The Effect of Illumination on the Malic Acid Content and Anion/Cation Balance of Mustard Leaves (*Sinapis alba*)

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1. Mustard plants have been grown under conditions in which the length of artificial day could be controlled. 2. Leaf samples were analysed for malic acid and citric acid, and for a number of inorganic anions and cations. A simple method is described by which sap was obtained from 0.5 g. samples of leaves. 3. In days of 16 hr. or more, malic acid was accumulated; the chief cation accumulated was calcium. 4. When the day-length was reduced the malic acid content decreased considerably but the calcium content remained the same. There was little change in the pH value of the sap, the balance of anions and cations having been maintained mainly by increases in citrate and nitrate contents. Analyses of the whole leaf still showed some deficiency in anion after sodium, potassium, calcium, magnesium, nitrate, sulphate, inorganic phosphate, chloride, malate and citrate had been accounted for. 5. Analyses at shorter intervals revealed a large diurnal variation in malic acid content, which increased during the first 5–6 hr. of the light period, and fell during darkness. 6. The significance of these findings is discussed, and it is suggested that malic acid accumulation is a by-product of photosynthesis, calcium being taken up irreversibly to maintain anion/cation balance, and hence creating a continuing need for anions to balance it.

Previous experiments (Palmer, DeKock & Bacon, 1963) have shown that leaves of mustard plants growing in normal conditions accumulate malic acid and calcium in approximately equivalent amounts. Fully expanded leaves were also shown to have a rough balance between major cations and anions, and a low nitrate content. Leaves growing in low light-intensity, however, showed a decreased accumulation of malic acid, an increased nitrate content, and a discrepancy between the sums of the major cations and anions which was apparent throughout the experimental period. To study further the effect of illumination on these constituents plants were grown in conditions in which the length of the light-period could be altered.

MATERIALS AND METHODS

Growth of plants. In all but one experiment plants were grown in nutrient solution as described by DeKock, Hall & McDonald (1960). The nutrient solution used, except where stated to the contrary, had a K/Ca ratio 3:10 and a phosphate/nitrate ratio 2:10 (K_2P_2 of De Kock *et al.* 1960). Iron was added as ferric EDTA at a concentration of 5 p.p.m. Plants were grown either in a growth room or a growth cabinet.

Growth room. This contained no windows and measured

approx. 10 ft. × 8 ft. 6 in. × 10 ft. high. Within it a metal framework, 8 ft. × 3 ft. × 4 ft. high, carried at its top eight 8 ft. warm-white 125 w fluorescent tubes mounted in pairs with reflectors and at the sides a total of eight 100 w tungsten lamps. The light-intensity achieved was 1000–1250 ft.-candles at a distance of 45 cm. from the tubes. Some control of temperature and a circulation of air was achieved by means of a small air-conditioning unit (Pacific model 25; Tempair Ltd., Maidstone, Kent) set high in the wall at one side of the room. The temperature during the light-period was 4–6° higher than that during the dark-period. There was no control of humidity, which in the light-period varied from about 40% when the plants were very small to about 55% when the plants were flowering. The crocks containing the plants were placed in a double row, standing on planks with spaces between them to allow circulation of air.

Growth cabinet. This was built by G. W. Webster (Caledonian Crescent, Glasgow, W.2) to the specifications of Dr P. C. DeKock. The internal dimensions were 4 ft. × 2 ft. × 3 ft. high. It was provided with a temperature control and a humidity control. There was no change in temperature between the light-period and the dark-period. The light-intensity was approx. 1250 ft.-candles 45 cm. from the bank of twelve 5 ft. warm-white 80 w fluorescent tubes. Air was circulated through the chamber, entering at the bottom and leaving at the top.

Analytical methods. Plants were sampled and the malic acid, citric acid, nitrate, phosphate, potassium, calcium, sodium and magnesium contents estimated as described by Palmer *et al.* (1963).

