

MOVEMENT OF PREVIOUSLY DEPOSITED ^{45}Ca IN SUBTERRANEAN CLOVER (*TRIFOLIUM SUBTERRANEUM* L.) BY FOLIAR INJECTIONS OF CERTAIN CATIONS

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Summary

^{45}Ca deposited in subterranean clover (cv. Dwalganup) either 9 days or 9 weeks previously showed movement following foliar injection into the plant of non-radioactive Ca^{2+} , Zn^{2+} , Mn^{2+} , or Mg^{2+} , but not water.

This movement resulted in differences in concentration or pattern of distribution or both of ^{45}Ca between individual leaflets of the same leaf sampled respectively immediately before and 24 hr after injection of the non-radioactive cations. These differences in ^{45}Ca were much greater than were found to occur between similar leaflets on uninjected plants.

The mobile ^{45}Ca detected in the leaflets came mainly from the petioles, and it is concluded that the exchange sites are non-selective.

A large proportion of the ^{45}Ca extracted by water, sodium chloride, acetic acid, and hydrochloric acid, respectively, from low-calcium plants was found to be exchangeable.

I. INTRODUCTION

Calcium has long been regarded as being very immobile in the plant. Reviews of the literature on this subject have been made by Williams (1955), Biddulph *et al.* (1958), Bollard (1960), and Zimmermann (1960). However, recent work in which radioactive calcium was used has indicated that calcium is not completely immobile within the plant.

Thus Ferrell and Johnson (1956) reported substantial movement of previously deposited ^{45}Ca into newly developed buds of western white pine; Vlasyuk and Grodzinskii (1958) found ^{45}Ca in leaves of lupins formed subsequent to transplanting to a non-radioactive medium; Kiselev (1961) noted movement of ^{45}Ca in several plant species, but not in beans, and Millikan and Hanger (1964) demonstrated the occurrence of ^{45}Ca in the new growth produced up to 9 weeks after previously calcium-deficient subterranean clover plants were transferred to non-radioactive, complete nutrient solutions.

Both Vlasyuk and Grodzinskii (1958) and Millikan and Hanger (1964) concluded that the mobile ^{45}Ca came mainly from root and hypocotyl tissues.

To obtain confirmation of the occurrence of exchangeable calcium in subterranean clover, the experiments described below were conducted. Use was made of the fact that previous work by Millikan and Hanger (1964, 1965*a*) had indicated that the patterns of distribution of ^{45}Ca in radioautographs of the three individual leaflets of any leaf of subterranean clover always appeared to be identical.

Comparisons were therefore made of both the patterns of distribution and concentrations of ^{45}Ca between individual leaflets from the same leaf sampled,

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respectively, immediately before and after the injection of non-radioactive calcium or other cations into another leaf of the plant.

II. METHOD

Subterranean clover plants (cv. Dwalganup) were grown in either normal or low-calcium nutrient solutions. The compositions of the solutions and the general procedure were as described by Millikan and Hanger (1964). Details of the ^{45}Ca injections (as $^{45}\text{CaCl}_2$) and of the non-radioactive injection treatments are given below.

For identification purposes, all leaves on each plant in experiments 2 and 3 were numbered consecutively with indian ink before sampling. All leaf injections were made by the method described by Millikan and Hanger (1965a).

All plant samples in experiments 2 and 3 were first photographed and then radioautographed (Millikan and Hanger 1964). Individual leaflets were then dried, weighed to the nearest 0.005 mg, and radioassayed. For this purpose a Berthold automatic sample-changer with associated scaler and recorder was used, and all samples were radioassayed at least twice, and many up to four times on separate days. All assays were corrected for the decay of the isotope, and were finally expressed as the mean number of counts per minute per milligram of dry matter. Details of each experiment follow.

(a) *Experiment 1*

This experiment was made to determine the magnitude of the natural variability in ^{45}Ca distribution and concentration between individual leaflets of subterranean clover leaves. For this purpose plants were grown in either normal or low-calcium solutions to which $20\ \mu\text{C}$ ^{45}Ca (containing a total of $13.4\ \mu\text{g}$ calcium) was added per pot. The normal and low-calcium solutions received a total of 4000 and $75\ \mu\text{moles}$ respectively of calcium per litre, and had specific activities of 0.0025 and $0.133\ \mu\text{C}$ per μmole calcium, respectively.

After 3 weeks the plants were transferred to non-radioactive solutions for 9 days and were then sampled and radioautographed. The leaves were numbered consecutively on the radioautographs and each leaflet was radioassayed separately for ^{45}Ca .

The mean counts obtained for the individuals in each trio of related leaflets were used to determine the mean count for the whole lamina, and the range between the counts. These values were then transformed to $\log(10 \times \text{mean value})$ and $\log(10 \times \text{range})$ respectively and were plotted to show their relationship and the limits of the probable error.

(b) *Experiment 2*

Plants were grown in non-radioactive normal or low-calcium solutions respectively. When each plant had at least five trifoliolate leaves, the second leaf was injected with a dose of $5\ \mu\text{C}$ ^{45}Ca containing a total of $3.1\ \mu\text{g}$ calcium in 0.05 ml plus $1440\ \mu\text{g}$ calcium (as CaCl_2) in 0.08 ml water plus a further 0.08 ml water. The treatment was duplicated in plants grown at each calcium level.

Sixty-one days later, one leaflet from each leaf was removed for radioautography and radioassay and each plant was immediately injected with 1440 μg calcium (as CaCl_2) in 0.08 ml water plus 0.16 ml water into a leaf different to that injected previously. Twenty-four hours later the plants were removed for radioautography and radioassay.

(c) *Experiment 3*

Subterranean clover plants were established in either normal or low-calcium solutions. One week later a radioactive dose consisting of 20 μc of ^{45}Ca with a total Ca^{2+} content of 13.4 μg was added to each pot. The calcium concentrations and specific activities of the solutions were identical to those in experiment 1.

After 3 weeks in the radioactive solutions, the plants were transferred to non-radioactive solutions of comparable calcium level for 9 days. A leaflet from each leaf was then removed for radioautography and radioassay, and the plants were immediately injected with one of the following non-radioactive treatments:

- (1) Ca^{2+} , 1226 μg (61.2 $\mu\text{-equiv.}$);
- (2) Zn^{2+} , 1000 μg (30.6 $\mu\text{-equiv.}$);
- (3) Mn^{2+} , 840 μg (30.6 $\mu\text{-equiv.}$);
- (4) Mg^{2+} , 372 μg (30.6 $\mu\text{-equiv.}$);
- (5) Distilled water.

The calcium and magnesium were as their chloride salts, the zinc and manganese as their sulphates. The total volume of each treatment was 0.2 ml, and each was duplicated. After 24 hr the plants were removed for radioautography and radioassay.

(d) *Experiment 4*

Plants were established in low-calcium solutions and a dose of 20 μc of ^{45}Ca with a total Ca^{2+} content of 13.4 μg was added to the solutions after 17 days growth. Final calcium concentration was 75 $\mu\text{moles/l}$, with a specific activity of 0.133 $\mu\text{c}/\mu\text{mole}$ calcium. On day 25, the plants were transferred to non-radioactive low-calcium solutions and by day 44, the petioles commenced to collapse. On day 45, plants were randomly harvested from each pot and samples of the oldest leaves and newly fully expanded young leaves were taken. Each sample was segregated into the following subsamples: lamina and distal and proximal halves of petioles.

Each subsample was cut up finely and an aliquot of 200 mg fresh weight was added to a Thomas hand-homogenizer and the tissue was thoroughly disrupted. This material was transferred to a sintered-glass filter (porosity 2) by repeated washing with cold water. The residue was then thoroughly leached in succession with 1% sodium chloride, 2% acetic acid, and 0.05N hot hydrochloric acid, and the leachate in each case was collected in a 50-ml beaker. The cold-water leachate was evaporated to near dryness and then transferred to an aluminium planchet for final drying. To the remaining leachates, 0.08 ml 0.5M calcium chloride (non-radioactive) was added, and calcium was precipitated as its oxalate by a modified method described by Vogel (1945). The precipitate was collected on a Millipore filter (porosity 8 μm)

which was transferred to a planchet for radioassay. The final residue was also transferred to a planchet for radioassay.

