EFFECTS OF GIBBERELLIC ACID ON *DATURA* SPECIES

Datura stramonium var. *tatula* (L.) Torr.

by

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SUMMARY OF THESIS TO BE SUBMITTED TO THE UNIVERSITY OF GLASGOW IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

The Department of Pharmacy,
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SUMMARY

This thesis incorporates results of some cultivation studies on plants of Datura stramonium var. tatula and in particular records some observations of the effects of gibberellic acid on these growing plants.

In cultivation studies variation of soil type and condition may lead to anomalous results therefore some of the plants used in this work were grown in a controlled hydroponic system supported in vermiculite. Vermiculaponic culture has been described for a variety of crops but no published information was available on its use for the growth of experimental plants; other plants were grown under field conditions and some in boxes of soil. Very healthy plants were obtained on the artificial medium and whereas young plants of corresponding age were similar in size when comparing garden-grown with those raised on vermiculite, the latter eventually gave a greater yield, the plants being taller and showing much more extensive root development. Similarly, in a small-scale experiment, plants grown in any one of three different nutrient solutions, supported in vermiculite, showed much better development than corresponding plants grown on John Innes compost. Whereas plants grown under field conditions tended to show the usual signs of chlorosis after gibberellic acid treatment, this effect was not observed in plants fed with nutrient solutions.

The effects of the growth-promoting gibberellins have been extensively studied on a wide variety of plants, including several of medicinal importance. In the latter group, studies have been made on plant growth and also on the production of important chemical constituents; some work has been done on alkaloid-yielding plants, including Datura species, but the results reported to date give information on total alkaloids only. In this present work an attempt was made to determine the effects of gibberellic acid treatment on the hyoscyamine/hyoscine ratio in the aerial parts of D. stramonium var. tatula at different stages of growth. On plants grown under both field and hydroponic conditions, the well known effect of gibberellic acid on stem elongation was further confirmed, most treated plants being taller than corresponding untreated controls; in general there was also an increased weight of aerial parts produced, although this was not so for some groups of garden-grown plants. The effect of treatment on root growth (dry weight) was variable and, since no conclusions can be drawn from the results obtained, no clarification of earlier controversial findings was achieved.

The separate assay of hyoscyamine and hyoscine in small samples of plant material presented considerable difficulty, the published method utilising partition chromatography being found to be unreliable. Attempts were made to use a method based on area measurements of the alkaloids separated on paper chromatograms and also to devise a

method utilising similar measurements of areas obtained on thin layer chromatograms. The latter method was found to be reliable using pure solutions of the alkaloids but some of the results on plant samples may be suspect since in certain cases the sum of the amounts of the two major alkaloids (hyoscyamine and hyoscine) were at considerable variance with the amount of total alkaloid as determined by the assay method described in the British Pharmacopoeia.

While it was quite clear that the total amount of alkaloid in the aerial parts was reduced following gibberellic acid treatment, thus confirming published results, only a tentative conclusion can be proposed with regard to the hyoscyamine/hyoscine ratio. For garden-grown plants, both untreated and treated, the total alkaloid increased but the proportion of hyoscine decreased as the plants matured. Thus gibberellic acid does not appear to exert any effect on the previously described ontogenetic production of these alkaloids. On the whole, the plants grown in vermiculite tended to be somewhat poorer in alkaloid content than the corresponding garden plants and the hyoscine appeared to form a higher proportion of the total alkaloid, particularly in the control plants; this proportion did not seem to decrease to any great extent as the plants matured. This relatively higher proportion of hyoscine in the aerial parts of plants fed by nutrient solutions, as compared with similar plants grown on soil,

is of interest in that it confirms a similar report on the proportions of root alkaloids in Datura innoxia grown under different conditions.

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PREFACE

Considerable interest has been shown in methods of improving yields and increasing the proportion of important constituents in growing medicinal plants. Numerous species have been used in these investigations, particularly alkaloid-yielding plants and, more especially, members of the Solanaceae, including *Datura* species.

Attention has focussed on internal genetic factors and on a number of external conditions, mainly climate and soil, and much of the earlier work was concerned with various manurial treatments, especially with inorganic fertilisers. More recent reports deal with effects of a wide range of organic compounds applied directly to the seeds or growing plants, in some instances to plants grown under artificial conditions such as hydroponic culture or tissue culture. Of interest in this connection is the effect of the growth-promoting gibberellins which have been shown to affect the morphology and the total alkaloidal content of several species when applied to growing plants. The majority of such species contain a complex mixture of alkaloids and while ontogenetic and diurnal variations in the proportions of individual bases have been demonstrated in normal plants, no reports have appeared on such variations in gibberellin-treated plants.

The scope of the present work was to investigate the effect of gibberellic acid (gibberellin A_3, gibberellin X) on the hyoscyamine/hyoscine ratio in plants of *Datura tatula* Linn. at different stages of development. At the same time comparisons were made between plants grown under normal field conditions and those grown in an artificial medium (vermiculite) where plant feeding was strictly controlled.

The separate determination of hyoscyamine and hyoscine in small quantities of plant material presents difficulties and most of the methods so far described can be criticised for being unreliable or time consuming. Available methods have been reviewed, some were examined, and an attempt has been made to devise a rapid and reliable method based on thin layer chromatography.

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INTRODUCTION

I N T R O D U C T I O N

PLANT GROWTH-PROMOTING SUBSTANCES

(a) Gibberellins.

The extensive literature which has built up on the isolation, chemistry and applications of this group of compounds has been well reviewed[1-4] and only a brief outline will be given, followed by some detail on the reported applications to medicinal plants.

In 1926, while studying the bakanae disease of rice seedlings, the Japanese pathologist Kurosawa discovered substances with a growth-promoting effect, the source of which was traced to the soil-borne ascomycetous fungus Gibberella fujikoroi (Saw.) Wr. (conidial state: Fusarium moniliforme Sheld.). Subsequently it was shown that a cell-free filtrate, obtained from a culture medium on which the fungus had been grown, possessed the same effects on rice as the fungus itself. However, it was not until 1939 that Yabuta and Hayaski isolated crystalline gibberellin A, later found to be a mixture of gibberellins.

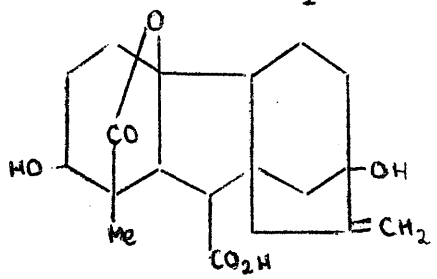
Pure gibberellic acid was first isolated by Brian in Great Britain and then independently, in the United States, by Stodola who called it gibberellin X. Japanese workers subsequently called it gibberellin A_3. Gibberellins A_1, A_2, and A_4 were also separated and identified but shown, by stem elongation studies on Dill[5] (Anethum graveolens Linn.), to be less potent biologically than gibberellic acid.

In 1960, Brian[6] discovered naturally-occurring gibberellin A_1 in immature seeds of the runner bean (Phaseolus multiflorus Willd.). Further discoveries[7,8] in other plants confirmed that the gibberellins

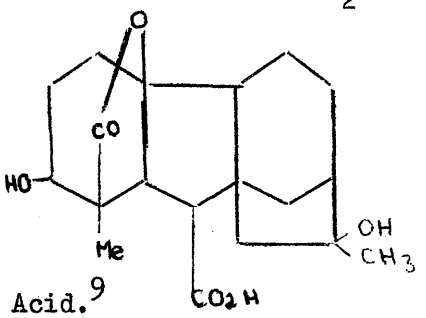
are natural plant hormones exerting their effects in extremely low concentrations. These investigations also led to the isolation of five new gibberellins; G. fujikuroi produced gibberellins A_7 and A_9, and various plant tissues A_5, A_6 and A_8.

The formulae of these different gibberellins are given below.

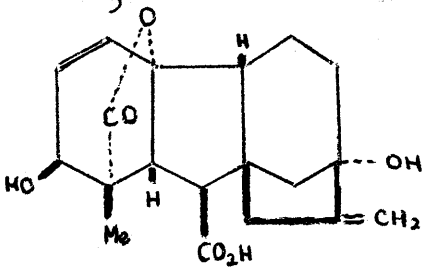
Gibberellin A_1.



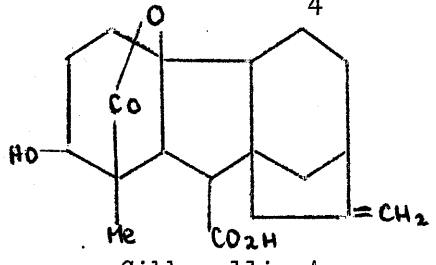
Gibberellin A_2.



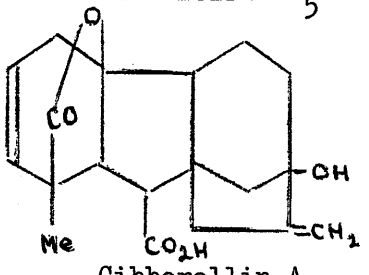
Gibberellin A_3, Gibberellic Acid.[9]



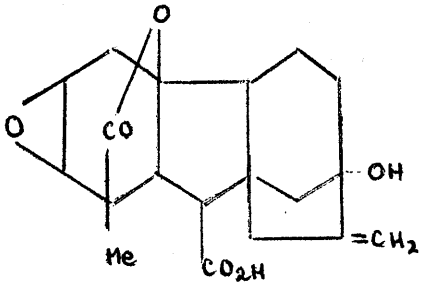
Gibberellin A_4.



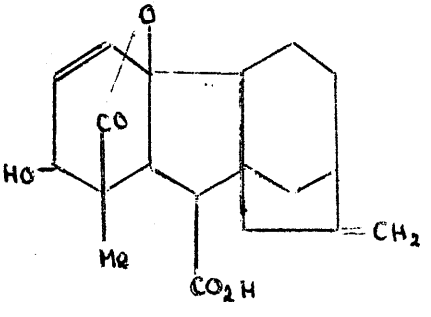
Gibberellin A_5.



