

ACID METABOLISM IN *ATRIPLEX*

I. REGULATION OF OXALATE SYNTHESIS BY THE APPARENT EXCESS CATION ABSORPTION IN LEAF TISSUE

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Summary

The leaves of *Atriplex* and eight related genera of the Chenopodiaceae from semi-arid Australia contained high levels of oxalate. Most of the oxalate was in the soluble form and was correlated with high cation content generally rather than with calcium level. In water-culture experiments calcium absorption and oxalate synthesis varied independently.

Other culture experiments with various levels of salts or over a time course showed that oxalate production was geared to the excess cation content of leaf tissue ($\Sigma \text{Na}^+ + \text{K}^+ - \text{Cl}^- - \text{NO}_3^-$). Oxalate balanced 75% of this cation excess in *A. spongiosa* leaves. Changes in the cation excess and insoluble nitrogen content of growing leaves were similar suggesting that cation excess arose principally from the metabolic incorporation of nitrate. Soluble nitrate levels remained low in growing leaves but exudation experiments showed that nitrate transport to the shoot was considerable, implying that nitrate was incorporated into organic form in leaf tissues.

These experiments indicate that oxalate is synthesized in *Atriplex* leaves in response to the cation excess created by metabolic incorporation of nutrient anions, such as nitrate, during growth. It is likely that oxalate and cations are accumulated to the vacuole of leaf cells.

I. INTRODUCTION

Olsen (1939) showed that the absorption of calcium by leaves of several species was related to the production of oxalate. Wood (1925) provided microscopic evidence for the presence of calcium oxalate deposits in saltbush leaves and considered the bulk of leaf calcium to be in this form. Quantitative evidence for high levels of oxalate in *Atriplex* was given by Mathams and Sutherland (1952). For these reasons it was considered important to examine the relationship between oxalate synthesis and ion absorption in *Atriplex*, particularly during the absorption of high levels of calcium (Osmond 1966).

This paper reports analyses for oxalate in a wide range of semi-arid Chenopodiaceae of which the genus *Atriplex* is typical. In an earlier paper (Osmond 1963) oxalate was shown to be the principal anion in *Atriplex* leaves and was largely responsible for cation balance. Experiments described here further expand the role of oxalate synthesis during ion uptake and growth of saltbush leaves.

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II. MATERIALS AND METHODS

(a) *Plant Material*

Leaf material from most genera of the semi-arid Chenopodiaceae growing in western New South Wales and Queensland was collected during field trips. Usually several bushes at each site were sampled and the mixed material used for analysis. Samples were stored at air temperature for several days before drying and analysis. Leaves were also taken from plants grown in the glasshouse. Two types of water-culture experiments were done:

- (1) Static experiments in which leaf material was removed from seedlings after a fixed growth interval on different cultures. Leaf material from *Atriplex inflata* and *A. spongiosa*, grown in experiments described earlier (Osmond 1966) was used. In addition, *A. spongiosa* seedlings were grown for 18 days in aerated culture solutions supplemented with 5, 10, 30, or 100 m-equiv/l of NaCl or NaNO₃ (base culture: 5 mM Ca(NO₃)₂, 2 mM KNO₃, 1 mM KH₂PO₄, NH₄H₂PO₄, and MgSO₄, with Fe-EDTA and micronutrients). Samples of young leaves and leaves which matured during the experiment were taken for analysis. Mature leaves were also taken from seedlings of the same species grown in culture solutions containing added chloride and nitrate in different ratios.
- (2) Dynamic experiments in which *A. spongiosa* seedlings were grown on a base culture solution (2.5 mM Ca(NO₃)₂ and KNO₃, 0.5 mM (NH₄)₂HPO₄, NaH₂PO₄, and MgSO₄ with Fe-EDTA and micronutrients) and harvested at intervals after transfer to the same nutrient solution, supplemented with 50 mM NaNO₃. At harvest (2, 5, 7, and 9 days after transfer) leaves from one plant from each of 5 or 10 replicated pots were separated into six categories. These categories were designated L₁–L₄ (L₁ being the first pair of leaves formed on the main axis, L₂ the second, etc.) and BL₁ and BL₂, the first two pairs of leaves formed on the branch in the axil of L₁. L₄ and BL₂ were too small for harvest at the start of the experiment.

Seedlings of the same species were grown to the six-leaf stage in a half-strength base culture solution and pots of four seedlings decapitated for collection of exudate. Exudation proceeded for several hours in a saturated atmosphere under a glass cover. The exudate was collected in 100-mm Microcap capillaries of 50 µl capacity at 10-min intervals. The volume collected in successive 30-min intervals was measured and transferred to test tubes for analysis. Exudates were collected from uniform plants at the same time at intervals of 2 or 3 days and the tops of plants used were dried and analysed for changes in ion content.

