THE UPTAKE OF SULPHUR, CALCIUM, AND MAGNESIUM AND THEIR DISTRIBUTION IN PHASEOLUS VULGARIS L. AS AFFECTED BY CYCLOHEXANECARBOXYLIC ACID

by

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ABSTRACT

Cyclohexanecarboxylic acid (CHCA) has one of the lowest molecular weights of the naphthenic acids, a group of compounds which have been recognized recently as stimulators of plant growth and metabolism. Potassium cyclohexanecarboxylate's (KCHC) effects on the mineral nutrition of bush bean plants (*Phaseolus vulgaris* L., cultivar Top Crop) were examined in four separate studies.

1. The foliage of two-week-old plants, growing in sulphur-free nutrient solution was treated with $1 \times 10^{-2}$ M KCHC solution and subsequently the roots were given a 4 hr exposure to $^{35}S_4$ in the nutrient solution.

Slightly more sulphur-35 was taken up by treated plants than untreated control plants, but the difference was not statistically significant. However, significantly more sulphur-35 was detected in the leaves of treated plants. The differences in distribution were attributed to metabolic changes brought about by treatment.

2. Plants were handled as above and the leaves were harvested one, two, and five days after feeding sulphur-35. The leaf tissue was separated, chemically, into sulphate, lipid, free amino acid, perchloric acid soluble, and protein fractions. A portion of the protein fraction was hydrolyzed to permit separation of the sulphur-amino acids by chromatography.

The bulk of the sulphur-35 was incorporated into the acid soluble fraction first and subsequently into the protein fraction. KCHC treated leaves contained significantly more
acid soluble S initially and more protein S finally than control leaves. Control leaves contained more sulphate S than treated leaves two days after sulphur-35 feeding. These results indicated that KCHC treatment stimulated the incorporation of sulphur into protein, and this was consistent with other reports of increased protein formation due to naphthenate treatment.

3. Bush bean plants growing in complete nutrient solution were treated and exposed to calcium-45. Treated plants took up slightly less calcium than controls, and they retained significantly more of it in their roots. These results are in contrast to the effect of treatment on sulphur-35 uptake and distribution.

4. Bush bean plants growing in complete nutrient solution were treated with KCHC solution at two weeks of age. One week later the distribution of sulphur, calcium, and magnesium was examined in leaf blades, stems plus petioles, and roots. No significant effect of treatment on uptake was found for any of the elements. Distribution of sulphur and calcium within the plant was not affected by treatment, but control plants contained significantly more magnesium in their leaves than treated plants.

The lack of significant effect on ion uptake indicated that the increased growth found in many studies, when plants were treated with naphthenic acids, apparently was not the result of improved uptake of sulphur, calcium, or magnesium.
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INTRODUCTION AND LITERATURE REVIEW

During the past few years, an increasing number of reports have been published documenting the effects of naphthenic acids as stimulators of plant growth. Much of the early work has dealt with the relative increases in yields that can be expected from various crop plants under treatment (80). In most cases, the concentration and mode of application of the naphthenate, and the plant's ontogenetic condition were described since these are critical factors affecting the response (110,112).

After the initial stages of determining growth responses, work in our laboratory has been aimed at understanding the basic physiological responses of the plants to naphthenate treatment. In this particular study, the effect of one of the naphthenic acids, cyclohexanecarboxylic acid, on some aspects of sulphur, calcium, and magnesium nutrition in bush beans has been examined. The sulphur status of the plants was looked at in some detail, while only the uptake and distribution of the other two elements were considered.

Naphthenic acids

Naphthenic acids are monocarboxylic acids of the naphthene (alicyclic) series of hydrocarbons (60). They occur in and are recovered from petroleum. There is a wide range of compounds which fall into this group, but they all share a general structure which may be written as \( R(CH_2)_nCOOH \), where \( R \) is
a cyclic nucleus composed of one or more rings. These rings may contain five, six, or seven carbon atoms (60). Many methods are available for separation of naphthenic acids from crude oil, but the simplest involves extraction with aqueous sodium hydroxide. The amount and composition of the naphthenic acid fraction vary with the source of the crude. As an example, gas chromatography, mass spectrometry, and distillation procedures were used to determine that an Austrian crude oil contained 120 different components (20). Eider (38) found as many as 60 components in Venezuelan crude oil.

Large amounts of naphthenic acids are recovered annually, and they are used commercially as lubricants, driers, catalysts, preservatives, corrosion inhibitors, emulsifiers, and napalm explosives (60).

The literature on the use of naphthenic acids in plant research has been extensively reviewed by Severson (83). The first known report of naphthenic acid used as a metabolic stimulant was in 1921 when Neuberg and Sandberg (75) found that potassium naphthenate stimulated the alcoholic sugar splitting activity of yeast, but credit for initiation of the present boom in this area belongs to workers in Bulgaria, Albania, and the USSR. Huseinov (57) reported the results of several experiments conducted in the Soviet Union. He noted that this work had begun about 1950, but his report to the 7th International Congress of Soil Science, held at Madison, Wisconsin in 1960, is one of the few references available that describe any of the early Russian work. Some of the results he reported then
will be incorporated into the following discussion.

Application of naphthenic acids to a wide range of crop plants has, in many cases, caused economically useful changes in their growth patterns. Reports of quite remarkable increases in the amount of potato tubers produced are noted. Krasnova et al (65) observed increases of 48% with some varieties. Abolina and Ataullaev (1) found 30 to 40% increases in production, and starch accumulation was up 20% when the soil was treated with 500 ml of naphthenate growth substances per hectare. Wort and Hughes (111) have shown that one treatment with a 5000 ppm foliar spray of the potassium salt of naphthenic acids (Knap) 33 days before harvest resulted in a significant increase (42%) in total fresh weight of tubers per plant, but there was no significant effect on the number of tubers per plant, the percentage of dry matter, or starch produced. Plants which received the first treatment of Knap as well as additional treatments of 2500 ppm of Knap 19 and five days before harvest showed no significant increase in any of the parameters measured, although the fresh weights of tubers per plant were higher than controls. The increased starch yield reported by the Russian workers was not found, and it was suggested that varietal differences may have been the reason.

Cotton's response to naphthenates has been examined by a number of workers. Naghibin (74) found that a seed-soak with naphthenic growth substances (NGS) gave 5 to 10% increases in yield while sprays at the rate of 250 g/ha caused
a 20% increase in yield. Other favourable influences such as accelerated ripening, earlier opening of bolls, and increased weight of cotton per boll were also noted.

In countries where regulations might require the approval of additives to food crop plants, the toxicity of naphthenates, or lack of it, will likely have to be determined before it is used commercially. However, even in these countries, large scale testing could reasonably be carried out on crops such as cotton and forest trees. In the USSR cotton in areas of over 200,000 hectares has been subjected to treatment with NGS (74).

In addition to the above, significant increases in the yield of bush bean pods (110,112), of carrots, cabbage, beet and onion (116), corn (113), rice (57), sugar beets (113), tomato (2,57), and wheat (86,113), have been found. Increases are noted for many other plants (see Severson, 83) but the examples above serve to illustrate the wide range of species which respond favourably to treatment.

In general, the treated plants appear to grow more rapidly than control plants. Frequently, the increased growth occurs in parts of the plant which are economically important, thus making Knap of interest from an agronomic point of view. Since it has been established that increased yields are quite a common occurrence, the questions that arise are physiological in nature. One wonders what changes occur in the plants to cause the responses. Certainly it appears that the plants are producing more building materials and are transporting them more rapidly. Increased activity implies that higher levels
of respiration and photosynthesis occur. Enzyme activity would be expected to increase, and this would be manifested in higher specific activities or in increased amounts of enzyme. The treated plants would be expected to absorb and utilize more mineral elements than the less active control plants. More complete understanding of these processes is being obtained slowly, since papers describing physiological effects are far outnumbered by reports considering the agronomic aspects. At this point, it might be well to summarize some of the relevant physiological information that is available.

Increased amounts of photosynthetic pigments have been reported by several authors (71,30,45). Although Chu (30) observed only minor increases of chlorophyll a and b, due to a 5000 ppm Knap spray on tomato, she reported increases of 1.4 to 13.8% in carotenoid pigments. Fattah (45) presented evidence of significant increases in the chlorophyll a content of bean leaves two and three weeks after treatment with Knap but only nonsignificant increases of chlorophyll b and carotenoids. He found a significant increase in total pigments three weeks after treatment.

Increased levels of protein have been found in several studies (77,78,62,114), and free amino acid levels were higher at the beginning and end of vegetative growth of maize treated with naphthenate growth substances (78). Severson also reported increased free amino acid levels (84), and Padmanabhan et al (77) have shown increased levels of several free amino acids.
Bazanova determined the auxin and inhibitor content of cotton seedlings treated with 0.005, 0.0025, and 0.00125% solutions of Sh-8 (an organic compound belonging to the cyclohexenyl butanols, 8)(10). He found evidence of redistribution of auxins and inhibitors in various organs. There was an increased translocation of growth regulating substances from vegetative to reproductive organs and an increased activity of the natural growth regulating substances in the reproductive organs. Huseinov (57) reported that petroleum growth helping substances (naphthenic acids) caused oat coleoptile bends of 9.7° to 12°, whereas heteroauxin caused bends of 6.8° to 8.4°, and hybberellic acid (gibberellic acid) 7.7° to 8.0°. These results, though interesting, are somewhat surprising and possibly suspect, because he reports an auxin effect for gibberellic acid which has not been shown to qualify as an auxin (82). Loh (72) has found several auxin-like properties associated with Knap. He found that Knap treatment caused elongation of pea stem segments and promoted the initiation of adventitious root formation by bean stem and azalea cuttings. Loh also found increased biosynthesis of IAA in dark-grown bean plants treated with Knap. The activity of enzymes associated with IAA destruction was also stimulated. These results suggest that, rather than being auxins, the naphthenic acids may stimulate auxin activity.

Several enzyme assays have been conducted to determine naphthenate effects. Russian workers report increased peroxidase activity (8) and increased catalase activity (63).
The ascorbic acid content of bean pods was 26.5% greater in Knap treated plants than controls (45), but decreased levels were found in tomato fruits (30). This could, of course, reflect differences in response by the two species.

Studies of enzymes in our laboratory have shown some positive effects due to 5000 ppm Knap sprays on bush bean leaves. Several nitrogen metabolizing enzymes were examined. Fattah and Wort (47) found variable results with nitrate reductase. In all cases the enzyme activity was increased, but only under certain conditions were the increases significant. Wort et al (114) determined that Knap treatment increased nitrate reductase activity 152% compared to control, when measured seven days after treatment. Under similar experimental conditions, Wort et al (114) found glutamic-oxaloacetic transaminase activity increased 10% over control, glutamine synthetase 17%, and glutamic dehydrogenase activity was unaffected. Fattah (45) discovered that glutamic-pyruvic transaminase activity was increased significantly under medium and high light conditions. A 72% increase in cytochrome oxidase activity was reported by Wort et al (114).

The presence of higher concentrations of nucleic acids in naphthenate treated plants (46, 77) and the greater mitotic activity that was found in some studies (50) indicate that naphthenates may be functioning at the genetic level (77).

Ejubov and Issaeva (39) reported increased availability of phosphorus and mineral nitrogen compounds in soil treated with naphthenates, as well as increased levels of nitrogen and phosphorus content in maize and lucerne plants. Yur'eva (115)
found an increased phosphorus content in the leaves of sugar beets. Babaev (8) determined that the amount of total P increased in initial growth phases in cotton treated with either a foliar or soil application of naphthenates. Severson (83) found that the uptake of phosphorus by beans grown in either complete or phosphorus free nutrient solutions was unaffected by Knap treatment. He did find that Knap treatment enhanced the acropetal movement of $^{32}$P from the roots of plants grown in a phosphorus free nutrient solution. Severson (83) also determined that naphthenate treatment increased the rate of incorporation of $^{32}$P into acid soluble (sugar phosphates, free nucleotides, phospholipids) and acid insoluble (nucleic acids, phosphoproteins) fractions of leaves. He did not find that naphthenate treatment affected the amount of P in the two fractions when compared with controls.

**Cyclohexanecarboxylic acid**

One of the simplest of the naphthenic acids, cyclohexanecarboxylic acid (CHCA) has been used extensively in our laboratory at the University of British Columbia. Many properties of this compound have made it attractive as a test material for research purposes. CHCA is a simple molecule for which the structure is known, while the naphthenic acids used in many of the studies are a complex mixture of chemical compounds.

![Cyclohexanecarboxylic acid](attachment:image.png)
CHCA can be obtained in a radioactive form, which is useful for studies of the metabolism of the compound itself. Stimulation of vegetative and reproductive growth of bush beans is very pronounced with this compound. Wort and Patel (113), reported increases of 23.5% in reproductive growth of bush beans with a 1 x 10^{-2} M foliar application of potassium cyclohexanecarboxylate (KCHC) two weeks after planting. In many trials the stimulative effects were clearly visible when the treated plants were placed beside their controls. Wort and Patel (113) pointed out that gas chromatographic analysis of naphthenic acids obtained from Venezuelan crude oil showed that low molecular weight compounds with carbon numbers close to that of CHCA were present in this fraction. They suggested that it was possible that the low molecular weight components of the naphthenate mixture could be the active agents.

In studies of the metabolism of cyclohexanecarboxylic acid by bean plants, Severson et al (85) found that CHCA formed conjugates with glucose and aspartic acid. Padmanabhan (76) has reported the appearance of a glucose ester of CHCA 0.125 hr after application of KCHC-7-^{14}C as droplets on the adaxial surface of the primary leaves, and the aspartate conjugate appeared after one hour. Free acid had disappeared within six hours of application. Considering these facts, Severson (83) has suggested that the conjugated form of CHCA rather than the free acid may be the growth stimulating form.

Severson (84) has also reported studies of the metabolism of 14C-glucose by bean root tips treated with naphthenic acids
and KCHC. Glucose incorporation by the roots was stimulated by both these substances, with KCHC having a somewhat stronger effect. The levels of radioactivity incorporated into 10 ethanol-soluble amino acids from \(^{14}\)C glucose were determined, and it was found that in all cases activity was greater in the treated roots. Serine and valine levels were significantly increased by both Knap and KCHC, whereas isoleucine/leucine was significantly increased only by naphthenate treatment.