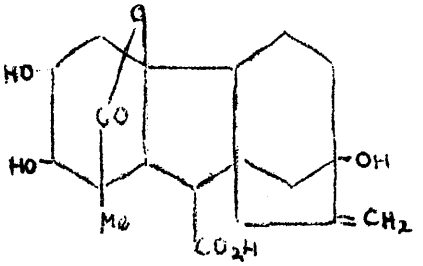
Gibberellin A_6.



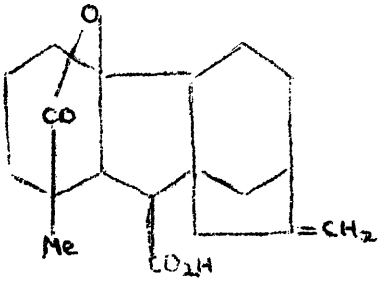
Gibberellin A_7.



Gibberellin A_8.



Gibberellin A_9.



The relative potencies of the gibberellins, as shown on various plant tissues, have been quoted[8] as follows:-

Dwarf pea stem growth: response/log. dose shows a linear relationship.

$A_3 > A_1 = A_7 > A_2 = A_5 = A_6 > A_4 > A_8 > A_9$

100 30 10 5 1 inactive.

Lettuce hypocotyl growth: response/log. dose shows a linear relationship.

$A_7 > A_3 > A_4 = A_9 > A_1 = A_2 = A_5 > A_6 > A_8$

200 100 33 4 1 inactive.

Cucumber hypocotyl growth: response/log. dose shows a linear relationship.

$A_4 = A_7 = A_9 > A_1 = A_2 = A_3 > A_5 = A_6 > A_8$

C5000 100 10 1

Lettuce seed germination: No obvious dose/response relationship

$A_4 = A_7 > A_3 > A_1 = A_5 > A_2 = A_6 = A_8 = A_9$

General Effects.

Interest in the biological effects of the gibberellins was slow to start but since 1952 numerous workers, in many parts of the world, have reported on gibberellins and their effects on a wide range of plant species. The main effects can be summarised as follows:-

(a) Stem elongation leading to increased plant growth.

(b) Induction of flowering and increased fruit set (often parthenocarpic).

(c) Breaking of seed, corm, tuber and bulb dormancies.

(d) Breaking of apical dominance.

Stem elongation is due mainly to internode extension. It is very marked in most species, especially those of dwarf habit many of which become normal plants after treatment. In a few cases there is also an increase in cell number (mitosis in Samolus parviflorus Nees.,[10,11] Hyoscyamus niger Linn.,[10,12] and Zea mays, Linn.).

Effects on Medicinal Plants.

With medicinal plants interest has attached mainly to the effects of gibberellic acid on Papaver somniferum Linn.,[13] Rauwolfia serpentina Benth.,[14] Atropa belladonna Linn.,[15-18] Hyoscyamus niger,[19] Datura species,[15, 17, 20-26.] Digitalis purpurea Linn.,[27, 28] D. lanata Ehrh., Fagopyrum esculentum Moench.,[29] Eucalyptus species,[30] Foeniculum vulgare Miller, Mentha piperita Linn., and M. spicata Linn.[31], and most of this work has been reviewed.[32]

The results of gibberellic acid treatment can be summarised under effects on morphology and on constituents.

Effects on morphology.

In all cases growth was stimulated and, initially, increased plant weight resulted; increased stem weight was reported for A. belladonna,[15] H. niger,[19] D. stramonium,[15] D. meteloides,[24] and F. esculentum.[29] Splitting of the stems in D. stramonium,[20] resulting

in the production of scar tissue, has been observed. Eucalyptus[30] stems were weaker; those of Spearmint[31] (M. spicata) were at first thread-like exhibiting vine-like characteristics, but later became thickened, very hard, rough, longitudinally wrinkled, and irregular in shape.

In the biennials H. niger[34] and D. purpurea[27] gibberellic acid replaced vernalization and induced flowering in the first year's growth, (an effect also observed in rosette species of Nicotiana[35]). It did not induce flower primordia in Spearmint[31], but gave earlier setting of seeds in R. serpentina[14]. Earlier flowering has also been reported for A. belladonna[15], D. stramonium[15], D. innoxia[23], and P. somniferum[13], but gibberellic acid did not replace the long-day requirement for F. vulgare[32].

Narrower leaves with a chlorotic appearance were apparent for A. belladonna[15], D. stramonium[15], M. spicata[31], R. serpentina[14] and D. lanata[32], and in D. purpurea[28] the petioles were elongated. Eucalyptus[30] had initially paler leaves, as had D. meteloides[24], the latter's stem becoming chlorotic at the final harvest. The treated leaves of M. piperita[32] were smaller, and had a reduced number of glandular trichomes. The leaves of Spearmint[31] and Pepper[33] were rougher. Stramonium[15], Belladonna[15] and Spearmint[31] leaves were also thinner. D. stramonium[20] leaves had reduced marginal indentation, while Nicotiana rustica[35] displayed curled margins with sun scalding at the tips. Younger leaves of Belladonna[15] appeared wrinkled and blistered, and the foliage generally was less succulent.

A decrease in weight of leaves and tops was noted in

A. belladonna[15], H. niger[19] and D. purpurea[32]. Increases reported in D. stramonium[15] were due mainly to increased stem weight. F. esculentum[29] had an increased number of leaves per plant.

Decreased root weight was produced in D. lanata[32], A. belladonna,[15], D. stramonium[15], and D. meteloides[24], but an increase was evident in R. serpentina[14], due to an increased number of secondary roots.

When applied to seeds of Stramonium[25], Belladonna[18], and Hyoscyamus[25], gibberellic acid has been reported to give a more uniform and increased rate, together with a higher percentage, of germination.

The effects of gibberellic acid on second year Belladonna[16] have also been noted. The treated plants appear smaller than control plants, but where a large concentration of gibberellic acid was applied during the first year, gibberellin growth-effects were again present. Second generation Stramonium[21] have been studied and generally there was decreased growth and dry weights.

Effects on constituents.

In all the Solanaceous species mentioned above, a decrease in total tropane alkaloids was recorded. The decrease was most pronounced in younger plants of Belladonna[17]. The percentage of total alkaloids in the leaves and tops were decreased in H. niger[19], D. stramonium[15], A. belladonna[15] and D. meteloides[24] but total stem alkaloids were increased in the latter two species. Alkaloids in the roots were decreased except in some cases with Stramonium, where no significant change was observed, and with D. innoxia[23] which had slightly increased root alkaloids. The

alkaloid content of Rauwolfia[14] was also decreased. It is of interest to note that the alkaloid content in both leaves and roots of Nicotiana species was also decreased but no change in the alkaloid composition was observed. In this report, however, no details were given of the method used to determine the composition of the alkaloid mixture.

In plants grown from gibberellic acid-treated seeds of Belladonna and Stramonium, a decrease in alkaloidal content was reported.[25]

The volatile oil content of Spearmint[31] and Peppermint was decreased. There was no change reported for carvone in Spearmint, but changes in physical and chemical constants in Peppermint[32] were noted although there was no difference in total menthol content. However, there was an increase in the amount of oil obtained from Chenopodium ambrioides Linn. var. anthelminticum Gray[36], following gibberellic acid treatment of the plant; the ascaridole content of the oil was not appreciably altered.

Although the total amount of rutin in plants of Buckwheat[29] was decreased, in D. purpurea[28] and D. lanata[32] increases in glycosides were reported along with increases in both total and reducing sugars. No difference in the percentage digitoxose was given for D. purpurea plants, but the concentration of starch in D. lanata was increased.

The percentage of total sugars was increased in Buckwheat[29] and Stramonium[26], and the glucose and sucrose content increased in Peppermint. Stramonium had also increased starch content.

Gibberellic acid treatment reduced the chlorophyll content in D. stramonium[26], D. meteloides[24] and M. spicata[31], but increases in chlorophyll have resulted in D. purpurea[28], M. piperita, F. esculentum[29]

and N. rustica[37].

Decreased water content was reported in Belladonna, Stramonium and Spearmint[31], but none was observed for Peppermint.

Gibberellic acid decreased the total petroleum ether extract of leaf tops of Stramonium[26], and also gave a qualitative difference to an ether extract, this being yellow-brown in colour as compared with the dark green of that of control plants.

Dosage and Methods of Application.

The concentration of gibberellic acid used for plant treatment ranges from one part per million to a maximum of 300 p.p.m., and variations in effects are produced with varying doses. Above a dose level of 300 p.p.m. no further growth increase is observed, and at 1000 p.p.m. toxic effects may result.

The method of application may also have a decided effect on plant response. The following methods have been used.

(1) Spraying of an aqueous solution. The whole of the plant receives treatment, therefore dosage can not be accurately controlled. Leaves of Belladonna, Stramonium and Spearmint treated in this way seem more sensitive to insect attack[15,31].

(2) Microdrop method. A small drop of an ethanolic solution is applied to a part of the plant, usually a leaf. Weather conditions must be favourable, without rain or wind, so that all the solution may be absorbed by the plant. A modification of this method, used by Scurfield and Moore[30] consists of cutting off the tip of the second or third leaf. The stump is inserted in a tube containing 0.1 ml. aqueous solution of gibberellic acid and the tube plugged with cotton wool to reduce evaporation.

(3) Lanolin paste. The gibberellic acid is mixed with anhydrous

lanolin and the paste applied to part of the plant. The lanolin may interfere with the absorption of gibberellic acid in this method.

(4) Powder. This method is used solely for seed dressing.

(5) Injection. An ethanolic solution is injected into the plant. This method, which was adopted in the present work, gives the advantages of accurate dosage and choice of the site of application.

(b) Other Compounds.

Natural Plant Auxins.