Also on day 45, the plants remaining in solution were injected with 1226 μg calcium (as its chloride) in 0.16 ml water into either the fifth oldest leaf or its neighbour. The plants were harvested on day 49 and treated as described above. Any leaves with petiole collapse were not included in the samples, and due to the continued senescence of some older leaves, the post-injection old-leaf sample may have been slightly younger than the corresponding pre-injection sample.

III. RESULTS

(a) *Experiment 1*

Radioautographs showing the typical distribution patterns of ^{45}Ca between individual leaflets of plants growing in either normal or low-calcium solutions to which ^{45}Ca was added are shown in Plate 1, Figures 1 and 2. It is apparent that the pattern of distribution of ^{45}Ca between the three leaflets of any leaf of either a normal or calcium-deficient plant is very uniform. This agrees with previous observations by Millikan and Hanger (1964, 1965a).

The relationships between the mean leaf count and the count range of the ^{45}Ca radioassays of the individual leaflets of a leaf of normal and low-calcium plants are shown in Figures 1(a) and 1(b). These results showed that the range of the natural variability in ^{45}Ca concentration between leaflets of any leaf of either a normal or low-calcium plant was predictable and dependent upon the total ^{45}Ca activity.

(b) *Experiment 2*

The radioautographs of one plant of each treatment are presented in Plate 2, Figures 1-3, and in Plate 3, Figures 1-6. It is evident that the second injection of 1440 μg of non-radioactive calcium caused appreciable movement of the previously injected ^{45}Ca into many leaves of both the normal and low-calcium plants. Not only was the pattern of distribution of the isotope in the leaflets appreciably altered but the ^{45}Ca concentration was increased. In Figures 2(a) and 2(b), respectively, the count difference between leaflets harvested before and after the injection treatment has been plotted against the leaflet count before injection for the normal and low-calcium series. The appropriate regression lines and \pm twice the standard error lines from Figure 1 have also been included in these figures. Thus any point falling above the positive standard error line indicates a significant increase ($P < 0.05$) in the count difference between leaflets due to treatment. Thus for the normal-calcium plants there was a general increase in the ^{45}Ca count range between the leaflets of the majority of leaves examined. Many of these increases were significant. In the low-calcium plants, however, ^{45}Ca movement within the plant following injection was very pronounced, with the majority of leaves having highly significant increases in the ^{45}Ca count range between leaflets.

It was not possible to determine from which tissues the mobile ^{45}Ca originated. From Plate 2, Figures 1-3, it is apparent that the leaves which were in existence at the time of injection of the ^{45}Ca were still highly radioactive when sampled for radioautography. The ^{45}Ca content of some of these leaves increased, whereas that of others decreased, following the second non-radioactive injection. However, the most notable

movement of ^{45}Ca induced by the non-radioactive injection was into the young leaves, particularly of the low-calcium plants. In the latter, many-fold increases in

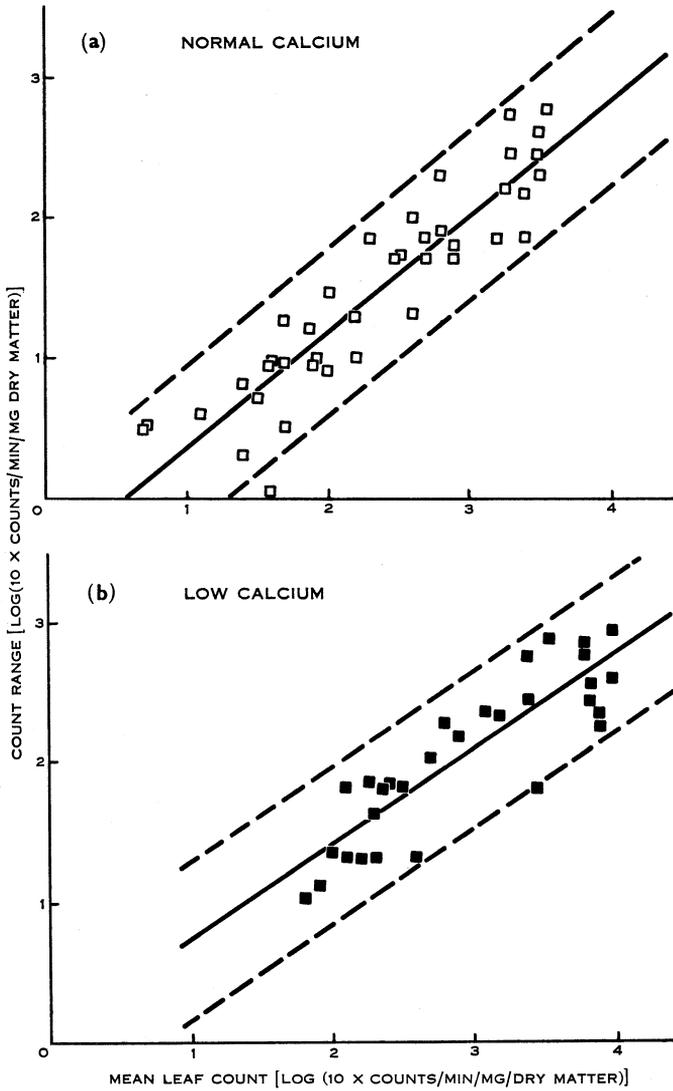


Fig. 1.—Experiment 1: relationship between the range and the mean ^{45}Ca concentration for the three related leaflets of subtterranean clover leaves grown in radioactive normal (a) and low-calcium (b) nutrient solutions. For the normal-calcium series the relationship is expressed by the regression equation

$$y = 0.818x - 0.446 \text{ (S.E. } \pm 0.30, r = +0.91),$$

and for the low-calcium series by

$$y = 0.660x + 0.074 \text{ (S.E. } \pm 0.28, r = +0.87),$$

where y is the range in counts and x is the mean leaf count, both expressed as shown on the figure. The dotted lines indicate twice the standard error for each regression.

^{45}Ca content were recorded (Plate 2, Figs. 1–3). Before the second injection many of these young leaves of the calcium-deficient plants had a very low concentration

or were completely devoid of ^{45}Ca , whereas young leaves of the plants receiving a normal supply of calcium all recorded the presence of the isotope (Plate 2, Figs. 1 and 2).

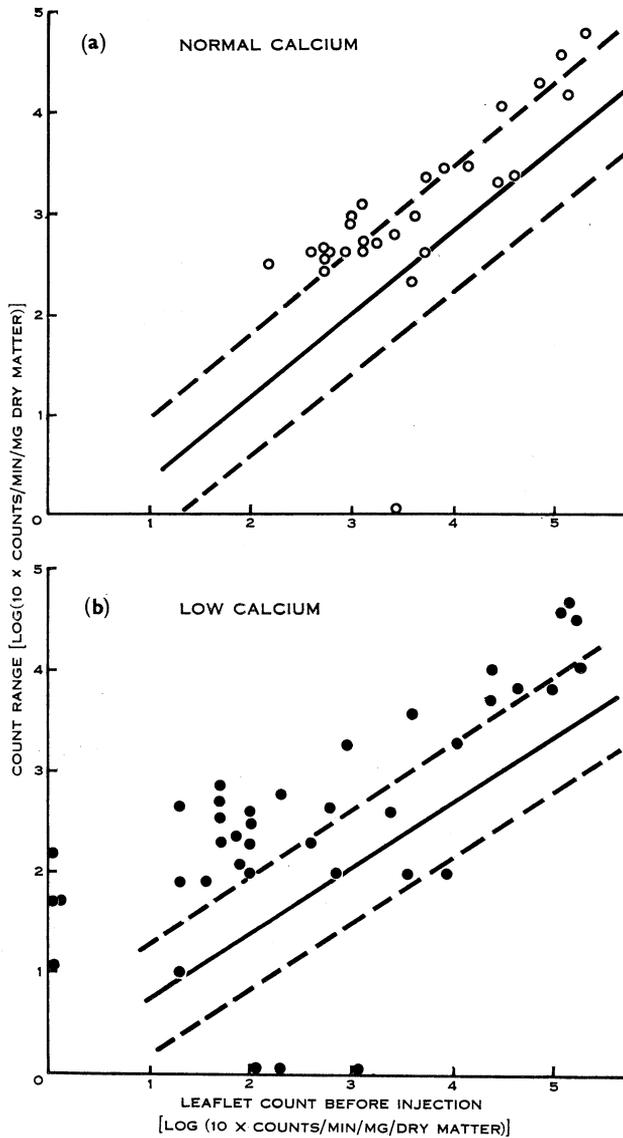


Fig. 2.—Experiment 2: relationship between the range in ^{45}Ca concentration of two related leaflets (one harvested prior to and the other after the second foliar injection of $1440\ \mu\text{g}$ calcium) and the ^{45}Ca concentration of the leaflet harvested prior to the second injection. The plants were previously grown in non-radioactive normal (a) or low-calcium (b) nutrient solutions. The regression lines (—) and twice the standard error lines (---) obtained in experiment 1 are also plotted.

