NITRATE ACCUMULATION AND REDUCTION IN PAPAW FRUITS

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Abstract

Nitrate reductase activity was present in roots, stems, leaves, and fruits of the papaw. In fruits, the location and activity of nitrate reductase varied with stage of maturation. Nitrate reductase activity was induced in the exocarp of green and mature green fruits by light and light plus exogenous nitrate. The endogenous nitrate in the exocarp of ripe fruit was not available for reduction and hence no induction occurred in the presence of light only. As fruit tissues commenced to show colour changes, there was a marked loss in their nitrate reductase activity. At the mature green stage of maturity, phloem transport appeared to predominate over xylem transport and this reduced the net influx of nitrate to fruit. The levels of nitrate observed in fruits at various stages of maturity may be explained in terms of the following: changes in nitrate reductase activity associated with fruit maturation; the availability of endogenous nitrate for reduction; factors controlling the net influx of nitrate into fruits and distribution of nitrate within fruits.

I. INTRODUCTION

The presence of relatively large amounts of an oxidizing agent, such as nitrate, in some canned food products causes a rapid detinning action on the internal can surface (Strodtz and Henry 1954). Nitrate in papaw fruits has caused a serious detinning problem in the tropical fruit salad pack (Kruger and Menary 1968). These authors report a direct relationship between nitrogen nutrition and nitrate level in papaw fruits, but attempts to solve the problem by controlled nitrogen nutrition in the field have had limited success.

The enzyme nitrate reductase has been studied extensively and several reviews have been published (Hewitt and Nicholas 1964; Kessler 1964; Beevers and Hageman 1969). The enzyme has been extracted from leaf tissue (Beevers, Flesher, and Hageman 1964), roots (Sanderson and Cocking 1964; Miflin 1968) barley aleurone layer, (Ferrari and Varner 1970), and tobacco pith cultures (Filner 1966). References in current literature make no mention of nitrate reductase activity in fruits. The reduction of nitrate in plants is susceptible to a range of factors such as light, drought, mineral nutrition, hormonal treatment, plant age, and genotype (Beevers and Hageman 1969). In most cases the control of nitrate reduction is mediated through the regulation of the enzyme nitrate reductase (Beevers and Hageman 1969). They concluded that most plant cells and tissues have the capacity to utilize nitrate and given an adequate supply of this substrate the presence of nitrate reductase can be demonstrated.

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The present study of the nitrate reductase system in papaws was undertaken to provide information on its location and activity in various plant parts. Nitrate reductase activity in various fruit portions was studied in relation to light and to endogenous and exogenous nitrate. An attempt has been made to explain nitrate accumulation in the papaw fruit by balancing the capacity of fruits to reduce nitrate against estimates of nitrate influx.

II. MATERIAL AND METHODS

(a) Plant Material

Papaws (*Carica papaya* cv. Sunnybank 7) were grown from seed in sand culture in 4-gallon plastic cans. The trees were watered daily and nutrients were supplied by adding Hoagland's solution (Hoagland and Arnon 1950) twice weekly at a rate of 1 gallon per time.

The plants were grown in a glasshouse in which the day temperature was 24°C and night temperature 18°C. Day length varied from a maximum of 15 hr in summer to a minimum of 9 hr in winter. The relative humidity was maintained above 50%. Flowers reached anthesis in midsummer and fruits were harvested in autumn.

Tissue samples for dry weight determination and calcium analysis were dried in a Dynavac freeze-drying unit (model F.D.16) for 1 week. The conditions maintained by the freeze-drying unit were $-80°C$ and 10^{-3} Torr.

The vascular system of the papaw fruit was traced using a $0 \cdot 1\%$ rhodamine dye. Fruits were halved longitudinally and the cut end of the pedicel of each half immersed in rhodamine solution until the dye reached the apex of the fruit. At this stage longitudinal sections were cut and photographed under ultraviolet light using 125 A.S.A. black and white film and ultraviolet filter [HOYA (Japanese) yellow, type Y (K2)].

(b) Nitrate Analysis

The nitrate content of fruit and plant parts was determined on a filtered homogenate using a nitrate-ion-activity electrode (Orion Research Institute, Cambridge, Mass., U.S.A.) and a Radiometer pH-meter. An extracting solution containing aluminium sulphate was used to eliminate interference from organic acid anions (Baker and Smith 1969).