(b) *Analytical Methods*

All samples were dried at 70°C and ground to a fine powder before analysis. Two methods of oxalate extraction were used:

- (1) Total organic acids were recovered by continuous ether extraction of the acidified dry plant material after the methods of Pucher, Wakeman, and Vickery (1941). The ether was evaporated and acids transferred to an

aqueous alkaline solution, aliquots of which were taken for total acid titration or oxalate precipitation.

- (2) Soluble oxalates were recovered by repeated hot water extraction of the dried material (Palmer 1955).

The oxalate content of either extract was estimated by permanganate titration of the hot, acidified calcium oxalate precipitate (Pucher, Wakeman, and Vickery 1941). Soluble protein in the water extract was removed by sodium phosphotungstate precipitation and calcium oxalate precipitated with calcium chloride-calcium acetate buffer (Baker 1952). Ether extraction gave complete recovery of added anhydrous calcium oxalate with duplicate variation of $\pm 3\%$, but variation during hot water extraction was $\pm 5\%$.

Cations were estimated after dry ashing or hot water extraction using methods described previously (Osmond 1966). Chloride was measured by potentiometric titration (Johnson and Ulrich 1959) and nitrate by the phenoldisulphonic acid method with precautions against chloride interference (Jackson 1958; Johnson and Ulrich 1959). These analyses showed $\pm 3\%$ variation between duplicates. Amino nitrogen in exudates was measured by the ninhydrin method of Yemm and Cocking (1955). Insoluble nitrogen in water-extracted plant material was estimated by the micro-Kjeldahl methods of McKenzie and Wallace (1954) which gave duplicate values to $\pm 2\%$. This insoluble nitrogen was taken as a measure of organically incorporated nitrate and was expressed as μ -equivalents of nitrate.

III. RESULTS

(a) *Static Experiments*

(i) *Oxalate Levels in the Leaves of Semi-arid Chenopodiaceae*

The effect of the delay between collection and analysis of field samples on the oxalate concentration of leaves was checked by measuring total oxalates in *A. nummularia* and *A. spongiosa* leaves kept at room temperature after sampling. Leaves kept for up to 14 days showed a 10% increase in oxalate concentration on a dry weight basis, presumably because changes in dry weight were greater than changes in oxalate. This suggests that oxalate may be relatively inert in *Atriplex* leaves, as it is in other tissues (Baker and Eden 1954).

Mature leaves from most semi-arid Chenopodiaceae were found to contain high levels of oxalate (Table 1). These values are in good agreement with those found in *A. semibaccata* and *Salsola kali* by Mathams and Sutherland (1952) and in the related genus *Halogeton glomeratus* (Morton, Haas, and Erikson 1959). High oxalate levels in the leaves of these plants were associated with high inorganic cation concentrations but there was no relation between calcium concentration and oxalate level (Table 2).

(ii) *Oxalate Synthesis and Calcium Absorption*

The extensive absorption of calcium from calcium chloride solutions by *Atriplex* spp. (Osmond 1966) was not accompanied by net synthesis of oxalate [Fig. 1(a)].

In *A. inflata* total oxalate level declined as calcium absorption increased and similar results were found in *A. vesicaria*.

TABLE 1
OXALATE IN THE LEAVES OF VARIOUS SPECIES OF SEMI-ARID CHENOPODIACEAE

| Species | No. of Samples | Mean Oxalate Concn.* | Species | No. of Samples | Mean Oxalate Concn.* |
|---------------------------|----------------|----------------------|--------------------------------|----------------|----------------------|
| <i>Atriplex angulata</i> | 2 | 2.69 | <i>Babbagia acroptera</i> | 2 | 3.56 |
| <i>A. bracteata</i> | 1 | 2.12 | <i>Bassia bicornis</i> | 1 | 3.32 |
| <i>A. conduplicata</i> | 2 | 2.66 | <i>B. birchii</i> | 1 | 2.08 |
| <i>A. inflata</i> | 2 | 3.49 | <i>B. divaricata</i> | 2 | 3.49 |
| <i>A. leptocarpa</i> | 1 | 2.42 | <i>B. lambagii</i> | 1 | 2.94 |
| <i>A. semibaccata</i> | 1 | 2.75 | <i>B. longicuspis</i> | 1 | 2.23 |
| <i>A. spinebractea</i> | 1 | 1.87 | <i>B. obliquicuspis</i> | 1 | 2.49 |
| <i>A. spongiosa</i> | 2 | 2.74 | <i>B. paradoxa</i> | 3 | 3.30 |
| <i>A. vesicaria</i> | | | <i>B. uniflora</i> | 1 | 2.97 |
| Broad-leaved form | 8 | 1.54 | <i>Chenopodium auricomum</i> | 2 | 4.16 |
| Narrow-leaved form | 9 | 1.38 | <i>Dysphania plantaginella</i> | 2 | 1.38 |
| <i>Kochia astrotricha</i> | 1 | 2.05 | <i>Enchylaena tomentosa</i> | 3 | 2.90 |
| <i>K. georgei</i> | 1 | 1.49 | <i>Rhagodia spinescens</i> | 3 | 3.05 |
| <i>K. pyramidata</i> | 1 | 3.14 | <i>Salicornia australis</i> | 2 | 0.68 |
| <i>K. sedifolia</i> | 1 | 3.55 | <i>Salsola kali</i> | 1 | 4.69 |
| <i>K. triptera</i> | 2 | 1.51 | | | |

