H. Breteler

*Department of Soils and Fertilizers, Agricultural University, Wageningen*

# Carboxylates and the uptake of ammonium by excised maize roots

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# Abstract

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The effect of carboxylates (organic acid anions) on $NH_4$ uptake was studied by changing the carboxylate level of roots prior to uptake experiments. Succinate was the most effective stimulator of ammonium uptake. The oxocarboxylates (α-oxoglutarate, oxaloacetate and pyruvate) and malate also promoted $NH_4$ entry. Preloading of roots with citrate, acetate, oxalate, glyoxylate or malonate reduced subsequent ammonium uptake. It is concluded that oxocarboxylates are important compounds in both the assimilation and the uptake process of ammonium. All carboxylates tested enhanced K uptake, but repressed $NO_3$ uptake, except citrate which increased nitrate absorption. Carboxylate stimulation of $NH_4$ entry showed metabolic as well as non-metabolic aspects.

Keywords: maize, excised roots, ammonium uptake, potassium uptake, nitrate uptake, carboxylates, organic acids, keto acids.

# Contents

# List of abbreviations

A = sum of inorganic anions ($Cl+H_2PO_4+SO_4+NO_3$) in meq/kg DM ($H_2PO_4$ stands for total P)
AA = ash alkalinity
AFS = apparent free space
ATP = adenosine triphosphate
C = sum of inorganic cations (Ca+Mg+Na+K) in meq/kg DM
C-A = cation excess in plants, meq/kg DM; data are exclusively $NH_4$ ions, unless otherwise stated
Ci = citrate
DFS = Donnan free space
DM = dry matter
F = fumarate
FAD = flavin adenine dinucleotide
FW = fresh weight
G = glyoxylate
M = malate
MO = malonate
NAD(P) = nicotinamide adenine dinucleotide (phosphate)
αOG = α-oxoglutarate
OXAC = oxaloacetate
PYR = pyruvate
SUCC = succinate
TCA = tricarboxylic acid(cycle)
WFS = water free space
WSC = water-soluble carbohydrates

In the text ionic species are usually represented by their chemical symbols, omitting charge signs, e.g. $NH_4$ instead of $NH_4^+$.

# 1 Introduction

The maintenance of tissue electroneutrality requires that any excess of inorganic cations (C) over inorganic anions (A) must be balanced by an equivalent amount of carboxylates (C-A). This fraction comprises mainly intermediates of the citrate and glyoxylate cycles, but also acetate, amino acids, sugar acids, uronides and other organic anions. Organic cations are of minor importance in plants (Harada et al., 1968; Takaki et al., 1968). For more than a century mineral nutrition has been reported as one of the factors that determine the size of the carboxylate pool in plants (Stohmann, 1864; Pfeffer, 1881; Wehmer, 1891 as cited by van Tuil, 1965).

Processes that generate carboxylates are cation uptake in excess of anion uptake, nitrate assimilation and sulphate assimilation (Ulrich, 1941, 1942; Jacobson & Ordin, 1954; Hiatt, 1967c; Dijkshoorn, 1969). Processes at the expense of the carboxylate pool are anion uptake in excess of cation uptake, and ammonium assimilation (Yemm & Folkes, 1954; Coïc et al., 1961, 1962; Houba et al., 1971). The effect of differential cation and anion uptake on the carboxylate (C-A) content of wheat plants is illustrated in Table 1. Cl is taken up in excess of Ca, the uptake rates for K and Cl are about equal and K is absorbed in excess of $SO_4$. Production of organic sulphur compounds plays a minor role, because the plants used were nitrogen-

Table 1. Effect of one-day exposure to 5 x $10^{-3}$ M solutions of $CaCl_2$, KCl and $K_2SO_4$ on the C-A content (in meq/kg DM) of tops and roots of 3-week-old wheat plants. In the KCl and $K_2SO_4$ media 5 x $10^{-5}$ M $CaCl_2$ was present.

| | Tops | Roots |
|---|---|---|
| $CaCl_2$ | 1098 | 210 |
| KCl | 1180 | 245 |
| $K_2SO_4$ | 1329 | 384 |

Table 2. C-A content (in meq/kg DM) of tops and roots of plants grown in ammonium or nitrate media. Maize 3 weeks, sugar-beet 6 weeks and wheat 3 weeks old (Breteler, 1973a,b, 1974b).

| | $NH_4$ | | $NO_3$ | |
|---|---|---|---|---|
| | tops | roots | tops | roots |
| Maize | 729 | 157 | 1272 | 226 |
| Sugar-beet | 992 | 598 | 3318 | 1018 |
| Wheat | 390 | 80 | 960 | 168 |

starved (Dijkshoorn & van Wijk, 1967).

Plants grown for several weeks with ammonium or nitrate as sole nitrogen source clearly reflect the effect of nitrogen metabolism on the size of the carboxylate pool (Table 2)[1].

Some authors (Jackson & Coleman, 1959; Hendricks, 1966; Cseh, 1972) put forward the crucial question whether carboxylates are the result or the cause of nutritional events. Many authors have described the influence of inorganic nutrition on the ionic balance and carboxylate content (Chouteau, 1960; Coïc et al., 1961; Noggle, 1966; Dijkshoorn, 1968; Kirkby, 1968).

Hypotheses on ion uptake mechanisms often include a function of carboxylates. This function has been thought mainly passive, e.g. by the supply of non-diffusible anions, by $H_3O^+$-$K^+$ and $HCO_3^-$-$NO_3^-$ exchange, or by accompanying cation passage to the vacuole, etc. (Ben Zioni et al., 1971; Blevins et al., 1974; Dijkshoorn et al., 1968; Haeder & Mengel, 1969; Mengel & Haeder, 1971; Hiatt, 1968; Hiatt & Lowe, 1967; Kirkby, 1974; Marschner, 1968, 1969; Osmond & Laties, 1969; Prins, 1974; Rains, 1972; Raven & Smith, 1973; Robertson et al., 1955a, b, 1958; Schaedle & Jacobson, 1965, 1966; van Steveninck et al., 1973; Torii & Laties, 1966b; Vervelde, 1952). Some authors also stress the metabolic function of carboxylates in ion absorption processes (Cseh, 1972; Hodges, 1973; Leggett, 1968; Neyra & Hageman, 1974; Prins, 1974; Pitman et al., 1971; Sutcliffe, 1962; Vervelde, 1952).

1. The data on sugar-beet plants include free $NH_4$ ions, the other data in this and the following tables are exclusively $NH_4$, unless otherwise stated.

Some information is available on the effect of carboxylates on ion absorption by micro-organisms (Goodman & Rothstein, 1957 in *Saccharomyces cerevisiae*; Budd, 1966 in *Neocosmospora vasinfecta*; Carrodus, 1966 in beech mycorrhiza; Okuda & Ida, 1966a,b in *Chlorella ellipsoidea;* Shaw & Miles, 1970 in *Schizophyllum commune* germlings). Publications on the effect of carboxylates on ion absorption by higher plants are still scarce (Koster, 1963; Ohyama et al., 1966; Jackson & Taylor, 1970; DeKock et al., 1973).

Better understanding of the regulation of nutrient absorption is needed in agriculture to predict fertilizer requirements in relation to production and quality of crops.

The aim of this study was to investigate the role of carboxylates in the uptake of ammonium ions by excised maize roots. In this research carboxylate means mainly aliphatic mono-, di- and tricarboxylic acids and their salts. It was tried to obtain relevant information by changing the carboxylate content of roots and investigating the effect on subsequent ion intake.

Ammonium was chosen because it is usually taken up faster than any other nutrient ion and because it is readily assimilated with the consequence that respiration and carboxylate consumption is enhanced (Beccari et al., 1969; Becking, 1956; Berner, 1971). Maize roots were taken as test material because, with the facilities available, maize plants were easy to cultivate throughout the year and because maize roots had been used for many other ion uptake studies, which could be used for comparison. Excised roots were taken to simplify the study to processes located in the root system.

# 2 Materials and methods

## 2.1 PLANT CULTIVATION

Seeds of maize (*Zea mays L.*, cv. CIV 2 'Prior') were germinated in sieves filled with coarse gravel and moistened with demineralized water. The sieves were placed in openings in the lid of a container with nutrient solution, the content of which was circulated and aerated by an electric pump (Slangen, 1971). The plants were alternately exposed to media without nitrogen (-N) and to complete nutrient solutions for periods of one week. The composition of these media is given in Table 3. Basically the complete solution is a 1/5 Hoagland & Arnon solution (Hewitt, 1962) with increased $NH_4$ and decreased $NO_3$ concentration. The pH of the media was kept between 4 and 7 by adding diluted NaOH or $H_2SO_4$.

After 6 to 7 weeks the plant roots were used in uptake experiments, which were always preceded by at least 3 days growth in the -N medium.

The plants were grown in a greenhouse throughout the year. From late autumn to early spring the greenhouse was heated and artificial light was provided by HPL lamps.

## 2.2 UPTAKE EXPERIMENTS

The whole root systems were detached and rinsed with demineralized water. Usually the roots were incubated in a carboxylate solution prior to ammonium uptake. Controls were incubated in 1 meq $CaCl_2$ $l^{-1}$. After incubation the roots were rinsed again and transferred to pots containing one litre uptake solution. About 25 g fresh roots (1-2 g DM) were used per pot. The conditions of carboxylate incubation and $NH_4$ uptake referred to as standard conditions in the text were as follows.

*Standard carboxylate incubation conditions:* roots in one litre (1 meq $CaCl_2$ with and without 50 mMol Na-carboxylate) $l^{-1}$ solutions, pH 5.5, aeration, room temperature, incubation period 16 hours.

*Standard ammonium uptake conditions:* roots in one litre (1 meq $NH_4Cl$ + 0.1

Table 3. Composition of the nutrient solutions in meq/l.

| | Na | K | Ca | Mg | $NH_4$ | Cl | $H_2PO_4$ | $NO_3$ | $SO_4$ |
|---|---|---|---|---|---|---|---|---|---|
| -K | 2.4 | 0 | 1.2 | 0.8 | 1.4 | 3.0 | 0.4 | 2.0 | 0.4 |
| -N | 0.4 | 2.0 | 2.0 | 0.8 | 0 | 4.0 | 0.4 | 0 | 0.8 |
| Complete | 0.4 | 2.0 | 1.2 | 0.8 | 1.4 | 3.0 | 0.4 | 2.0 | 0.4 |

Trace elements: 0.5 ppm B, 0.5 ppm Mn, 0.4 ppm Fe, 0.05 ppm Zn, 0.02 ppm Cu, 0.01 ppm Mo, 10 ppm N-serve (2-chloro-6-(trichloromethyl)pyridine).

meq $CaCl_2$) $l^{-1}$ solutions, aeration, room temperature, experimental period 4 hours.

Aeration was by compression of air, room temperature 21-23°C. After an experiment the roots were cleaned, dried at 70°C for 24 hours or used immediately, depending on whether fresh or dried material was analysed.

## 2.3 CHEMICAL ANALYSIS

### *2.3.1 Ion uptake*

*Ammonium uptake* was measured from depletion of the ambient solution. In uptake experiments with 1 meq $NH_4$ $l^{-1}$, aliquots of 0.200 ml of the well-mixed solution were pipetted into previously controlled N-free test tubes. At each desired time-interval, solution samples were taken in triplicate and uptake experiments consisted of at least 2 pots per treatment. Samples were assayed for ammonium by the indophenol blue method (Berthelot reaction, c.f. Novozamsky et al., 1974; Rommers & Visser, 1969; Beecher & Whitten, 1970; Brown, 1973; Kempers, 1974). The coefficient of variation of this method was less than 2% while the coefficient of variation for the ammonium uptake, calculated per unit dry weight, was less than 6%. Under standard conditions efflux, if any, of organic N-compounds did not affect the $NH_4$ determination. This was checked by comparison with steam distillation at pH 10.2. The use of an ion-specific $NH_4^+$ electrode (Philips) proved to be unsuccessful as both $K^+$ and $H_3O^+$ release severely interfered.

*Potassium uptake* was estimated by adding a negligible amount of $^{86}Rb$ to (1 meq KCl + 0.1 meq $CaCl_2$) $l^{-1}$ uptake solutions and measuring ambient radioactivity decrease. A Nuclear Chicago Mark I Liquid Scintillation

Counter was used to measure activity in 10 ml aliquots. Keltjens (in prep.) showed that the present maize roots do not discriminate between K and Rb, so that analysis for $^{86}Rb$ properly evaluates uptake of K. The coefficient of variation of the $^{86}Rb$ scintillation counting was less than 1%, while the coefficient of variation of the calculated K (Rb) uptake (3 pots per treatment, on dry matter basis) was less than 6%.

*Nitrate uptake* Several methods to determine $NO_3$ uptake, e.g. from depletion of ambient nitrate were tested. An ion-specific $NO_3$-electrode (Orion) gave bad results probably because organic substances, lost during uptake interfered with the electrode membrane. For the same reason direct measurement of the 210 nm nitrate absorption peak (Cawse, 1967) and UV-spectrophotometry after colourization with the di-sodium salt of chromotropic acid (West & Lyles, 1960) were not suitable methods. Reduction of nitrate to nitrite and determination of nitrite with sulphanylamide and N-1-naphtylethylenediamine (van 't Riet et al., 1968; Middleton, 1959) was rejected because the reduction was neither quantitative nor reproducable.

It was decided to estimate $NO_3$ absorption by assay of $^{15}N$ in the roots after uptake from a labelled solution of 1 meq $l^{-1}$ $Ca(NO_3)_2$ enriched with 53 atom % $^{15}N$. Atom excess percentage $^{15}N$ in the dried roots was determined after digestion in $H_2SO_4$-$H_2O_2$-salicylic acid. $NH_4$ was converted to $N_2$ by NaOBr. A Statron NOI-4 $^{15}N$-Analysator (emission spectrograph) was used (Leicknam et al., 1968). Atom % $^{15}N$ was calculated from the spectrograms using the $^{14}N^{15}N$ and $^{14}N^{14}N$ peaks as described by Ferraris & Proksch (1972), Method 1. The coefficient of variation of the $^{15}N$ atom excess determination was less than 2%, while the coefficient of variation of the calculated nitrate uptake (2 pots per treatment, on DM basis) was less than 6%.

### *2.3.2 Plant analysis*

Inorganic constituents, nitrogen, carboxylates, ash alkalinity, free ammonium, amides and amino acids, starch and water-soluble carbohydrates were determined as described previously (Breteler et al. 1972; Breteler, 1973b; Breteler & Wittich, 1973). Oxocarboxylates in the roots were estimated after extraction of fresh material with cold 10% perchloric acid in a Bühler homogenizer with an ice-water cooled container (40 000 rpm, 1 minute). The extracts were filtered through glass wool and centrifuged (40 000 *g*, -5°C, 15 minutes). An aliquot of the supernatant was neutralized with NaOH

and diluted. In this solution oxocarboxylates were assayed enzymatically (Bergmeyer, 1962), with consumption of reduced NAD as a measure. Oxaloacetate was reduced to malate by malate dehydrogenase, α-oxoglutarate to glutamate by glutamate dehydrogenase + $NH_4$ and finally pyruvate to lactate by lactate dehydrogenase. All reactions proceeded at pH 7.6 in the same cuvette for periods not exceeding 15 minutes.

## 2.4 STATISTICAL ANALYSIS

The statistical significance of the results was checked by a Student's t-test (Snedecor & Cochran, 1967). Data, that differ significantly from the control data are marked with a single (P = 0.05) or double (P = 0.01) asterisks.

Effects of treatments are always expressed as % of the control (without that particular treatment).

## 2.5 LITERATURE RETRIEVAL

Both current awareness and retrospective literature search was by computer service, as a part of the Aladin project of the Centre for Agricultural Publishing and Documentation (Pudoc) at Wageningen. The files of the Bibliography of Agriculture (CAIN) and of Biological Abstracts were investigated using a program with the logic: A * B * C - D. Keywords were divided into 4 categories of synonyms and specifications:

Group A: carboxylates
Group B: uptake
Group C: plants
Group D: keywords to exclude combinations of A * B * C outside the scope of the research.

The CAIN file comprised the volumes 1970 till present and the Biological Abstracts file included 1964 till present. This means that all relevant publications concerning the subject in the indicated period are recorded in the reference list, provided their titles or keywords were matched by the computer program.

# 3 Entry of carboxylates into roots and their effect on ammonium uptake

When investigating the effect of carboxylates on ion uptake, the first problem is how to obtain roots that differ appreciably in carboxylate content. In the introduction some processes regulating the size of the carboxylate pool have been mentioned. First these processes were used to establish different carboxylate levels. The effects of $NH_4$ or $NO_3$ assimilation could not be used because subsequent ammonium uptake would not only be an effect of carboxylates but also of the nitrogen history. Sulphate reduction also depends on the nitrogen history of the plant (Dijkshoorn & van Wijk, 1967), thus the only remaining nutritional method was to treat the roots with solutions of salts, in which the cation and anion absorption rate were unequal; e.g. $K_2SO_4$, $CaBr_2$, $Na_2SO_4$, KCl and $CaCl_2$.

This treatment was tried out but finally proved to be unsuccessful with the salt concentrations used ($10^{-4}$-$10^{-3}$M). The results for changes in C-A were sometimes fair (Table 1). However, the differences were not consistent and maximum and minimum carboxylate contents of the roots usually differed by not more than 100 meq/kg DM, unless exposure to the mono-salt solutions was continued for several weeks, by which time nutritional disorders had occurred. No reproducable relation between pretreatment and subsequent ammonium uptake was found during 4-hour periods. Trials to infiltrate the sodium salts of succinic and α-oxoglutaric acid at pH 4 and 6 into excised root systems with a syringe (about 500 mmoles carboxylate/g DM) did not increase subsequent $NH_4$ uptake either. Analysis of the roots did not show appreciable changes in carboxylate (C-A) content.

Koster (1963) reported that addition of 25 mM succinate to the nutrient solution increased respiration and ammonium uptake by ringed soyabean plants. Because of root permeability he introduced succinate at pH 3.5-4.0.

At the same low pH level the entry of succinate into excised maize roots and its effect on ammonium uptake were studied. During 4-hour incubation experiments at pH 3.5 succinate, added as the sodium salt, entered the roots especially at high concentrations (50 mM). No straightforward relation was observed between root carboxylate content and succinate incubation con-

centration, probably because small pH changes during incubation caused large differences in root permeability. Subsequent $NH_4$ uptake was about 25% lower than that of roots treated with $CaCl_2$ only, except for the first 30 minutes. Simultaneous addition of succinate and ammonium to the uptake solution at a somewhat higher pH (4.0) resulted in 10% less ammonium absorption in 4 hours by the presence of succinate (Experiment 1, Figure 1).

*Exp. 1: Standard $NH_4$-uptake conditions, with 50 mM succinate (pH 4.0 with NaOH) or without succinate (pH 4.0 with HCl).*

$NH_4$ uptake in Experiment 1 was exceptionally high compared with other results in this report. Graphical analysis of Figure 1 shows an apparent free space of about 300 meq $kg^{-1}$ DM, which means, assuming a DM percentage of 5, ca 15 litre per litre. These unrealistic AFS values will be commented on in Chapter 7.

The suppression of $NH_4$ uptake can probably not be attributed to monovalent cation uptake inhibition induced by sodium as the succinate pretreated roots absorbed only 50 meq Na/kg DM during the experiment.

The effect of ambient Na concentration on $NH_4$ uptake was studied in

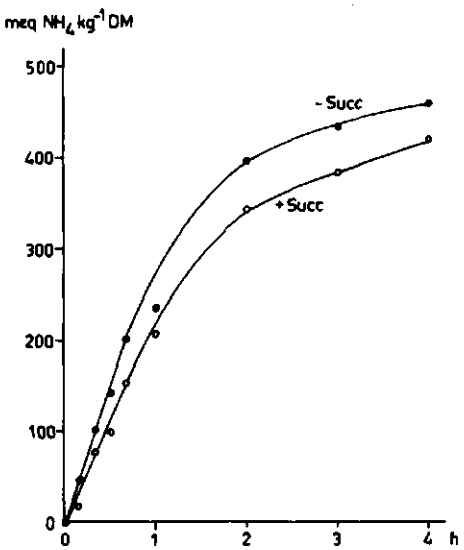


Fig. 1. Ammonium uptake at pH 4.0 in the presence or absence of 50 mM Na succinate. Experiment 1.

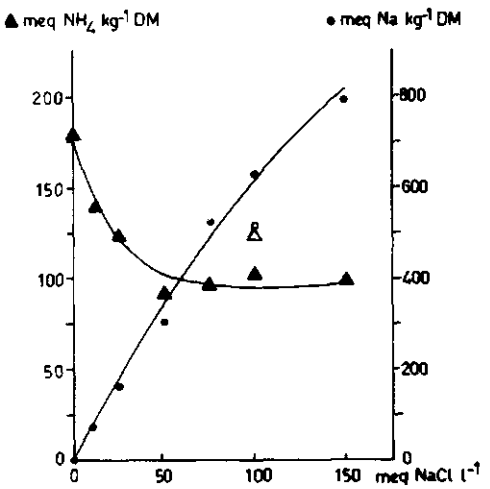


Fig. 2. Sodium and ammonium uptake from a 1 meq $NH_4$ $l^{-1}$ solution as a function of Na concentration. Open symbols refer to uptake in the presence of 50 mM Na succinate at pH 5.5. Experiment 2.

Experiment 2 (Figure 2). Below 50 meq Na $l^{-1}$, uptake of 50 meq Na corresponded with a fall in $NH_4$ uptake of 15 meq and above that concentration Na uptake had no more effect on $NH_4$ absorption. However this experiment was performed at pH 5.5.

*Exp. 2: Standard $NH_4$-uptake conditions with 0-150 meq NaCl $l^{-1}$. Na uptake was calculated by subtraction of the Na content of the control roots from the Na content of the roots after 4 hours of $NH_4$ uptake in the presence of Na.*

Contents of the other inorganic cations (Ca, Mg and K) were not altered during ammonium uptake. C-A values of the roots before the experiment and after the uptake with or without succinate were 206, 257 and 149 meq/kg DM, respectively. These figures indicate a decrease because of ammonium assimilation and an increase due to sodium-balanced carboxylate uptake.

As any effect of succinate on $NH_4$ uptake could depend on the internal supply of organic acid anions originating from carbohydrate metabolism, the next experiment was carried out with roots of plants kept at 35°C (high respiration rate) for the preceding night. Results are presented in Figure 3.

*Exp. 3: Experimental conditions as in Exp. 1, roots of plants subjected to 35°C in the night, preceding the uptake experiment.*

Ammonium uptake was about 50% of the uptake measured in Experiment 1, especially due to decreased uptake during the first 2 hours. Net uptake

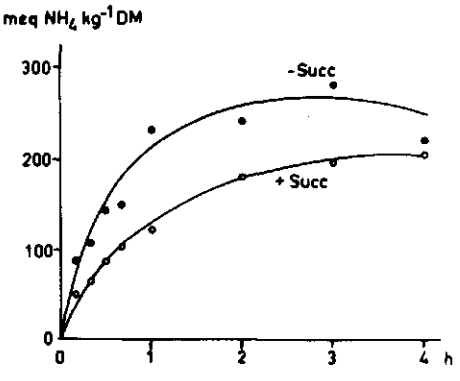


Fig. 3. Ammonium uptake at pH 4.0 in the presence or absence of 50 mM Na succinate. Roots poor in carbohydrates. Experiment 3.

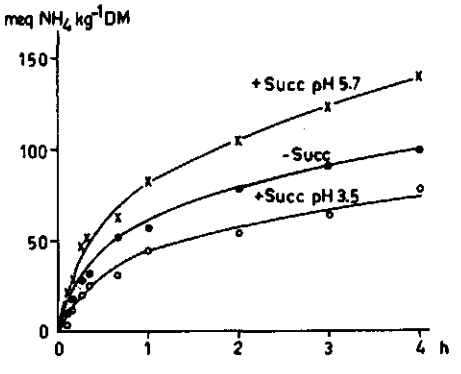


Fig. 4. Ammonium uptake after 22h incubation in 50 mM succinic acid, titrated with NaOH to pH 3.5 and 5.7. Experiment 4.

after 3 hours was very low. The negative succinate effect however, was even more pronounced. In this experiment the roots exposed to succinate absorbed 40 meq Na/kg DM.

From Experiments 1 and 3, it was concluded that stimulation of $NH_4$ uptake as obtained by Koster was absent for maize roots under the experimental conditions adopted.

In the next experiment a different way of succinate preloading was studied. Roots were incubated for a longer period of time (22 hours) in 50 mM Na-succinate solutions at pH 3.5 and 5.7 and subsequent ammonium uptake was determined (Figure 4).