It is concluded, therefore, that although the second injection demonstrated that portion of the previously injected ^{45}Ca in both the normal and low-calcium plants

was still in an exchangeable form, the uptake of calcium by the roots of deficient plants between these two injections was sufficient to permit only a very limited recirculation of ^{45}Ca to new leaves by an exchange mechanism.

(c) *Experiment 3*

In Figures 3(a)–3(f) the count range between leaflets of the normal and low-calcium series which were harvested before and after treatment has been plotted against leaflet count prior to treatment with the various cations or distilled water. The appropriate regression lines and \pm twice the standard error lines from Figure 1 have also been included.

Radioautographs of whole plants from the low-calcium series are shown in Plate 4, Figures 1 and 2, and Plate 5, Figures 1 and 2. Enlargements of the radioautographs of leaflets of selected leaves sampled before and after injection, from the low- and normal-calcium series, are shown in Plate 6, Figures 1–6, and Plate 7, Figures 1–6, respectively.

In the low-calcium series, the injection not only of Ca^{2+} , but also of the Mn^{2+} (840 μg) or Zn^{2+} (1000 μg) caused large increases in ^{45}Ca concentrations, and differences in the patterns of its distribution in certain leaves. In each case the isotope accumulated in the leaf margin, sometimes as “islands” of high activity, and tended to move out of the midrib (Plate 4, Figs. 1 and 2; Plate 5, Fig. 1; Plate 6, Figs. 1–6). Relatively smaller but non-significant increases in ^{45}Ca concentration resulted from the Mg^{2+} (372 μg) injections, but they were nevertheless associated with obvious changes in the pattern of ^{45}Ca distribution in some instances (Plate 6, Fig. 6). The injections of distilled water caused no differences in ^{45}Ca distribution (Plate 5, Fig. 2).

The radioautographs in Plate 4, Figures 1 and 2, and Plate 5, Figures 1 and 2, also reveal a considerable difference in the distribution of ^{45}Ca in the petioles of the plants injected with either Ca^{2+} , Zn^{2+} , or Mn^{2+} when compared with the plants injected with distilled water. In the latter plants the petioles of the older leaves showed a high concentration of ^{45}Ca which was a comparable type of distribution to that of the uninjected plant shown in Plate 1, Figure 2. By contrast, there was considerably less evidence of a similar high ^{45}Ca concentration in the petioles of leaves receiving the Ca^{2+} , Zn^{2+} , or Mn^{2+} injections respectively.

It is concluded, therefore, that the marked increase in ^{45}Ca in certain leaves following these cation injections was due to the movement of previously deposited ^{45}Ca out of the petioles.

Further evidence that outward movement of ^{45}Ca along the petioles occurred following injection of Ca^{2+} or Zn^{2+} but not water is afforded by the radioautographs which show a leaf with petiole collapse occurring on one plant in each of these treatments (Plate 4, Figs. 1 and 2; Plate 5, Fig. 2). Radioassays of the proximal and distal (collapsed) portions of the petioles concerned gave the following results:

Injection Treatment	^{45}Ca in Petiole (as counts/min/mg dry matter)	
	Proximal	Distal (collapsed)
Distilled water	310	140
Ca^{2+} (1226 μg)	60	370
Zn^{2+} (1000 μg)	140	270

These radioautographs and radioassays show that petiole collapse in the case of the water-injected plant was associated with a higher concentration in the proximal than the distal (collapsed) portion. On the other hand, the collapsed petioles on the Ca^{2+} - and Zn^{2+} -injected plants had much higher concentrations of ^{45}Ca in the distal (collapsed) regions than in the proximal parts.

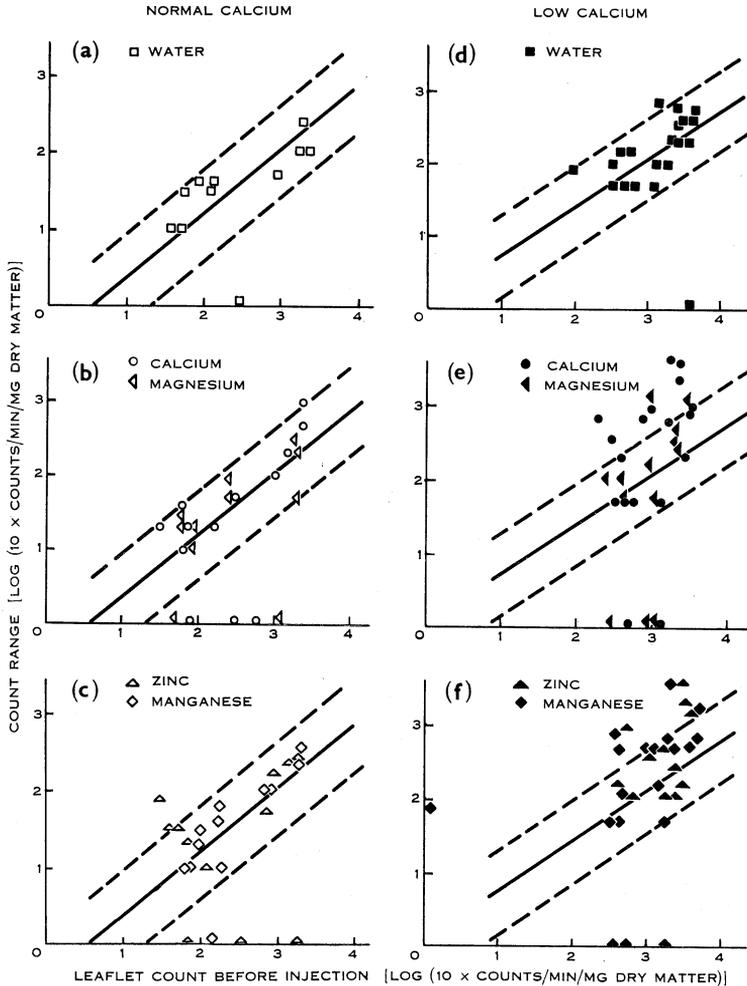


Fig. 3.—Experiment 3: relationship between the range in ^{45}Ca concentration of two related leaflets (one harvested prior to, and the other after, foliar injection) and the ^{45}Ca concentration in the leaflet harvested prior to injection. The plants were previously grown in normal [(a)–(c)] or in low-calcium [(d)–(f)] nutrient solutions. The regression lines (—) and twice the standard error lines (---) obtained in experiment 1 are also plotted. Quantities of cations injected in each case are listed in Section II(c).

In contrast with the results for the low-calcium series, injections of non-radioactive cations into the normal-calcium plants resulted in a trend towards a reduction in ^{45}Ca concentration in certain leaves. However, although Figures

3(a)–3(c) show that for the normal-calcium series there were no significant differences in range except for one leaflet following the Zn^{2+} injection, there were still some marked changes in the pattern of distribution of ^{45}Ca in leaflets following the Ca^{2+} , Zn^{2+} , and Mn^{2+} injections, as is shown in Plate 7, Figures 1–6.