Sulphate was estimated by the method of Butters &

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Chenery (1959) on extracts of dried samples (0.5g.): the dried material was ground to a powder, 20ml. of 5N-HCl added, the mixture evaporated to dryness on a hot plate, and the residue dissolved in hot water and filtered. Chloride was estimated on boiling-water extracts of powdered dried material by the potentiometric method of Sanderson (1952). Carbohydrate was measured on extracts deproteinized with 5% (w/v) trichloroacetic acid, by the anthrone procedure of Fairbairn (1953); a reaction time of 12min. was used and the unknowns were compared with glucose standards. Uronic acids were determined by the method of Tracey (1948).

Extraction of sap. Sap was extracted from samples (0.5g.) of chopped leaves after a preliminary treatment with ether. The sample was transferred to a small weighed screw-topped glass bottle standing in an atmosphere of ether. After 10min. the bottle was screwed on to the top of a Hemmings filter (A. Gallenkamp and Co. Ltd.), which was covered with a circle of Whatman no. 42 paper. Another weighed bottle was screwed on to the bottom of the filter and the system centrifuged for 10min. at 2500 g. A clear pale-yellow sap was recovered in the bottom bottle. Both bottles were then weighed and the weights of sap and residue obtained. Between 50 and 65% of the original weight of the sample was recovered as sap. The pH of the sap was measured by using a micro-electrode assembly with a general-purpose pH-meter (W. G. Pye and Co. Ltd., Cambridge), after which the volume was adjusted to a convenient value with water and samples were taken for the analysis of various constituents. The leaf residue was dried and used for the estimation of cations as described by Palmer *et al.* (1963).

RESULTS

The results given here are drawn from experiments in which plants were grown from seed on 24 separate occasions. As in our previous work practically all analyses were made on the second true leaf; exceptions are noted in the text. The term 'day-length' is used throughout for convenience, to indicate the number of hours in each 24 during which the plants were illuminated; it cannot be assumed that exposure to natural days of the same length would necessarily produce identical responses.

Changes in malic acid content with illumination. When mustard plants were grown in the growth room with illumination during 18hr. of each day the changes in malic acid, citric acid, calcium and potassium contents of the leaves resembled those found earlier in the greenhouse (Palmer *et al.* 1963). Fig. 1 shows that during a period of observation of 14 days the malic acid concentration rose from 108 to 320 m-equiv./kg. fresh wt., and the calcium concentration from 140 to 350 m-equiv.; potassium concentration fell slowly from 135 m-equiv. to 82 m-equiv. In this experiment citric acid was not measured but in similar experiments it changed little.

The earliest experiments in the growth room were carried out with plants grown in John Innes no. 2 compost. The leaves of these plants accumulated

Fig. 1. Accumulation of malic acid and calcium in 18hr. days. Plants grown in the growth rooms in K_2P_2 nutrient (June 1963) were sampled at the beginning of the light-period on the days indicated. ○, Uronic acids; Δ, malic acid; ●, potassium; ▲, calcium; ■, nitrate.

Fig. 2. Effect of reduction of day-length on various constituents of leaves. Plants were grown on K_2P_2 nutrient in the growth room in 18hr. days. Sampling was begun 13 days after sowing and the day-length was reduced to 6hr. on the tenth day of observation. This is Expt. 3 of Table 2. ○, Citric acid; Δ, malic acid; □, sulphate; ●, potassium; ▲, calcium; ■, nitrate.

more potassium and less calcium and malic acid than those grown on the nutrient solution (K_2P_2 of DeKock *et al.* 1960). By transfers between the growth room, in which the day-length had been reduced to 6hr., and the greenhouse in late July (day-length about 16hr.) it could be shown that the

Table 1. *Change in leaf composition in the early part of the light-period*

In Expts. 1 and 2 plants were grown in the growth room (November–December 1963), in Expt. 3 in the growth cabinet at 18° and 60% humidity (February 1964). Day-length was 18hr. Throughout the nutrient was K_2P_2 . Methods of sampling and analysis are described in Palmer *et al.* (1963) and in the text. Sample A was taken at the beginning of the light-period, sample B 6hr. later. —, Not measured.

