Changes in the Concentrations of Malic Acid, Citric Acid, Calcium and Potassium in the Leaves during the Growth of Normal and Iron-Deficient Mustard Plants (*Sinapis alba*)

By M. J. PALMER, P. C. DEKOCK AND J. S. D. BACON

Departments of Biochemistry and Plant Physiology, Macaulay Institute for Soil Research, Aberdeen

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The mineral composition of leaves is subject to great variations, which are often accompanied by changes in the content of organic acids. Much still remains to be discovered about the relationship between the inorganic and organic constituents, although a few general statements can be made. For example, several workers have found that the content of malic acid or oxalic acid in leaves is related to that of calcium (Gilbert, Shear & Gropp, 1951; Rasmussen & Smith, 1961).

At this Institute DeKock, Hall & MacDonald (1960) showed that the leaves of mustard plants made chlorotic by iron-deficiency had a high ratio of potassium to calcium and that this ratio decreased as the concentration of iron in the nutrient solution was increased. In another study DeKock & Morrison (1958) examined several species and found that chlorotic leaves often contained more citric acid and always distinctly less malic acid or oxalic acid than green leaves; the ratio citric acid/(malic acid + oxalic acid) was thus always higher in chlorosis. DeKock (1958) put forward a hypothesis that accounts for both observations in terms of the parts that these constituents might be expected to play in the metabolism of the leaf as a whole.

In the present work we have studied the changes in the two major organic acids of mustard leaves, malic acid and citric acid, under a variety of nutritional conditions, including iron-deficiency. Simultaneous measurements were made of the major inorganic constituents, to ascertain whether changes in these could be correlated in any way with the changes in the organic acids. A preliminary account has been published (Bacon, Palmer & DeKock, 1961b).

MATERIALS AND METHODS

Plants were grown in the greenhouses or gardens of this Institute as described by Bacon, DeKock & Palmer (1961a). Leaves were picked shortly before use and carried to the laboratory in a dry beaker surrounded by ice in a vacuum flask. In the experiments in which leaves were sampled at various stages of the growth of the plants, one leaf was removed from one plant from each pot or crock, as close to the main stem as possible. The petioles were removed and weighed and the rest was sliced into small pieces,

from which 0.5 g. samples were taken for acid estimations and the remainder was weighed in a silica crucible and dried at 80° to constant weight. The dry material was ashed at 480° and potassium, calcium, sodium and magnesium were determined on hydrochloric acid extracts of the ash (DeKock & Hall, 1955). Plants were grown in nutrient solution with differing concentrations of iron, potassium, calcium, phosphate and nitrate as described by DeKock *et al.* (1960). All extractions before March 1961 were made at room temperature with ice-cold solutions and after that date were made in a cold room at 5°.

Estimations. Malic acid was estimated by the polarimetric method of Krebs & Eggleston (1943) for α -hydroxy acids. Mustard leaves contain only malic acid and citric acid in any appreciable concentration (DeKock & Morrison, 1958), and so the use of this relatively unspecific method was considered justified. [Qualitative examination by paper chromatography and occasional quantitative separations on silica-gel columns (DeKock & Morrison, 1958) were made on selected samples, to confirm that no other organic acids were present.] The leaf sample (0.5 g.) was disintegrated in distilled water in an all-glass homogenizer and then placed in a boiling-water bath for 5 min. The coagulated residue was centrifuged off and the pellet washed twice with small volumes of water such that, after recentrifuging, the total volume of supernatant and washings was 4 ml. This was divided into two equal portions. To one was added 2 ml. of water, 1 ml. of M-sodium citrate, 0.5 ml. of acetic acid and 4.5 ml. of freshly prepared 29% (w/v) ammonium molybdate. The other tube had water in place of the ammonium molybdate solution, and was used as a blank; this blank reading never exceeded the error of reading the polarimeter ($\pm 0.02^\circ$), which corresponded in most cases to ± 4 m-equiv./kg. fresh weight of leaf, the solutions being read in a 1 dm. tube.

Another 0.5 g. leaf sample was disintegrated in water, stored in the frozen state, and later thawed in the presence of trichloroacetic acid for estimation of citric acid by the method of Taylor (1953).

On certain occasions ascorbic acid and nitrate were also estimated. A 0.5 g. leaf sample was extracted with 2% (w/v) metaphosphoric acid, the solution treated with dithizone to remove traces of copper (Hewitt & Dicks, 1961) and the ascorbic acid titrated with 2,6-dichlorophenol-indophenol (Roe, 1954). Nitrate was estimated colorimetrically on extracts of dried samples (0.5 g.), by interaction with phenol-2,4-disulphonic acid, as described by Humphries (1956). The samples for this purpose were dried at 100° to hasten the process and hence prevent the increases in nitrate that are claimed to occur in wilting leaves. Under these conditions the small amount of material dried rapidly (20–30 min.).

Phosphate was estimated by the method of Berenblum & Chain (1938) as modified by Ernster, Zetterström & Lindberg (1950) on samples deproteinized with 5% (w/v) trichloroacetic acid.

Chlorophyll was extracted with acetone from fresh leaf samples and measured spectrophotometrically (cf. Arnon, 1949). Potassium, calcium and sodium were estimated by flame photometry, the method having an accuracy of ± 3 to $\pm 5\%$ with our samples, which were low in aluminium and phosphate (Ure, 1958). Magnesium was estimated by direct photometry by a porous-cup-spark method with a coefficient of variation of $\pm 2-3\%$ (Scott & Ure, 1958).

