room temperature for the major changes in starch and organic acids to occur is rather closely adapted to the period of the normal diurnal variation in illumination. The range through which the concentration of these components may move in response to the diurnal alteration of light and darkness is moderately constant for any given lot of leaves and at ordinary temperature is not increased by prolonged exposure either to light or to darkness.

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CHLORINE—A MICRONUTRIENT ELEMENT FOR HIGHER PLANTS[1]

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This article presents evidence of the essential nature of chlorine for the growth of higher plants and the proposal for its classification with the micronutrient elements. The experiments serve to support many past observations suggesting beneficial effects derived from fertilizers containing chlorine. Of particular interest are the controlled culture solution experiments of Eaton (2) and Raleigh (6) which showed highly significant increases in yields of tomatoes and cotton, and of beets, respectively, when supplied with additional chlorine. It also supports earlier work of Lipman in 1937 (4) who, after directing his attention specifically to chlorine as a growth factor for buckwheat concluded that "if chlorine is not essential, it is certainly highly beneficial."

Evidence of chlorine as a plant micronutrient offered in this paper seems conclusive beyond doubt, since inherent chlorine contaminations in culture solutions were controlled well enough to produce the nutritional disease in severe form, showing leaf symptoms of wilt, chlorosis, necrosis, and an unusual bronze discoloration which in combination are characteristic of no other known nutritional or pathological[3] disease of tomatoes. It has also been possible to maintain control at different levels of chlorine so that other significant data have been made available including yield versus chlorine supply, and the chlorine contents of roots, stems, and leaves at the various levels of chlorine deficiency. Further observations have been made

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as to rates of recovery of diseased plants after supplying chlorine, three to four days being required to arrest advance of the disease. Other points of interest are that after adding chloride ion to deficient culture solutions, new growth appears healthy and remains so and moderately injured leaf areas progressively recover from chlorosis and resume growth; but leaf areas where chlorosis has progressed into bronzing, although they regain some of their original color, remain static and do not expand.

The present data on the chlorine nutrition of plants were an outgrowth of intensive work on cobalt, which was of interest because of its recognized need in the life of animals, but whose essentiality has not been critically demonstrated for plant life. In beginning this work, it appeared that cobalt offered a special challenge for investigation, particularly because of the very small amounts known to be involved. Consequently, nutrient salts of analytical reagent grade were treated chemically to remove cobalt as the sulfide along with copper or iron sulfides. As an improvement in the degree of sensitivity of detection and estimation of cobalt, a known amount of high specific activity radio-cobalt was added to the salt solutions undergoing purification, and its residual activity was used as an indicator of the degree of removal of this element. The most satisfactory removal of cobalt from culture solutions was attained through alkaline sulfide co-precipitation with either copper or iron salts as carriers. Measurements of the residual radioactivity of Co showed that the purified nutrient salts contributed less than 2×10^{-4} micromoles (0.01 μg) cobalt per liter of culture solution. Presumably the same process which allowed this degree of removal of cobalt would have similar merit for other sulfide precipitable metals. It is expected that details of these procedures will be reported elsewhere.

Using the salts thus purified, experiments were designed to test plant growth responses to cobalt at a level of 0.085 micromoles (5 μg) per liter of culture solution. One of the striking observations of the first experiment was that provision of cobalt at 5 μg per liter (also 5 μg per plant) resulted in a 50 % increase in shoot yield—statistically significant at the 5 % level, strongly suggesting cobalt as a growth factor. However, in following experiments it became apparent that culture solutions made from these salts, even with additions of cobalt up to 1.7 micromoles (100 μg) per liter, would not allow plants to grow satisfactorily as compared to growth made on similar culture solutions of technical grade nutrient salts. Because of this, the question was raised as to whether purification procedures had resulted in the addition of a toxic material or whether significant amounts of another unknown element necessary for growth of the tomato plant had been eliminated. There also remained the question as to why the 5 ppb cobalt addition of the previous experiment showed an increased yield and it was speculated that the cobalt salt itself might have contained the unknown growth factor. Whatever the case, it was obvious that further progress could not be made until it was found how to grow plants on these purified culture solutions. The idea of toxicity seemed unlikely. The only toxic materials added to the salts during purification were copper salts and H_2S, and auxiliary experiments with added H_2S or copper did not produce these symptoms. Also, similar separations had been used many times in our laboratories for producing plants deficient in Mo, Cu, Zn, Mn, or Fe without previous evidence of toxicity. Therefore, it was assumed that the stock greenhouse salts contained a growth factor which was not present in the purified salts in sufficient quantity to meet the needs for growth of the tomato plant and work was directed toward its identification. Pending identification, it became known as "element X."