Expt. no.	Time after sowing (days)	Average fresh wt. of leaf (g.)	Leaf constituents (m-equiv./kg. fresh wt.)											
			Malic acid		Citric acid		Calcium		Potassium		Nitrate		Sulphate	
			A	B	A	B	A	B	A	B	A	B	A	B
1	{ 15	0.05	50	107	55	40	—	—	—	—	64	56	—	—
	{ 22	0.20	129	170	78	60	225	206	114	123	47	73	—	—
2	{ 14	0.07	58	108	52	40	148	143	117	146	61	47	33	33
	{ 20	0.20	202	254	62	65	210	236	160	136	42	20	22	24
3	{ 17	0.12	71	104	64	55	123	134	132	149	53	39	30	24
	{ 30	0.41	227	248	77	66	230	268	115	97	18	19	5	5

malic acid content responded by rising when the day-length was increased and falling when it was decreased. A similar effect was produced with plants growing in solution culture in the growth room by reducing the day-length when malic acid accumulation had proceeded for 1–2 weeks. Fig. 2 shows a typical experiment, in which the malic acid concentration fell from 285 to 206 and 139 m-equiv./kg. fresh wt. respectively 3 and 5 days after the day-length had been reduced from 18 to 6hr. Under growth-room conditions a rather long day was needed to promote malic acid accumulation; thus in one experiment malic acid concentration rose from 69 to 130 m-equiv./kg. fresh wt. during 13 days of 14hr. duration and subsequently to 275 m-equiv. in 8 days when the day-length was increased to 18hr.

A closer examination of the response to illumination showed that it had a diurnal character. The malic acid content of leaves sampled at 3 p.m. was always higher than in those sampled at 9 a.m. (shortly after the lights were switched on) (Table 1). A more detailed analysis of the phenomenon is given in Fig. 3. This and other experiments showed that the malic acid content rose and fell during each light-period. The length of the light-period made it difficult to follow the whole time-course as a routine, but Fig. 4 shows the result of one experiment lasting for 24hr.; it suggests that most of the increase occurs during the first 5hr. in light. If this is always so it probably explains why in Expt. 3 of Table 1 in Palmer *et al.* (1963) a statistical examination failed to show any difference between malic acid contents of samples taken in the mornings and the afternoons, because under winter conditions these were taken 4hr. and 9hr. after the supplementary lighting was switched on.

The increase also occurred in plants exposed to a 6hr. day in the growth room, suggesting that the failure of these plants to accumulate malic acid is due to the subsequent fall being as great as the in-

Fig. 3. Fluctuation in concentrations of malic acid and citric acid during a succession of 18hr. days. Sampling was begun (December 1963) 14 days after sowing. Experimentally determined points are joined to show the trends in composition, not to indicate the actual course of variations. ○, Citric acid; △, malic acid; ■, nitrate.

crease. For example, when plants were grown in the growth cabinet, with constant temperature (18°) and humidity (60%), samples taken in 18hr. days showed rises during the first 5hr. of light of 71 to 104 and 130 to 184 m-equiv./kg. fresh wt. respectively on the 17th and 24th day from sowing. On the 29th day malic acid content rose from 223 to 244 m-equiv./kg. in 5hr. and the lights were then turned off for 18hr. The malic acid content then rose from 227 to 248 m-equiv./kg. in a 6hr. light-period, and after a further 18hr. dark-period rose from 202 to 230 m-equiv./kg. in 6hr. light.

In another experiment in the growth cabinet (temperature 18°; humidity 60%) changes during the last 5hr. of several 18hr. light-periods were examined. Judged by the content at the end of each dark-period these plants were augmenting their calcium content very little (increase from 163 to 208 m-equiv./kg. fresh wt.), and malic acid not at all, during the 16 days of observation. The malic acid

Fig. 4. Changes in leaf composition during a single day, with 18 hr. illumination. Sampling was begun (March 1964) in the dark-period 13 days after sowing, 9 days after transfer to K_2P_2 nutrient. The plants had received 18 hr. days previously. The results are expressed in terms of $\mu\text{equiv./leaf}$ because some increase in fresh weight took place during the experimental period. The comparatively young shape growth is indicated by the high potassium content (cf. Fig. 1). ○, Citric acid; △, malic acid; ●, potassium; ▲, calcium; ■, total water-soluble carbohydrate.

content fell from 147 to 112, 147 to 94, 138 to 94 and 121 to 112 m-equiv./kg. fresh wt. in the last 5 hr. of the four days examined (19, 26, 32 and 35 days from sowing).