Paper chromatography of organic acids. The leaf sample (0.5 g.) was ground in a mortar with 2 g. of Celite no. 545 (Johns-Manville Co.) and 0.2 ml. of 3N-sulphuric acid. A further 1 g. of Celite was added, and the powder was packed in a small glass tube and eluted with chloroform-2-methylbutan-2-ol (1:1, v/v). The first 5 ml. of effluent was collected, 5 ml. of ether and 1 ml. of water were added, and the mixture was shaken in a glass-stoppered tube. The aqueous layer was removed with a Pasteur pipette and 5 μ l. spotted on Whatman no. 1 paper. The chromatogram was developed with butan-1-ol-formic acid-water (10:3:10, by vol.) overnight at 20–22°, allowed to dry for at least 3 hr. in a fume cupboard at room temperature, and sprayed with 0.1% bromocresol green in 75% (v/v) ethanol. The procedure is based on those of Vickery & Wilson (1958) and Lugg & Overell (1948).

RESULTS

Influence of variations in the composition of the nutrient solution on the concentration of malic acid and citric acid in leaves of plants grown in solution culture

Three experiments were set up as described by DeKock *et al.* (1960). Sampling was begun at about the time when the first flower buds were showing, usually about 3 weeks after sowing. Each crock was sampled separately and eight leaves of position 1 or 2 (i.e. the first and second leaf counting from the base of the stem), depending on the experiment, were taken for analysis. The whole set was sampled in about 8 days. Expts. 1 and 3 were carried out with supplementary illumination and Expt. 2 with natural illumination. In all three experiments the leaves of plants grown in solutions with the lowest iron supplement had lower concentrations of malic acid than those grown in the solutions containing more iron, but the concentration of citric acid was affected only in Expts. 1 and 3 (Table 1). The effect of the high K/Ca ratio on the concentration of malic acid (Table 2) was statistically significant in Expts. 1 and 3 when the values for both P/N ratios

Table 1. *Effect of concentration of iron in the nutrient solution on malic acid and citric acid in mustard leaves*

Plants were grown in 5 l. glazed crocks supported on a plastic circle of gauze containing washed silica sand. The nutrient solutions contained four different ratios of potassium to calcium, three concentrations of iron and two different ratios of phosphate to nitrate; the experiments were arranged in a $4 \times 3 \times 2$ factorial design, each treatment being repeated twice, and the treatments were randomized in two blocks of 24. Fe 1, 2, 3 represent 0.2, 1.0, 5.0 p.p.m. respectively of iron as ferric EDTA. Samples (0.5 g.; 8 leaves) were taken from crocks when the plants were 3–4 weeks old. Each figure is the mean of analyses of sixteen samples, and is expressed as m-equiv. of acid/kg. fresh wt. Expt. 1 is the same as that described in Table 2, Bacon *et al.* (1961a).

Expt. no.	Date	Fe 1		Fe 2		Fe 3		S.E.M.	
		Malic acid	Citric acid	Malic acid	Citric acid	Malic acid	Citric acid	Malic acid	Citric acid
1	April 1959	236	98	273	87	272	77	± 11	± 6
2	August 1959	32	69	133	73	127	71	± 9	± 4
3	January 1960	87	91	104	64	102	46	± 5	± 3

Table 2. *Effect of concentration of potassium and calcium in the nutrient solution on malic acid and citric acid in mustard leaves*

These results are from the experiments described in Table 1. Each figure is the mean of analyses from 12 samples.

Expt. no.	Ratio K/Ca in total of 13 m-equiv./l.				S.E.M.
	1/12	3/10	6/7	10/3	
	Amount of acid (m-equiv./kg. fresh wt.)				
Malic acid					
1	280	297	270	194	± 12
2	100	106	102	83	± 10
3	107	106	98	78	± 6
Citric acid					
1	86	80	93	91	± 6.5
2	67	72	73	71	± 4.5
3	69	65	67	67	± 3.4

were taken together. In Expt. 2 the concentration of malic acid in leaves from this treatment was also noticeably lower, but the difference was not statistically significant. In no experiment was there an effect of K/Ca ratio on the concentration of citric acid in the leaves.

The concentrations of malic acid reached in the three experiments were noticeably different and there were indications that the concentration of malic acid increased during the period of sampling. This observation prompted an approach to the problem by a different type of experiment, in which the amounts of malic acid, citric acid, calcium and potassium were measured as leaves matured. It was thought that, if the concentrations of some or all of these constituents changed, metabolic connexions between them might become apparent.

Changes in malic acid, citric acid, calcium and potassium contents of leaves of growing plants

The earliest experiments of this type were made on plants growing in soil; later a series of experiments were carried out on plants growing in solution culture, since this allowed control to be exercised over the accumulation of some of the leaf constituents.

Estimations were made on leaves from a fixed position on the plant (leaf 2) and samples were taken at intervals during plant growth. In most of the experiments described below, the manner of collecting the sample permitted the results to be expressed as m-equiv./unit fresh wt., as m-equiv./unit dry wt., or as μ equiv./leaf. The results are expressed as μ equiv./leaf in all the experiments where this was possible and in m-equiv./kg. fresh wt. in the others. The average weight of a leaf usually increased during the first few samplings and mature leaves weighed 0.15–0.25 g. Thus 10 μ equiv./leaf is equivalent to about 40–70 m-equiv./kg. fresh wt.

The results given below refer to analyses of the leaf after removal of the petiole. The petiole constituted about one-quarter of the fresh weight of the leaf, and this proportion did not alter very much as the leaf matured. The concentrations of sodium and magnesium are not given in detail, because they did not change significantly during the period of sampling except in two experiments. In one (Expt. 3, Table 3) the Na/K ratio (12/3) in the nutrient solution was considerably higher than that (2/3) used for all other experiments; the concentration of sodium was high at first sampling, and a slow but continuous rise (from 80 to 130 m-equiv./kg. fresh wt.) took place over the experimental period of 18 days. In the other (Expt. 2, Table 3) the K/Ca ratio of the nutrient solution was higher than usual. Here the concentration of

sodium rose at the beginning of the experimental period from 19 to 36 m-equiv. and thereafter remained at 40 ± 5 m-equiv.; that of magnesium was 40 ± 5 m-equiv. for the greater part of the period, but rose to 60 m-equiv. at the end. Otherwise the concentration of sodium was about 24 m-equiv./kg. fresh wt. (range 20–30) in plants grown in solution culture, and about 10 m-equiv. in soil-grown plants, the limits of variation in a single experiment being ± 5 m-equiv. or less. The concentration of magnesium was about 30 m-equiv. (range 20–40) in both soil- and solution-culture-grown plants, an exception being Expt. 5 (Table 3), where it was about 55 m-equiv. From the discussion below it will be seen that the concentrations of calcium, potassium, malic acid and citric acid were in most cases considerably higher and were subject to changes as great as 300 m-equiv./kg. fresh wt., i.e. ten times the total content of sodium or magnesium.