These changes differed from those induced by similar injections into low-calcium plants (Plate 6, Figs. 1–6). Thus, in the normal-calcium plants ^{45}Ca activity increased in the midrib, but not uniformly in the leaf margin. Instead, “islands” of high ^{45}Ca activity appeared in the radioautographs, particularly following the Zn^{2+} and Mn^{2+} injections. There was also a decrease in ^{45}Ca activity in the interveinal tissues around the proximal half of the midrib in some leaflets (Plate 7, Figs. 1–6).

The injection of Mg^{2+} or distilled water into the normal-calcium plants caused no changes in ^{45}Ca distribution.

(d) Experiment 4

The results of this experiment are presented in Table 1. It is seen that virtually all the ^{45}Ca in the tissues was removed by successive extractions with water, sodium chloride, acetic acid, and hydrochloric acid respectively. Irrespective of whether the samples were taken before or after injection, the lamina and petiole subsamples of the older leaves each had a higher ^{45}Ca concentration than the corresponding parts of the young leaves.

In the young leaves, the lamina of both samples contained higher concentrations of ^{45}Ca than their associated petioles, and the distal half of each petiole had a comparable or slightly higher concentration than the proximal half. The injection of non-radioactive calcium resulted in a small increase in the total ^{45}Ca level in each young leaf part. There was a consistent increase in the amount of ^{45}Ca extracted in the water-soluble and sodium chloride-soluble fractions, whereas the acetic acid-extractable calcium decreased in the lamina and distal petiole, and the hydrochloric acid-extractable calcium decreased in the lamina and proximal portion of the petiole.

In the pre-injection sample of old leaves, the distal petiole subsample was slightly higher in ^{45}Ca than the lamina, and almost twice as high as the proximal petiole sample. The injection of non-radioactive calcium caused a drop in the total ^{45}Ca concentration in the petiole which amounted to 90% in the distal half and 50% in the proximal half. These changes resulted in a reversal of the pre-injection ratio in ^{45}Ca concentration between these two sections of the petiole. The decreases in total ^{45}Ca concentration were recorded in each extract fraction. The injections evidently moved some of the ^{45}Ca from the petiole into the lamina, as there was a large concomitant increase in the total concentration of the isotope in the latter. This increase was recorded in all fractions except that extracted by hydrochloric acid.

Irrespective of leaf age or part, the injection of non-radioactive calcium caused an increase in the percentage of ^{45}Ca found in the water-soluble fraction. Following the injection, the percentage of ^{45}Ca extracted by sodium chloride also increased in all the young leaf parts, whereas in the old leaves this extract showed a decrease in the lamina, and a slight increase in the proximal petiole fraction. The percentage of acetic acid-soluble calcium fell in all leaf subsamples except the lamina of the old

leaf. Similarly, a fall was recorded for hydrochloric acid-soluble calcium for all subsamples except in the distal portion of the petiole from the old leaf.

IV. DISCUSSION

It has been demonstrated herein that previously deposited ^{45}Ca may be made to recirculate to a marked degree by injection into the plant of non-radioactive Ca^{2+} , Zn^{2+} , or Mn^{2+} and, to a lesser degree, by Mg^{2+} , but not by water. Thus differences were induced in the pattern of distribution and concentration of ^{45}Ca

TABLE I
EXPERIMENT 4: RESULTS OF RADIOASSAY OF ^{45}Ca IN VARIOUS EXTRACTS OF THE LAMINA AND PETIOLE OF SUBTERRANEAN CLOVER (CV. DWALGANUP)
Results are for 200-mg (fresh weight) samples from plants grown at the low-calcium level and sampled either before or after injection with non-radioactive calcium. Percentage ^{45}Ca in each extract shown in parenthesis

Leaf Part and Extract	^{45}Ca Activity (counts/min/mg)			
	Young Leaf		Old Leaf	
	Before Injection	After Injection	Before Injection	After Injection
Lamina				
Water	471 (29.6)	857 (51.2)	3139 (40.2)	5800 (49.6)
Sodium chloride	436 (27.4)	606 (36.2)	2910 (37.3)	3898 (33.3)
Acetic acid	117 (7.3)	50 (3.0)	877 (11.2)	1336 (11.4)
Hydrochloric acid	550 (34.5)	159 (9.5)	884 (11.3)	664 (5.7)
Residue	19 (1.2)	1 (0.1)	2 (0)	3 (0)
Total	1593	1673	7812	11701
Petiole distal				
Water	66 (38.8)	157 (52.0)	2124 (23.9)	302 (34.0)
Sodium chloride	30 (17.6)	68 (22.5)	3621 (40.8)	349 (39.3)
Acetic acid	18 (10.6)	13 (4.3)	2053 (23.1)	91 (10.2)
Hydrochloric acid	56 (33.0)	63 (20.9)	1068 (12.0)	146 (16.4)
Residue	0 (0)	1 (0.3)	18 (0.2)	0 (0)
Total	170	302	8884	888
Petiole proximal				
Water	51 (31.3)	97 (45.5)	1063 (22.6)	827 (34.4)
Sodium chloride	43 (26.4)	67 (31.5)	2016 (42.9)	1087 (45.2)
Acetic acid	24 (14.7)	24 (11.3)	1044 (22.2)	339 (14.1)
Hydrochloric acid	44 (27.0)	24 (11.3)	579 (12.3)	152 (6.3)
Residue	1 (0.6)	1 (0.5)	0 (0)	0 (0)
Total	163	213	4702	2405

between individual leaflets of the same subterranean clover leaf samples, respectively, immediately before and 24 hr after the cation injection. These differences were, in many instances, very much greater than were found to occur naturally between similar leaflets with comparable ^{45}Ca concentrations from uninjected plants.

One corollary which appears implicit in these results is that those ions which were active in displacing calcium are themselves subject to movement and displacement in quite the same way as calcium itself. Millikan and Hanger (1965*b*) have postulated the occurrence of non-selective negatively charged sites of fixation in subterranean clover. Evidently there is mutual displacement of cations on such sites within the tissue, subject presumably, like all such reactions, to the effects of mass action and the position of the cation in the lyotropic series.

However, some factor, at present unexplained, may affect the movement of the isotope following the injection of non-radioactive cations. It appears to be related to the calcium level and the time the plant is allowed to take up non-radioactive calcium via the roots between the end of the radioactive treatment and the non-radioactive injection into a leaf.

Thus, where the ^{45}Ca was injected with an additional 1440 μg of non-radioactive calcium into plants grown in non-radioactive solutions, a second injection of non-radioactive calcium 9 weeks later caused outward movement of the ^{45}Ca in both normal- and low-calcium plants. However, where the ^{45}Ca was added to the nutrient solution and the plant subsequently transferred to a non-radioactive solution, an injection of non-radioactive cations only 9 days later caused marked movement of ^{45}Ca out of the petioles and into the laminae of the low-calcium plants but not of the normal-calcium plants. Instead, the injection of the latter plants resulted in a tendency for the ^{45}Ca to move out of the lamina.

It is known that the basic metabolism of leaves changes as they age. Also, Millikan and Hanger (1964) have shown that the ratio of water-soluble to other calcium fractions in subterranean clover varies appreciably with the age of the leaf and the calcium level in the plants.

The mobility of ^{45}Ca demonstrated by the experiments described above is in accord with the results of Millikan and Hanger (1964). They found that ^{45}Ca , which was previously immobile in acutely calcium-deficient subterranean clover plants, moved into the young leaves when the plants were transferred to non-radioactive solutions containing sufficient calcium to induce new growth. A similar movement of ^{45}Ca into the young leaves of the calcium-deficient plants occurred in experiment 2 following injection of the non-radioactive calcium (Plate 2, Figs. 1-3).

An indication that outward movement of ^{45}Ca from the proximal towards the distal part of the petiole, presumably by exchange with non-radioactive calcium, occurred subsequent to the transfer of the low-calcium plants from radioactive to non-radioactive solutions, is provided by the radioautograph of the non-injected plant (Plate 1, Fig. 2) and the water-injected plants (Plate 5, Fig. 2). In these plants the proximal part of the petiole was lower in ^{45}Ca than either the distal half or the lamina. This relative distribution of ^{45}Ca between the leaf parts is different from that of plants grown continuously in low-calcium solutions containing ^{45}Ca . Millikan and Hanger (1964) have shown that in such plants the isotope occurs at first in higher concentration in the proximal than the distal half of the petiole, and that as the leaf ages, the concentration becomes uniform along its length, and is higher than in the lamina.