Changes in citric acid concentration. In the long term citric acid concentration changed little in plants receiving an 18 hr. day. However, the diurnal changes in malic acid and the fall in malic acid concentration induced by a reduction of day-length to 6 hr. were usually accompanied by opposing changes in citric acid concentration (see e.g. Fig. 1 and Table 1). The exception was the fall in malic acid content at the end of the light-period (see above), which was accompanied by smaller falls in citric acid concentration (66 to 58, 60 to 51, 73 to 65 and 71 to 58 m-equiv./kg. fresh wt. respectively).

Changes in calcium and other cations. Table 2 shows the results of three experiments in which the composition of leaves was followed for about 3 weeks. In the middle of this period the day-length was reduced from 18 to 6 hr.; at this point the concentrations of malic acid and calcium were almost equivalent. The calcium content continued to increase slightly after the change to 6 hr. days. In fact, no situation was ever found in which the calcium content decreased.

The other major cation present was potassium; in plants grown in John Innes no. 2 compost its concentration exceeded that of calcium in the first samples taken, but did not increase significantly during the period of observation (4 weeks). In solution culture the potassium concentration was close to that of calcium in the earliest samples taken but tended to fall thereafter (cf. Table 2). The pattern of growth of each leaf was such that the total potassium usually increased considerably during the first week of sampling, but then became almost constant at a figure of about 20 $\mu\text{equiv./leaf}$. The only exceptions to this were seen when the day-length was reduced to 6 hr. (Table 2); in Expts. 1 and 2 the potassium fell to 11 and 12 $\mu\text{equiv./leaf}$ respectively from maxima of 20, but in Expt. 3 the fall was less marked, to 16 from a maximum of 25.

The concentrations of sodium and magnesium were smaller and showed little change with time in the 20 experiments analysed. Sodium concentrations lay within the range 15–35 m-equiv./kg. fresh wt., and did not usually vary by more than 10 m-equiv. in a single experiment. When a trend was discernible it was in an upward direction and seemed to be associated with slower-growing plants. Magnesium concentrations were more variable, in the range 20–45 m-equiv./kg. fresh wt., and any upward trend was very slight. It may be concluded from this that contamination of the surfaces of the leaves contributed little to the accumulation of calcium and other cations.

Anion/cation balance. Since the reduction of day-length from 18 to 6 hr. did not produce any considerable changes in cation content the disappearance of a large part of the malic acid should have led either to a rise in pH or to the introduction of compensatory amounts of other anions. Samples of ether sap were obtained by the method described and showed no significant changes in pH when illumination was altered. Thus in Expt. 1 of Table 1, with 18 hr. days throughout, the values measured at 2-day intervals were: 5.8, 5.5, 5.6, 5.6, 5.5, 5.6, 5.6 and 5.7; and in Expt. 2 of Table 2, 5.7 on the day the light-period was reduced to 6 hr., and 5.7, 5.6, 5.5, 5.5, 5.6 thereafter. Numerous other measurements gave no values above 5.9 or below 5.4.

Changes in other anions were therefore sought. Measurements of sulphate were rather more variable

Table 2. *Effect of shortening the day on leaf constituents*

Plants were grown in K_2P_2 nutrient, in the growth room, in January (Expt. 1), March (Expt. 2) and October 1963 (Expt. 3). Tungsten lamps were used in Expt. 3, but not in the others. In each experiment after a preliminary period (17, 14 and 13 days from sowing respectively) an initial sample was taken. Exposure to 18hr. days was continued for a period (6, 7 and 10 days respectively), an intermediate sample was taken and the day-length reduced to 6hr. After a further period (15, 11 and 7 days respectively) a final sample was taken. In Expt. 2 sap samples were also analysed; these weighed 45% (initial), 42% (intermediate) and 57% (final) of the leaf samples from which they were extracted, and the values given express the contribution of the sap sample to the composition of the whole leaf.