(c) Nitrate Reductase Assay

The activity of the enzyme was assayed by two techniques. An *in vivo* method involved vacuum infiltration of tissue disks with a solution containing potassium nitrate ($0 \cdot 1$M) in phosphate buffer ($0 \cdot 005$M) at pH $7 \cdot 0$. The nitrite produced after incubation at 27°C in the dark for 1 hr in this solution was determined (Randall 1969). The *in vitro* method was carried out on cell-free extracts which were prepared by homogenizing tissue in $0 \cdot 1$M Tris–HCl buffer, pH $7 \cdot 5$, containing 10^{-2}M cysteine and 3×10^{-4}M EDTA. Extracts were assayed for nitrate reductase activity using the method of Hageman and Flesher (1960).

For enzyme assays on roots, lateral roots approximately 2 mm in diameter were sampled at random and sectioned into disks. Assays were made on two portions of the stem, one midway between the fruits and the roots, the other immediately above the point of attachment of the youngest fruit. All assays were replicated six times.

Nitrate reductase activity was measured in leaves at four stages of development. The four stages were: emerging leaves, pale green in colour with a "claw-like" appearance; rapidly expanding leaves, intermediate in development between emerging and fully expanded leaves; fully expanded leaves, dark green in colour and had just reached the fully expanded condition; senescing leaves, commencing to develop marginal interveinal chlorosis. Assays were replicated six times.

Fruits at three stages of maturity were used. "Green" fruits had white seeds, endocarp, and mesocarp and green exocarp; "mature green" had black seeds, green exocarp, and the endocarp and mesocarp had commenced to change colour; "ripe" had black seeds, yellow-green exocarp,

and the endocarp and mesocarp were orange-coloured. Assays were made on the four parts of each fruit at the three stages of maturity and these assays were replicated three times. The tissue disks used were 4 mm in diameter and 2 mm thick. They were prepared by punching 4-mm disks from slices that were cut to a constant thickness of 2 mm.

To establish a time course for enzyme induction in the presence of light and nitrate substrate, disks of exocarp, mesocarp, and endocarp of mature green fruit were incubated in buffered nitrate ($0 \cdot 1$M) substrate illuminated at 16,000 lux for 0, $\frac{1}{2}$, 1, 2, 4, and 6 hr prior to the measurement of nitrate reductase activity. Dark controls were set up for each period of induction as described below.

The kinetics of the light response was investigated by incubating exocarp tissue for 4 hr at 27°C in light intensities of 0, 2000, 4000, 8000, and 16,000 lux prior to the measurement of nitrate reductase activity. A light intensity of 2000 lux was equivalent to the intensity recorded under the tree canopy.

For induction studies, the tissue disks were vacuum-infiltrated with distilled water or buffered $0 \cdot 1$M KNO_3 and incubated in the light (16,000 lux) for 4 hr at 27°C. These disks were washed in deionized water, allowed to stand in buffered nitrate in the light for 20 min, and then incubated in the dark for 1 hr to determine the amount of nitrite produced. Dark controls were prepared as above except that the disks remained in the dark for the appropriate period prior to the estimation of nitrate reductase activity. The activities recorded in dark controls were subtracted from the corresponding activities obtained from light induction treatments to measure the response due to light.

To estimate the rate of reduction of nitrate in whole fruits, pedicels were immersed in water and fruits incubated for a maximum period of 72 hr. Nitrate determinations were made at intervals of 12 hr during the incubation period. Tissue isolates were taken from fruits at the conclusion of the experiment to check for the presence of microorganisms.

(d) Calcium

Calcium was determined on a Techtron AA100 atomic absorption spectrophotometer. Plant extracts were prepared by wet digestion of dried material with perchloric–nitric acid. Strontium chloride was added to reduced phosphate interference (Parker 1963) and isopropanol to increase sensitivity (Allan 1961).

(e) Microorganisms

To check the possibility that microorganisms were producing a significant amount of nitrate during incubation, a control solution was retained from the buffered nitrate substrate on which tissue disks had been incubated in light for 4 hr. This solution was incubated for 1 hr at 27°C with the test solutions and then tested for nitrite in the usual way.

(f) Statistical Analysis

Results were analysed using analysis of variance and means were compared using Duncan's multiple range test (Steele and Torrie 1960).