*Exp. 4: Standard $NH_4$-uptake conditions and standard carboxylate incubation conditions, except that the incubation time was 22 hours and the pH 3.5 or 5.7.*

After 4 hours, roots preincubated with succinate at pH 5.7 or 3.5 had absorbed 139% and 87%, respectively of the $NH_4$ absorbed by control roots. Stimulation and inhibition by the pretreatments was obvious after only 5 minutes of ammonium uptake. Table 4 shows that the pH during incubation

Table 4. Ammonium uptake, and inorganic chemical composition of roots before (0h) and after four hours (4h) of ammonium uptake. Before the uptake period, roots were incubated in solutions with succinate at pH 3.5 and 5.7, and without succinate. Experiment 4. Contents and uptake in meq/kg DM.

| | - succ | | + succ pH 3.5 | | + succ pH 5.7 | |
|---|---|---|---|---|---|---|
| | 0h | 4h | 0h | 4h | 0h | 4h |
| $NH_4$ uptake | | 100 | | 78* | | 139* |
| $Na^+$ | 233 | 239 | 219 | 180 | 685 | 460 |
| K | 373 | 319 | 333 | 310 | 232 | 290 |
| Ca | 108 | 93 | 68 | 45 | 70 | 89 |
| Mg | 84 | 80 | 56 | 51 | 52 | 79 |
| C | 798 | 731 | 676 | 586 | 1039 | 918 |
| Cl | 109 | 250 | 163 | 155 | 98 | 266 |
| $SO_4$ | 107 | 162 | 104 | 119 | 67 | 187 |
| $H_2PO_4$ | 221 | 202 | 158 | 142 | 179 | 207 |
| A | 437 | 614 | 425 | 416 | 344 | 660 |
| C-A | 361 | 117 | 251 | 170 | 695 | 258 |
| Δ(C-A) | | -244 | | -81 | | -437 |

The nitrate content of the roots in the present experiments was usually below 15 meq/kg DM and neglected. Δ(C-A) = difference in C-A before and after an uptake experiment.

influences carboxylate absorption as well as $NH_4$ entry. Roots incubated at the higher pH contained about 450 meq more carboxylates per kg DM than those incubated at the lower pH. Incubation at pH 3.5 even lowered the C-A value compared with the $CaCl_2$ treatment. C-A increase of 444 meq by incubation at pH 5.7 instead of 3.5 was mainly accounted for by sodium uptake. At the same time loss of K, Ca and Mg occurred. During $NH_4$ uptake efflux of Na and K was observed, while Cl was taken up. The observed increase in sulphate content is hard to explain. Initial and remaining C-A values as well as the magnitude of the change of this characteristic $\Delta$(C-A) were correlated with the amount of absorbed ammonium ions.

An effect of acidity level during incubation on sodium, succinate and subsequent ammonium uptake was observed in Experiment 4. In the next experiment the effect of an increased pH (5.5) on the action of succinate, added to the uptake solution, on $NH_4$ absorption was studied. Under the same conditions the effect of citrate was examined.

*Exp. 5: Standard $NH_4$-uptake conditions, with 50 mM succinate or citrate (pH 5.5 with NaOH) or without carboxylates.*

Presence of carboxylates at pH 5.5 (Figure 5) caused a small depression (7%) of $NH_4$ uptake by sodium succinate and a severe depression (54%) caused by sodium citrate. It will be shown later that under conditions where $NH_4$ absorption was stimulated by succinate pretreatment (no Na added to the uptake medium) citrate pretreatment was also inhibitory. In Experiment 1 (Figure 1) where succinate was added to the uptake solution at pH 4.0 a negative effect of 10% was found. In contrast to Experiment 1 however, sodium uptake in Experiment 5 was considerable (Table 5); 370 and 257 meq Na/kg DM in the presence of citrate and succinate respectively, a quotient

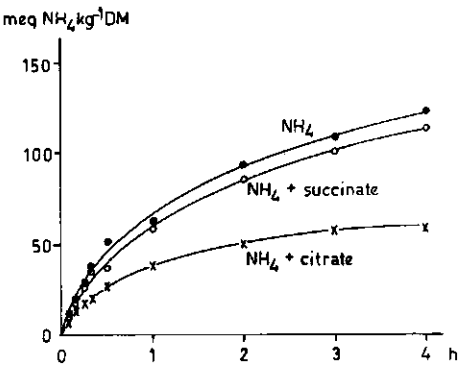


Fig. 5. Ammonium uptake in the presence or absence of succinate or citrate (50 mM Na-salt, pH 5.5). Experiment 5.

Table 5. Ammonium uptake, and inorganic chemical composition of roots before and after (0h, 4h) four hours of ammonium uptake with or without 50 mM succinate or citrate, titrated to pH 5.5 with NaOH. Experiment 5. Contents and uptake in meq/kg DM.

| | 0h | $NH_4$ 4h | $NH_4$+succinate 4h | $NH_4$+citrate 4h |
|---|---|---|---|---|
| $NH_4$ uptake | | 123 | 114 | 57** |
| Na | 182 | 149 | 439 | 552 |
| K | 583 | 494 | 484 | 443 |
| Ca | 68 | 56 | 46 | 43 |
| Mg | 52 | 53 | 49 | 47 |
| C | 885 | 752 | 1018 | 1085 |
| Cl | 355 | 339 | 345 | 310 |
| $SO_4$ | 154 | 132 | 136 | 139 |
| $H_2PO_4$ | 258 | 228 | 234 | 233 |
| A | 767 | 699 | 715 | 682 |
| C-A | 118 | 53 | 303 | 403 |
| Δ(C-A) | | -65 | 185 | 285 |

of 1.44. The quotient of sodium concentrations initially present in the media (number of carboxylate groups per molecule to be neutralized with NaOH) had about the same value. Obviously inhibition of $NH_4$ uptake by Na uptake was of more importance than enhancement by exogenously supplied carboxylates (cf. Fig. 2). At pH 5.5 the roots accumulated Na and carboxylates during $NH_4$ uptake. The C-A of the control roots decreased by 65 meq/kg DM, while the presence of citrate and succinate during ammonium uptake increased this value by 285 and 185, respectively. Uptake of $NH_4$ was at the expense of Na and K in the roots and to a lesser extent Ca and Mg were exchanged for $NH_4$ and Na. The several treatments also resulted in a lower content of inorganic anions after 4 hours of $NH_4$ uptake.

SUMMARIZING the data obtained so far I observed that in only one treatment did exogenously supplied carboxylates stimulate ammonium uptake; pretreatment of the roots with 50 mM succinate for 22 hours at pH 5.7.

To obtain reliable standard conditions for carboxylate (succinate) entry for the following test, it was decided to study the factors regulating entry of carboxylates in more detail.

The effect of the succinate concentration in the incubation medium on succinate uptake by excised roots was investigated in Experiment 6.

*Exp. 6: Succinate incubation (succinic acid titrated to pH 5.5 with NaOH) for 8 hours at 1 meq $CaCl_2$ $l^{-1}$ with 0, 10, 25, 50 and 100 mM succinate.*

The size of the carboxylate pool kept approximately pace with the succinate concentration (Figure 6). When succinate entered the roots both succinate and malate accumulated. Both fractions constitute the bulk of the determined carboxylates. Fumarate and citrate were present in lower concentrations and their content was hardly affected by the medium succinate concentration. Both the carboxylate pool and the C-A (Figure 7) were increased by about 500% (or by 400-500 meq/kg DM) when the succinate concentration was raised from 0 to 100 mM. Uptake of succinate was almost balanced stoichiometrically by Na uptake.

A comparison of 100 with 0 mM succinate showed the carbohydrate content of the roots (starch plus water-soluble carbohydrates) to be unchanged (Table 6). Reduced consumption of endogenous sugars or synthesis of carbohydrates from organic acid anions was not observed (Canvin & Beevers, 1961; Beevers et al., 1966; Hofstra, 1966).

After 8 hours of succinate uptake at different concentrations, there was no substantial change in the roots of the content of free $NH_4$, gluta-

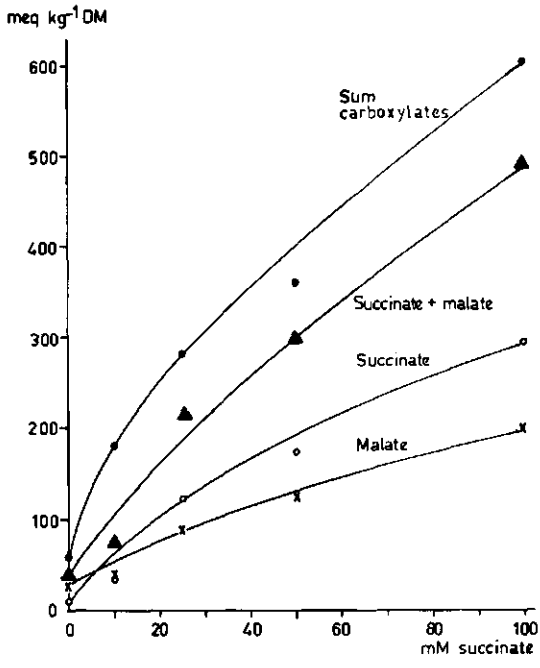


Fig. 6. Effect of succinate incubation concentration on total carboxylate, succinate and malate content. Incubation at pH 5.5 for 8 h. Experiment 6.

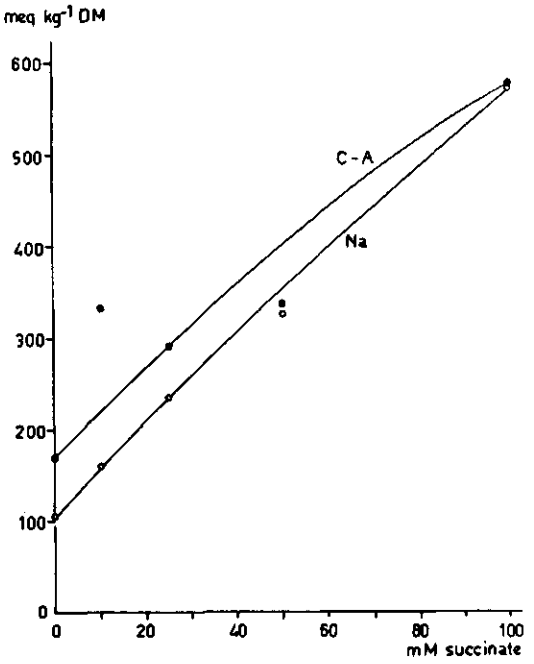


Fig. 7. Effect of succinate incubation concentration on C-A and Na content. Incubation at pH 5.5 for 8 h. Experiment 6.

Table 6. Contents of some free nitrogenous compounds, total nitrogen and inorganic constituents in roots after 8 hours incubation in 0 and 100 mM succinate. Experiment 6.

| | 0 | 100 mM succinate |
|---|---|---|
| $NH_4$ | 10 | 16 mmol/10 kg FW |
| Glutamine | 9 | 6 " |
| Asparagine | 13 | 17 " |
| Free amino acids | 62 | 55 " |
| Carbohydrates (WSC+starch) | 5.0 | 4.8 % DM |
| K | 907 | 888 meq/kg DM |
| Ca | 60 | 52 " |
| Mg | 128 | 124 " |
| $SO_4$ | 200 | 230 " |
| $H_2PO_4$ | 442 | 410 " |
| $N_{total}$ | 1069 | 1053 mmol/kg DM |

mine, asparagine and amino acids, total N, K, Ca, Mg, $SO_4$ and $H_2PO_4$ (Table 6). During this and following incubations, an increase in pH of the incubation medium was sometimes measured, indicating that net uptake of succinate may exceed net uptake of Na. Figures 6 and 7 show that the sum of the carboxylates and C-A expressed as ion equivalents are of the same magnitude

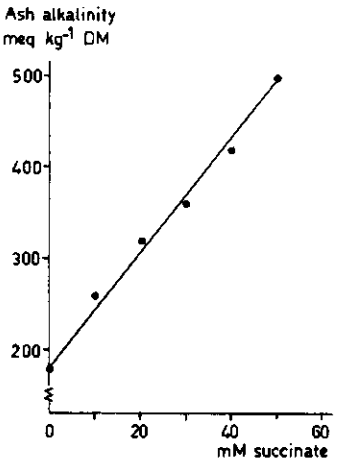


Fig. 8. Effect of succinate incubation concentration on ash alkalinity. Incubation at pH 5.5 for 16 h. Experiment 7.

and that contents of total carboxylates, succinate, malate, C-A and Na increase with the succinate concentration. The increase is linear when the concentration exceeds 25 mM. In Experiment 7 the ash alkalinity of the roots was determined after 16 hours incubation in 0-50 mM Na-succinate at pH 5.5. Although the incubation period was twice as long, the results agree with those of Experiment 6 (Figure 8).

*Exp. 7: Standard carboxylate incubation conditions, succinate concentration 0, 10, 20, 30, 40 and 50 mM.*

The effect of the incubation time on succinate uptake was investigated in Experiment 8 (Figure 9).

*Exp. 8: Succinate incubation for 0, 2, 4, 8 and 24 hours. 1 meq $CaCl_2$ $l^{-1}$ and 50 mM succinic acid titrated to pH 5.5 with NaOH.*

Carboxylates and succinate contents in the roots increased with time up to approximately 8 hours. The sum of the carboxylates was numerically equal to the C-A values. As time proceeded changes in C-A were not only brought about by Na influx. Efflux of K and minor losses of Ca and Mg also occurred (Figure 10).

The effect of the pH in the incubation medium on succinate entry was studied in Experiment 9.

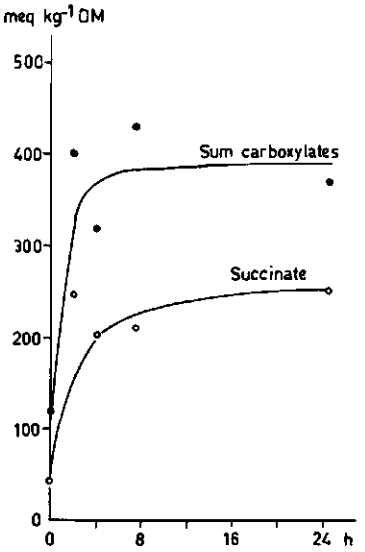


Fig. 9. Effect of succinate incubation time on total carboxylate and succinate content. Incubation at pH 5.5, 50 mM succinate. Experiment 8.

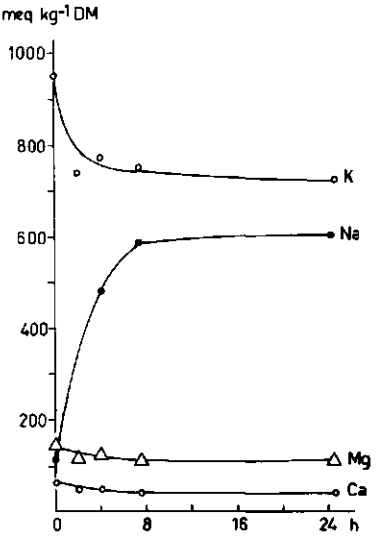


Fig. 10. Sodium, potassium, calcium and magnesium contents of roots incubated in 50 mM succinate at pH 5.5 in the course of time. Experiment 8.

*Exp. 9: Succinate incubation for 7 hours with 1 meq $CaCl_2$ $l^{-1}$ and 50 mM succinic acid tritrated to pH 3, 4, 5, 6 and 7 with NaOH.*

When the pH is raised from 3 to about 5 the carboxylate content is increased; the same is found for the malate and to a lesser extent for the succinate fraction (Figure 11).

Results of Experiment 9 indicate preferential uptake of the carboxylate anion compared with the undissociated acid molecule. Calculation of the succinate species present at each pH (Figure 12) shows that the curve of carboxylate content in Figure 11 and the curve indicating the sum of the dissociated succinate ion species (broken line in Figure 12) are similar.

SUMMARIZING the experiments concerning succinate entry conditions I concluded - assuming that the factors concentration, time and pH are not interdependent - that incubation in 100 mM succinate, neutralized with NaOH to a pH in between 5 and 7, for 8 to 24 hours resulted in the highest carboxylate level in the roots. To avoid osmotic pressure (Table 7) and Na contents being too high, the standard conditions for succinate pretreatment were chosen as follows: 50 mM sodium salt of succinic acid, pH 5.5, 16

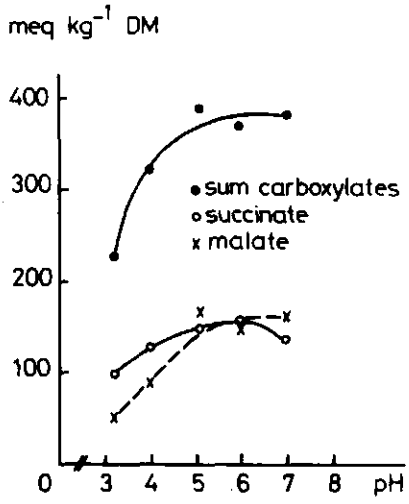


Fig. 11. Effect of pH during succinate incubation on total carboxylate, succinate and malate content. Incubation in 50 mM succinate for 7 h. Experiment 9.

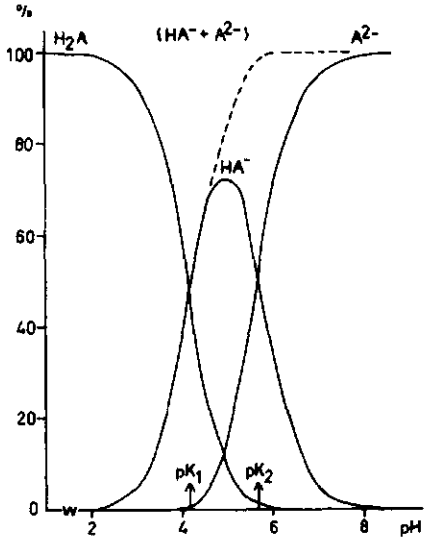


Fig. 12. Relative distribution of succinate ion species as a function of pH at 25°C.

Table 7. Dissociation (%) and osmotic pressure (atmosphere) of several 50 mM carboxylate solutions at pH 5.5 and room temperature.

| | $H_3A$ | $H_2A$ | HA | $H_2A^-$ | $HA^-$ | $HA^{2-}$ | $A^-$ | $A^{2-}$ | $A^{3-}$ | O.P. |
|---|---|---|---|---|---|---|---|---|---|---|
| Acetate | | | 15 | | | | 85 | | | 2.2 |
| Pyruvate | | | 0 | | | | 100 | | | 2.4 |
| Glyoxylate | | | 1 | | | | 99 | | | 2.4 |
| Oxaloacetate | | 0 | | | 44 | | | 56 | | 2.8 |
| α-Oxoglutarate | | 0 | | | 24 | | | 76 | | 3.3 |
| Succinate | | 2 | | | 55 | | | 43 | | 2.9 |
| Fumarate | | 0 | | | 8 | | | 92 | | 3.5 |
| Malate | | 0 | | | 29 | | | 71 | | 3.3 |
| Malonate | | 0 | | | 61 | | | 39 | | 2.9 |
| Oxalate | | 0 | | | 5 | | | 95 | | 3.5 |
| Citrate | 0 | | | 7 | | 41 | | | 52 | 4.1 |

hours (overnight). In the experiments to be reported here the carboxylate (C-A) contents of the roots after the standard conditions described were usually 150-200 meq/kg DM for the control (-succinate) and 400-500 for the +succinate roots. In Experiment 10 the entry of several carboxylates into roots was studied by determining the ash alkalinity, the C-A and the carboxylate contents of the roots after incubation. Oxo-carboxylate contents are dealt with in Chapter 5.

*Exp. 10: Standard carboxylate incubation conditions (50 mM Na-salt, pH 5.5, 16 h) applied to oxaloacetate, citrate, α-oxoglutarate, succinate, fumarate, malate, pyruvate, malonate and glyoxylate.*

Table 7 gives the dissociation percentages and the osmotic pressure of some carboxylate solutions used. It is clear that the term 'organic acids' should be abandoned. Ash alkalinity, C-A and carboxylate data are presented in Table 8 and the inorganic composition in Table 9. Many more anions of oxaloacetic and pyruvic acid were absorbed than of the accompanying sodium ions, but sodium was taken up in excess of glyoxylate and fumarate, as inferred from pH changes in the media. All carboxylates penetrated and resulted in

Table 8. Ash alkalinity, C-A and carboxylates in the roots after incubation. Contents in meq/kg DM. Experiment 10.

| | AA | C-A | Sum | Ci | Succ | F | M | MO | O |
|---|---|---|---|---|---|---|---|---|---|
| $CaCl_2$ | 160 | 222 | 165 | 28 | 24 | 4 | 27 | 11 | 71 |
| Oxaloacetate | 640 | 664 | 188 | 30 | 46 | 12 | 7 | 57 | 36 |
| Citrate | 500 | 487 | 454 | 296 | 38 | 38 | 49 | 15 | 18 |
| α-Oxoglutarate | 560 | 381 | 282 | 22 | 140 | 36 | 33 | 21 | 30 |
| Succinate | 460 | 441 | 452 | 32 | 256 | 30 | 97 | 0 | 37 |
| Fumarate | 540 | 576 | 502 | 38 | 10 | 320 | 31 | 39 | 64 |
| Malate | 540 | 545 | 500 | 30 | 64 | 40 | 315 | 21 | 30 |
| Pyruvate | 400 | 464 | 210 | 0 | 69 | 34 | 72 | 0 | 35 |
| Malonate | 480 | 543 | 429 | 8 | 42 | 20 | 17 | 289 | 53 |
| Glyoxylate | 400 | 421 | 310 | 0 | 36 | 38 | 63 | 29 | 144 |

Ci = citrate, Succ = succinate, F = fumarate, M = malate, MO = malonate, O = oxalate

Table 9. Inorganic constituents, C-A and nitrogen in roots after incubation in several carboxylate solutions. Contents in meq or mmol (N)/kg DM. Experiment 10.

| | $CaCl_2$ | OXAC | Ci | αOG | Succ | F | M | PYR | MO | G |
|---|---|---|---|---|---|---|---|---|---|---|
| Na | 117 | 674 | 520 | 374 | 381 | 433 | 474 | 390 | 447 | 346 |
| K | 708 | 615 | 668 | 670 | 714 | 799 | 796 | 757 | 714 | 736 |
| Ca | 84 | 62 | 42 | 46 | 50 | 52 | 50 | 66 | 54 | 52 |
| Mg | 122 | 122 | 86 | 80 | 90 | 100 | 106 | 112 | 114 | 112 |
| C | 1031 | 1473 | 1316 | 1170 | 1235 | 1384 | 1426 | 1325 | 1329 | 1246 |
| Cl | 351 | 387 | 398 | 378 | 384 | 376 | 423 | 421 | 362 | 409 |
| $SO_4$ | 162 | 164 | 146 | 142 | 144 | 154 | 156 | 160 | 150 | 146 |
| $H_2PO_4$ | 296 | 258 | 285 | 269 | 269 | 278 | 302 | 280 | 274 | 270 |
| A | 809 | 809 | 829 | 789 | 797 | 808 | 881 | 861 | 786 | 825 |
| C-A | 222 | 664 | 487 | 381 | 438 | 576 | 545 | 464 | 543 | 421 |
| N | 798 | 780 | 770 | 736 | 822 | 779 | 802 | 782 | 773 | 907 |

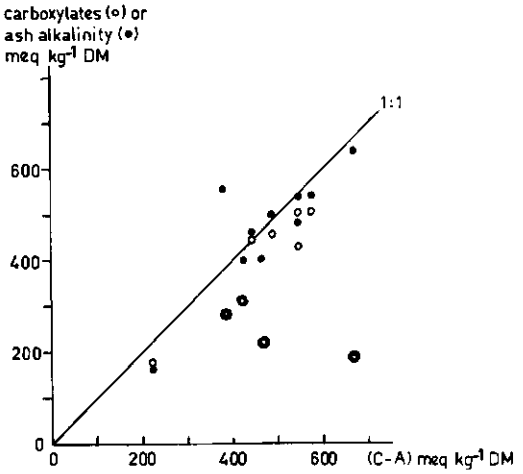


Fig. 13. Relation between (C-A) values and ash alkalinity or carboxylate contents of roots after incubation in several carboxylate solutions. Double-ringed symbols refer to α-oxocarboxylate pretreated roots. Experiment 10.

increase of the determined carboxylate pool of 240 to 480 meq/kg DM (ash alkalinity). Only glyoxylate and pyruvate incubation resulted in relatively low carboxylate contents. Compounds used in the pretreatments accumulated in the roots. Main fractions of the carboxylate pools after the incubations were nearly equal in size, showing concentrations not far from 300 meq/kg DM. In general there is a good agreement between (C-A), carboxylate and ash alkalinity (Figure 13). The values for (C-A) and AA usually exceed the sum of the determined carboxylates. The discrepancy between carboxylate, C-A and ash alkalinity values in α-oxo-carboxylate pretreated samples (double rings in Figure 13) has the following background. During carboxylate analysis, decarboxylation of oxo-acids easily occurs (Titus et al., 1968; Harborne, 1973). If this is the explanation for the gaps found in Table 8, it should be assumed that oxo-acids are not only decarboxylated themselves, but also stimulate decarboxylation of other carboxylic acids or other processes that make them unavailable for the carboxylate analysis used, which was essentially the method described by Freeman (1967); see van Egmond (1975). When oxo-acids were added to synthetic carboxylate mixtures or plant samples the temperature of 70°C, used to dry the roots after sampling, the presence of glucose and of root material, but not of cellulose, led to a reduced recovery of carboxylates. Quantitatively oxo-carboxylates are of minor importance (Chapter 5). After incubation with other carboxylates reliable analytical results were obtained.