Soil-grown plants. Plants were grown in John Innes no. 2 mixture (a mixture of 7 parts of soil, 3 parts of leaf mould and 1 part of sand, with additions of about 2.5 g. of superphosphate, 2.5 g. of hoof and horn, 1 g. of potash, and 0.5 g. of lime/l.). Preliminary experiments of this nature (May and June 1960), in which only acids were estimated, showed that the concentration of malic acid increased rapidly for about 10 days (from 30 to 170 m-equiv./kg. fresh wt.) and was then maintained at about the same value for a further 10 days. The concentration of citric acid fell from 74 to 49 m-equiv. in the first 10 days, then rose to 89 m-equiv. during the remaining period. An experiment in July 1960 gave similar results; the concentration of malic acid increased from 90 to 200 m-equiv. in 12 days and then remained at about the same value for a further 12-day period. The concentration of calcium followed that of malic acid closely. It increased from 100 to 190 m-equiv. during the first 12 days and then was steady during the next 12 days. The concentration of citric acid increased slightly (60–100 m-equiv.) during the sampling period, and that of potassium varied within the range 80–125 m-equiv.

Mustard plants grown in garden soil in a cold frame in September–October 1960 did not reach the flowering stage; the leaves sampled showed a gradual increase in the amount of malic acid from 9 to 75 μ equiv./leaf over 4 weeks. The amount of calcium also increased steadily (11–81 μ equiv./leaf) over the same period. Citric acid varied between 12 and 29 μ equiv. during the sampling period. The amount of potassium in the young leaves was high (35 μ equiv.). This fell to 20 μ equiv. in 10 days and then varied between 20 and 27 μ equiv., with the exception of one value of 36 μ equiv.

Plants grown in pots in the greenhouse without supplementary light during October and November

1960 also did not reach the flowering stage. During the sampling period of 6 weeks, the amount of malic acid changed only slightly. After an initial small rise during the first 10 days (4–20 μ equiv.) it fluctuated widely within the range 6–22 μ equiv. during the next 5 weeks. Calcium behaved in a similar way. It increased from 7 to 22 μ equiv. in the first week and then varied between 16 and 27 μ equiv. for the next 5 weeks. The amount of citric acid varied only slightly throughout the period of sampling (from 5 to 11 μ equiv.). The amount of potassium was much higher than that of citric acid; it increased from 9 to 32 μ equiv. in the first 12 days and then varied between 24 and 34 μ equiv. (with one sample 40 μ equiv.).

Plants growing in nutrient solutions containing sufficient iron. The nutrient solutions used fell into two main groups. One contained only nitrate as a source of nitrogen; this was the solution with K/Ca ratio 3/10 and phosphate/nitrate ratio 2/10 used in the experiments described above (Table 1; K₂P₂ of DeKock *et al.* 1960). The other nutrient solutions had part of the nitrogen supply as NH₄⁺ ions. The earliest experiments (June 1960) were made with plants grown in a solution of ammonium nitrate. The concentration of malic acid increased steadily with time (from 50 to 280 m-equiv./kg. fresh wt. in 16 days); the concentration of citric acid varied from 35 to 55 m-equiv. during 14 days and increased to 80 m-equiv. in the last 2 days of the sampling period.

The results of subsequent experiments are summarized in Table 3. They show the effects of the form in which nitrogen is given, and of variations in the K/Ca ratio. The increases in amounts of malic acid and citric acid, and of calcium, were more or less continuous over the periods stated, but potassium always rose sharply during the first 3–5 days to a value that approximated to the final value, and sometimes exceeded it. During this early period the fresh weight of the leaf was still rising fairly fast, and an accompanying fall in the concentration of potassium was usually insufficient to counterbalance it.

(a) *Effect of supplying part of the nitrogen as ammonium ions.* The results of Expts. 1 and 4 (Table 3) conform to many previous reports on this subject, the accumulation of organic acids being greater when all the nitrogen is supplied in the form of nitrate. There was a close equivalence between calcium and malic acid in both cases. A direct comparison was made in Expt. 2, plants being grown simultaneously in 12 crocks of each nutrient solution, and the results were similar. In these three experiments the K/Ca ratios and the total nitrogen contents of the two solutions were not the same, 5 m-equiv. of Ca²⁺/l. in the nitrate solution having been replaced by 5 m-equiv. of

Table 3. *Changes in the composition of leaves of plants growing in solution culture with sufficient iron*

The nutrient solutions, one having all its nitrogen as NO₃⁻ ions, the other part as NH₄⁺ ions, are described in detail in the text.

Expt. no.	Date of expt.	Nutrient soln.	Duration of expt. (days)	Constituents (μ equiv./leaf)							
				Malic acid		Calcium		Citric acid		Potassium	
				Start	Finish	Start	Finish	Start	Finish	Start	Finish
1	Aug.-Sept. 1960	NH ₄ NO ₃	22	10	52	12	53	8	9	16	17
2	Mar. 1961	NH ₄ NO ₃ ; K/Ca 3/5 NO ₃ ⁻ ; K/Ca 3/10	18 19	8 18	45 85	8 17	40 75	3 5	11 18	10 16	14 9
3	June 1961	NH ₄ NO ₃ ; K/Ca 3/5* NO ₃ ⁻ ; K/Ca 3/5*	18 18	3 5	27 76	4 7	32 71	2 2	9 27	5 5	9 9
4	July-Aug. 1961	NO ₃ ⁻	19	2	73	4	77	2	19	3	28
5	Aug. 1961	NO ₃ ⁻ ; K/Ca 10/3	19	5	42	7	31	3	14†	12	19

* Compensatory adjustment of cation-anion balance made with Na⁺ and SO₄²⁻ ions.