Constituent	Expt. no.	Anions (m-equiv./kg. fresh wt.)			Constituent	Expt. no.	Cations (m-equiv./kg. fresh wt.)		
		Initial sample	Intermediate sample	Final sample			Initial sample	Intermediate sample	Final sample
Malic acid	1	163	213	71	Calcium	1	210	238	295
	2	121	252	73		2	176	250	280
	2 (sap)	(90)	(129)	(49)		2 (sap)	(72)	(100)	(125)
	3	66	285	133		3	171	304	329
Citric acid	1	59	52	143	Potassium	1	119	111	60
	2	59	71	92		2	138	113	75
	2 (sap)	(38)	(37)	(52)		2 (sap)	(41)	(28)	(38)
	3	54	81	172		3	121	129	89
Nitrate	1	22	22	59	Total	1	329	349	355
	2	33	13	83		2	314	363	355
	2 (sap)	(17)	(8)	(54)		2 (sap)	(113)	(128)	(163)
	3	49	39	70		3	292	433	418
Sulphate	3	45	9	32					
Total	1	244	251	273					
	2	213	336	248					
	2 (sap)	(145)	(174)	(155)					
	3	214	414	411					

than those of cations, suggesting that the method was not altogether reliable. Most of the values obtained were in the range 15–25m-equiv./kg. fresh wt. and there was no evidence for any considerable increase when the day-length was reduced. In one experiment in which K_2P_2 nutrient was used at half its usual concentration there was an upward trend of sulphate concentration amounting to about 10m-equiv./kg. fresh wt. during 10 days of 6hr. illumination. Determinations of inorganic phosphate and of chloride were made in a few experiments. The values obtained were small (5–10m-equiv./kg. fresh wt. for phosphate and 10–15m-equiv. for chloride) and neither showed an obvious trend with age of leaves.

The most likely compensatory anion (cf. Palmer *et al.* 1963) seemed to be nitrate, which was determined in whole leaf samples in all the experiments under review. Since sodium and magnesium (total 35–80m-equiv./kg. fresh wt.) roughly balanced sulphate, phosphate and chloride (total 30–50m-equiv./kg. fresh wt.) an approximate idea of the anion/cation balance could be obtained by comparisons of malic acid, citric acid and nitric acid against calcium and potassium. In the whole series of experiments in the growth room and growth

cabinet, and also in earlier experiments in the greenhouse there was an excess of cations, calculated in this way, in the youngest leaf samples (cf. Table 3). As the leaves became older the discrepancy was reduced and in some cases the anion total came to exceed the cation total, but some excess of anions like malate and citrate would be expected at pH 5.6, and if it is assumed that one-quarter of the malic acid is un-ionized at this pH the true discrepancy is seen to change little with age.

Similar analyses and comparisons on sap samples showed a slight excess of anions throughout the sampling period (Table 2), suggesting that the deficiency of anions shown by whole-leaf analyses was due to some constituent of the leaf residue. The same was also true of leaf samples taken after the reduction of day-length in Expt. 2 of Table 1; in both this experiment and Expt. 1 there was a large discrepancy in the final analyses of whole leaves, but this did not seem always to be the case; thus in Expt. 3, although there was the usual deficiency in the earliest samples, the fall in malic acid content on transfer to 6hr. days was balanced by the combination of a rise in nitrate content similar to that in the other two experiments and a particularly large rise in citric acid content.