Incubation with most carboxylates slightly increased contents of other citrate cycle intermediates as well (Figure 14), usually due to a precursory

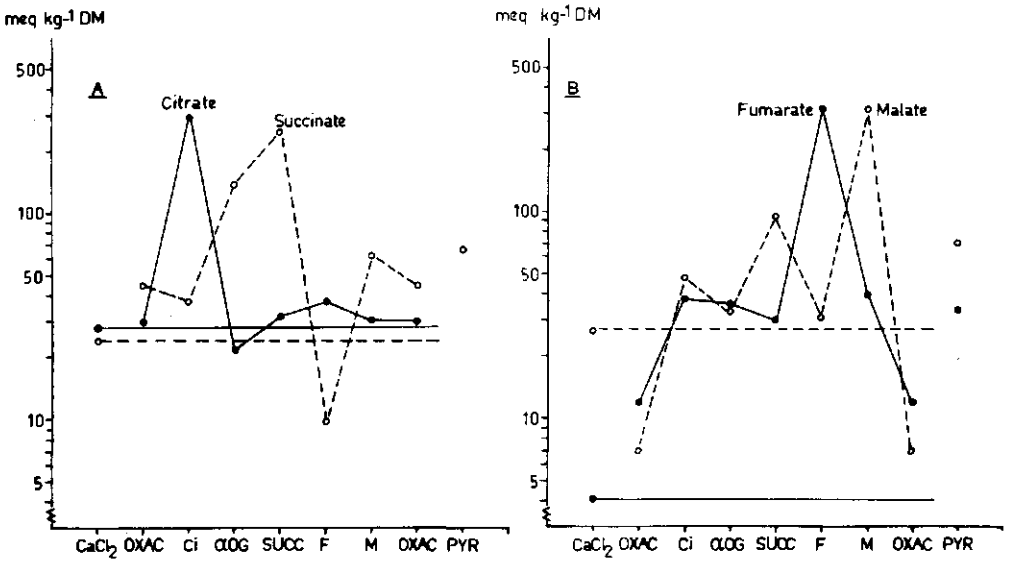


Fig. 14. Effect of incubation with several carboxylates (abscissa) on citrate and succinate (A) and fumarate and malate (B) content of roots (ordinate). Carboxylates on the abscissa in the sequence of the TCA-cycle. Experiment 10.

function. Pretreatment with α-oxoglutarate increased the succinate content and decreased the citrate content, due to inhibition of citrate synthase (Sarkissian & Boatwright, 1974). Malate was increased by succinate pretreatment. Fumarate hardly accumulated in the tissue except after fumarate incubation. Malate and pyruvate caused a higher succinate level, succinate and glyoxylate also a higher malate content. The inhibition of succinate dehydrogenase by oxaloacetate (Gimpel, 1973) can be seen in Figure 14 (low fumarate content). Formation of malonate from oxaloacetate by α-decarboxylation probably also occurred (de Vellis et al., 1963; Shannon et al., 1963). Beside those of the citrate cycle, enzymes of the glyoxylate pathway were probably present as indicated by the glyoxylate stimulated oxalate content (Chang & Beevers, 1968; Richardson & Tolbert, 1961). Bonner (1973), however, was unable to detect glyoxysomes in plant roots and therfore doubts the existence of a glyoxylate pathway in these organs. Effect of the pretreatments on the contents of α-oxoglutarate, oxaloacetate and pyruvate will be discussed in Chapter 5. Major variations in the mineral contents after incubation are found in Na, K, Ca, Mg and thus C content (Table 9), A being constant. The composition of control roots contrasts with data on maize of Coïc et al. (1961), Torri & Laties (1966b), Jackson & Coleman (1959) and

Jacobson & Ordin (1954), who found the bulk of the carboxylates in the aconitate or malate fraction.

## CONSLUSION AND DISCUSSION

Preloading roots with carboxylates under 'standard conditions' proved to be a suitable method for the purpose of ion uptake because:

1. All carboxylates investigated were accumulated at a similar rate (Experiment 10).
2. The osmotic pressure of the incubation media did not exceed the ca. 4 atmosphere threshold (Table 7) where severe damage to maize roots may start (Wadleigh, 1947; Richards, 1954; Lapina & Bikmukhametova, 1972).
3. Stimulation by succinate of subsequent $NH_4$ uptake was reporduced in a large number of trials. The carboxylate contents of the roots were also consistently changed by the succinate treatment (Table 4).
4. Chemical characteristics, except Na and C-A, were not substantially altered during incubation (Table 6).
5. Supply of succinate at 50 mM to the uptake solution had the disadvantage that concomittantly supplied sodium ions hampered $NH_4$ uptake (Experiment 2 and 5).

Sodium pretreatment as such did not affect $NH_4$ uptake, as shown by Experiment 11 (Table 10).

*Exp. 11: Standard carboxylate incubation conditions and incubation in 100 meq $l^{-1}$ NaCl and $Na_2SO_4$. Standard $NH_4$-uptake conditions.*

Sodium contents of all kinds of Na-pretreated roots before or after 4 hours of $NH_4$ uptake were equal. Succinate stimulation of ammonium uptake was 57%.

Several authors have reported on the effect of pH on carboxylate entry: Simon & Beevers (1952) in baker's yeast, Laties (1949), Beevers et al. (1966), Jackson & Taylor (1970), Jackson et al. (1970) in barley roots and Kornberg (1959) and Budd (1966) in bacteria. Jackson & Taylor suggested that membrane permeability is affected by organic acids and that aliphatic monocarboxylic acids exert more effect than dicarboxylic and hydroxylic acids. The least effect was at high pH values, where the concentration of undissociated acids is low. Meyer (1971) found that malate affects the permeability of rat liver mitochondrial inner membranes. Jackson et al. working with $^{14}C$ carboxylates, found that a pH increase from 5 to 7 decreased succinate uptake from 1 meq $l^{-1}$ Na succinate. Their data suggest parallelism

Table 10. Ammonium uptake, and changes in Na and K content of roots incubated in $CaCl_2$, Na succinate, NaCl and $Na_2SO_4$. Experiment 11. Uptake and contents in meq/kg DM.

| | $CaCl_2$ | NaSucc | NaCl | $Na_2SO_4$ |
|---|---|---|---|---|
| $NH_4$ uptake | 210 | 330** | 199 | 222 |
| % | 100 | 157 | 95 | 106 |
| Na(0h) | 18 | 306 | 320 | 317 |
| Na(4h) | 17 | 139 | 141 | 131 |
| ΔNa | -1 | -167 | -179 | -186 |
| ΔK | -211 | -296 | -256 | -137 |
| Δ(Na+K) | -212 | -463 | -435 | -323 |

between the concentration of the monovalent hydrogen succinate species ($HA^-$) and succinate uptake. At higher concentrations, however, the pH effect decreased. Kornberg reported that oxidation of exogenous succinate by *Corinebacterium creatinovorans* and *Brucella abortus* was considerably more rapid in acid solutions than at neutral pH. Laties and Simon & Beevers concluded that the concentration of the undissociated species is the major factor in determining carboxylate entry.

It seems logical that undissociated molecules penetrate cells easier than charged compounds. The results so far, however, show that uptake and stimulation of subsequent $NH_4$-uptake is best accomplished with succinate in the ionic form. Notwithstanding the differences between the chosen standard incubation pH of 5.5 and the pK values for several carboxylates, it can be concluded from the results of Table 8 in combination with Table 7 that all carboxylates investigated were mainly taken up in the anionic form. There is possibly more than one mechanism involved in carboxylate entry. At higher concentrations (e.g. 50 mM) permeability as such may be less of a barrier to carboxylate penetration than at the low concentrations used by the investigators cited. Cram & Laties (1974) consider the plasmalemma rate-limiting for malate uptake at 1 mM, whereas other barriers may exist at higher concentrations.

Results obtained in this chapter supply only indirect information about the question of dual carboxylate pools (Lips & Beevers, 1966ab), one of which is not immediately available for TCA-cycle metabolism. It was observed that incubation with the various carboxylates stimulated the formation of other carboxylates, but also that the compounds used for incubation, were recovered in large quantities. Steer & Beevers (1967) found that for succinate no pool-storage of the 'malate type' was found in maize roots.

# 4 The stimulatory succinate effect

## 4.1 INTRODUCTION

Some insight into the penetration of carboxylates, especially succinate, into excised roots of maize and its effect on ammonium absorption was obtained in Chapter 3. More information will be given about the effect of succinate incubation on the stimulation of subsequent ammonium uptake in this chapter.

First the depencency of the observed effect on experimental conditions adopted was investigated, e.g. duration of the uptake period (4.2) and exposure of the roots to a nitrogen-free medium several days prior to trials on $NH_4$ uptake (4.3).

During the last decade the so-called dual isotherm or mechanisms I and II have received considerable attention in plant nutrition (Epstein, 1972). Mechanism I is the high-affinity, low concentration system and Mechanism II is the low-affinity, high concentration system. The systems operate in parallel or in series and the barriers are thought to be at the plasmalemma membrane (I) and at the tonoplast membrane (II). In the experiments described in Section 4.4, the effect of succinate stimulation was studied at several $NH_4$ concentrations.

One of the features of the dual isotherm - the anion dependent rate of cation uptake in Mechanism II (Torii & Laties, 1966a) was investigated in relation to the succinate effect (4.5).

The succinate and carboxylate content of the roots was varied and the effect on subsequent $NH_4$ uptake measured (4.6). How the chemical composition of succinate incubated roots as compared with control roots changes during ammonium uptake was described in Section 4.7.

The next sections are concerned with the influence of the carbohydrate status and respiration of the roots. In Section 4.8 the effect of glucose on ammonium uptake is described and this glucose effect is compared with the succinate effect in Section 4.9. Factors related to root respiration, e.g. oxygen supply, temperature and a respiratory inhibitor, were used in

Section 4.10 to see how $NH_4$ uptake and the succinate effect are affected.

In the last section of this chapter (4.11) the effect of inhibitors of protein synthesis is described to check relationship between succinate stimulation of $NH_4$ uptake and protein metabolism.

## 4.2 DURATION OF THE SUCCINATE EFFECT

In Experiment 4 there was a positive succinate effect for a 4-hour uptake period. In Experiment 12, $NH_4$ uptake was determined for as long as net uptake existed (Figure 15).

*Exp. 12: Standard succinate incubation conditions and standard $NH_4$-uptake conditions with extended experimental time.*

Although there was a lack of data between 8 and 24 hours, the lines were drawn in between these points because it could be inferred from parallel trials that net uptake in this time interval is small. Net uptake was positive for at least 15 hours and for more than 30 hours +succinate roots absorbed about twice as much $NH_4$ as -succinate roots. It seems that the +succinate roots retained their capacity for ammonium uptake longer than the -succinate roots. In excised beech mycorrhiza's ammonium uptake declined after about 12 hours, due to glucose exhaustion (Carrodus, 1966).

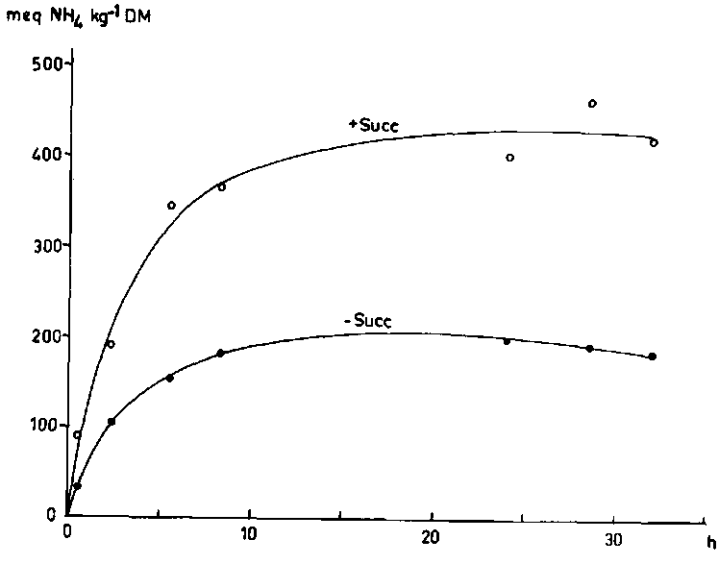


Fig. 15. Effect of succinate pretreatment on ammonium uptake in the course of time. Experiment 12.

Table 11. Sodium, potassium and C-A content (in meq/kg DM) of roots after 24 or 53 hours $NH_4$ uptake. Data refer to different tests. Experiment 12.

| | 24h | | 53h | |
|---|---|---|---|---|
| | -succ | +succ | -succ | +succ |
| Na | 20 | 93 | 26 | 133 |
| K | 502 | 531 | 319 | 248 |
| C-A | -132 | -73 | -20 | +16 |

After 32 hours a net efflux of $NH_4$ seemed to exist. This observation could also be due to analytical failure. Under standard conditions excretion of organic N substances, if any, did not interfere with the N determination (see 2.3.1). It is known that amino compounds may interfere with the phenol-blue method (Breteler et al., 1972). If the roots accumulate free N-compounds for 32 hours or more, efflux of free amino or other organic compounds may interfere with the $NH_4$ assay.

Succinate stimulation continued for more than 4 hours after which the experiments were stopped. The roots were then in an active phase of both ion uptake and succinate stimulation. Chemical composition of the roots after 24 and 53 hours of $NH_4$ uptake is given in Table 11. After one-day exposure of excised roots to $NH_4$ solutions, the roots showed very low C-A contents and the residual effect of succinate pretreatment was small; only 59 meq was left of the normal difference of about 300 at zero time. In another test this difference was only 36 meq after 53 h of $NH_4$ uptake. Negative C-A values are explained in Section 4.3.

## 4.3 EFFECT OF NITROGEN STARVATION

All experiments (except 13) were carried out with roots from plants that were nitrogen-starved for at least 3 days. It is possible that the positive succinate effect is accounted for by the low nitrogen status of the roots. Experiment 13 was done with roots of plants that were exposed to a nitrogen-free medium for 2 weeks. Part of the plants was transferred to a $NH_4$ medium on the day before the incubation and uptake experiment.

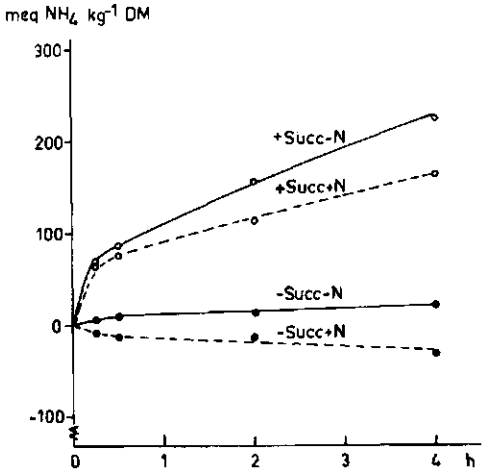


Fig. 16. Effect of nitrogen status and succinate pretreatment on ammonium uptake. Experiment 13.

*Exp. 13: Standard $NH_4$-uptake conditions and standard succinate incubation conditions. Part of the roots from plants grown in $NH_4$ nutrition (-N medium + 3 meq $NH_4Cl$ $l^{-1}$) on the day prior to incubation.*

Figure 16 shows that the succinate effect strongly overshadows the effect of the N status. Roots with a low N status absorbed about 60 meq $NH_4$/kg DM more than roots with a high N status, while succinate pretreatment resulted in about 200 meq more $NH_4$ uptake. In the +N control roots, net uptake seemed negative. Because roots were transferred from a 3 meq $NH_4$ $l^{-1}$ medium to the 1 meq $l^{-1}$ uptake solutions via succinate, net efflux of $NH_4$ ions may have occurred. Interference with the $NH_4$ assay as described in Section 4.2 is also possible. Ammonium uptake by the -N control roots (-succ-N) was very low. Roots that had absorbed N shortly before the test were able to absorb

Table 12. Ammonium uptake, and changes in Na and C-A content of roots (in meq/kg DM) of different nitrogen status, exposed to succinate incubation and ammonium nutrition. Experiment 13.

| | -succ | | +succ | |
|---|---|---|---|---|
| | -N | +N | -N | +N |
| $NH_4$ uptake | 20 | -35* | 225** | 164** |
| Na(0h) | 18 | 30 | 575 | 645 |
| Na(4h) | 13 | 23 | 183 | 162 |
| ΔNa | -5 | -7 | -392 | -483 |
| C-A(0h) | 29 | -93 | 571 | 620 |
| C-A(4h) | -29 | -59 | 44 | -2 |
| Δ(C-A) | -58 | 34 | -527 | -622 |

$NH_4$ due to carboxylate pretreatment.

Chemical composition of the roots after incubation and after $NH_4$ uptake is given in Table 12. $NH_4$ nutrition of the +N roots was interrupted by 16 hours of succinate incubation. In this period assimilation of previously absorbed $NH_4$ ions may have been responsible for the low C-A in the -succ+N roots. During succinate incubation (+succ+N), Na accumulation was obviously of more importance than $NH_4$ assimilation. Negative C-A values come from the omission of free $NH_4$ ions in the ionic balance, as will be shown in Chapter 5. Apparent loss of $NH_4$ by the -succ+N roots occurred together with inorganic anion efflux (mainly phosphate) resulting in a slight C-A increase.

## 4.4 EFFECT OF AMMONIUM CONCENTRATION

Standard $NH_4$-uptake experiments were done with 1 meq $l^{-1}$ $NH_4Cl$. This concentration has the advantage that the $NH_4$ uptake system is highly saturated at this point (Lycklama, 1963; Koster, 1973). Hence changes in concentration caused by depletion of ambient $NH_4$, hardly affect the uptake rate. A disadvantage of this concentration is that it may be in between two uptake mechanisms (I and II). The aim of Experiment 14 was to study ammonium uptake and the succinate effect at several $NH_4$ concentrations.

*Exp. 14: Standard succinate incubation conditions and standard $NH_4$-uptake conditions with 0.10, 0.25, 1 and 10 meq $NH_4Cl$ $l^{-1}$. The same analytical techniques were used throughout the experiments, only the root: solution ratio was changed, when necessary.*

As the comparison was made in separate experiments, the succinate effect as such is more relevant than the absolute height of $NH_4$ uptake. Average results of the several tests are plotted in Figure 17. It seems that the effect of succinate is small in the concentration range of Mechanism I and greater in the second part of the isotherm if it occurs (Mechanism II). No proof for the existence of a dual isotherm for ammonium uptake has been presented till now (Epstein, pers. comm.). The data of Berlier et al. (1969), Fried et al. (1965), Tromp (1962) and Picciurro et al. (1967) that seem to be in favour of a dual isotherm were obtained in the absence of Ca, which introduces a measure of uncertainty (Epstein, 1972) by its effect on membrane stability. In an extensive reanalysis of uptake kinetic data from literature, Nissen (1973a,b) demonstrated that the dual isotherm handling of uptake data for most ions can as well be replaced by a multiphasic single isotherm

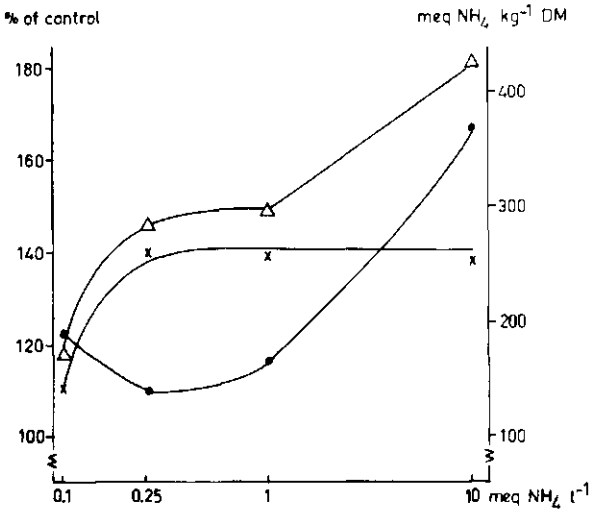


Fig. 17. Ammonium uptake by succinate pretreated roots (Δ) and control roots (x) as a function of $NH_4$ concentration (right-hand ordinate). On the left-hand ordinate the uptake of +succinate roots as % of the control (●). Experiment 14.

approach. From Figure 17 it seems that only the +succinate roots show a transition at about 1 meq $l^{-1}$. This finding is in agreement with the concept, that "organic acid" synthesis interferes only with the low-affinity part of the uptake isotherm (Torii & Laties, 1966a,b; Marschner, 1969). Curves like the one obtained with -succinate roots support the single isotherm. Curves of similar shape were found for maize up to 1.75 meq $l^{-1}$ (van den Honert & Hooymans, 1961) or 1.5 meq $l^{-1}$ (Becking, 1956), for potato disks up to 40 meq $l^{-1}$ (van den Honert & Hooymans, 1961), for perennial ryegrass up to 1 meq $l^{-1}$ (Lycklama, 1963), for bean plants up to 3 meq $l^{-1}$ (Hentschel, 1970) and for soyabean up to 2.1 meq $l^{-1}$ (Koster, 1973). In the cited experiments attempts were made to exclude the free space effect. If we assume that the contribution of the AFS to the $NH_4$ uptake in 4 h is about one half at 1 meq $l^{-1}$ and about two thirds at 10 meq $l^{-1}$ (graphical analysis of Figs. 4, 5, 18B and 22) and we correct Figure 17 for these data, there is no reason to believe that steady state uptake at 10 meq is higher than at 1 meq. AFS data in the low concentration range are lacking, because no time studies were made. Major changes in inorganic composition after 4h uptake are found in root samples pretreated with succinate. With increased $NH_4$ concentration the C-A decreases and the uptake of $NH_4$ increases, partly balanced by efflux of Na and K ions.

## 4.5 EFFECT OF ANIONS

In Experiments 15 and 16 the effect of succinate incubation on subsequent ammonium uptake was compared for several inorganic ammonium salts at concentrations of 1 and 10 meq $l^{-1}$. The former concentration was standard and the latter was chosen because it was inferred from the results of Section 4.4 that high succinate effects were found at this concentration.

*Exp. 15: Standard succinate incubation conditions and standard $NH_4$-uptake conditions with $NH_4Cl$, $NH_4H_2PO_4$, $(NH_4)_2SO_4$, $NH_4HCO_3$ and $(NH_4)_2CO_3$.*
*Exp. 16: Standard succinate incubation and $NH_4$-uptake conditions with 10 meq $NH_4$ $l^{-1}$ as $NH_4Cl$, $NH_4H_2PO_4$, $(NH_4)_2SO_4$ and $(NH_4)_2CO_3$.*

At a concentration of 1 meq $l^{-1}$ most $NH_4$ was taken up from $HCO_3^-$ and $CO_3^{2-}$ salts. Carbonate and bicarbonate of ammonium solutions are alkaline[1], which favours cation uptake in general and $NH_4$ entry due to $NH_4OH$ diffusion (Tromp, 1962; Lycklama, 1963; van den Honert & Hooymans, 1961; Barker et al., 1966). Simultaneous conversion of bicarbonate and carbonate anions to $CO_2$ ($H_2CO_3$) at the prevailing cell sap pH and discharge of $NH_4$ cations by metabolism may facilitate ammonium entry. Carbon from $HCO_3^-$ or $CO_3^{2-}$ is used in root cell metabolism to synthesize carboxylate, mainly reported to be malate. This dark fixation reaction serves as an alternative source for carboxylate production (Gauch, 1972; Splittstoesser, 1966). If dark $CO_2$ fixation occurred, a high $NH_4$ uptake rate from (bi)carbonate solutions would be an indirect indication for the importance of carboxylates in the uptake mechanism. An effect of succinate pretreatment is then expected to be small.

The average effect of succinate tended to increase in the order $H_2PO_4^-$, $SO_4^{2-}$, $Cl^-$, $CO_3^{2-}$ and $HCO_3^-$ (123*, 128*, 131*, 133*, 145**% resp. of the control). The uptake of $NH_4$ (mean of + and -succinate) increased in the same sequence up to 200%. Lycklama (1963) found increasing uptake in the order $SO_4^{2-}$, $H_2PO_4^-$, $Cl^-$ with intact seedlings of perennial ryegrass at pH 5.5-6.0 and 1 meq $NH_4$ $l^{-1}$. The difference between (C-A) values of roots pretreated with succinate or with $CaCl_2$ after 4 hours of ammonium uptake was positively correlated with the amounts of absorbed $NH_4$ and with the effect of succinate preloading on the absorption. Analysis of time-uptake graphs yielded AFS values of about 160 (-succ) and about 220 (+succ) meq $NH_4$/kg DM.

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1. Negligible amounts of $NH_3$ were lost from control pots without roots.

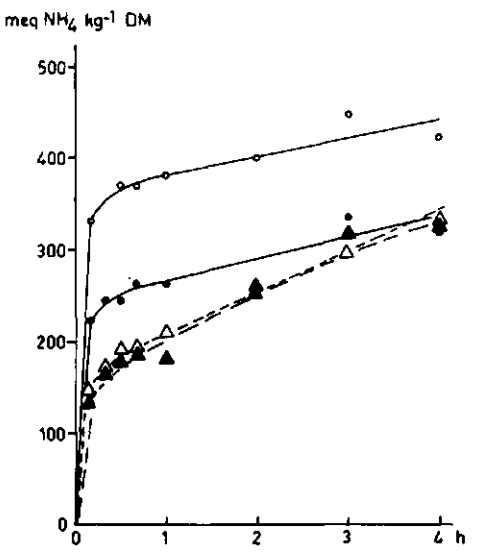


Fig. 18A. Time curves of $NH_4$ absorption from 10 meq $l^{-1}$ $NH_4$-salt solutions; $NH_4Cl$ (o) and $NH_4H_2PO_4$ (Δ). Solid symbols = controls, open symbols = succinate pretreated roots. Experiment 16.