† Interpolated value.

NH_4^+ ; this made the K/Ca ratio higher (3/5 instead of 3/10) and the total nitrogen 15 instead of 10 m-equiv. A second experiment (Expt. 3) was therefore designed in which these discrepancies did not occur; but to maintain the anion-cation balance the nitrate solution was given a higher concentration of sodium (12 instead of 2 m-equiv./l.) and the ammonium nitrate solution a higher concentration of SO_4^{2-} ions, and both contained additional Cl^- ions. The results were similar to those of Expt. 2, except that the concentrations at the start of the period of sampling were much closer together for the two treatments. Once again there was close correspondence between the figures for calcium and malic acid, irrespective of the magnitude of the accumulation.

(b) *Effect of K/Ca ratio.* In the experiments described in Table 2 it was shown that when the K/Ca ratio of the nutrient solution was at its highest (10/3) the concentration of malic acid in the leaves was lower than in the other treatments. A growth experiment (Expt. 5, Table 3) was set up with this nutrient solution. In this the accumulation of malic acid exceeded that of calcium, and after the fifth day the excess became constant at about 12 $\mu\text{equiv.}$ The increase in citric acid all took place during the first 7 days, as did that of potassium. Therefore the potassium remained at about 10 $\mu\text{equiv.}$ higher than the citric acid, so that the balance of these anions and cations was preserved.

Plants growing in nutrient solution containing insufficient iron. The experiments described in Table 1 showed that the concentration of malic acid in the leaves of iron-deficient plants was lower than that in green leaves. This observation was confirmed and extended by the following five experiments. In each, 24 crocks of mustard plants were grown deficient in iron; after sampling, 12 were given extra iron in the nutrient solution and the other 12 were left iron-deficient. Leaves from plants receiving extra iron are referred to below as greening leaves and those growing in low iron concentration as iron-deficient leaves. To obtain sufficient material from the iron-deficient plants both leaf 1 and leaf 2 were taken from each set. The five experiments were carried out at the following times: (a) October–November 1960; (b) April 1961; (c) September 1961; (d) December 1961–January 1962; (e) February 1962. It might have been expected that correction of the deficiency would allow an additional increase in the fresh weight of the greening leaves, but there was an equal increase in fresh weight in the iron-deficient leaves in Expts. (a), (d) and (e). In (c) the fresh weight of both sets was unchanged, and only in (b) did the fresh weight of the iron-deficient leaves remain constant, whereas that of the greening leaves increased. Changes in dry-matter content also

followed no regular pattern: the percentage dry weight was somewhat lower in iron-deficient than in greening leaves in Expts. (a) and (c), about the same in (b), and somewhat greater in (d) and (e).

The results of analyses for cations and organic acids are shown in Figs. 1 and 2 and Table 4. Figs. 1 and 2 are constructed from the average values for the first 13 days of all five experiments. Table 4 shows the average values of initial amounts, changes during 13 days and the final amounts in each of the five experiments, expressed as m-equiv./kg.

Fig. 1. Changes in the contents of calcium and malic acid during recovery from iron-deficiency. The values are averages from analyses made during five experiments. Plants were first grown in one of the nutrient solutions used for the experiments of Table 1, having K/Ca ratio 3/10 and phosphate/nitrate ratio 2/10, with 0.2 p.p.m. of iron as ferri EDTA. At the beginning of the experimental period the solution in 12 of the 24 crocks was changed to one containing 5 p.p.m. of iron. Other details are given in the text. ●, Calcium in greening leaves; ○, calcium in iron-deficient leaves; ▲, malic acid in greening leaves; △, malic acid in iron-deficient leaves.

Fig. 2. Changes in the contents of potassium and citric acid during the experiments of Fig. 1. ●, Potassium in greening leaves; ○, potassium in iron-deficient leaves; ▲, citric acid in greening leaves; △, citric acid in iron-deficient leaves.

fresh wt. and as $\mu\text{equiv./leaf}$. The experiments were usually sampled for longer than 13 days; but figures are given only for this period, which seemed to be all that was required for recovery from iron-deficiency, as judged by the rise in chlorophyll content, most of which took place in the first week.

The results show that in iron-deficient leaves the increases in calcium and malic acid were approximately equivalent, although there was always a much higher concentration of calcium than of malic acid at the beginning and hence at the end of the sampling period. They also show that when iron-deficient plants were given extra iron in the nutrient solution the concentrations of malic acid and calcium reached approximate equivalence by the end of the sampling period. There was very little difference between the amounts of potassium in the iron-deficient leaves and the green leaves, but citric acid increased more in the iron-deficient leaves.

Taken singly the experiments showed considerable variation, although except for a slight fall in calcium in one experiment the changes in all four constituents were always positive. In Expt. (a) very little increase occurred in the amount of malic acid. In the iron-deficient leaves it increased from 3 to 8 $\mu\text{equiv./leaf}$ and in the greening leaves from 2 to 9 $\mu\text{equiv.}$ During the same period calcium increased from 8 to 23 and from 7 to 18 $\mu\text{equiv.}$ respectively. The plants in this experiment had a very low dry-matter content, but the amount of chlorophyll increased steadily in the leaves receiving extra iron (from 0.6 to 1.4 $\mu\text{moles/g. fresh wt.}$).

In Expt. (b) malic acid increased in the greening leaves (from 5 to 33 $\mu\text{equiv.}$) to a much greater extent than in the iron-deficient leaves (from 3 to 7 $\mu\text{equiv.}$). Calcium changed from 12 to 27 $\mu\text{equiv.}$ and 11 to 23 $\mu\text{equiv.}$ respectively.

