STUDIES ON A GROWTH INHIBITOR IN GUAYULE

Thesis by

SIDNEY GOTTLIEB

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PREFACE

The purposes of the studies to be described in the following sections were twofold. The first object was to establish the existance of a substance arising from the roots of *Parthenium argentatum*, the guayule rubber plant, which was inhibitory to the growth of other guayule plants. A further aim was to ascertain the chemical nature of this inhibiting principle.

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CHAPTER I
INTRODUCTION

Before discussing the experimental work on *Parthenium* it will be necessary to consider in some detail the historical development of the question of toxic excretions of roots. The problem of whether plants give rise to toxic substances in the media in which they grow will be taken up first, and the rather meager researches on whether these substances are actually excreted from plant roots will be dwelt on later. Brief mention will also be made of growth-promoting substances arising from organic matter in the soil.

A. Toxic Substances

The first clear statement that toxic substances arise from the growth of plants was made by DeCandolle (21). However, even before this, Brugmans (70) had alleged that he observed drops of liquid to exude from the roots of *Viola arvensis* and that he observed small fragments of material at the extremities of the roots of certain other plants which he regarded as excretions. Despite the lack of good scientific evidence for these statements at this early date, they seem to have been widely accepted by the naturalists of that time, including von Humboldt and DeCandolle.

Decandolle, on the basis of extensive observation of plant life and some experiments, maintained that plants excrete from their roots substances which are deleterious to continued growth. He believed that these excreted substances had an unfavorable effect when absorbed from the soil by other plants belonging to the same natural order as the plants from which the excretions had come, but that their excretions would be harmless or even beneficial to plants belonging to a different order. He carried this idea further and used it to explain the apparent antagonisms between certain plants, explaining that they injured their neighbors by the substances exuded from their roots into the soil. He cited the antagonisms between Euphorbias and flax, tares and wheat, and thistles and oats.

A contemporary of DeCandolle, Macaire-Prinsep (70) carried out experiments which seemed to offer strong support to DeCandolle's theory. He grew Chondrilla plants in rain water and reported that after eight days the water had a yellow tint, a strong odor, gave a bitter taste, and yielded a precipitate with lead acetate. Also, he said that water in which peas had been growing was toxic to other plants of the same species, but not to wheat.

Using these experiments and ideas as a basis, DeCandolle formulated a theory to explain the well-known benefits of crop rotation. He expressed the belief that the harmful effects arising when a soil was continually cropped with the same species are due to an accumulation of toxic substances. However, the substances excreted by the roots of one order of plants were not supposed to be harmful to the roots of plants belonging to other natural orders. According to him, they might even be beneficial. He cited many plant associations and successions in nature in support of this, pointing out especially that in the majority of cases good yields of all crops are obtained from soil when plants belonging to different natural orders are grown in succession.

These ideas were not allowed to pass unchallenged and soon controversial articles began to appear. Gyde (27) obtained small amounts of organic matter from the water solutions in which plants had grown and applied these to soil in which other plants were growing. He observed no harmful effects on either the same plants or other species and concluded that excretions of plants were not harmful to their own kind, but that the necessity of rotation of crops arises from a depletion of the soil of mineral elements.

The curious regular growth of certain fungi on soil in ever widening circles, known as "fairy rings," was the subject of a controversy on whether it was deleterious excretions of the fungi which caused the phenomenon. The common explanation was that the soil within the ring was so depleted of nutrients that it was unable to support growth of the fungus. Lawes and Gilbert (42, 43) investigated the chemistry of the soil within and outside the rings and found such slight defferences in the total nitrogen, carbon and other elements that they could not possibly explain the total absence of fungi within the ring. Way (91), earlier had admitted in a paper published in 1847, that "by far the most intelligent solution of the question is that which was based on DeCandolle's theory of the excretions of plants."

Special mention must be made of Liebig (44) and his attitude towards this question. At first he pronounced the DeCandollian theory of crop rotations to be the only one "resting on a firm basis." He particularly favored the experiments of Macaire-Prinsep as positive proof of this. However, when, after his extensive studies on the ash constituents of plants, he formulated the theory of mineral requirements, his ideas on the subject of crop rotations changed. After developing the idea that it is absolutely essential for all higher plants to be supplied with a certain ratio of mineral constituents in the soil, as well as a certaim minimum amount, he re-interpreted the practices of crop rotations in these terms. He reasoned that since some plants take mineral elements out of the soil in established ratios, they must in time destroy the necessary ratio of these elements in the soil; but when another plant drawing its ash constituents in a different ratio is substituted, it obtains a sufficient supply of nutrients, and the soil is thus relieved of exhaustion.

Due to Liebig's tremendous prestige, his theory of crop rotations in terms of mineral exhaustion achieved almost universal acceptance, and the theory of DeCandolle was practically abandoned. For about fifty years after Liebig's investigation, the mineral elements in plant and soil claimed paramount attention, and biological and organic chemical factors connected with soil problems were almost entirely neglected. A notable exception to this trend was an essay which appeared shortly after Liebig's pronouncement, by John Coleman (13) in 1855 in which he aptly pointed out the inadequacy of the theory of mineral requirements alone to explain the productivity of soils.

As can be seen from the discussion above, most of the interest in the problem of toxic secretions arose from a consideration of the "soil sickness" due to one-crop agriculture, and from the attempt to alleviate this condition by crop rotation. In addition to these injurious after-effects, there are many cases of harmful interactions of plants growing adjacent to each other. The effect of grasses on adjacent fruit trees has been discussed at length in the literature (10, 34, 59). Plants most frequently reported as injurious to their neighbors are walnut tress, corn, rye, buckwheat, thistles, squash, turnips, sesame, rutabagus, mangles and potatoes (17, 60, 63, 71). On the other hand, certain plants have been consistantly observed to be easily injured by various neighboring crops. Among these are fruit trees, grapes, onions, alfalfa, tomatoes, potatoes and sesame (22, 83, 95).

A remarkable renewal of interest in the subject of soil toxicity took place in the years 1900-1910, when three independant groups of investigators undertook extensive investigations in the field. Bedford and Pickering in England, Livingston at the University of Chicago, and a group of investigators in the U.S.D.A. Bureau of Soils, including Whitney, Cameron, Reed, Schreiner, Skinner, and others all made important contributions in this period.

Bedford and Pickering (4), beginning in 1903, made the first definite mention of the modern theory of active soil toxicity. They found that young apple trees were severely injured when grass was allowed to grow about them. This effect was attributed successively to the removal of plant food by the grass, the removal of water by the grass, the effect of CO_2 liberated by the grass, the effect of exclusion of O_2 from the vicinity of the tree roots, and to the packing brought about by the sod formed by the grass. However, each of these tentative explanations was ruled out on the basis of careful experiments, and finally, after seven years of work, they concluded that the pernicious effect of the grass could be due only to some action on the tree like that of direct poisoning. They left the question open as to whether this action is due to direct excretions of the grass or to a changed bacterial action in the soil induced by the presence of the grass.

Livingston (45), in 1905 studied the physiological properties of water expressed from saturated bog soils of the Northern Central States, with the resulting conclusion that such soils commonly contain toxic substances. He showed that these toxic substances varied in their nature, being sometimes colloidal and sometimes volatile. Their inhibiting principles could all be absorbed by substances like tannins and charcoal, and the toxic properties of the expressed bog water could thus be alleviated. Livingston (46), working with Whitney and Cameron in the U.S. Bureau of Soils, in 1905 published an important series of studies on an unproductive soil, indicating that its infertility was due to toxic organic compounds.

Perhaps the most convincing demonstration of toxicity due to organic compounds in soils is due to the extensive work of the group of U.S. Bureau of Soils scientists headed by O. Schreiner (74, 75, 76, 77, 78, 79). These investigators showed that from certain infertile soils could be obtained water extracts which were toxic to the growth of wheat seedlings and that this toxicity could be alleviated by a sufficient dilution of the extracts. These simple experiments immediately cast doubt on the contention that the unsuitability of the extracts was due to mineral nutrient deficiency, since, if this were the case, dilution should have aggravated the situation. But even more convincing evidence was accumulated; in the course of attempts to isolate the specific organic compounds which were causing the toxicity, the group reported the isolation of four crystallizable compounds which were markedly toxic in low concentration to the growth of wheat seedlings. The compounds were picoline carboxylic acid, dihydroxy stearic acid, salicylaldehyde, and vanillin. A remarkably good correlation could be set up between the infertility of certain soils and the presence of one or more of these compounds in them. In another interesting series of experiments, this group was able to offer good evidence that these compounds may arise in most soils, fertile and infertile, but that the fertile soils have the proper factors, both chemical and biological, for making these toxic factors disappear.

With the publications of the work of these three groups, particularly that of the U.S. Bureau of Soils, agronomical investigators began to take notice of this rebirth of the old DeCandollian theory, although the general reaction was quite unfavorable. Groups of investigators took sides violently in the debate during the years 1910-25. An interesting account of the way the question of soil toxicity even became a political issue is given in an address by Livingston (45) to a Symposium on Soil Toxicity. In this period many papers were published, some of which pointed to toxic compounds as the cause of soil infertility, and others which indicated other causative factors.

Thus, Fletcher (24, 25) had early reported a chemical investigation on the antagonism between Zea Mays and Sesamum indicum, in which he first thought the causative agent to be alkaloidal in nature, but later reported that its solutions behaved like solutions of the sodium salt of dihydroxy stearic acid. No isolation was reported, however. Also Dandeno (19) in the year immediately following the work of Schreiner et al. published evidence in support of the contention that antagonisms between plants are due to bacterial action, the bacteria and fungi decomposing dead plant matter to give toxic substances.

In 1917, Bedford and Pickering (4, 61, 62), stimulated by their previous investigations on the antagonism between grass and apple trees, developed a technique for growing two plants in a single pot, one in a layer of soil contained in a tray which is superimposed on the soil in which the other plant is growing, with the water applied to the trays so that the lower soil receives the drainage from the upper one. In this way, they could study the effect of one plant on another. They claimed that all combinations of plants so tested were reciprocally deleterious to the growth of each other. Crops varying as widely as tobacco, tomatoes, barley, clover, many varieties of fruit and forest trees, and sixteen varieties of grasses were used. Their conclusions were at variance with those of Schreiner et al. only in that Bedford and Pickering believed that the observed deleterious influence of one crop on another is due to some toxic substance formed in the soil which had only a very temporary existance in the soil and does not accumulate at all. Most of the substances isolated by Schreiner et al. were quite resistant, and they were believed by the investigators to accumulate in the soil.

Pryanichnikov (64), although admitting that his own experiments on the subject indicated the presence of toxic material arising from the roots of plants, preferred to attribute the lowering of yields after successive cropping to factors like the packing of the soil, decomposition of roots left in the soil giving rise to denitrification and anaerobiosis, and a change of reaction of the soil due to selective ion absorption. He also pointed out that a factor in the necessity for crop rotation is the presence of injurious organisms associated with specific plant hosts, like Fusarium and Asterocystis on flax and nematodes on beets. Molliard (53, 54) took exception to this view on the basis of experiments done with successive plantings of young seedlings in sterile nutrient solutions. These showed suppression of growth in many cases, which Molliard attributed to toxic organic substances, having arranged his experiments so as to rule out other factors.