This outward movement of ^{45}Ca was greatly enhanced by the cation injections. The radioautographs of the cation-injected, low-calcium plants shown in Plate 4, Figures 1 and 2, and Plate 5, Figure 1, reveal that the petioles of the old leaves were quite unusual as they were very much lower in ^{45}Ca than their associated laminae. Also, the high ^{45}Ca concentration in the collapsed compared with the proximal half of petioles of the Ca^{2+} - and Zn^{2+} -injected, but not the water-injected, plant (Plate 4, Figs. 1 and 2; Plate 5, Fig. 2) is further evidence of outward movement of ^{45}Ca induced by the cation injections. Millikan and Hanger (1964) have shown that the distal collapsed portion normally had a much lower concentration of ^{45}Ca than the proximal part of the same petiole. Further, Millikan and Hanger (1965c) were able to make ^{45}Ca move in an outward direction through the collapsed portion of petioles of subterranean clover by injection of ^{45}Ca plus non-radioactive Ca^{2+} into another leaf of the plant.

The occurrence of outward movement of ^{45}Ca through the petioles of low-calcium plants is further indicated by the results in Table 1.

It is thus concluded that a significant proportion of the calcium in subterranean clover, and particularly that in the petioles, may be in an exchangeable form. Also the sites of this exchangeable calcium are non-selective in that the introduction of divalent cations other than calcium can cause movement of the previously deposited calcium.

Further, a large proportion of the ^{45}Ca extracted by water, sodium chloride, acetic acid, and hydrochloric acid, respectively, was found to be exchangeable, as the total amount in each of these extracts showed marked decreases in both the distal and proximal halves of the petioles of the old leaves of the low-calcium plants following the non-radioactive injection (Table 1). The concomitant increase in ^{45}Ca concentration in the laminae during the 24 hr after injection also occurred in the water, sodium chloride, and acetic acid fractions. At this stage, however, the import of calcium into the laminae of the young as well as the old leaves caused a decrease in the total amount of the isotope in the hydrochloric acid extract.

Recently, Petrov-Spiridonov (1964) has described the re-utilization of individual calcium extracts by plants. His results are not entirely in accord with those described above, as he found that the water, sodium chloride, and, to a lesser extent, the acetic acid fractions, but not the hydrochloric acid fraction, were re-utilized by plants.

Previously, Sveshnikov (1953) had investigated the distribution of calcium between various fractions extracted from red clover tissues. He concluded that no stable organic complexes of calcium occurred in that plant, since it was all removed with water, sodium chloride, acetic acid, and hydrochloric acid, as in the experiment with subterranean clover described above.

The mechanism of the exchange reactions shown to occur in the experiments described in this paper is evidently similar to that suggested by Epstein (1962), whereby the movement of cations attached to negatively charged sites in stems of plants is accelerated by the presence of competing ions. Likewise, Biddulph, Nakayama, and Cory (1961) and Bell and Biddulph (1963) showed that calcium ascended the stems of bean plants by displacing adsorbed calcium from exchange sites. In this

process, the xylem acts as an exchange column. They concluded that the entry of ^{45}Ca into stem tissue showed two phases, namely a reversible "exchange" phase which was completed within 3 hr, followed by an irreversible accumulation phase. However, previous results by Millikan and Hanger (1964) and the results described above indicate that in subterranean clover calcium persists in an exchangeable form for at least 9 weeks.

V. ACKNOWLEDGMENTS

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EXPLANATION OF PLATES 1–7

PLATE 1

Figs. 1 and 2.—Experiment 1: radioautographs of subterranean clover plants grown for 21 days, and then 9 days, in radioactive and non-radioactive solutions, respectively. Exposure 10 days. Note that the distribution pattern and concentration of ^{45}Ca within each related leaflet appears to be identical. Plant grown in a complete nutrient solution (Fig. 1); in a low-calcium solution (Fig. 2).

PLATE 2

Figs. 1–3.—Experiment 2: radioautographs of subterranean clover plants and leaflets (inserts). The plants were grown initially in non-radioactive solution. At the fifth leaf stage, the second oldest leaf (denoted by circled 1 in Fig. 3) was injected with ^{45}Ca plus $1440\ \mu\text{g}\ \text{Ca}^{2+}$. After 61 days a leaflet was removed from each leaf for radioautography, and the plants were then foliar-injected with $1440\ \mu\text{g}$ non-radioactive calcium into the leaf denoted by circled 2 in Figure 3. The plants were harvested 24 hr later. Exposure 7 days.

Fig. 1.—Leaflets removed prior to second injection from plant in Figure 3, *upper*.
The numbers indicate from which leaf the leaflet was removed.

Fig. 2.—As for Figure 1, except that leaflets were removed from plant in Figure 3, *lower*.

Fig. 3.—*Upper*: Plant grown in a low-calcium solution.

Lower: Plant grown in a complete nutrient solution.

PLATE 3

Figs. 1-6.—Experiment 2: radioautographs of selected leaves from subterranean clover plants in Plate 2 to show changes in ^{45}Ca distribution and concentration following the second injection with non-radioactive calcium. In each figure the leaflet on the left-hand side was removed prior to injection. On the right-hand side is shown the remainder of the leaf after injection. Leaf numbers correspond with those in Plate 2.

Fig. 1.—Leaf 3	} Low-calcium plants	Fig. 4.—Leaf 6	} Normal-calcium plants
Fig. 2.—Leaf 7		Fig. 5.—Leaf 7	
Fig. 3.—Leaf 10		Fig. 6.—Leaf 19	

PLATE 4

Figs. 1 and 2.—Experiment 3: radioautographs of leaflets and of subterranean clover plants. The plants were initially grown in non-radioactive, low-calcium solution for 7 days and were then transferred to comparable radioactive solutions for 21 days, after which they were returned to non-radioactive solutions. After 9 days in the latter solution, a leaflet was removed from each leaf and radioautographed. The plants were then foliar-injected with various cations, and harvested after 24 hr. Injected leaves denoted by circled numbers. In the inserts, the leaflet of the injected leaf is indicated by an arrow. Exposure 4 days. *PC*, petiole collapse due to calcium deficiency.

Fig. 1.—Leaves injected with $1226 \mu\text{g Ca}^{2+}$. Inserts are radioautographs of leaflets removed prior to injection. The leaflet number identifies its source leaf on the plant.

Fig. 2.—As for Figure 1, leaves injected with $1000 \mu\text{g Zn}^{2+}$.

PLATE 5

Figs. 1 and 2.—Experiment 3: identical with Plate 4, except that plants were injected with $840 \mu\text{g Mn}^{2+}$ (Fig. 1); distilled water (Fig. 2). Injected leaves denoted by circled numbers. In the inserts, the leaflet of the injected leaf is indicated by an arrow.

PLATE 6

Figs. 1-6.—Experiment 3: radioautographs of selected leaves from the low-calcium series of subterranean clover plants, grown and treated as described in Plates 4 and 5. In each figure the left-hand side leaflet was removed prior to injection. On the right-hand side is the remainder of leaf after injection. Plants injected as follows:

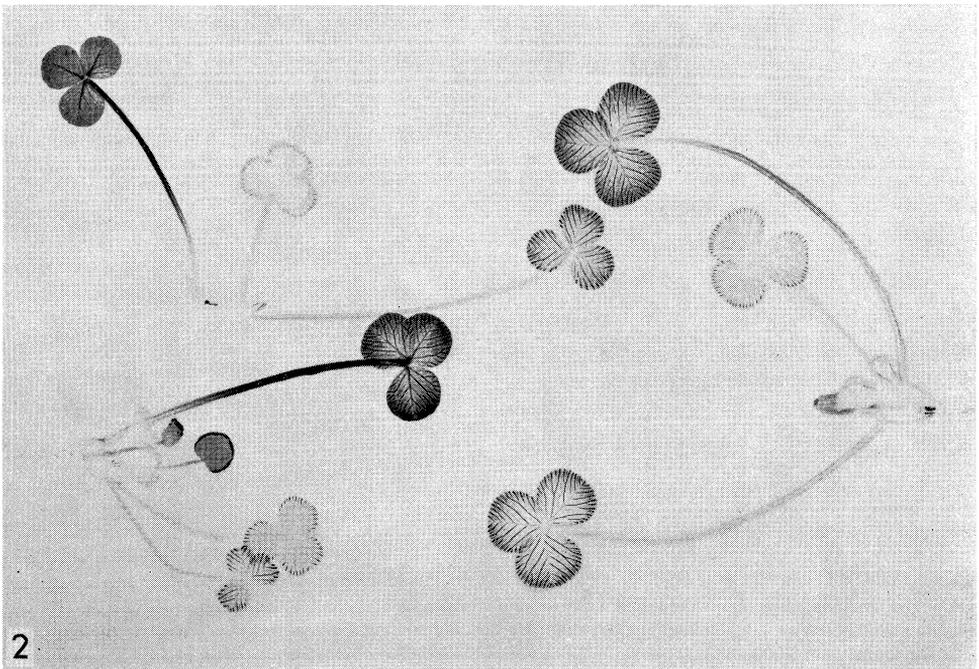
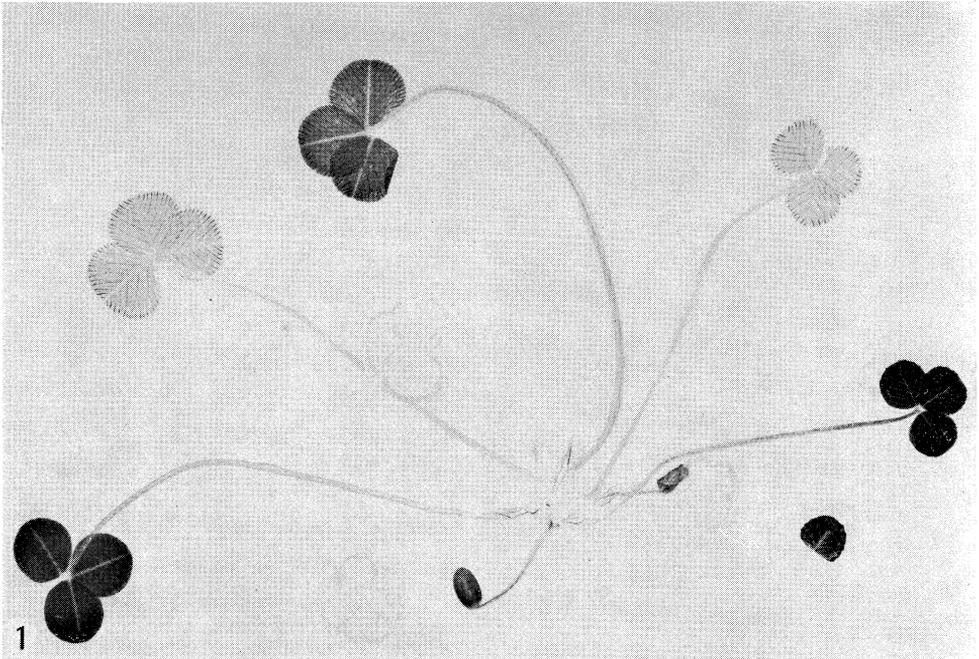
Fig. 1.— $1226 \mu\text{g Ca}^{2+}$.	Fig. 3.— $1000 \mu\text{g Zn}^{2+}$.	Fig. 5.— $840 \mu\text{g Mn}^{2+}$.
Fig. 2.— $1226 \mu\text{g Ca}^{2+}$.	Fig. 4.— $1000 \mu\text{g Zn}^{2+}$.	Fig. 6.— $372 \mu\text{g Mg}^{2+}$.

PLATE 7

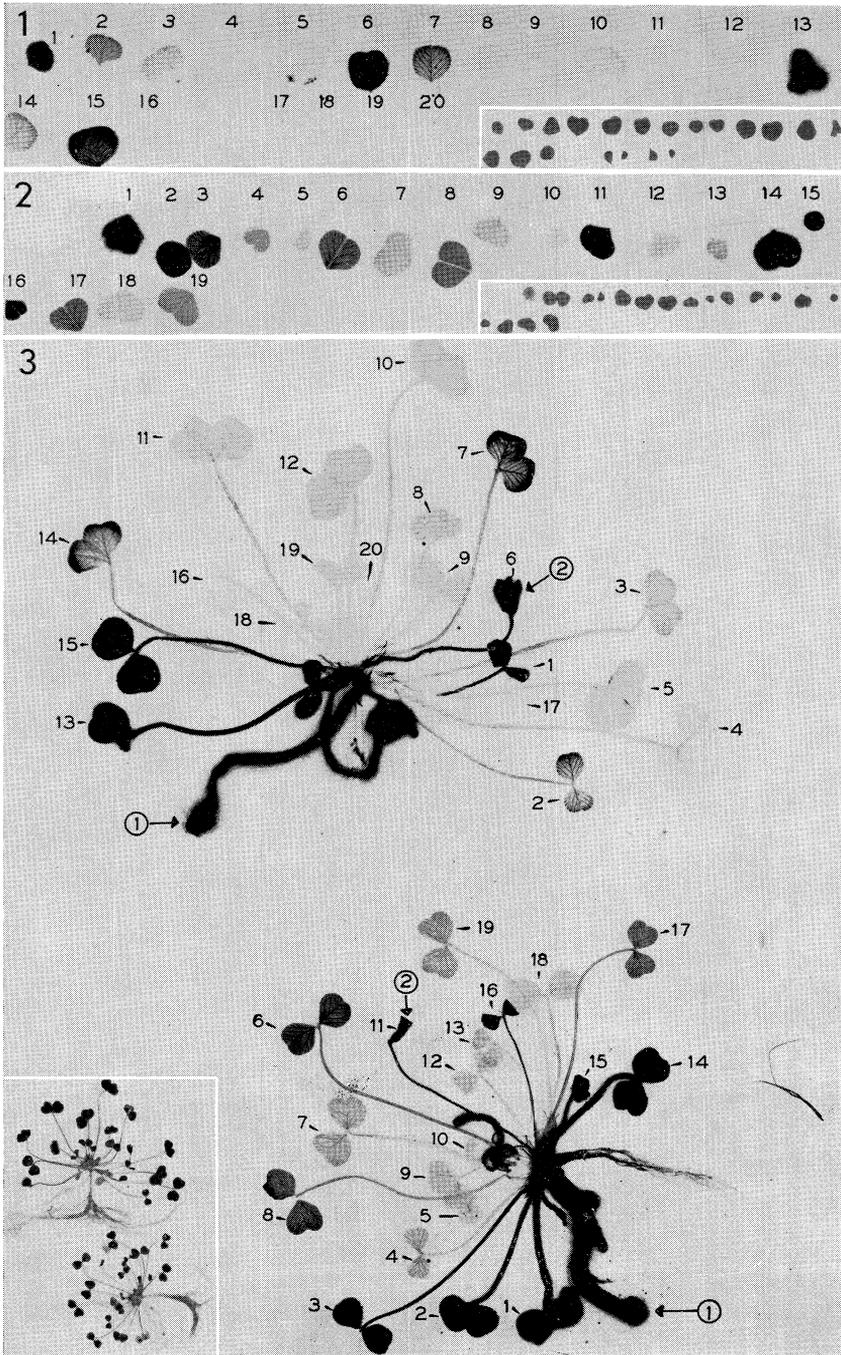
Figs. 1-6.—Experiment 3: radioautographs of selected leaves from the normal-calcium series of subterranean clover plants grown and treated as described in Plate 4, except that plants were grown in complete nutrient solutions. In each figure the leaflet on the left-hand side was removed prior to injection. On the right-hand side is the remainder of leaf after injection. Plants injected as follows:

Fig. 1.— $1226 \mu\text{g Ca}^{2+}$.	Fig. 3.— $1000 \mu\text{g Zn}^{2+}$.	Fig. 5.— $1000 \mu\text{g Zn}^{2+}$.
Fig. 2.— $1226 \mu\text{g Ca}^{2+}$.	Fig. 4.— $1000 \mu\text{g Zn}^{2+}$.	Fig. 6.— $840 \mu\text{g Mn}^{2+}$.