The auxins isolated and identified are auxentriolic acid (Auxin A), auxenolonic acid (Auxin B), and heteroauxin (β indolylacetic acid)[38]; the latter is the most important, being widespread in the plant kingdom. The effects of the gibberellins have been compared particularly with those of the natural plant auxins, and the greatest difference between the two classes is that gibberellic acid gives growth-promotion only in the presence of other inherent growth-promoting substances, whereas auxins produce effects without other growth regulators being present. Gibberellic acid and auxin (indolylacetic acid or indole-3-acetic acid) stimulate different parts of plants, and the former, which exerts its effect chiefly at the sub-apical zone of the stem, has a greater effect on elongation than the latter, which acts at a lower level in the stem. When both are present, there is a synergistic effect. If 2,3,5-tri-iodobenzoic acid (TIBA) is applied at a point on the plant below where gibberellic acid treatment is given, no gibberellin effects are apparent except above the treated part, TIBA inhibiting the translocation of auxin and hence the effects of gibberellic acid.[39] Light has an inhibiting effect on auxin stimulation but does not alter that of gibberellic acid. Gibberellins increase leaf growth in some

grass types, replace vernalin (but not florigen) in some flowering plants[39], and break dormancy in reproductive organs, but these properties are not shared by auxins. It has been reported[40] that dilute auxin sprays can induce formation of female flowers on male plants of Cannabis sativa Linn. at sites which would normally be occupied by male flowers. Auxins cause mitosis of the cambium layer leading to thickening of stems, an effect not given with gibberellic acid, the stems often becoming more slender after treatment.

Miscellaneous Compounds.

Synthetic auxins, including substituted phthalamic and benzoic derivatives, have some similar effects to gibberellic acid, such as increasing flower production and fruit set, and on a molar basis are more effective for this purpose than gibberellic acid[41]. The benzoic derivatives retard plant growth, giving more compact plants which may be more useful for floral display.

2,4-dichlorophenoxyacetic acid (2,4-D.), aminocaproic acid, hexamethyltrimethine-thiazolocyanine iodide[42], hydrocyanic acid[43], and potassium ferricyanide[44] all stimulate germination of Datura seeds. Watering with a solution of potassium ferricyanide has also an effect on growing plants, lengthening the period of flowering and giving increases in leaf yield and of total alkaloids in plant and seeds[45]. 2,4-D when applied to stems of Datura stramonium[46] produces stunted growth, malformation of stem structure, folding of leaves and retardation of flower development. Naphthalene acetic acid[47] gives increased dry and fresh weights in Datura species and, as the sodium salt, methyl ester, or amide gives a 30-50% increase in volative oil content of Mentha piperita,

the menthol content of the oil being increased by 4.5-9%[48,49]. Penicillin[50] and vitamin K[47] both give increased weights in Datura, but the former results in decreased alkaloid content in D. stramonium. Colchicine, by inducing tetraploidy, produces larger plants and a greater alkaloid content[51].

Long-wave ultraviolet irradiation (365 mμ) of plants for 30 minutes daily[52], and thorium X[53] in water sprinkled on the roots, both increase alkaloid content of D. stramonium. Total alkaloid content of leaves, stems, and roots is also increased by injection of D. stramonium plants with adipic acid; methylamine injections increase the alkaloids in the roots only[54].

In cultural experiments on Datura species, glutamic acid[55] and acid-hydrolysed casein increase growth[56].

Coumarin has been reported[57] to have an effect on phosphorus metabolism in D. stramonium, and it has been suggested as a naturally-occurring growth regulator. Audus[38] classes coumarin along with vitamin C. as an "anti-auxin". Kinetin[24] (6-(furfurylamino)-purine) has an effect on nitrogen metabolism and gives an increased percentage of chlorophyll in Datura meteloides.

An interesting discovery[58] has been made by infecting Datura plants with X virus (strain H 19/17, a ring spot strain), Y virus, and mixed X and Y strains. Increased alkaloid production was effected in the virus infected plants, probably due to an increased rate of development.

With most growth-promoting substances, optimum and maximum concentrations have been observed; if this latter level is surpassed

their effect resembles that of the growth retarding compound, maleic hydrazide[38], a competitive auxin inhibitor which causes stunted growth and malformation of the treated plants, often accompanied by scorching of the leaves.

There exists a vast literature pertaining to cultivation conditions for alkaloid-bearing plants, dealing with external and internal factors affecting both plant and alkaloid yields.

In the present work differences due to genetic variations were eliminated by using seeds grown from a pure strain of Datura tatula, developed by Blakeslee.[59]

The most important external factors which must be considered are climate and soil, including manurial treatment.

Climate.

In Datura species, excessive rain has a detrimental effect on alkaloid content,[60] while water deficiency definitely retards growth and development, giving an initial increase in alkaloids but a subsequent low level.[61]

Datura stramonium, although hardy, is adversely affected by low temperatures, the alkaloidal content being greatly reduced by frost.[62] There are no available data on wind effects. The effect of light is complex. Long-day plants, for example D. stramonium and D. tatula, produce more alkaloids under continuous illumination, and alkaloid production and accumulation run parallel to the duration of exposure.[63] However, it appears that increased production of alkaloids is often accompanied by some retardation of growth.[64] Optimum growth, as well as increased alkaloids should be taken into account.

Various reports on diurnal variation of alkaloids in Datura have reached different conclusions.[62, 65 - 67] It has been reported that the alkaloids decrease from 7 to 23 hours and increase again

during the subsequent hours of night.[66] Hegnauer[67] suggested that the following processes should be considered: the accumulation of root alkaloids in the leaves, diurnal variations in the content of dry matter in the leaves, synthesis of alkaloids in the leaves themselves, and the migration of the alkaloids out of the leaves. These processes may be influenced by weather conditions, age of plant, age of particular leaves and the characteristics of the species. In the present work, to eliminate possible diurnal variations, plants in groups to be compared were all collected at the same time of day.

Soil and Fertiliser Treatment.

The effects of nitrogen, phosphorus, and potassium have been widely studied, and in earlier work it was reported that not all alkaloid-producing plants respond to specific sources of these elements, that is particular fertiliser compounds, in the same way. Differences among the tropane group of the Solanaceae were quite marked in this respect.[68,69]. Latterly it has become apparent that the ratios of N, P, and K made available to the plant are important.[70,71].

Nitrogen generally stimulates growth, but its form is important; NO_3^- stimulates growth but does not affect the alkaloid content, while NH_4^+ may retard growth and increase the production of alkaloids.[72]

Phosphorus, potassium, and calcium have a much less marked effect although results using different amounts of P and K are very varied. This is probably due to the differences in soil type used in the investigations and this has made difficult the interpretation of effects on growth and alkaloid production. K is known to favour protein synthesis, and hence reduce alkaloid production, while P usually

increases growth and may or may not increase the alkaloid production. Calcium increases alkaloid production and retards growth although, again, differing reports of K and Ca effects have been noted.[69] Magnesium and sulphur are also important major elements in fertilisers, but in addition a number of trace elements are necessary for optimum plant growth, and these include boron, iron, manganese, copper, zinc, molybdenum, and sometimes cobalt. In particular the advantageous use of boron[73] has been reported for Datura cultivation.

The pH of the soil is also relevant, but no definite conclusions have been reached, alkaloids being formed in plants growing within the range pH4 to 8.[69]

Because soil type and condition contribute to the variation in results, some workers[74,75,76] have used sand, humus, peat, gravel, sawdust, compost, or mixtures of these. In most cases, organic material played an important but, again, probably variable part in the nutrition of the plants and the usefulness of a hydroponic system became evident.

Artificial growth media.

Water Culture.

The growing of plants in nutrient solutions has been carried out in the laboratory for many years. Inorganic salts were dissolved in water, this solution being used for plant nutrition, the roots being unsupported. Apart from solutions of inorganic salts, water culture has provided a useful method for feeding precursors of an organic nature to plants in studies on biosynthesis of alkaloids. An extension and modification of this method has been carried out using stone chips, gravel, broken bricks, leached cinders, peat, and sand to provide root

support in this technique of hydroponic culture.

Synthetic Resins (Amberlites).

Synthetic resins have also been used as media for artificial culture: this work[77] is still in the early stages of development but results are very promising. Because of the ion-exchange properties of these resins, it is possible to vary single ion concentration with simultaneous variation of oppositely charged ions. The resins have been mixed in definite ratios together with supporting materials in hydroponic troughs, watering being carried out with known strengths of KNO_3 solutions. Another advantage of these resins is that they can be regenerated and used indefinitely in cultivation work.

Vermiculite.

Vermiculite has also been recommended[78] for use in hydroponics and a wide range of crops have been cultivated using this vermiculaponic system.

Vermiculite is chemically similarly to mica, being a complex containing a mixture of the oxides of silicon, aluminium, iron, titanium, manganese, calcium, magnesium, sodium and potassium, together with water.[79]

After being mined it is heated to 2,000° F. This causes the material to exfoliate and become very porous. It has an extremely large water absorbing capacity but when moist still contains entrapped air between the expanded lamellae of the fragments, this being useful for aeration of roots. It is chemically inert, clean and free from soil organisms and weed seeds; also light and loose, permitting free development of roots which it insulates from extremes of temperature.

Natkruit vermiculite, mined in Northern Transvaal, is the best

grade for horticultural use since it has a natural pH range (6.2-6.8) suitable for maximum plant growth.

Since there are no available nutrients in this inert support, all necessary elements must be added in the required proportions by regular feeding with balanced nutrient solutions.

Vermiculite has been recommended for growing the medicinal plants Stramonium, Belladonna, Digitalis, Chamomile, Ipecacuanha, Aconite, Ephedra, Gentian, Dandelion, Ginger, Calumba, and Turmeric. Special nutrient solutions for such plants grown in vermiculite have not been published but the following general vermiculaponic rules were advocated by Bentley.[78]

(i) For the promotion of osmosis and diffusion, the nutrient solution should be kept within the range of 0.75 to 1.25 atmospheres, 1 atmosphere being the most advantageous. 2,000 p.p.m. is the maximum amount of solids permissible and this should take into account the solids present in the water used to make up the nutrient solution.