As a first step in localizing "element X," greenhouse stock salts (KNO_3, KH_2PO_4, $Ca(NO_3)_2$, and $MgSO_4$) were examined separately for presence of the growth factor by adding the single salts to complementary groups of culture solutions otherwise compounded from the purified salts known to give rise to the disease. For comparison, tomato plants were grown on both the purified cultures and stock greenhouse cultures. As before, diseased plants developed on cultures made entirely from the purified salts and healthy plants grew on the technical grade salts. However, healthy plants were also obtained when either KNO_3 or KH_2PO_4 from the technical stock was added to the purified cultures. Addition of technical grade $Ca(NO_3)_2$ or $MgSO_4$ did not correct the disease. From these experiments it was concluded that the growth factor was contained in each of the technical grade potassium salts. The question was then raised as to the possibility of a significant association of the unidentified growth factor with the potassium salts. Potassium chloride is the parent raw material of most other potassium salts of commerce and in the processing of crude KCl a great many important contaminants are recognized. However, for our purposes, the task of analyzing for all the many possibilities seemed too great, particularly after unfruitful exploratory analyses of the potassium salts with the hydrogen flame spectrophotometer failed to reveal any prominent differences between those having and those not having the growth factor.

Consequently, a supply of the U.S.P. KNO_3, now known to contain the growth factor, was taken from the technical stock and subjected to fractionation by a variety of chemical processes, including recrystallization. The latter method was the only one which showed promise of concentrating the growth factor. For comparison with the U.S.P. KNO_3 which contained the growth factor, a sample of commercial fertilizer grade KCl [4] was similarly processed. Tests of the mother liquors and the recrystallized salts for presence of the growth factor were then made by growing tomato plants in culture solutions wherein the potassium component was provided from the above fractions of potassium salts; otherwise, the culture solutions were prepared from the purified salts.

[4] Supplied by the Pacific Guano Company as representative of commercial KCl produced at Searles Lake, California.

Thus, healthy plants would show the presence of "element X" in the substituted potassium salt.

For these tests, a new lot of reagent grade salts was purified as before, since the original stock had become depleted. For five weeks of the growth period it was a matter of concern that no evidence of the disease assigned to "element X" deficiency appeared, and it was concluded that the elaborate purification procedure for metal separation was not responsible for the purity of the earlier batches of salts. The plants were continued for another week and having learned from past experience how to detect early stages of the disease, we were rewarded by recognition of its appearance in mild form in some of the cultures. From the cultures showing the disease we concluded—though not very firmly—that recrystallized KNO_3 had lost "element X" while its mother liquor had not. It also seemed reasonable to conclude tentatively that the growth factor was supplied by a) the fertilizer grade KCl; b) the recrystallized KCl; and also c) the mother liquor from the recrystallized KCl. Thus, it appeared that chloride ion might be the missing growth factor. Further evidence from the experiments to be discussed proved that it was.

Meanwhile, ten single tomato plants, each on a separate culture solution composed of our earlier set of salts of low "X" content were grown with the hope of procuring seed more dilute than normal with respect to "element X." However, blossoms would not set, except on one plant which produced two single fruits. By comparison, another set of tomato plants grown at the same time upon the technical grade nutrient solutions blossomed profusely and produced a heavy set of fruit. The latter plants were judged to be high yielding as compared with commercial standards.