Since no nitrate reductase activity was recorded for seed of different ages, these were excluded from the analysis.

The error variance for endogenous nitrate reductase activity was low and a separate analysis was carried out for this determination.

III. Results

(a) Nitrate Reductase Assays

Papaw plants and fruits contain significant amounts of papain (Kimmel and Smith 1957). In cell-free extracts of papaw tissue, papain is likely to reduce nitrate reductase activity through inactivation of the enzyme. When the *in vitro* technique

was used on seed, endocarp, mesocarp, and exocarp of green, mature green, and ripe fruit, the endocarp of green fruit was the only tissue that gave activity, presumably due to the absence of papain in this tissue.

Assays were conducted on lettuce leaf tissue in the presence and absence of papain to check interference by papain. In the absence of papain, and using the *in vivo* technique (Randall 1969), a nitrate reductase activity of 2 μmoles nitrite per gram per hour was recorded.* Using the *in vitro* technique of Hageman and Flesher (1960) on the same type of tissue, an enzyme activity of 6 μmoles nitrite per gram per hour was recorded. This difference is in agreement with results published by Ferrari and Varner (1970) who found that activities in whole tissues of barley aleurone layers gave 40% of cell-free activity. When a papain extract was added to the cell-free extracts of lettuce leaves the nitrate reductase activity was reduced to zero. However, the presence of papain did not affect activities as determined by the *in vivo* technique. Consequently, the latter technique was adopted for subsequent nitrate reductase assays on papaw tissues.

(b) Microbial Reduction of Nitrate

There was no contribution of nitrite from microorganisms in the control solutions which were incubated in the dark for 1 hr at 27°C.

(c) Location and Activity of Nitrate Reductase

(i) Roots

A mean activity of 12$\pm$2·6 nmoles nitrite per gram per hour was recorded for papaw roots.

(ii) Stems

The mean activities recorded for the two portions of stem were 38$\pm$4 and 45$\pm$6 nmoles nitrite per gram per hour respectively.

(iii) Leaves

A mean activity of 1·3$\pm$0·4 μmoles nitrite per gram per hour was recorded for emerging leaves, but for rapidly expanding, fully expanded, and senescing leaves the activities were 6·2$\pm$0·9, 6·9$\pm$0·6, and 6·1$\pm$0·4 μmoles nitrite per gram per hour respectively.

(iv) Fruits

Each fruit was divided into seed, endocarp, mesocarp, and exocarp. The boundary between the three inner tissues was well defined on the basis of colour and texture. The boundary between the mesocarp and the exocarp was quite diffuse and for this reason the exocarp was taken to include the outer layer (2 mm thick) of the ovary wall which contained the bulk of the chloroplasts.

* Throughout paper enzyme activities will be given on a fresh weight basis.

Fresh weights of fruits and the percentage of tissues in fruits at different stages of maturity are given in the following tabulation:

Stage of maturity	Fresh weight (g)	Percentage contribution by each portion			
		Seed	Endocarp	Mesocarp	Exocarp
Green	443	9	10	71	10
Mature green	616	11	12	67	10
Ripe	716	11	12	67	10

The proportion of tissues in fruit did not vary with age.

Nitrate reductase activities in fruit at the three stages of maturity are given in Figure 1. The ripe fruit exhibited low activities in all four portions. In the mature
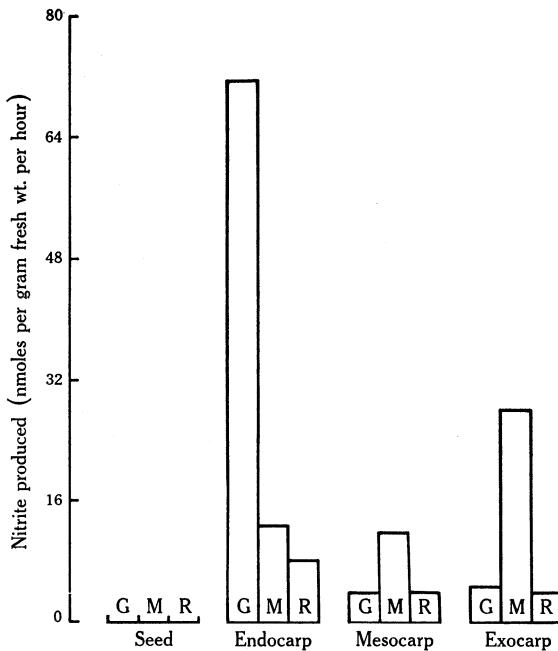