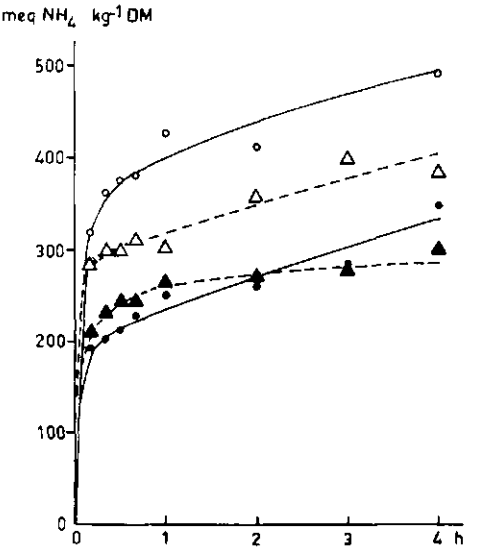


Fig. 18B. Time curves of $NH_4$ absorption from 10 meq $l^{-1}$ $NH_4$-salt solutions; $(NH_4)_2SO_4$ (o) and $(NH_4)_2CO_3$ (Δ). Solid symbols = controls, open symbol = succinate pretreated roots. Experiment 16.

At 10 meq $l^{-1}$, uptake of $NH_4$ from a carbonate solution (pH 8) was not higher than that from solutions at common pH with chloride, sulphate or phosphate as anion and no effect of succinate pretreatment was observed. The succinate effect tended to increase in the order $CO_3^{2-}$, $H_2PO_4^-$, $SO_4^{2-}$, $Cl^-$ (97, 132*, 138* and 161**% of the control resp.). The uptake (mean of + and -succinate) also increased in the same order, just as observed in Experiment 15. Bicarbonate or carbonate ions can neutralize the acidity produced by ammonium assimilation (Dijkshoorn, 1969) and in this sense they may save carboxylate breakdown, the process that normally balances $NH_4^+$ discharge to $N_{org}$. At the higher concentration (10 meq $CO_3^{2-}$/l) this system was inoperative or was not involved in stimulation of $NH_4$ entry. According to Hiatt & Hendricks (1967) plant respiration yields enough $CO_2$ for carboxylate formation. Cram & Laties (1974) reported that enough $CO_2$ for malate synthesis is available at 1 meq $HCO_3^-$ $l^{-1}$. High $CO_2$ concentrations are toxic for plants and repress ion uptake (Jacobson et al., 1967). Carrodus (1966) experimenting with excised mycorrhiza's of beech, found reduced $NH_4$ uptake from a 10 meq $NH_4HCO_3$ $l^{-1}$ medium, as compared with $NH_4Cl$. Above 7.5 meq $l^{-1}$, bicarbo-

nate no longer stimulated ammonium uptake.

Time course of ammonium uptake in Experiment 16 is shown in Figure 18A and B. It seems that differences in AFS (+succinate roots show higher AFS values than -succinate roots) and in the ratio passive (AFS): active uptake exist. The effect of carboxylates on the AFS will be discussed later.

## 4.6 EFFECT OF SUCCINATE CONTENT OF THE ROOTS

Stimulation of $NH_4$ uptake after incubation in 50 mM succinate was found in Experiments 4, 11, 12, 13, 14 and 15. To investigate the effect of other succinate concentrations on subsequent $NH_4$-uptake, roots were preloaded with a variety of succinate concentrations (Experiment 17) and in the next 4 hours the effect on ammonium absorption was measured.

*Exp. 17: Standard succinate incubation conditions with 0-100 mM succinate. Standard $NH_4$-uptake conditions.*

Experiment 6 showed that the carboxylate and succinate content of roots keeps pace with the succinate incubation concentration (Figure 6). Figure 19 shows that $NH_4$ absorption is stimulated by succinate concentrations up to 100 mM, but the effect increases only slightly if the concentration exceeds 25 mM.

*Exp. 18: Standard succinate incubation conditions with 0-50 mM succinate. Standard $NH_4$-uptake conditions.*

In Experiment 18 the effect of succinate in a lower concentration range was investigated (Figure 20). The succinate effect was not unexpectedly high at

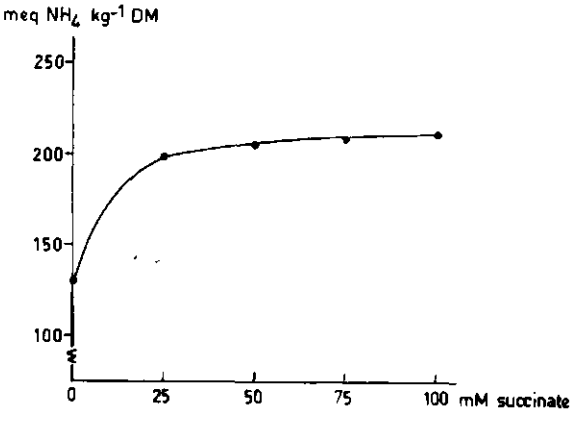


Fig. 19. Effect of succinate incubation concentration on subsequent $NH_4$-uptake. Experiment 17.

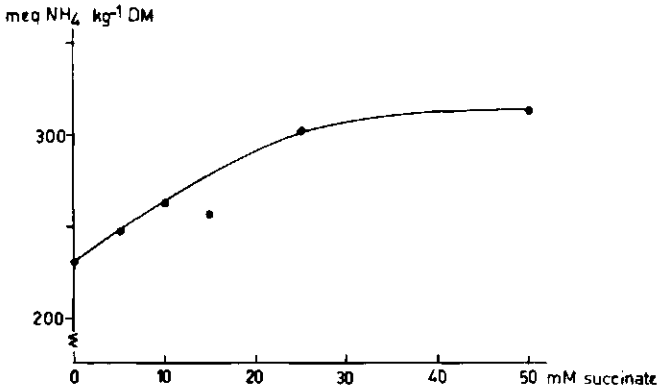


Fig. 20. Effect of succinate incubation concentration on subsequent $NH_4$-uptake. Experiment 18.

low concentrations, but increased steadily up to 25 mM. Again the effect increased only slightly at concentrations over 25 mM. C-A values before and after $NH_4$-uptake and changes in C-A and succinate content (Table 13) parallelled the succinate incubation concentration.

It is possible that the succinate effect is caused by succinate as such or by the formation of compounds derived from succinate in the citrate cycle, e.g. α-oxocarboxylates or its consequences. It is known that α-oxocarboxylates acids are the first step in the organization of mineral nitrogen (Prianishnikov, 1951). In Experiments 19 and 20 succinate metabolism was inhibited by malonate (Lips et al., 1966) and in Experiment 21 the effect of the incubation with an α-oxocarboxylate (α-oxoglutarate) was compared with the effect of succinate.

Table 13. Ammonium uptake, and (changes in) C-A and succinate content (in meq/kg DM) after incubation in several succinate concentrations and subsequent $NH_4$-uptake. Experiment 17.

| mM succinate | C-A | | | Succinate | | | |
|---|---|---|---|---|---|---|---|
| | 0h | 4h | Δ(C-A) | 0h | 4h | Δsucc | $NH_4$ uptake |
| 0 | 268 | 170 | -98 | 22 | 14 | -8 | 135 |
| 25 | 334 | 216 | -118 | 63 | 24 | -39 | 198* |
| 50 | 467 | 220 | -247 | 121 | 26 | -95 | 205* |
| 75 | 800 | 351 | -449 | 234 | 42 | -192 | 208* |
| 100 | 837 | 364 | -473 | - | 53 | - | 211* |

*Exp. 19: Standard succinate incubation conditions. Standard $NH_4$-uptake conditions, with or without 25 mM Na-malonate (pH 5.5).*

*Exp. 20: Standard carboxylate incubation conditions with succinate or succinate+25 mM malonate. Standard $NH_4$-uptake conditions.*

*Exp. 21: Standard carboxylate incubation conditions with succinate or α-oxoglutarate. Standard $NH_4$-uptake conditions.*

The effect of malonate added during $NH_4$ uptake (Table 14) was a 16% depression and the effect of succinate incubation a 23% stimulation. The effects were independent of succinate pretreatment and malonate addition, respectively. Succinate stimulation thus was not prevented by the presence of malonate, and $NH_4$ uptake by both the control and the succinate pretreated roots was on a lower level due to ambient Na ions (cf. Figure 2). C-A data of the roots after the absorption period reflect the effect of malonate uptake (cf. Table 8) and/or succinate accumulation.

Malonate added during succinate incubation before $NH_4$ uptake rapidly promoted $NH_4$ entry (Figure 21). It is possible that the succinate dehydrogenase block was incomplete or overcome under the present conditions (Table 8, Lips et al., 1966) and that the plants benefitted by extra succinate present at the start of uptake. Literature data on malonate inhibition are conflicting (Laties, 1949; Laties & Hoelle, 1965). Corn root segments showed only 20% decrease in oxygen consumption in a 6-hour period following malonate (100 mM, pH 5.0) addition (Lips et al., 1966). A pathway to overcome malonate blockage has been suggested by Lips & Beevers (1966). In aged potato tissue discs, the addition of malonate (50 mM, pH 5.6) stimulated $O_2$ uptake for about 6 hours and stimulated sulphate entry for some 20 hours (Hanebuth et al., 1974). Pretreatment with malonate in the absence of succinate severely depressed $NH_4$ uptake (Chapter 5).

Table 14. Ammonium uptake, and Na and C-A in roots (in meq/kg DM), that took up $NH_4$ in the absence or presence of 25 mM malonate. Experiment 19.

| | -succinate | | | +succinate | | |
|---|---|---|---|---|---|---|
| | $NH_4$ uptake | Na | C-A | $NH_4$ uptake | Na | C-A |
| -malonate | 227 | 27 | 41 | 278 | 200 | 147 |
| +malonate | 191 | 227 | 258 | 234 | 402 | 370 |

| Succinate effect | | Malonate effect | |
|---|---|---|---|
| -malonate | 123%* | -succinate | 84%* |
| +malonate | 123%* | +succinate | 84%* |

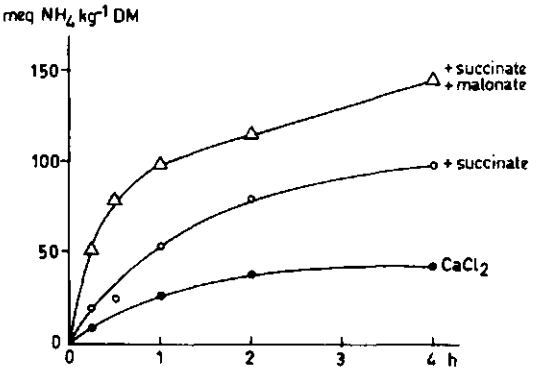


Fig. 21. Ammonium uptake after incubation in $CaCl_2$, succinate and succinate + malonate media. Experiment 20.

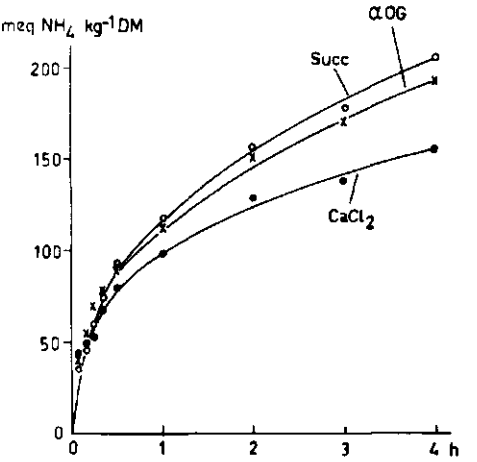


Fig. 22. Ammonium uptake after incubation in $CaCl_2$, succinate and α-oxoglutarate. Experiment 21.

Fig. 22 shows that not just succinate but also compounds metabolically derived from it - e.g. α-oxoglutarate - can stimulate ammonium absorption. Roots incubated in succinate or in α-OG took up respectively 31 and 23% more $NH_4$ than the control roots.

From the experiments in this section it is deduced that, beside succinate, an α-oxocarboxylate like α-OG stimulates $NH_4$-uptake. The effects of succinate incubation and malonate present during $NH_4$-uptake were not interdependent, suggesting that the succinate stimulus is not completely accounted for by oxidation of succinate itself. As shown in Figure 6 and Table 8, succinate incubation alters not only the succinate content of roots. Accumulation and metabolism of other compounds possibly enhances $NH_4$-uptake.

## 4.7 SUCCINATE AND CHANGES IN ROOT COMPOSITION DURING AMMONIUM UPTAKE

Some aspects of inorganic root composition before and after 4-hour uptake trials have been treated in preceding experiments.

*Exp. 22: Standard conditions for succinate incubation and $NH_4$-uptake.* In Experiment 22 changes in chemical composition were followed during the whole period of ammonium uptake and analyses were extended to some organic components as well. $NH_4$ uptake was stimulated 20% by succinate pretreatment

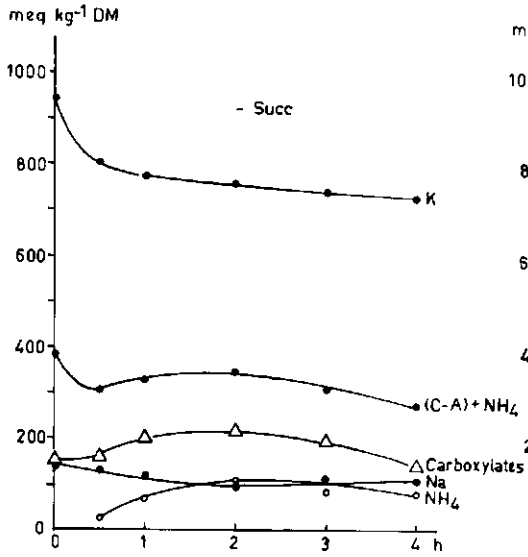


Fig. 23A. Contents of K, Na, $NH_4$, carboxylates and C-A (incl. $NH_4$) in $CaCl_2$ pretreated roots during $NH_4$ uptake. Experiment 22.

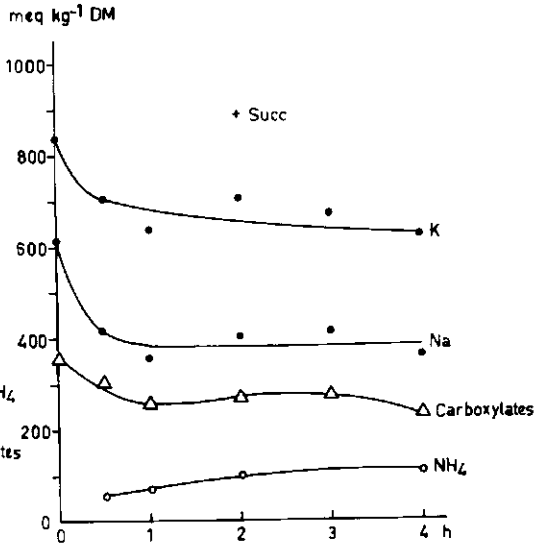


Fig. 23B. Contents of K, Na, $NH_4$ and carboxylates in succinate pretreated roots during $NH_4$ uptake. Experiment 22.

and the curves of $NH_4$ uptake versus time were essentially as Figures 4 and 22. Inorganic constituents are plotted in Figure 23A and B. In the course of time Na and K are lost. Succinate pretreated roots showed a higher initial content and efflux to the $NH_4$ medium of sodium, a further decrease in carboxylate (C-A) content, and a higher carboxylate level throughout the experiment. Free ammonium ions concentrated in the tissue up to values of about 100 meq/kg DM. Accumulation of $NH_4$ was somewhat higher in +succinate roots than in the control roots. Enough ammonium ions were present to explain the negative C-A values that were sometimes found in the roots. Throughout the experimental period (Figure 23A), the C-A data including ammonium were about 100 meq higher than the amounts of determined carboxylates.

Absorbed ammonium was readily incorporated into free amino compounds (Figure 24). After about 60 minutes the size of the free amino acid pool decreased and, because $NH_4$ uptake continued, protein synthesis must have accelerated and its rate overtaken the synthesis rate of free amino acids and amides. Initially and after 2 hours, diversification of the soluble N-pool was small as it comprised mainly amides. Yemm & Willis (1956) obtained the same results with nitrogen-starved barley roots grown for 27

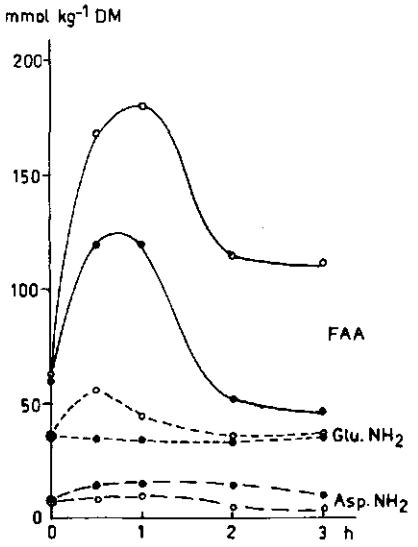


Fig. 24. Contents of free amino acids (FAA), asparagine and glutamine during $NH_4$ absorption. Open symbols = succinate pretreated roots, solid symbols = control roots. Experiment 22.

hours in a $NH_4$-medium. Ivanko & Maksianova (1968) and Ivanko (1971) also recovered the bulk of assimilated N in the amide fraction of plant roots. Synthesis of free amino acids occurred at the highest rate in +succinate roots, which also showed the highest $NH_4$-uptake rate. In both kinds of roots glutamine was the prevailing amide and it was somewhat more readily synthesized in +succinate than in -succinate roots. Amino acid exudation by detopped tomato plants grown with ammonium was increased by the addition of 25 mM K succinate at pH 4.5 (Hofstra, 1966).

In the control roots more carbohydrate (starch + water-soluble carbohydrates) was consumed during $NH_4$ uptake than in the succinate preloaded roots, indicating that carboxylates can save carbohydrate breakdown. The carbohydrate content of the roots started to decrease after about 2 hours of ammonium uptake in both cases.

## 4.8 GLUCOSE AND AMMONIUM UPTAKE

Carboxylates are derived from carbohydrates, e.g. through glycolysis and the citrate cycle. Stimulatory action of carbohydrates like glucose and sucrose on ion uptake has often been reported (Hoagland & Broyer, 1936; Budd & Harley, 1962a; Okuda & Ida, 1966a; Carrodus, 1966; Breteler, 1973c). In this section the effect of glucose on $NH_4$ uptake will be described. The effect of glucose will be compared with that of succinate in the next section. Entry of glucose and the relationship between the sugar content of

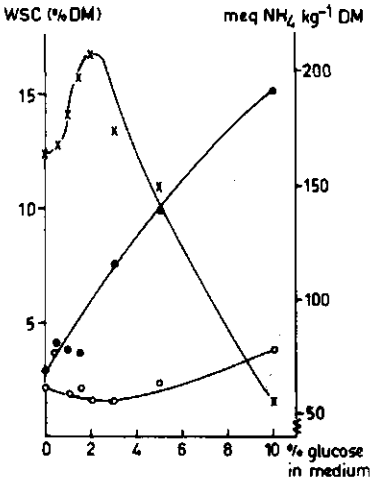


Fig. 25. Water-soluble carbohydrate content of roots after 4h of incubation in glucose solutions of different concentration (●) and after 4h of subsequent $NH_4$-uptake (○). $NH_4$ uptake (x) indicated on the right-hand ordinate. Experiment 23.

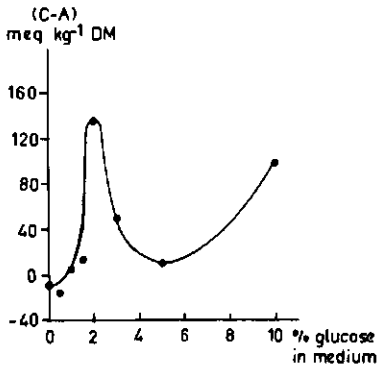


Fig. 26. C-A content of roots after 4h $NH_4$ uptake as a function of the glucose concentration during incubation, prior to $NH_4$ uptake. Experiment 23.

the roots and $NH_4$ uptake will be described in this section.

The amount of soluble sugars in the excised roots of maize increased as the glucose concentration in the medium increased (Experiment 23, Figure 25).

*Exp. 23: Excised roots were exposed to glucose solutions (0-10% w/v + 1 meq $CaCl_2$ $l^{-1}$) for 4 hours, room temperature, aeration. After this period part of the roots was analysed and the rest subjected to standard $NH_4$-uptake conditions.*

As far as stimulation of $NH_4$ uptake is concerned there exists a clear optimum effect of incubation with 2% glucose (0.11 M), corresponding with 6% water-soluble carbohydrates in the roots. Depression of ammonium uptake after pretreatment at increased glucose concentrations results from too high osmotic pressures, for example 5% glucose (0.28 M) = 6.6 at. After four hours of $NH_4$ absorption, the carbohydrate content of the roots was decreased, due to dissimilation and efflux of carbohydrates to the $NH_4$ medium, especially from roots pretreated in high glucose concentrations. Ammonium uptake increased and residual WSC content decreased in roots that were incubated in glucose concentrations up to 2%. Increase in residual WSC content

between 2 and 10% obviously had no function in stimulating $NH_4$ uptake. Figure 26 demonstrates that roots with the highest ammonium absorption also showed the highest residual carboxylate (C-A) content. A high carboxylate content of roots incubated with 10% glucose was not effective in stimulating $NH_4$ entry.

## 4.9 GLUCOSE AND THE SUCCINATE EFFECT

The effect of succinate pretreatment on ammonium absorption with and without glucose was studied in Experiment 24, the results of which are presented in Figure 27 and Table 15.

Table 15. Effect of glucose addition and succinate pretreatment, in the absence or presence of each other, on $NH_4$ uptake. Experiment 24 with 2% glucose, and Experiment 26 with 1% glucose and roots of etiolated plants.

| Experiment | Effect of glucose | | Effect of succinate | |
|---|---|---|---|---|
| | -succinate | +succinate | -glucose | +glucose |
| 24 | 125*% | 104% | 155**% | 129*% |
| 26 | 116% | 109% | 120*% | 112% |

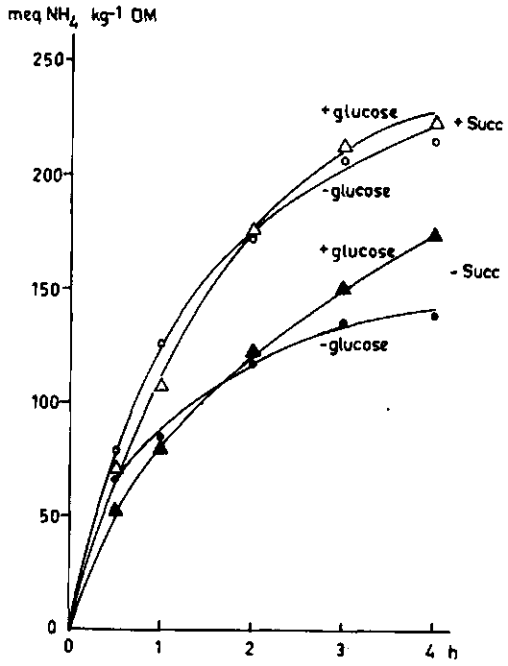


Fig. 27. Ammonium uptake in the presence and absence of 2% glucose. Open symbols = succinate pretreated roots, solid symbols = control roots. Experiment 24.

*Exp. 24: Standard succinate incubation conditions. Standard $NH_4$-uptake conditions with and without 2% (w/v) glucose added to the uptake solution.*

There is a clear interaction between glucose and succinate supply because the effect of glucose is non-significant in high carboxylate roots, while without glucose the effect of succinate is twice that with glucose. The effect of succinate preloading is more pronounced than the effect of glucose addition. Absorbed glucose can be considered as a buffer, able to supply carboxylates, where and when needed and it seems that the effect of both substances is more or less complementary. Laties (1949) showed a higher pyruvate stimulation of respiration in carbohydrate-depleted than in normal barley roots.

The succinate-glucose interaction was tested in a concentration range of 0-1% glucose present during $NH_4$ uptake in Experiment 25 (Figure 28).

*Exp. 25: Standard succinate incubation conditions. Standard $NH_4$-uptake conditions with 0-1% glucose (w/v) added to the uptake solution.*

Basically the tendencies of Experiment 24 (2% glucose) are confirmed over a whole range of glucose concentrations stimulating uptake. With increased glucose concentration the succinate effect declines, while the glucose

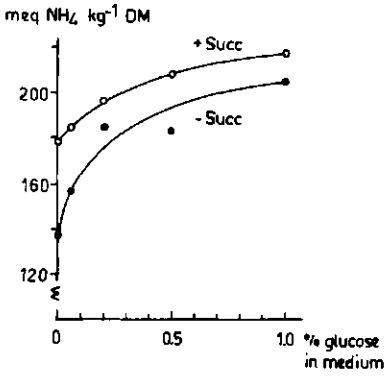


Fig. 28. Ammonium uptake as a function of glucose concentration, present during $NH_4$ uptake. Experiment 25.