Thus, it became known that the first lot of purified salts was too low in "element X" to meet the nutritional requirement demanded of it for the continuation of the life cycle of the tomato plant, simply because blossoms would not mature or set fruit. To test the now very strong suspicion with respect to chlorine as "element X," a large scale experiment was designed, but in this instance salts were pre-treated with silver ion to remove the silver precipitable halides, after which the second purification for heavy metals was undertaken.

Materials and Methods

Experimental Procedure Testing the Requirement for Chlorine in the Growth and Development of the Tomato Plant: On the assumption that chlorine was the growth factor, an experiment of 64 cultures was designed to find the levels required for the adequate chlorine nutrition of the tomato plant. With the exception of 4 cultures using technical grade, all salts used for making the cultures were subjected either to recrystallization (especially $(NH_4)_2HPO_4$) or to halide precipitation with $AgNO_3$, the excess silver being removed subsequently by coprecipitation with copper as the sulfide.

The culture solutions contained the following nutrient ions in millimoles per liter: Ca, 4; Mg, 1; K, 6; NH_4, 2; H_2PO_4, 2; SO_4, 1; and NO_3, 14. They were purified as salt solutions of $Ca(NO_3)_2$, KNO_3, $Mg(NO_3)_2$, $(NH_4)_2HPO_4$, and K_2HPO_4. H_2SO_4 was then added to the phosphate salts to bring them to the dihydrogen equivalent. The relative proportion of the ammonium to nitrate ion as nitrogen sources was chosen because with this proportion the hydrogen ion concentration of the solutions remained reasonably near a pH value of 6 throughout the growth period.

The amounts of micronutrients used are generally expressed in micromoles per liter or per culture. The odd amounts reflect the customary round number concentrations traditionally given in parts per million or parts per billion. Six micronutrient elements were added in micromoles per liter as follows: Fe, 86; B, 46; Mn, 9.1; Zn, 0.76; Cu, 0.31; Mo, 0.10. The chlorine treatments were made in series, with the lowest concentration being the chlorine remaining in the salts after the purification process. The residual chlorine was determined by analysis to be 3.0 micromoles per liter. Considering the residual chlorine found by analysis, the total chlorine in the other members of the series was respectively 3.7, 6.5, 20.6, and 105 micromoles per liter. Cultures made from the technical grade salts contained 11.7 micromoles per liter.

The added chlorine supplements were taken from 6 different sources, namely: 1) reagent grade KCl; 2) technical grade KCl; 3) recrystallized technical grade KCl; 4) mother liquor from the recrystallized KCl; 5) reagent grade $CaCl_2$; and 6) reagent grade $MgCl_2$. Each supplement was duplicated, making a total of 12 cultures having received the same amount of chlorine. Later on, it became clear that the symptoms were related specifically to the amount of chlorine supplied to the cultures irrespective of source.

Iodine and bromine were also tested for possible interrelations with chlorine, but the experiments were less elaborate, having but two replications per treatment. To the 3 micromoles of residual chlorine per liter, bromine was added as KBr at levels of 0.31 and 7.8 micromoles per liter, and iodine as KI at levels of 0.20 and 4.9 micromoles per liter. Although there were but two replications for each treatment, the duplicate cultures responded uniformly as judged by visual observation. The plant response to iodine or bromine was unquestionably different from that obtained with chlorine at equivalent levels. Both iodine treatments appeared to be toxic, and the eventual yields were lower than for any other plants. Also, yields of plants treated with 4.9 micromoles of iodine were slightly lower than those receiving 0.20 micromoles per liter. At early growth stages the higher iodine treatment showed the more severe effect.