(ii) The solution should be as dilute as possible.

(iii) $(NH_4)NO_3$ is not recommended as a source of nitrogen, as plants do not appear to thrive when it is used.

(iv) Nitrogen as NH_4+ should not exceed 25 per cent of the total nitrogen.

(In this work the appearance of Datura tatula plants did not seem to alter when this ratio was not observed).

(v) K and N ratio is very important and should be correctly balanced. If the K level is too low, excess nitrogen is taken up by the plant. (Again with Datura tatula, the ratio range seems to be large, as N:K 1:1; 3:2; 2:1 all gave good plant development).

Nicol[80] forwarded a theory for ionic balance of fertilisers, i.e. cation-anion balance by weight. The best balance is approximately 1:2.4 but there is considerable latitude on either side and all ions must be considered, irrespective of their nutrient value.

In formulating nutrient solutions for the vermiculaponic section of the present work account was taken of some recommendations made for growing Stramonium under field conditions using fertiliser dressings.[70] The amounts of trace elements used were those reported in a successful water culture of Nicotiana spp.[81]

METHODS OF ASSAY FOR HYOSCYAMINE AND HYOSCINE

A vast literature has accumulated on the extraction, separation, identification and assay of tropane alkaloids. Assay methods vary from a simple field technique[82] to critical microtechniques. Much of the earlier work has been carried out with a view to determining the total alkaloids present, but in biogenetic studies isolation of individual alkaloids and their separate assay have become more important. The ultimate determinations, whether of total alkaloids or of separated individual alkaloids, are usually similar, hence methods of separation will be reviewed briefly followed by general assay techniques.

Separation Techniques.

Tropane alkaloids have been successfully separated by adsorption chromatography on alumina[83] or activated silica[84] and also by partition column chromatography on kieselguhr[85 - 88].

Paper chromatography has also been found very satisfactory for the separation of the Solanaceous alkaloids, using both descending[89 - 91] and ascending[92] techniques on different papers, including succinyl cellulose paper[93] with a variety of solvent mixtures. Paper buffered with citrate or phosphate[94], or oxalic acid[95], have proved satisfactory, and a technique of "over-flow" paper chromatography on many differently - buffered paper strips has been described[96]. A microchromatographic technique in acid media[97] has been successfully used for the separation of hyoscine, atropine and hyoscyamine. Paper electrophoresis[98, 99], ion-exchange resins[100-103], and counter-current distribution[104-106] have also been successfully applied to the separation of the tropane alkaloids.

Assay Techniques.

Titration Methods.

There are many variations including direct titration[88, 107-109], back titration[110,111], titration in non-aqueous media[112,113], or potentiometric titration[114]. A hydrolytic method is based on the liberation of tropic acid after boiling the neutralised alkaloid solution with excess alkali, back titrating with phenolphthalein as indicator[115]. A more complicated titration technique[90], using 100g. of material and including a descending chromatographic technique on phosphate buffered paper, gives values for total alkaloids and also for hyoscine and hyoscyamine. A method reported[116] for the separate determination of hyoscyamine, atropine and hyoscine in large plant samples (30g.) involved titration to determine the total alkaloid, separation of the hyoscine in chloroform at pH 8.5 followed by its separate titration, and estimation of the hyoscyamine by measurement of optical rotation; the amount of atropine was obtained by difference.

Colorimetric Methods.

This comprises a large group of which the most commonly used is based on the Vitali-Morin reaction in which polynitrobenzene derivatives give a blue colour with alkalis. At least two nitro-groups are introduced into the benzene ring located in the tropic acid part of the alkaloid molecule[117]. The absorbance of the coloured solution is measured spectrophotometrically. There have been a number of modifications[118,121] to this method, which has the advantage of using only small quantities of material.

Colour complexes with ammonium reineckate[122,124], p-dimethylaminobenzaldehyde[91,125 126], cupric sulphate and naphthenic acid[127], oleic

acid and cupric salt[128], and the molybdenum blue released by reduction from an alkaloid-silicomolybdate complex[129] have all been used. The acidic dye bromocresol purple forms a salt-like compound with some tropine alkaloids and the dye component has been estimated colorimetrically[130] to give a determination of the amount of atropine and hyoscyamine present.

When separation of the individual alkaloids is carried out using a paper chromatographic technique, the separated bases can be eluted off and estimated colorimetrically[131, 132]. Other assay methods have been applied whereby the alkaloids are estimated directly[133] on the paper by direct comparison with standard alkaloid solutions run on the same paper; area estimates[134] or densitometric readings[135,136] have both been used but the former gives only approximate results. It has been found[137] that there is a relation between the amount of alkaloid present and the Rf values obtained, and this has been used to give a very approximate determination of the amount of alkaloid present.

Other Methods.

Both gravimetric and volumetric methods for the determination of alkaloids have been reported using tetraphenyl borate[138]; the ratio of atropine and hyoscyamine was checked by measuring the specific rotation of the borate in hydrochloric acid.

A nephelometric method has been described based on the insoluble complexes formed by the alkaloids with iodine solution[139], and a polarographic determination[140] has been attempted.

For routine analysis, with an adequate amount of material available, the titrimetric assay of the British Pharmacopoeia is usually carried out, but when the supply of material is limited a Vitali-Morin assay is often

used. Both these methods have the disadvantage that only the total alkaloids present are determined, and in the present work the ratio of hyoscine and hyoscyamine was required.

Despite the many and varied methods already proposed, none proved of appeared to be satisfactory for the reasonably rapid and accurate separation and estimation of these two major alkaloids in small plant samples, hence the thin layer chromatographic technique described in the experimental section of this thesis was developed.

EXPERIMENTAL

E X P E R I M E N T A L

CULTIVATION OF PLANTS

The seeds used were from a pure strain of *D. tatula* L. (*D. stramonium* var. *tatula* (L.) Torr.) supplied by Dr. W.C. Evans, University of Nottingham.

The following methods of inducing germination in seeds of *D. tatula* were carried out on a small scale:-

1. soaking in water,
2. chipping the testa and soaking in water,
3. leaving at $0^{\circ}C$ for 4 days and then soaking in water,
4. exposing to sunlight for one day and then soaking in water,
5. treating with concentrated sulphuric acid for one minute then chipping and soaking in water.

It was found that chipping the testa followed by soaking in water gave the best results, all seeds germinating within a two day range of each other. This was the method adopted.

On 13th April, 1962, seeds were germinated in darkness, and on the 30th April, 100 seedlings were potted in vermiculite and watered with $\frac{1}{4}$ strength nutrient solution (see Appendix page 77). By 14th May all had their first true leaf, and were 4-6.5 cm. in height. The plants were hardened off from 4th - 14th June, during which period they were watered with $\frac{1}{2}$ strength nutrient solution then divided into 2 main groups of 50, one batch being planted in vermiculite in the hydroponic tank (see Appendix and Plate 6) and the other in well-worked soil which had been liberally enriched with farmyard manure. Each batch was planted in 5 rows of 10, allowing 18 inches between garden plants and 9 inches between plants in vermiculite (spacing can be reduced since this is a forcing system[78].)

During the week that they were left to establish, adverse weather conditions caused serious damage to 10 plants in the tank. Each of the remaining 40 plants was assigned to one of eight groups and random numbers were used to determine the position of individual plants in each row; 40 of the garden plants were similarly arranged (Table 1).

During the whole period of cultivation of the plants exfloration was carried out, as required, since this gives an increased content of alkaloids, delaying the general decrease noted in the Solanaceae after flowering[141-144]. The garden grown plants received no additional fertiliser treatment. Nutrient solution was applied when necessary to the tank to maintain the vermiculite in a slightly damp condition.

On 2nd July, plants in groups C, D, G and H, were each injected with 100 µg gibberellic acid. A solution containing 5 mg. gibberellic acid in 0.1 ml. absolute ethanol was made, and each plant received 0.002 ml., by means of a micrometer syringe, into the base of a young leaf.

Four weeks later, on the 30th July, the heights of the plants in groups A, B, C and D, were measured (Table VI) and the plants lifted (early collection). The roots were washed and separated and then the aerial parts and roots were dried at 55°C in a forced draught oven[145]. Stems over 5 mm. in diameter were separated from the aerial parts (only 2 per cent of stems over 5 mm. in diameter are permitted in the monograph for Stramonium in the British Pharmacopoeia); the remainder was finely powdered and weighed and then placed in air-tight, screw-capped jars, with silica gel, until required. Stems over 5 mm., and roots were similarly treated and weighed. (Table VII)

At this time, plants in groups G and H were given a further 100 µg. gibberellic acid and those together with plants in groups E and F were, on

TABLE I.

Designation and Arrangement of Plants during Cultivation.

1962 Crop.

Tank.

Row 1	Row 2	Row 3	Row 4	Row 5
F	A	B	G	B
G	C	G	A	D
C	D	F	C	H
H	B	D	H	A
A	H	A	F	E
B	G	C	E	C
E	E	H	D	F
D	F	E	B	G

Garden.

D	F	E	G	G
E	E	H	D	F
B	G	C	E	C
A	H	A	F	E
H	B	D	H	A
C	D	F	C	H
G	C	G	A	D
F	A	B	B	B

The groups, each containing 5 plants, were used as follows:-

A and B,	duplicate	groups	for early collection,	controls.
C and D,	"	"	" " "	treated.
E and F,	"	"	" late "	controls.
G and H,	"	"	" " "	treated.

27th August, measured, (Table VI), uprooted, (late collection) dried, powdered and weighed, (Table VII) as above.

During this programme a further 20 plants were raised to replace earlier losses and these were used to form groups I and J in both tank and garden. Two weeks after injection of the J groups the I and J plants were uprooted and dried (very early collection).