* As m-equiv. oxalic acid per gram dry weight.

Independent changes in the total oxalate and calcium concentration in *A. spongiosa* leaves were found in other experiments. With increasing calcium nitrate concentration, leaf calcium level remained constant but oxalate level declined appreci-

TABLE 2
CATIONS AND TOTAL OXALATE IN LEAVES OF VARIOUS SEMI-ARID CHENOPODIACEAE
Values in milliequivalents per gram dry weight

| Species | Na ⁺ | K ⁺ | Ca ²⁺ | Mg ²⁺ | Oxalate |
|--------------------------------|-----------------|----------------|------------------|------------------|---------|
| <i>Bassia divaricata</i> | 5.94 | 0.42 | 0.41 | 0.25 | 2.07 |
| <i>Bassia uniflora</i> | 6.00 | 0.70 | 0.87 | 1.37 | 2.97 |
| <i>Chenopodium auricomum</i> | 3.61 | 0.37 | 0.96 | 1.29 | 4.43 |
| <i>Dysphania plantaginella</i> | 0.16 | 1.25 | 0.94 | 0.50 | 1.64 |
| <i>Enchylaena tomentosa</i> | 4.51 | 0.95 | 0.41 | 0.29 | 2.36 |
| <i>Kochia pyramidata</i> | 3.93 | 0.70 | 0.39 | 0.26 | 2.85 |
| <i>Kochia triptera</i> | 3.59 | 0.95 | 0.26 | 0.23 | 1.75 |
| <i>Rhagodia spinescens</i> | 3.90 | 1.16 | 0.77 | 0.95 | 2.77 |
| <i>Salsola kali</i> | 2.08 | 1.25 | 1.91 | 0.96 | 4.69 |

ably [Fig. 1(b)]. The leaves of seedlings grown in solutions with increasing sodium nitrate concentration increased in oxalate concentration while calcium level remained constant [Fig. 1(c)]. Oxalates represented 60–70% of the total titratable organic

anions in these leaves and changes in total acids were largely due to changes in oxalate level. These changes were similar to changes in total cation concentration in leaf tissue [Figs. 1(b) and 1(c)].

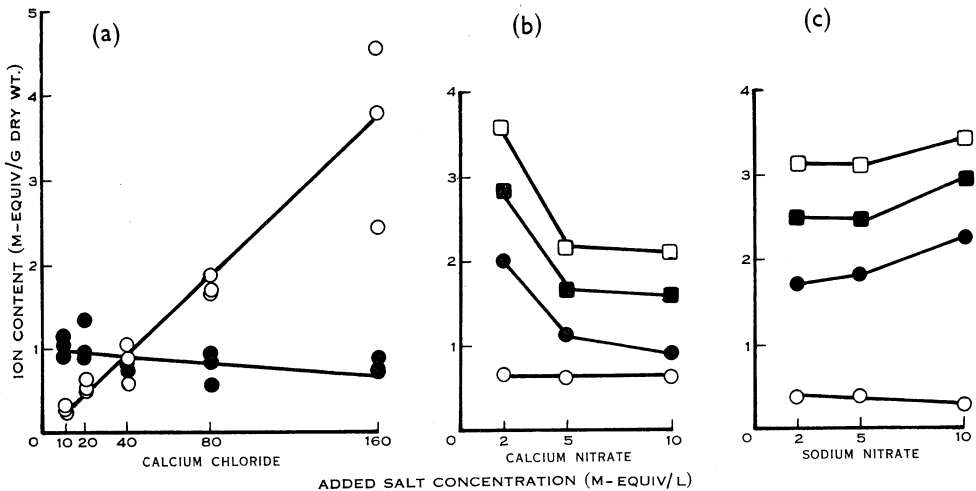


Fig. 1.—Oxalate synthesis and calcium absorption in leaves of *Atriplex nummularia* (a) and *A. spongiosa* (b, c) grown in different culture solutions. □ Total cations. ○ Ca^{2+} . ● Oxalate. ■ Total acids.