Mention must also be made of the extensive work done by Hartwell and his collaborators at the Rhode Island Experiment Station (16, 30, 31, 32, 56, 57) over a period of eight years on the effect of certain crops on those which follow. They interpreted the deleterious effects in terms of the inorganic substances in the soil, even though they found that it was not universally true that the crops which removed the largest amount of nutrients from the soil were the ones which had the most depressing effect on the yield of the succeeding crop. They indicated that among the main causative factors were changes in the reaction of the soil and presence of toxic soluble aluminum in the soil.

An interesting and typical controversy on this subject arose in 1923, when Sewell (82) investigated the well-known injurious effect of sorghum stubble on various plants. He found that although the sorghum did remove more nutrients from the soil than most crops, this was not enough to explain the depressed growth of the succeeding crop. Since leachings from whole sorghum plants did not effect the growth of corn while those from topped sorghum plants did, he concluded that the effects were due to decomposition products from the crop residues. Breazeale (9), supported this hypothesis, maintaining that the toxicity was due to a substance which was either decomposed or volatilized in a short time. He claimed part of the effect was due to a toxicity of these decomposition products on the CO_2-producing flora of the soil, with a subsequent change in various inorganic equilibria in the soil. However, several years later Conrad (14) took exception to this explanation, pointing out that sorghum roots are extremely rich in sugars. He reasoned that the sugars from the sorghum roots will diffuse into the soil shortly after the plant is killed and then various kinds of soil bacteria will utilize this sugar as an energy source and multiply rapidly. These vigorously growing organisms would compete with the succeeding crop put into the soil for nitrogen, bringing about a suppression of the plants growth. When the sugar was all used up, the organisms would die, returning the nitrogen to the soil, and the deleterious effect would thus be alleviated. In support of this theory, Conrad pointed out that the effect of sorghum stubble on various crops could be at least partially alleviated by proper fertilization with nitrates. Wilson and Wilson (97) soon offered further evidence for the plausibility of this hypothesis. They actually found that soil cultures containing ground sorghum roots made available nitrogen disappear more rapidly than did soil without sorghum roots. They further found that an unclassified soil organism increased greatly in the sorghum cultures and that the soil containing sorghum roots gave off more CO_2 than did soil not containing sorghum roots. Thus, in this case, the available experimental evidence would make it appear as though a competition for nitrogen was the causative factor in the deleterious effect of sorghums on succeeding crops.

The injurious effect of black walnut trees on various other plants has been reported often in the literature (11, 15, 20, 50, 73). Cook (15) noticed that the area over which this effect was exerted coincided very closely with the spread of the root system. Davis, (20) in a chemical investigation of the problem, sought to identify the toxic principle with juglone (5-OH, naphthoquinone,) which is found in all upper parts of the plant. He obtained a substance by extracting the roots of the tree according to a method for obtaining juglone. The crystallized substance was identical with synthetic juglone, and was extremely toxic when injected into alfalfa and tomato plants. On the basis of these experiments, the author was satisfied that juglone was the causative agent in black walnut tree antagonisms.

Luchetti (48), in a study on the effect of growing seedlings of Vicia faba in nutrient solutions in which one crop of the plant had already grown, concluded that toxic undetermined organic substances are formed by the anabolism of the plant and by bacteria and fungi.

A recent indication of an active toxic substance in soils was published by Benedict (5) in 1941. In the course of studies in bromegrass, *Bromus inermis*, he was led to suspect the presence of a substance arising from the bromegrass and accumulating in the substratum to a concentration where it was toxic to the bromegrass. Varying amounts of dried root tissue of *Bromus* were placed in the vicinity of growing young bromegrass plants watered with complete nutrient solution. The plants were found to be inhibited in their growth to an extent proportional to the amount of dried roots added. Tests showed a sufficiency of available nitrogen present at the end of the experiment and no pathogenic organisms could be found. Benedict suggests that the well-known "sod-bound" condition with pure stands of bromegrass, where thick stands tend to thin out after a few years, may be in part due to the accumulation of growth inhibiting substances in the soil. This phenomenon had always been attributed to nitrogen-deficiency in the soil and Meyer and Anderson (52) in 1942 contended that this was indeed the principle factor. They found that application of nitrogen fertilizer could partially alleviate the inhibition of growth shown when various grasses were grown on brome-grass soil, and hence concluded that nitrogen was the limiting factor, not the accumulation of toxins. However, their data clearly show that nitrogen deficiency could not possibly explain the entire inhibition since the fertilized brome soils still gave a good deal less yield than did the soil on which no bromegrass had grown.

Additional evidence for root substances as the cause of soil infertility is presented in a research by Proebsting and Gilmore (68) on the problem of the difficulty in reestablishing peach trees in old peach orchards. After experimentally ruling out nutrient deficiency and pathogenic organisms as possible factors, it was found that the incorporation of peach roots into virgin soil of peach seedlings could reproduce the inhibiting effect. Moreover, an alcohol extraction of these peach roots gave an extract which was toxic and a residue which was non-injurious. The specific compounds concerned were not identified.

Recently several prominent workers in the field have expressed themselves very definitely as opposed to the idea that soluble toxins are of much practical importance. Loehwing (47), and Russell (72) believe that soluble toxins do not contribute much to the fertility picture in well-aerated soils which are provided with sufficient drainage and fertilizer. Waksman (91) maintains that many instances of soil infertility previously attributed to toxic excretions are actually due to an increase of useless or pathogenic microorganisms at the expense of beneficial forms.

What conclusions can be reached from this survey of scattered investigations on soil toxicity? Firstly, notwithstanding the opinions expressed in the preceding paragraph, it cannot be denied that cases of toxic organic substances arising from plants have been well-established. On the other hand, certain other cases of soil infertility seem to be definitely due to other causes. Secondly, it is apparent that each worker or reviewer is strongly inclined to favor as the crucial factor in soil fertility that aspect of soil science in which he is most interested. Thus, Waksman leans toward a bacteriological interpretation, while Russell, concerned principally with the mineral reactions in soil, believes infertility can be explained chiefly on a mineral basis. Similarly, Whitney, in 1905, probably exagerrated the case for organic toxins when he started his work. All of these workers probably attempted to apply their results too generally. Nevertheless, it seems to the writer that not enough credence has been given to the possibility that many cases of soil infertility which have as yet not been proven to be due either to mineral deficiency or to the presence of pathogenic organisms may arise from organic toxins in the soil which originate from higher plants. It is with this idea in mind that the following investigations on Parthenium argentatum were commenced.

B. Root Secretions

The early work of Brugmans (70), in which he alleged that he observed drops of liquid to exude from the roots of Vicia faba, has already been mentioned. Macaire-Prinsep's (70) work should also be recalled here. His observation of the changes taking place in rain water in which plants had grown pointed to the possibility of root excretions. In this early period there were serious objections to both of these works. Unger, and his pupil Walser (70), pointed out that, if Macaire's statements were correct, it should be possible to demonstrate the existence of organic material in the soil similar in composition to that existing in the plant which had grown upon it. Braconnot (8), making the same assumption, tried to demonstrate the existence of opium-like bodies by washing the soil on which plants of the poppy family had grown for several years. He obtained a solution of inorganic compounds, with only traces of organic compounds and concluded that organic secretions could not yet be considered as well-established. While it is obvious that these investigators lacked any idea of the difficulties of working with soil organic matter, they nevertheless deserve credit for showing that Brugmans had entirely misinterpreted the death of the root-hairs and the decortication of the growing roots and had assumed that this material was solid excretory matter from the living root.

Gyde (27) in 1846, reported that on growing various plants in distilled water, the roots imparted to the water soluble substances. In some instances the water acquired an odor which was separable on the application of heat. Plants like the bean and cabbage imparted an odor to the water similar to that which characterizes their leaves.

In the researches of the U.S. Bureau of Soils group not much attention was given to the problem of exactly how the toxic substances are given off by the root. Although the term "root excretions" is used loosely throughout the whole research, the workers always alluded to "decomposing plant debris" as the principle source of the compounds which they isolated. Whitney and Cameron's (75) original suggestion that the roots of crop plants actually secrete toxic substances to a significant degree was pure conjecture and no attempt was made to substantiate it by experiments.

However, later work seems to show that, at least under certain conditions, organic root secretions actually do occur. The acidification of the environment of the root by growing plants was a phenomenon that had long been known. Czapek (18) studied the problem in 1896 and decided that besides H_2CO_3, some of the lower acids might be secreted. Kunze (41) presented evidence to show that free mineral acids do not exist in the root excreta of higher plants. He thought that the acidity produced by roots was due to excretions of organic acids, but that in many plants the amounts "were so small as to be undetectable by litmus."

There followed then a long series of investigations (39, 58) on the excretion of organic acids by roots, motivated by a desire to explain the solution of insoluble phosphate in the soil by roots. Stoklasa (84) early claimed that under aerobic conditions, only CO_2 is excreted, but when O_2 is lacking in the vicinity of the roots, lower organic acids are secreted. Aberson (1) concurred in this view, claiming that under normal conditions only the CO_2 excreted from the roots plays a part in dissolving the inorganic phosphate. However, several researches produced results at variance with Stoklasa's contention. Schulow (81) and a little later Herke (35) reported malic acid in the aerated medium in which roots were bathed. Frey-Wyssling (26) and Ratsek (69) reported the isolation of oxalic acid as a secretory product of roots of Oxalis repens. On the other hand, Haas (28) contended, in agreement with the early findings of Stoklasa, that only CO_2 is excreted.

The excretion of sugars by roots has been reported by several investigators. Maze, (51) Schulow (81), Herke (35), and Knudsen (38) all contended that reducing sugars were excreted by roots. Knudsen tried to find a secretion of amylase by roots, but he could not substantiate this by experiment. The excretion of ammonia by roots is well established by the researches of Pryanichnikov (65, 66, 67) and others. Lyon and Wilson (49) in 1921 reported the presence of organic nitrogen in the sterile nutrient in which peas or maize were growing. Later, the famous researches of Virtanen and collaborators (81, 88, 90) showed beyond a doubt that, with legumes at least, a sizeable excretion of amino acids took place from the root nodules into the surrounding medium.

We can conclude, then, that, in certain cases at least, organic matter is excreted from plant roots. In at least two cases, those of the legumes and of Oxalis repens, the excretion takes place to a considerable degree. The conditions under which these excretions are increased or minimized are as yet quite obscure. As to whether the toxic organic substances arising from roots are true excretions, little can be said at this time, due to the complete lack of crucial experiments to test this point.

C. Organic Stimulatory Substances

In connection with some preliminary observations on organic stimulatory substances in the growth of guayule seedlings, brief mention should be made of the literature on this subject. We will only consider those substances which stimulate growth in concentrations lower than 50 mg. per liter. After many suggestive observations and experiments by early workers, Schreiner and collaborators (79) in the early part of the century reported the isolation of several nitrogenous substances from the soil which were stimulatory to seedlings in very low concentration. Bottomly (7), and later his student Mockeridge, claimed that small amounts of organic matter were essential for the growth of several aquatic plants. Many later researches on this problem, as those of Clark (12) and Ashby (3), refuted the claim for essentiality of these organic substances but agreed on their stimulatory effect.