Due to the very small yield of plant obtained for groups I and J, (Table VII) it was decided to repeat this part of the experiment in 1963. A sufficient quantity of seeds were chipped and soaked on the 7th June. This was done later in the season with the hope that the weather would be better than that of 1962. After germination and hardening off of the plants, the following were planted in tank and garden on 26th July; groups K, L, M, N, O, P, Q and R, the plants for each group again being chosen at random. (Table II).

On the 6th August, plants in M, N, Q and R groups were each injected with 100μg. gibberellic acid; those in groups K, L, M and N were removed two weeks after this treatment. It was hoped to observe the seasonal difference with the controls O and P and the treated plants Q and R after 4 weeks as compared to the groups A, B, C and D in 1962, but weather conditions again interrupted the study and the 4 sets of plants had to be removed 3 weeks after treatment.

The hydroponic system control plants of 1962 had appeared very sturdy, and even those treated with gibberellic acid had maintained a healthy appearance but it was decided to vary the concentrations of N, P and K on a few plants in a small-scale experiment to see if any differences occurred using different strengths of nutrient solution. Forty plants,

TABLE II

Designation and Arrangement of Plants during Cultivation.

1963 Crop.

Tank.

Row 1	Row 2	Row 3	Row 4	Row 5
M	K	K	R	Q
L	Q	R	N	M
N	L	L	L	K
Q	P	M	O	P
K	O	P	P	O
O	M	O	K	R
P	R	Q	M	N
R	N	N	Q	L

Garden.

K	L	N	P	P
O	R	L	O	L
Q	O	Q	L	K
M	N	K	K	Q
P	K	M	Q	M
L	Q	R	M	N
N	P	O	R	R
R	M	P	N	O

The groups, each containing 5 plants, were used as follows:-

K and L,	duplicate	groups	for	early	collection,	controls.
M and N,	"	"	"	"	"	treated.
O and P,	"	"	"	late	"	controls.
Q and R,	"	"	"	"	"	treated.

brought on with those of the groups K - R, were planted in 8 Vencel (expanded polystyrene) troughs, each 37 inches long by 8.5 inches wide by 7 inches deep. Six troughs contained 1 inch of granite chips, 1 inch of sand and a 5 inch layer of vermiculite; the remaining 2 troughs contained 1 inch of granite chips topped with John Innes Compost No. 2. The 6 vermiculite-filled troughs were divided into 3 groups of 2 and the 10 plants in each group were fed with one of the following nutrient solutions:-

1. N, P and K, each 200 p.p.m. (tank formula).
2. N 300; P and K each 200 p.p.m. (see Appendix page 78).
3. N and P each 200, K 100 p.p.m.

The plants grown in John Innes Compost were watered regularly, but did not receive any additional fertiliser.

All the above plants were grown for 14 weeks after transplanting and half the number in each group were harvested in the morning, and the remainder in the afternoon, of 23rd September.

By appearance, the plants grown in synthetic media were the more healthy, and there seemed to be no difference among the plants receiving the different concentrations of N, P and K. On the other hand, those in John Innes Compost were decidedly smaller, not quite so bushy, and yet they looked more completely mature. All these plants were measured (Table XII), dried, powdered and weighed as previously described (Table XIII).

ANALYSIS OF DRIED PLANT MATERIAL

Three methods were tried for the separation and subsequent assay of the alkaloids hyoscine and hyoscyamine using column, paper, and thin layer (film) chromatography.

Partition Chromatographic Method[88].

The sample (5g.), in a moderately fine powder, was moistened with water (3 ml.) and left overnight in a well-closed container. Calcium hydroxide (1g.) was added and thoroughly mixed with the plant material which was then transferred to a 100 ml. cylindrical separating funnel with the aid of solvent ether (approx. 50 ml.). The mixture was shaken continuously for 1 hour, the liquid allowed to drain off, and the marc compressed. Percolation to exhaustion was carried out using solvent ether. The ether was removed in a rotary film evaporator, and the residue dissolved in carbon tetrachloride (2 ml.) (Sometimes 1 or 2 drops of ether had to be added to effect complete solution.)

A partition column was prepared by intimately mixing "Hyflo-Supercel" kieselguhr (10 g.) with 0.25M phosphate buffer, pH 5.9 - 6.2, (3.2 ml.). This mixture was then packed tightly into a glass column in the presence of carbon tetrachloride; unbuffered kieselguhr (1 g.) was added at the top, and the column was washed with carbon tetrachloride (previously equilibrated with buffer solution). The plant extract in carbon tetrachloride was carefully added to the column and development started with carbon tetrachloride (equilibrated) at a rate giving 3 ml. eluate per minute (positive pressure was applied to give this rate). The chlorophyll was quickly eluted and then, when the eluate became pale yellow, solvent

ether (equilibrated with buffer solution) was used for development. The fraction was collected into bromocresol green solution, adjusted to its transition point (pH 4.4), until the eluate became negative. Chloroform (previously equilibrated) was then used to develop the column and elution continued until the eluate again became negative to bromocresol green.

The ether fraction contained hyoscine and this was titrated directly with 0.005N H_2SO_4 using adjusted bromocresol green indicator.

1 ml. 0.005N H_2SO_4 ≡ 0.00152 g. hyoscine.

The chloroform fraction contained the hyoscyamine. Most of the chloroform was removed (to give a clear end point), solvent ether was added, and the solution titrated using bromocresol green as indicator.

1 ml. 0.005N H_2SO_4 ≡ 0.00145 g. hyoscyamine.

To check the efficiency of the method it was first used to separate known amounts of pure hyoscine and hyoscyamine from a solution of chloroform (omitting the carbon tetrachloride stage of the partition column separation) and, later, for separating the same known alkaloids from a solution in carbon tetrachloride to which "chlorophyll" had been added.

Separation of the two alkaloids was sharp but, while the recovery of hyoscine was satisfactory (92 - 96%), the recovery of hyoscyamine was variable and usually poor, sometimes being only about 50 per cent of the amount applied. It was noted, that as soon as chloroform was applied to the column, a marked "wetting" of the kieselguhr occurred in the upper part of the column. It was thought that temperature fluctuations may have been responsible for throwing out water from solution in the equilibrated chloroform, but results were not improved by carrying out column packing and elution in a constant temperature room.

In an attempt to minimise this "wetting" of the column, which was thought to be responsible for the retention of the hyoscyamine, a step-wise gradient elution was used. After elution of hyoscine with ether, 20 ml. a mixture of 95% ether and 5% chloroform were added, then 20 ml. of 90% ether and 10% chloroform, and so on, gradually increasing the amount of chloroform until this was being used alone as eluant. No improvement was noted; indeed this procedure greatly increased the tendency to column "cracking".

Two modifications[146] to the technique were used:

1. Solvents were not equilibrated with buffer solution.
2. The column was packed in petroleum ether (b.p. 40 - 60° C) and this solvent was also used to remove chlorophyll.

Analyses were then made of plant material of which the percentages of individual alkaloids were known. (This powdered Stramonium was kindly supplied by Dr. W.C. Evans, Nottingham University). Results were very much improved, there being an 87 - 88% recovery of hyoscyamine (Table III).

TABLE III.

Assay of Stramonium by Partition Chromatographic method.

Hyoscine		Hyoscyamine	
% present	% recovered	% present	% recovered
0.126	0.119	0.41	0.355
	0.120		0.356

Although concordant duplicate results were also obtained on 10

individual plant samples taken from the 1962 crop (Table X), with many other samples the method proved unsatisfactory due to the presence of an unidentified acidic principle which was eluted along with the end of the hyoscine fraction and the early part of the hyoscyamine fraction. This strongly acidic material turned the bromocresol green indicator yellow and, since no method of eliminating this interfering compound from the column was found, the method had to be abandoned.

Paper Chromatographic Method.

This method has been successfully adopted[147] for the assay of Duboisia myoporoides R. Br. and D. leichardtii F. Moeller using comparisons with a standard solution of hyoscine/hyoscyamine in the ratio 1:2 (0.5%: 1.0%), the proportions usually found in these species.

To obtain experience of the technique it was first tried on a sample of Duboisia and, subsequently, on a sample of Stramonium. Powdered Duboisia (25 g.) was mixed with lime (2.5 g.) and distilled water (10 ml.) and set aside for 30 minutes. The mixture was extracted continuously with hot benzene (300 ml. approx.) for four hours. The benzene extract was then shaken with successive quantities of a mixture of 10 per cent sulphuric acid (12 ml.) and absolute ethanol (5 ml.) until complete extraction of the alkaloids had been effected, (usually 5 quantities were sufficient). The combined acid layers were then washed with chloroform and the chloroform layer washed with dilute sulphuric acid (10 ml.). The acid fractions were combined, made alkaline with dilute solution of ammonia and then extracted with successive quantities of chloroform (20 ml.) until complete extraction had been effected; each chloroformic solution was washed with the same 10 ml. of water. The combined chloroform extract was reduced in

volume and adjusted to exactly 25 ml. with chloroform. The solution was then ready for applying to paper. A standard solution of hyoscine and hyoscyamine (0.5 per cent and 1.0 per cent respectively) in Chloroform B.P. was made.

A litre of a mixture of distilled water, Analar butanol and glacial acetic acid in the ratio 5:4:1 was made up, thoroughly shaken, and allowed to separate. The bottom layer was placed at the bottom of a chromatographic tank, and the upper layer was used as a solvent for the downward development of the papers.

Whatman No. 3 paper was cut into sheets, $4\frac{1}{2}$ inches by 22 inches, and each paper received 3 spots, two of unknown and one of standard solution as follows:-

0.005	(U)	0.005	(S)	0.007 (U)	ml.
0.006	(U)	0.005	(S)	0.008 (U)	ml.

A microsyringe was used for the applications and the outer spots were placed 1 inch from the edge of the paper, leaving 1.25 inches between adjacent spots. Care was taken so that the areas receiving the spots were small, and of the same size.