(iii) Oxalate and the Balance of Inorganic Ion Absorption

The ion content of young and mature leaves of *A. spongiosa* grown in solutions with increasing sodium nitrate or sodium chloride concentration is shown in Figure 2.

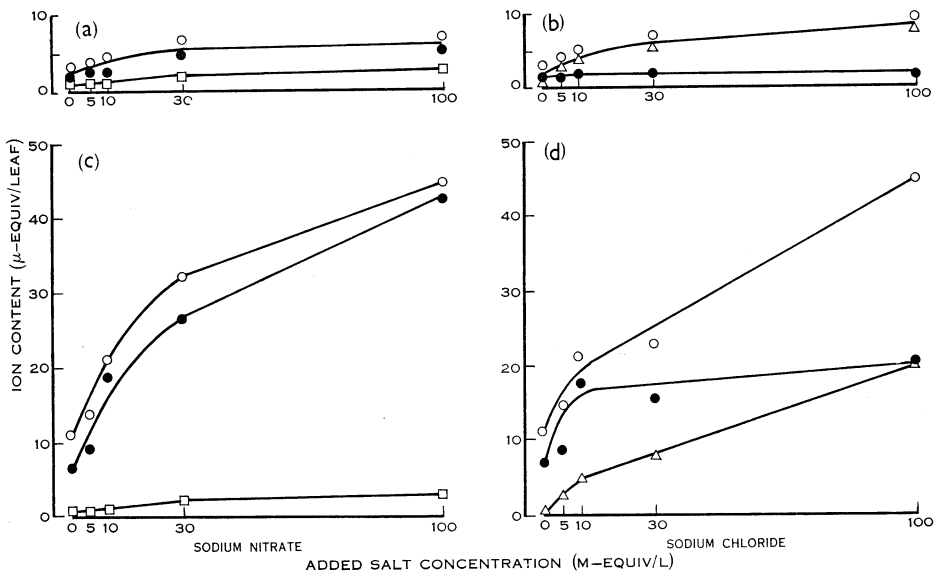


Fig. 2.—Oxalate synthesis and ion content of young (a, b) and mature (c, d) *A. spongiosa* leaves after growth in different culture solutions. ○ $\text{Na}^+ + \text{K}^+$. □ Nitrate. ● Oxalate. △ Chloride.

In mature leaves the ion content increased over the whole range but young leaves showed little increase above 10–30 m-equiv/l of either salt. Leaves from plants grown on the same concentration of nitrate or chloride had about the same ion content but the balance of ions was different. In plants grown on nitrate cultures the sodium and potassium content was largely balanced by soluble oxalate, the small deficit being made up by nitrate. A similar ion balance was found in leaves of plants on cultures of low NaCl concentration. At 30 and 100 m-equiv/l NaCl, however, a large proportion of cation content was balanced by chloride and the contribution of oxalate to ion balance was reduced.

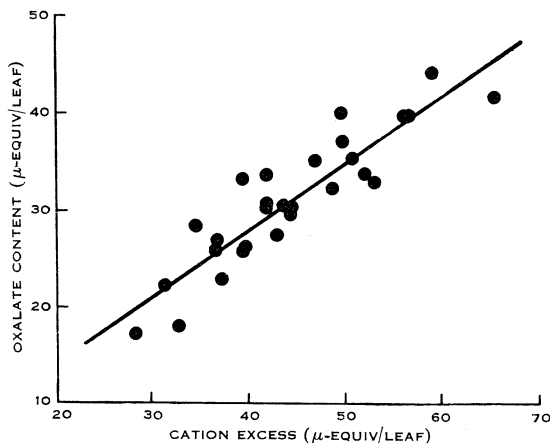


Fig. 3.—Relationship between oxalate content and cation excess ($\Sigma \text{Na}^+ + \text{K}^+ - \text{NO}_3^- - \text{Cl}^-$) in mature *A. spongiosa* leaves.

A similar difference in ion balance was found in young leaves. In plants grown on sodium nitrate cultures, cations were balanced by oxalate and these leaves contained the same amount of nitrate as mature leaves. Chloride balanced most of the cations in the young leaves of plants grown on sodium chloride cultures and oxalate content remained constant over the whole range.