In the case of hydrolysates of ethanol-insoluble material (the protein amino acids), KCHC significantly increased the level of radioactive aspartic acid, glutamic acid, and alanine, while Knap significantly stimulated incorporation of alanine. This effect of the naphthenates and KCHC on protein amino acids is not surprising when it is recalled that protein levels are increased under their influence (77).
Sulphur compounds

A comprehensive list of the sulphur-containing organic compounds present in plants was given by Freney (49). The metabolically important ones included the amino acids cysteine, cystine, and methionine, and the vitamins thiamine and biotin as well as S-adenosyl-methionine, lipoic acid, coenzyme A, and the tripeptide glutathione. Quite a number of other compounds containing sulphur have been identified in plants, but their metabolic role has not been established. These include the mustard oil glucosides of some Cruciferae (49) and the sulpholipid of spinach (14).

Sulphur-containing compounds are extremely important in plants both structurally and metabolically. Tertiary protein structure is determined, in a large part, by the cross linkage and folding caused by the formation of the covalent disulphide bonds of cystine (4). Sulphydryl groups may be the site for attachment of divalent cations which also affect the conformation of proteins (4). The hydrophobic, thioether groups of methionine, by interacting with other hydrophobic groups, can affect the tertiary structure of proteins. Sulphydryl groups may also act as points of attachment for substrates or coenzymes associated with an enzyme (4). Thus, sulphur compounds as components of proteins, and therefore enzymes, are involved in a wide variety of plant activities. Allaway and Thompson (4) reviewed some of the other metabolic activities of nonprotein sulphur compounds. These include roles in fatty acid biosynthesis, oxidation of keto acids, acetylation reactions,
carboxylations, and methyl transfer.

Sulphur uptake

Plants generally obtain sulphur from the soil as sulphate (19,109), but sulphur-containing amino acids can also be an important source for some plants (9). Sulphur exists in the soil as sulphate, organic sulphur compounds, and sulphur-containing minerals such as pyrite (FeS₂), sphaerite (ZnS), chalcopyrite (CuFeS₂), and cobaltite (CoAsS) (21), but in humid and semi-arid regions most of the sulphur is in organic forms (105).

Oxidation of weathered minerals and airborne SO₂, and metabolic degradation of the organic forms by various soil bacteria and fungi, make sulphur available to the plants (49). The microorganisms are strong competitors for sulphur, and if sufficient amounts of carbohydrate are present in the soil to supply them with energy, the microbes can tie up so much sulphur that plants will experience deficiency conditions (49).

Sulphur may enter the soil with rain water (19, 58) and by the addition of fertilizers. Until recently, the use of sulphur-containing pesticides contributed significant amounts of sulphur to the soil, but organic substitutes for these pesticides have almost eliminated this source (19, 40). Near industrial centers sulphur may be obtained as SO₂ through the leaves (43, 102). The sulphur dioxide dissolves in the moist surface of mesophyll cells producing sulphurous acid which is neutralized by inorganic and organic bases, such as amino groups and other nitrogenous compounds, or it may unite with aldehydes to form addition products. These products are subsequently
oxidized to form sulphate (95). Excess sulphur dioxide can be injurious to vegetation (43), and the recent ecological consciousness has caused industries to use low-sulphur containing fuels and to take steps to remove SO$_2$ from smokestack emissions. These changes in practice, along with the increasing use of high analysis fertilizers which contain very little sulphate (40), are expected to produce sulphur deficient conditions in many areas within a few years.

In one of the few studies done with higher plant tissue, Leggett and Epstein (69) found that uptake of sulphate by excised roots of barley exhibited typical enzyme kinetics for the range of external sulphate from 0.005 meq/l to 0.05 meq/l. The uptake was apparently metabolically dependent since anaerobic conditions stopped it. Wedding and Black (107) reported that low temperatures inhibited the uptake of sulphate by Chlorella. Leggett and Epstein (69) found that the sulphate uptake was competitively inhibited by selenate but not by nitrate or phosphate, which indicated considerable specificity on the part of the carrier. They reported, in addition, that double reciprocal plots (Lineweaver-Burk plots) gave a curved line as the concentration of substrate increased. This indicated the presence of additional uptake sites having different affinities for sulphate.

In plants exposed to conditions of sulphur sufficiency ranging from deficient to just adequate, most of the sulphur is present in the form of protein (40). When excess sulphur is available in the soil or nutrient solution, sulphate will
be accumulated (81,88).

**Sulphur translocation**

Once sulphur, as sulphate, was absorbed by the roots, it was apparently translocated to other parts of the plant via the xylem (100). The younger developing leaves of Red Kidney bean were found to accumulate a relatively greater quantity of labelled sulphur than older primary leaves (15). Crafts and Crisp (32) noted that root application of $^{35}$S to bean plants resulted in uniform labelling of the whole plant with slightly higher concentrations in stem apices and margins of leaves. They said that this indicated an apoplastic type of transport through the nonliving continuum of cell walls and xylem vessels.

Levi (70) found that $^{35}$S moved directly out of bean leaf midribs into mesophyll areas with no special accumulation in the veins.

Sulphur translocated into the leaves of a growing plant was very rapidly trapped in newly formed protein leaving very little available for recirculation (15). This phenomenon was probably responsible for the early concept that sulphur was relatively immobile in the plant (109). Kylin (66) found that deseeded wheat plants fed $^{35}$S until the first leaf was half formed and then transferred to S deficient nutrient solution had relocated only small amounts into the deficient third leaves. However, several studies (16,23,98) have shown that sulphur was readily relocated when it was present in excess. Biddulph *et al* (16) using radioactive tracer, have shown that a portion of the total sulphur within the Red Kidney bean
remained mobile and moved freely from one organ to another, and they have suggested that the rate of downward movement of sulphur in the phloem of the stem was similar to that for phosphorus, and exceeded 40 cm/hr. That it does travel down via the phloem was shown by S.F. Biddulph (18), when autoradiographs of stem cross sections from plants fed $^{35}$S via the leaf were superimposed on photomicrographs. The activity was clearly associated with the phloem connected to the treated leaf. Thomas et al (98) have also shown that $^{35}$SO$_4$ is recirculated within the plant. Using an alfalfa plant with several shoots, they dipped the leaves at the tip of one of these shoots into a solution of $^{35}$SO$_4$ for one, two, or five days. The sulphate was absorbed by the immersed leaves and translocated to other parts of the plant. At harvest, a higher concentration of sulphur-35 was found in the leaves of undipped shoots than in the roots of the same plant. Because of the structure of the alfalfa plant, it was clear that the sulphur-35 must have been transferred down the shoot which had been exposed to the radioactive material, through the root or root crown, and up into the unexposed shoots.

Bouma (23) determined that the retranslocation depended largely on the sulphur status of the plant. Mobility increased as sulphur deficient plants recovered from their deficiency and, in addition, mobility was greater in plants grown in nutrient solutions containing sulphur than in plants that suffered sulphur deficiency. It is likely that the nutritional status of the plant is the major factor controlling sulphur mobility. Because of the nature of the compounds containing sulphur, very
little can be mobilized for transfer even when other parts of the same plant are experiencing severe deficiency. Only when there is excess sulphur present, as sulphate or small molecular weight organic compounds, can the plant transfer sulphur from one organ to another.

**Sulphur distribution**

In a review, Thomas *et al* (97) considered the range of sulphur levels normally found in plants. In addition to the values obtained from others, they reported the results of nearly 1000 of their own measurements. They found, in general, that total sulphur was quite variable. The availability of sulphur from the source, either soil or atmosphere, had a significant effect on plant sulphur content. Sulphate values paralleled the totals while organic sulphur levels varied only by a small amount, usually 0.2 to 0.4% on a dry weight basis. Total sulphur for some plants was given as follows: soybeans 0.20 to 0.30% of dry weight, clover 0.21 to 0.30%, alfalfa 0.18 to 0.24%, conifer needles normally had as little as 0.1%, and some plants such as cabbage, rutabagas, rape and radish had 0.40 to 0.60% or more (97). Thomas *et al* (96) found that under adequate sulphur conditions alfalfa tops contained 0.30% sulphur, leaves 0.60% sulphur, and roots 0.20% sulphur. Ensminger and Freney, (40) claimed that the critical percentage (i.e. the minimum concentration of a mineral nutrient in a plant at which there was unrestricted growth) of sulphur probably varied little for a given species under widely varying conditions. They reported the critical percentage for alfalfa
as 0.20% sulphur, and the probable range for most other species as 0.15% to 0.30%.

Thompson et al (99) claimed that over 90% of the organic sulphur in plants was present as cysteine and methionine. However, this was probably not the case for some of the Cruciferae (and Liliaceae) mentioned earlier, in which there were quite large amounts of sulphur-containing organic compounds of no apparent function other than to supply odour or taste. Thomas et al (95) found 60 to 80% of the radioactive sulphur in the plant was translocated to the grain of barley and wheat. Beaton (11), in his review, stated that sulphur was largely present in the leaves of plants before flowering, but at the time of fruit development, the organic sulphur of leaves was converted to sulphate and translocated to the seeds where it was recombined in organic form.

**Sulphate reduction**

Allaway (3) suggested that "the synthesis of sulfur amino acids from sulfates in plants is one of the key reactions in biology, comparable in importance to the reduction of carbon in photosynthesis." Sulphate reduction must occur before cysteine and methionine can be synthesized. The following is a proposed scheme for this reduction (99). For abbreviations see page x.
\[ \text{ATP} + \text{SO}_4^= \rightarrow \text{APS} \]  
\[ \text{APS} + \text{ATP} \rightarrow \text{PAPS} \]  
\[ \text{PAPS} + (\text{Pr(SH)}_2) \rightarrow 3'\text{PAP} + \text{SO}_3^= + (\text{PrS}_2) \] (yeast)  
\[ \text{SO}_3^= \text{ sulphite reductase} \rightarrow \text{S}^= \]  
\[ \text{S}^= + 0\text{-acetylserine} \xrightarrow{\text{sulphydrase}} \text{cysteine} + \text{acetate} \]  

The formation of sulphite as described for yeast in reaction 3 has never been demonstrated in higher plants. It was thought that the sulphite may remain bound to an enzyme complex before undergoing final reduction to \( \text{S}^= \), but free sulphite could also act as a substrate for sulphite reductase. NADPH\(_2\) probably functioned as the electron donor for regeneration of the protein dithiol and for sulphite reductase. Serine was thought to be the acceptor for sulphide, but it has been shown that 0-acetylserine may be a more likely candidate. It was found to be more active than serine, and plants contained a serine acetylase (99). Also, it was found that cysteine inhibited the serine acetylase, and this was taken as evidence for a feedback control within the system.
Calcium

Calcium is frequently the most abundant cation in the soil. Plants obtain calcium either from the soil solution or by direct contact of the roots with the soil particles to which the calcium is bound (91).

Biddulph et al (15) found that calcium moved through the root system more slowly than sulphur. They suggested that the delay could be due to the activity of the absorption system, and the mechanism of transfer across the cortex may be more complex than for sulphur. Absorption of calcium into the nonvacuolated tips of corn roots was found to be nonmetabolic, but uptake in vacuolated sections was strongly temperature dependent and therefore largely metabolic (51). Higinbotham et al (55) showed that the electrochemical gradient for calcium (and magnesium) was from the outside to the inside of the cell in pea and oat roots and concluded that this ion would move passively into the cells. They suggested that calcium may either be excluded from the cells by some mechanism, or if active transport was involved, it would pump the ions out.

A commonly held concept of ion translocation from the root to the leaves is that, after the minerals are loaded into the xylem, the flow of water will carry them along until they are dumped at the site of transpirational loss of the water (33). Biddulph et al (17) and Bell and Biddulph (13) have conducted a number of experiments which contradicted this concept and suggested another mode of transport at least for calcium. They found that the calcium did not move directly
through the xylem vessels of Red Kidney bean, but rather moved as though the xylem and surrounding tissues were acting as an exchange column. Calcium-45 was found in the bark as well as in the xylem area, but only a small amount of this activity became fixed in the bark (17). It was shown that when unlabelled calcium followed the tracer up the stem, exchange activity resulted in movement of the tracer on up the stem to be replaced by cold calcium (17). One other significant observation which argued against the hypothesis of mass flow was that the calcium did not move at the same rate as labelled water (17). Bell and Biddulph (13) found that there was a rapid uptake phase of calcium-45 in their test tissue followed by a slow accumulation phase. When radioactive feedings were followed by cold calcium, the bulk of the radioactive material absorbed during the rapid uptake phase was displaced from this tissue. This was considered to be good evidence for exchange. The mode of translocation proposed by these workers was seen to provide greater control of ion movement, since characteristics of the ion and its exchange sites, utilization of the ion, and growth could all influence the destination of the ions, whereas the mass flow hypothesis offered no means of directing the flow.

Biddulph et al (17) found that calcium-45 fed through the roots of Red Kidney beans was transported most rapidly to the youngest trifoliate leaf set, while the second and oldest trifoliates received progressively less and the primaries least. After six hours, there was less spread in absolute amount of
tracer in each of the sets of leaves, but when expressed as activity on a fresh weight basis, the younger leaves had a definite advantage in securing calcium-45.

Bukovac and Wittwer (28) found that calcium-45 applied to leaf tissue was readily transported out into adjacent parts of the leaf but not to any other organ of the plant. Biddulph et al (15) showed that calcium was almost completely immobile in the phloem of kidney bean. Since calcium remained in the plant part to which it was transported initially, it meant that calcium would have to be supplied to plants continuously to ensure that newly formed tissues received sufficient amounts (15).