In Expt. (c) the amount of malic acid again increased more rapidly in the greening leaves (from 4 to 16 $\mu\text{equiv.}$) than in the iron-deficient leaves

(from 2 to 7 $\mu\text{equiv.}$). There was a slight fall in calcium in the plants given extra iron (from 20 to 15 $\mu\text{equiv.}$). In the iron-deficient leaves it increased from 11 to 23 $\mu\text{equiv.}$

In Expt. (d) malic acid increased rapidly in both sets of plants (iron-deficient leaves from 6 to 53 $\mu\text{equiv.}$, greening leaves 8 to 27 $\mu\text{equiv.}$) and the increases of calcium, from 17 to 57 and from 21 to 34 $\mu\text{equiv.}$ respectively, were similar. The iron-deficient leaves in this experiment had a higher chlorophyll concentration than usual (about 1.2 $\mu\text{moles/g. fresh wt.}$) and this increased to 1.7 μmoles in the leaves receiving extra iron.

In Expt. (e) malic acid again increased rapidly in both sets of plants (iron-deficient leaves from 4 to 22 $\mu\text{equiv.}$ and greening leaves from 6 to 54 $\mu\text{equiv.}$). The increase of calcium was similar to that of malic acid in the iron-deficient leaves but much less in the greening leaves (from 10 to 26 $\mu\text{equiv.}$ and from 12 to 31 $\mu\text{equiv.}$ respectively).

The differences in potassium and citric acid between the five experiments were less marked than those in calcium and malic acid. The amount of potassium was appreciably higher than that of citric acid in Expts. (a) and (e), especially in the plants receiving extra iron. There was more citric acid in the iron-deficient leaves than in the greening leaves at the end of the sampling period in all experiments except (e).

Changes in acids and cations during the period of yellowing of the leaves

In several of the experiments with normal plants samples of yellowing leaves were taken. Sometimes the leaves had not begun to turn yellow at the end of the normal sampling period (about 4 weeks) and by this time there was insufficient material to extend the analyses to this period of growth. This always happened with plants grown with ammonium nitrate; when yellowing did occur in these plants it was accompanied by rapid wilting and abscission and they were thus impossible to sample.

Table 4. *Changes in potassium, calcium, citric acid and malic acid during recovery from iron-deficiency*

Plants were grown in 24 crocks of nutrient solution containing 0.1 p.p.m. of iron as ferric EDTA. In the nutrient solution nitrate, phosphate, potassium and calcium were present at 10, 2, 3 and 10 m-equiv./l. respectively. From the two-leaf stage the plants in 12 crocks were given extra iron as ferric EDTA to 5 p.p.m. and analyses were begun. Concentrations are expressed as m-equiv./kg. fresh wt. and amounts as $\mu\text{equiv./leaf}$.

	Calcium		Malic acid		Potassium		Citric acid	
	Concn.	Amount	Concn.	Amount	Concn.	Amount	Concn.	Amount
Iron 0.1 p.p.m.								
Initial value	173	12	54	4	117	8	58	4
Increase in 13 days	116	18	111	15	9	7	46	7
Total after 13 days	289	30	165	19	126	15	104	11
Iron 5 p.p.m.								
Initial value	170	13	61	5	111	8	51	4
Increase in 13 days	43	12	165	22	14	6	23	4
Total after 13 days	213	25	226	27	125	14	74	8

At the beginning of this phase of leaf development the amounts of malic acid and calcium were always high and citric acid and potassium usually low. During the period of yellowing there was always an inversion in the concentrations of the two acids. For example, in the preliminary experiment (soil-grown plants, May–June 1960) the concentration of malic acid on the 17th day of sampling was 196 m-equiv./kg. fresh wt. The concentration of citric acid was 84 m-equiv. On the 27th day the concentration of malic acid had fallen to 22 m-equiv. and citric acid had risen to 198 m-equiv. On the 21st day of sampling of plants grown in nitrate solution (March 1961) the amount of malic acid was 74 μ -equiv./leaf and citric acid 21 μ equiv. By the 31st day malic acid had fallen to 51 μ equiv. and citric acid increased to 54 μ equiv. This experiment was the only one in which the cations also showed an inversion. Calcium fell from 88 to 21 μ equiv. and potassium increased from 10 to 24 μ equiv. from the 21st to the 31st day of sampling. In the experiment of July–August 1961 the amount of malic acid was 73 μ equiv. and citric acid was 19 μ equiv., on the 19th day of sampling. After 32 days malic acid had fallen to 33 μ equiv. and citric acid increased to 42 μ equiv. Calcium fell slightly during the same period (from 77 to 61 μ equiv.), but the amount of potassium also fell (from 28 to 8 μ equiv.).

Changes in cations and organic acids in the leaves of the lesser celandine, Ranunculus ficaria L.

During the spring of 1962 leaves of the lesser celandine were sampled and the organic acids and cations estimated in the same way as in the experiments with mustard. The plant was chosen because it has a short period of growth and because the leaves turn yellow slowly at the end of the growth cycle. Over the whole sampling period (83 days) there was very little change in the amount of calcium in the green leaves taken at random from plants growing in a shaded position. It varied from 47 to 70 m-equiv./kg. fresh wt. of leaf. The amount of malic acid varied similarly (from 45 to 81 m-equiv.) during the first 36 days of sampling, then rose to a peak (105 m-equiv.) just before yellowing was detected. There was little change in the amount of citric acid (from 13 to 27 m-equiv.), but potassium was much higher than citric acid and varied to a greater extent (from 124 to 177 m-equiv.). Nitrate was looked for in some samples but at no time was any detected; there was thus a large discrepancy between the major cations and organic acids measured. After 48 days the leaves began to lose their green colour; after 83 days some were white and no chlorophyll was measurable. During this period the amount of malic acid decreased to about 18 m-equiv., but although citric acid was slightly higher in the yellow leaves the

difference was very small. There was no consistent difference between the amounts of cations in the green and yellow leaves.