Subramanyan (85), Virtanen and v. Hausen (89), and Nath (55) agree that substances contained in yeast extract have a marked stimulatory effect on the growth of various higher plants.

Various specific organic substances, having a known chemical constitution and active in low concentrations, have been investigated for their effect on the growth of higher plants. Among those for which favorable growth responses have been obtained by more than two workers are pantothenic acid (96), ascorbic acid (33), thiamine (6), and estrogenic hormones (98).

CHAPTER II
PHYSIOLOGICAL EVIDENCE FOR A GROWTH INHIBITOR

Having reviewed many cases of inhibiting influences on various plants, it is planned in this section to present some experiments indicating the possibility of such a phenomenon in the guayule plant. The experimental data presented herein are taken from an unpublished preliminary report prepared by Dr. James Bonner.

A situation had been repeatedly observed at the guayule nursery at Salinas, California whereby seedlings did not grow satisfactorily unless they were spaced a very considerable distance apart. The same phenomenon was noticed, although to a lesser extent, when the seedlings were planted out in the field. A number of physiological experiments were carried out which indicated that the production of an unfavorable environment by the roots of guayule plants might possibly explain this situation. These experiments will be described below.

Hoagland's nutrient solution (2) was used throughout, either in the standard concentration, on in 1-1 dilution of this solution with distilled water. Other experiments indicated that this was the optimal concentration for growth in height and accumulation of dry weight under the conditions used. Also, it was observed that dimunition of the nutrient concentration to one half of the standard level did not significantly affect growth. Further, the micro-elements were included in the nutrient solution in the concentration recommended by Hoagland and Arnon (2). This was despite the fact that it was observed under the conditions of the experiments that growth was not impaired when the micro nutrients were left out. It is therefore highly improbable that micro element deficiencies developed during the experiments.

Three kinds of experiments were carried out, which will be presented under the headings of leaching experiments, recirculation experiments, and experiments on the growth of seedlings under older plants, in the order named.

A. Leaching Experiments

In these experiments, guayule seedlings weighing less than 0.1 gram per plant were planted in four mesh gravel which was contained in one gallon cans painted with a non-toxic asphaltum paint. Good drainage was provided for, and the cans were placed on a well lighted greenhouse bench which was similarly well drained. After about a week, when new growth began to appear, these plants were used in the experiments. Eighty fairly uniform cans of plants were placed in rows of four along a greenhouse bench. Each alternate row served as a control. Each culture was given 250 ml. of Hoagland's solution three times a week and on the remaining days was supplied with 250 ml. of water. The other experimental rows were similarly given 250 ml. of nutrient solution, but this nutrient solution had previously been poured through 2 gallon cans of gravel which contained vigorously growing older guayule plants. (Nutrient obtained this way will be called guayule leachate hereafter.) On the remaining days of the week water which had been flushed through the older guayule plants was given to this series.

After six weeks measurements of the total height of all the plants were made, and after two months the plants were harvested and their dry weights ascertained. The results are given below on Table I.

TABLE I GROWTH OF GUAYULE PLANTS IN GRAVEL CULTURE

Each plot consists of 4 control and 4 experimental cultures. Each figure is a mean from 4 plants

	Height-cm/plant after 6 wks		Weight-gm/plant after 9 wks	
Plot Number	Control (nutrient and water)	Experimental (leached nutrient and water)	Control (nutrient and water)	Experimental (leached nutrient and water)
1	22.8	16.2	4.70	2.05
2	21.2	19.0	4.45	2.72
3	21.2	15.2	4.15	1.90
4	21.8	18.0	4.65	2.20
5	22.0	15.2	4.48	2.12
6	21.5	14.0	4.28	2.18
7	21.8	13.2	3.68	1.78
8	21.0	15.2	4.65	2.18
9	21.0	16.8	4.42	1.90
10	22.0	18.2	4.48	2.65
Ave.	21.6±.172	16.1±.584	4.39±.096	2.17±.098

Analysis of the guayule leachate showed no significant reduction in any of the major nutrients. It can be seen that the plants which received guayule leachate in place of fresh nutrient are significantly retarded in both height and weight.

B. Recirculation Experiments

The technique used here was to allow nutrient solution to drip through gravel contained in containers in which 6-12 month old guayule plants were growing and then to percolate this leached nutrient through similar containers containing guayule seedlings. The rate of flow was about 500 ml. per hour. After each draining through the crocks, the nutrient was collected and recirculated through the system. Control seedlings were grown in a similar crock into which was dripped nutrient solution which had not previously been leached through containers containing older plants. The nutrient supply was adjusted so that the concentration of nutrient remained relatively constant. After three months the height and dry weight of the plants were measured. The results from one such experiment can be seen in Table II. The seedlings receiving the nutrient which had been recirculated over older guayule plants were significantly retarded in their growth as compared to the controls.

TABLE II. GROWTH OF GUAYULE SEEDLINGS AS AFFECTED BY RECIRCULATION OF NUTRIENT SOLUTION FOR 3 MONTHS

	# of plants used	Height of plant cm/plant	Dry weight of plant gm/plant
Nutrient Recirculation over Seedlings only	18	24.3±0.70	3.45
Nutrient Recirculation over Seedlings and 8 month old plants	18	17.1±1.76	1.29

C. Growth of Seedlings Under Older Plants

Another type of experiment was to sink a glass container of about 500 ml. capacity into the gravel surrounding a 5-9 month old guayule plant, all contained in a 2 gallon glazed crock. The glass container was provided with drainage at the bottom and was filled with fresh gravel. Four seedling plants were placed under the older plant, two inside the glass container and two outside. The two sets of seedlings were therefore subjected to the same environment, except that the set

TABLE III. EFFECT OF 5 AND 9 MONTH OLD GUAYULE PLANTS ON THE GROWTH OF SEEDLING PLANTS IN THE SAME CROCK

Group I (5 month old plants)

	# of plants	# of plants surviving	Ht. in cm. after 5 wks.	Dry wt. gm./plant
Seedling plants in separate jars under larger plants.	8	7	6.5±0.86	0.28±0.44
Seedling plants under larger plants	8	7	3.1±0.44	0.08±.058

Group II (9 month old plants)

	# of plants	# of plants surviving	Ht. in cm. after 5 wks.	Dry wt. gm./plamt
Seedling plants in separate jars under larger plants	40	26	51.3±3.76	———
Seedling plants under larger plants	40	7	21.6±2.66	———

Group III (7 month old plants)

	# of plants	# of plants surviving	Ht. in cm. after 5 wks.	Dry wt. gm./plant
Seedling plants in separate jars under larger plants	50	49	42.2	———
Seedling plants under larger plants	50	50	30.3	———

outside the glass container was in addition exposed to influences of the culture medium of the older plant. Table III shows the growth of the three sets of seedlings after a period of five weeks. Those outside the glass containers are obviously retarded in their growth; there was also greater mortality in the plants outside the glass containers in one experiment.

Mention should also be made here of the fact that in the case of the first type of experiment, out of six experiments tried, five showed growth retardation similar to that shown in Table I but a sixth did not show any retardation. In the recirculating type of experiment results similar to those in Table II were obtained for two experiments while two additional experiments did not show retardation. The explanation and implications of the relatively poor reproducibility will be dwelt on later, in the discussion section.

These experiments then, provide good evidence that some influence is exerted by the roots of guayule plants which is unfavorable to the growth of guayule seedlings. In review, the evidence consisted of the obtaining of a nutrient solution which was unfavorable for the growth of guayule seedlings by pouring this nutrient over the root system of an adult guayule plant; of the gradual accumulation of a toxic principle by constantly recirculating nutrient over the roots of adult guayule plants; and of the inhibition of seedlings grown in a similar environment except for the fact that their root systems were not exposed to the influences exerted by the root system of the older plant.

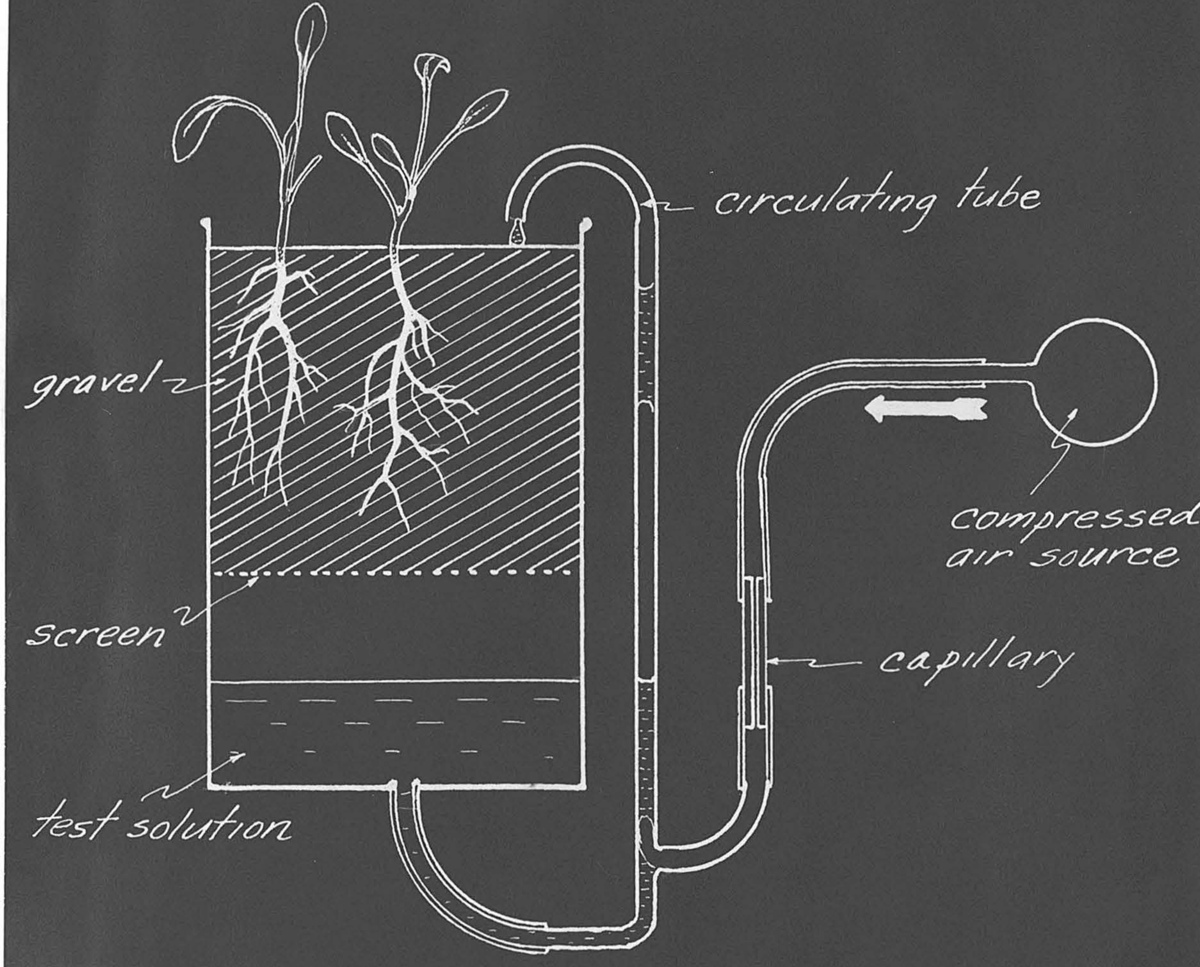


Fig. 1. CIRCULATING UNIT FOR
TYPE 1 ASSAY
(Longitudinal section.)
(2/3 actual size)

CHAPTER III
DEVELOPMENT OF ASSAY

In the preceding section it has been shown that nutrient solutions acquire growth inhibiting properties when leached through gravel cultures of guayule plant. Two methods for the semi-quantitative assay of the inhibiting principle will be described in this section.