Calcium is an essential element for plant growth, and usually a concentration of about 0.5%, on a dry weight basis is considered to be adequate for most plants (82). Concentrations of several percent may be found in the foliage of legumes (2 to 3%) tomato, tobacco (3 to 4%), and other dicotyledonous plants, while cereals and grasses contain from 0.2 to 0.5 percent (61). In both groups, the roots have less calcium than the tops. Although it is present in macro quantities, the established requirement for calcium is so low that it could be considered a micronutrient (29). Epstein (41) noted that algae required very low levels of calcium. Higher plants have been grown successfully in nutrient solutions containing only 0.05 mM calcium (Hoagland solution may have as high as 5.0 mM), but other divalent cations also had to be kept at low concentrations (106). Under normal circumstances, the higher level of calcium may be necessary to offset the toxic effects of some
of the other divalent metals (106).

Calcium has a very weak biochemical identity and has been identified as a component of only one metalloenzyme, α-amylase (41). Even here it apparently can be substituted for by magnesium or strontium (29), and the binding of calcium to the enzyme found in barley was quite weak (41).

Tagawa and Bonner (93) showed that calcium treatment of *Avena* coleoptile tissue repressed the plasticizing effect of indoleacetic acid (IAA) on the cell wall. In contrast, potassium treatment augmented IAA's softening effect. Calcium and potassium had the same effect on the physical properties of pectic materials as they did on cell wall plasticity. Therefore, Tagawa and Bonner concluded that the pectic materials of the cell wall, in association with calcium, may be of importance in determining the mechanical strength of the tissue, and may also have a role in regulating growth (91).

Formation of calcium oxalate crystals may be a method of inactivating toxic organic acids (91).

Epstein (41) considered in some detail the essential role of calcium in maintenance of healthy membranes. Calcium deficiency rapidly led to break down of the physical structure of membranes and to the loss of the membranes' ability to exert control over the movement of other elements and compounds. Epstein thought that this was probably one of the most significant activities of calcium in the plant.

Jones and Lunt (61), Burstrom (29), and Epstein (41) provide more extensive reviews of the present level of understanding of calcium function in plants.
Magnesium

Magnesium is present in the soil as a component of the soil particles (27), as the divalent cation adsorbed to soil particles, or in the soil solution. Magnesium is apparently taken up actively but not at the same sites as calcium, barium, or strontium (42).

Bell and Biddulph (13), in a study of calcium transport in the xylem (discussed earlier) noted that magnesium was able to exchange for calcium in the bean stem. This would suggest that magnesium might move up through the stem by a mechanism similar to that proposed for calcium, but apparently no work has been done in this area yet.

Bukovac and Wittwer (28) found that $^{28}\text{Mg}$ would move into adjacent leaf tissue when applied as a drop but was apparently not translocated to other parts of the plant. On the other hand, Tammes and Van Die (94) found measurable quantities of magnesium in the phloem exudates of Yucca inflorescences, while calcium levels were predictably very low. Steucek and Koontz (89) reexamined this problem and concluded that magnesium was quite mobile in the phloem and was exported from bean leaves at rates similar to those for sulphur. They suggested that one of the reasons, among others, for the failure of Bukovac and Wittwer to observe phloem mobility was that they did not apply enough $^{28}\text{Mg}$ to the leaves. Like sulphur, magnesium is bound into immobile fractions from which it cannot be readily retranslocated even if a deficiency does occur elsewhere in the plant. This factor was also considered to be one of the reasons for confusion over magnesium mobility (89).
An adequate supply of magnesium in most plants is considered to be about 0.2% of dry weight (82). The only major stable compound containing magnesium is chlorophyll which is a magnesium porphyrin (41). Only 10 percent of the leaf magnesium is tied up in chlorophyll, but the chloroplasts contain 50 percent or more of the total magnesium in the leaf. Magnesium is a very important activator of many enzymes including, in particular, those which act on phosphorylated substrates, so its presence in chloroplasts in forms other than chlorophyll is not surprising (41). In many instances of enzyme activation, manganese and some other divalent cations can substitute for magnesium.
MATERIALS AND METHODS

Uptake and Distribution of Sulphur-35

A. Growth of plants

Uniform seeds of the dwarf bush bean plant, *Phaseolus vulgaris* L., cultivar Top Crop, were sown in vermiculite contained in wooden flats, and the flats were placed on benches in a growth room. The seedlings were watered with tap water. Throughout the experiment, conditions in the growth room were maintained as follows: 16.35 kilolux at the top of the plants, a 14 hour photoperiod, a day/night temperature regime of 22 ± 2.5 °C/20 ± 2 °C, and a day/night relative humidity range of 50 to 65%/55 to 70%. The light in the growth room was supplied by cool-white fluorescent tubes (Westinghouse) and 60 watt incandescent lamps.

On the ninth day after sowing, the roots of the seedlings were washed free of vermiculite, and 12 uniform plants were placed in each of four, 20 cm X 25 cm X 15 cm deep plastic trays. The trays were painted black on the outside to reduce algal growth, and the plants' stems were wrapped with cotton and supported in slotted, opaque plastic stoppers sitting in holes cut in the lid of the tray. Four liters of continuously aerated sulphur-free Hoagland-Arnon's solution was put in each tray (Table I).
TABLE I  Volumes of molar stock solutions of major ions used for preparing one liter of a 1X nutrient solution. Modified after Hoagland and Arnon (56).

<table>
<thead>
<tr>
<th>Stock solution</th>
<th>complete</th>
<th>-S</th>
</tr>
</thead>
<tbody>
<tr>
<td>KH₂PO₄</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>KNO₃</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Ca(NO₃)₂.4H₂O</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>2</td>
<td>---</td>
</tr>
<tr>
<td>NaCl (0.1M)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>---</td>
<td>2</td>
</tr>
<tr>
<td>Fe EDTA (5mg/ml)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>A-5b</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

a  deionized water (0.2 ppm, as NaCl)

b. The A-5 micronutrient solution was prepared by dissolving the following in 1 liter of distilled water: 2.86 g H₃BO₃, 1.81 g MnCl₂.4H₂O, 0.22 g ZnSO₄.5H₂O, 0.08 g CuSO₄.5H₂O, and 0.02 g Na₂MoO₄.H₂O.
B. Preparation of, and treatment with, cyclohexanecarboxylic acid solution

A 1 X 10^-2 M potassium salt solution of cyclohexane-carboxylic acid (KCHC) was prepared by dissolving 0.56 g of KOH in 750 ml of water followed by the addition of 1.28 g of CHCA. After stirring for several hours, 3 g of the wetting agent, Tween 20 (polyoxyethylene sorbitan monolaurate) was added, and the volume of solution was brought to one liter.

On the thirteenth day after sowing, the leaves of the bean plants in two trays were sprayed to drip with the 1 X 10^-2 M solution of KCHC. Plants in the other two trays were sprayed with 0.3% w/v solution of Tween 20.

C. Exposure to 35S

Twenty-four hours after spraying, the roots of both treated and control plants were immersed in a continuously aerated, complete nutrient solution containing 35S as Na_2SO_4 (New England Nuclear, Boston, Mass.). For the three sulphur uptake and distribution experiments reported, the level of activity fed was 34 to 37 μc/l.

After exposing the roots for four hours, the radioactive solution was siphoned off into a large receiving flask, and room temperature tap water was poured into the trays. The complete rinsing sequence lasted for one hour and consisted of four changes of tap water, one change of 0.004 M magnesium sulphate solution, and a final change of tap water. After rinsing, the sulphur-free nutrient solution was added to the trays.
D. **Harvest**

At each of the four harvest times (4, 8, 12, and 24 hours after the conclusion of radioactive loading), two plants were taken from each tray. They were quickly separated into three parts: roots, stems plus petioles, and leaf blades. Like parts of both plants from one tray were combined, weighed, and analyzed as a unit. The plant fractions were then placed in separate 100 ml Kjeldahl flasks for digestion.

E. **Digestion**

Fresh or dry plant tissue was placed in a 100 ml Kjeldahl flask and heated gently with 4 ml of \( \text{HNO}_3 \) until the tissue disintegrated. One and one-half ml of \( \text{HClO}_4 \) was added to the mixture and heated (115 to 125°C) until a colourless or slightly yellowish liquid remained. The digest was transferred to a centrifuge tube and neutralized by the addition of KOH pellets to precipitate the perchloric acid as \( \text{KClO}_4 \). When necessary, the solution was brought to pH 6 or less by the addition of small amounts of HCl. The solution was centrifuged at 3000 X g (Sorvall RC2 centrifuge, 4.25 head) for 10 minutes, and the \( \text{KClO}_4 \) precipitate was washed three times with distilled water. The combined supernatants were usually brought to 10 ml by evaporation. To minimize the amount of \( \text{KClO}_4 \) carried over in solution, all the wash steps were carried out at 4°C or less.

As an alternative to the tedious centrifugation procedure, it was later found that filtration through a 4.25 cm Buchner funnel using Whatman #42 filter paper was equally satisfactory.
The precipitate trapped on the filter paper was stirred and washed several times with ice cold water. Under circumstances where the volume of the final solution could be 20 ml or larger, the precipitate could be left in the bottom of the solution.

F. **Radioactive Counting**

A routine procedure was developed for counting radioactive sulphur which provided reliability, convenience, and the efficiency necessary for handling hundreds of samples. A sheet of Whatman #1 filter paper, (4 cm x 7.5 cm) was pleated across its width to form five, 4 cm x 1.5 cm panels (101). The paper was held by forceps or a paper clip attached to one corner, and 0.3 ml of digested sample was pipetted evenly over the surface. The paper was supported upright on a bench by the paper clip and left to dry completely. When dry, the paper was inserted into a scintillation vial filled to the shoulder with a scintillation fluid consisting of 4 g of PPO (2,5-diphenyloxazole) per liter of toluene. The samples could be counted immediately.

The expense of prepared standards and the relatively short half life of $^{35}$S prompted an alternative method of determining counting efficiency. Five leaf samples were digested as described and various amounts of KOH were added ranging from none to complete neutralization. The volumes were brought to 10 ml, and 5 ml of each of these solutions was mixed with 5 ml of a water solution containing a high level of $^{35}$S activity ($6 \times 10^5$ cpm/ml). A sixth was prepared by combining 5 ml of water and 5 ml of the radioactive solution. Duplicate 0.20 ml samples were pipetted onto filter papers, placed in vials as described,
and counted. It was found that a smooth curve was obtained when efficiency was plotted against the values obtained by channels ratio counting. The most efficiently counted sample was considered to be 100%, and all the others were plotted in relation to this one. A long term decay curve was prepared, and each time the "standards" were counted, the count rate was related back to the day they were prepared. Only very minor fluctuations from true decay values were noted over a period of ten months.

All radioactive samples were counted in the same Nuclear Chicago, 720 series, scintillation counter. Sulphur-35 was counted at the same settings as used for carbon-14.

When the activity in a sample was adjusted to 100% with respect to the quench curve, this value was designated dpm (disintegrations per minute) to distinguish it from the cpm (counts per minute) obtained directly from the machine. Although these were not true dpm, the fact that all samples counted were related to one standard value put them on the same relative scale. True counting efficiency for $^{35}\text{S}$ was in the 63 to 65% range.

The use of filter paper, as a carrier of the radioactive sample, offered many advantages and no apparent disadvantages. A very serious problem associated with counting $^{35}\text{S}$ in solution was its tendency to adsorb to the glass walls of the scintillation vials (108). This changed its geometry with respect to the scintillation fluor and resulted in an uncontrolled and unpredictable decline in measured activity (103,108). Calcium-45 and phosphorus-32 were also apparently susceptible to this
error (79). One solution for this problem has been to use a thixotropic gel, such as Cabosil, which prevented the migration of the ions to the glass (108). This seemed to be satisfactory but was messy to handle. Another was to siliconize the scintillation vials to prevent adsorption (79). The $^{35}$SO$_4^-$, immobilized on the filter paper, was surrounded by scintillator and activity declined according to normal decay patterns. Calcium-45 also behaved normally when counted in this way.

Pure toluene based scintillators can not be used for samples containing water (34). The addition of ethanol to the mixture allowed incorporation of limited amounts of water, but efficiency dropped due to quenching from both the water and the ethanol (34). Dioxane based mixtures could accommodate a larger proportion of water (24), but this was a relatively expensive mixture. Use of the filter paper permitted evaporation of the water from a sample, and then the simplest and most efficient scintillator could be used. It was possible to load 0.3 ml of sample on the paper at one time, and larger amounts could be put on if the paper was allowed to dry between loadings (101). The choice of a more absorbent paper, such as some of the fiber-glass types, permitted the addition of larger amounts at each time, but the effects of these thicker papers on counting efficiency have not been examined thoroughly in this investigation.

When the filter paper was removed from a vial, the radioactive sample was removed with it, and the vial usually counted at background levels. It could then be used for a second
sample, resulting in a great saving of cleaning time as well as materials.

The filter paper procedure described was used for counting $^{35}$S ions, $^{45}$Ca$^{++}$ ions, $^{14}$C labelled leucine, $^{35}$S labelled cystine and methionine, and $^3$H labelled DNA. Of these, only methionine was found in the scintillator after the paper was removed, and this activity was barely above background. It appeared that this procedure could be used for a wide variety of isotopes either in the form of inorganic or organic compounds (see also Tonzetich, 101).

Perchloric acid was a strong quenching agent and elimination of it by precipitation improved counting efficiency dramatically. NaOH neutralization was also effective, but it left a large amount of salt dissolved in the solution. POPOP 1,4-Bis-(2-(5-phenyloxazole))-benzene, commonly a constituent of scintillation mixtures, turned green in the presence of small amounts of perchloric acid causing serious colour quenching problems. The beta particles emitted by sulphur-35 have high enough energy that POPOP's contribution to counting efficiency was minimal (103). Therefore, it was omitted from the mixture to avoid the difficulty that could arise if some perchlorate remained in the samples.

Precipitation of sulphate as barium sulphate by the addition of barium chloride, followed by suspension of the precipitate in a Cabosil, dioxane based scintillator (Bray's solution, 24), has been suggested as a means of counting $^{35}$S from large samples of vegetation (108). With small samples,
a considerable loss of barium sulphate occurred when using this procedure. Duplicate samples were impossible to obtain. This was probably due to the fact that very small aggregates of BaSO\textsubscript{4} particles are colloidal in nature (108) and could not be centrifuged into a pellet in the round bottom tubes of the Sorval RC-2 centrifuge.