DISCUSSION

The results reported here pose three main questions: What is the nature of the relationship between malic acid and calcium in mustard leaves? Is there a corresponding relationship between citric acid and potassium? What explanations are there for the large discrepancy between the sums of these cations and anions in leaves grown in certain conditions?

Relationship between malic acid and calcium

Biddulph, Cory & Biddulph (1959) concluded that once calcium had been taken up by a leaf it remained there irrespective of changes in the concentration of calcium in the nutrient solution. Our results do not contradict this finding, for at no time was there a fall in the amount of calcium per leaf during the period of sampling, so long as the leaves remained green.

Gilbert *et al.* (1951), using tung leaves, and Rasmussen & Smith (1961), using Valencia-orange leaves, have shown a correlation between the concentration of calcium and that of oxalic acid. Similarly, Rasmussen & Smith (1961) and Iljin (1951) (working with several species, mainly trees) have shown a correlation between calcium and malic acid. Even an exact equivalence between the values for calcium and malic acid could not be taken to mean that every malic acid molecule in the leaf was combined with a Ca^{2+} ion; obviously a part of the calcium will be associated with the cell structure. For example, Sinclair & Eny (1947) found that a small proportion of the insoluble calcium (0.222 out of a total of 1.923 m-equiv./g. dry wt.) was not associated with oxalic acid in Valencia-orange leaves. Nevertheless in our experiments calcium and malic acid were accumulated at almost identical rates under a variety of conditions. This was true whether the processes were very vigorous (e.g. in plants grown in solution culture with nitrate as the sole source of nitrogen) or very feeble (soil-grown plants under short-day conditions), and it is difficult to avoid the conclusion that one must depend on the other.

Jacobson & Ordin (1954) showed that malic acid was used to maintain an ionic balance during the uptake of ions by excised roots. Jacobson (1955) further found that roots taking up excessive cations fixed about seven times as much $^{14}\text{CO}_2$ from the solution as those taking up excessive anions and that malic acid accounted for a large portion of the ^{14}C present in the tissues. He considered that the malic acid was formed mainly by dark-fixation of

carbon dioxide. We have no evidence to indicate the source of the malic acid formed in the present experiments.

Although a close equivalence of the concentrations of malic acid and calcium was often found, this was not true of leaves from iron-deficient plants (see below), nor from plants grown in a nutrient solution with a high K/Ca ratio. The latter accumulated calcium at a steady rate, about half that of plants grown with lower K/Ca ratios. The concentration of malic acid increased quickly in the first five days, but thereafter the rate fell to a value similar to that for calcium. Thus even in this experiment, where the absolute values for the two constituents differed by about 12 μ equiv./leaf, their accumulation seemed to be related.

In seeking a mechanism that would explain the association of calcium with malic acid one meets difficulties. The calcium must obviously come from outside the leaf, but some of the malic acid is almost certainly formed within it. Although the amount accumulated is often a surprisingly large proportion of the dry matter, its formation would require only a small fraction of the normal metabolic resources of the leaf. Conditions that limit the capacity of the leaf to form malic acid could also affect its synthesis elsewhere in the plant. In the experiment of October–November 1960, in which plants were grown in soil without supplementary light, neither the calcium nor the malic acid increased during six weeks of sampling. The plants did not reach the flowering stage, and although the average weight of the leaves was normal (about 0.2 g.) they had a dry-matter content falling to 7%, which suggests a very low carbohydrate content. This could affect both the production of malic acid and the uptake of calcium in the roots, even if this were an energy-requiring process not involving the formation of malic acid.

The relationship between the two constituents can be treated as a simple neutralization reaction. According to this explanation malate happens to be the most readily available anion and Ca^{2+} the most readily available cation, and any tendency for one to accumulate is counterbalanced by absorption or formation of the other. The chief objection to such a mechanism is its rejection of the specificity usually found in biological reactions. It seems likely that the accumulation in the cytoplasm of large concentrations of either calcium or malate would not be tolerated, as both must have other specific biochemical functions to perform. Alternatively a mechanism may exist by which malic acid is accumulated in the vacuole, comparable with the inwardly directed chloride pump shown by MacRobbie & Dainty (1958) to be present in the tonoplast of large algal cells, and calcium is transported with malic acid. No direct evidence for

accumulation of malic acid in the vacuole seems to exist, but Laties (1961) has mentioned the existence of more than one pool of organic acids in slices of potato tubers. Specific association of calcium with malic acid during its accumulation in the vacuole would not preclude the possibility that in conditions such as the experiment with the high K/Ca ratio referred to above, where there is an induced deficiency of calcium, some calcium could leak back into the cytoplasm and be replaced by another ion, such as K^+ .

Relationship between potassium and citric acid

Although K^+ is often the major cation in fruits, and a close relationship has been shown to exist between it and the organic acids, in particular the citric acid content (cf. Bradley, 1957), there is little evidence for an association between potassium and citric acid in green leaves. Cooil (1948) found that citric acid was higher in leaves taken from guayule plants grown in nutrient solution containing a high concentration of potassium.

In the present experiments there was usually little change in the amounts of potassium and citric acid in the leaves. Young expanding leaves often had a high concentration of potassium which fell as the leaves matured, but the amount per leaf remained the same or even increased a little because this fall coincided with an increase in leaf weight. The fact that potassium and citric acid hardly changed could suggest some relationship between them, but the absolute amounts present varied considerably. In some experiments they were almost equivalent. In soil-grown plants (September 1961), for example, citric acid varied from 18 to 29 μ equiv./leaf and potassium from 20 to 27 μ equiv. In the plants grown in winter without supplementary light (October–November 1960) citric acid varied from 6 to 11 μ equiv. and potassium from 20 to 34 μ equiv., and in plants grown in nutrient solution with NO_3^- ions as the source of nitrogen (July 1961), citric acid and potassium gradually increased in amount (from 2 to 19 μ equiv. and from 7 to 28 μ equiv. respectively), the increase here being approximately equivalent.