In another experiment with *A. spongiosa* grown in solutions of different nitrate and chloride concentrations the soluble oxalate content of mature leaves was closely related to the excess inorganic cation content [$\Sigma(\text{Na}^+ + \text{K}^+ - \text{Cl}^- - \text{NO}_3^-)$ μ -equiv/leaf] as shown in Figure 3. The slope of this curve shows that oxalate balances about 75% of the cation excess measured by these analyses.

(b) Dynamic Experiments

(i) Oxalate, Cation Excess, and Nitrogen Incorporation during Growth

The growth and changes in ion content of different leaves of *A. spongiosa* seedlings are shown in Figure 4. Old leaves (L_1) which did not increase in dry weight during the experiment showed little change in oxalate or cation excess. Leaves L_2 , L_3 , and BL_1 increased in dry weight during the experiment but at declining relative growth rate. Increases in cation excess and oxalate were more or less parallel in these leaves and in rapidly growing leaves L_4 and BL_2 . As shown in Figure 5(a), increments in the oxalate content of leaves between harvests were related to increments

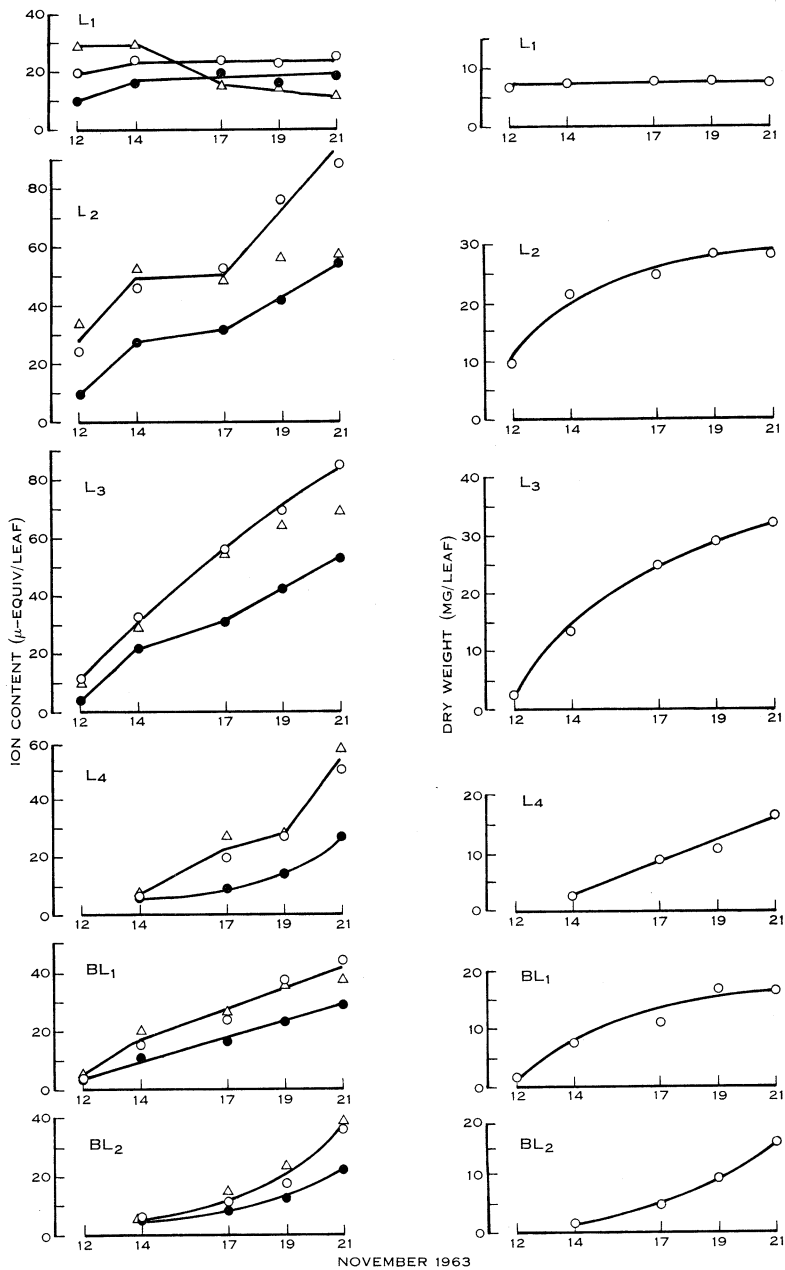


Fig. 4.—Changes in ion content and dry weight of *A. spongiosa* leaves during growth. \circ Cation excess ($\Sigma \text{Na}^+ + \text{K}^+ - \text{NO}_3^- - \text{Cl}^-$). \bullet Oxalate. \triangle Insoluble nitrogen. Leaf categories L₁–L₄, BL₁, and BL₂ as in text [see Section II(a)].

of cation excess in the same interval. The curve is drawn for a 75% balance of cation excess by oxalate, a value found in other experiments, and is a reasonable fit to the data of this experiment.