A. Type I Assay

This type of assay was so devised that a solution to be tested for its inhibiting properties could be continually circulated over guayule seedlings. Metal containers of approximately 500 ml. capacity were divided into two parts by a screen resting on three nails driven through the side of the container. This gave a lower and an upper section, the former having about one-half of the volume of the latter. The entire container was painted with a layer of non-toxic asphalt paint. Washed ten-mesh gravel was placed in the top compartment and the solution to be tested was poured into the bottom compartment. A hole, placed in the center of the container, was tightly fitted with a short piece of rubber pressure tubing. To the end of this tube was fitted a circulating tube blown from pyrex glass and constructed in such a way that, upon the application of air pressure at the side, the solution in the lower compartment could be made to rise in the tube and drip into the sand in the top. This arrangement is shown in Figure 1. These circulating units were arranged in rows of 5 each,

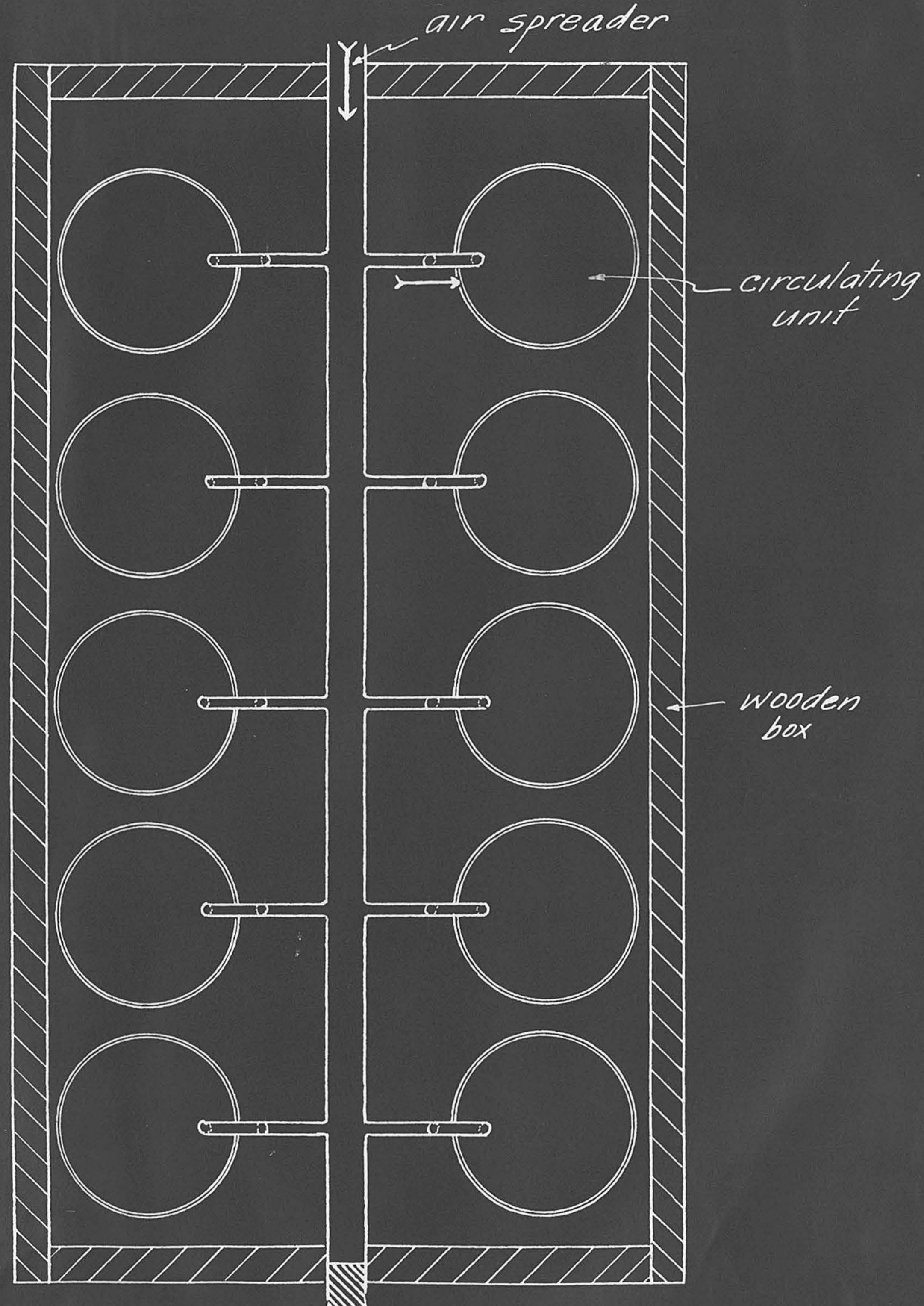


Fig. 2. 10 UNIT ASSEMBLY FOR TYPE 1 ASSAY
(top view)
(1/3 actual size)

contained in boxes having bottoms of wire mesh. Each box contained 2 rows of 5 circulating units, and was fitted with a spreader which distributed the air pressure to each individual unit. The pressure on each unit was equalized by short lengths of very fine capillary tubing placed in the rubber tubing connecting the air spreader to the individual recirculating tubes. Two 4-8 week old guayule seedlings were then placed in each unit, so that each row of 5 units contained 10 seedlings. Figure 2 shows the assembly of 10 circulating units to give 2 rows of 10 plants each, while Figure 4 is a photograph of a series of such assemblies, ready to begin the assay.

The nutrient solution recommended by Arnon and Hoagland (2), diluted 1-1 with distilled water, was used as the nutrient medium for the control plants. The extracts to be tested were dissolved in a nutrient solution of the same composition and concentration. Such a dilution of Hoagland's solution gave a total solute concentration of 700 mg. per liter, and the fractions to be tested were used in such concentrations that they never added more than 50 mg. per liter to the total solute concentration. One row of 10 seedlings was used to test each of the three dilutions made up for a single fraction, and one control series of 10 plants was used for each 3 dilutions. Every solution was tested in 3 concentrations, each 3 times more dilute than the preceding. The dilutions were made with one-half strength Hoagland's solution, so that the nutrient concentration remained approximately the same for all dilutions and for the control, but the fraction to be tested was diluted 3 times and 9 times, respectively. The assay boxes were then placed on a well-lighted greenhouse bench. A considerable amount of water was lost by transporation and evaporation. In order to keep the amount and concentration of the solutions at a

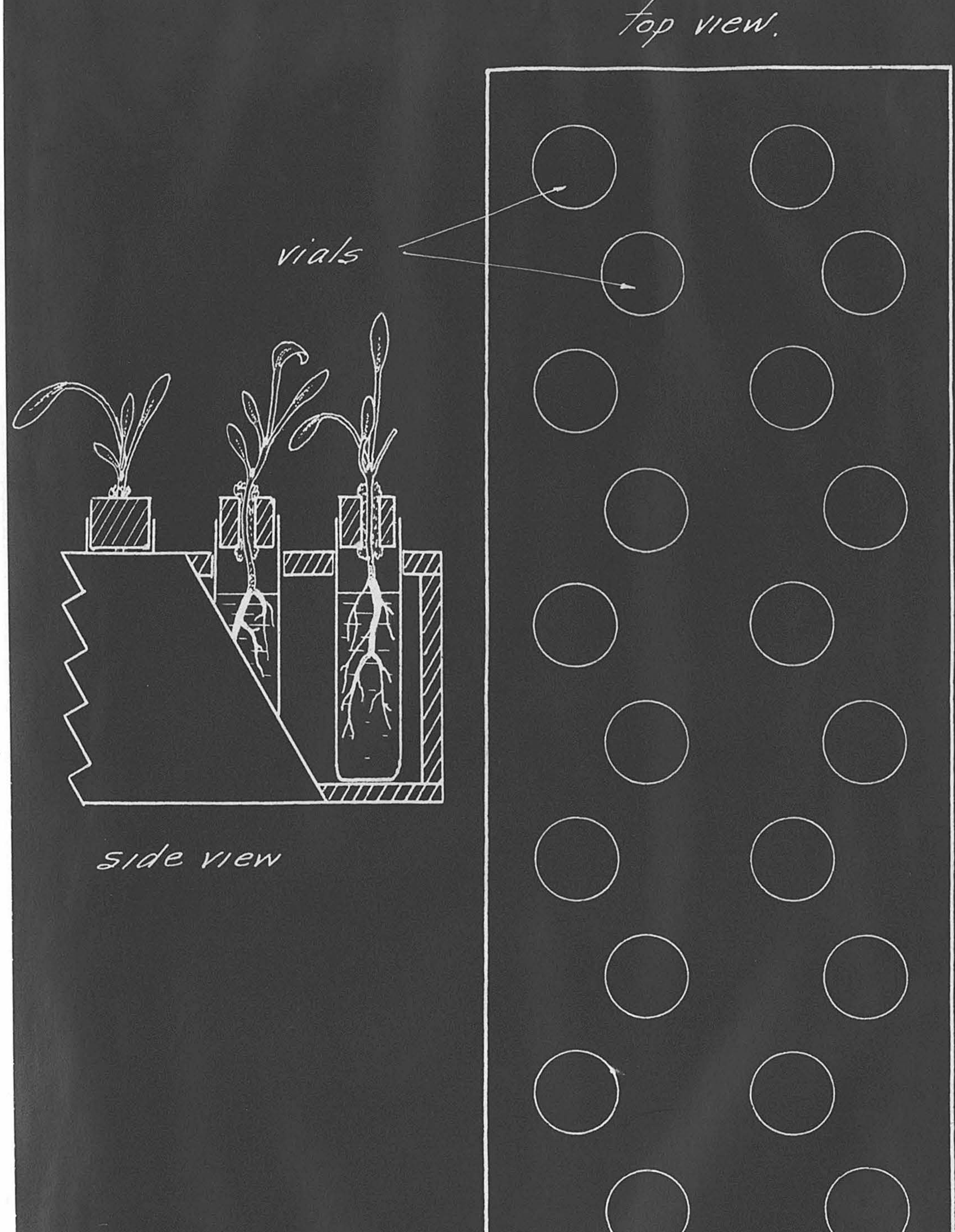


Fig. 3. ASSEMBLY FOR TYPE 2 ASSAY (½ actual size).