Fig. 1.—Endogenous nitrate reductase activity in fruits at three stages of maturity: G, green; M, mature green; R, ripe. Differences according to Duncan's multiple range test: $p = 2, R_p = 17 \cdot 4$; $p = 3$, $R_p = 18 \cdot 3$.

green fruit, there is a trend towards higher activity in the exocarp as compared with other parts; however, the exocarp contributes only 10% of the total weight of the fruit (see above tabulation). The mesocarp activity is only half that of the exocarp but this tissue contributes 67% of the total weight of the fruit and, therefore, is more significant in regard to the total reducing capacity of the fruit. In green fruits, the endocarp is the site of greatest nitrate reductase activity, being 72 nmoles nitrite per gram per hour. Again, this tissue comprises only 10% of the fresh weight of the fruit but its activity is more than 18 times that in the mesocarp (comprising 67% of the fresh weight). In fact, the endocarp accounts for approximately 70% of the activity in green fruits.

(d) Induced Activity

In the time course study, nitrate reductase activity did not increase in the mesocarp or endocarp of mature green fruits in the presence of light and nitrate substrate. However, enzyme activity in the exocarp did increase in the presence of light and nitrate. Activity increased from an endogenous level of 28 to 320 nmoles nitrite per gram per hour after an induction period of 2 hr. Activities remained constant for induction periods of 2, 4, and 6 hr.

When disks of exocarp tissue were incubated for 4 hr in the presence of nitrate substrate with light intensities of nil, 2000, 4000, 8000, and 16,000 lux there was an increase in activity from the nil light treatment to a light intensity of 8000 lux. Light saturation occurred above this intensity. This result is similar to those of Chen and Ries (1969) and Travis, Jordan, and Huffaker (1970).

Using the above data an incubation time of 4 hr and a light intensity of 16,000 lux were adopted for light induction studies on fruit tissues.

The effect of light (L) and light plus exogenous nitrate (LN) on nitrate reductase activity in four types of tissue at three stages of maturity were measured. In tissues other than exocarp there was no significant increases in the presence of L or LN and activities remained at the endogenous levels which are presented in Figure 1.

Enzyme activities in exocarp of fruits at the green and mature green stages of maturity showed significant increases above endogenous levels due to induction by L and LN. In the green fruit (Fig. 2) enzyme activity was 111 and 176 nmoles nitrite
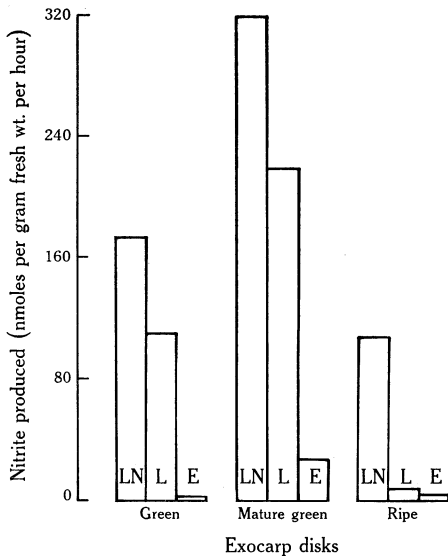

Fig. 2.—Endogenous and induced activities of nitrate reductase in papaw fruits at three stages of maturity: E, endogenous nitrate reductase activity; L, nitrate reductase activity in presence of light only; LN, nitrate reductase activity in presence of light plus exogenous nitrate. Differences according to Duncan's multiple range test: $p = 2$, $R_p = 91 \cdot 3$; $p = 3$, $R_p = 95 \cdot 8$.

per gram per hour for the L and LN treatments respectively. The activities in exocarp of mature green fruit were approximately double those of the green fruit for the equivalent treatments (Fig. 2). The presence of exogenous nitrate had no significant effect on the induction by light in exocarp from green fruit, but exogenous nitrate did produce a significant increase in activity in exocarp from mature green fruit.

In ripe fruit, L did not give a significant increase in activity above the endogenous level of nitrate reductase activity (Fig. 2). However, the tissue did show a significant increase in activity in the presence of LN, the activity being approximately one-third of that obtained for mature green fruit.