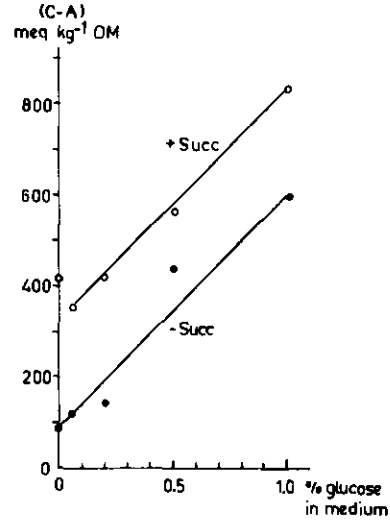


Fig. 29. C-A values of roots after 4h of $NH_4$ uptake in the presence of glucose as a function of the glucose concentration. Experiment 25.

effect is less pronounced in roots with a high carboxylate content than in low carboxylate roots. Exogenous carbohydrate replenishes the carboxylate pool (Figure 29), a phenomenon already shown in Figure 26. Differences in the amounts of carboxylate present after N uptake in the sets of roots represented in both figures come from higher initial carboxylate (C-A) contents of the roots in Experiment 25 and different ways of glucose administration (before or during $NH_4$ uptake).

*Exp. 26: Conditions as in Experiment 24. Roots of plants that have been kept for 1 week in the dark.*

Finally the effect of succinate on ammonium absorption was studied in roots with different carbohydrate situations. In roots of etioled plants the same tendencies as observed in Experiment 24 were found (Experiment 26, Table 15). Compared with 'normal carbohydrate status' roots, (Experiment 24) the roots of etiolated plants absorbed less $NH_4$ ions; the effects of both glucose addition and succinate preloading were depressed, except that glucose tended to have slightly more effect in succinate pretreated roots.

*Exp. 27: Standard conditions for succinate incubation and $NH_4$ uptake. Roots of plants cultivated for 3 weeks on 0.5 meq $CaSO_4$ $l^{-1}$.*

In roots of plants cultivated on a gypsum medium, a negative effect of succinate pretreatment was found (21% less $NH_4$ uptake than in the control). This is not in contradiction with the other results of this section as the roots showed an extremely high carbohydrate content. A net efflux of carbohydrates to the $NH_4$ medium was measured during nitrogen uptake. Sugar loss from low salt roots to a high salt solution has also been reported by Pitman et al. (1971). This finding should be taken into account when interpreting results of ion uptake studies with roots of plants cultivated on gypsum solutions (low salt roots) (Hoagland & Broyer, 1936; Overstreet et al. 1942; Marschner, 1968; Pitman et al., 1971; Leigh & Wyn Jones, 1973; Louwerse, 1967). During 3 weeks of growth on $CaSO_4$ mineral deficiencies may also have occurred.

## 4.10 RESPIRATION AND THE SUCCINATE EFFECT

The citrate cycle is always an active part of aerobic repiration. Respiration rate, and thus the intensity of carboxylate metabolism, is influenced by various environmental factors. Experiments were conducted to re-

veal whether the carboxylate (succinate) stimulus is affected in the same way by environmental factors as respiration is.

*Exp. 28: Standard succinate incubation conditions. Standard $NH_4$-uptake conditions with air and $N_2$ gassing.*

The effect of aeration on $NH_4$ uptake and the succinate effect was studied in Experiment 28. The nitrogen gas was of a technical grade and contained not more than 50 ppm $O_2$. Under these circumstances glycolysis continues, but the citrate cycle is inoperative. Replacement of air by $N_2$ reduced ammonium uptake by 39% in the +succinate and by 59% in the -succinate roots (Table 16). Hence the succinate effect is not completely of a respiratory nature. Malonate inhibition tests (Section 4.6) left this possibility open. The uptake of many ions has been reported to be repressed by anaerobiosis (Hoagland & Broyer, 1936).

*Exp. 29: comprises experiments with standard succinate incubation and standard $NH_4$-uptake conditions at 2, 10, 20 and 30°C.*

In Experiment 29 ammonium uptake stimulation by succinate was studied as a function of temperature. Uptake data are presented in Table 17 and Figure 30, and the (C-A) values before and after $NH_4$ absorption in Table 18. In the temperature range investigated, the effect of succinate preloading increased with temperature. Respiration rate is known to increase in the same direction.

Figure 31 shows the carboxylate (C-A) contents of roots after $NH_4$ uptake. To get an idea about changes affected by temperature in the carboxylate pool of excised roots without interference of nitrogen uptake and assimila-

Table 16. $NH_4$ uptake (in meq $NH_4$/kg DM) by succinate pretreated and control roots from solutions, that were gassed with air or nitrogen. Experiment 28.

| | Air | $N_2$ |
|---|---|---|
| -succinate | 78 | 32* |
| +succinate | 195** | 118** |

| Effect of $N_2$-gassing | | Effect of succinate | |
|---|---|---|---|
| -succinate | 41% | air | 250% |
| +succinate | 61% | $N_2$ | 369% |

Table 17. Ammonium uptake in meq/kg DM by succinate pretreated and control roots at 2 and 20°C. Experiment 29.

| | 2°C | 20°C |
|---|---|---|
| -succinate | 109 | 127 |
| +succinate | 108 | 199 |

| Succinate effect | | Temperature effect | |
|---|---|---|---|
| 2°C | 99% | -succinate | 117% |
| 20°C | 157%* | +succinate | 184%* |

Table 18. C-A (meq/kg DM) of succinate pretreated and control roots after incubation and after 4 h of subsequent $NH_4$-uptake at 2 and 20°C. Experiment 29.

| -succinate | | | +succinate | | |
|---|---|---|---|---|---|
| 0h | 4h, 2°C | 4h, 20°C | 0h | 4h, 2°C | 4h, 20°C |
| 293 | 66 | -4 | 578 | 163 | 53 |

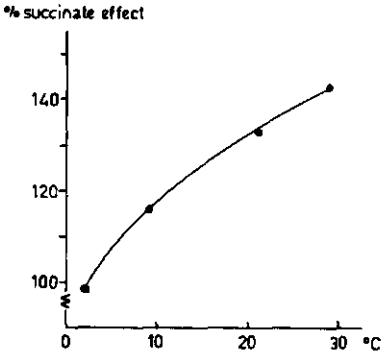


Fig. 30. Effect of temperature on the succinate effect (uptake by +succinate roots as % of control) on $NH_4$ uptake. Experiment 29.

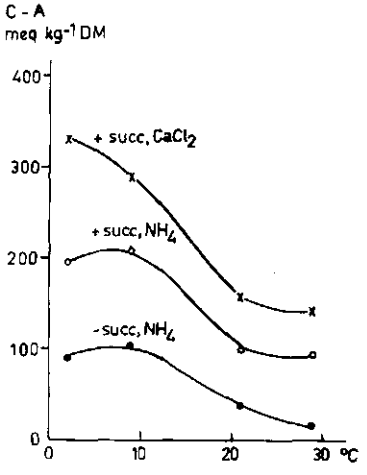


Fig. 31. C-A of succinate pretreated and control roots after 4h in (1 meq $NH_4Cl$ + 0.1 meq $CaCl_2$) $l^{-1}$ as a function of temperature. Experiment 29.

tion, a parallel test was done with succinate pretreated roots in the absence of $NH_4$. The effect of $CaCl_2$ as such on the carboxylate pool can be considered as small under the prevailing conditions. More organic acid anions are lost as temperature increases. As a consequence of $NH_4$ assimilation, the carboxylate content was lowered after 4 hours of $NH_4$ uptake and both lines concerning carboxylates after ammonium absorption (Figure 31) show a decreasing tendency with increase in ambient temperature. Combining Figures 30 and 31 it is concluded that the more carboxylates are lost, the higher the stimulus of succinate preloading on $NH_4$ uptake. Data on changes in (C-A) of Table 18 are quantitatively in accordance with Figure 31.

*Exp. 30 comprises experiments with standard succinate incubation conditions and standard $NH_4$-uptake conditions with or without 1 mM $NaN_3$.*

The effect of sodium azide, an uncoupler of oxidative phosphorylation, (Bryant, 1971; Ikuma, 1972) on $NH_4$ ion uptake was investigated in Experiment 30. Azide in the uptake solution resulted in a very low $NH_4$-uptake rate after the 'free space shoulder' (15 minutes) of the bottom curve in Fig. 32. In a parallel experiment the effect of azide on $NH_4$ uptake by -succinate roots was included (Table 19). Some stimulation of succinate pretreatment seemed to be exerted in the presence of azide, another indication that the succinate effect is not completely metabolic. Repression of $NH_4$ uptake by $NaN_3$ was similar in both experiments. A balance sheet for the ionic composition before and after $NH_4$ uptake is given in Table 20. Pretreatment with succinate resulted in higher Na, C, A and C-A contents. For changes in inorganic composition $NH_4$ uptake presents the well-known picture. Na de-

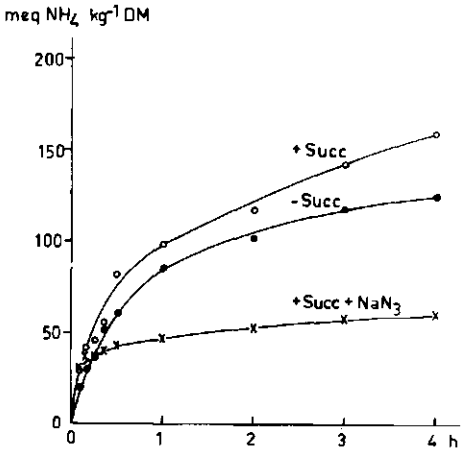


Fig. 32. Ammonium uptake of control and succinate pretreated roots in the presence or absence of 1 mM $NaN_3$. Experiment 30.

Table 19. Effect of $NaN_3$ and succinate pretreatment, in the presence and absence of each other, on $NH_4$ uptake. Experiment 30.

| Effect of succinate | | Effect of sodium azide | |
|---|---|---|---|
| $+NaN_3$ | 115% | +succinate | $34^{**}$% |
| $-NaN_3$ | $138^{*}$% | -succinate | $37^{**}$% |

Table 20. Ammonium uptake, and chemical composition (in meq/kg DM) of roots before and after pretreatment of roots in succinate solution and after 4 hours of $NH_4$-uptake in the presence or absence of 1 mM $NaN_3$. Experiment 30.

| | -succinate | | +succinate | | +succinate+$NaN_3$ | |
|---|---|---|---|---|---|---|
| | 0h | 4h | 0h | 4h | 0h | 4h |
| $NH_4$ uptake | | 125 | | 159 | | 59 |
| $Na$ | 195 | 171 | 476 | 266 | 476 | 293 |
| K | 546 | 461 | 531 | 431 | 531 | 497 |
| Ca | 84 | 66 | 60 | 64 | 60 | 68 |
| Mg | 48 | 53 | 56 | 50 | 56 | 50 |
| C | 873 | 751 | 1123 | 811 | 1123 | 908 |
| Cl | 292 | 314 | 367 | 340 | 367 | 315 |
| $SO_4$ | 139 | 133 | 131 | 115 | 131 | 135 |
| $H_2PO_4$ | 226 | 217 | 216 | 215 | 216 | 190 |
| A | 657 | 664 | 714 | 670 | 714 | 640 |
| C-A | 216 | 87 | 409 | 141 | 409 | 268 |
| Δ(C-A) | | -129 | | -268 | | -141 |

creases, even in the presence of sodium azide. Efflux of previously absorbed Na may have been lowered by the presence of ambient Na. During nitrogen uptake from $NH_4Cl$ potassium decreases and $CaCl_2$ pretreated plants accumulated some Cl. Presence of $NaN_3$ in the uptake solution resulted in a higher residual C-A possibly because a part of the previously accumulated carboxylates was inactive in the $NH_4$ absorption process.

## 4.11 INHIBITORS OF PROTEIN SYNTHESIS AND THE SUCCINATE EFFECT

Carboxylates supply the carbon skeletons for the incorporation of N in amino acids and proteins. Drainage of carboxylates in $NH_4$ assimilation is related to the availability (uptake) of nitrogenous ions. In this section some aspects of protein and nucleic acid synthesis in relation to the succinate effect are considered. Puromycine, cycloheximide (actidion), (D-threo-)

chloramphenicol and actinomycine $C_1$ (actinomycine D) were used as inhibitors, acting in various stages of protein and nucleic acid metabolism (Bücher & Sies, 1969; Briggs, 1973).

*Exp. 31: Comprises experiments with standard succinate incubation conditions. Standard $NH_4$-uptake conditions; 2 ppm cycloheximide, 20 ppm actinomycine $C_1$, 25 ppm puromycine or 50 ppm chloramphenicol added.*

Ammonium uptake experiments in the presence of the inhibitors yielded non-significant succinate effects in the presence of actinomycine $C_1$ or chloramphenicol (Table 21). Succinate history stimulated uptake from control media and from media containing cycloheximide or puromycine.

The negative effect of puromycine on ion uptake was overcome by succinate pretreatment. Actinomycine and chloramphenicol depressed $NH_4$-uptake more in +succinate than in -succinate roots.

Biochemical interpretation of the results without data on concomitant metabolic changes is dangerous. It has often been reported that the inhibitors used are involved in many reactions directly or indirectly coupled to synthesis of proteins, e.g. oxidative phosphorylation, mitochondrial electron transport, enzyme synthesis (Sutcliffe, 1973; Skogqvist, 1973; Wara-Aswapati & Bradbeer, 1974; Barash et al., 1974; Kylin, 1967b; Ellis & MacDonald, 1970). Moreover, the biochemical effect of the inhibitors depends on concentration, plant age, species and type of tissue (Böszörményi et al., 1972).

Table 21. Ammonium uptake (meq $NH_4$/kg DM) by succinate pretreated and control roots in the absence or presence of cycloheximide, puromycine, actinomycine $C_1$ or chloramphenicol. Experiment 31.

| | $NH_4$ | $NH_4$ + cycloh. | $NH_4$ + purom. | $NH_4$ | $NH_4$ + actinom. | $NH_4$ + chloram. |
|---|---|---|---|---|---|---|
| -succinate | 102 | 116 | 38 | 166 | 160 | 145 |
| +succinate | 161 | 175 | 161 | 229 | 144 | 142 |
| effect of succinate (%) | 158** | 151** | 424** | 138* | 90 | 98 |

| Effect of (in %) | -succinate | +succinate |
|---|---|---|
| cycloheximide | 114 | 109 |
| puromycine | 37** | 100 |
| actinomycine $C_1$ | 96 | 63* |
| chloramphenicol | 87 | 62* |

Puromycine is a very effective terminator of proteosynthesis by inhibition of polypeptide formation (Mummery & Valadon, 1973). It can be used to demonstrate a possible relationship between succinate stimulus and protein synthesis. With a large carboxylate pool at hand, blockage of protein synthesis did not hamper $NH_4$ absorption, while only 37% of the uptake capacity of the control roots was found in low carboxylate roots treated with puromycine.

The other results are ambiguous. The only conclusion, that can be drawn is that the studied inhibitors - except cycloheximide (a terminator of protein synthesis after amino acid activation or transfer of amino acids to tRNA) - all interfere with $NH_4$ uptake and that +succinate roots are more affected by actinomycine and chloramphenicol, while -succinate roots are severely repressed by puromycine.

## 4.12 CONCLUSIONS

1. Succinate incubation concentrations up to 25 mM promoted subsequent $NH_4$-absorption. Above this concentration the effect hardly increased (Experiments 7, 18). Inhibition of succinate metabolism by malonate did not destroy the pretreatment effect of succinate on ion uptake, indicating that succinate as such can be involved (Experiments 19, 20). Succinate pretreatment enhanced $NH_4$ uptake for at least 30 hours. The effect is fully developped in 4 hours and is independent of the nitrogen nutritional history of the roots (Experiments 12, 13). Although there is no reason to assume a dual isotherm for $NH_4$ uptake, the highest succinate effects were found in the high concentration range of Mechanism II (Experiment 14). The uptake of $NH_4$ is anion sensitive (1-10 meq $l^{-1}$) and the succinate effect is related to both concentration and anion species (Experiments 15, 16).
2. During ammonium entry succinate preloaded roots lost more Na and K, and contained more free ammonium and amino acids than control roots (Experiment 22).
3. Pretreatment of roots in glucose concentrations up to 2% increased the sugar content of the roots, subsequent $NH_4$-uptake and the carboxylate (C-A) level after the absorption period (Experiments 23, 25). The effect of glucose on $NH_4$ uptake was negligible in roots that were pretreated with succinate (Experiments 24, 26). All data indicate interrelation between carboxylate and carbohydrate consumption.
4. The succinate effect has non-metabolic and metabolic aspects (Experiments

28, 29, 30). $N_2$ gassing reduced $NH_4$ uptake but succinate pretreatment was still effective. The effect of succinate increased with the temperature during $NH_4$ uptake. The presence of sodium azide reduced both $NH_4$ uptake and the succinate effect. In the presence of inhibitors of protein synthesis, the effect of succinate preloading was never found to be significantly negative (Experiment 31).

# 5 The effect of other carboxylates on ammonium uptake

In Chapter 3 succinate stimulation of $NH_4$ uptake was described and some aspects of this phenomenon specified in Chapter 4. In order to judge generality of conclusions the reaction of ammonium uptake upon pretreatment with other carboxylates was investigated in Experiment 32. Results of Experiment 10 (Table 8) revealed that all carboxylates were accumulated at almost equal rates.

*Exp. 32: comprises experiments with standard carboxylate incubation conditions (50 mM or 10 mM) with oxaloacetate, citrate, α-oxoglutarate, succinate, fumarate, malate, pyruvate, malonate, oxalate, glyoxylate, oxalosuccinate or cis-aconitate. Standard $NH_4$-uptake conditions.*

Ammonium uptake after the several pretreatments is given in Table 22. After standard incubation conditions (Table 22, first column), succinate and α-oxoglutarate stimulated and citrate tended to depress $NH_4$ uptake (cf. Figures 5 and 22). Other stimulating carboxylates are oxaloacetate, malate,

Table 22. Ammonium uptake (in meq $NH_4$/kg DM) after incubation of roots in several carboxylate solutions. Experiment 32.

| | Incubation concentration | |
|---|---|---|
| | 50 mM | 10 mM |
| control | 143 | 218 |
| oxaloacetate | 193* | 232 |
| citrate | 123 | |
| α-oxoglutarate | 204** | |
| succinate | 233* | 230* |
| fumarate | 122 | |
| malate | 190* | |
| pyruvate | 180* | |
| malonate | 56** | |
| glyoxylate | 44** | |
| oxalate | 40** | |
| oxalosuccinate | | 119** |
| *cis*-aconitate | | 249* |

pyruvate and cis-aconitate, while glyoxylate, malonate, oxalate and oxalosuccinate showed depressive action.

Carrodus (1966) working with mycorrhizal roots of beech, found that $NH_4$ entry was stimulated by the presence of (10 mM, pH 6, 20h uptake period) succinate, fumarate and malate. Oxo-carboxylates exerted small effects, while citrate, acetate and aconitate repressed N uptake. Negative effects or absence of stimulation was ascribed to lack of carboxylate entry or enzyme inhibition. Malate and succinate were most effective, the magnitude of their effect being rather variable.

Budd (1966) reported that $NH_4$ uptake in mycelium of *Neocosmospora vasinfecta*, growing in a glucose-containing medium, was stimulated by the addition of malate, succinate, citrate, α-oxoglutarate, pyruvate and acetate. Acetate was the most potent stimulator, while citrate stimulation was only low.

Okuda & Ida (1966b) found acetate to decrease oxygen uptake and $NH_4$ entry in nitrogen-starved cells of *Chlorella ellipsoidea* grown in the dark. Glucose and glycolate acted stimulatively. After 3 hours illumination the effect of acetate on ammonium uptake became positive. Glycolate worked also positively under photosynthetic conditions and its effect depended on glycolate oxidation. Michael et al. (1970) found that malate exerted a stimulatory effect on $NH_4$ uptake by bean plants. It is recalled that a considerable succinate effect on $NH_4$ uptake in soyabean was reported by Koster (1963).

Addition of carboxylates to plants grown in $NH_4$ nutrition does not only influence $NH_4$ uptake but can also have a drastic effect on plant growth.

Shaw & Miles (1970) found that $NH_4$ inhibition on the development of *Schizophyllum commune* germlings was reversed by the presence of α-oxoglutarate. Citrate, succinate, fumarate and malate were also effective deinhibitors, and so were - to a lesser extent - acetate and pyruvate. Their results were interpreted in terms of drainage of α-oxoglutarate to such an extent, that TCA-cycle disruption occurred.

Gamborg (1970) and Gamborg & Shyluk (1970) reported satisfactory growth of plant cell suspension cultures grown with ammonium, provided Krebs-cycle intermediates were added, e.g. citrate, malate, fumarate or succinate. Bad results were obtained with carbonate, shikimate, tartrate, acetate and pyruvate. Hewitt (1962; cited by Snaydon, 1969) observed that tomato plants utilized $NH_4$ if succinate was provided, and that plants grew better with ammonium citrate than with inorganic $NH_4$ salts.

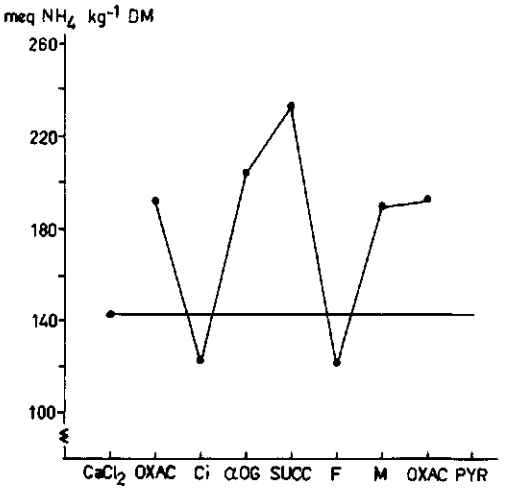


Fig. 33. Effect of incubation with several carboxylates on subsequent $NH_4$-uptake. Experiment 32.

Ammonium toxicity in cucumber leaves was decreased by the addition of carboxylates (Matsumoto et al., 1971). Rice callus tissue produced better growth with $NH_4$ citrate than with $NH_4NO_3$ or $(NH_4)_2SO_4$ (Yatazawa & Furuhashi, 1968).

In synthetic media containing $NH_4$ as the sole N source, *Phytophthora* species grew well, when sufficient calcium and a suitable organic acid were supplied (Lin & Liang, 1965).

From the plot of $NH_4$ uptake in the sequence of the citrate cycle (abscissa Figure 33) three peaks are observed at or near the salts of the α-oxo acids: pyruvic, α-oxoglutaric and oxaloacetic. This finding drew my attention to the α-oxo acids.

Figure 34 gives the α-oxocarboxylate contents of roots after incubation with several carboxylates in Experiment 10.

The oxaloacetate level of the roots was increased by oxaloacetate and all other citrate cycle metabolites. An increase in α-oxoglutarate was found in roots pretreated with α-oxoglutarate and also with citrate, whereas the pyruvate level was high after incubation in pyruvate and also with oxaloacetate. Total oxocarboxylates (Figure 34B) showed peak contents after preloading with salts of α-oxo acids. Between Figures 33 and 34 there are similarities - high contents of α-oxocarboxylates correspond with high subsequent $NH_4$ uptake-, and dissimilarities - citrate and glyoxylate incubation results in repression of $NH_4$ uptake, but in increased α-oxocarboxylate contents. The reverse was found for malate and succinate.

Oxocarboxylates, take part in (trans)amination reactions with the other

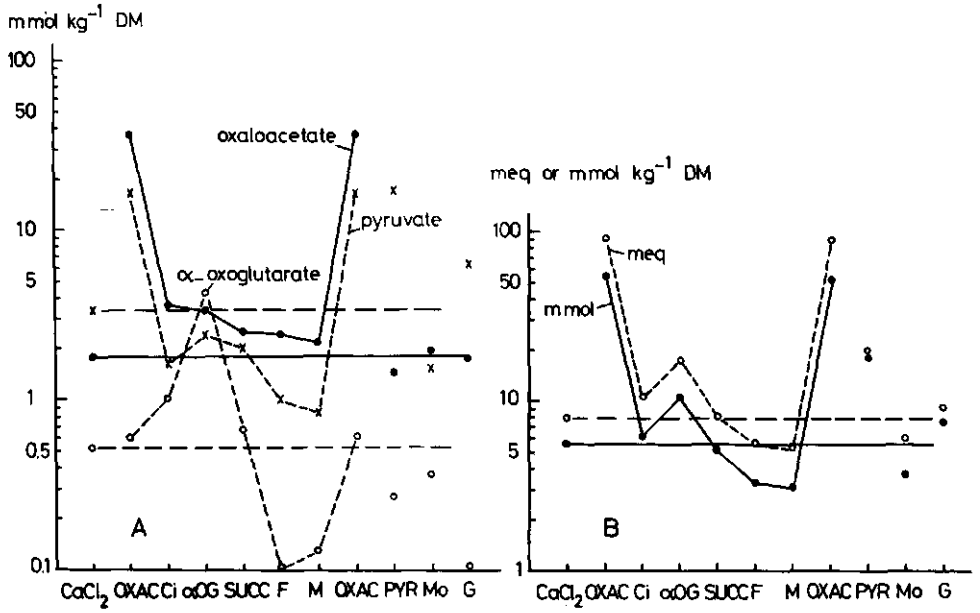


Fig. 34. Effect of incubation with several carboxylates on the levels of α-oxoglutarate, oxaloacetate and pyruvate (A) and the sum of these α-oxo acid salts (B) of roots. Sum in mmoles and meq. Experiment 10.

oxocarboxylates, amino acids or amides (Bidwell, 1963). This explains why incubation with the salt of one oxocarboxylate leads to accumulation of another, e.g. oxaloacetate and glyoxylate save pyruvate consumption. Pyruvate-oxaloacetate interconversion is also possible via phosphoenolpyruvate or $CO_2$ fixation (Gimpel, 1973). Citrate may have a precursory effect on the α-oxoglutarate content, whereas succinate may restrict metabolic consumption of absorbed α-oxoglutarate. The same phenomenon plays a role in the effect of citrate on oxaloacetate turnover. Malonate or glyoxylate preloading resulted in α-oxocarboxylate levels somewhat lower or somewhat higher than in control roots.