Fig. 4. Series of Assay Units
Type 1 Assay

constant level throughout the testing period, the water was replaced several times daily.* The initial weight of the seedlings was obtained by taking every 5th seedling from the planting material and drying and weighing them. At the beginning and at the end of the 10-day testing period the height of the plants was measured, and then the entire plant was harvested, dried and weighed. A statistical analysis of the height and dry weight measurements will be given in the latter part of this chapter.

B. Type 2 Assay

Although the type of assay described above gave satisfactory results, it required a considerable amount of attention over the 10-day testing period, and various considerations made it necessary to devise an assay which could maintained with a minimum of tending.

To this end, a type of assay was developed wherein guayule seedlings, age 4-8 weeks, were grown in individual vials of 18 ml. capacity. Wooden boxes were constructed in such a way that each vial fitted closely into a hole at the top of the box, and only the top 2-3 mm. of the vial protruded above the top of the box. This left the solution in the vial in darkness, but permitted the seedling to be exposed to light. Twenty such holes were made in each box, giving 2 rows of ten guayule seedlings each. This arrangement is shown in Figure 3. The plants were placed in slits made in the cork stoppers which fitted tightly into each vial. One-fourth strength Hoagland's solution was used for the control series, and the fractions and dilutions were made up as described for the Type 1 Assay. In each vial was placed 14 ml.

*Orin C. Russel rendered valuable assistance in the operation of Type 1 Assay.

of solution to be tested. At the end of one week all the vials were filled up to their original level with one-fourth strength Hoagland's solution. Initial dry weights and heights were obtained as in Type 1 Assay. At the end of the two weeks the height of the plants was measured, and the plants were harvested, dried, and weighed. Additional measurements were made in some assays on the amount of water transpired by each seedling during the first week's growth.

C. Variability of the Assay

In order to determine the percentage inhibition of the treated plants as compared to the control which could be considered as significant, two statistical analyses on the increase in height and dry weight over the assay period were carried out.

Table IV shows the increase in height and weight of 5 groups of control plants for a typical Type 1 and Type 2 Assay. The best estimate of the standard errors and difference needed for significance are shown in the table.

A more complete analysis on the assay measurements was made for a larger number of assay groups. In comparing the growth of groups of 10 seedlings as to increase in both height and weight, treatment by the analysis of variance showed that in both cases the variation within groups of 10 is, in general, not significantly different from the variability between replicate groups of 10 plants. Table V summarizes this statistical analysis for two experiments. It can be seen that in Type 1 Assay, for the height measurements the treated plants must grow at least 59.7% of the controls to be significantly retarded at the 5% level, and a growth of 46.9% of the controls is necessary for significance at the 1% level. For the weight measurements in

Type 1 Assay, a growth of 90.4% of the controls is necessary at the 5% level, and a growth of 81.6% of the controls at the 1% level. For Type 2 Assay, the corresponding growth percentages necessary are for height measurements 73.2% and 62.2% at the 5% level and 1% level respectively, and for weight measurements, 92.9% and 89.4%. Considering the general agreement between the results of these two analyses and several other calculations of variability (made at random,) the growth of guayule seedlings shall be considered as significantly inhibited when the growth is 50% of the controls.

TABLE IV. VARIABILITY OF THE ASSAY

Height Measurements

replicate group / plant	Type 1 Assay: Growth in mm/pl in 10 days — 1	2	3	4	5	Type 2 Assay: Growth in mm/pl in 14 days — 1	2	3	4	5
1	2	8	5	8	8	6	4	4	3	3
2	4	4	7	6	7	2	4	4	2	3
3	6	4	6	11	7	3	4	4	3	5
4	10	3	7	9	6	3	3	5	3	3
5	10	5	6	6	6	5	4	3	2	0
6	7	7	6	10	3	4	1	3	5	3
7	10	7	7	11	7	1	2	4	5	3
8	13	3	3	6	3	5	3	3	4	4
9	5	9	8	6	6	4	3	3	6	4
10	7	7	9	3	7	3	4	4	4	3
Ave.	7.0	5.8	6.4	7.7	5.9	3.6	3.2	3.7	3.7	3.1
S.E.m	±1.32	±0.45	±0.52	±0.72	±0.64	±0.48	±0.33	±0.21	±0.51	±0.41

Type 1 Assay: Best est. of std. error for diff. betw. 2 gps. of 10 = ±1.1

Diff. in % of mean needed for sig. @ 1% level-44%; @ 5% level-33%

Type 2 Assay: Best est. of std. error for diff. betw. 2 gps of 10 = ±0.57

Diff. in % of mean needed for sig. @ 1% level-43%; @ 5% level-33%

Weight Measurements

replicate group / plant	Type 1 Assay: Growth in mg/pl in 10 days — 1	2	3	4	5	Type 2 Assay: Growth in mg/pl in 14 days — 1	2	3	4	5
1,2	23	35	24	20	28	46	33	42	41	59
3,4	32	24	26	50	25	43	58	49	36	36
5,6	37	38	23	39	57	68	61	56	41	48
7,8	37	31	27	33	26	34	50	43	47	59
9,10	27	42	32	36	52	66	22	32	34	59
Ave.	32	27	27	36	38	51	46	46	40	54
S.E.m	±2.9	±3.1	±1.6	±6.3	±6.9	±6.7	±7.5	±2.3	±2.3	±4.6

Type 1 Assay: Best est. of std. error for diff. betw. 2 gps. of 10 = ±6.1

Diff. in % of mean needed for sig. @ 1% level-49%; @ 5% level-37%

Type 2 Assay: Best est. of std. error for diff. betw. 2 gps. of 10 = ±7.6

Diff. in % of mean needed for sig. @ 1% level-44%; @ 5% level-33%

TABLE V. SUMMARY OF RESULTS FROM THE TREATMENT OF TWO EXPERIMENTS BY THE ANALYSIS OF VARIANCE

For the height measurements in Type 1 Assay, 16 groups of 10 plants each were considered; for the height measurements in Type 2 Assay, 20 groups of 10 plants each were considered. For the weight measurements in Type 1 Assay, 16 groups of 10 plants each were used, but the plants were weighed in pairs, so that a total of 80 measurements were considered. Similarly, for the Type 2 Assay weight measurements, 24 groups of 10 plants each were used, giving a total of 120 measurements.

Analysis	Mean growth of 10 controls (cm. or mg.)	Best estimate of standard error of mean of 10 plants	Difference between 2 means of 10 plants necessary for significance at 5% level		Difference between 2 means of 10 plants necessary for significance at 1% level	
			Absolute	Percentage	Absolute	Percentage
Increase in height Type I Assay	6.4 cm	± .841	2.5 cm	39.1%	3.4 cm	53.1%
Increase in weight Type I Assay	190 mg	±5.586	18.2 mg	9.6%	26.5 mg	13.9%
Increase in height Type II Assay	7.4 cm	± .681	2.0 cm	27.1%	2.8 cm	38.2%
Increase in weight Type II Assay	233 mg	±5.186	16.9 mg	7.3%	24.6 mg	10.6%

CHAPTER IV
COLLECTION OF MATERIAL

With the development of a suitable assay for the detection of inhibiting principles on the growth of guayule seedlings, it was possible to commence an investigation of the chemical properties of the substance or substances responsible for the inhibition. In order to execute this study, it was necessary to collect large quantities of nutrient solution containing the inhibiting principle. This was accomplished by the following adaptation of one of the physiological experiments described in Chapter II.

Guayule plants, age from 6 months to 2 years, were cultured in 2 gallon glazed crocks filled with loose gravel and provided with good drainage. Twenty such crocks were arranged into a unit so that 5 gallons of half-strength Hoagland's nutrient solution was allowed to drip through the plants at a rate of about 500 ml. per hour. The nutrient solution was adjusted at weekly intervals so that the concentration of minerals remained relatively constant. The pH of the solution at the end of the recirculating period was between 6 and 7. During the months in which highly active leachate could be obtained, the plants in the glazed crocks took on a sickly appearance, suggesting that they were themselves being affected by the material which was accumulating in the nutrient solution.

Usually 220 liters of leachate were collected each month from 12 of the collection units described above. Table VI shows the activity of leachate collected during the successive months. The reasons for the inactivity of the leachate collected during December, January, and February will be discussed later.

TABLE VI. ACTIVITY OF LEACHATES COLLECTED DURING SUCCESSIVE MONTHS

Month Collected	Type Assay used	not diluted cm/10 pl	not diluted % of control	dilutions 3 x diluted cm/10 pl	dilutions 9 x diluted cm/10 pl	control cm/10 pl
*June 1942	1	5.7	52%	7.2	10.7	10.7
*July 1942	1	3.4	48%	4.0	7.4	7.0
*Aug. 1942	1	0.9	26%	1.4	4.1	3.4
*Sept. 1942	1	3.4	71%	4.0	4.8	4.8
*Oct. 1942	1	1.9	61%	2.2	2.5	3.1
*Nov. 1942	2	2.8	56%	3.1	4.2	5.0
Dec. 1942	2	3.3	81%	3.7	4.5	4.1
Jan. 1943	2	2.4	100%	3.1	2.5	2.4
Feb. 1943	2	7.6	98%	9.5	7.2	7.8

* represents active leachate

All the material so collected was subject to a concentration in large vacuum stills. The stills each had a capacity of 18 liters, and the concentration was run at pressure of from 18-35 mm. mercury, with the temperature inside the stills never exceeding 60 degrees Centigrade. Under these conditions, each of the two stills used could deliver 6-8 liters of distillate per hour. The 220 liter batches which were collected each month were concentrated in this way to 2-4 liters, and this concentrate, together with the material which precipitated out during the concentration was used for the chemical studies to be described in the next chapter.

CHAPTER V
CHEMICAL STUDIES ON THE INHIBITOR

A. Preliminary Experiments

In commencing the chemical studies on the inhibiting principle, a prime consideration was the question of whether the substance was organic or inorganic in nature. Indications were that we were not dealing with inorganic deficiencies, because the physiological experiments had shown that the major nutrients were not deficient in the toxic leachates. Also, as mentioned before, a micronutrient supplement was added even though no deficiency symptoms for any of these micronutrient could be induced under the conditions used. Further, it was seen that dilution of the leachate with distilled water alleviated the toxicity, which is difficult to explain on the basis of a mineral deficiency. The possibility of toxicities induced by excessively high total salt concentrations was ruled out by determining the optimum salt concentration for the plants to grow in the assay. Thus, for Type 2 Assay, the optimum salt concentration was found to be one-fourth strength Hoagland's solution. Table VII gives the results of two typical experiments on salt concentration for Type 2 Assay. Figure 5 depicts these same results graphically.