The papers were then placed in the tank, and after equilibration with the vapour of the aqueous phase had been obtained, they were developed with solvent for approximately 30 hours.

The papers were removed, allowed to dry, and then sprayed with a modified Dragendorff's reagent (Appendix, page 79), and the stained alkaloidal areas were outlined immediately. The papers were allowed to dry, and rectangles were constructed over the coloured spots to give the areas which, in any one determination, were taken to be proportional to the

amounts of alkaloid present, those for the alkaloids in the unknown sample being directly compared with the areas of the standards. Hyoscyamine was the further displaced alkaloid. The mean of four results was taken as the strength of the unknown solution.

The method as attempted on these plant samples was time-consuming, and the results somewhat variable (Table IV).

TABLE IV.

Assay of Plant Samples by a Paper Chromatographic Method.

Material	Assay No.	Hyoscine %	Hyoscyamine %
Duboisia leichardtii	1	0.62	1.52
	2	0.46	1.33
Datura stramonium	3	0.08	0.25
	4	0.07	0.20
	5	0.11	0.27
	6	0.08	0.23

Direct application of the green extract to the paper was suggested[147], but this was found to be even less successful, separation not always being achieved, probably due to interference by the chlorophylls. The use of Whatman papers No. 1 and No. 4 was also tried but was of no advantage.

The paper technique was checked to determine its reproducibility and accuracy by using pure alkaloids, a solution containing a mixture of

pure hyoscine (0.1 per cent) and hyoscyamine (0.2 per cent) being spotted on No. 3 paper in volumes ranging from 0.005 to 0.050 ml. From the point of view of detection and separation best results were obtained over a 0.015 to 0.035 ml. range. Below 0.015 ml. the hyoscine areas were difficult to determine and above 0.035 ml. separation was not always complete.

More favourable distribution conditions in many systems have been obtained[148] by respotting the application area with stationary phase from the solvent mixture. This modification was carried out on a series of papers which were all spotted with 0.025 ml. alkaloid solution; the subsequent application of 0.002 ml. of stationary phase gave improved results.

During this work, using different amounts of pure alkaloids, planimetric readings of all areas obtained were recorded. Gradation was apparent but not always precise, the results becoming approximate at both ends of a short range.

When the same amounts of pure alkaloid were spotted and run on large chromatographic sheets, the range of discrepancy between the areas measured was 10 to 15 per cent. However, as some relationship between area and concentration of alkaloids present was apparent, it was decided to persevere with this type of technique. The greatest drawback of the paper method was the time taken for development and, therefore, the much faster thin layer (film) technique was tried.

Thin Layer Chromatographic Separation.

As with the other separation techniques attempted, a standard solution in chloroform containing hyoscine and hyoscyamine in the ratio 1:2 was used in the preliminary work.

Series of silica gel and aluminium oxide plates, heated at different temperatures for varying periods of time, were prepared; these were spotted with the standard alkaloid mixture, by means of a micrometer syringe, and developed in various solvents. Development at different temperatures was also tried. Three detecting reagents were tested; Dragendorff-Munier[149], Dragendorff Jatzkewitz[149], and the modified Dragendorff reagent as used for the paper chromatographic technique. The modified Dragendorff reagent was adopted because of its high sensitivity and the very light background obtained after spraying.

With silica gel plates heated at 122° C for two hours and developed in dry chloroform, no separations were achieved. "Caps" of hyoscine were visible above the main hyoscyamine spot, but these proved too faint for area tracing.

Aluminium oxide plates were heated for periods of one to four hours, from temperatures of 115° to 280° C. Plates heated for two hours at 138° C gave the best results for the solvent systems attempted. (The range 120° - 140° C however gave good results). Dry ether and dry chloroform, separately and mixed in different proportions, absolute alcohol with dry chloroform, and Chloroform B.P. were used. Ether and chloroform were unsatisfactory, no separation occurring. Chloroform with ethanol in high

proportions gave distortion of the spots, but Chloroform B.P. was found to give good separation.

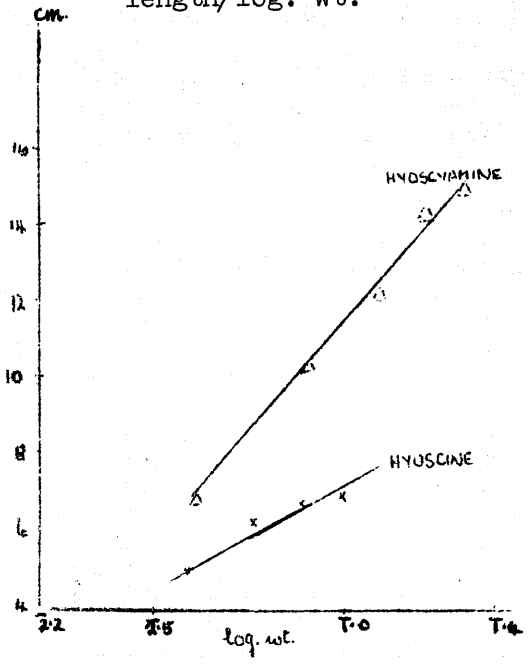
Temperature of development was very critical and to obtain free-flowing alkaloidal areas the plates had to be developed in a completely lined, constant-temperature tank. A 20 by 20 by 5 cm. chromatographic tank was used and this was almost totally immersed in a constant-temperature water-bath which was provided with a close-fitting, insulated lid. Different development temperatures, ranging from 20° to 40° C were used with aluminium oxide plates and Chloroform B.P. At higher temperatures there was no separation, or separation only with very small concentrations of alkaloids and the spots were badly distorted. A temperature of 22° C was found to give good separation and development took only 30 to 35 minutes for a 10 cm. length.

Having obtained a good separation, evaluation of the concentration of the applied solution was attempted by area measurement. The area of spots was traced on to paper and determined both planimetrically and by the rectangle method described under the paper chromatographic technique. Graphs of area/log. wt., square root of area/log. wt., length/log. wt., and log. length/log. wt. were drawn, and over a limited range the correlation was quite good. (Fig. I). The square root of area/log. wt. gave the best straight line.

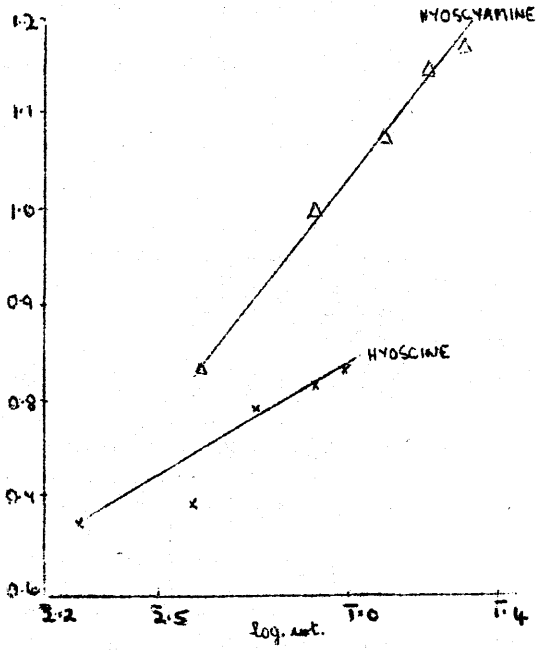
The method described by Truter[149] for thin layer chromatographic estimation was applied and this was found to be the most successful method for determination of both hyoscine and hyoscyamine when strict attention to detail was paid as follows. Three solutions were required; firstly a standard solution of hyoscine and hyoscyamine, in which the ratio of alkaloids was known, secondly a strong test solution (unknown strength) of a

Fig. I

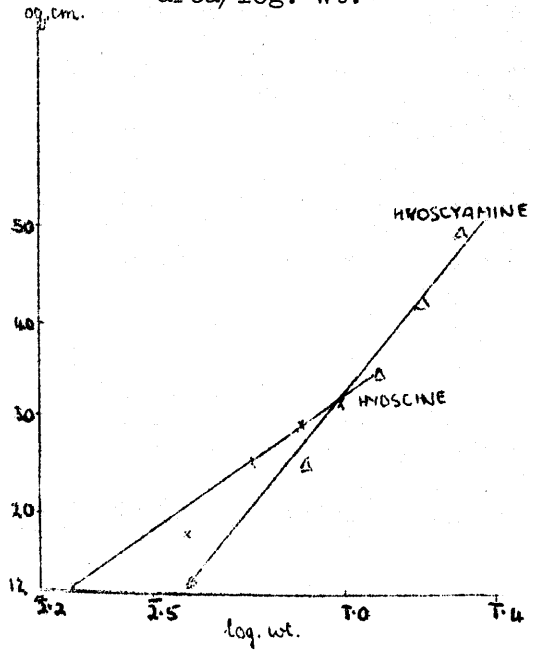
length/log. wt.



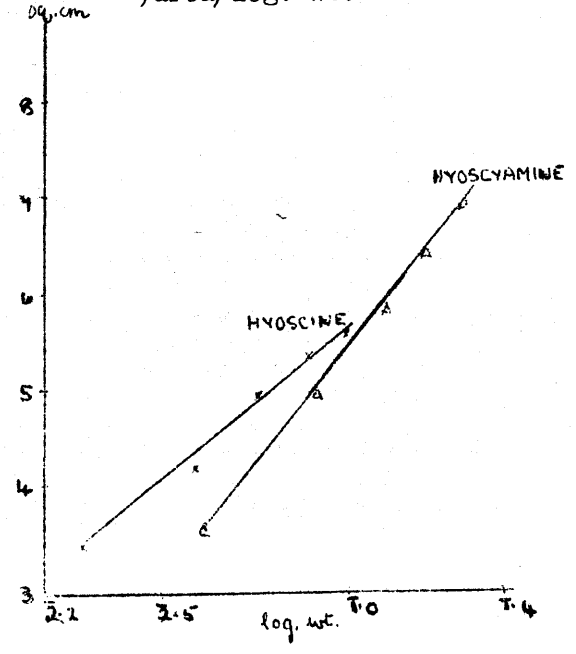
log. length/log. wt.



area/log. wt.