Rasmussen & Smith (1961) found no correlation between potassium and citric acid in citrus leaves, but rather between potassium and oxalic acid. Analogous results were obtained in our experiment in which plants were grown in nutrient solution containing a high K/Ca ratio. Here the higher amount of potassium present in the leaves was not correlated with extra citric acid but with an excess of malic acid over calcium. These results suggest that citric acid is usually present in mustard leaves in an approximately constant amount, and is not affected by excess of potassium. Rasmussen & Smith (1961) showed that the oxalic acid in citrus

leaves was present as calcium oxalate and they considered that calcium was released from some other mechanism or fraction to neutralize the oxalic acid formed in response to the presence of excess of potassium.

Iron-deficient leaves

Several workers have found that chlorotic leaves contain a somewhat higher K/Ca ratio than green leaves (DeKock *et al.* 1960). Iron-deficient leaves also contain more citric acid and less malic acid than green leaves (Iljin, 1951; DeKock & Morrison, 1958).

A direct comparison between previous results and those reported here cannot be made. The results of DeKock *et al.* (1960), for example, are analyses of all the leaves from mature plants and are expressed as percentages of the total ash. Iljin (1951) studied other species, mainly trees, suffering from lime-induced chlorosis and measured only the constituents in the sap. In our present experiments only the oldest leaves from mustard plants were sampled, and although the results allow the calculation of concentration per unit fresh or dry weight, or amount per leaf, the yields of ash were not recorded.

DeKock (1958) suggested that the abnormal metabolism of iron-deficient leaves is due to a depression of the activity of aconitase when the supply of ferrous iron is low. He considered that this causes an accumulation of citric acid and a depression of malic acid formation in the leaves. The ratios of citric acid to malic acid and of potassium to calcium are assumed to be linked metabolically; hence the rise in citric acid/malic acid ratio in iron-deficient leaves should be accompanied by a rise in K/Ca ratio.

The results in Tables 1 and 2 are comparable with those of DeKock *et al.* (1960) except for the method of sampling, and agree with the hypothesis at least as far as the effect of iron-deficiency on the organic acids is concerned; the concentration of malic acid is depressed in all three experiments, and that of citric acid raised in two out of three. Alteration of the K/Ca ratio of the nutrient solution had no effect on the concentration of citric acid, but at the highest value of K/Ca the malic acid was depressed, giving a higher citric acid/malic acid ratio.

The experiments described in Fig. 1 and Table 4, in which recovery from iron-deficiency was studied, agree with the hypothesis only in the effect on the organic acids. Thus during the period of recovery the rate of accumulation of malic acid is greater and that of citric acid less than in the iron-deficient controls (Fig. 1). The citric acid/malic acid ratio thus decreases as the leaves recover. However, the concentration of potassium changes little and the

rate of accumulation of calcium is less than in the iron-deficient controls, so that at the end the K/Ca ratio is higher in the green leaves than in the controls. Under these conditions the main effects of giving iron are to depress the uptake of calcium and increase the formation of malic acid. This would appear to argue against the close connexion between the two constituents discussed above, until it is seen that as a consequence of these effects calcium and malic acid reach the position of near equivalence that is typical of normal leaves.

In all the plants, iron-deficient or green, the changes in malic acid, citric acid and calcium are great and far exceed the differences between treatments of the plants growing on the same basic nutrient solution in the experiments of DeKock *et al.* (1960). The effect of iron on the K/Ca ratio in these conditions seems to be determined by two factors: (1) the potassium concentration is not affected by the concentration of iron in this nutrient solution (DeKock *et al.* 1960); (2) the concentration of calcium tends to follow that of malic acid and to reach near-equivalence with it in normal leaves. DeKock *et al.* (1960) showed that as the K/Ca ratio was increased there was a rise in leaf potassium (as percentage ash) and a fall in leaf calcium. The latter is probably responsible for the fall in malic acid (Table 2). The failure of the concentration of citric acid to rise with the increase in potassium in these conditions indicates that there is no simple metabolic connexion between them.

Discrepancy between major cations and anions

In iron-deficient leaves there was no approximate equivalence between the major cations and organic acids. The main difference was an excess of calcium over malic acid (about 8–12 μ equiv./leaf). There was no evidence that other organic acids were present in large amounts in these leaves; for instance, the concentration of ascorbic acid was similar in iron-deficient and normal leaves and did not exceed 2 μ equiv. Iron-deficient leaves contain and maintain quite high amounts of nitrate (about 5–7 μ equiv./leaf), but this was not sufficient to account for the whole of the difference between cations and organic acids. In one experiment (February 1962) inorganic phosphate was measured and in iron-deficient leaves was about 2.5 μ equiv./leaf, slightly higher than in green leaves (about 1.5 μ equiv.) but too small to be important in the cation-anion balance. Other inorganic ions, e.g. Cl^- and SO_4^{2-} , are present in plants and may be at a higher concentration in iron-deficient leaves. Another possible explanation is that these leaves contain a larger amount of structurally bound calcium, which may be released as the leaves turn green and used to balance the malic acid that is formed at a greater rate under these conditions.

Another experiment in which there was a marked difference between cations and organic acids was that in which plants were grown in soil without supplementary light (October–November 1960). The amount of potassium increased during the first 9 days until it was about three times that of citric acid (about 10 μ equiv./leaf). Plants grown under normal conditions do not usually accumulate potassium, although its concentration is often high in young expanding leaves. The concentration falls as the leaves expand and the calcium increases, but in this experiment the calcium did not increase. We found in subsequent experiments that plants growing under these conditions, like those deficient in iron, accumulate nitrate (up to 25 μ equiv./leaf).