Special consideration was taken to avoid possible sources of chlorine contamination while setting up the experiment. For example, our laboratory custom of washing all beakers with 10 % HCl as part of the cleaning procedure for micronutrient element studies received special attention. As a part of the regular rinsing procedure a jet of redistilled water was sprayed around the walls of the beakers. When about

50 ml of water had accumulated, it was poured off and and its pH was tested with the glass electrode. This rinsing process was repeated until the pH of the discarded rinse water was the same as the original. In all subsequent experimentation 1 % HNO_3 was substituted for HCl.

PLANT CULTURE METHOD: Marglobe tomatoes were germinated over Pyrex glass trays on cheesecloth. They were watered with a one tenth strength culture solution without added micronutrients. They were transplanted in the cotyledon stage to Pyrex glass beakers of 4 liters capacity having paraffined plaster of Paris covers. The roots were shielded from light by double lined cloth skirts, white outside and blue denim inside. Solutions were aerated continuously. Five plants were set out on each culture and these were thinned to the four most uniform, after one week.

Molar stock solutions of the macronutrient salts were purified by the precipitation of halides with silver nitrate and subsequent removal of the excess silver by an alkaline copper sulfide co-precipitation. Heated stock solutions, to which a small excess of silver nitrate was added, were allowed to cool overnight and then filtered. The filtrate was heated to boiling and treated with 10 mg copper (as $CuSO_4$) and 1 gm of low-halide $CaCO_3$, per liter of solution. Hydrogen sulfide was then passed into the solution and continued for half an hour while the solution slowly cooled. After cooling overnight, the solution was again filtered, brought to a boil, and water pumped nitrogen bubbled through the boiling solution to remove excess hydrogen sulfide. Solutions prepared in this manner are sufficiently low in halides and heavy metals including iron, manganese, zinc, copper, and molybdenum to be used in solution culture studies involving these elements.

Recrystallization of reagent grade salts is also effective in removing halides and has been used in some of these studies. The technique involves the use of the first fraction of crystals as the halides appear to concentrate in the supernatant.

Halide determinations on solutions were done by one of two methods depending on the amounts present. For very dilute concentrations or where size of samples was limited the microdiffusion method of Conway (1) was used. For larger amounts, in excess of 10 micromoles halide per sample, a modification of the potentiometric method of Kolthoff and Kuroda (3) was used. Plant samples were ashed at 500° C, after treatment with freshly prepared low-halide calcium oxide as recommended by Piper (5). Complete details of analytical methods and results will be reported in another paper.

GROWTH AND DEVELOPMENT OF THE TOMATO PLANT AT DIFFERENT LEVELS OF CHLORINE SUPPLY: The reward for the above precautions was an exceedingly uniform set of plants in all 64 beakers of the series. No differences in health or growth, as judged by visual observation, could be detected in any of the cultures until 24 days after transplanting. On that day the first signs of abnormality appeared as the characteristic "low chlorine" wilt of leaf tips, as manifested by the tomato plant. The treatments affected were a) each of the four pots having received iodine supplements at lower and higher levels; b) each of the two pots receiving the lower bromine supplement (1.25 micromoles Br per culture) and c) two of the four replicates to which no halide was added but which contained as an unremoved impurity, 12.0 micromoles chlorine per 4 liters of culture solution. Two days following the first sign of the deficiency disease all four of the lowest chlorine cultures showed similar symptoms. Two days later the two higher level bromine treatments (31.2 micromoles Br per culture) became affected. Also on this last date all of the 12 treatments having received the lowest added supplement of chlorine (2.8 micromoles per culture) acquired the wilt symptom. None of the remaining cultures at higher chlorine levels was affected. It appeared at that time that 31.2 micromoles of bromine was as effective as 2.8 micromoles of chlorine in delaying onset of the disease.

The next set of plants to show the disease was the 12 cultures at the next higher chlorine level (14.1 micromoles added chlorine per culture). Upon the 10th day following the first indication of disease on the lowest chlorine level, each and every one of the cultures which received 14.1 micromoles chlorine became affected, but at the same time none of the cultures having chlorine levels above this showed the disease, nor did they show it during the remaining ten days of the experiment.