Whole green fruits without added nitrate substrate were incubated at 27°C, one sample in the presence of light, the other in the dark. Nitrate contents were measured over the period 24–72 hr after the commencement of treatments. The calculated amounts of nitrate reduced per gram fresh weight of fruit per hour in the presence and absence of light were 11·5 and 7·0 nmoles respectively. Internal fruit tissues were found to be sterile at the conclusion of the incubation period.

(e) Nitrate Levels in Stems, Bark, Leaves, and Fruit

Sap extracts from stems, bark, and leaves contained 8, 5, and 3 μmoles nitrate per millilitre respectively. The nitrate content of fruit parts at the three stages of maturity are given in Figure 3. Nitrate levels in seed and mesocarp of green fruits
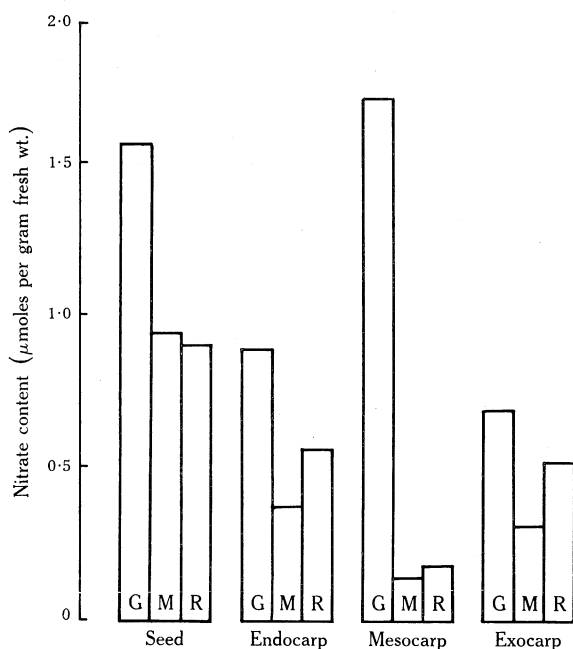

Fig. 3.—Nitrate content of fruit tissues at three stages of maturity: G, green; M, mature green; R, ripe. Differences according to Duncan's multiple range test: $p = 2$, $R_p = 0·38$; $p = 3$, $R_p = 0·40$.

were significantly higher than corresponding portions of the more mature fruits, the mesocarp of green fruits being approximately 10 times higher than that of mature green and ripe fruits. On an absolute basis, the amount of nitrate in the mesocarp of green, mature green, and ripe fruits was 538, 58, and 86 μmoles respectively. In mature green and ripe fruits the nitrate content was approximately 1 μmole/g in seed and 0·16 μmole/g in mesocarp. There were no significant differences between the nitrate contents of the endocarp, mesocarp, and exocarp of mature green and ripe fruits.

(f) *Nitrate Movement within Fruits*

Translocation patterns to various portions of the fruit were demonstrated using rhodamine dye (Fig. 4). Similar distribution patterns were obtained at the three stages of maturity. As shown by the intensity of the rhodamine fluorescence, nitrate entering the pedicel would have ready access to the endocarp. The vascular system branched into the mesocarp and seed and this would facilitate lateral distribution of materials transported into the endocarp. The main vascular bundles located in the mesocarp do not appear to branch and may not be effective in distributing solutes in the mesocarp and exocarp.
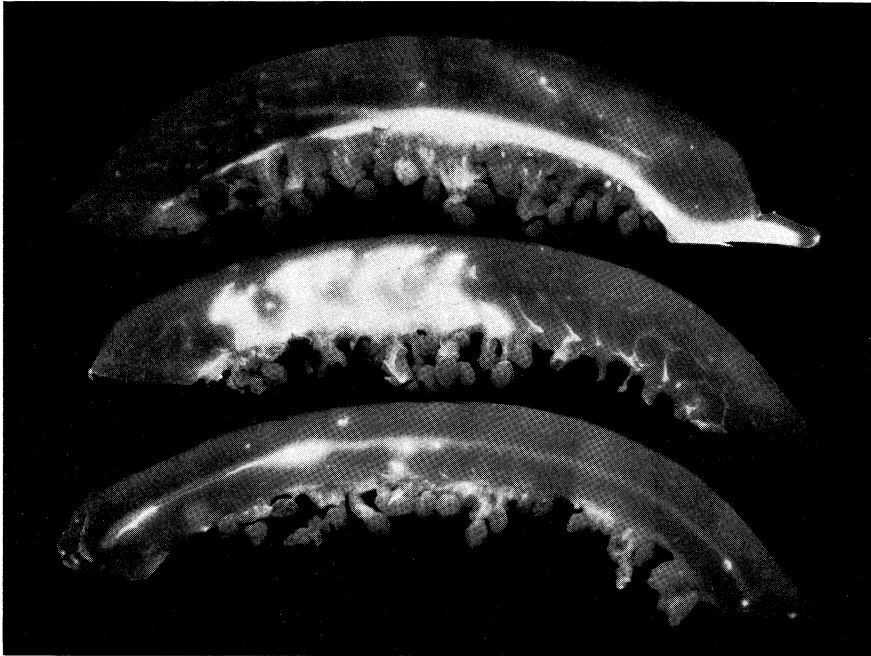