$\overline{\text{area}}$/log. wt.



mixture of hyoscine and hyoscyamine, and thirdly a known dilution of this strong test solution. Equal volumes of each of these three solutions were spotted on to the starting line of 10 by 20 cm. plates carrying a 250 μ layer of alumina (Merck Alumina G. after Stahl for T.L.C.), previously heated for 2 hours at 138° C and subsequently cooled in a desiccator. Applications were made by means of a micrometer syringe mounted on a vertically-adjustable screw stand, the use of which permitted careful application without the danger of damaging the surface of the adsorbent. The size of the spot must be constant irrespective of the total volume of solution applied, hence 0.001 ml. was delivered in repeated applications, allowing the area to dry between applications, until a total volume of between 0.005 ml. and 0.035 ml. had been applied. The plates were developed in Chloroform B.P. at 22° C and, after running for exactly 10 cm., allowed to air dry. The plates were then lightly sprayed with the modified Dragendorff's reagent and the areas immediately magnified and drawn. The coloured areas were magnified by means of projection equipment on to a ground glass screen and the enlarged areas traced. Surface illumination from a strong light source had to be used, as the aluminium oxide film was too opaque to transmit light. The magnified areas were measured by means of a planimeter, the average of five readings for each area being taken.

Using 0.02 ml. of a solution containing 0.1% hyoscine and 0.2% hyoscyamine in chloroform as standard, and similar volumes of the same solution and a 1 in 2 dilution of this solution as the "unknown" strong and weak solutions respectively, the amounts of hyoscine and hyoscyamine were calculated in the "unknown" strong test solution from the mean results

of four plates using the following formula:

$$\log. w = \log. w_s + \frac{\sqrt{A} - \sqrt{A_s}}{\sqrt{A_x} - \sqrt{A}} \cdot \log. d$$

w_s = wt. of alkaloid (hyoscyamine or hyoscine) in 0.02 ml. standard solution.

w = wt. of alkaloid in 0.02 ml. strong test solution.

A_s = magnified area given by 0.02 ml. standard solution.

A = magnified area given by 0.02 ml. strong test solution.

A_x = magnified area given by 0.02 ml. weak test solution.

d = dilution of strong test solution to give weak test solution.

This formula applied to plates spotted with pure alkaloid mixture was found to give very accurate results. (Table V).

TABLE V

Results for pure Alkaloids obtained by Thin Layer Chromatography.

Wt. of Hyoscine (μg.)		Wt. of Hyoscyamine (μg.)	
Applied	Calculated	Applied	Calculated
20.56	20.63 20.27 21.34 20.97	41.04	40.21 40.16 41.91 43.25

Thus for alkaloids in pure solution the average results for hyoscine were calculated as 101.17 per cent, and for hyoscyamine 100.83 per cent of the amounts actually present in the "unknown" solution.

When the determination was repeated using a test solution which

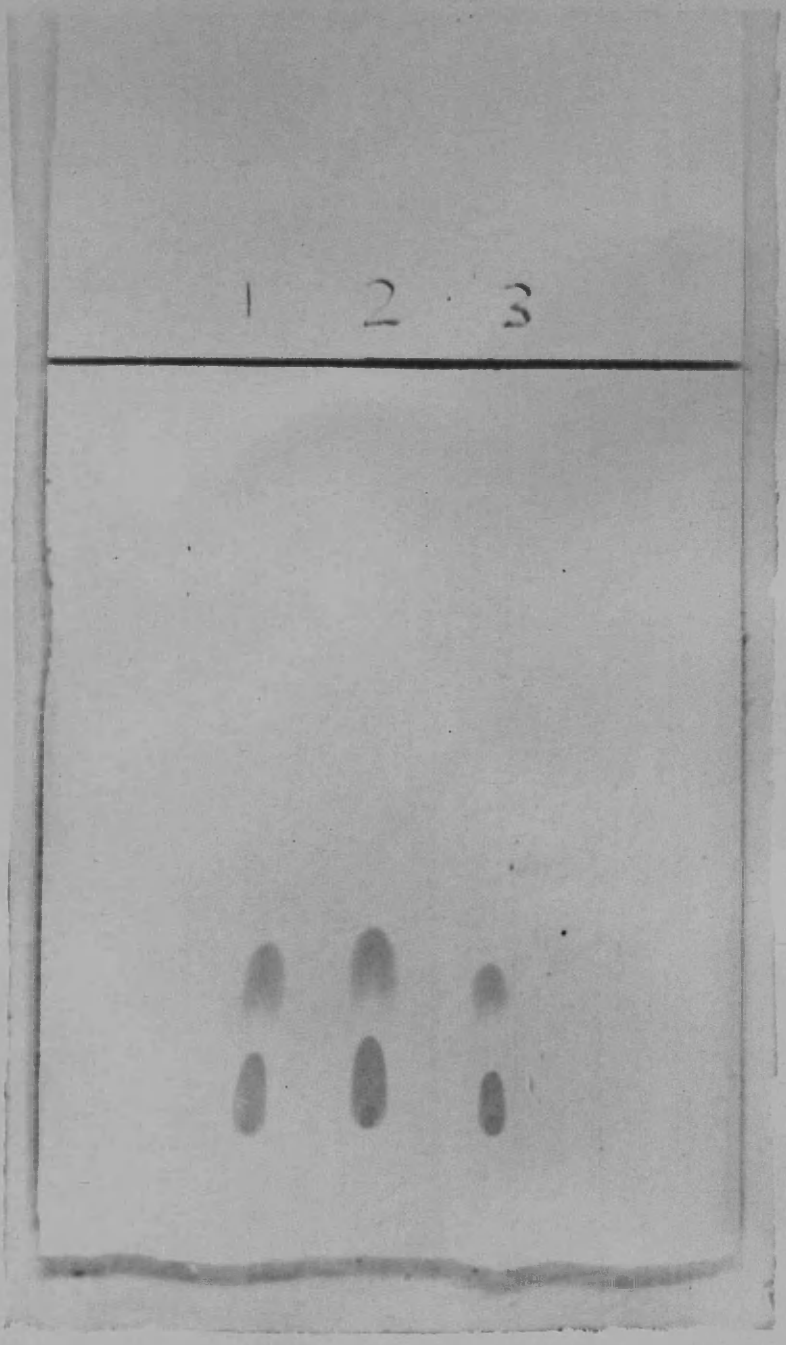
contained chlorophyll, in addition to the alkaloids, some distortion of the areas on chromatograms took place and the results were not so good, there being a discrepancy of ± 15 to 20 per cent. between the calculated and actual amounts of alkaloid present. Thus for the determination of plant samples it appeared imperative to remove the colouring matter and carry out the determination on a solution of the separated total alkaloids. Concurrently a determination of total alkaloids was performed using the official pharmacopoeial method on an aliquot of the same extract.

The powdered plant material (10 g., being 2 g. of each individual plant in a group of five) was accurately weighed and the B.P. 1958 extraction procedure carried out. The final chloroformic solution was made up to exactly 50 ml. 10 ml. was pipetted out and evaporated at 40° C to form a syrup using a rotary film evaporator. The residue was made up to exactly 2 ml. with dry chloroform and this solution was used in the thin layer estimation. The remaining 40 ml. was evaporated to dryness, and the B.P. method was continued to give an estimation for total alkaloids in 8 g. plant material (Tables VIII and XIV).

In a few groups of plants the smallest individual plant weighed less than the required 2 grammes when dried: in such cases, this entire plant together with the same weights of other dried plants in the group constituted the sample used for assay. In the I and J groups of plants grown in vermiculite, the yields were not sufficient to enable a pharmacopeial assay to be performed and, therefore, these were assayed by the thin layer technique only.

For the thin layer determination, 0.02 ml. of the prepared solution was spotted on a plate. 0.5 ml. of this chloroform solution was diluted with dry chloroform to give 1 ml. (dilute test solution) and

0.02 ml. was applied to the plate. An equal volume of the standard chloroformic solution of hyoscine (0.1%) and hyoscyamine (0.2%) was also applied. Four plates were used for each individual assay, the relative position of spots being varied on different plates which were developed simultaneously in the same tank. After air drying, the plates were lightly sprayed with the Dragendorff's reagent (Plate 1) the areas immediately enlarged and traced. The means of five planimetric readings for each area were substituted in the formula above and the amounts of hyoscine and hyoscyamine in the plant extract were thus separately determined.


1 2 3

RESULTS & DISCUSSION

RESULTS AND DISCUSSION

Results obtained from the 1962 crop showed a difference between plants grown under field (garden) conditions and those grown in the artificial medium. The vermiculite-grown control plants had, on the whole, a lower percentage of alkaloids than the corresponding soil-grown plants. Further investigation of this effect the following season led to a series of plant groups being grown under varying nutrient conditions in vermiculite, and comparison of the results produced against a similar group grown in John Innes Compost No. 2. Hence the work carried out can be divided into two aspects; first, the effects of gibberellic acid treatment on both soil-grown and vermiculite-grown plants, and secondly, the effect of different fertiliser treatments on the growth of Datura stramonium var. tatula.

EFFECTS OF GIBBERELLIC ACID TREATMENT

Effects on Morphology.

Injection of gibberellic acid into the bases of young leaves of Datura stramonium var. tatula, a dichasially branched species, gave plants with the injected side exhibiting the characteristic gibberellic acid effects while the other side of the plant appeared normal. (See Plates 2 - 5). This effect has already been reported[20]. However, confirmation of the presence of extensive scar tissue on stems of treated plants was not obtained. Chlorosis was apparent in leaves of treated plants, some leaves showed reduced indentation at the margins, and were narrower than those of control plants. Chlorosis quickly disappeared in treated plants grown in vermiculite but older leaves of such treated plants were more prone to abscission than corresponding garden plants. All treated plants lost their older leaves more quickly than control plants.