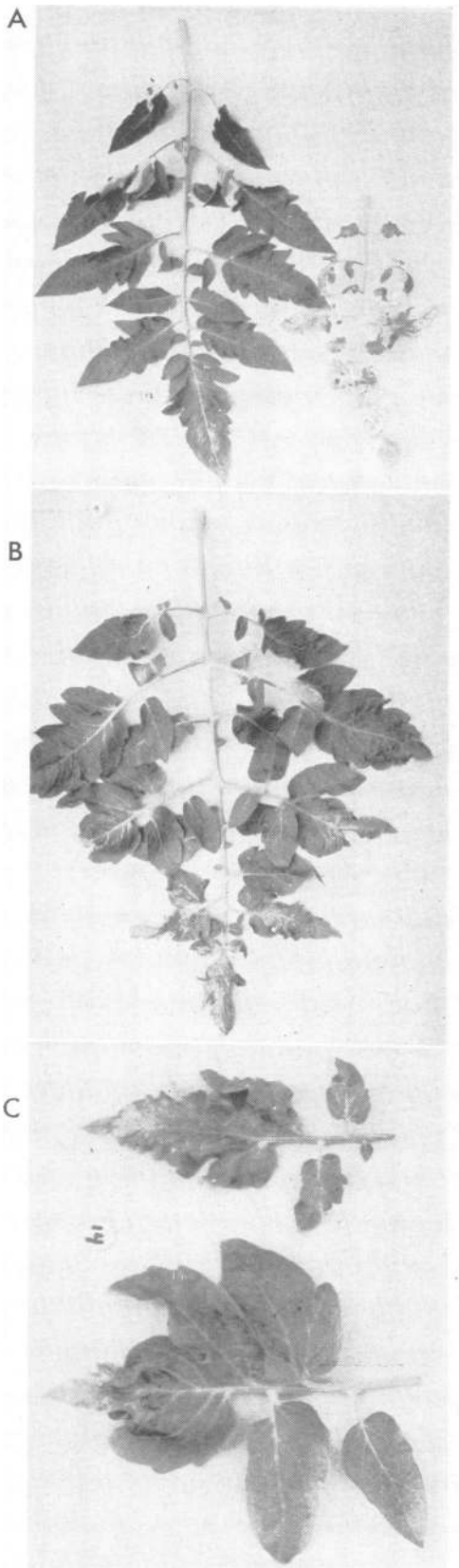
RECOVERY OF CHLORINE DEFICIENT PLANTS FOLLOWING ADDITION OF REAGENT GRADE KCl: It appeared that the delaying of symptoms was related to addition of chlorine and not to the kind of chloride salt containing it, that is reagent grades $CaCl_2$, $MgCl_2$, and KCl, technical grade KCl, mother liquor KCl, and recrystallized KCl. Thus it was considered worth while to divide the 12 diseased replicates which had been supplemented with 2.8 micromoles chlorine per culture, and from 6 sources of chloride salts, into two lots by selecting one culture from each of the salt types. One lot was given an additional supplement of 352 micromoles per culture as reagent grade KCl, i.e., approximately 3 parts per million in the culture solution. All six of the treated cultures recovered completely, the last symptom of wilt disappearing on the sixth day after treatment. By contrast, the six cultures which did not receive the extra chlorine supplement progressed in their symptomatology into chlorosis, bronzing, and necrosis of leaves. They also became most obviously restricted in growth. At harvest time, 13 days after treatment with KCl, the treated plants showed a large difference in yield which was statistically significant at better than 1 % with respect to leaves, stems, roots, or total yield. The weights of the recovered vs. diseased plants were: 65.3 vs. 42.7 for leaves, 58.6 vs. 41.4 for stems, and 23.4 vs. 15.0 grams dry weight for roots.