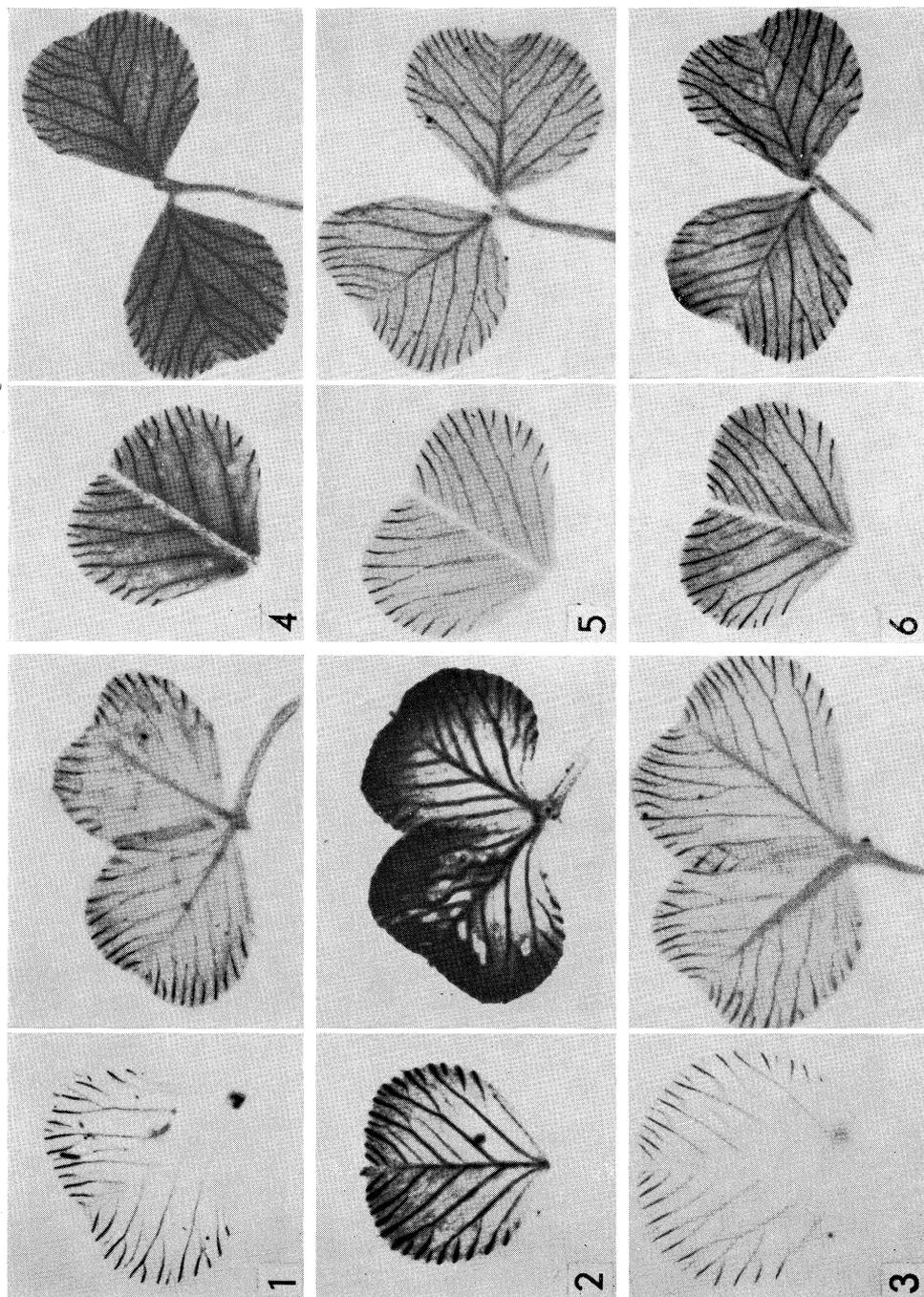
MOVEMENT OF ^{45}Ca IN SUBTERRANEAN CLOVER