Table 3. *Anion/cation balance in whole leaf and in sap during growth in 18 hr. days*

Plants were grown in K_2P_2 nutrient in March 1963 (Expt. 1) and September 1963 (Expt. 2), and in a 1:1 dilution of this nutrient in February 1964 (Expt. 3). The experimental period began 13, 20 and 16 days respectively after sowing, and lasted for 16, 17 and 13 days in the three experiments. Analyses were made at 2–3-day intervals, but only the values for initial and final samples are given, the range of variation being indicated if it lay outside these limits. The values for sap analyses are not expressed as actual concentration but as the contribution made by the sample to the composition of the leaf material from which it was extracted; in Expt. 1 the sap samples were 48% (initial) and 55% (final), and in Expt. 2 56% (initial) and 59% (final) of the weight of the leaf samples from which they were derived. —, Not measured.

Constituent	Expt. no.	Composition of whole leaf (m-equiv./kg. fresh wt.)			Contents of sap sample of whole leaf (m-equiv./kg. fresh wt.)		
		Initial	Final	Range	Initial	Final	Range
Malic acid	1	73	310		49	183	
	2	123	332		112	214	
	3	129	297		—	—	
Calcium	1	136	270		38	133	
	2	190	278		84	147	
	3	141	306		—	—	
Citric acid	1	41	50	(41–57)	29	29	(28–36)
	2	58	61	(53–67)	31	35	(29–41)
	3	60	109		—	—	
Potassium	1	133	107		51	51	(39–51)
	2	140	78		79	41	
	3	138	108		—	—	
Nitrate	1	45	17	(49–10)	27	9	(34–9)
	2	38	16	(47–16)	27	13	(33–9)
	3	12	1		—	—	
Sulphate	2	44	14		—	—	
	3	21	12		—	—	
Total cations	1	269	377	(393)	89	184	
	2	330	356		163	188	
	3	279	414		—	—	
Total anions	1	159	377	(382)	105	221	(227)
	2	263	423		170	262	
	3	224	419		—	—	

Cell walls were prepared from leaves (position 3) by the method of Bean & Ordin (1961); duplicate samples (0.5 g.) were used and the homogenate was centrifuged through sucrose solutions of concentration 1.0M, 0.75M and 0.5M. The final cell-wall fraction, which was still slightly green, contained only 9% of the leaf calcium.

Since the rise in malic acid content in the first few hours of a light-period was often as great as its progressive fall during a succession of 6 hr. days it seemed that a temporary daily excess of anions might be created. Analyses were therefore made of sap samples taken on four occasions 0 and 6 hr. from the beginning of an 18 hr. light-period. At 0 hr. anions were equal to or slightly exceeded cations, but at 6 hr. there was a substantial excess of anion; the pH changed little, falling by about 0.1 unit in three cases, and rising by 0.2 unit in the fourth (Table 4). Changes in sodium, sulphate and inorganic phosphate were negligible.

Mr A. H. Knight kindly provided analyses of the total uronic acid content of dried leaf samples from a few experiments. Some of these are plotted on Fig. 1; similar values were obtained in other experiments. From their experience of similar materials Mr Knight and Dr W. M. Crooke suggest that only one-third to one-half of the uronide would contribute free carboxyl groups.

Changes of other leaf constituents with illumination. A few analyses were made of other constituents. Expected results were a rise in soluble sugars during the first 6 hr. of the light-period in plants accumulating malic acid, and a decrease in Kjeldahl nitrogen concentration as the leaves aged.

Analyses of free amino acids showed a decrease in glutamic acid content, almost exactly balanced by an increase in glutamine, and also a distinct increase in serine, during the first 6 hr. of the light-period. These changes were about 1–2 m-equiv./kg. fresh wt.

Table 4. *Changes in sap composition with illumination*

Plants sown in January 1964 were grown in K_2P_2 nutrient in 18hr. days. Wet- and dry-bulb temperatures were followed daily, dry-bulb temperatures being 20–20.5° at 8.30a.m. (dark) and 22–23.5° at 5p.m. (after 8hr. light), humidity about 45–55% in the dark and 40–50% in the light. Samples were taken at the beginning of the light-period (9a.m.) and 6hr. later, and the sap was analysed. Results are expressed as the contribution of sap constituent to the composition of the whole leaf sample, in m-equiv./kg. fresh wt.