Yellowing leaves

When the leaves turn yellow the concentration of malic acid falls and that of citric acid increases, but their sum changes very little; it is therefore possible that malic acid is being converted into citric acid. Pucher & Vickery (1949) showed that when detached tobacco leaves were cultured in water in the dark ('starving' leaves) malic acid decreased considerably and a corresponding amount of citric acid was formed. The yellowing mustard leaves might be regarded as 'starving' leaves if it could be shown that there was no exchange of substances between them and the rest of the plant. Samples taken during the period of yellowing were rather variable, but there was a tendency for calcium and potassium to decrease; so it must be accepted that some movement takes place, but on a scale much smaller than the changes in acid concentrations.

The extent to which the changes in acid concentrations are attributable to a breakdown in cellular organization cannot be judged from present knowledge about their metabolic connexions. In the leaves of celandine there was a fall in the concentration of malic acid, but no corresponding rise in that of citric acid. The plant is, however, storing up reserves for the next season's growth.

An indication that the yellowing leaf may still have high metabolic organization is the contrast between our observations and those of Biddulph *et al.* (1959), who found that calcium applied to leaves was quickly transported away from them after their exposure to an atmosphere of diethyl ether.

SUMMARY

1. Mustard plants (*Sinapis alba*) have been grown in soil and on various nutrient solutions. Measurements were made of the major leaf anions, malate and citrate (and occasionally also of ascorbate, nitrate and phosphate), and of certain cations (sodium, potassium, calcium and magnesium).

2. While green the leaves accumulated malic acid and calcium in equivalent amounts under most of the conditions studied. The content of citric acid, and of potassium, sodium and magnesium, changed relatively little.

3. The content of malic acid was lower than that of calcium in iron-deficiency and in conditions of poor illumination. It was depressed by a high potassium/calcium ratio in the nutrient solution, but not to the same extent as the calcium content. The content of citric acid was increased by iron-deficiency, but not much affected by other variations in conditions.

4. Usually the sum of the content of malic acid and citric acid balanced the sum of the principal cations, calcium and potassium, but when the malic acid content was depressed a discrepancy existed that could be only partly explained by accumulation of nitrate.

5. When the leaves turned yellow the content of malic acid fell and that of citric acid rose correspondingly until it became the major component. There were no consistent changes in cation content.

6. The results are discussed with special reference to the significance of the potassium/calcium and citric acid/malic acid ratios in normal and deficient leaves.

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The Aerobic Inhibition of Glycolysis Associated with Brain Mitochondria

By R. BALÁZS

Neuropsychiatric Research Unit, Medical Research Council, Woodmansterne Road, Carshalton, Surrey

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In recent years several attempts have been made to clarify the mechanism of the aerobic control of glycolysis by using cell-free preparations (Terner, 1954; Graffi & Schneider, 1956; Aisenberg, Reinafarje & Potter, 1957; Aisenberg & Potter, 1957; Von Korff & Twedt, 1957; Tiedemann & Born, 1958; Sisakian & Pinus, 1958; Von Korff, 1959; Gatt & Racker, 1959; Cremer, 1960; Schwartz & Lee, 1960; Tiedemann & Born, 1960). Only in some of these studies was evidence obtained of an interaction between respiration and glycolysis, as implied by the so-called 'Pasteur effect'.

In previous work from this Laboratory it was confirmed that about 10% of the glycolytic activity of whole-brain homogenates prepared with a blender is associated with the mitochondrial fraction obtained by the usual differential-centrifuging procedures. The aerobic inhibition and the site of inhibition of lactate formation in the glycolytic chain were also studied with this preparation (Balázs & Lagnado, 1959; Balázs, 1959).

In this paper results are given of an investigation of the interaction of glycolysis with respiration; experiments on the effects of respiratory inhibitors and uncouplers of oxidative phosphorylation are also described. It is concluded that more than one mechanism is operative in the aerobic control of glycolysis associated with brain-mitochondrial preparations. One mechanism involves a specific aerobic inhibition of glyceraldehyde phosphate dehydrogenase; another mechanism appears to be closely connected with the processes of oxidative phosphorylation. Part of this investigation has been published (Balázs & Richter, 1960a).

EXPERIMENTAL

Abbreviation. BAL, 2,3-Dimercaptopropanol.

Preparation of brain-mitochondrial fraction of rat brain. The method previously described (Balázs, 1959; Balázs & Lagnado, 1959) was followed. The two hemispheres were homogenized in a NIRD blender (Nelco Ltd., Shalford, Surrey), in 2 vol. of 0.25 M-sucrose containing 0.025 M-nicotinamide, for 45 sec. at 10 000 rev./min. The sucrose solution was then added to make a 10% homogenate and the suspension was homogenized for a further 30 sec. at the same speed. The nuclear fraction was sedimented at 1500g for 10 min. in a MSE 20 refrigerated centrifuge. The supernatant was centrifuged again at the same speed and time. The mitochondrial fraction was collected and washed once at 18 000g for 10 min. The final pellet was suspended in the nicotinamide-containing sucrose solution.

Media. The basic medium was prepared freshly from stock solutions and contained 6 mM-MgCl₂, 2 mM-EDTA (Na salt, pH 7.2), 25 mM-nicotinamide, 10 mM-KH₂PO₄, 20 mM-tris (adjusted to pH 7.2 with HCl) and sufficient KCl to bring the osmolarity to 0.3 after the addition of all the constituents to the final incubation medium. The pH of the basic medium was adjusted to 7.2 after the addition of the substrates. The pH of the medium was maintained generally by prior adjustment of the pH of the additives when necessary. A few compounds were used in alkaline solutions: sodium 5-ethylamylbarbiturate (Amytal), 2-n-heptyl-4-hydroxyquinoline N-oxide and dicoumarol. In these cases the pH of the basic medium was adjusted to 7.2 after the addition of these substances. The final volume in the main compartment of the Warburg flask in the absence and presence of the cofactors (2 mM-ADP and 0.33 mM-NAD⁺) was 1.5 and 2.0 ml. respectively.

Special chemicals and reagents. Sodium Amytal was a gift from Eli Lilly and Co. Ltd. Dicoumarol was a gift from Organon Laboratories Ltd. 2-n-Heptyl-4-hydroxyquin-