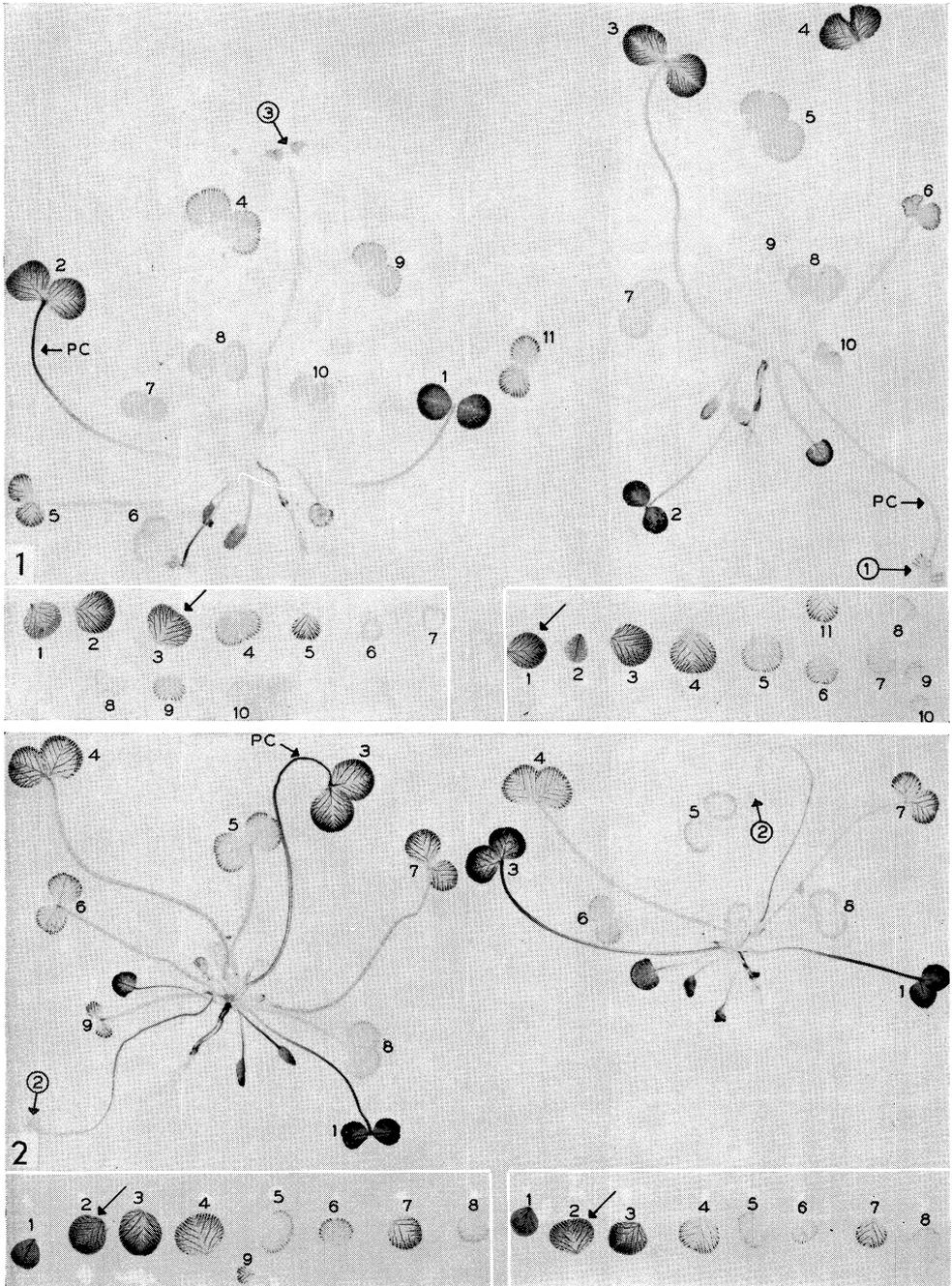
MOVEMENT OF ⁴⁵Ca IN SUBTERRANEAN CLOVER



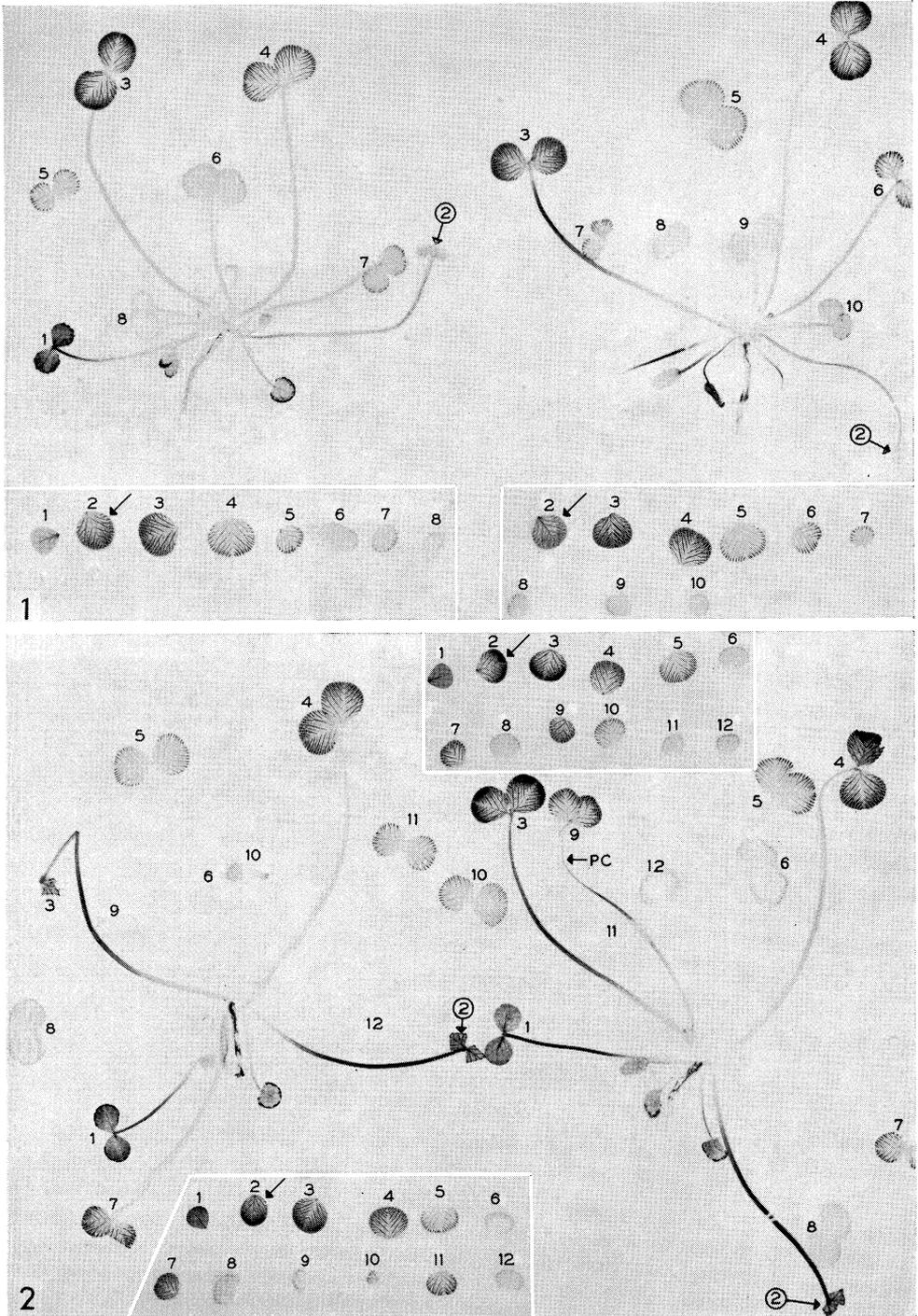
MOVEMENT OF ^{45}Ca IN SUBTERRANEAN CLOVER



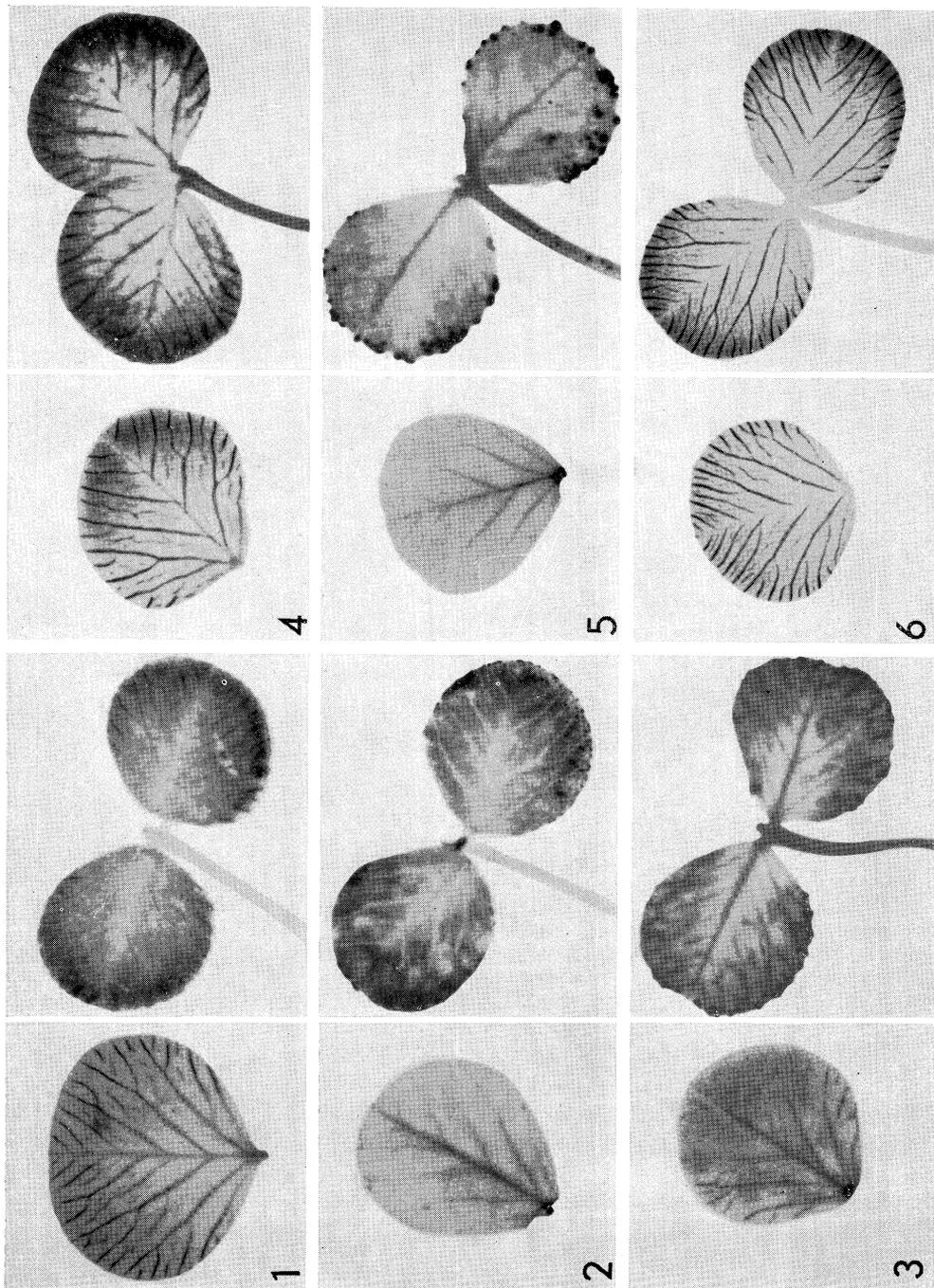
MOVEMENT OF ^{45}Ca IN SUBTERRANEAN CLOVER



MOVEMENT OF ^{45}Ca IN SUBTERRANEAN CLOVER



MOVEMENT OF ^{45}Ca IN SUBTERRANEAN CLOVER



MOVEMENT OF ^{45}Ca IN SUBTERRANEAN CLOVER