A preliminary study of some of the properties of the inhibitor furnished some information on its volatility, its stability towards heat, and its stability on drying. Table VIII shows the activity of an unconcentrated leachate, of an aliquot of the concentrate after

TABLE VII. EFFECT OF SALT CONCENTRATION ON GROWTH OF GUAYULE SEEDLINGS

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 14 days
Type 2 Assay

Experiment	Strength of Hoagland Solution Used					
	Full str.	1/2	1/4	1/8	1/16	0
1	21	23	29	19	16	—
2	—	28	39	25	20	21

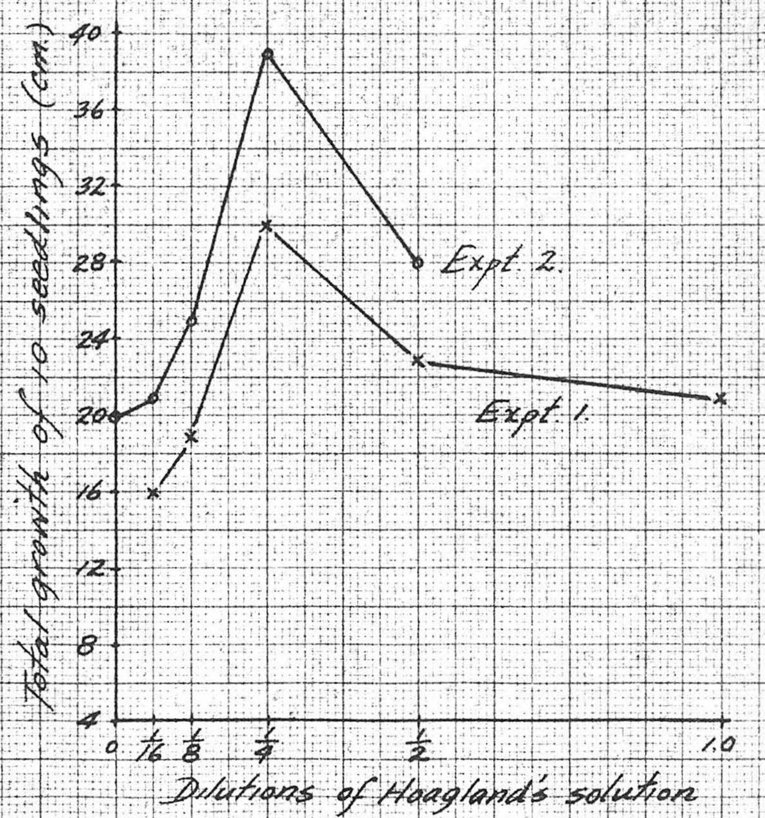


Fig. 5. Effect of salt concentration on growth of Guayule seedlings.

TABLE VIII. ACTIVITY OF THE INHIBITOR DURING CONCENTRATION

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 10 days
Type 1 Assay

	not diluted		dilutions		control
			3 x diluted	9 x diluted	
Experiment	cm/10 pl	% of control	cm/10 pl	cm/10 pl	cm/10 pl
*unconc. leachate	12	30%	14	30	40
*aliquot of conc. leachate	16	34%	34	36	47
distillate from conc.	24	96%	24	28	25

* indicates active fractions.

concentration as described in Chapter IV, and of the distillate obtained during the concentration. The aliquot of the concentrate was diluted in the original dilution to such an extent that the salt concentration was the same as that of the unconcentrated leachate. The distillate was tested by using it instead of distilled water to make up the standard one-half strength Hoagland's solution used in the assay. It can be seen that little activity is lost during the concentration and that the distillate is inactive. The active principal is therefore not volatile under the conditions of the concentration.

Table IX gives the results of a typical experiment on the effect of autoclaving and of drying the aliquots of concentrated leachate. It can be seen that the activity is not significantly reduced either by autoclaving at 15 pounds pressure for 30 minutes or by drying. All of these experiments have been repeated at least four times with consistent results.

TABLE IX. EFFECT OF AUTOCLAVING AND DRYING ON ACTIVITY OF INHIBITOR

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 10 days
Type 1 Assay

	not diluted		dilutions		control
			3 x diluted	9 x diluted	
Experiment	cm/10 pl	% of control	cm/10 pl	cm/10 pl	cm/10 pl
*aliquot of unconc. leachate	11	50%	15	18	22
*identical aliquot autoclaved 30 minutes	10	44%	18	19	23
*aliquot of conc. leachate	24	51%	25	34	47
*identical aliquot dried and redissolved	15	44%	24	36	34

* indicates active fraction

These results did not preclude the possibility of the presence of a toxic inorganic substance. To determine the presence or absence of such toxic inorganic ions four experiments were carried out in which different guayule leachates were evaporated to dryness and ashed thoroughly in a porcelain crucible. The residue after ashing was dissolved in dilute HCl or HNO_3 and neutralized. In every case, the activity of the leachate disappeared on ashing. Table X shows the results of a typical ashing experiment. It was obvious, then, that the toxic principle was either volatilized or combusted during the ashing process. This evidence pointed to an organic substance, but there still remained the possibility that an inorganic principle was either volatilized or changed to an inactive form during the ashing.

TABLE X. EFFECT OF ASHING
ON GUAYULE LEACHATE

Each figure represents total growth in dry weight (mg.)
of 10 guayule seedlings in 10 days
Type 1 Assay

Experiment	not diluted mg/10 pl	not diluted % of control	dilutions 3 x diluted mg/10 pl	dilutions 9 x diluted mg/10 pl	control mg/10 pl
*aliquot before ashing	128	51%	167	187	252
identical aliquot after ashing	225	98%	239	235	230

* indicates active fraction

That this was not the case will be shown in the next paragraph, where the solubility of the toxic principle in various organic solvents will be described.

B. Extraction with Organic Solvents

In Table XI are given the results of an experiment in which identical aliquots of an active concentrated leachate were subjected to extractions with various organic solvents. In the acetone and ethyl alcohol extractions, the aliquots were thoroughly dried under vacuum and exhaustively extracted in a sohxlet extractor. The other extractions were liquid-liquid extractions, all continued until no more material could be extracted. The acid-ether extraction was carried out by first acidifying the aqueous concentrate to pH 2.1 with dilute H_2SO_4 and then extracting with ether. The other extractions were done from neutral solutions. Each extraction was repeated two or more times, and the results were consistent with those given in the table. It

TABLE XI. SOLUBILITY OF INHIBITOR IN VARIOUS ORGANIC SOLVENTS

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 10 days
Type 1 Assay

	not diluted		dilutions		control
Experiment	cm/10 pl	% of control	3 x diluted cm/10 pl	9 x diluted cm/10 pl	cm/10 pl
*original conc. leachate	24	51%	25	34	47
*96% ethyl alcohol extract	27	58%	32	45	46
residue after ethyl alcohol extraction	46	107%	41	44	43
*acid ethyl ether extract	26	40%	41	55	65
residue after acid ethyl ether extraction	51	94%	47	53	54
neutral ethyl ether extract	42	78%	52	45	54
acetone extract	40	91%	48	43	44
petroleum ether extract	54	110%	52	43	49
ethyl acetate extract	39	98%	43	41	40
chloroform extract	69	92%	65	64	75

* indicates active fraction

can be seen that the active material is extractable by 96% alcohol from neutral solution and by ethyl ether from acid solution, leaving the residual aqueous solutions inactive. The inhibitor is not extractable in appreciable amounts from neutral solution by ethyl ether, acetone, ethyl acetate, chloroform, or petroleum ether. Thus, the fact that the material responsible for the activity is extractable by ether from acid solution leaving the residual salt solution inactive can be accepted as conclusive evidence that we are dealing with an organic substance, possibly acidic in character.

An acid-ether extraction was adopted as the standard procedure for obtaining an active organic fraction from the concentrated leachates. The alcohol extraction, although equally effective in extracting the active principle, undoubtedly carried some inorganic salts along with the organic matter and it was therefore not used. In some experiments the acid-ether extraction was carried out by concentrating the leachate completely to dryness and acidifying the dry salts with dilute H_2SO_4, and then exhaustively extracting with ether in a sohxlet extractor. This procedure was found to be as effective as the liquid-liquid extraction in separating the active material.

C. Amount of Organic Matter Collected

With the development of the acid-ether extraction, it was possible to quantitatively determine the amount of acid-ether extractable organic matter arising from an adult guayule plant. Thus, it was found that, under the conditions described in Chapter IV for the collection of leachate, a one year old guayule plant weighing from 10 to 50 grams, gives rise, over a period of 30 days, to an average of about 100 gammas of acid-ether extractable organic matter per day. This quantity varies somewhat from month to month, having been 95 gamma per plant in March 1942, 85 gamma in December 1942, and 140 gamma in January 1943. It should be borne in mind that these figures were obtained by considering the total acid-ether extracted material arising from the roots of 240 guayule plants over a period of 30 days. This gives a total of 6200 plant-days of leachate, and the amount of material obtained from the month's leachate divided by 6200 gives the average quantity arising from each plant per day. Therefore, we have no idea as yet as to the variability from plant to plant in the production of this organic material. Also, it is possible that there is a variation in the daily production of this material as the leachate became more toxic towards the end of the month's recirculation period. Further, the acid-ether extractable organic material is undoubtedly only a part of the total organic matter in the leachate. For one month's leachate, the volatile material was caught in a CO_2 trap and the distillate was exhaustively extracted with ether to give an additional 100 gammas of organic matter per plant per day. Therefore, a figure of 200 gamma or organic matter per day per plant can be taken as a minimum quantity for the total organic matter produced.

D. Fractionation

With an organic fraction at hand, free of inorganic salts, it was possible to begin studies on the isolation of the factor responsible for the toxic activity. While this end has not been accomplished as yet, it is possible to report several promising experiments in this direction. They will be described in the following paragraphs.

The effect of charcoal on the active principle was early considered, and it was found that when charcoal was added to an aliquot of active concentrated leachate the filtrate retained the activity, although the original yellow color of the leachate was lost. The charcoal adsorbate when eluted with 60% acetone containing 2½% NH_4OH, yielded an eluate which was either non-toxic or sometimes stimulatory. A

TABLE XII. EFFECT OF CHARCOAL ON THE INHIBITOR

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 10 days
Type 1 Assay

	not diluted		dilutions		control
			3 x diluted	9 x diluted	
Fraction	cm/10 pl	% of control	cm/10 pl	cm/10 pl	cm/10 pl
A. *aliquot of conc. leachate	28	56%	36	47	50
*filtrate from charcoal adsorbtion of A.	11	58%	24	30	19
activity of eluate 53 mg/l.	36	95%	31	40	38
B. *acid-ether extract in H_2O 37 mg/l.	25	57%	35	37	44
*filtrate from charcoal adsorbtion of B. 18 mg/l.	16	57%	16	22	28
activity of eluate 18 mg/l,	24	266%	13	12	9

* indicates active fraction

similar result was obtained when the acid-ether extract was dissolved in water and charcoal allowed to adsorb from this solution. Table XII gives the results with two different starting materials concerning the effect of charcoal on the inhibitor.