No straightforward relationships were found between $NH_4$-uptake data and the content of (individual and sum of) α-oxocarboxylates.

Inorganic chemical composition of roots after pretreatment with several carboxylates and 4h of $NH_4$ absorption is given in Tabel 23. Correlation between $NH_4$ uptake and residual C-A value was poor. After $NH_4$ uptake the values for C-A in roots incubated in carboxylate solutions were still higher than in the control, another indication for the entry of carboxylates under the prevailing conditions (c.f. Table 8). Different C-A levels re-

Table 23. Inorganic chemical composition of roots (in meq/kg DM) after pretreatment with several carboxylates (50 mM) and subsequent $NH_4$-uptake. Experiment 32.

| | $CaCl_2$ | OXAC | Ci | α-OG | SUCC | F | M | PYR | MO | G | O |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Na | 22 | 334 | 275 | 223 | 208 | 238 | 240 | 171 | 169 | 156 | 305 |
| K | 916 | 651 | 786 | 849 | 990 | 788 | 886 | 830 | 820 | 893 | 322 |
| Ca | 124 | 127 | 94 | 127 | 118 | 112 | 116 | 116 | 100 | 102 | 170 |
| Mg | 96 | 98 | 95 | 101 | 96 | 101 | 105 | 97 | 98 | 93 | 80 |
| C | 1158 | 1210 | 1250 | 1300 | 1412 | 1239 | 1347 | 1214 | 1187 | 1244 | 877 |
| Cl | 471 | 443 | 485 | 508 | 536 | 478 | 552 | 483 | 450 | 477 | 235 |
| $SO_4$ | 187 | 195 | 198 | 178 | 208 | 201 | 208 | 196 | 176 | 171 | 112 |
| $H_2PO_4$ | 349 | 298 | 324 | 341 | 357 | 277 | 338 | 308 | 275 | 269 | 193 |
| A | 1007 | 936 | 1007 | 1027 | 1101 | 956 | 1098 | 987 | 901 | 917 | 540 |
| C-A | 151 | 274 | 243 | 273 | 311 | 283 | 249 | 227 | 286 | 327 | 337 |
| $i_{NH_4}$ | 143 | 193 | 123 | 204 | 233 | 122 | 190 | 180 | 56 | 44 | 40 |

sulted mainly from differences in Na, K and Cl content. Interpretation of data for single components is useless as they reflect both the effect of incubation and ammonium uptake. However, the composition of roots after $NH_4$ uptake following oxalate incubation was interesting because Na and Ca were higher and K, Mg and C lower than in the other samples of Table 23. DeKock et al. (1973) found that oxalate stimulates calcium uptake into *Lemna*. These results may be related to calcium oxalate precipitation in the cells. The contents of anions were also lower, resulting in a low A, but a normal to rather high C-A.

Similar data as in Table 23 are given for roots incubated in other carboxylates or different concentrations in Table 24. Pretreatment under these circumstances also led to higher residual C-A values.

Finally the effect of acetate incubation on ammonium uptake was investigated in Experiment 33.

*Exp. 33: Standard carboxylate incubation conditions with succinate and acetate. Standard $NH_4$-uptake conditions.*

Succinate and acetate pretreated roots took up 164 and 31%, respectively of the $NH_4$ absorbed by the control.

In Experiment 32 there were indications that α-oxocarboxylates play an important part in the $NH_4$ absorption process. Roots incubated in several succinate uptake concentrations were assayed for α-oxocarboxylates in Experiment 7 (Figure 35). Higher contents of pyruvate, α-oxoglutarate and

Table 24. Inorganic chemical composition of roots after pretreatment with several carboxylates and 4 h of subsequent $NH_4$ uptake. Meq/kg DM. Experiment 32.

| | $CaCl_2$ | Succ 50 mM | Succ 10 mM | Oxalosuccinate 10 mM | Cisaconitate 10 mM | OXAC 10 mM |
|---|---|---|---|---|---|---|
| Na | 29 | 143 | 40 | 34 | 50 | 78 |
| K | 803 | 791 | 756 | 870 | 806 | 701 |
| Ca | 62 | 76 | 72 | 52 | 68 | 90 |
| Mg | 91 | 84 | 88 | 76 | 78 | 84 |
| C | 985 | 1094 | 956 | 1032 | 1002 | 953 |
| Cl | 587 | 594 | 555 | 501 | 569 | 549 |
| $SO_4$ | 98 | 66 | 80 | 82 | 81 | 78 |
| $H_2PO_4$ | 288 | 262 | 274 | 263 | 290 | 273 |
| A | 973 | 922 | 909 | 846 | 940 | 900 |
| C-A | 12 | 172 | 47 | 186 | 62 | 53 |
| $i_{NH_4}$ | 218 | 272 | 230 | 119 | 249 | 232 |

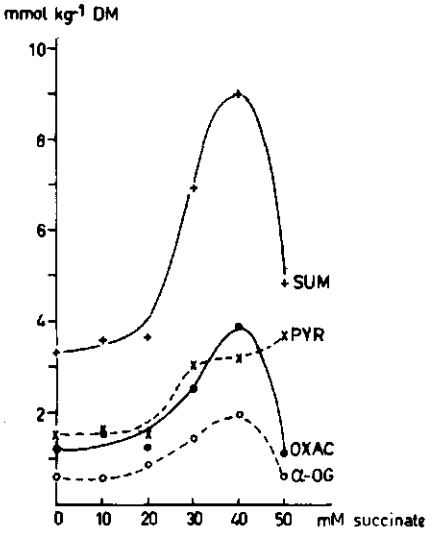


Fig. 35. Contents of oxaloacetate (●), pyruvate (x), α-oxoglutarate (0) and sum of these components (+) in roots as a function of the succinate incubation concentration. Experiment 7.

oxaloacetate were found as succinate incubation proceded at concentrations up to 40 mM, but the level of the latter two compounds decreased between 40 and 50 mM. The relatively small difference of about 1 mmol total α-oxo-carboxylates per kg DM, between control and standard incubated roots is in accordance with Figures 34 and 36. In Chapter 4 it was demonstrated that succinate concentration during incubation increased subsequent $NH_4$-uptake, although the effect of an increase from 40 to 50 mM was negligible (Figures 19 and 20).

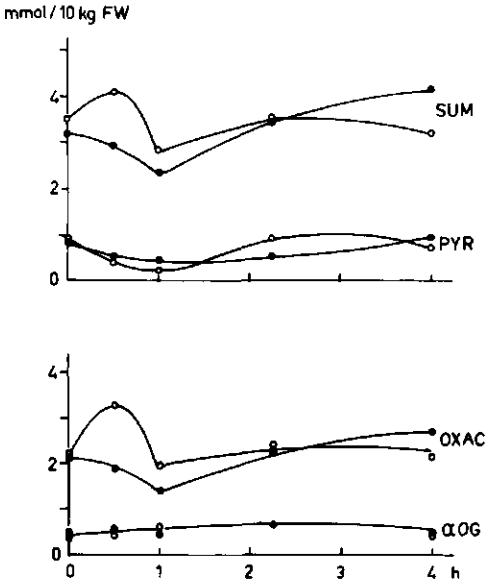


Fig. 36. Contents of α-oxocarboxylates in the course of ammonium uptake by succinate pretreated (0) and control (●) roots. Experiment 34.

Some information has been gained on the effect of several carboxylates and of succinate concentration on the α-oxocarboxylate level prior to $NH_4$ uptake. The question how these contents behave during ammonium entry is dealt with in Experiment 34 (Figure 36).

*Exp. 34: Standard conditions for succinate incubation and $NH_4$ uptake. Determination of α-oxocarboxylates in the roots in the course of $NH_4$ uptake.*

Up to two hours the sum of pyruvate, α-oxoglutarate and oxaloacetate was somewhat higher in roots pretreated with succinate than in control roots. Mainly oxaloacetate determined the shape of the curves. Contents of α-oxoglutarate and pyruvate were low and apparently not related to the total oxocarboxylate level. Oxaloacetate and total α-oxocarboxylate levels showed minimum concentrations after 1 hour. During the first 60 minutes of $NH_4$ ion uptake the oxaloacetate content dropped steadily in -succinate roots but first increased and than decreased in +succinate roots. In long-term experiments with tomato root systems, amino acid secretion was found at the expense of α-oxoglutarate (van Die, 1960). Hofstra (1966) found somewhat more oxocarboxylates in succinate treated (25 mM, K-salt, pH 4.5 during uptake) than in control roots of detopped tomato plants.

If the (salts of) α-oxo acids are key-substances in the $NH_4$ ion uptake

process, as some results suggest, there are several modes of action by which oxo acid stimulation could be achieved. A very simple explanation would be that the α-oxo salts, due to their primary action on ammonium assimilation, keep the $NH_4$ level in the cells low, thus maintaining a certain concentration gradient, which can be considered as a driving force in the $NH_4$ uptake process. This hypothesis was checked in Experiment 35, where roots were incubated with solutions of several carboxylates prior to exposure to a 10 meq $l^{-1}$ $NH_4Cl$ solution.

*Exp. 35: Standard carboxylate incubation conditions with pyruvate, citrate, α-oxoglutarate, succinate, fumarate or malate. $NH_4$ uptake from (10 meq $NH_4Cl$ + 0.1 meq $CaCl_2$) $l^{-1}$. Analysis of free $NH_4$, amides and amino acids after 1 and 4 hours.*

A higher nitrogen concentration than usual was chosen to stimulate the free $NH_4$ level in the roots. Experiment 13 (Figure 17) showed a stimulatory succinate effect at this $NH_4$ concentration. Although oxaloacetate was not included in this part of the research the resemblance between Figures 37 and 33 is striking. Preloading with compounds that enhance $NH_4$ uptake was found to coincide with the highest free ammonium levels in the roots in the period of ammonium uptake following carboxylate incubation. Plant roots with the highest uptake capacities also showed the highest increase in $NH_4$ content in the 1-4 hour interval. The concentration gradient hypothesis is

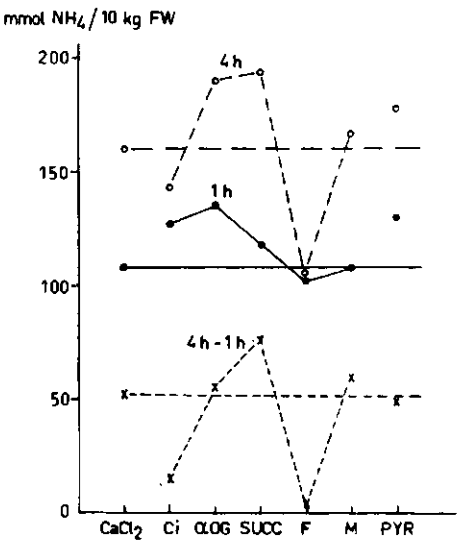


Fig. 37. (Changes in) free ammonium ion content of roots after pretreatment with several carboxylates (abscissa) and 1 or 4h of $NH_4$ uptake (10 meq $NH_4$ $l^{-1}$). Experiment 35.

therefore rejected. Calculation of free $NH_4$ in the cell sap (6% DM in the fresh roots) yields values higher than the ambient concentration. It is known that plant roots are able to absorb ions like Na and K against a concentration gradient and it seems that this is also true for $NH_4$ ions, at both 10 and 1 meq $NH_4$ $l^{-1}$ (Figure 19). Several authors report decreased internal $NH_4$ levels in the presence of glucose or carboxylates (Budd & Harley, 1962a; MacMillan, 1956; Matsumoto et al., 1971) However, their data do not apply to plant roots.

As far as the amide contents are concerned (Figures 38 and 39), the picture is not as simple as for free $NH_4$ accumulation. After 1 and 4 hours of ammonium absorption, highest glutamine concentrations are found in roots pretreated with succinate, malate or pyruvate while α-oxoglutarate (the first step towards glutamine synthesis) preloading was followed by low glutamine contents. Preloading with the three components mentioned before resulted in the same or a drop in glutamine content between 1 and 4 hours.

Asparagine contents were maximum after 1 and 4 hours of $NH_4$ uptake

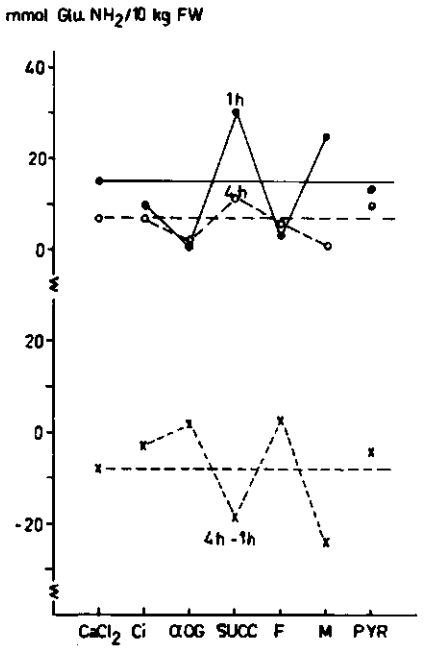


Fig. 38. (Changes in) free glutamine content of roots after pretreatment with several carboxylates (abscissa) and 1 or 4h of $NH_4$ uptake (10 meq $NH_4$ $l^{-1}$). Experiment 35.

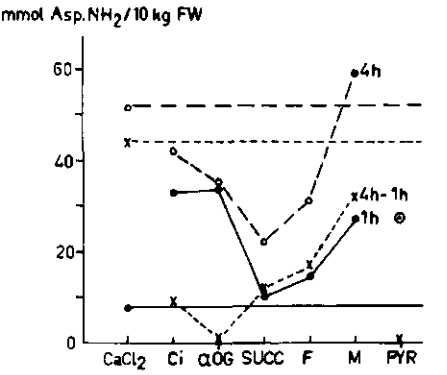


Fig. 39. (Changes in) free asparagine content of roots after pretreatment with several carboxylates (abscissa) and 1 or 4h of $NH_4$ uptake (10 meq $NH_4$ $l^{-1}$). Experiment 35.

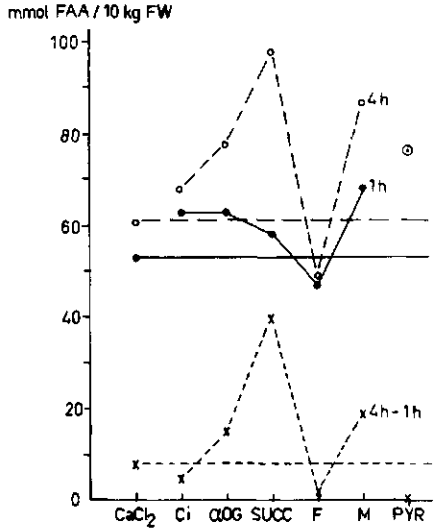


Fig. 40. (Changes in) free amino acid content of roots after pretreatment with several carboxylates (abscissa) and 1 or 4h of $NH_4$ uptake (10 meq $NH_4$ $l^{-1}$). Experiment 35.

after incubation with malate, citrate and α-oxoglutarate and the lowest contents occurred in roots that were succinate pretreated. Succinate inhibits the amination reaction of oxaloacetate to aspartate but the same is true for α-oxoglutarate (Michal, 1972). In contrast to other treatments, the α-oxoglutarate and pyruvate pretreated roots showed no increase in asparagine between 1 and 4 hours.

Free amino acids (Figure 40) occurred in the highest quantities in roots preloaded with malate, pyruvate, α-oxoglutarate and succinate.

Total free amino compounds, asparagine and ammonium accumulated between 1 and 4 hours of ammonium uptake, while glutamine sometimes decreased. Accumulation of free amino acids and asparagine in this time interval followed roughly the trend of the absolute contents after 1 and 4 hours. Hence roots with a high content of these compounds after 1 hour accumulated more of these compounds in the next three hours than roots with low initial contents.

## CONCLUSIONS

1. Beside succinate there are other carboxylates which, if applied to roots, stimulate subsequent ammonium absorption. Carboxylates with the highest stimulating effect are the α-oxocarboxylates (excepted glyoxylate) or compounds close to them in the citrate cycle. Under the chosen conditions there

are also carboxylates (citrate cycle intermediates and others) that reduce $NH_4$ uptake (Experiments 32, 33).

2. Some pretreatements that stimulate $NH_4$ entry coincide with high contents of α-oxocarboxylates at the start of $NH_4$ uptake, but there are also compounds that - after incubation - yield oxaloacetate, pyruvate and α-oxoglutarate levels not proportional to $NH_4$ uptake stimulation (Experiment 34).

3. During 4 hours of ammonium uptake differences in contents of α-oxo compounds were small. Obviously, the α-oxocarboxylate level does not determine $NH_4$ uptake by maize roots (Experiment 34).

4. Carboxylate pretreatments that enhanced subsequent entry of ammonium ions at the same time created higher levels of free ammonium and amino acids in the root tissue, after 1 and 4 hours of ammonium nutrition. Increase in $NH_4$ and free amino acids in the 1-4 h interval showed in general the same trend as $NH_4$ uptake (Experiment 35). A hypothetical mechanism of oxocarboxylate stimulation due to low internal $NH_4$ levels and thus keeping up a concentration gradient, is therefore rejected. High $NH_4$-levels probably are not the cause of a low but the result of a high absorption rate.

# 6 The effect of carboxylates on the uptake of other ions

It seems logical to consider the uptake of nutrients by plants in relation to their physiological role. Till now most ion uptake theories have focussed on general concepts, valid for all ions or valid for cations or anions (Brouwer, 1965; Lundegårdh, 1960; Epstein, 1972). In this chapter the validity of the results for ammonium uptake was checked for two other ions: potassium and nitrate. K was chosen to test the possibility that carboxylate mediation is cation specific. The physico-chemical properties of the ions $K^+$ and $NH_4^+$ are rather similar. Nitrate was included to check whether the observed phenomena are typical for the uptake of nitrogenous ions.

## 6.1 POTASSIUM ABSORPTION

As indicated in Chapter 2, K uptake was estimated by measuring the decrease in ambient radioactivity of a $^{86}Rb$ labelled KCl solution in Experiment 36. Roots were detached from plants grown for 3 days on a medium without potassium (Table 3).

*Exp. 36: Standard carboxylate incubation conditions with succinate, oxaloacetate, α-oxoglutarate, malate, citrate, fumarate or NaCl. K (Rb) uptake from a 1 meq KCl $l^{-1}$ solution, conditions as for $NH_4$ uptake.*

To check the effect of sodium during incubation on subsequent potassium absorption the treatments also comprised incubation with 50 mM NaCl. Potassium uptake is given in Table 25 and presented in Figure 41 as a function of the

Table 25. Potassium uptake (in meq K/kg DM) by roots after pretreatment with several carboxylates. Experiment 36.

| | | | |
|---|---|---|---|
| control ($CaCl_2$) | 44 | α-oxoglutarate | 71** |
| NaCl | 46 | succinate | 56** |
| oxaloacetate | 75** | fumarate | 65** |
| citrate | 76** | malate | 64* |

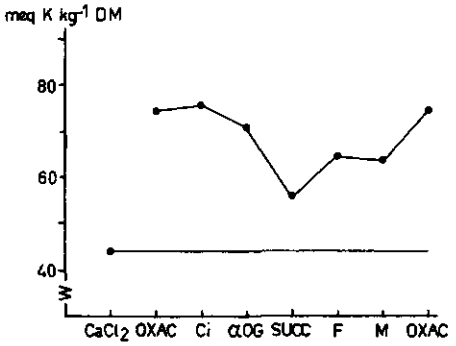


Fig. 41. Effect of incubation in several carboxylate solutions on subsequent potassium uptake. Experiment 36.

carboxylate position in the citrate cycle. The picture is quite in contrast with carboxylate stimulation of $NH_4$ ion uptake. K uptake is strongly stimulated after incubation with citrate, a repressor of ammonium uptake (Figure 5 and 33), whereas the least stimulus was exerted by succinate. All carboxylates enhanced subsequent K-uptake by at least 27%. Incubation in a high sodium concentration in the absence of carboxylates (NaCl) did not affect the subsequent absorption of K. Uptake of potassium was about 20 percent of that of ammonium under comparable conditions. A similar experiment with roots of low salt and high sugar content (cf. Experiment 27) yielded a 22% depression of K(Rb) uptake by succinate pretreatment. The effect of carboxylates on K uptake was obviously also related to the carbohydrate status of the roots.

Dark fixation of $CO_2$ into excised bean roots stimulated carboxylate production and K and Ca uptake (Jackson & Coleman, 1959). Nobel (1970) showed that light-dependent K-entry into pea leaf fragments was stimulated by bicarbonate and, to a lesser extent, by acetate, formate, butyrate, propionate and pyruvate. The K uptake rate was proportional to the pK and the uptake of carboxylate anions.

Jackson & Taylor (1970) observed salt efflux from roots of barley plants grown in the dark, after the addition of acetate, propionate, formate and glutarate (10 meq/l, pH 5). The total inorganic cation content of high salt roots was increased in the presence of glycolate. Combined supply of glycerate and succinate yielded 20-50% more cation accumulation than in the control roots. Introduction of formate and propionate to the medium at pH 7 stimulated K, Na and Cl uptake. The results are explained by assuming changes in permeability caused by interaction of membrane structure and (undissociated) organic acids.

Ohyama et al. (1966), also using barley roots as test material, found that adding acetate and glycolate to the medium accelerated K accumulation. No effect was exerted by glyoxylate, oxalate and formate. Schaedle & Jacobson (1965, 1966b) stated that cation entry into *Chlorella pyrenoidosa* is coupled to the generation of negative charges. Carboxylate synthesis or other metabolic reactions, that create anions, should limit K uptake.

## 6.2 NITRATE ABSORPTION

Heavy-nitrogen enrichment in the roots after uptake from a labelled nitrate solution was taken as a measure of $NO_3$ uptake in Experiment 37. The roots were from plants grown for 4 days in the -N medium.

*Exp. 37: Standard carboxylate incubation conditions with succinate, oxaloacetate, α-oxoglutarate, malate, citrate, pyruvate or fumarate. $NO_3$ uptake from a 1 meq $Ca(^{15}NO_3)_2$ $l^{-1}$ solution, conditions as for $NH_4$ uptake.*

Table 26 deals with $NO_3$ uptake by the roots after the various pretreatments and Figure 42 presents the data in TCA-cycle orientation. Nitrogen uptake was low, about ten times less than uptake in the form of $NH_4$ under comparable conditions. Incubation with most carboxylates depressed subsequent nitrate uptake by about 20%. Exceptions are citrate that enhanced $NO_3$ uptake by 16% and pyruvate (68% depression). Nitrate uptake was low and so was nitrate assimilation. The resemblance between Figure 42 and Figure 43 indicates that, because the initial $NO_3$ content of the roots is negligible, most of the absorbed nitrate ions are accumulated. This implies that the carboxylate effect was investigated independent of any effect on nitrate reduction or further N assimilation. Some authors have suggested an assimilation-uptake link for nitrate (Lycklama, 1963; Jackson, 1973; Breteler, 1973c, 1974a; Breteler & Smit, 1974). An explanation for the relatively

Table 26. Nitrate uptake (meq $NO_3$/kg DM) by roots after pretreatment with several carboxylates. Experiment 37.

| | | | |
|---|---|---|---|
| control | 20.9 | succinate | 15.9** |
| oxaloacetate | 15.4** | fumarate | 15.0** |
| citrate | 24.2** | malate | 16.4* |
| α-oxoglutarate | 16.4** | pyruvate | 6.7** |

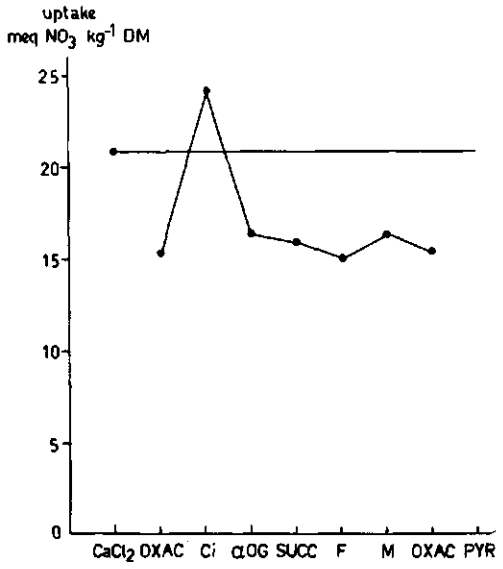


Fig. 42. Effect of incubation in several carboxylate solutions on subsequent nitrate uptake. Experiment 37.