Dr. J. Tonzetich, of the Faculty of Dentistry at the University of British Columbia, had no difficulty with the precipitation of sulphate as BaSO\textsubscript{4} from saliva. In this case, he used conical centrifuge tubes in a swinging bucket, International centrifuge. This combination of equipment was apparently suitable for bringing down the precipitate. Observation of his procedure led directly to the method developed for counting radioactive samples in this study.

Tonzetich "dissolved" the BaSO\textsubscript{4} in a small amount of ethylenediaminetetraacetic acid (EDTA) solution. The EDTA-BaSO\textsubscript{4} complex was placed on the folded filter paper, dried, and counted as described earlier.

Precipitation of sulphate as BaSO\textsubscript{4} from plant digests, followed by complexing with EDTA would be a way of separating the sulphate from other interfering ions, and the concentration of activity could easily be regulated by varying the amount of EDTA solution used to complex the BaSO\textsubscript{4}. However, it was unnecessary to take these additional steps when the simpler procedure of putting plant digests directly on the filter paper worked satisfactorily.
Uptake and Distribution of Calcium-45

Except for the changes noted below, the methods used for this experiment were the same as those for the sulphur-35 uptake and distribution experiments. In this case, complete Hoagland-Arnon's solution (Table I) was supplied to the plants both before and after the feeding of radioactive calcium.

Harvests were made at 12, 24, and 48 hours after radioactive calcium feeding ceased. At each harvest time, four plants were taken at random from each of the four trays and each plant was analyzed separately. Consequently, there were 8 treated and 8 control plants from each of the harvest times. The plants were separated into roots, stems plus petioles, and leaf blades and weighed immediately. The parts were then sandwiched between paper towels and dried in a fan oven at 60°C for 24 hours. Dry weights of the parts were recorded.

The plant parts were digested as described in section E. After neutralization with KOH, about 0.5 ml concentrated HCl was added to the digest to strongly acidify it, and thus prevent the formation of insoluble calcium hydroxide.

The method used for counting calcium-45 was virtually the same as used for sulphur-35. Calcium standards were prepared as before except that solutions of increasing concentration of perchloric acid were used instead of the plant digests (section F). Both methods produced suitable quench curves.
Total Uptake and Distribution of Nonradioactive Sulphur, Calcium, and Magnesium

G. Growth of plants

Bush bean seeds were sown as described in section A, and placed in a greenhouse on August 9, 1971. Seventy-two liters of complete Hoagland-Arnon's solution was prepared in one large container, and four liters of this solution was placed in each of 18, one-gallon crocks. Nine days after sowing, two of the bean plants were transferred from the vermiculite to each of the crocks. They were supported by cotton batting wrapped around their stems and held in a slot in a wooden lid. The solutions were aerated continuously.

When the plants were two weeks old, uniform specimens were selected and placed one per crock. The leaves of one half of the plants were sprayed to drip with $1 \times 10^{-2}$ M KCHC in 0.3% Tween 20 solution while the leaves of the other half were sprayed with Tween 20 solution. The treated and control plants were placed alternately in two parallel rows along one bench in the greenhouse.

H. Harvest

Three weeks after sowing, five treated and five control plants were harvested, separated into roots, stems plus petioles, and leaf blades. The roots were rinsed in two changes of tap water, blotted to uniform dryness, and the fresh weights of all three organs recorded. The material was dried in a fan oven for 24 hours at 60°C, weighed, and stored in individual plastic bags.
The tissue was ground to a powder with a pestle in a mortar and placed in small vials. The powder was dried and weighed. Approximately 0.4 g aliquots of this powder were taken for digestion and analysis.

A second experiment with some modifications was begun August 27, 1971. Nine-day-old plants were transferred from vermiculite to trays (section A) containing 0.5 X Hoagland-Arnon's solution, and kept in the growth room until they were two weeks old. While still in the trays, the plants were treated with KCHC or Tween 20 as before. Two plants were then transferred to each of the one-gallon crocks and grown in aerated 0.5 X Hoagland-Arnon's solution.

Two plants were harvested from each of eight treated and control crocks one week after treatment. The two plants were combined to give one sample and the same harvesting, drying, and grinding procedure was used as before.

I. Digestion

After digestion with HNO₃ and HClO₄ was complete, 6.0 ml 6N HCl was added to the flask and boiling was continued until all the HCl evaporated. Neutralization with KOH and separation of the insoluble perchlorates was done as described (section E). Reacidification with 0.5 ml concentrated HCl was carried out immediately after neutralization to ensure that no insoluble calcium or magnesium hydroxides were formed. The volume of each digest was brought to 25 ml with water.

Only high nitrate and high perchlorate concentrations in the wet ashed samples were known to interfere with the measurement of total sulphate (59). Perchloric acid was
effectively removed by the precipitation step, and nitrate was apparently converted to volatile nitrosyl chloride by the 6N HCl added at the end of the digestion sequence (59).

J. Analysis of sulphur

The method used for sulphur analysis was a modification of Johnson and Nishita's technique (59). Dean (35) developed a method in which bismuth nitrate was used as the colour reagent rather than the methylene blue used by Johnson and Nishita. Kowalenko and Lowe (64) have modified the equipment to provide simpler handling and more reliable results.

One ml of plant digest was heated in a distillation flask with an hydriodic acid reducing mixture (12). H₂S produced was swept up through a condenser by a stream of nitrogen and trapped in 10 ml of 1N NaOH in a receiving tube. Five ml of bismuth nitrate reagent (12) was added to the NaOH-H₂S solution and bismuth sulphide was formed as a clear brown colloidal dispersion. The absorbance of the bismuth sulphide colloid was measured at 400 nm in a Spectronic 20 colorimetric photometer. Amounts of sulphur were determined from a standard curve prepared from sodium sulphate standards.

K. Measurement of calcium

Calcium was measured at 4227 A with a Perkin Elmer, Model 303 atomic absorption spectrophotometer. Except for digestion, the procedures followed were as described in the machine manual (6).

One-half ml of plant digest and 5 ml of a solution containing 5% w/v lanthanum were combined and brought to a
total of 25 ml. Therefore, the final concentration of lanthanum was one percent. The machine was zeroed with a one percent lanthanum solution. A 10 μg/ml calcium standard was also prepared in one percent lanthanum. Measurements were taken directly from a digital readout device attached to the spectrophotometer which, when adjusted to the standard, gave values expressed as μg/ml.

I. Magnesium measurement

Magnesium was measured with the atomic absorption spectrophotometer at a wavelength of 2852 Å. Magnesium solutions were prepared by diluting one ml of plant digest to 50 ml with distilled water. The machine was zeroed with distilled water. Stock magnesium standards were prepared from magnesium metal turnings as described in the atomic absorption spectrophotometer manual (6). Standards of 2 μg/ml were used to calibrate the measurements.
Distribution of Sulphur-35 in Leaf Fractions

M. Growth of plants

Bean plants were grown, treated with KCHC, and exposed to radioactive sulphur as described in sections A, B, and C. However, there was only one tray of treated plants and one tray of control plants.

N. Harvest

One day after radiotracer loading, three control and three treated plants were harvested. The leaf blades were removed and weighed. They were then placed in an aluminum-foil-lined mortar, frozen with liquid nitrogen, and ground with an aluminum-foil-covered pestle. The foil prevented the ground plant material from sticking to either the mortar or pestle. When grinding was complete, the aluminum-foil was folded slightly and placed in a 400 ml beaker. The beaker was covered with Saran Wrap and stored at -10°C until all plants had been harvested. From the cold storage, the beakers were placed in a freeze-drying unit and left for one day. After freeze drying, the material was collected, weighed, placed in a vial and stored in a desiccator. Additional harvests were made 2 days and 5 days after 35S loading.

A second batch of plants was grown, treated, labelled and harvested. Harvest days were two and three days after 35S labelling.

O. Fractionation of the Leaf Material

a. Approximately 50 milligrams of dry leaf material was weighed accurately, placed in a Kjeldahl flask, and digested
as described in section E. The activity in this sample was used to determine total sulphur (Total S).

b. The remainder of the tissue was weighed, placed in a 20 ml Virtis "45" blender cup with 15 ml of water, blended for three minutes at high medium speed, and poured into 65 ml of boiling 95% ethanol. After boiling 5 to 10 minutes, the material was transferred to a centrifuge tube and centrifuged at 12,000 X g for 10 minutes. The supernatant was decanted and saved, and the residue was washed and centrifuged three times with 80% ethanol. This was a modification of the procedure used by Forest and Wightman (48) for the extraction of free amino acids from bush bean seedlings.

c. The supernatants from part b were combined, and the ethanol was evaporated on a steam bath. The material remaining was transferred to a separatory funnel by rinsing with water and chloroform. The water and chloroform-soluble fractions were separated.

d. The chloroform-soluble fraction from part c was poured into a Kjeldahl flask and the chloroform evaporated. The material was then digested and called the Lipid S fraction (67).

e. The water soluble portion of part c was poured into a Dowex-50 (16-50 mesh) cation exchange column in the H+ condition. The column (a cylinder, 4 cm x 13 cm) contained about 125 ml of resin and was washed with 250 ml of water after the sample had been poured in it. The eluate was collected, reduced in volume by heating on a steam bath, and either
digested or counted directly as the Sulphate S fraction. The column was then eluted with 200 ml of 2N NH₄OH followed by 200 ml of water. The eluate was reduced to dryness in a fan oven at 60°C, transferred to filter paper with a little water, and counted as the Free Amino Acid S fraction (48).

f. About 25 ml of 0.3 M HClO₄ was added to the centrifuge tube containing the residue from part b. The tube was held in a water bath at 75°C for 30 minutes, cooled, and centrifuged. The residue was washed once with 1% HCl, centrifuged, and the two supernatants combined. The material was either digested and counted or counted directly. This was called the Acid Soluble S fraction (67).

g. The residue of part f was considered to be the protein fraction. It was freeze-dried, weighed, and stored in vials in a desiccator until needed. Three, 10-mg aliquots of this material were weighed, and placed in separate small test tubes. Six normal HCl was added, and the tubes were evacuated and sealed. The material was hydrolyzed, in vacuo, at 110°C for 24 hours. The tubes were opened, and the HCl evaporated under vacuum in a desiccator. The contents of two or three of the tubes were combined by rinsing them out with water and filtering all the liquid through a one-cm Buchner funnel. The filtrate was freeze-dried, taken up in a small amount of water, and placed as a 6 cm-long, transverse band about 8 cm from one end of a 7.5 cm x 46 cm strip of Whatman #1 chromatographic paper. The paper was hung in a closed chromatocab, and the chromatogram was developed with a
phenol-water (80:20) mixture in a descending direction. Forest and Wightman (48) developed their chromatograms in 80% phenol in the presence of ammonia vapours. However, the ammonia was omitted here because it gave an undesirable, blue colour reaction with the phenol, and its omission did not interfere with the separation of the sulphur-containing amino acids. After developing for about 24 hours, the chromatogram was dried in a fume hood for two days. It was then cut transversely into 2-cm sections which were pleated and counted in the toluene-PPO scintillator. Authentic methionine, cysteine, and cystine were run concurrently in the same system, and their positions were indicated by dipping the paper strip in a cadmium acetate-ninhydrin reagent (54). Peaks of radioactivity corresponded very well with the location of these amino acids.

h. The remainder of the dried residue from part f was weighed, placed in a Kjeldahl flask, digested, and counted. The results of this step were used to calculate total Protein S.
Fig. 1. Flow sheet for separation of S-35 containing compounds in bean leaves

leaf tissue → weigh 40 mg → place in a Kjeldahl flask

weigh the remainder of the tissue

group with water in a small blender cup, 3 min

pour into boiling 95% ethanol

boil, 5 min

centrifuge, 12,000 × g, 10 min

wash with 80% ethanol, centrifuge (repeat 3X)

residue

CHCl₃ into Kjeldahl flask, evaporate all CHCl₃

digest

count (Lipid S)

reduce volume

digest

count (Sulphate S)

Continued overleaf
Fig. 1. cont.

residue

heat residue with 0.03 M HClO₄, 70°C, 30 min

collect the supernatants

digested

count (Acid soluble S)

centrifuge, 12,000 x g, 20 min

residue

add water and freeze solid

10 mg of dry residue in a small pyrex test tube

freeze dry

10 mg of dry residue in a Kjeldahl flask

add 6N HCl

evacuate and seal the tube

digest

heat at 110°C for 24 hr

count (Protein S)

add water and freeze solid

open the tube and dry in a desiccator

residue in a Kjeldahl flask

digest

filter hydrolysate

freeze dry the filtrate

transfer filtrate to chromatogram

develop with 80% phenol

cut the chromatogram into strips transversely

count the strips (Amino acid S in protein)
RESULTS

A. **Total uptake of sulphur-35 by bean plants**

The roots of fourteen-day-old control and cyclohexane-carboxylic acid-treated bean plants which had been growing in sulphur-free nutrient solution were transferred to a complete nutrient solution containing $^{35}\text{SO}_4^-$. After four hours in the radioactive solution, the roots were rinsed several times with tap water over a period of one hour and returned to sulphur-free nutrient solution. The rinsing was necessary to remove adsorbed $^{35}\text{SO}_4^-$ from the root surfaces. In preliminary experiments, the nutrient solution was checked on the harvest day and no activity was found above background, so it can be assumed that $^{35}\text{SO}_4^-$ had been removed completely and that there was no significant efflux of activity from the plants.

Table II is a summary of the total activity data obtained from three runs of the experiment described in the materials and methods sections A, B, C, and D. CHCA treatment had no significant effect ($p = 0.05$) on total uptake of sulphur-35 from the nutrient solution, although treated plants contained about 6.3 percent more activity than controls.

B. **The distribution of sulphur-35 within bean plants expressed as activity per gram of fresh weight**

In most cases there was a progressive increase in the weight of the organs from one harvest to the next (Table III), but only the weight of treated leaves and stems had increased significantly ($p = 0.05$) over the 24 hour period. The increasing weight of the plant parts due to growth will have
TABLE II

The effect of KCHC on total uptake of sulphur-35 by bush bean plants at various harvest times on a per plant basis.*

<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Total sulphur-35 activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>868,453&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>917,838</td>
</tr>
<tr>
<td>12</td>
<td>890,099</td>
</tr>
<tr>
<td>24</td>
<td>953,813</td>
</tr>
</tbody>
</table>

Mean                        907,813            965,117

Percent of control          106.3

---

*Plants grown in a minus sulphur nutrient solution before and after they were exposed for four hours to a sulphur-35 nutrient solution. Two plants were combined and treated as one.