RECOVERY OF PLANTS FOLLOWING ADDITION OF NH_4Cl PREPARED BY COLD GAS PURIFICATION: Although the above experiments seemed thoroughly con-

clusive that the element responsible for relieving symptoms of injury was chlorine, there was a question brought forth that some other element might have fortuitously followed chlorine throughout the chemistry of the manufacturing processes for each of the salts used as a source of chlorine and that this postulated contaminating element might be the effective one rather than chlorine. Therefore, a new salt of ammonium chloride was prepared from ammonium hydroxide and hydrochloric acid, by a cold distillation process. Nitrogen was passed through concentrated reagent grade HCl and bubbled into glass redistilled water until the resultant solution was approximately 0.1 *N* with respect to HCl. Similarly, nitrogen was passed through reagent grade NH_4OH, and discharged into the 0.1 *N* HCl solution to neutralize the latter. Aliquots of the NH_4Cl so prepared were analyzed for chlorine content. This method of preparation was designed to obviate inclusion of heavy metal and other nongaseous contaminants which might have been present in the aqueous stocks of reagent grade NH_4OH or HCl.

To test the effectiveness of the NH_4Cl in removing the symptoms under observation, the 12 cultures of injured plants having received an added 14.1 micromoles chlorine per culture were divided into two sets in the same way as described for the recovery set just discussed. As with the earlier recovery experiment in which reagent grade KCl was used, the added amount of chlorine was 352 micromoles per culture (approximately 3 ppm Cl in the culture solution), but derived from the cold gas-purified NH_4Cl. This recovery experiment was started 10 days before harvest. Six days later 23 of the 24 affected plants had recovered from symptoms. Nine days later all 24 had recovered completely. By contrast, all 24 plants on the 6 cultures which did not have the NH_4Cl supplement, progressed into symptomatology of wilt in severe form, and 23 of them acquired the advanced symptoms of bronzing. Effectiveness of this chloride treatment was also shown in an increased weight of the chloride treated plants. The dry weights of the recovered versus the non-recovered plants were: leaves 71.4 vs. 58.8, stems 63.2 vs. 54.6, roots 27.4 vs. 22.1 grams dry weight. The increased yield was significant in each instance at the 1 % level.

Recovery of Chlorine Deficient Tomato Plants through Injection: From the four replicates of lowest halide plants, one culture was selected for recovery experiments through injection. There were four plants growing on the single culture. All plants were severely injured and in the stage of advanced leaf necrosis. Three of the four plants were injected respectively with a) redistilled H_2O, b) 0.003 *N* KNO_3, and c) 0.003 *N* KCl. A hypodermic needle was inserted into leaf-stem junctions directed steeply downward to bring its point within the pithy region of the stem. Five junctions were punctured, and a total of 0.056 micromoles (2 μg) of chlorine were thus introduced within the stem tissue of one plant. As controls, similar volumes of solution were injected into two of the other plants, one being KNO_3



and the other H_2O. The fourth plant was not injected.

Three days after injection, growing tips of the chloride injected plant appeared to grow without injury, whereas all other plants showed necrotic spots. This early pattern continued. However, further injections of equal volume were made 2 weeks later using H_2O, 0.1 *N* KNO_3 and 0.1 *N* KCl. The chlorine administered at this time was 0.56 micromoles (20 μg). New growth developed rapidly and in a seemingly normal fashion only with the plant injected with KCl. Since that time, many other recovery experiments have been run, and for this reason, we are assured that the reactions shown by the KCl injected plant are typical, and the result of chlorine being added. Older leaves badly affected did not recover, but mildly affected ones did. The pattern of the general recovery is of sufficient interest, that some of its features are included in figure 1.