Fig. 4.—Distribution of vascular elements in longitudinal slices of green papaw fruit. Rhodamine in the vascular tissue is shown fluorescing under ultraviolet light. Top slice shows concentration of rhodamine in the endocarp; middle slice, the lateral distribution from endocarp to seeds and mesocarp; lower slice, the lack of lateral distribution from the vascular strand in the mesocarp.

(g) *Water Loss from Fruits*

Excised green fruits with pedicels immersed in distilled water were held in a growth cabinet for 48 hr. The cabinet temperatures were 18°C night and 24°C day, relative humidity 50%, light intensity 16,000 lux ,and day length 12 hr. Water was transpired at the rate of 12 ml/day.

(h) *Water Movement into Fruits*

Estimates of water uptake via the xylem were calculated from calcium content of green fruits and a measured xylem sap concentration of $3 \cdot 2$ μmoles/ml. This calculation was made according to the method described by Wiersum (1966). Calcium

determinations were made on the various fruit portions. Concentrations of calcium
on a whole fruit basis were computed from known moisture contents and the percent-
age contribution of each portion of fruit [see tabulation, Section III(c)(iv)]. The
calcium contents on a fresh weight basis for green, mature green, and ripe fruit were
15·4, 13 and 9 μmoles/g respectively. From fresh fruit data [tabulation, Section
III(c)(iv)] it was apparent that the decrease in calcium concentration with increasing
maturity from the green stage was due to growth dilution. A mean volume of water
of 19 ml/day would need to be transpired to account for the calcium accumulated.
This is a mean value over the 108 days from fruit set to the green stage of maturation.
Actual water loss from excised green fruit was 12 ml/day. On this basis, the theoretical
estimate of water influx to green fruit would appear to be a reasonable estimate of
water loss from attached fruit.

IV. Discussion

The induction of nitrate reductase in the exocarp of green and mature green
fruit by L is evidence that a low light intensity due to shading from the leaf canopy
is limiting nitrate reductase activity. This is further supported by light measurements
taken under the canopy (2000 lux) that were below the light saturation value of 8000
lux. The role of light in inducing the enzyme is a matter of controversy (Beevers
et al. 1965; Chen and Ries 1969; Travis, Huffaker, and Key 1970). These authors
suggest that light affects nitrate absorption and translocation or the maintenance of
protein synthesis via the formation of polyribosomes. Since no exogenous nitrate was
present in the L treatments, the intracellular movement of nitrate may have been
affected. The results of Hageman, Flesher, and Gitter (1961) suggest that nitrate may
be accumulated under shady conditions, being retained in vacuoles and not available
as an inducer. The fact that LN gave an increase in activity over L for mature green
fruit is evidence that nitrate substrate was limiting but this effect was probably due to
the low level of endogenous nitrate in mature green exocarp (0·3 μmole/g fresh wt.).

The endogenous level of nitrate in the exocarp of ripe fruit was similar to that
in mature green fruit. However, L did not induce nitrate reductase activity in the
exocarp of ripe fruit and it was only in the presence of LN that significant induction
occurred. This suggests that the accumulated nitrate in senescing cells, presumably
in vacuoles, is not free to move into the cytoplasm under the influence of light. This
phenomenon has been reported in non-photosynthetic tissue by Heimer and Filner
(1971) and Lycklama (1963). They found that accumulated nitrate was not involved
in nitrate reduction. High levels of endogenous nitrate will accumulate in the meso-
carp of ripe fruits when grown under field conditions (Kruger and Menary 1968).
Should the endogenous nitrate be unavailable for reduction, this condition would
preclude effective reduction of nitrate reserves during post-harvest storage of fruit
prior to canning.