<sup>b</sup> Disintegrations/minute/plant.

<sup>c</sup> Control: 0.3% Tween 20 spray; Treated: 1 X 10\(^{-2}\)M KCHC spray.

<sup>d</sup> Average of six values.
### TABLE III  Fresh weights of plant parts in sulphur-35 uptake and distribution experiments\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Leaf blades</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>5.745\textsuperscript{b}</td>
<td>5.770</td>
<td>2.752</td>
</tr>
<tr>
<td>8</td>
<td>6.065</td>
<td>5.952</td>
<td>2.862</td>
</tr>
<tr>
<td>12</td>
<td>6.472</td>
<td>6.453</td>
<td>2.947</td>
</tr>
<tr>
<td>24</td>
<td>6.592</td>
<td>6.822</td>
<td>2.945</td>
</tr>
</tbody>
</table>

\textsuperscript{a} See Table II

\textsuperscript{b} Average of 6 values.

\textsuperscript{c} See Table II

A continuous vertical line to the right of a set of numbers indicates values which do not differ significantly from each other at the 0.05 level.
diluted the activity, and values calculated on a per gram basis were sensitive to these changes.

The distribution of sulphur-35 among the three organs, roots, stems plus petioles, and leaf blades, was measured four, eight, 12, and 24 hours from the time $^{35}\text{SO}_4^-$ loading concluded. Because loading lasted four hours, plants at the four hour harvest, for example, had been translocating radioactive sulphur for eight hours. Sulphur-35 activity in leaf blades, expressed as activity per gram of fresh weight, was greater in treated plants than in control plants at each of the harvest times and significantly greater ($p = 0.05$) at the 4 hr and 12 hr harvest (Table IV and Fig. 2). There was no significant differences due to treatment in any of the values associated with roots and stems, or in leaf blades at the 8 hr and 24 hr harvests.

The level of activity at the 24 hr harvest was greater than the activity at the four hour harvest for control leaves, and control and treated stems. In addition, there was a significant decline in sulphur-35 activity in control and treated roots between each of the four harvest times. These results indicated that sulphur was being translocated from the roots to the stem and leaves. They also indicated that some of the activity was becoming incorporated into stem tissue as time passes.

C. The distribution of sulphur-35 within bean plants expressed as activity per plant organ

The total values for activity on a per plant basis are shown in Table II. When these were broken down to give the
<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Leaf blades</th>
<th></th>
<th>Stems</th>
<th></th>
<th>Roots</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>4</td>
<td>72,705^d</td>
<td>83,627*</td>
<td>13,928</td>
<td>14,733</td>
<td>81,889</td>
<td>85,427</td>
</tr>
<tr>
<td>8</td>
<td>76,286</td>
<td>85,058</td>
<td>15,243</td>
<td>14,205</td>
<td>74,906</td>
<td>73,554</td>
</tr>
<tr>
<td>12</td>
<td>74,791</td>
<td>91,711*</td>
<td>15,801</td>
<td>16,571</td>
<td>67,059</td>
<td>67,613</td>
</tr>
<tr>
<td>24</td>
<td>88,073</td>
<td>90,918</td>
<td>22,724</td>
<td>20,468</td>
<td>52,407</td>
<td>51,258</td>
</tr>
<tr>
<td>Mean</td>
<td>77,963</td>
<td>87,578*</td>
<td>16,924</td>
<td>16,494</td>
<td>69,064</td>
<td>69,463</td>
</tr>
<tr>
<td>Percent of control</td>
<td></td>
<td></td>
<td>112.3</td>
<td>97.4</td>
<td>100.6</td>
<td></td>
</tr>
</tbody>
</table>

*a* See Table II.

*b* Disintegrations/minute/gram fresh weight.

*d* Average of six values.

*Significantly different from the comparable control value at the 0.05 level.

Vertical line, see Table III
FIGURE 2 The effect of KCHC treatment on sulphur-35 distribution in bean plants at various harvest times. Graphic illustration of the data in Table IV.
amount in each organ and analyzed statistically, it was found that there was no significant difference in activity between comparable treated and control plants for any of the organs at any of the harvest times (Table V). However, when expressed in this way, there was a significant effect due to harvest time. Leaves and stems steadily accumulated sulphur-35 while roots lost it. There was a significant difference in the level of activity in the 24 hr harvest in every case when compared to the 4 hr harvest. The treated roots showed a significant decline only between 12 and 24 hr. Expressing the data in this way gave an idea of the absolute activity present in each organ. These data were not sensitive to changes in the weight of the organs. It was noted that the level of activity, as indicated by the means, showed that the treated leaves contained more sulphur-35 than controls while roots and stems were almost identical to the controls. It appeared that the extra sulphur-35 taken up by the treated plants (Table II) had been moved to the leaves. This suggested a slightly more rapid movement of sulphur-35 within the treated plants.
<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Leaf blades</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>4</td>
<td>417,080d</td>
<td>481,236</td>
<td>38,671</td>
</tr>
<tr>
<td>8</td>
<td>463,689</td>
<td>494,814</td>
<td>43,593</td>
</tr>
<tr>
<td>12</td>
<td>482,718</td>
<td>589,363</td>
<td>46,877</td>
</tr>
<tr>
<td>24</td>
<td>584,923</td>
<td>614,322</td>
<td>67,129</td>
</tr>
<tr>
<td>Mean</td>
<td>487,102</td>
<td>544,934</td>
<td>49,067</td>
</tr>
</tbody>
</table>

Percent of control:

<table>
<thead>
<tr>
<th>Leaf blades</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td>111.9</td>
<td>99.0</td>
<td>100.1</td>
</tr>
</tbody>
</table>

---

a c See Table II.

b Disintegrations/minute/plant part.

d Average of 6 values.

Vertical line, see Table III
D. The distribution of sulphur-35 among the plant organs as a percent of the total activity in the plant

The percent of total plant activity present in treated leaves at the 8 and 12 hr harvests, as well as the averages of these values, was significantly greater than in the control leaves. Also, roots of treated plants, on the average, contained a significantly lower percentage of the total sulphur-35 in the plant. This means that the treatment had stimulated the rate of translocation of sulphur-35 from the roots to the leaves (see also results, section B). The data are presented in Table VI and Fig. 3.

E. Uptake and distribution of total sulphur

For an explanation of how the plants were grown, treated, and harvested, see materials and methods sections G, H, I, and J.

Analysis for total sulphur according to the method of Kowalenko and Lowe (64) gave the results shown in Table VII. Since there was a difference in the size of the plants between the two experiments, the data from each experiment, expressed as mg of sulphate per plant (or plant part), were presented separately. These data were not analyzed statistically. When the data were expressed as mg of sulphate per g dry weight, the results of both experiments were combined and analyzed statistically. There was no apparent effect of treatment on total uptake of sulphur by these plants or on the distribution of sulphur among the organs.
TABLE VI  The effect of KCHG treatment on sulphur-35 distribution within bean plants at various harvest times on a percent per plant part basis\(^a\).

<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Leaf blades</th>
<th></th>
<th>Stems</th>
<th></th>
<th>Roots</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C(^c)</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>4</td>
<td>48.11(^d)</td>
<td>49.37</td>
<td>4.42</td>
<td>4.16</td>
<td>47.46</td>
<td>46.47</td>
</tr>
<tr>
<td>8</td>
<td>50.49</td>
<td>54.74*</td>
<td>4.87</td>
<td>4.45</td>
<td>44.64</td>
<td>40.81*</td>
</tr>
<tr>
<td>12</td>
<td>54.06</td>
<td>58.23*</td>
<td>5.28</td>
<td>4.89</td>
<td>40.67</td>
<td>36.88*</td>
</tr>
<tr>
<td>24</td>
<td>61.22</td>
<td>63.73</td>
<td>6.96</td>
<td>6.57</td>
<td>31.83</td>
<td>29.70</td>
</tr>
<tr>
<td>Mean</td>
<td>53.47</td>
<td>56.51*</td>
<td>5.38</td>
<td>5.02</td>
<td>41.15</td>
<td>38.47*</td>
</tr>
</tbody>
</table>

\(^a\) See Table II
\(^c\) See Table II
\(^d\) Average of six values
* See Table IV

Vertical line, see Table III
FIGURE 3 The effect of KCHC treatment on percentage distribution of sulphur-35 in bean plant organs at various harvest times. Data from Table VI.
TABLE VII The effect of KCHC treatment on total sulphur uptake and distribution in three week old bean plants.

<table>
<thead>
<tr>
<th>Source of data</th>
<th>Plant part</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
<td>4.61</td>
<td>4.71</td>
</tr>
<tr>
<td>mg/plant part</td>
<td>Stem</td>
<td>1.12</td>
<td>1.16</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>1.76</td>
<td>1.94</td>
</tr>
<tr>
<td>Experiment a</td>
<td>Total/plant (mg)</td>
<td>7.53</td>
<td>7.81</td>
</tr>
<tr>
<td>One</td>
<td>Leaf</td>
<td>61.7</td>
<td>60.5</td>
</tr>
<tr>
<td>%/plant part</td>
<td>Stem</td>
<td>14.9</td>
<td>14.8</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>23.4</td>
<td>24.7</td>
</tr>
<tr>
<td>Experiment b</td>
<td>Total/plant (mg)</td>
<td>4.79</td>
<td>4.65</td>
</tr>
<tr>
<td>Two</td>
<td>Leaf</td>
<td>56.6</td>
<td>60.3</td>
</tr>
<tr>
<td>%/plant part</td>
<td>Stem</td>
<td>15.0</td>
<td>13.1</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>28.4</td>
<td>26.6</td>
</tr>
<tr>
<td>Combined Expt. 1 + Expt. 2</td>
<td>mg/g dry wt.</td>
<td>Leaf</td>
<td>2.82</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>2.14</td>
<td>1.91</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>4.20</td>
<td>4.32</td>
</tr>
</tbody>
</table>

a Plants grown in 1X Hoagland-Arnon's solution for one week after treatment.

b Plants grown in 0.5X Hoagland-Arnon's solution for one week after treatment.

c See Table II
F. The distribution of sulphur-35 among various fractions of bean leaves

These experiments were carried out in an attempt to find whether CHCA treatment had any effect on the distribution of sulphur among various compounds in bean leaves. It was decided to analyze only leaves, because 24 hours after feeding sulphur-35, over 60% of the total activity of the plant was found in the leaves (Table VI). The plants were grown in sulphur-free nutrient solution so that the only sulphur available to the plant had come from the seed. There was no indication of sulphur deficiency in two-week-old plants in these experiments or in any of the other experiments in which plants were grown in sulphur-free nutrient solution. However, it was likely that the plants were sulphur deficient to some extent.

The plants were grown, fed $^{35}$SO$_4^-$, harvested, and the leaves fractionated as described in materials and methods sections M, N, and O. Plants were grown on two separate occasions, and these were referred to as the first and second run of the experiment. For the first run, harvest I, II, and III were taken 1, 2, and 5 days, respectively, after the plants were fed radioactive sulphate. Only harvest II was used from the second run of the experiment.

Table VIII shows the distribution of the activity in each of the fractions at the various harvest times. The activity in each fraction was expressed on the basis of the dry weight of the leaf material. There was significantly ($p = 0.05$) more sulphate S in the control leaves than treated
TABLE VIII The effect of KCHC treatment on sulphur-35 distribution among several fractions of bean leaf tissue at various harvest times.

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Harvest I&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Harvest II&lt;sup&gt;e&lt;/sup&gt;</th>
<th>Harvest III&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Sulphate</td>
<td>83,216</td>
<td>93,596</td>
<td>242,789</td>
</tr>
<tr>
<td>Lipid</td>
<td>24,158</td>
<td>23,346</td>
<td>20,219</td>
</tr>
<tr>
<td>Free amino acid</td>
<td>23,333</td>
<td>21,644</td>
<td>7,982</td>
</tr>
<tr>
<td>Acid soluble</td>
<td>695,159</td>
<td>821,943*</td>
<td>449,028</td>
</tr>
<tr>
<td>Protein</td>
<td>236,695</td>
<td>259,155</td>
<td>378,057</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sulphur-35 activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Harvest I&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>83,216</td>
</tr>
<tr>
<td>Harvest II&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>242,789</td>
</tr>
<tr>
<td>Harvest III&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>26,120</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plants grown in -S nutrient solution before and after a 4 hr feeding of S-35 in a complete nutrient solution.

<sup>b</sup> Disintegrations/minute/gram dry weight.

<sup>c</sup> See Table II.

<sup>d</sup> Average of 3 values.

<sup>e</sup> Average of 6 values.

*Indicates a significant difference between treated and control values at the 0.05 level.

Harvests I, II, and III are 1, 2, and 5 days respectively after sulphur-35 feeding.
FIGURE 4 The effect of KCHC treatment on sulphur-35 distribution among some fractions of bean leaf tissue at various harvest times.
leaves at harvest II. Treated leaves had significantly more acid-soluble sulphur than control leaves at harvests I and II. Treated leaves contained more protein S than control leaves at each harvest, with significantly greater amounts in harvest II and III. Lipid S and free amino acid S values were not significantly different for control and treated leaves at any harvest. However, it was interesting to note that there was a steady decline in the amount of activity in the lipid and free amino acid fractions as time passed. This could be partly due to the diluting effect of growth (see Table IX).

Figure 4 illustrates the distribution of the activity in the three major fractions. It was apparent that protein S content increased at the expense of the acid soluble S and sulphate S fractions. It was also clear from this graph that the treated plants had a higher concentration of sulphur-35 than controls. This was in agreement with the information in results, section B.