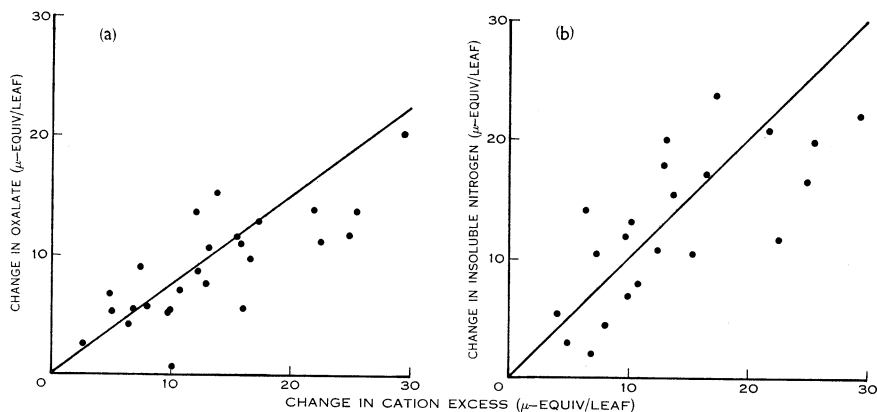


Fig. 5.—(a) Changes in oxalate and cation excess in *A. spongiosa* leaves between harvests. Theoretical curve for 75% balance of cation excess by oxalate. (b) Changes in insoluble nitrogen and cation excess in growing *A. spongiosa* leaves between harvests. Theoretical curve for 1:1 relationship. Data taken from Figure 4.

Changes in the insoluble nitrogen content of leaves are also shown in Figure 4, plotted as equivalents of nitrate nitrogen. These changes were related to the growth

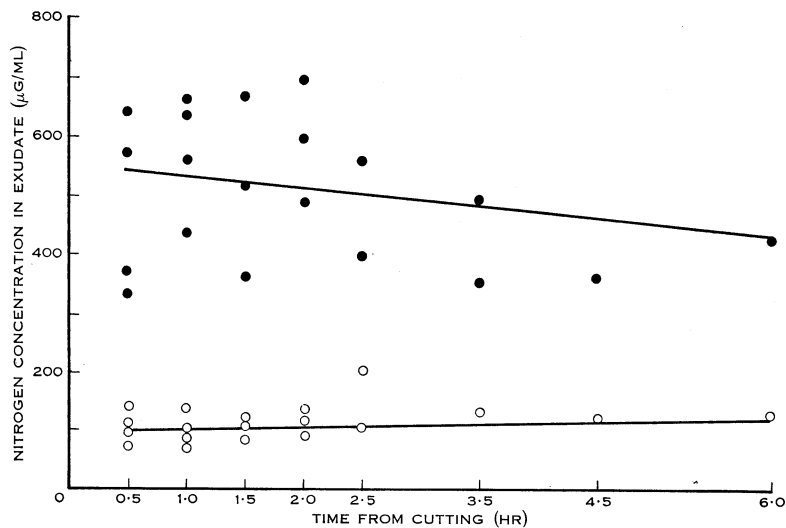


Fig. 6.—Concentration of nitrogen in exudate collected from decapitated *A. spongiosa* seedlings. ● Nitrate nitrogen. ○ Amino nitrogen.

characteristics of the different leaves. Insoluble nitrogen increased in rapidly growing leaves (L_4 , BL_1 , BL_2); increased to a steady level in leaves which were declining in growth rate (L_2 , L_3); and declined throughout in non-growing leaves (L_1). These

changes were also related to changes in ion content. In leaves which increased in dry weight, cation excess, insoluble nitrogen, and oxalate increased similarly (L_4 , BL_1 , BL_2). When growth slowed oxalate and cation excess continued to increase independently of insoluble nitrogen (L_2 , L_3) or remained steady while insoluble nitrogen declined (L_1). Changes in the insoluble nitrogen and cation excess of leaves which increased in dry weight were calculated from data in Figure 4 and plotted in Figure 5(b). The curve is drawn to a 1 : 1 relationship and is a reasonable fit to the data of this experiment.

