analysis cannot distinguish between constituents of the cell, for example proteins, which are present as storage materials, and those which are present as protoplasmic components. Other types of measurement must be developed to measure the shift from storage to active compounds within any particular class of compounds.

The changes in phosphate compounds are closely correlated with the changes in respiratory activity previously noted (7). During after-ripening at 5°C the efficiency of utilization of the respiratory system, as measured by the respiratory increase in the presence of 2,4-dinitrophenol, increases. During 25°C after-ripening respiratory efficiency remains constant or declines. These results might be explained on the basis of an increasing supply of phosphate or phosphate acceptors during 5°C after-ripening, and not at 25°C. The data presented in this paper are consistent with this hypothesis. During after-ripening sufficient to break rest, phosphate is translocated to the growing organs; this translocation is in excess of the rate of growth, and so the effective phosphate concentration increases.
gressing toward the growing condition, phosphorus tends to shift from the normal compounds such as nucleic acids and accumulate as inorganic phosphate. These data are in agreement with previous observations on respiratory rates and pathways. Although these data suggest that the rest period may be associated with a block in the phosphate metabolism of the cells, this interpretation cannot be fully substantiated.

Literature Cited


STUDIES OF REST PERIOD. III. RESPIRATORY CHANGES IN LEAF PRIMORDIA OF MAPLE BUDS DURING CHILLING

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Respiration is one of the physiological processes frequently measured in relation to the rest period. In general, respiration increases slowly with the shift from the resting to the potentially growing condition, and then very rapidly with the beginning of active growth. Obviously there is a limitation to respiration during early rest; this limitation is removed as exposure to low temperature removes the rest period block.

Few data are available in which the changes occurring in the growing organs can be separated from changes in the storage or protective organs, and in which an analysis of limiting factors has been attempted. Pollock (5) investigated respiratory changes in bud scales and growing regions of Acer platanoides buds, and concluded that respiration is limited by oxygen diffusion through the thick, living, respiring layer of bud scales. Working with Pinus strobus buds in which the rest period was already broken, Kozlowski and Gentile (3) noted a similar limitation to oxygen diffusion imposed by the nonliving scales. In addition, they noted a relationship between oxygen uptake and water content of the tissues, with a deficiency of water acting to limit oxygen uptake.

In the case of seeds, work on the respiration of cherry embryonic leaf primordia showed a respiratory limitation possibly associated with phosphate metabolism. Using 2,4-dinitrophenol, it was shown (6) that the respiratory efficiency of such leaf primordia changes with after-ripening; respiratory enzyme utilization seems to become more complete as the rest period is broken.

A number of workers (1, 2) have considered that the mechanism of rest in seeds (embryo dormancy) and buds is similar if not identical. It therefore seemed desirable to make a direct comparison between leaf primordia of a seed and leaf primordia of a bud to see whether or not similarities could be noted. Ac-