G. Cysteine/cystine and methionine incorporation into bean leaf protein

Protein hydrolysates were placed as bands near the end of long strips of chromatographic paper (see materials and methods section 0, part g) and developed with 80% phenol solution for a distance of 25 to 35 cm. All samples from one harvest were run identical distances. When these strips were cut into 2 cm sections and the activity on these sections determined by counting in a liquid scintillation counter, it was found that
### TABLE IX  
Leaf weights, total activity, and recovery of activity in the leaf fractionation experiment.

<table>
<thead>
<tr>
<th>Harvest</th>
<th>Harvest</th>
<th>Leaf dry weight (g)</th>
<th>Total sulphur-35 activity/g&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Sum of five fractions, sulphur-35 activity/g&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Percent recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C&lt;sup&gt;c&lt;/sup&gt;</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>I</td>
<td>run one</td>
<td>.3866</td>
<td>.4194</td>
<td>1,144,163</td>
<td>1,262,266</td>
</tr>
<tr>
<td>II</td>
<td>run one</td>
<td>.4072</td>
<td>.4462</td>
<td>1,148,436</td>
<td>1,290,376</td>
</tr>
<tr>
<td></td>
<td>run two</td>
<td>.4507</td>
<td>.4720</td>
<td>1,243,194</td>
<td>1,208,031</td>
</tr>
<tr>
<td>III</td>
<td>run one</td>
<td>.5892</td>
<td>.5872</td>
<td>733,355</td>
<td>691,550</td>
</tr>
</tbody>
</table>

<sup>b</sup> Disintegrations/minute/gram.

<sup>c</sup> See Table II

<sup>d</sup> See Table VIII
two regions of activity existed. The curves in figure 5 were from harvest II of the first run but were representative of the situation at other harvests as well. Each point was the average of three determinations and the figure illustrated the distribution of the activity on the chromatogram and the relationship of treated to control. According to the positions of the authentic compounds, the two peaks corresponded to the cysteine/cystine group and the methionine group (methionine, methionine sulphone, and methionine sulphoxide can occur). It was arbitrarily decided to separate these two groups at the point between the two which showed the minimum activity. The methionine region occasionally appeared as a sharp peak (methionine) or as a more diffuse peak (a combination of the three possible forms). Attempts to convert all the cysteine/cystine to cysteic acid and all the methionine to methionine sulphone by oxidizing with \( \text{H}_2\text{O}_2 \) and molybdate (36), or with performic acid were generally unsuccessful. Both treatments tended to smear the origin over a wide area and neither was able to convert all of the material into just two clearly separated compounds. The best separation, with the least trouble, was obtained when protein hydrolysate was chromatographed directly.

From Table X it was apparent that KCHC treatment had some effect on the incorporation of sulphur-35 into protein amino acids. The sulphur-35 activity in cysteine/cystine and methionine was greater in control leaves than treated leaves
FIGURE 5 The effect of KCHC treatment on sulphur-35 incorporation into protein amino acids. Data from experiment one, harvest II.
**TABLE X**  
The effect of KCHC treatment on cysteine/cystine and methionine incorporation into the protein fraction of bean leaf tissue.

Sulphur-35 activity

<table>
<thead>
<tr>
<th>Harvest</th>
<th>Cysteine/cystine</th>
<th>Methionine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>I</td>
<td>75,781</td>
<td>65,189</td>
</tr>
<tr>
<td>II</td>
<td>57,790</td>
<td>67,904</td>
</tr>
<tr>
<td>III</td>
<td>69,739</td>
<td>105,270</td>
</tr>
<tr>
<td>Mean</td>
<td>65,275</td>
<td>76,567</td>
</tr>
<tr>
<td>Percent of control</td>
<td>117.3</td>
<td></td>
</tr>
</tbody>
</table>

a  b  See Table VIII

c  See Table II

* Indicates a significant difference between treated and control values at the 0.05 level.

Harvests I, II, and III are 1, 2, and 5 days respectively after sulphur-35 feeding.
at harvest I, but at harvest II and III, the treated leaves contained a greater amount of activity in both amino acids. There was no significant difference between comparable control and treated values at any harvest. The mean value for methionine activity was significantly greater ($p = 0.05$) for treated than control. Total sulphur-35 activity in the protein amino acids of treated leaves was 15.3% greater than in control leaves.

In summary, it was found that KCHC treated bean plants took up slightly more sulphur-35 than control plants and accumulated significantly higher concentrations of sulphur-35 in their leaves than controls. When the activity in each organ, at each harvest time, was expressed as a percent of total activity in the plant, it was clear that treated plants were moving sulphur from the roots to the shoots more vigorously than control plants. Total sulphur uptake over a period of one week after treatment was not significantly affected by the treatment. Treated leaves had higher concentrations of sulphur-35 in the acid soluble and protein fractions, while at the second harvest, control leaves had higher concentrations of sulphate. This indicated that treated plants were converting sulphate into metabolically active compounds more quickly than control plants. Cysteine/cystine and methionine levels were higher in treated leaves than control leaves suggesting the presence of more protein.
H. Total uptake of calcium-45 by bean plants

Control plants accumulated more calcium-45 than treated plants, but the difference was not significant (p = 0.05) (Table XI).

I. The distribution of calcium-45 within bean plants expressed as activity per gram dry weight

Bean plants were grown, treated, fed calcium-45, harvested, and analyzed as described in materials and methods section titled "Uptake and distribution of calcium-45". A treatment effect was found only in the level of activity in the roots (Table XII). The treated roots at 48 hr contained a greater concentration (p = 0.05) of activity than control roots. No significant difference was observed in the level of activity in leaves or stems at any of the harvest times. A significant drop in the level of activity in the leaves at the 48 hr harvest can probably be explained in terms of growth dilution. Relatively small amounts of calcium-45 remained to be translocated into the leaves from the roots and stems after twenty four hours. The increase in the weight of the leaves over this period was sufficient to account for the decline in activity expressed on a per gram basis. The dry weight of leaves at the 48 hr harvest was considerably greater than at the 12 or 24 hr harvests (Table XVII). For example, in the case of the treated plants, there was a 37% increase in weight from the 12 hr harvest to the 48 hr harvest and a 23% drop in calcium-45 activity. The difference between these two numbers indicated that additional calcium-45 was imported by the leaves, but the rate of accumulation between 24 and 48 hr was considerably below the earlier periods.
TABLE XI  

The effect of KCHC treatment on total uptake of calcium-45 by bush bean plants at various harvest times on a per plant basis.

<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Control$^c$</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>203,311$^d$</td>
<td>197,494</td>
</tr>
<tr>
<td>24</td>
<td>217,400</td>
<td>203,796</td>
</tr>
<tr>
<td>48</td>
<td>214,649</td>
<td>200,843</td>
</tr>
<tr>
<td>Mean</td>
<td>211,787</td>
<td>200,711</td>
</tr>
</tbody>
</table>

Percent of control 94.8

---

$^a$ Plants grown in a complete nutrient solution before and after they were exposed for four hours to a calcium-45 nutrient solution. Plants were handled individually.

$^b$ Disintegrations/minute/plant.

$^c$ Control - 0.3% Tween 20 spray; Treated - 1 X 10^{-2}M KCHC spray.

$^d$ Average of eight values for all but 12 hour harvest, C and T leaves, six values.
TABLE XII The effect of KCHC treatment on the distribution of calcium-45 within bean plants at various harvest times on a per gram basis.

<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Leaf blades</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>12</td>
<td>515,188d</td>
<td>514,271</td>
<td>259,364</td>
</tr>
<tr>
<td>24</td>
<td>541,945</td>
<td>561,487</td>
<td>171,709</td>
</tr>
<tr>
<td>48</td>
<td>447,236</td>
<td>418,191</td>
<td>116,665</td>
</tr>
<tr>
<td>Mean</td>
<td>501,451</td>
<td>497,978</td>
<td>182,578</td>
</tr>
<tr>
<td>Percent of control</td>
<td>99.3</td>
<td>91.5</td>
<td>133.9</td>
</tr>
</tbody>
</table>

a See Table XI
b Disintegrations/minute/gram.
c See Table XI
d Average of eight values for all but 12 hour harvest, C and T leaves, six values.

Vertical line, see Table III.
J. The distribution of calcium-45 within bean plants expressed as activity per plant organ

The absolute levels of calcium-45 in the various organs of the plant are given in Table XIII. A significantly greater amount of calcium-45 was found in the stems of control plants than treated plants at the 12 and 24 hr harvest and in the mean value for the stems. The leaves of control plants contained more calcium than leaves of treated plants. On the other hand, the mean value for the roots at all harvests showed significantly greater activity in the treated plants than in the controls. The situation here appeared to be just opposite to that for sulphur uptake and distribution. Leaves continued to accumulate calcium-45 up to the 48 hr harvest (Table XIII). These data expressed the amount of activity in the organ and were, therefore, insensitive to changes in the weight of the organ. However, it was clear that the increase in activity in the leaves over the final 24 hours of the experiment was minimal. Significant decreases in the level of activity in the stems from the 12 hr harvest to the 48 hr harvest suggested that the stems were contributing the calcium being accumulated by the leaves. Very little decline in root levels occurred over the period of the experiment.
TABLE XIII  The effect of KCHC treatment on calcium-45 distribution within bean plants at various harvest times on an activity per plant part basis\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Leaf blades</th>
<th></th>
<th>Stems</th>
<th></th>
<th>Roots</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
<td>C</td>
<td>T</td>
</tr>
<tr>
<td>12</td>
<td>162,273\textsuperscript{d}</td>
<td>157,726</td>
<td>32,131</td>
<td>28,325\textsuperscript{*}</td>
<td>8,276</td>
<td>10,465</td>
</tr>
<tr>
<td>24</td>
<td>186,076</td>
<td>172,048</td>
<td>24,112</td>
<td>20,863\textsuperscript{*}</td>
<td>7,996</td>
<td>10,089</td>
</tr>
<tr>
<td>48</td>
<td>188,250</td>
<td>173,785</td>
<td>18,920</td>
<td>17,302</td>
<td>7,479</td>
<td>9,756</td>
</tr>
<tr>
<td>Mean</td>
<td>178,866</td>
<td>167,853</td>
<td>25,054</td>
<td>22,163\textsuperscript{*}</td>
<td>7,917</td>
<td>10,103\textsuperscript{*}</td>
</tr>
</tbody>
</table>

Percent of control: 93.8 88.5 127.6

\textsuperscript{a} See Table XI

\textsuperscript{b} Disintegrations/minute/plant part.

\textsuperscript{c} See Table XI

\textsuperscript{d} See Table XI

Vertical line, see Table III
K. The distribution of calcium-45 among the plant organs as a percent of the total activity in the plant

The data from Table XIII were recalculated to give the percentage distribution of calcium activity in each organ. When viewed in this manner (Table XIV), the same pattern of distribution and translocation appeared as in Tables XII and XIII. The steady accumulation of calcium-45 by the leaves was largely at the expense of the stems, while the roots apparently contributed very little. The very minor decrease in activity of the roots from 12 to 48 hr suggested that some of the calcium-45 became incorporated into the root tissue.

L. The uptake and distribution of total calcium

Plants were grown, treated and harvested as described in materials and methods sections G, H, I, and J. The effect of KCHC treatment on total calcium uptake and distribution was detailed in Table XV. The data from Experiment One and Experiment Two, expressed as mg of calcium per plant (or plant part), were presented separately in the table. These data were not analyzed statistically. When the amount of calcium in the plants was expressed as mg/g dry weight, the data from both experiments were combined and analyzed statistically. There were no apparent differences in the amount of calcium present in the plant tissue due to treatment regardless of how the data were expressed.

The percentage distribution of ordinary calcium within the plant was not unlike that for the radioactive calcium at the 12 hour harvest. As time passed, a greater percentage of the radioactive calcium was accumulated by the leaves. The
TABLE XIV  The effect of KCHC treatment on calcium-45 distribution within bean plants at various harvest times on a percent basis\(^a\).

<table>
<thead>
<tr>
<th>Harvest time in hours</th>
<th>Leaf blades</th>
<th>Stems</th>
<th>Roots</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C(^c)  T</td>
<td>C  T</td>
<td>C  T</td>
</tr>
<tr>
<td>12</td>
<td>79.6(^d) 79.5</td>
<td>16.1(^c) 15.0</td>
<td>4.2 5.5</td>
</tr>
<tr>
<td>24</td>
<td>85.1 84.0</td>
<td>11.3(^c) 10.6</td>
<td>3.6 5.5</td>
</tr>
<tr>
<td>48</td>
<td>87.6 86.3</td>
<td>9.0(^c) 8.7</td>
<td>3.4 5.0</td>
</tr>
</tbody>
</table>

\(^a\) See Table XI
\(^c\) See Table XI
\(^d\) See Table XI

Vertical line, see Table III
TABLE XV The effect of KCHC treatment on total calcium uptake and distribution in three week old bean plants.

<table>
<thead>
<tr>
<th>Source of data</th>
<th>Plant part</th>
<th>Control c</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
<td>43.885</td>
<td>41.801</td>
</tr>
<tr>
<td>mg/plant part</td>
<td>Stem</td>
<td>6.693</td>
<td>7.150</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>3.022</td>
<td>2.968</td>
</tr>
<tr>
<td>Experiment a</td>
<td>Total/plant (mg)</td>
<td>53.580</td>
<td>51.919</td>
</tr>
<tr>
<td>One</td>
<td>Leaf</td>
<td>81.9</td>
<td>80.5</td>
</tr>
<tr>
<td>%/plant part</td>
<td>Stem</td>
<td>12.5</td>
<td>13.9</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>5.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Experiment b</td>
<td>Total/plant (mg)</td>
<td>34.489</td>
<td>33.459</td>
</tr>
<tr>
<td>Two</td>
<td>Leaf</td>
<td>78.3</td>
<td>79.3</td>
</tr>
<tr>
<td>%/plant part</td>
<td>Stem</td>
<td>13.6</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>8.2</td>
<td>7.2</td>
</tr>
<tr>
<td>Combined Expt. 1 + Expt. 2 mg/g dry wt.</td>
<td>Leaf</td>
<td>27.480</td>
<td>26.273</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>13.500</td>
<td>13.024</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>8.248</td>
<td>7.809</td>
</tr>
</tbody>
</table>

a b See Table VII

c See Table II
differences may be explained by the fact that the radioactive data represented the distribution of calcium obtained over one short period of time, while the distribution of cold calcium was the result of all calcium movements from the time of germination.