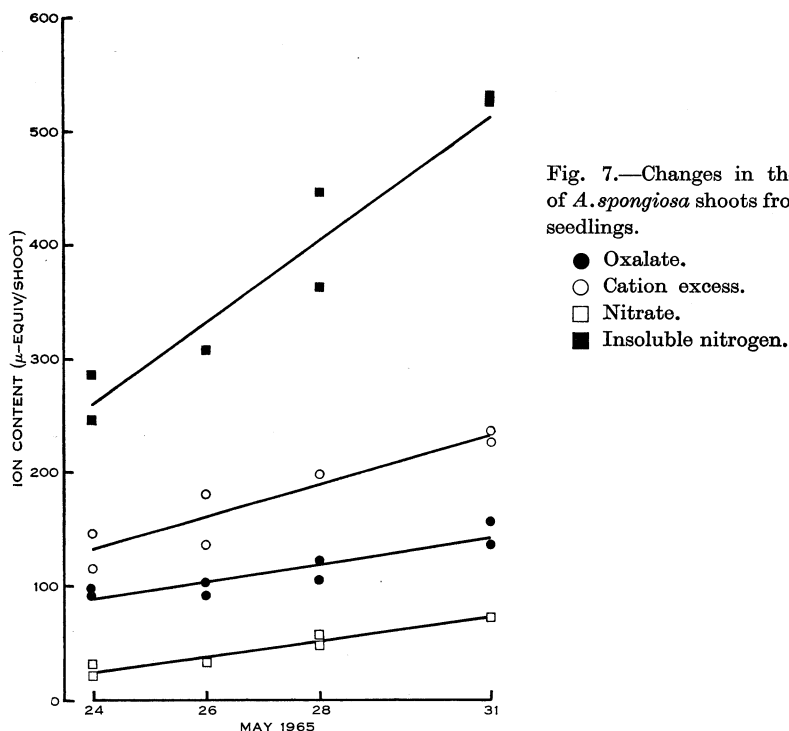


Fig. 7.—Changes in the ion content of *A. spongiosa* shoots from decapitated seedlings.

- Oxalate.
- Cation excess.
- Nitrate.
- Insoluble nitrogen.

(ii) Exudation Experiments

In these experiments the nitrogen composition of stem exudates was determined and compared with increases in the nitrogen content of shoots. Exudation occurred only in the morning and rate of exudation declined rapidly after cutting. Variation in rate was large and was independent of seedling age. The composition and concentration of nitrogen in exudates did not change appreciably with declining rate. As shown in Figure 6, the concentration of nitrate was always 3–5 times greater than amino nitrogen.

At maximum exudation rates ($15 \mu\text{l/plant/hr}$) and average nitrogen concentrations ($500 \mu\text{g}$ nitrate, $100 \mu\text{g}$ amino nitrogen/ml; i.e. $43 \mu\text{-equiv. nitrogen/ml}$), each plant received approximately $15 \mu\text{-equiv. nitrogen per day}$, only 35% of the observed rate of increase in the nitrogen content of the decapitated portion of the plants

shown in Figure 7 ($36 \mu\text{-equiv. insoluble nitrogen} + 7 \mu\text{-equiv. nitrate per day}$). This discrepancy emphasizes the difficulty in evaluating the relationship between exudation and translocation in the transpiring plant.

However, these experiments show high rates of nitrate exudation, and support the notion that oxalate may be produced in response to the incorporation of nitrate in leaf tissues.

IV. DISCUSSION

These experiments confirm the importance of oxalate in the balance of cations in *Atriplex* leaves (Osmond 1963) and demonstrate the widespread occurrence of this phenomenon in leaves of the semi-arid *Chenopodiaceae*. Black (1960) attributed the balance of cation excess in *Atriplex* leaves to a Donnan equilibrium. This suggestion implied an exchange capacity of some tenfold that normally found in plant tissues.

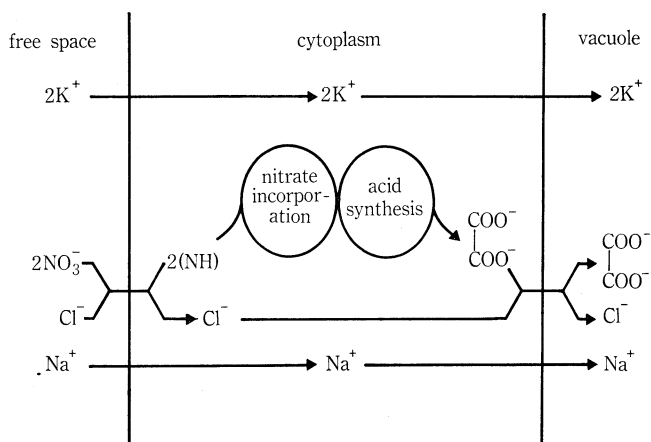


Fig. 8.—Postulated relationship between ion absorption, nitrogen incorporation, and oxalate synthesis in *Atriplex* leaf cells.