YIELD OF TOMATO PLANT AS A FUNCTION OF CHLORINE SUPPLY: The tomatoes were grown for 45 days after transplanting, then harvested. The plants were separated into three fractions—leaf blades, stems including petioles, and roots, and were weighed after oven drying at 70° C. In figure 2 the average dry weight of each plant part per culture is plotted against the known total amount of chlorine supplied per culture in micromoles. The total chlorine supplied includes 3.0 micromoles per culture as residual salt contamination which could not be removed in the purification process, but which was shown upon analysis for chlorine. In figure 2 the points shown by open circles are the ones from cultures having had halogens removed either by salt recrystallization or by precipitation with silver ion and the excess silver subsequently removed as Ag_2S coprecipitated with CuS. The black points are the yields of leaves, stems and roots obtained from plants grown on the technical grade salts regularly used for making culture solutions in our greenhouses. These latter salts were analyzed for chlorine and were shown to contain enough impurity to provide 47 micromoles of chlorine for the 4 liters of the culture solution. As earlier suggested, the content of "element X" in the greenhouse salts was sufficient to give good growth by customary standards. From these data (fig 2) it appears that chlorine supplements even to these greenhouse salts might have resulted in significantly greater yields, depending upon care taken to exclude chlorine contaminations from other sources.

FIG. 1. Leaf development under adequate and limiting chlorine supply and recovery following subsequent application.

A. Normal leaf (left) produced after injecting plant with 22 μg Cl. This photograph was taken 22 days following the first injection of 2 μg and 8 days after the second injection of 20 μg Cl. This leaf was the 14th, counting from the base. Cl-deficient leaf (right), 8th from the base of the plant injected with KNO_3. This leaf is very similar to the 8th leaves on the water injected plant and on the uninjected control. For the 8th leaf of the KCl injected plant compare figure 1-B.

B. Recovered leaf, 8th from the base of the KCl injected plant. The terminal leaflets were moderately bronzed at the time of injection and never recovered. Some leaflets only mildly bronzed, grew basipetally, but not acropetally beyond the bronzed area. The tips of the leaflets which were wilted but unbronzed, recovered turgor, but failed to expand. Compare with 8th leaf, figure 1-A, right.

C. Leaflets from water (upper) and KCl (lower) injected plants. These are corresponding leaflets taken from the 8th leaf. The lower leaflet of this figure is the one shown in figure 1-B, the 4th down on the left. The basipetal expansion as contrasted with restricted growth of the tip is very characteristic of the recovery pattern of mildly bronzed leaves of Cl-deficient plants after recovery.

DISCUSSION

The chlorine requirement is not small as compared to other micronutrients, for in the leaves of tomato plants suffering from chlorine deficiency disease, the chlorine concentration is in the order of 7 micromoles per gm dry weight (250 ppm). Data bearing on this point will be presented in another paper. By comparison, the presently recognized micronutrient which is required in the least amount is molybdenum and plants showing an equivalent degree of visual stress by reason of molybdenum deficiency would have in the order of 1.4×10^{-3} micromoles per gram (0.1 ppm) or less of molybdenum. Thus, on a mole basis, the minimal quantity of chlorine required in plant tissue is several thousand times greater than for molybdenum. Plant requirements for Fe, B, Mn, Zn, and Cu are intermediate, and reasonably within the order given.

In recognizing chlorine as a plant nutrient, it is with the realization from this experiment that Br can substitute at least partially in plant functions normally dependent on Cl, in a way reminiscent of the sparing effect of Na for K or Sr for Ca. Whether or not Br can substitute completely for Cl must remain a subject for future investigation.

SUMMARY

1. A severe nutritional deficiency disease was observed in tomato plants when the known halide supply was limited.

2. The nutritional deficiency is visibly characterized in its early stages, by a wilting of leaflet blade tips; progressively, by chlorosis, bronzing and necrosis basipetally in areas proximal to the wilting.

3. Severely diseased plants failed to produce fruit.

4. Growth was correlated with chlorine supply in the culture solution.

5. Adequate additions of chlorine as chloride to culture media entirely prevented the disease; severely deficient plants resumed satisfactory growth after chlorine was applied to the culture solution or through injection into stems.