The plants analyzed in this experiment accumulated calcium for two weeks before treatment with KCHC and for one week after treatment. If there was going to be an effect due to treatment, it had to occur during the third week. When the dry weight data from the two-week-old plants used in the calcium-45 uptake and distribution experiments (12 hr harvest) were compared with the dry weights for the three-week-old plants used in this experiment (Table XVII), a three to five fold increase in weight was noted for the whole plant during the third week. It seems reasonable that the uptake of minerals during the third week would also amount to several times as much as was accumulated in the first two weeks when the plants were quite small. If treatment had a direct effect on uptake, it might be expected to show up during the third week, but this was not evident.

In summary, it was found that treated plants took up somewhat less calcium-45 than control plants, but retained significantly more of it in their roots. Total calcium uptake by control plants over a period of one week after treatment was not significantly greater than in treated plants. The distribution of calcium within these plants was also very similar for treated and control.
M. The uptake and distribution of total magnesium

The same plants used for total sulphur and calcium uptake were analyzed for magnesium content. For details, see materials and methods sections G, H, I, and J.

The data from Experiment One and Experiment Two, expressed as mg of magnesium per plant (or plant part), are presented separately in Table XVI. These data were not analyzed statistically. When the amount of magnesium in the plants was expressed as mg/g dry weight, the data from both experiments were combined and analyzed statistically.

There was slightly more magnesium in control plants than treated plants. When expressed as mg of magnesium/g dry weight, there was significantly more in control leaves than treated leaves.

N. Comparison of fresh weight, dry weight and dry weight as a percent of fresh weight

Table XVII contains average values for fresh weight, dry weight, and dry weight as a percent of fresh weight for the experiments in which dry weight was determined. For the total sulphur, magnesium, and calcium experiments, it was clear that the plants in Experiment One weighed more than those in Experiment Two. The plants in the second experiment were grown on 0.5X Hoagland-Arnon's solution as opposed to the 1X strength solution used in Experiment One. In addition to this, the plants of Experiment Two were grown later in the season when light levels were not as high as for Experiment One.
TABLE XVI  The effect of KCHC treatment on total magnesium uptake and distribution in three week old bean plants.

<table>
<thead>
<tr>
<th>Source of data</th>
<th>Plant part</th>
<th>Control</th>
<th>Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaf</td>
<td>9.517</td>
<td>9.110</td>
</tr>
<tr>
<td>mg/plant part</td>
<td>Stem</td>
<td>1.025</td>
<td>1.121</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>1.519</td>
<td>1.690</td>
</tr>
<tr>
<td>Experiment a</td>
<td>Total/plant</td>
<td>12.059</td>
<td>11.920</td>
</tr>
<tr>
<td>One (mg)</td>
<td>Leaf</td>
<td>78.9</td>
<td>76.4</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>8.5</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>12.6</td>
<td>14.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>6.031</td>
<td>5.912</td>
</tr>
<tr>
<td>mg/plant part</td>
<td>Stem</td>
<td>7.58</td>
<td>7.47</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>1.690</td>
<td>1.466</td>
</tr>
<tr>
<td>Experiment b</td>
<td>Total/plant</td>
<td>8.479</td>
<td>8.125</td>
</tr>
<tr>
<td>Two (mg)</td>
<td>Leaf</td>
<td>71.2</td>
<td>73.1</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>9.4</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>19.9</td>
<td>17.9</td>
</tr>
<tr>
<td>Combined</td>
<td>Leaf</td>
<td>6.069</td>
<td>5.783*</td>
</tr>
<tr>
<td>Expt. 1 +</td>
<td>mg/g dry wt.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Expt. 2</td>
<td>Stem</td>
<td>2.063</td>
<td>2.126</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>4.721</td>
<td>4.611</td>
</tr>
</tbody>
</table>

\[\text{a} \quad \text{See Table VII.}\]

\[\text{b} \quad \text{See Table II.}\]

\[\text{c} \quad \text{Significantly different from the comparable control value at the 0.05 level.}\]
TABLE XVII  A comparison of fresh weight, dry weight, and the dry weight as a percent of fresh weight of plants used in total uptake and distribution of sulphur, calcium, and magnesium, and the uptake and distribution of calcium-45 experiments.

<table>
<thead>
<tr>
<th>Experiment description</th>
<th>Plant part</th>
<th>Fresh Weight (g)</th>
<th>Dry Weight (g)</th>
<th>Dry weight as a percent of fresh weight</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>C</td>
<td>T</td>
<td>C</td>
</tr>
<tr>
<td>Total Ca, S, and Mg.</td>
<td>Leaf</td>
<td>16.01</td>
<td>15.63</td>
<td>1.74</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>7.23</td>
<td>7.06</td>
<td>.58</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>9.45</td>
<td>9.70</td>
<td>.46</td>
</tr>
<tr>
<td>Expt. 1.</td>
<td>Leaf</td>
<td>9.90</td>
<td>10.11</td>
<td>.95</td>
</tr>
<tr>
<td></td>
<td>Stem</td>
<td>4.47</td>
<td>4.66</td>
<td>.33</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>6.33</td>
<td>6.01</td>
<td>.30</td>
</tr>
<tr>
<td>Calcium-45 uptake and distribution 12 hour</td>
<td>Leaf</td>
<td>3.01</td>
<td>2.83</td>
<td>.3109</td>
</tr>
<tr>
<td>harvest</td>
<td>Stem</td>
<td>1.56</td>
<td>1.44</td>
<td>.1248</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>2.39</td>
<td>2.25</td>
<td>.1477</td>
</tr>
<tr>
<td></td>
<td>Leaf</td>
<td>3.43</td>
<td>3.07</td>
<td>.3417</td>
</tr>
<tr>
<td>24 hour harvest</td>
<td>Stem</td>
<td>1.76</td>
<td>1.52</td>
<td>.1417</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>2.46</td>
<td>2.33</td>
<td>.1574</td>
</tr>
<tr>
<td>48 hour harvest</td>
<td>Stem</td>
<td>1.99</td>
<td>1.92</td>
<td>.1628</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>2.70</td>
<td>2.51</td>
<td>.1698</td>
</tr>
</tbody>
</table>
The dry weight as a percent of fresh weight for the roots was quite consistent in both runs of the total calcium, sulphur, and magnesium uptake experiment, but the stem and leaf values were lower in run two. The difference in water content of the shoots may have been the result of different conditions which existed at the harvest times. This notion was strengthened when examining the data for percent dry weight from the three harvests of the calcium-45 uptake and distribution experiments. The values for the leaves of 24 and 48 hr harvests were very similar but were lower than the value for the 12 hr harvest. The 12 hour harvest was made just at the end of the daily light cycle when temperatures were lower, while the other two were harvested about midway through the cycle. The conditions at the different times of the daily cycle could have an influence on the water content of the tissue.

Although these data were not analyzed statistically, it appeared that treatment with KCHC had no effect on the dry weight as a percent of fresh weight.
DISCUSSION

For this discussion, it is assumed that the effect of cyclohexanecarboxylic acid on plant metabolism is representative of the effect of naphthenic acid treatment. This is a reasonable assumption since CHCA qualifies as a naphthenic acid on the basis of structure (60), and it has been found in the naphthenic acid mixture (38). Moreover, studies of plant responses to CHCA treatment have revealed that differences are in degree rather than in character (84,113).

Sulphur uptake

Active accumulation of ions by roots from soil or a solution culture is accomplished by expenditure of energy obtained from the respiratory activities of root cells (91).

Naphthenic acid treatment has been shown to stimulate processes which are associated with increased energy levels in the plant. Increased rates of photosynthesis and dark respiration were found in the shoots of Knap treated beans at 7, 14, and 21 days after treatment (47), and increased photosynthesis was reported for treated grape (63). Increased production of photosynthate can be implied from studies which showed significant increases in vegetative or reproductive growth due to naphthenate (47,112,113) or CHCA treatment (113).

In addition to increased levels of metabolites, the activity of some enzymes associated with energy production has been stimulated by naphthenates. Fattah and Wort (47) reported stimulated phosphorylase and phosphoglycerate kinase activity due to Knap treatment and increases have also been found for cytochrome oxidase (114). It was suggested that increased
rates of photosynthesis would lead to a greater supply of reduced nucleotides, ATP, and photosynthate for use in biochemical processes, and the hexose phosphate supply would be increased due to the stimulated phosphorylase activity (47).

Increased cytochrome oxidase activity implies more active electron transport. It is likely that increased metabolic activity of the shoot causes stepped-up rates of transport of metabolites to the roots where they are utilized in the energy requiring process of ion uptake.

It would not be surprising to find that CHCA treatment had stimulated uptake of sulphur-35 from the nutrient solution. However, the increase in uptake, when compared to the control, amounted to only 6.3 percent and did not differ significantly from the amounts accumulated by control plants. There were several possible reasons for these results. The increased metabolic activities mentioned above have all been reported for plants at least seven days after treatment. It was entirely possible that not enough time had passed to allow the treatment to become fully effective. There was the second possibility that the shoots were in a better competitive position with respect to increased metabolites for the first two or three days after treatment. Padmanabhan (76) has found that after one week 72% of the $^{14}$C activity remained in the primary leaves of bean plants which had received a foliar application of KCHC-$^{14}$C. She also observed that only a very small amount of the KCHC-$^{14}$C activity spotted on primary leaves of bean had been translocated to the roots. As yet it is not known
how much CHCA must be present in a tissue to be effective as a stimulator of metabolic activity. The fact that there is only a small amount of CHCA in an organ is not necessarily a reason for low activity, but it could be.

**Sulphur distribution**

Under the conditions of the experiments, a significantly greater amount of sulphur-35 was translocated to the leaves of treated plants than control plants. This was true when the data were expressed as activity per gram fresh weight or as a percent of total plant content. When the total activity in control and treated leaves was compared, the treated leaves had more, but the difference was not significant. The lack of significance in the latter figures may indicate greater variability among leaf fresh weights than among the values for concentration of activity in each leaf. These results suggested that the sulphur absorbed by treated plants over and above the amount obtained by the control plants was moved to the leaves rather than being distributed evenly among the three organs.

Since sulphate moves by mass flow in the xylem stream (33), translocation to the leaves would be a function of the rate of xylem loading in the roots, if transpirational loss of water were constant. The rate of xylem loading would be a function of the availability of sulphate and the metabolic activity of the cells responsible for loading. Since treated plants may have higher metabolic levels and they did take up more (not significantly more) sulphate than control plants,
it might be expected that sulphate was more available to the xylem sap of treated plants. When this extra sulphate reached the leaves, the small difference in uptake became a significant difference in sulphur levels in the leaves.

Severson (83) reported an experiment in which the rate of transpiration was decreased in Knap-treated plants. It is not known whether KCHC treatment has the same effect. If it does, it would likely slow the rate of sulphate movement to the leaves in the transpiration stream rather than accelerating it. However, rate of flow of the xylem sap is only one factor which determines translocation rates. The concentration of the sulphate would also affect delivery rates, and conceivably, the sulphur-35 levels in xylem sap might have been somewhat higher in treated than in control plants. It would be interesting to compare the respective levels of activity in the xylem sap to test this idea.

Naphthenate treatment had a significant effect on the amount of $^{32}$P translocated from the root to the shoot of bean plants (83). Severson suggested that phosphorus translocation could have occurred in the symplast where the increased energy production associated with naphthenate treatment could contribute to more rapid phosphorus translocation. A strong sink effect due to phosphorus deficiency in the leaves was considered to be an important factor in this mechanism (83). Certainly, the same explanation could be put forward for the increased rate of movement of sulphur to the leaves, but it does not seem necessary.
Sulphur-35 distribution within leaf fractions

Within twenty-four hours of feeding sulphur-35 to two-week-old bean plants which had been growing in sulphur-free nutrient solution, 60 to 65% of the sulphur was present in the leaves (Table VI). This sulphur activity could be separated into five fractions.

Extraction with 80% v/v ethanol was expected to remove free amino acids (90), sulphate (107), and lipid associated sulphur (107). Subsequent extraction of the residue with a mixture of hot ethanol and toluene (3:1) (67) confirmed that all lipid associated with sulphur-35 had been extracted by the 80% ethanol. After the ethanol was evaporated, the lipid associated sulphur was separated from the other two fractions by extraction with chloroform.

The water soluble portion of the ethanol extract was passed through a cation exchange column to separate the free amino acids, which were trapped on the resin, and the sulphate which had passed through (87).

The residue from the ethanol extraction was heated with 0.3 M HClO₄ to 75°C for 30 minutes (67). The supernatant contained something called sulphur bound to DNA, or DNA sulphur, by Hase et al (52, 53). They had extracted DNA with hot perchloric acid and had found sulphur in the extract. In this investigation large amounts of activity were found in this fraction at some harvests, but separation of DNA by Marmur's method (73) revealed that very little sulphur-35 was associated directly with DNA. As the protein was removed from the DNA in purification steps, the level of activity also
declined. Consequently, sulphur in this fraction was simply referred to as acid-soluble sulphur.

The residue remaining was considered to be protein sulphur (67). Hydrolysis of this material yielded ninhydrin responsive substances and the only sulphur-containing compounds in the hydrolysate were apparently cysteine/cystine and methionine.

The bulk of the sulphur-35 activity was found in the protein, acid soluble, and sulphate fractions, and there were major changes in the amounts present in each of these fractions from day to day. From figure 3 it can be seen that sulphur was moving from the acid soluble fraction into the protein fraction. Sulphate levels increased until day two and fell thereafter.

It is difficult to determine where sulphate sulphur fits into the sequence of transfer. Sulphur is apparently delivered to the leaves as sulphate (100). It must then be reduced and incorporated into cysteine from which it may be converted into methionine or some other forms (99). The cysteine and methionine formed will presumably become part of the free amino acid pool until incorporated into protein or metabolized in other ways. There are at least two possible sequences for the movement of sulphur through the various fractions.