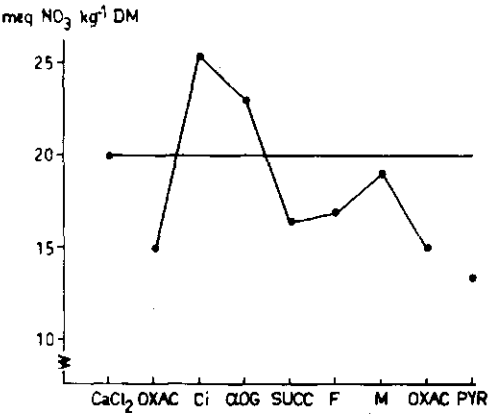


Fig. 43. Effect of incubation in several carboxylate solutions on nitrate content of roots after 4h of $NO_3$ uptake. Experiment 37.

small uptake, assimilation and carboxylate effect could be that induction of the adaptive nitrate reductase enzyme took too long. About 4 h induction time is not unlikely (Beevers et al. 1965). A lag-time for (accelerated) transport of $NO_3$ into cells, varying from 3 to 30 hours after transfer of $NO_3$-depleted plants to a nitrate solution seems to exist for several plant tissues, including detached roots (Jackson et al, 1972; Kopp et al, 1974). If this phenomenon depends on the onset of nitrate reductase activity, by its effect on the concomittant generation of carboxylates, the roots that were carboxylate pretreated should absorb nitrate faster than control roots. For other anions (phosphate, nitrite, sulphate) a similar lag-phase is reported by Ferrari & Nisato (1971), White (1973), Jackson et al. (1974a,b), Lin & Hanson (1974) and Hanebuth et al. (1974).

Low rates of uptake and assimilation of nitrate mean that the effect of exposure to $NO_3$ on inorganic plant composition and carboxylate metabolism is negligible. For this reason no data are given.

Nitrogen-starved cells of *Chlorella ellipsoidea* grown in the dark, showed a small increment in nitrate uptake in the presence of acetate (Okuda & Ida, 1966b). Absorption of urea and ammonium was depressed under the same circumstances. Under photosynthetic conditions nitrate entry was

slightly stimulated by glycolate. Ben Zioni et al. (1971), Dijkshoorn et al. (1968), Kirkby (1974) and Purvis et al. (1974) suggest a key-role of carboxylates in the $NO_3$ uptake process, through decarboxylation and $HCO_3^-$ - $NO_3^-$ exchange. In contrast, Izawa et al. (1966) found that carboxylates inhibited $NO_3$ uptake by barley and rice seedlings. Their results can be explained in terms of nitrate-carboxylate uptake competition.

Some data are available on carboxylate mediation in the entry of other nutrients. DeKock et al. (1973) reported enhanced Ca absorption into *Lemna* after the addition of oxalate. Calcium deficiency symptoms were aggravated by oxalate.

Goodman & Rothstein (1957) showed inhibition of phosphate uptake by lactate and pyruvate in baker's yeast. Phosphorus accumulation stopped completely in the presence of acetate. It was concluded that P absorption depends to a large extent on glycolysis and not on citrate cycle respiration. In phosphate uptake experiments with excised barley roots the addition of 100 mM succinate decreased P uptake. The effect of β-hydroxybutyrate was even worse (Hagen et al., 1957).

Okuda & Ida (1966a) reported enhanced respiration rates due to addition of acetate, propionate, butyrate and pyruvate in phosphorus- and sugar-depleted cells of *Chlorella ellipsoidea*. None of these carboxylates promoted phosphate uptake.

Indications for a relation between malate content and chloride influx were found in barley roots and washed carrot-tissue (Cram, 1973). Bean roots with a high carboxylate content showed lower chloride absorption rates than control roots (Jackson & Coleman, 1959). This effect can be due to the high pH caused by the presence of bicarbonate. After a stimulation of 20 hours malonate (pH 5.6) added to the medium repressed sulphate absorption by potato tissue discs (Hanebuth et al., 1974). Citrate has been reported to depress sulphate uptake by *Scenedesmus* (Kylin, 1967a).

Citrate seems to be involved in the translocation and possibly also in the uptake of iron (Brown & Chaney, 1971; Clark et al., 1973; Tiffin, 1965).

## CONCLUSION

The effect of several carboxylates on uptake of the three investigated ions is summarized in Table 27. None of the studied compounds affects ion uptake in general. K uptake seems to be stimulated, independent of the car-

Table 27. Effect of carboxylate incubation on $NH_4$, K and $NO_3$ uptake. +, -, 0 mean positive, negative and no significant effect, respectively. (Experiments 32, 36 and 37).

| | $NH_4$ | K | $NO_3$ | | $NH_4$ | K | $NO_3$ |
|---|---|---|---|---|---|---|---|
| oxaloacetate | + | + | - | pyruvate | + | | - |
| citrate | - | + | + | malonate | - | | |
| α-oxoglutarate | + | + | - | glyoxylate | - | | |
| succinate | + | + | - | oxalate | - | | |
| fumarate | 0 | + | - | oxalosuccinate | - | | |
| malate | + | + | - | acetate | - | | |
| cis-aconitate | + | | | | | | |

boxylate species. Lack of stimulation or depression of $NH_4$ uptake by some carboxylates prove that there is no overall effect of carboxylates on cation uptake. Citrate-stimulated nitrate entry means that - in general - carboxylates neither depress anion uptake, nor affect the uptake of cationic and anionic nitrogen in the same way. Except for fumarate, all observations show that the effects on the absorption of the two nitrogen forms are opposite.

# 7 Discussion

## 7.1 EXPERIMENTAL TECHNIQUE

Prior to speculations about the metabolic (Section 7.4) or non-metabolic (Section 7.3) nature of the effect of carboxylates on ion uptake, the consequences of the employed methods for the interpretation of results is discussed in Section 7.1. The interference of one of the facets of the experimental approach, the apparent free space or initial phase uptake, will be treated separately in Section 7.2.

At the start of this chapter the influence of some factors that are intimately related to the adopted experimental techniques will be discussed.

The process that was called absorption or uptake of ions is in fact only the net influx, because the excised roots were submerged in the test media. The xylem was in open connection with the medium. An advantage of the procedure used is that ion transport via xylem to the shoot cannot interfere; a disadvantage of the method is that the uptake capacity of the roots is underestimated. Release of ions, in unchanged or metabolized form (organic N compounds) via the xylem vessels to the medium may depend on the carboxylate status of the roots.

All plant material was treated and maintained under non-sterile conditions. The question is what part do rhizosphere micro-organisms play in ion uptake and the carboxylate effect. Maintenance of maize plants for about 2 months under axenic conditions is very difficult (Barker & Broyer, cited by Hewitt, 1950). Separation of roots from adhering or partly penetrated microflora is impossible (Barber, 1969). Working with sterile roots does not facilitate interpretation of results as these roots differ from normal roots, to which most ion uptake studies refer (Koster, 1973). Comparison of ion uptake by sterile and non-sterile roots is usually done at very dilute nutrient concentrations (Barber, 1968, 1969, 1971). For $NH_4$ uptake at 0.01 meq $l^{-1}$ it was found that sterile barley plants absorbed some 30% more $NH_4$ than non-sterile plants. In Chapters 5 and 6 it has been recalled that the respiration and ion uptake rate of some micro-organisms are stimulated by

exogenous carboxylates.

Carboxylate incubation resulting in e.g. 400 meq increase in C-A is followed by accumulation of the carboxylates in the root cells. Otherwise, the small amount of dry matter represented by the micro-organisms (0.01 - 0.8%, according to Epstein or Barber, 1974) would contain amounts of carboxylates, that were improbably large. For this reason the effect exerted by carboxylates is due to the higher plant and not to increase in respiration and ion uptake in the rhizosphere microflora.

Experiments with antibiotics (4.11) showed in general a decrease in ammonium uptake, but the succinate effect was not consistently prevented. Apart from an effect on root metabolism, this result is another indication for the minor role of micro-organisms.

The results given in Chapters 3 and 5 indicate why the effect of preloading roots with e.g. $10^{-4}$ or $10^{-3}$ M $CaCl_2$, KCl or $K_2SO_4$ salts (Chapter 3) was poor. Total carboxylate accumulation in a short preloading period is small and the level of specific carboxylates in the roots is of more importance than the total C-A value.

If one considers all experiments together, variation in $NH_4$ uptake and the succinate effect was considerable. Excluding some extremes like 115% in Experiment 14 and over 1000% in Experiment 13, the average standard $NH_4$-uptake of standard succinate preloaded roots was about 150% of the control. The variability can be ascribed to differences among sets of plants, plant age, and climatic variation during plant culture. Carrodus (1966) found a huge variation in the effect of succinate and malate (126-174%) on $NH_4$ uptake in mycorrhizal beech roots. Under favourable weather conditions day to day variation in uptake and succinate effect was reasonable.

Uptake studies lasted the whole period of 4 hours after transfer of the roots to a $NH_4$-medium. This period includes the rapid initial phase of ion uptake, which is dealt with in the next section.

## 7.2 APPARENT FREE SPACE

The contribution of the apparent free space to 4h of ion uptake interferes with the physiological and physical interpretation of the results. In the present studies it was not useful to restrict observations to the phase of active accumulation, that occurs after about 1h of contact with the uptake medium, because succinate effect(s) over the whole uptake period were within the scope of the research. Moreover, filling of the AFS before an up-

take experiment introduces a measure of uncertainty because this sometimes takes 15 minutes (Figures 16 and 18) and sometimes a few hours (Figures 1 and 3).

The effect of the AFS was checked in two ways; by graphical analysis of time-uptake curves in Experiment 38 and preceding experiments, and by washing roots in $CaCl_2$ solutions (Experiment 39).

*Exp. 38: Standard carboxylate incubation conditions, with succinate, oxaloacetate, α-oxoglutarate, malate, citrate or fumarate. Standard $NH_4$-uptake conditions. Uptake solutions sampled 0, 5, 10, 15, 20, 30, 60, 120, 180 and 240 minutes after initiation of $NH_4$ uptake. AFS calculated as indicated in Figure 44.*

Graphical analysis of the free space (Table 28) showed it to be rather apparent (> one litre/litre root, see Chapter 3) and gave unreliable results, mainly due to variable uptake patterns (cf. Lüttge, 1973b). It is generally assumed that the AFS constitutes not more than about 10-30% of the root volume (Bernstein & Nieman, 1960; Brouwer, 1965; Epstein, 1972). The contribution of AFS to 4h of $NH_4$ absorption after pretreatment with several carboxylates ranged between 43 and 60%. The AFS was 60-80 meq and 4h of uptake was 95-190 meq/kg DM. Treatments followed by high uptake tended to show slightly higher AFS values than those followed by low $NH_4$-uptake. Citrate - a repressor of $NH_4$-uptake - had the highest value for the ratio AFS: 4h uptake, followed by the control and fumarate, a compound that seems not to affect $NH_4$ uptake. Ighe & Petterson (1974) showed a linear relationship between initial binding of Rb and the subsequent active uptake rate. They considered initial binding of cations in the free space as a process, that is closely linked to the mechanism of active ion uptake. The question rises whether

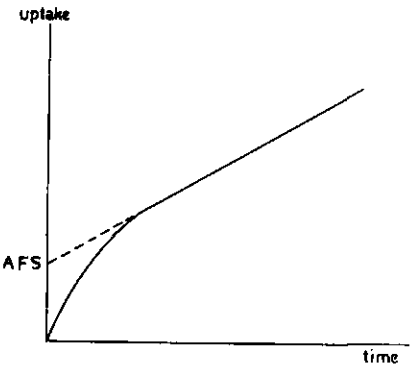


**Fig. 44. Graphical determination of the apparent free space in an uptake-time plot.**

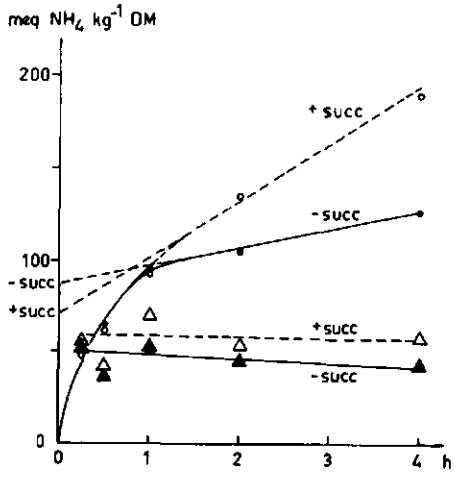


Fig. 45. Ammonium uptake (circles) and apparent free space ammonium ions (triangles) in the course of time. Open symbols = succinate pretreated roots, solid symbols = control. Experiment 39.

initial binding is the cause or the result of active uptake. Brouwer (1965) stated that no relation exists between the amount of adsorbed ions and the uptake rate.

In Experiment 39 succinate stimulation was 65% (Figure 45) and the uptake curves differed only after 60 minutes of ammonium nutrition. Graphical AFS determination yielded an AFS of 70-85 meq.

*Exp. 39: Standard conditions for succinate incubation and $NH_4$ uptake. After 15, 30, 60, 120 and 240 minutes the roots were blotted dry and rinsed for 1 minute in 0.1 N $CaCl_2$. Ammonium was determined in the contact solution.*

About 15 meq more AFS was found in -succinate than in +succinate roots. Drawing the curve extension to the ordinate by hand gives an error of the same magnitude. In this experiment and in all other tests presented here, the contribution of AFS (graphical) to 4h of $NH_4$ uptake was higher in control than in succinate pretreated roots (cf. Table 28). As the variation in AFS is small, this indicates that succinate stimulates the active phase of ammonium uptake. AFS determined after washing with calcium chloride was about 50 meq, independent of the sampling time. Succinate preloaded roots had 10-20 meq more free space than control roots. Some overestimation by the wash-exchange method is likely, as no correction for fluid adhering to the roots was possible. Results of Experiments 39 and 22 are in agreement with the observation of Budd & Harley (1962), who found a ratio of exchangeable : non-exchangeable $NH_4^+$ of about 1:1 in mycelium of *Neocosmospora vasinfecta*.

Table 28. Contribution (%) of the apparent free space to 4h of $NH_4$ uptake by roots after pretreatment with several carboxylates. AFS calculated as in Figure 44. Experiment 38.

| | | | |
|---|---|---|---|
| Control | 52 | Succinate | 49 |
| Oxaloacetate | 46 | Fumarate | 50 |
| Citrate | 60 | Malate | 43 |
| α-oxoglutarate | 46 | | |

Variations in AFS are of minor importance compared with the difference after 4h of $NH_4$ absorption. Both the graphical and the exchange method of rediffusible $NH_4$ assay yield AFS values of 3-4 litre per litre of root volume, reflecting that beside a Water Free Space, a considerable Donnan Free Space exists. A carboxylic nature of DFS anions, as sometimes suggested (Cseh, 1972), facilitates the understanding of metabolic regulation of AFS or DFS. Grobler (1959) described initial entry of ions as a passive process, kept at a steady state by metabolism. Brouwer (1965) suggested metabolic DFS regulation via the amount of negative charges. The pH effect on ion uptake can be due to dissociation of carboxyl groups (van den Honert & Hooymans, 1961).

In contrast to Figure 45, large differences in AFS (grapical method) were found in roots that absorbed $NH_4$ from sulphate and chloride salts at 10 meq $NH_4$/l. All experiments, however, in which the time course of $NH_4$ uptake was studied at 1 meq/l (except Experiment 13, Figure 16) show diffrences in AFS between +succinate and -succinate roots of less than 20 meq. The former usually had higher AFS values than the latter. In the temperature experiment (28) no succinate effect existed at $2^oC$, indicating identical free spaces.

Whether carboxylate anions are involved in the AFS or DFS depends on their cellular localization. There is no evidence that the succinate pulse alters the DFS or AFS substantially. Moreover, completely non-metabolic AFS is doubtful as for example Figure 32 shows that the presence of sodium azide reduces active ammonium uptake as well as the AFS phase. A study of $NH_4$ uptake by potato tuber tissue discs by Bange & van Gemerden (1961) led to the question whether free space uptake ($0^oC$) is completely passive. AFS data obtained from Experiments 15 and 16 show highest values for salts of those anions from which $NH_4$ is most readily absorbed. At 10 meq/l there was no tenfold increase in AFS (in meq) as compared with 1 meq per litre, and the size of the AFS seems to be related to carboxylate pretreatment and

type of ammonium salt. Succinate accumulation due to malonate inhibition enhanced the AFS in Figure 21.

Concluding, at 1 meq $NH_4$/l, the AFS of the roots is hardly affected by carboxylate pretreatments. Thus the carboxylate effect on ammonium uptake is on the steady state or active phase of absorption.

## 7.3 NON-METABOLIC CARBOXYLATE EFFECT

Evidence for non-metabolic aspects of carboxylate (succinate) stimulation of $NH_4$ entry has been presented in Chapter 4. Lack of oxygen, and sodium azide or malonate inhibition did not completely rule out the succinate effect.

Permeability of roots for ions in general is probably not involved as the effect of the investigated carboxylates differed for $NH_4$, K and $NO_3$ ions (Chapter 6). As far as $NH_4$ uptake is concerned, there is no proof of differential interaction of the more lipophilic monocarboxylic and more hydrophilic dicarboxylic acids with cell membranes, as suggested by Jackson & Taylor (1970).

Presence of carboxylates inside the outer cell membranes can act as a negative charge to attract cations (Schaedle & Jacobson, 1965, 1966; Hiatt & Lowe, 1967; Hiatt, 1968; Marschner, 1968, 1969; Blevins et al., 1974; Robertson, 1958).

This kind of attraction is not pure physico-chemical because passage of biological membranes is involved. Influx of $NH_4$ and efflux of e.g. Na and K can exist without AFS or DFS interference (Figure 23). Sodium succinate preloaded roots lost far more sodium during $NH_4$ nutrition than non-succinate pretreated roots. Loss of K was unaffected by pretreatment. Available data on Na-$NH_4$ exchange do not permit a quantitative conclusion.

Increase in root potential difference by negative charges of carboxyl groups can also stimulate cation entry (Higinbotham, 1967). Differential behaviour of K and $NH_4$ after e.g. citrate preloading is against the exchange hypothesis.

Sodium and potassium release often exceeds $NH_4$ intake (Tables 4, 10, 12 and 20). This observation led to Experiment 40, in which the test solution was assayed for carboxylates after the uptake period.

*Exp. 40: Standard conditions for succinate incubation and $NH_4$ uptake.* During $NH_4$ uptake, carboxylates (initially mainly succinate and malate) de-

Table 29. Carboxylate content (in meq/kg DM) of succinate pretreated roots before and after four hours of ammonium uptake, and carboxylates recovered in the $NH_4$ medium after uptake. Experiment 40.

| | Root composition | | | Recovered |
|---|---|---|---|---|
| | 0h | 4h | 0h-4h | |
| Fumarate | 28 | 44 | -16 | 0 |
| Succinate | 168 | 40 | 128 | 174 |
| Oxalate | 10 | 0 | 10 | 0 |
| Malate | 122 | 96 | 26 | 0 |
| Citrate | 24 | 0 | 24 | 25 |
| Sum | 352 | 180 | 172 | 199 |
| $NH_4$ uptake 254 meq/kg DM | | | | |

clined and only some fumarate accumulated. Succinate accounted for the bulk of the decrease in carboxylates which is not only due to succinate metabolization as shown by the recovery of succinate in the solution (Table 29).

To maintain tissue electroneutrality assimilation of each equivalent ammonium ($NH_4^+ \rightarrow N_{org}$) requires the concommittant disappearance of one equivalent carboxylate. This is regulated by metabolic consumption (amino acid synthesis, decarboxylation) or by efflux to the medium.

Differential cation-anion absorption (mainly $NH_4^+$-$Cl^-$) is balanced by anion uptake ($HCO_3^-$, $OH^-$) or cation ($Na^+$, $K^+$, $H_3O^+$) release. Concurrent efflux of organic anions implies that more alkali cations can be released during $NH_4$ uptake. Thus the decrease in Na+K and carboxylates (C-A) may be in excess of $NH_4$ uptake (Tables 4, 10, 12, 13 and 20).

In the present trials pH changes in the medium were not always as expected. The balance of ion uptake from a monosalt solution is complex because more than 2 ions are involved. Loss of $RCOO^-$ may lead to withdrawal of free hydronium ions in solution, because most carboxylates are salts of weak acids (Table 7). Changes in carboxylate content during salt uptake by high and low carboxylate roots were studied with a number of salt solutions in Experiment 41.

*Exp. 41: Standard succinate incubation conditions. Standard uptake conditions (including $CaCl_2$, where necessary) from 25 meq/l solutions of $K_2SO_4$, $KCl$, $CaCl_2$, $Ca(NO_3)_2$ or $NH_4Cl$.*

Table 30 shows that in all treatments a great part of the carboxylates, accumulated during incubation, are effluxed. No indication for carboxylate release was found in the control roots. Salt losses stimulated by carboxy-

Table 30. Carboxylates in succinate pretreated and control roots (in meq/kg DM) before and after 4h of salt absorption from 25 meq/l solutions of $K_2SO_4$, KCl, $CaCl_2$, $Ca(NO_3)_2$ or $NH_4Cl$. Experiment 41.

| | -succ | +succ |
|---|---|---|
| 0h | 148 | 532 |
| 4h $K_2SO_4$ | 158 | 432 |
| KCl | 146 | 282 |
| $CaCl_2$ | 132 | 138 |
| $Ca(NO_3)_2$ | 132 | 296 |
| $NH_4Cl$ | 82 | 126 |

late have also been reported by Jackson & Taylor (1970) for detached barley roots. They found an efflux of titratable anions, enough to cover excess cation loss. Hiatt (1967b) reported carboxylate balanced $K^+$ loss by barley roots under anaerobiosis. Conway & Brady (1950) suggested $H_3O^+$ release from fermenting yeast cells in the form of undissociated organic acids. Cseh (1972) considered a compensatory function of organic anion efflux from the roots of higher plants as unlikely. Van Steveninck et al. (1973) observed leakage of organic acids from fresh beet root discs.

Physico-chemical processes are obviously involved in the stimulation of carboxylate preloading on $NH_4$ uptake. Explanations for the ion and carboxylate specificity of the effect, however, cannot be obtained from physico-chemical considerations.

## 7.4 METABOLIC CARBOXYLATE EFFECT

Some results suggest participation of succinate as such in the stimulation of ammonium absorption (Section 4.6). Data on the carboxylate composition of roots after incubation with several carboxylates (Table 8) and $NH_4$ uptake after incubation under identical conditions (Table 22) show that the relationship between initial succinate level and $NH_4$ uptake is striking (Figure 46). Such an effect of succinate is difficult to understand without involvement of succinate metabolism. For other carboxylates, including alpha oxocarboxylates, no relation between initial content and subsequent ion influx was found.

Evidence for metabolic aspects of the stimulation of ammonium absorp-

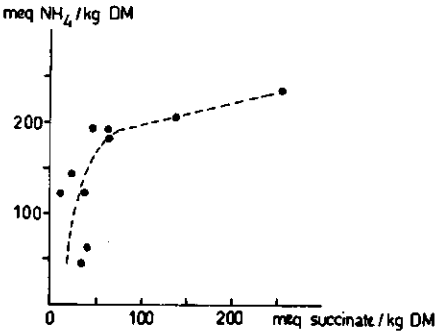


Fig. 46. Relationship between succinate content of roots after incubation in several carboxylate solutions (Table 8) and subsequent $NH_4$ uptake (Table 22). Experiments 10 and 32.

tion by carboxylates (succinate) comes from the Experiments 24, 25 and 26 (complementary succinate-glucose effect), 29 (temperature dependent succinate effect), 30 (no significant succinate effect in the presence of azide) and 31 (repression of the succinate effect by some inhibitors of protein synthesis).

Succinate metabolism results in at least four processes, which may affect ion uptake.

1. Delivery of carbon skeletons, that enhance nitrogen assimilation.
2. Supply of reducing power by the formation of reduced pyridine nucleotides. This change in redox potential may increase the assimilation rate of absorbed N by its action on reductive amination.
3. Supply of high energy compounds, like ATP, due to oxidative phosphorylation after carboxylate oxidation in the citrate cycle. Energy gained in this way may be used in various processes, e.g. N assimilation or ATP (ase) driven uptake mechanisms.
4. Electron transport from carboxylates to oxygen via the cytochrome chain.

Prior to a discussion of the 4 processes in relation to literature data and the present work, some general remarks on the cellular localization of carboxylates and different behaviour of particular carboxylates in these processes are necessary.

In a review on intermediary metabolite compartmentation, Oaks & Bidwell (1970) concluded that the turnover pools of TCA cycle intermediates are too large to be completely in the mitochondrial fraction. At least three different pools of malate exist, located respectively in the vacuole, the cytoplasma and inside mitochondria. The cytoplasma pool is in equilibrium with external malate, and a rapid exchange between mitochondrial and cytoplasmic malate is possible (cf. Cram & Laties, 1974). For succinate, citrate and pyruvate, the cytoplasmic and mitochondrial pools are not separated. A

large active pool of succinate exists, while aconitate occurs mainly in an inactive (vacuolar?) pool.