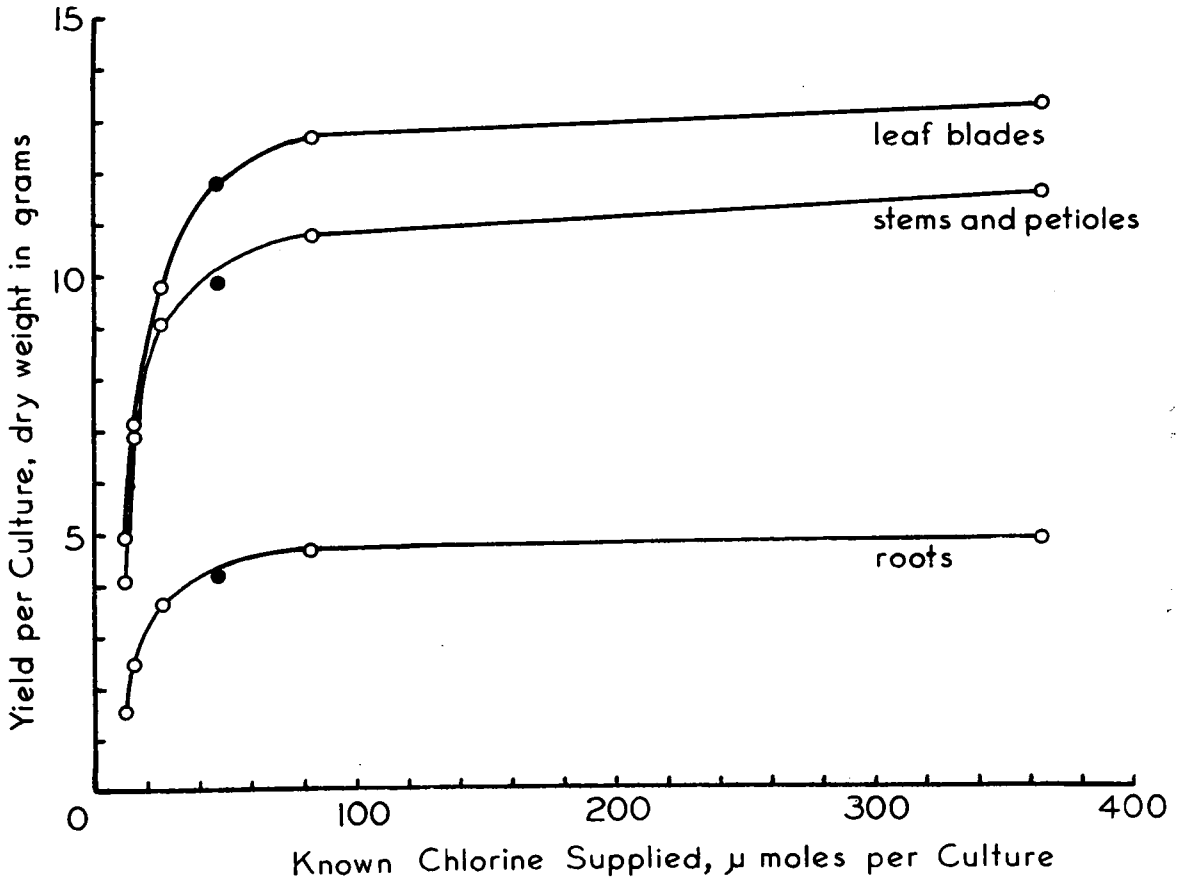


FIG. 2. Relation between yield of plant material and total known chlorine supply. Open circles indicate values from repurified salt solutions; closed circles, from technical grade salts.

6. Bromine appears to complement chlorine when supplied at about ten times the required chlorine levels. Possible effects of iodine are difficult to assess because of its toxicity.

It is concluded that chlorine is a nutrient element, certainly the naturally occurring essential halide.

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