However, other experiments (Osmond, unpublished data) show that the exchange capacity of the Donnan free space in *Atriplex* leaf tissue is similar to that of other plant tissues ($15\text{--}20 \mu\text{-equiv/g fresh wt.}$). Donnan adsorption may bind some of the cations in these tissues but the bulk of the cation excess is balanced by soluble organic anions, principally oxalate.

Although *Atriplex* seedlings may absorb high levels of calcium the absorption of this divalent cation was not regulated by a simple system dependent on the precipitation of oxalate as suggested by Olsen (1939) and Burström (1945). In most species examined only a small proportion of the oxalate could have been present in the insoluble form. However, it is probable that most of the calcium in *Atriplex* was bound in this way, as suggested by Wood (1925). The extensive deposits of calcium oxalate crystals are readily seen in cleared leaves but the exact location of the crystals is difficult to determine. Although there is no direct evidence, it is probable that the soluble oxalates are in the vacuole of leaf cells. If soluble oxalate were restricted to the cytoplasm of *A. spongiosa* leaf cells the concentration would be

about 3M, but if the vacuole were occupied as well the concentration would be about 0.2M. Similar calculations applied to other plant tissues suggest that a large proportion of most organic anions are in the vacuole (MacLennan, Beevers, and Harley 1963).

The role of oxalate in ion balance in *Atriplex* leaves is comparable to that of malate in many other plants (Pierce and Appleman 1943; Burström 1945). However, as Dijkshoorn (1958, 1962) emphasized, the excess cation absorption in growing plants is largely due to the metabolic incorporation of nutrient anions such as nitrate and phosphate. The problem is similar to ion absorption from bicarbonate solutions (Jacobson and Ordin 1954; Hurd 1958) where the bicarbonate absorbed may be directly incorporated into malate, the balancing organic anion. When *Atriplex* was grown in cultures rich in nutrient anions the level of nitrate in leaves was low and did not increase with external concentration or leaf age. Experiments showed that nitrate was the most abundant form of nitrogen in exudates as would be expected if incorporation of nitrate into organic form occurred primarily in leaf tissue. This could then explain the close relationship between changes in insoluble nitrogen, cation excess, and oxalate production in growing leaves.

This *in situ* relationship is opposed to current observations on nitrogen metabolism and transport in other plants, where nitrate is not usually detectable in exudates (Bollard 1957) and it is generally accepted that nitrogen reduction occurs mainly in the roots. Figure 8 shows a model of the suggested interactions between ion absorption, metabolism, and organic acid synthesis in *Atriplex* leaf cells, which receive the bulk of their nitrogen via the xylem in the form of nitrate. It does not distinguish the nature of ion absorption processes at different cell membranes. Although incorporation of nitrate into organic form and oxalate synthesis presumably occur in the cytoplasm of leaf cells, the separation and movement of oppositely charged ions through the cytoplasm is unknown. This separation is important in the case of calcium and oxalate and will determine the site of crystal deposition. The nature of factors controlling acid synthesis in relation to cation excess are unknown and may be related to the accumulation of ions to the vacuole. Accumulation of organic anions from the cytoplasm to the vacuole via an anion pump, similar to that believed to operate for chloride in other tissues (MacRobbie 1964), is probable. These movements of organic anions should be open to investigation by flux techniques similar to those used for inorganic ions.

Organic anion accumulation to the vacuole is implied in a wide range of plant tissues (MacLennan, Beevers, and Harley 1963) and, as shown above, is related to the utilization of nutrient anions during normal growth. De Wit, Dijkshoorn, and Noggle (1963) controlled the growth of cereals via different nutrient regimes and showed depression of growth was reflected in lower organic acid levels. Leaves of plants which grow poorly under saline conditions have a lower organic acid concentration (Bernstein 1961; Osmond, unpublished data). Although the lower organic anion level may account in part for the increased osmotic potential of leaf tissues (Bernstein 1961), another important implication is in regard to growth. Reduced growth and low organic acid levels reflect reduced uptake and incorporation of nutrient anions and suggests that competition between nutrient anions and chloride during absorption

may limit growth in a saline environment. Although the concentration of incorporated nitrogen and phosphorus may be little altered by salinity (Gauch and Eaton 1942), the amount of nutrient ions absorbed is much reduced. Interactions of chloride during the absorption and incorporation of nutrient anions have not been investigated and characterization in simple systems, e.g. excised roots, may be rewarding.

Other important implications of the relationships suggested in Figure 8 have been investigated. These are concerned with the nature of ion absorption processes in leaf tissue and with the biosynthesis of oxalate in *Atriplex* leaves and will be discussed elsewhere.

V. ACKNOWLEDGMENTS

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