Sample			Cationic constituents			pH of sap	Anionic constituents			
Time from sowing (days)	Exposure to light (hr.)	Sap as % of whole leaf	Potassium (a)	Calcium (b)	Total (a+b)		Total (c+d+e)	Malic acid (c)	Citric acid (d)	Nitrate (e)
14	0	55	55	58	113	5.6	108	38	34	36
	6	48	51	50	101	5.8	112	67	16	29
21	0	55	49	101	150	5.4	189	134	40	15
	6	57	52	104	156	5.3	218	161	36	21
22	0	56	58	93	151	5.8	196	140	40	16
	6	55	62	132	194	5.7	243	192	43	8
24	0	58	58	145	203	5.8	217	161	36	20
	6	62	71	132	203	5.7	243	191	30	22

DISCUSSION

Although these experiments were begun in order to investigate the accumulation of malic acid and calcium over relatively long periods of time the results have drawn attention to the large changes that can occur within a few hours. Long-term accumulation of malic acid is seen to be the result of diurnal cycles, in which gains always exceed losses. Accumulation of calcium, on the other hand, would seem to be continuous, although it must be admitted that the methods employed would not distinguish between this and a stepwise process.

The pH of ether sap samples showed little or no change as a result of the daily increases in malic acid, so it must be concluded that compensatory changes in cations and other anions occurred. Of these the most striking was the decrease in nitrate.

A smaller decrease in citrate was noticed in some experiments, and is reminiscent of the changes seen in the yellowing mustard leaf (Palmer *et al.* 1963) and in the excised leaves of tobacco studied by Vickery and his colleagues (see e.g. Vickery & Zelitch, 1962).

There seem to be few references to studies of diurnal changes in the organic acids of non-succulent plants. Buckley (1961) measured malate, citrate and succinate in spinach leaves during a series of 24hr. periods, with the object of relating changes in organic acids to the photo-induction of flowering. His results show that malate concentration was low and falling slightly during sequences of 15–60 8hr. days; with 12hr. days it changed little, but with 16hr. days it rose considerably, especially at higher temperatures (27–34°). During the light-period malate concentration always rose, under

some conditions by 100m-equiv./kg. fresh wt., but Buckley (1961) distinguished three types of behaviour. In the first (characteristic of all plants at the beginning of treatment and retained in 8hr. days), the malate rose slowly throughout the light-period and fell throughout the dark-period. In the second (apparently associated with the flowering response), there was little change, sometimes even a fall, in malate concentration during the first 6–10hr. of light, but then a sharp rise until the period ended when a steady fall occurred in the dark. In the third, malate concentration rose from the beginning of the light-period to a maximum at 7–10hr. and then began to fall, the onset of darkness producing no immediate acceleration of the fall. In vegetative plants citrate showed changes smaller but otherwise similar to those of malate; changes in photo-induced plants were 'bewildering' (Buckley, 1961).

These results show a number of similarities to our own: (a) long-term accumulation of malate required a succession of long days; (b) all plants showed a diurnal cycle of accumulation and loss of malate; (c) under some circumstances malate concentration fell during the light-period. They illustrate the complexity of the processes by which malate is produced and consumed.

A tendency for the formation of malate to be more pronounced in the light was shown also in a series of experiments by Graham & Walker (1962). They fed ^{14}C -labelled acids to mung-bean leaves and found that more label appeared in malate in the light than in the dark. The reverse was true of citrate, and also of aspartic acid; the latter could have been a consequence of oxaloacetate formation, followed by exchange transamination. (In our experiments the total amount of aspartic acid fell slightly during the

period of illumination, and an increase in organic nitrogen consisted chiefly of a conversion of glutamic acid into glutamine.)