It was found that a purification of the acid-ether extract could be effected by taking the material up in boiling water, using approximately one ml. of water to one mg. of extract. In this way the insoluble resinous fraction could be separated from the soluble material by filt-

TABLE XIII. FRACTIONATION OF ACID-ETHER EXTRACT BY AQUEOUS SOLUBILITIES

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 10 days
Type 1 Assay

		not diluted		dilutions		control
				3 x diluted	9 x diluted	
Experiment	conc.	cm/10 pl	% of control	cm/10 pl	cm/10 pl	cm/10 pl
*acid-ether extract	16mg/ liter	10	50%	17	23	20
hot H_2O insoluble (resins)	32mg/ liter	27	69%	29	30	39
*cold H_2O insoluble (crystalline)	3.5mg/ liter	11	55%	13	18	20
cold H_2O soluble	30mg/ liter	17	121%	16	10	14

* indicates active fraction

ering the hot solution through a wet filter paper. The resinous material remained on the filter paper, and the clear hot solution filtered through. On cooling, a precipitate formed which was active, while the mother liquor had little or no activity. In one experiment, this precipitate was crystalline in form, and was very toxic. The crude crystals, when thoroughly dried, melted at 60-65 degrees. The activities of the various fractions from this experiment are given in Table XIII. The total amount of crystalline material obtained in this way amounted to four mg. This amount was necessary for the determination of activity and it could therefore not be studied further. Attempts are now in progress to collect enough of this crystalline material for a structural determination. It will be noticed from the table that the resin had some activity; this activity of the resin fraction seemed to vary from month to month, being completely inactive in some months and quite active in others.

Precipitation with lead and attempts to fractionate the activity in the resinous fractions by molecular distillation did not yield clear-cut results. In the first case, the major part of the activity was apparently not precipitable by lead, and in the second, both the distillable part and the residue were inactive. This inactivation was possibly caused by the exposure of the material to excessively high temperatures during the molecular distillation.

E. pH Effect

Consideration was given to the possibility that the various fractions which showed a significant growth-retarding activity might have exerted their influence because of an effect on the pH of the nutrient solution. Table XIV shows the pH of the various typical fractions, both active and inactive, determined at the beginning and at the conclusion of the assay. It can be seen that there is very little variation in the pH from fraction to fraction, and that no correlation exists between pH

TABLE XIV. pH AND ACTIVITY OF VARIOUS FRACTIONS

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 10 days
Type 1 Assay

Fraction	pH of original dilution beginning	end	not diluted cm/10 pl	% of control	dilutions 3 x diluted cm/10 pl	9 x diluted cm/10 pl	control cm/10 pl
*acid-ether extract	5.3	6.1	10	59%	14	19	17
neutral ether extract	5.6	6.9	27	159%	23	19	17
*acid-ether extract	6.0	6.4	10	50%	15	20	20
extracted conc. leachate	6.0	6.2	17	81%	21	17	21
unconc. leachate	5.9	7.0	20	111%	26	14	18

* indicates active fraction

and activity. Similar pH determinations were carried out on five other assay groups, with results similar to those in the table.

F. Stimulatory Fractions

At various times during the chemical work on the inhibitor, there would appear a fraction, which instead of being toxic or inactive when tested in the assay would significantly increase the growth of the test plants over the controls. While the purpose of this investigation was not to investigate this aspect of the leachate material, it was nevertheless considered of interest. Table XV shows the stimulatory activity of several fractions. All the fractions listed were

TABLE XV. STIMULATORY ACTIVITY
OF LEACHATE FRACTIONS

Each figure represents total growth in height (cm.)
of 10 guayule seedlings in 10 days

		not diluted		dilutions		control
				3 x	9 x	
	Type			diluted	diluted	
Fraction	Assay	cm/10 pl	% of control	cm/10 pl	cm/10 pl	cm/10 pl
A 2 mg./l.	1	30	167%	26	18	18
B 36 mg./l.	1	27	159%	23	19	17
C 8 mg./l.	1	33	150%	32	30	22
D 18 mg./l.	1	24	267%	13	12	9
E *50 mg./l.	2	126	143%	124	117	88

* Type 2 Assay is run for 14 days

organic material free of inorganic salts. Fractions A,B, and C are neutral ethyl ether extractions of concentrated leachates from different months, while fractions D and E are the eluates from the charcoal adsorbtion procedure mentioned above. It should be noticed that these fractions are stimulatory in concentrations where they could not possibly be regarded as nutrient material. Since the amount of actual substance is only a fraction of this concentration, it is possible that we are dealing with true accessory growth factors.

G. Activity of Pure Compounds

In the course of these studies, it was deemed advisable to test the activity of various synthetic mono- and dicarboxylic acids. With the exception of one organic acid, cis-9,10 dihydroxy stearic acid, all the acids were non-toxic to the guayule seedlings. Those acids tested and found to be inactive were the following:

acetic	margaric
propionic	stearic
valeric	undecylenic
iso-valeric	oleic
caproic	glutaric
iso-caproic	adipic
caprylic	sebacic
capric	pelargonic
undecylic	pyruvic
lauric	itaconic
tridecylic	tiglinic
myristic	mandelic

These were all tested at a concentration of 10 mg. per liter. The dihydroxy stearic acid was markedly toxic at the concentration as shown in Table XVI.

TABLE XVI. TOXICITY OF cis-9,10 DIHYDROXY STEARIC ACID

Each figure represents total growth in height (cm.) of 10 guayule seedlings in 10 days
Type 1 Assay

	not diluted		dilutions		control
Experiment	cm/10 pl	% of control	3 x diluted cm/10 pl	9 x diluted cm/10 pl	cm/10 pl
dihydroxy stearic acid 10 mg./l.	5	29%	8	13	17

We can conclude from the chemical studies, then, that we are dealing with an organic inhibitor which is possibly acidic in character. It is active in concentrations of 3.5 mg. per liter. The growth retarding activity of the inhibitor is not due to pH effects on the nutrient solution. Also, a growth promoting substance is present in the leachate which is active in concentrations as low as 2 mg. per liter.

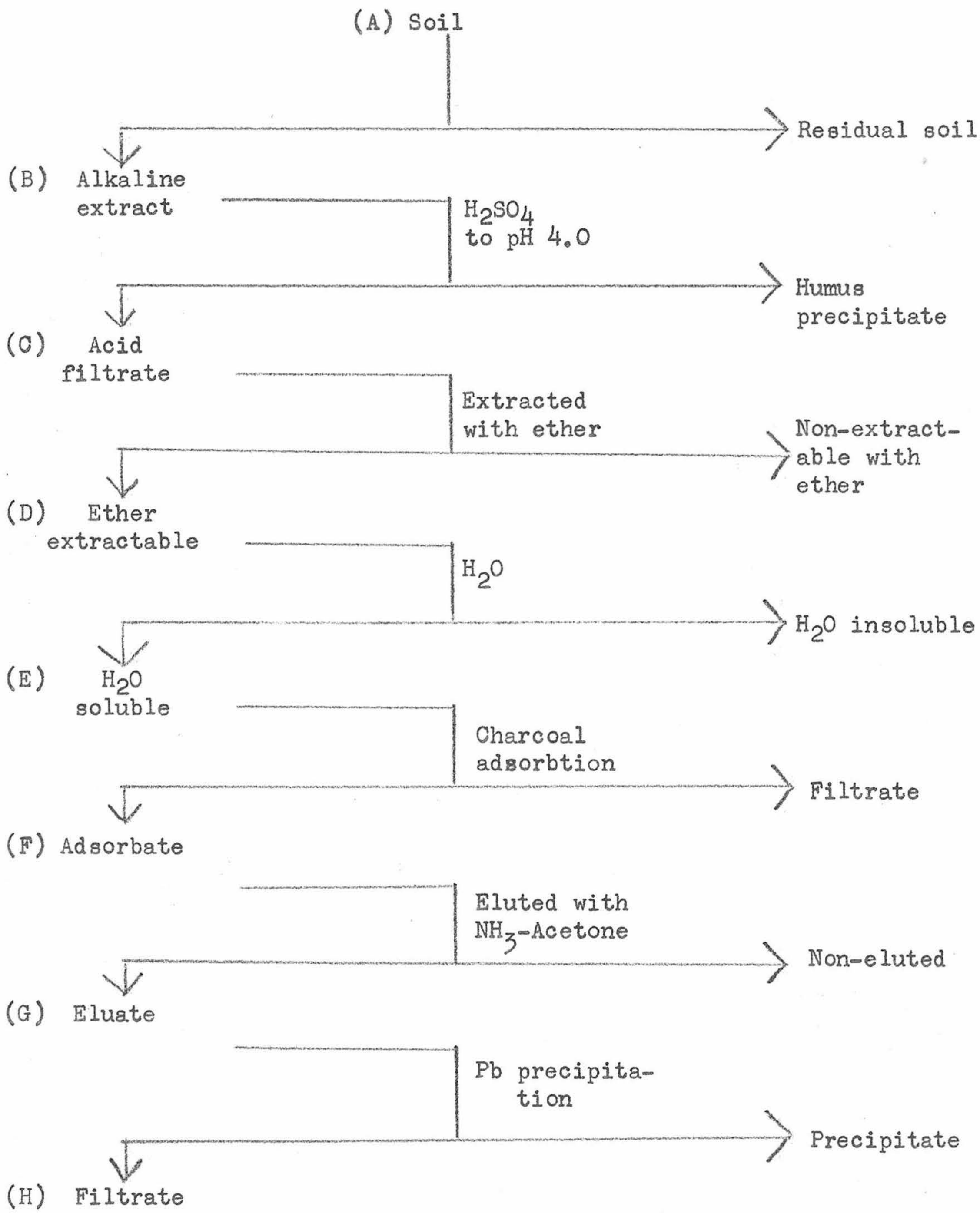
CHAPTER VI
CHEMICAL FRACTIONATION OF GUAYULE SOIL

During the course of the experiments on guayule leachates, it became of interest to know whether similar inhibiting substances were present in the soil in which guayule plants had been growing. To this end, an investigation was carried out on various fractions of the organic matter of guayule soil in the manner described below. It is recognized that this experiment should have been carried out together with a similar treatment of a similar soil in which no guayule plants had grown. However, these experiments were only of a preliminary nature and are presented as such. The fractionation procedure used is essentially that of Schreiner and Lathrop, (74) with several modifications.

Guayule plants had been grown for a year and a half on a Hanford light sandy loam soil at the California Institute of Technology Experimental farm at Arcadia, California. When these plants were harvested, 27 kilograms of soil were taken from the top eight inches of soil in the region where guayule roots had penetrated, and submitted to a chemical fractionation. Figure 6 diagrams the fractionation scheme used. The capital letters used to designate the fractions in the description below refer to the corresponding letters in the fractionation scheme.

The 27 kilograms of soil (A) was extracted with 70 liters of 2% NaOH for three days, using vigorous mechanical stirring. The alkaline

FIGURE 6. FRACTIONATION OF
SOIL CROPPED TO GUAYULE



extract was separated from the residual soil by filtering through thick layers of dicalite. 68 liters of dark red alkaline extract were thus obtained.