I. 

\[
\text{Sulphate S} \xrightarrow{\text{reduction}} \text{Acid soluble S} \xrightarrow{} \text{Sulphate S} \xrightarrow{} \text{Free amino acid S} \xrightarrow{} \text{Protein S}
\]
II. Sulphate S $\xrightarrow{\text{reduction}}$ Acid soluble S $\xrightarrow{\text{reduction}}$ Free amino acid S $\xrightarrow{}$ Protein S

Scheme I gives the acid soluble portion the role of storage sulphur. Remembering that the plants in this investigation were initially, and subsequently, low in sulphur, it was possible that during the sulphur feeding period, there was a very rapid influx of sulphur and the leaves may have needed to find a way to store it. Sulphate levels increased slightly as acid soluble levels dropped drastically between day one and two, and then both declined together for the next three days. These observations would be consistent with scheme I.

Scheme II assigns a more significant role to the acid soluble fraction. A rate-limiting step in the reduction of sulphate or formation of cysteine could have caused the pile up of an acid-soluble intermediate compound. Apparently there is a known feedback mechanism in which cysteine inhibits the formation of O-acetylserine (99) thus regulating its own formation. This mechanism would keep cysteine levels low and slow up preceding steps. If the acid soluble sulphur was one of the intermediates in the reduction of sulphate, it could have increased in quantity until protein formation caught up with the supply. Chromatography of the free amino acid fraction revealed that cysteine levels were quite low in these leaves.

There have been other reports of significant amounts of acid-soluble sulphur, although the extraction procedures were not exactly as they were here (68,95). Moreover, it should be emphasized that the amount of acid-soluble sulphur-35
reported here (70% of the total at harvest I) was much larger than in the other studies. The high level of acid-soluble sulphur-35 was likely a result of the particular experimental conditions experienced by these plants. Identification of these compounds promises to be a very interesting area for future research especially since CHCA treatment had such a pronounced effect on their formation.

At harvest II, the leaves of control plants contained significantly less acid-soluble S and protein S. This indicated that treatment had caused a more rapid conversion from the metabolically inactive sulphate form to more useful organic forms. Conversion of sulphate to protein (either via the acid-soluble fraction or directly) would require considerable expenditure of energy. From previous discussion, it may be recalled that treated leaves were probably in a better position to supply this energy, and it might be expected that they would incorporate sulphur into the amino acids more rapidly than control leaves. In treated plants the higher level of sulphur in protein and lower level in sulphate agreed with this concept. The increased rates of photosynthesis and respiration mentioned earlier (47) would contribute NADPH$_2$ and ATP which are needed for the reduction of sulphate.

At harvest III, five days after sulphate feeding, the sulphate S and acid-soluble S fractions were nearly depleted. The protein fraction contained more activity than it did at the second harvest and the treated plants once again showed a greater amount of activity than controls. However, the total amount of activity in the leaves had dropped when measured on
a per gram basis. It was clear that some of this drop was due to the diluting effect of growth, but at the same time there was also a decrease in the absolute amount of sulphur-35 in these leaves. The appearance of the plants and the presence of some sulphate in the leaves showed that they had not yet used up all their sulphur, but there was probably a sulphur stress building again. Some of the decline in activity in the leaves could be attributed to mobilization of sulphate and its retranslocation to other parts of the plant, but it was not known if this was the case. Plants grown for seven days under the conditions of these experiments were beginning to show visible signs of sulphur deficiency.

It appeared from these results that KCHC treatment did have a positive effect on the rate of conversion of imported sulphate into protein. It was not likely though, that it was this activity which accounted for the general growth effects of KCHC found in other studies. Instead, they were probably a reflection of stimulated activity occurring elsewhere. A growing body of evidence obtained in our laboratory and elsewhere (46) points to the likelihood that naphthenates stimulate metabolic activity at the genetic level. This activity in turn would promote the rate of formation of enzymatic protein and subsequently most other cellular activities (77). The increased incorporation of sulphur-35 in protein observed here was an obvious manifestation of this activity. The fact that protein formation is so vitally dependent upon sulphur (37) makes it a good test parameter for studies of naphthenate
effects on protein formation.

Recovery of sulphur-35 activity from the fractions was not 100% when compared to a value obtained for the total activity in the fraction (Table IX). Recoveries were usually of the order of 90% or better, but the fate of the other 10% was not known. It was assumed that all fractions were affected equally. Certainly, all were treated with equal care. One source of difficulty could be that the small sample taken for total sulphur measurements amounted to less than 10% of the leaf material. A small error in this measurement could have been magnified when calculations were made to convert the value to total activity. Variations in recovery between treated and control leaves affect the numbers slightly but not the general conclusions.
The effect of KCHC treatment on the incorporation of sulphur amino acids into protein

KCHC-treated leaves incorporated more radioactive cysteine and methionine into protein than control leaves. The average incorporation of methionine for the three harvests was significantly greater in treated than in control. Several studies have found that protein levels were higher in treated plants than controls (62, 77, 78, 114), and the treated plants in this study were no exception. The higher level of sulphur-35 in the protein fraction of treated leaves compared to control leaves implied that there was more protein in the treated leaves on a per gram basis. Again, the effect of treatment could be indirect. If naphthenate treatment caused increased production of DNA and RNA, it almost surely would lead to increased protein production. Increased levels of enzymatic protein would cause increased metabolic activity.
Calcium-45 uptake and distribution

Calcium-45 uptake is shown in Table XI. Since the feeding time was the same for each plant, it was possible to make a comparison of total uptake. In this case, control plants took up 5.2% more calcium than treated plants, but the difference was not significant.

Distribution of calcium-45 within the plant gave results which were in contrast to the sulphur-35 data. When the concentration of activity was considered (i.e. calcium-45 activity per gram dry weight) the leaves of treated and control contained equal amounts of calcium-45, on the average, although there was some variation from one harvest to the next. The stems of control plants contained more calcium-45 than the stems of treated plants, and the roots of treated plants have retained significantly more calcium-45 than the controls. Either the roots of control plants used less calcium than the roots of treated plants, or it was transported out of control roots faster than treated roots. The decrease in level of calcium-45 in roots from 12 to 48 hours was very slight. Apparently the relocation observed at these harvest times and for these experimental conditions was mainly from stem to leaf.

The calcium content of stems constituted a relatively high percentage of the total calcium at the 12 hour harvest and declined as time passed, whereas sulphur-35 content of stems increased with time. The calcium in stems was probably exchangeable calcium and was moved slowly up the stem as non-radioactive calcium came up from below to replace it. This was in agreement with the concept of calcium translocation.
described by Bell and Biddulph (13).

The significantly greater amounts of calcium-45 retained by the treated roots may be due to increased trapping or utilization. If treatment caused increased formation of organic acids, or stepped-up secretion of hydrogen ions, cations such as calcium would become associated with these negatively charged acids and be retained in the tissue.
Uptake of nonradioactive sulphur, calcium and magnesium

Foliar application of $1 \times 10^{-2}$ M KCHC had no effect on the total amount of sulphur, calcium, and magnesium taken up by bush beans from a complete nutrient solution over a period of one week. These results were consistent with the earlier observation that neither sulphur-35 nor calcium-45 uptake over a four hour period was affected by a KCHC treatment applied 24 hours before the radioactive material was administered to the roots. Similarly, Severson (83) found that a 5000 ppm foliar application of Knap had no effect on the uptake of $^{32}\text{P}$ when the plants were grown either in phosphate-free or complete nutrient conditions. In contrast, workers in Bulgaria and the USSR have found that various naphthenate treatments have stimulated the uptake of mineral elements from soil.

Increased uptake of nitrogen and phosphorus, due to naphthenate treatment, has been reported for cabbage (7), cotton (8), potato (1), and tomatoes (2). Peterburgsky and Karamete (78) found that naphthenates' effect on N, P, and K uptake was greatest for maize plants growing in solution culture, somewhat less in sand culture, and least in soil. These results would appear to be a direct contradiction of the results of Severson (83) and the others mentioned above. Differences in species, treatment, growing periods, and environmental factors could all be reasons for these differences, but the uncertain nature of our knowledge in this area is evident.

If treatment with KCHC stimulated metabolic activity, it may be asked why there was no evidence of this increased activity in higher levels of inorganic ion uptake. Setting aside
Peterburgsky and Karamete's findings for the moment and considering the results of this study and some others, it may be possible to offer some explanation. Normally, plants will accumulate more ions than they need. This is quite possible from a nutrient solution in which ions are present in readily available forms. Therefore, increased metabolic activity due to CHCA treatment would not necessarily produce a sink effect which would lead to noticeably different rates of ion uptake by plants growing in solution culture. Furthermore, measurement of the total amount of an ion present in a tissue is no indication of its metabolic involvement. It is necessary to determine the chemical form in which the element exists. For example, sulphur, as sulphate, probably would not contribute to the metabolic activity of the plant whereas sulphur in protein would. Two similar plants could contain the same amount of sulphur, but the more active metabolically would probably have more in the form of protein.

Although there is now much evidence that naphthenate and CHCA effects occur within the plant, several years ago Huseinov (57) expressed the idea that naphthenate effects were due to its influence on soil activity. When plants growing in soil are treated with foliar sprays of naphthenic acids or CHCA, some of these compounds enter the soil. Also, it is possible that they could enter the soil by being excreted from the roots. Voinova-Raikova (104) has reported that naphthenic growth substances improved the nitrogen conditions in the soil by stimulating the activity of nitrogen fixing bacteria and depressing the activity of denitrifying bacteria. He concluded that this
improved the nitrogen situation in the soil in two ways: 1) by increasing the amount of nitrogen in forms available to plants, and 2) by reducing the activity of organisms that convert nitrogen into readily leached forms. In this way naphthenates may have an important effect on the mineral nutrition of plants. However, when plants grow in solution culture where, presumably, no element is limiting, it would be impossible for naphthenate treatment to have an important influence on ion uptake in the way mentioned above.

In a review, Brian (25) noted that there was no clear understanding of how herbicides may affect ion uptake, but he offered three suggestions. Auxin herbicides could depress ion uptake by reducing transpiration and cell permeability, but these effects could be offset by a concurrent increase in respiration which would contribute to ion uptake.

Very low concentrations of 2,4-D were found to stimulate uptake of phosphate but the results were not always consistent (26). Cooke (31) found that 2,4-D application to bean leaves caused a stimulation of ion uptake shortly after treatment, but an inhibition after 24 hours. The difference was attributed to the fact that, in the early stages, a low concentration of the 2,4-D had accumulated in the roots and was acting as a stimulus to respiration and ion uptake, but as the concentration increased in time, the effect was reversed.

This effect was not very likely for Knap or CHCA treatment at the concentrations being administered. It has not been shown that concentrations of Knap from 0 to 5000 ppm or CHCA
up to $1 \times 10^{-2}$ M have anything other than stimulative properties (110).

Swenson and Burstrom (92) believed that auxins do not affect the uptake of ions either directly or as a result of their growth inhibition, but rather by their effect on some property of the cells conducive to both cation and water absorption.

The lack of an immediate effect of CHCA or Knap treatment on ion uptake would indicate that they do not affect ion uptake directly either. Increased uptake of nitrogen and phosphorus reported by the Russian workers was likely the result of a long term effect. The increase in metabolic activity and growth due to treatment eventually brings about increased uptake, but the effect may be indirect.

In a study of phosphorus metabolism, Fang and Butts (44) reported that 2,4-D, indoleacetic acid, indolebutyric acid, and naphthalene acetic acid all caused changes in the pattern of $^{32}$P distribution within bean plants. CHCA treatment was also responsible for changes in the distribution patterns of the elements examined in this study. Sulphur-35 accumulated in excess in the leaves and calcium-45 in the roots of treated plant. Magnesium concentration was lower in the leaves of treated plants than controls. No particular pattern emerges from these results, but perhaps that is not surprising when Brian (25) comments, after 20 years of intensive study of 2,4-D, that it is possible to do little more than catalogue its effects on the distribution of elements within plants.
SUMMARY

Four separate studies were carried out with bean plants to determine the effect of cyclohexanecarboxylic acid (CHCA) treatment on: 1. uptake and distribution of sulphur-35, 2. uptake and distribution of calcium-45, 3. uptake and distribution of total sulphur, calcium, and magnesium in plants grown for one week after treatment, and 4. distribution of sulphur-35 within compounds of bean leaves.

Several conclusions may be drawn from the results of these experiments.

1. Uptake of sulphur-35 and calcium-45, measured one day after treatment, and sulphur, calcium, and magnesium uptake measured one week after treatment was not directly affected by CHCA treatment.

2. Distribution of the elements within the plant was affected by treatment. Sulphur-35 was moved more rapidly to the leaves of treated plants than controls, while calcium-45 was retained in excess by the roots of treated plants. Control plants accumulated more magnesium in the leaves than treated plants.

3. Treatment significantly affected the incorporation of sulphur-35 into organic compounds of bean leaves. One and two days after supplying $^{35}\text{SO}_4^-$, the activity in acid-soluble compounds was significantly greater in treated than control leaves, and the level of sulphate was lower in treated than control on the second day. Treated plants had significantly more activity in protein than controls.
Furthermore, the level of activity in cysteine/cystine and methionine also increased over the level in controls as time passed.

Evidence has accumulated in favour of the concept that naphthenates activate nucleic acid metabolism, which in turn would stimulate the formation of enzymatic protein. Increased metabolic activity may be expected to follow. The results of this study agree with this concept. Increased levels of sulphur in protein, and protein amino acids, suggested stepped-up metabolic activity. The more rapid conversion of sulphate into organic sulphur compounds by treated leaves compared to control leaves was consistent with the protein increases. Distribution effects due to treatment, especially in the sulphur-35 distribution experiment, suggested changed metabolic patterns. These plants were somewhat deficient in sulphur, and the more rapid transport to the leaves may have been the result of an increased sink effect due to the stimulation of metabolic activity by CHCA. The retention of calcium in treated roots could also be due to increased activity. A reasonable explanation for the reduced levels of magnesium in treated leaves was not evident.

The lack of a significant effect of treatment on ion uptake suggested that CHCA does not stimulate plant growth by improving the uptake of sulphur, calcium, or magnesium.