Estimates of potential nitrate reduction in whole green fruit can be made from
the in vivo estimations of nitrate reductase activity in the endocarp, mesocarp, and
exocarp (Fig. 1) and the proportions of tissue contributed by each type [tabulation,
Section III(c)(iv)]. Based on these calculations, 10 nmoles of nitrate could be reduced
per gram per hour. In the presence of light the rate would be increased to

11 nmoles nitrate per gram per hour (Fig. 2). The actual rates of reduction recorded in whole green fruits in the presence of light and in the dark were 11·5 and 7 nmoles nitrate per gram per hour, respectively. Although there is a discrepancy between these activities, this might be expected on the basis of altered distribution patterns of nitrate between mesocarp, exocarp, and endocarp in excised fruits as compared with attached fruit. The pattern of distribution of rhodamine (Fig. 4) in whole fruits provides such evidence.

Similar estimates of nitrate reduction on mature green fruits indicate that they have three times the capacity of green fruit to reduce nitrate in the light. The three stages of maturity that were chosen corresponded with the pre-climacteric, climacteric, and post-climacteric stages of physiological maturity. The mature green fruit being in the climacteric phase of maturation would have the highest rates of respiration and protein synthesis and, therefore, the greatest potential for nitrate reduction.

Normal Hoaglands solution has a nitrate content of 15 μmoles/ml. In spite of this high level, the nitrate reductase activity in roots and stems was low. This low activity would favour accumulation of nitrate in fruit direct from the transpiration stream. The presence of 8 μmoles nitrate per millilitre of xylem sap supports this view.

Calcium content of fruits provides evidence that xylem transport to fruits ceases after fruits reach the green stage of maturity. Mature green fruits are, therefore, receiving nitrate via the phloem only. If one assumes a mass flow hypothesis for the movement of nitrate into fruits via the phloem, and that xylem sap contains more nitrate than phloem sap, then influx of nitrate into mature green fruits would be less than that for green fruit. In addition phloem transport of nitrate into green fruits may be operative. Therefore, the lower net influx of nitrate to mature green fruit and their higher nitrate reductase activity particularly in the light as compared with green fruit could explain the lack of nitrate accumulation in the mature green fruit as compared with green fruit.

It is well known that the inorganic composition of the xylem sap will vary with changes in environmental conditions. Accepting this known variability there is some justification for calculating net influx of nitrate into fruits in order to check the validity of *in vivo* estimates of the nitrate-reducing capacity of fruits. Using the measured value of 8 μmoles/ml as the concentration of nitrate in xylem sap and a daily water uptake of 19 ml per green fruit, the rate of influx of nitrate to the fruit would be 6·3 μmoles/hr. The total capacity of each green fruit to reduce nitrate, based on *in vivo* estimates in the light, is 5 μmoles/hr which is inadequate to cope with the influx of nitrate. Each mature green fruit has a capacity of 8 μmoles/hr but as nitrate influx is via the phloem the amount reaching the fruit would be 4 μmoles/hr. Thus mature green fruits would appear to have sufficient capacity to reduce incoming nitrate.

As fruits mature from the green to the ripe condition, the source of nitrate, its accessibility, and the location of sites of reduction show marked changes. In green fruit, the highest nitrate reductase activity is in the endocarp, but when this tissue shows the first sign of colour development as in mature green fruit, its activity falls. This loss may be associated with senescence. In mature green fruit, the highest induced activity occurs in the exocarp. In the presence of light, whole mature green

fruits have three times the capacity of green fruit to reduce nitrate. This could explain the effective depletion of nitrate in mature green fruit.

If one assumes that the fruit is a sink for nitrate, the amount of accumulation will be determined by the balance between the activity of the nitrate reductase system in the fruit and the rate of influx of nitrate from roots and shoots and the accessibility of accumulated substrate. The evidence reported in this paper suggests that changes in location and activity of nitrate reductase in relation to fruit maturation could explain either accumulation or depletion of nitrate in green or mature green fruit, while in ripe fruit the nitrate which accumulated in the exocarp may be unavailable for reduction.

V. ACKNOWLEDGMENTS

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