The alkaline extract (B) was neutralized with H_2SO_4 and enough excess acid was added to bring the solution to pH 4.0. The humus precipitate which formed was allowed to settle over night. The next morning the clear supernatant liquid was decanted off the precipitate, and the remainder of the filtrate was obtained by filtering the wet precipitate on a dicalite mat. The decantate and filtrate were combined (C) and used in the next step.

The acid filtrate (C) was then exhaustively extracted with ether in a continuous liquid-liquid extractor to give 250 mg. of ether extractable material (D).

This ether-soluble fraction was dissolved in 250 ml. of boiling water and the water allowed to cool slowly. This gave 105 mg. of resinous precipitate and a water soluble fraction (E).

To the water soluble fraction was added 5 grams of activated charcoal (Norite A) and the charcoal allowed to adsorb for 24 hours with occasional stirring. The charcoal adsorbate (F) contained most of the material leaving only 8 mg. in the filtrate.

The 5 grams of charcoal containing the adsorbed material was eluted with 100 ml. of 60% acetone containing $2\frac{1}{2}$ ml. of concentrated NH_4OH. The eluate (G) left on evaporation of the solvent, 98 mg. of dark brown residue. During the evaporation of the solvent, however, 2 mg. of white crystalline material were deposited in the neck of the flask. These crude crystals had a melting point of 62-75 degrees Centigrade. They were used to test in the assay, proving to be inactive, and were not further characterized.

The final step in the fractionation was a precipitation with lead wherein a saturated solution of neutral lead acetate was added to a water solution of the 98 mg. of eluate (G). This caused a precipitate to form, which was decomposed with concentrated H_2SO_4 and the decomposition mixture exhaustively extracted with ether to give 22 mg. of material. The filtrate on similar decomposition and extraction yielded 72 mg. of material (H).

Fractions E,G, and H were all active in concentrations of 10 mg. per liter. The other fractions which were retained were also active, and the discarded fractions had little or no activity. Fraction F was found to be stable to autoclaving at 20 lbs. pressure for 30 minutes.

It can therefore be concluded that in the soil under examination there exists a substance or substances which are inhibitory to the growth of guayule seedlings. Any attempt to identify the inhibitor in the guayule soil with that in the guayule leachate would be premature until the isolation of the substances involved, but the chemical evidence at hand indicates that they might at least belong to the same class of substances, since they are both of acidic character, non-precipitable by lead, and stable to heat. It will be noted, however, that the substances behave differently in their adsorbability on charcoal.

CHAPTER VII
DISCUSSION

The establishment of the fact that an inhibitory organic substance arises from gravel cultures of guayule plants suggests several questions, which shall be considered below.

It was mentioned in the chapter on Collection of Material that the production of inhibitor seemed to decline during the winter months. This phenomenon had also been suspected as the cause for the lack of reproducibility of certain of the experiments mentioned in Chapter II, since every experiment which did not yield a positive result was done in the winter. This tendancy towards a periodic production of inhibitor, although very troublesome from the standpoint of obtaining active material for the chemical studies, is of considerable interest. It implies that the production of the inhibitory influence is in some way connected with the period of greatest metabolic activity in the plant. Thus, during the winter months, when the plant does not make any new growth and is in a relatively dormant state, the production of this inhibitor for some reason decreases. This suggests that the inhibitor is in some way intimately connected with the physiology of the plant's growth, rather than a product of dead plant residues. A controlled study of the influences of various external factors on the production of the inhibitor should afford more insight into the relation of the inhibitor to the metabolism of the plant.

In connection with the question of the origin of the inhibitory substances the method of collecting the material used in this study should be kept in mind. In the other studies reported in the literature where a search was made for the substance involved in inhibition phenomena, the source of the inhibitory material was such that the origin of the material could not be decided unambiguously. In the work of Schreiner et al. on toxic substances found in soils (78) it is not at all clear from whence their material originated. They worked with soil organic matter and this could have come from any one of a great number of sources, such as dead plant residues, metabolic products of soil bacteria or fungi, excretory products of the living root system, etc. In the study by Davis on the toxicity of black walnut trees (20), the supposedly active principle was obtained by extracting parts of the walnut tree. It is conceivable that an organic substance could be found in the walnut tree which is toxic to the growth of other plants, and yet is not the one responsible for the detrimental effect of the tree to surrounding vegetation. It would first have to be proven that this substance escapes from the tree and persists in the surrounding soil. The same objection could be raised to the work of Proebsting and Gilmore on peach root toxicity (68), for they obtained their active fractions by extracting dried peach roots. Benedict's investigation (5) on bromegrass toxicity also utilized dried bromegrass roots. The various substances inside of the plant can have no influence until they are in some way transferred from the plant into the medium surrounding the plant. In the material which was used for this study, however, these difficulties are largely overcome. By constantly washing the roots with nutrient solution, material is obtained which more nearly represents that which would be expected to be effective under field conditions. Since the material was not collected under sterile conditions, the possibility still remains that the effective substances are formed by bacteria at the expense of sloughed-off cells.

An objection to this method of collecting material that can be raised is that the plants supplying the organic matter to the nutrient solution are artificially exposed to an accumulation of toxic material. In the soil, this toxic material would be leached away from the plant or exposed to influences which would tend to decompose it. This artificial accumulation of toxic material may cause the plants to act differently in their production of organic matter than they would under natural conditions. Therefore, the results obtained from such studies on plants grown in nutrient solution cannot be applied to natural situations without further experiment.

Little can be said as to whether the toxic organic matter under consideration is a true secretion of the roots or not. One method of attacking this problem would be to cut the top of a series plants in the collection units described in Chapter IV. If the toxic substances arise from decomposing root tissue, such topped plants would be expected to yield greatly increased amounts of inhibitor. On the other hand, if the inhibitor is a secretion of the root system, topping should decrease the amount of toxic material produced. Such studies are under way at the present time. However, the data presented on the total amount of organic matter produced per plant might give some indication as to the possibility of a root secretion. Considering the minimum value of 0.2 mg. per day per plant for the organic matter arising from the roots, it is possible that practically all of this material arises from sloughed-off root material in the growing process. Certainly the quantity involved is not inconsistent with such an idea. The determination of the organic matter arising from a plant in a day is only a suggestive preliminary investigation. Experiments designed towards the end of quantitatively measuring the variations in the production of this organic matter under different external conditions should shed much light on the problem of root secretions.

As to the specificity of the guayule inhibitor, preliminary experiments had shown that guayule leachate was not toxic to tomato seedlings, and vice-versa. Aside from this observation, no other data is at hand concerning this question. It would be quite useless to speculate on the meager data at hand, but an investigation on the effect of guayule leachate on plants closely related to guayule and on plants not related to guayule should prove informative as to its relative specificity.

Some consideration should be given to the growth-promoting fractions encountered during the studies on guayule leachate. The presence of such stimulatory activity together with the inhibitory activity indicates a much more complicated picture than was at first assumed. This means that the growth response obtained with the guayule leachate might have been resultant of the inhibitory and stimulatory activities. Possibly the failure to obtain a consistant relation between concentration and response for the inhibitory principle is due to the presence of a growth-promoting substance whose activity dilutes out at a different rate from that of the inhibitor. Such questions can be decided only when good chemical methods for the separation of the inhibitory and stimulatory activities are devised.

Another aspect of the stimulatory and inhibitory activities of the guayule leachates is the possible bearing that such organic substances have on various ecological and agronomical phenomena. Kooper (40), studying the tendency of various tropical weeds to grow in association with each other, could not correlate this association with any known physical or chemical factor. Went (93) describes various associations between trees and epiphytes in the forests of Java and maintains that although various physical factors play a part in this association, the critical factor is the kind of plants involved. A similar tendency for certain desert plants to always grow near one another is described by Went (94). It is at least a possibility that such associations are conditioned by the influence that one plant has on another due to the stimulatory or inhibitory substances which it gives up to the soil. The well-known beneficial effect of interplanting legumes with many crops may be dependent on other factors besides the return of nitrogen to the soil. With the sizeable excretion of amino acids that some legumes have shown to be capable of (90), it would be interesting to investigate the effect of excreted accessory growth factors. Also many common agronomical practices in which non-legumes are planted in association with or following other crops may depend on stimulatory organic substances left in the soil by the non-legumes.

Finally, we may consider what could possibly be done towards alleviating the effect of an organic inhibitory substance of the type described in the preceding chapters. Earlier, Schreiner and co-workers (77), had found that the application of fertilizer of various types was helpful in alleviating the toxicities induced by the toxic compounds in soils. Liming was especially effective in this regard. Common agricultural practices, such as deep tillage, allowing lands to be fallow, etc. also were effective in bringing about conditions under which the toxic substance could be decomposed. These remedies, however, do not exhaust the possibilities. A study of the structure of the compound or compounds responsible for the toxic effect might yield a specific chemical treatment which would decompose the inhibitor.

It is interesting in this regard that Subramanyan and co-workers (36,86) found that application of oxidizing compounds, like MnO_2 and H_2O_2, to soil in which plants are growing, give beneficial growth responses. This might be due to the destruction of toxic factors which are limiting to the growth of the plants. Another possibility of alleviating a soil toxicity induced by organic compounds would be to find a bacteria in soil which could metabolize the compound involved, and then determine the conditions necessary to enrich the soil with this organism. However, it is obvious that such studies must await the isolation and chemical identification of the specific substances responsible for the toxicity.

SUMMARY

1. Evidence is presented to show that when guayule plants (6 months to 2 years of age) are grown in gravel culture, an influence is exerted which is inhibitory to the growth of guayule seedlings.

2. The development of two types of semi-quantitative assay for the detection of the inhibiting principle is described.

3. A method is described for the collection of large quantities of nutrient solution containing the inhibiting principle, and for the subsequent concentration of this material to small volume. It is pointed out that the production of the inhibiting principle decreases sharply during the winter months.

4. Evidence is presented to show that the inhibiting principle is an organic substance. The critical evidence consists of the disappearance of activity on ashing and of the extractability of the inhibitor by organic solvents.

5. It is estimated that a guayule plant (6 months to 2 years of age), growing in gravel culture gives up to the nutrient medium, on the average, a minimum of 200 gammas per day of organic matter.

6. Various chemical properties of the inhibitor are given. It is shown to be relatively stable to heat, stable to drying, non-volatile, non-adsorbable by charcoal, and probably non-precipitable by lead.

7. An experiment is described in which a crude crystalline material was obtained which was very active.

8. Evidence is presented to show that the effect of the inhibitor

is not due to an influence on the pH of the nutrient solution.

9. The occurance of growth stimulatory organic substances in the guayule leachate is described. These stimulatory fractions were active at concentrations as low as 2 mg. per liter.

10. A number of pure synthetic organic acids were tested for inhibitory activity, of these only one, cis-9,10 dihydroxy stearic acid was found to be active. This compound caused marked inhibition in concentrations as low as 3.5 mg. per liter.

11. The chemical fractionation of a soil on which guayule plants had grown is described and it is shown to contain inhibitory organic substances having some chemical properties similar to those of the substances in the guayule leachates.

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