Graham & Walker (1962) suggested that photosynthesis kept a greater proportion of NAD in the reduced state and thus with the participation of malate dehydrogenase diverted more of the label reaching oxaloacetate into the malate pool. In the dark, metabolism would shift to a more oxidative condition and formation of oxaloacetate (aspartic acid) and citrate would be favoured. Our present observations, which show that the accumulation of malate is somehow associated with photosynthesis, and that it is freely reversible, could be explained by this hypothesis. The essence of the explanation, however, is that malate is a product of an excess of reducing power. If this is so, it is difficult to see why nitrate nutrition, which consumes some of this reducing power, should increase the accumulation of organic acids (cf. Palmer *et al.* 1963).

A more oxidative origin for malate would be through the action of malate synthetase. Suggestions that in photosynthetic systems malate originates through $C_2 + C_2$ condensation have been made by Tolbert, Clagett & Burris (1949), Schou, Benson, Bassham & Calvin (1950), Calvin & Bassham (1962), and most recently by Ranson (1965). No definitive experiment seems yet to have been performed, but the plausibility of the hypothesis is supported by a number of observations.

Glycollate and malate are both early products of photosynthesis. Glycollate formation is favoured by high light-intensities and low carbon dioxide concentration; it disappears very rapidly in the dark (Benson & Calvin, 1950). Incorporation of label from glycollate into malate is less in the dark than in the light (Schou *et al.* 1950). Glycollate fed to tobacco leaves in the dark is extensively converted into citrate, and malate could be an intermediate (Vickery & Palmer, 1956). When inhibitors of glycollate oxidation are fed to detached leaves the glycollate concentration increases considerably in the light, particularly at high light-intensities; fixation of $^{14}CO_2$ in glycollate and malate is about equal in untreated leaves, but in the presence of the inhibitor fixation in glycollate rises and that in malate falls, in each case about fivefold (Zelitch, 1959). Experiments with tomato fruit in the dark support the view that malate synthetase is operating in the conversion of glycollate into malate (Doyle, Huff & Wang, 1960), and the enzyme has been found in leaves of various plants by Yamamoto & Beevers (1960). The case for the participation of glyoxylate and glycollate in the transformations of organic acids accompanying photosynthesis has been argued in detail by Zelitch (1964).

Oxalic acid is probably formed from glycollate in photosynthetic tissues. There are therefore con-

siderable attractions in a theory that proposes a common origin for the two acids most often accumulated in large amounts in green tissues. Whichever explanation is correct, both visualize malate formation as a by-product of photosynthetic reactions, and the accumulation of calcium would consequently be a compensatory process.

Since the pH changes little when the illumination is reduced, and calcium appears never to leave the leaf (Biddulph, Cory & Biddulph, 1959), some other anions must take the place of malate. This may explain why the malate disappearing is not oxidized to carbon dioxide, but accumulated at the intermediate stage of citrate.

Although the anion/cation balance in the ether sap sample is fairly well accounted for by the substances we measured it would seem that there is an unidentified anion fraction in the leaf residue. It is difficult to believe that this can consist entirely of uronic acid residues. In the cases where uronic acid content was measured the extent of this discrepancy would allow none of the uronide to be esterified, whereas the existence of the 'acid drift' in minced leaves (Holden, 1945) and measurements of cation-exchange capacity (Knight, Crooke & Inkson, 1961) suggest that a considerable proportion must be masked in this way.

If our interpretation is correct malic acid accumulation would appear not to be essential, but merely the consequence of excessive illumination. Much of the calcium taken up by the plant would fall into the same category, and the effect of nitrate nutrition on cation uptake would be seen to have no connexion with the actual absorption process in the root system.

Note added in proof. Since this paper was submitted Hatch & Slack (1966) have shown that when leaves of sugar cane are photosynthesizing at high light-intensities radioactivity from $^{14}CO_2$ appears very rapidly in malate (about 250 times as fast as in the dark). From a study of the kinetics of incorporation they conclude that the first product of carbon dioxide fixation is a C_4 dicarboxylic acid, and suggest that label is later transferred to carbohydrate by a transcarboxylation reaction, perhaps involving ribulose 1,5-diphosphate. This would provide a much more direct explanation than ours for the connexion between illumination and malate formation.

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