Harley & Beevers (1963) and Lips & Beevers (1966a) expressed the opinion that the turnover pool of citrate cycle products comprises only a fraction of total cellular carboxylates. In maize roots, 60% of the malate was not in equilibrium with the mitochondrial turnover pool. For citrate and succinate no separation of pools was demonstrated. Steer & Beevers (1967) suggested that the entire citrate, pyruvate and succinate pools are accessible for citrate cycle metabolism.

Carboxylates in plant cells do not only differ with regard to their compartmentation. The accessibility to metabolic pools also differs. Succinate entered pea root mitochondria much more readily than any other carboxylate, as inferred from oxygen consumption data. Oxygen absorption decreased in the order succinate, α-oxoglutarate, glutarate, fumarate and citrate (Childress & Stein, 1965).

Mitochondria isolated from mung bean hypocotyls had an apparent $K_m$ value for $O_2$ uptake that was 7.5 times higher for malate than for succinate (Ikuma & Bonner, 1967). Maize top mitochondria also readily oxidized pyruvate and succinate, in contrast to citrate (Kenefick & Hanson, 1967) and mitochondria from several organs of the rat were difficult to penetrate for citrate and fumarate (Meijer, 1971). A higher oxygen uptake rate with succinate than with malate as substrate was shown by Miller & Miller (1974) in mitochondria extracted from soyabean hypocotyls and Robertson (1955b) also measured the highest rates of oxygen intake by carrot and beet tissue, when succinate was added.

For a proper evaluation of the effect of carboxylate preloading in ion uptake processes, information about pooling and about the metabolization of internally and externally supplied carboxylate by the tissue is needed. Otherwise one cannot associate the observed phenomena directly with one of the 4 processes described before. An important question is whether exogenously added carboxylates and internal carboxylates behave symmetrically.

*Supply of carbon skeletons* As soon as carboxylate carbon enters the nitrogen assimilation pathways, the rate of nitrogen metabolism and nitrogen uptake may be altered. Budd (1966) and Budd & Harley (1962a) explained the effect of carboxylates on ammonium absorption as a contribution of C to $NH_4$ metabolism. Van Die (1962) even suggested that the uptake of ammonium and its assimilation are two aspects of the same chemical reaction. Increased

nitrogen supply rises the glutamate dehydrogenase activity of plants, thus more α-oxoglutarate can be consumed (Eppley & Renger, 1974; Gamborg & Shyluk, 1970).

If delivery of C compounds to nitrogen assimilation is responsible for carboxylate stimulation of $NH_4$ entry, it seems logical that the ports of entry (Prianishnikov, 1951) of mineral nitrogen, the oxocarboxylates, exert most effect. Results of Figure 33 suggest oxocarboxylate mediation. Other trials in Chapter 5, however, showed a positive relation between the content of free ammonium and amino acids and the rate of $NH_4$ absorption. A straightforward relationship between the concentration of α-oxocarboxylates in the tissues, prior to or during $NH_4$ uptake, and the nitrogen absorption rate could not be demonstrated (Experiment 34). Because average cellular oxocarboxylate contents were determined, the possibility that higher contents of these compounds are present at sites of active amino acid synthesis (the mitochondria, Yemm & Folkes, 1958) cannot be excluded.

Neither the cellular localization of carboxylates, nor of ammonium is known. It is possible that the bulk of the free $NH_4$ is present in the vacuole. Tonoplast passage can be balanced by organic anions, as suggested by Torii & Laties (1966b), Osmond & Laties (1969) and van Steveninck et al. (1973) for cations in the Mechanism II concentration range (Experiment 14). This implies that cytoplasmatic ammonium levels could be low and function as a sink for external ammonium. Differences in $NH_4$ assimilation rate could thus explain the carboxylate specific effect on $NH_4$ entry.

*Supply of reducing power* Reducing power is required for the reductive amination of oxocarboxylates, e.g.

$$\alpha\text{-OG} + (NH_3) + NAD(P)H + H_3O^+ \xrightarrow[\text{dehydrogenase}]{\text{glutamate}} \text{GLUTAMATE} + NAD(P)^+ + H_2O$$

Of all N sources, especially ammonium is known to decrease the level of reduced coenzymes in the cell (Gamborg, 1970; Vines & Wedding, 1960; Gamborg & Shyluk, 1970; Ohmori & Hattori, 1974). Reducing equivalents originate from hydrogen carriers formed during citrate cycle metabolism.

If reduced pyridine nucleotides are rate limiting in the primary step of $NH_4$ assimilation, with repercussions for the uptake rate, one would expect those compounds, that are readily oxidized, to have most effect. The particular position of succinate in this respect draws the attention. All carboxylates enter the root at almost equal rate (Figure 14), but mitochon-

dria oxidize compounds like succinate and α-oxoglutarate much more readily than fumarate or citrate.

Redox reactions may also be involved in the uptake process per se. If this aspect of respiration is responsible for ion uptake, the effects on K and $NH_4$ uptake should be similar. As this was not the case, reducing power is probably involved in the assimilation phase and possibly indirectly in the uptake process of ammonium ions.

*Generation of high energy compounds* Nowadays many plant physiologists tend to believe that energy derived from ATP hydrolysis is essential in the uptake process of cations and possibly of anions as well, whereas electron flow is involved in anion transport. Because their results depend mainly on the results of inhibitor studies, interpretations are often ambiguous.

Johansen & Lüttge (1974) stated that ion uptake by green plant cells is powered by ATP, electron transport or reducing equivalents. The source (respiration or photosynthesis) is difficult to be proven. Light-stimulated chloride uptake into *Chlorella pyrenoidosa* was independent of photophosphorylation, but this does not hold for K uptake (J. Barber, 1968). Indications for ATP as energy source for cation uptake into algal cells were also obtained by Fisher et al. (1970). They found a linear relationship between total adenosine triphosphatase activity and Rb intake from 1-50 mM solutions. According to Nobel (1970) high energy compounds are required for potassium uptake. Photosynthetic electron flow alone was not found responsible for K uptake.

Prins (1974) studied the effect of photosynthesis on Rb and Cl uptake by leaf strips of *Vallisneria spiralis*. He encountered many interpretational problems and eventually tended to indicate ATP as driving force, not excluding a possible role of other metabolic processes. In his 1973 review Hodges arrives at the conclusion that ATP(ase) driven cation translocators and anion exchange for respiratory $OH^-$ or $HCO_3^-$ present the main picture, as far as passage of the plasma membrane is concerned, but that both cation and anion uptake can proceed with ATP as energy source. Aerobic chloride uptake into leaf cells of *Tradescantia albiflora* seems to depend on phosphorylation (Johansen & Lüttge, 1974). In Beevers (1961) concept not electron flow but phosphorylation couples respiration to salt uptake.

In literature the least doubt exists about the role of ATP in phosphate uptake. Incorporation of phosphate groups into ATP or NADP has been related to the P uptake reaction (Humble et al., 1969; Okuda & Ida, 1966a; Raven,

1974; Lin & Hanson, 1974).

In aerobic metabolism the majority of ATP is derived from carboxylate metabolism. Apart from any effect on the $NH_4$ uptake mechanism, ATP is involved in nitrogen metabolism. The activity of glutamate dehydrogenase (a key-enzyme in the $NH_4$ assimilation process) is regulated by the availability of ATP (Batt & Brown, 1974) and ATP is involved in various steps leading to protein synthesis.

Carboxylates may be stored in pools with different turnover rates and the accessibility of mitochondrial pools differs from component to component. Another complication is that the oxidation of carboxylates via the citrate cycle and the cytochrome chain yields different amounts of energy. Malate has a higher ADP/O quotient than succinate (Miller & Miller, 1974). Oxidation of the latter compound in soyabean mitochondria yielded considerably less energy than oxidation of other TCA cycle intermediates (Beevers, 1961). ADP/O quotients of 2 for succinate, 3 for malate, isocitrate and pyruvate and 4 for α-oxoglutarate have been reported (Ikuma, 1972; Hall et al., 1974).

Results of MacMillan (1956), Budd & Harley (1962a), Carrodus (1966) and of Experiment 30 reveal that uncoupling of oxidative phosphorylation by sodium azide or dinitrophenol severely reduces $NH_4$ uptake as well as succinate stimulation, indicating that ammonium entry is intimately linked to respiratory-chain phosphorylation.

Because no data on metabolization of carboxylate by the present roots are available, the biochemical mechanisms are only a matter of speculation. For instance the low ADP/O quotient of succinate can be compensated for by a higher uptake rate into the mitochondria. A compound like α-oxoglutarate is known to enter mitochondria readily and have a high ADP/O quotient at the same time. Pretreatment with citrate, a carboxylate that enters mitochondrial pools difficulty and has a lower ADP/O quotient, yields low $NH_4$ uptake rates. These two extremes are in favour of ATP as energy source for $NH_4$ uptake.

It is unlikely that the generation of ATP interacts with the $NH_4$ uptake process per se, because ATP originating from carboxylate turnover would then stimulate K and repress $NH_4$ uptake in some cases. An indirect effect of ATP via $NH_4$ assimilation seems more probable, assuming that the K and $NH_4$ transport processes are of essentially the same metabolic nature.

*Electron transport* The famous work of Lundegårdh & Bürström (1933) on anion respiration has been criticized (Sutcliffe, 1962; Lüttge, 1973b) because it does neither explain the phenomenon of selectivity, nor include anaerobic salt uptake, nor cope with spatial separation of nutrient uptake e.g. through the plasmalemma and mitochondrial electron transport. Recently alternative biochemical and biophysical models have been presented to link ion uptake with electron flow (Anderson, 1973). Most results indicate that anion uptake depends on electron transfer. Data, that relate electron flow to cation transport have also been produced.

In giant algal cells the uptake of K and Cl appeared intimately linked to electron flow (J. Barber, 1968). Fisher et al. (1970) described anion uptake in algae with electron flow as energy source. Polya & Atkinson (1969) attributed Na, K and Cl ion uptake in the Mechanism I concentration range by aged beet parenchyma cells to electron flow and rejected the concept of direct involvement of ATP as energy source.

When the terminal electron acceptor ($O_2$) is removed, as in Experiment 28, the uptake of $NH_4$ is reduced, especially in the control roots, but succinate pretreatment is still effective in the stimulation of ammonium uptake. The succinate effect is even more pronounced under anaerobic than under aerobic conditions. This is at variance with the view of Gamborg & Shyluk (1970), who ascribed the stimulation of $NH_4$ uptake in plant cell cultures by ambient carboxylates to increased electron flow. Crofts (1967) concluded that $NH_4$ uptake into chloroplasts was related to electron flow and ATP hydrolysis, the first possibility being more probable than the latter. In terms of the anion respiration theory (Lundegårdh, 1960) an additional flow of electrons towards the end of the cytochrome pathway would be expected to stimulate anion entry. For nitrate this was not observed.

As far as specific effects of carboxylates and ion specific effects are concerned, the arguments are the same as for the 'ATP explanation'.

If the effect of carboxylates on $NH_4$ entry is mainly by the raising of the succinate level in the roots and its consequences, the negative effect of some carboxylates on ammonium uptake needs explanation. Citrate obviously is a potent repressor of $NH_4$ uptake (Figure 5 and 33). The effect of high citrate levels on respiratory metabolism has been reported to be on the enzyme phosphofructokinase, leading to inhibition of the initial steps in glycolysis and thus the substrate level (pyruvate) for the citrate cycle

(Passoneau & Lowry, 1963; Parmeggiani & Bowman, 1963; Davies, 1973). Negative effects of preloading with malonate, acetate, glyoxylate and oxalate may originate from citrate cycle blockage and lack of matabolization and its consequences.

Positive or negative effects of carboxylates on the uptake of K and $NO_3$ are only discussed in comparison with $NH_4$ accumulation, because no information about the regulation of their effects is available. It is possible that carboxylates have an effect on salt entry, both on uptake and on ion assimilation. This requires coupling between the uptake and assimilation mechanisms. As metabolization of K is out of order, one can conclude that the carboxylates used (Figure 41) favour the K accumulation process per se. From the rather high correlation coefficients between K uptake (Table 25) and C-A or ash alkalinity values after preloading (Table 8); + 0.71 and + 0.87 respectively, it can be inferred that carboxylate specific effects, as observed for $NH_4$ uptake, are not likely for K absorption.

Metabolization of nitrate proceeded at a very low rate under the conditions adopted in Experiment 37 (Figure 42) so that it can be concluded that the carboxylates studied defavour the $NO_3$ uptake process per se. An exception is citrate that showed peak absorption of nitrate ions. Available data support no conclusion about nitrate reduction in citrate preloaded roots. Ferguson & Knypl (1974) reported induction of nitrate reductase (NR) in cucumber cotyledons when exposed to organic acids at pH 3. Because the tissue and the media contained no nitrate, the carboxylic compounds could not affect nitrate availability. Of all carboxylates tested, citric acid induced the highest NR activity. NRA in tissue pretreated with citric acid was even 34% higher than that of plants incubated with $NO_3$ at pH 3. Incubation with other carboxylates resulted in lower NRAs than in nitrate-induced cotyledons. Tingey et al. (1974) found citrate (pH 7.5) to stimulate in vivo NRA in young expanding soyabean leaves, while succinate was ineffective.

Although the experiments described here were carried out at physiological pH, nitrate assimilation could be induced by citrate and may explain the stimulation of nitrate absorption.

## CONCLUSION

Ion uptake is probably related to many physiological processes in the plant root. Most of these processes depend to a certain extent on carboxy-

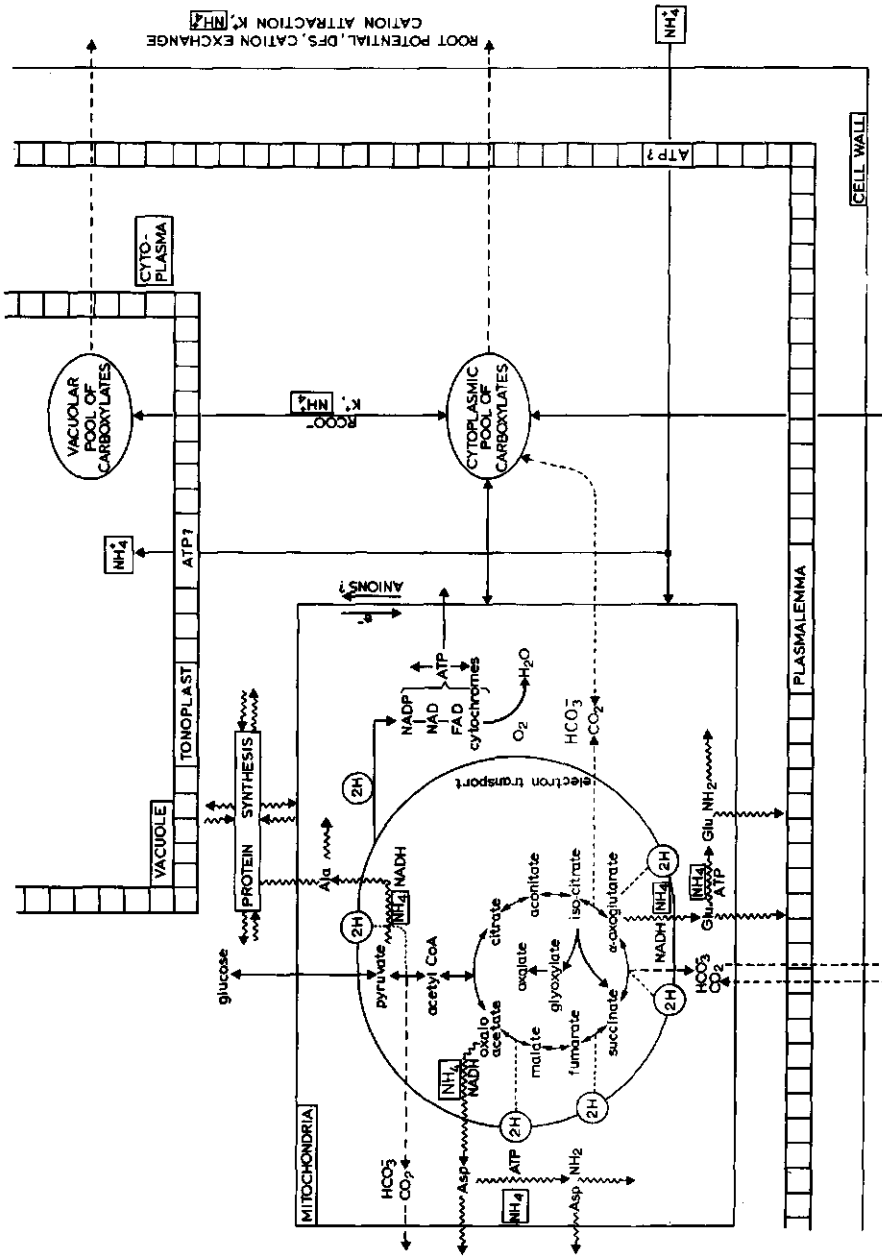


Fig. 47. Simplified scheme of the citrate cycle, in relation to ion uptake.

late metabolism. Therefore a single mechanism relating carboxylates to ion uptake will not be found. Carboxylates differ in their (bio)chemical properties and uptake mechanisms for K, $NH_4$ and $NO_3$ probably also differ because ion assimilation is involved in the latter two processes.

Referring to the initial question whether ion uptake regulates the size of the carboxylate pool or the reverse, the answer cannot be as simple as the question.

An incomplete and simplified model of citrate cycle metabolism in relation to nutrient uptake is presented in Figure 47. The three main cellular compartments of carboxylates (vacuole, cytoplasma and mitochondria), and the two main cell barriers (plasmalemma and tonoplast) for ion uptake are drawn. Primary assimilation patterns of ammonium are shown in the (enlarged) mitochondrion. Zig-zag lines indicate the transport of organic N compounds from mitochondria to the cytoplasma. Respiratory chain phosphorylation and electron flow are drawn on the right-hand side of the mitochondrion. Outside the cell some factors of the carboxylate effect on ion uptake, that do not depend on carboxylate turnover are indicated. For details the reader is referred to the several sections of the discussion.

The central position of carboxylates in the chemistry of the living cell can be a good background for the study of ion uptake processes. Thus carboxylates may provide the link in several hypotheses, stressing the role of metabolism in salt accumulation.

# Summary

Plants contain more charge-equivalents of mineral cations than of mineral anions. Cation excess is balanced by carboxylate groups of organic acid molecules. The question rises whether carboxylates are the result or the cause of nutritional events.

Various environmental factors, including inorganic nutrients, have been investigated in relation to (changes in) the size of the carboxylate pool. Relatively little effort has been made to study the effect of the carboxylate status of the roots on the ion uptake process (Chapter 1). The purpose of this publication is to gain information on the effect of carboxylates, especially succinate, on mineral uptake, with special reference to the ammonium ion.

After the experimental sections of Chapter 2, the entry of carboxylates into excised maize roots is described in Chapter 3. It is concluded that most carboxylates enter the root cells readily at pH 5.5 and a concentration of 50 mM as sodium salt. To avoid high salt concentrations during $NH_4$ uptake, the roots were treated with carboxylates prior to uptake experiments. Under the circumstances described before, the mean stimulation of $NH_4$ uptake by succinate preloading was about 50% in a 4-h uptake test.

In Chapter 4 it was shown that the effect of succinate preloading was independent of a number of adopted experimental conditions. Nitrogen-starved roots and roots from plants precultured in complete nutrient media were both stimulated in their ammonium uptake rate by succinate pretreatment (Section 4.3). The positive effect of succinate lasted as long as net uptake of $NH_4$ was measured (Section 4.2). Succinate incubation concentrations up to 25 mM promoted $NH_4$ absorption. Above this concentration the effect rose only slightly (Section 4.6). The effect of succinate on ammonium uptake appeared to depend on both $NH_4$ concentration and anion species (Sections 4.4 and 4.5).

Glucose addition to the nutrient media proved that the effect of succinate depended on the carbohydrate level of the roots (Section 4.9).

Both metabolic and non-metabolic aspects of succinate incubation were encountered. Nitrogen gassing reduced $NH_4$ uptake but succinate pretreatment was still effective. With increase of temperature in the nutrient solution, the effect of succinate preloading increased. Sodium azide, an uncoupling agent, repressed both uptake and the succinate effect (Section 4.10).

In Chapter 5 the effect of root incubation with a variety of carboxylates on $NH_4$ uptake was investigated. Succinate proved to be the most effective stimulator, followed by α-oxoglutarate, oxaloacetate, malate and pyruvate. Preloading with citrate, acetate, malonate, glyoxylate and oxalate resulted in lower $NH_4$ uptake than in control roots. Consideration of the effects of carboxylates and their position in the tricarboxylic acid cycle revealed that the availability of oxocarboxylates might be involved in the enhancement of $NH_4$ absorption.

The effect of carboxylate incubation on the uptake of potassium and nitrate was checked in Chapter 6. All carboxylates tested stimulated K entry, but the absorption of $NO_3$ was repressed, except by citrate.

After a discussion of factors inherent in the chosen experimental approach; such as the use of detached roots, non-sterility and variability of the plant material (Section 7.1) and the determination of $NH_4$ uptake, including the initial phase (Section 7.2), an attempt was made to unravel non-metabolic and metabolic effects of carboxylates on ion uptake in the Sections 7.3 and 7.4, respectively.

Non-metabolic or passive stimulation of ion entry can be caused by the presence of negative charges within the cell membranes. Stimulation of K and repression of $NO_3$ uptake are in accordance with this view. The observation that citrate preloading inhibits ammonium, but stimulates potassium and nitrate uptake is at variance with this concept. It is concluded that the picture is incomplete if ion uptake and ion assimilation are considered as independent processes.

Metabolic or active stimulation of ion entry results from carboxylate turnover, which may result in carbon skeletons for nitrogen incorporation, high-energy compounds, reducing power and electron transport. A possible role of each of these processes in the uptake and assimilation process of ions is discussed. An unambigous picture could not be presented, but the causes therefore are known and discussed in Section 7.4.

# Samenvatting

Planten bevatten meer anorganische kationen (C) dan anorganische anionen (A). Het overschot aan positieve lading (C-A) wordt gecompenseerd door carboxylgroepen van organische anionen, kortweg carboxylaten genoemd.

Het carboxylaatgehalte van de plant wordt door een aantal groeifactoren bepaald. Enkele aspecten van de minerale voeding, zoals de vorm waarin stikstof wordt opgenomen en de verhouding tussen de opname van kationen en anionen, spelen hierbij een grote rol. De vraag rijst of gebeurtenissen die samenhangen met de minerale voeding het gevolg of de oorzaak zijn van het carboxylaatniveau in de plant. Wijzigingen van het carboxylaatgehalte, teweeggebracht door de anorganische voeding van de plant, zijn in de literatuur uitvoerig beschreven. Over het omgekeerde is betrekkelijk weinig bekend.

Dit verslag behandelt de invloed van het carboxylaatniveau in afgesneden maiswortels op de opname van ammonium. Het ammonium-ion wordt snel opgenomen en vervolgens via een proces, dat ten koste gaat van het carboxylaatgehalte, ingebouwd in organische stikstofverbindingen.

Om verschillende carboxylaatgehalten te verkrijgen werden de wortels in de nacht voorafgaand aan de opnameproef blootgesteld aan een 50 mM Na-carboxylaatoplossing in zwak zuur milieu (pH 5,5). De meeste experimenten werden gedaan met barnsteenzuur, omdat uit eigen en andere gegevens bleek dat dit de $NH_4$-opname sterk bevordert. Gedurende opnameperioden van ieder 4 uur was de $NH_4$-opname door met succinaat voorbehandelde wortels gemiddeld 50% hoger dan door wortels zonder succinaat-voorbehandeling. In alle levende organismen is de oxydatie van barnsteenzuur een onderdeel van de aerobe ademhaling. Stimulatie van de ammoniumopname door voorbehandeling met barnsteenzuur bleek ook op te treden onder omstandigheden waarbij de omzetting van deze stof werd geremd.

Naast barnsteenzuur werd het effekt van een aantal andere carboxylaten op de opname van ammonium, kalium en nitraat onderzocht.

Uit de ammonium-opnameproeven werd geconcludeerd dat de ketozuren een belangrijke functie kunnen hebben bij het opnameproces. Omdat ketozuren de

verbindingen zijn waarmee opgenomen ammonium-ionen in eerste instantie reageren, ligt het voor de hand dat er een verband bestaat tussen enerzijds de verwerking en anderzijds de opname van ammonium-ionen.

Voor kalium, een element dat niet in organische stoffen wordt omgezet, werd gevonden dat alle carboxylaten de opname bevorderen, waarbij een verband bestaat tussen het (C-A) gehalte van de wortels aan het begin van een opname-proef en de opgenomen hoeveelheid kalium ionen.

De opname van nitraat werd, met uitzondering van citraat, door alle onderzochte carboxylaten geremd. Er zijn aanwijzingen dat de nitraatverwerking onder de gekozen omstandigheden gering is en dat citraat ook in dit opzicht een uitzonderingspositie inneemt.

De slotconclusie is dat carboxylaten, onafhankelijk van het soort carboxylaat, bij de opname van ionen die niet of nauwelijks door de stofwisseling van de wortel omgezet worden de kationenopname bevorderen en de anionenopname remmen. Voor ammonium is de situatie ingewikkelder, daar de processen van opname en verwerking van $NH_4$ nauw samenhangen.

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