Table VI records the effect of gibberellic acid treatment on plant height.

PLATE 3

GARDEN - GROWN TREATED PLANT.

PLATE 4

VERMICULITE - GROWN CONTROL PLANT.

PLATE 5

VERMICULITE - GROWN TREATED PLANT.

TABLE VI

Effect of Gibberellic Acid on Plant Height.

Season	Period between treatment and collection (wks.)	Treatment	Group	No. of Plants in group	Plant heights (cm.)
1962 (early sowing)	4	Co	(A+B)g	10	15 - 21.1 - 25
			(A+B)t	10	50 - 60.5 - 66
		T	(C+D)g	10	20 - 29 - 34
			(C+D)t	10	38 - 59.9 - 67
	8	Co	(E+F)g	10	46 - 52.7 - 64
			(E+F)t	10	75 - 96.4 - 112
		T_2	(G+H)g	10	64 - 80.9 - 104
			(G+H)t	10	99 - 113 - 124
1962 (late sowing)	2	Co	Ig	5	22 - 37.2 - 53
			*It	5	10 - 12 - 18
		T	Jg	5	22 - 42.6 - 72
			*Jt	5	13 - 17.4 - 24
1963	2	Co	(K+L)g	10	17 - 22.3 - 27
			(K+L)t	10	18 - 21.5 - 25
		T	(M+N)g	10	20 - 26.7 - 32
			(M+N)t	10	24 - 26.2 - 32
	3	Co	(O+P)g	10	26 - 29.4 - 33
			(O+P)t	10	25 - 29.5 - 34
		T	(Q+R)g	10	29 - 33.1 - 44
			(Q+R)t	10	24 - 32 - 39

The symbols used are explained on the following page.

T = 1 treatment (100 μg) of gibberellic acid
T_2 = 2 treatments (each 100 μg) of gibberellic acid

g = garden plants
t = tank plants
Co = control plants
* Affected by adverse weather conditions.

With the exception of group (C+D)t, all treated groups showed the characteristic height gain due to increased internode length effected by gibberellic acid. The garden plants, with a height increase of approximately 27.5 per cent for 1 treatment (100 μg) and 34.5 per cent for two treatments of gibberellic acid (each of 100 μg) confirmed previous workers' findings that increased dosage gives increased height within this dose range[15].

Effect of Gibberellic Acid on Yield of Plant Material.

Table VII records the weights obtained for the dried aerial parts, roots, and stems (greater than 5 mm.) for both garden and tank plants.

In the garden-grown plants the weight of dried aerial parts increased with gibberellic acid treatment in most groups, although with the small number of plants used, the increases could not be shown to be statistically significant. Contrariwise, however, in garden plants left standing for eight weeks after treatment, the controls showed a substantial gain in weight over the treated plants, this possibly due to an eventual impairment of vitality in plants sometime after gibberellic acid treatment leading to a considerable premature leaf drop. In the corresponding plants grown in vermiculite, there was no significant weight difference between treated and untreated plants.

The dried root weights were rather variable, some groups showing a tendency to weight loss, while others showed a tendency to weight gain after

TABLE VII

Effect of Gibberellic Acid Treatment on Plant Dry Weight Yields.

Season	Period between treatment & collection (wks.)	Treatment	Group	No. of plants in group	Plant Dry Weight Yields. (g.)		
					Aerial parts	Roots	Stems 5+ mm.
1962 early sowing	4	Co	(A+B)g	10	1.38 - 3.28 - 4.5	0.25 - 0.90 - 1.74	none
			(A+B)t	10	5.33 - 8.67 - 11.66	2.34 - 3.27 - 4.15	1.66 - 3.37 - 4.39
		T	(C+D)g	10	1.96 - 3.45 - 4.27	0.52 - 0.86 - 1.09	none
			(C+D)t	10	6.41 - 9.40 - 15.74	1.36 - 3.13 - 4.02	0.95 - 3.27 - 4.36
	8	Co	(E+F)g	10	14.46 - 22.5 - 31.22	2.88 - 4.18 - 6.05	5.16 - 10.26 - 16.16
			(E+F)t	10	14.37 - 21.82 - 37.75	3.11 - 7.17 - 12.63	11.0 - 17.85 - 25.87
		T_2	(G+H)g	10	8.66 - 14.42 - 22.08	2.59 - 4.39 - 6.70	4.07 - 10.06 - 18.02
			(G+H)t	10	16.9 - 22.61 - 26.74	6.10 - 10.05 - 13.85	18.05 - 23.05 - 30.43
1962 late sowing	2	Co	Ig	5	2.91 - 4.95 - 7.87	0.87 - 1.78 - 3.03	0 - 1.87 - 5.08
			*It	5	0.22 - 0.70 - 1.58	0.05 - 0.12 - 0.21	none
		T	Jg	5	2.85 - 5.24 - 7.25	0.64 - 1.35 - 1.95	0 - 1.66 - 4.12
			*Jt	5	0.66 - 1.07 - 1.69	0.13 - 0.22 - 0.31	none

Table VII is continued on the following page; the symbols used are explained at the end of the table.

TABLE VII (Contd.)

Season	Period between treatment & collection (wks.)	Treatment	Group	No. of plants in group	Plant Dry Weight Yields. (g.)		
					Aerial parts	Roots	Stems 5+ mm.
1963	2	Co	(K+L)g	10	0.95 - 1.79 - 2.51	0.38 - 0.63 - 0.81	none
			(K+L)t	10	1.19 - 2.93 - 5.13	0.86 - 1.11 - 1.51	none
		T	(M+N)g	10	1.18 - 2.06 - 3.26	0.51 - 0.69 - 0.97	none
			(M+N)t	10	2.14 - 3.21 - 4.76	0.74 - 1.10 - 1.60	0 - 0.16 - 0.85
	3	Co	(O+P)g	10	2.64 - 4.43 - 6.43	0.21 - 0.90 - 1.41	none
			(O+P)t	10	3.48 - 5.45 - 7.44	1.04 - 1.75 - 2.28	0 - 0.70 - 1.57
		T	(Q+R)g	10	2.46 - 3.59 - 5.44	0.73 - 0.96 - 1.69	none
			(Q+R)t	10	3.48 - 5.54 - 7.12	0.83 - 1.92 - 2.79	0 - 1.17 - 2.02

Co = control plants

T = 1 treatment with gibberellic acid (100 μg.)

T_2 = 2 treatments with gibberellic acid (each 100 μg.)

g = garden plants

t = tank plants

* affected by adverse weather conditions.

treatment; no firm conclusions could be drawn from the results. Other workers[15,17,20] also have observed this wide variation in dried root weights of plants treated with gibberellic acid.

Again, with stems greater than 5 mm. diameter, the results were not entirely consistent. However, it is of interest to note that with one exception ((C+D)t groups) all treated groups grown in vermiculite had increased stem weight as compared with the corresponding untreated controls and these results thus confirm earlier findings on the effect of gibberellic acid on stem weight.[32]

Effect of Gibberellic Acid on Plant Constituents.

Table VIII shows the results obtained on bulked material from the five plants in each group. At each stage in the cultivation studies duplicate groups were treated; thus plants in group Ag had conditions identical with those for plants in group Bg, Ct corresponded exactly with Dt, and so on. Because of limited amounts of material for each group, only one assay was performed by the method of the British Pharmacopoeia to give total alkaloids, (although, as indicated above, there were two groups representing each phase of the work so that results were duplicated) and from an aliquot of each extract quadruplicate results were obtained for the hyoscyamine and hyoscine content of each group using the thin layer method of assay previously described.

Table IX shows the mean values for total alkaloids as obtained by both methods of assay and also the average values for hyoscyamine and hyoscine in the plants combined in groups according to treatment, being

TABLE VIII

Effect of Gibberellic Acid Treatment on Plant Constituents.

Detailed Results of Analyses

Season	Period between treatment and collection (wks.)	Treatment	Group	No. of plants in group	B.P. Assay Total Alkaloids %	Thin Layer Method	
						Hyoscyamine %	Hyoscine %
1962	4	Co	Ag	5	0.30	0.16; 0.15; 0.15; 0.15	0.12; 0.11; 0.12; 0.12
			At	5	0.30	0.07; 0.07; 0.08; 0.08	0.07; 0.07; 0.07; 0.07
			Bg	5	0.29	0.10; 0.10; 0.11; 0.11	0.13; 0.14; 0.13; 0.14
			Bt	5	0.25	0.11; 0.11; 0.10; 0.10	0.10; 0.10; 0.12; 0.11
		T	Cg	5	0.16	0.13; 0.13 0.13; 0.13	0.10; 0.10; 0.10; 0.10
			Ct	5	0.25	0.07; 0.06; 0.06; 0.07	0.09; 0.09; 0.09; 0.09
			Dg	5	0.16	0.14; 0.14; 0.15; 0.14	0.11; 0.10; 0.10; 0.11
			Dt	5	0.18	0.15; 0.14; 0.14; 0.15	0.11; 0.12; 0.12; 0.12

Table VIII is continued on the following page; the symbols used are explained at the end of the table.

TABLE VIII (Contd.)

Season	Period between treatment and collection (wks.)	Treatment	Group	No. of plants in group	B.P. Assay Total Alkaloids %	Thin Layer Method	
						Hyoscyamine %	Hyoscine %
1962	8	Co	Eg	5	0.51	0.27; 0.27; 0.27; 0.27	0.15; 0.16; 0.16; 0.16
			Et	5	0.42	0.22; 0.22; 0.21; 0.22	0.19; 0.19; 0.19; 0.19
			Fg	5	0.52	0.21; 0.21; 0.21; 0.21	0.13; 0.12; 0.13; 0.11
			Ft	5	0.33	0.20; 0.21; 0.21; 0.21	0.18; 0.19; 0.17; 0.18
		T_2	Gg	5	0.49	0.26; 0.26; 0.26; 0.25	0.13; 0.14; 0.14; 0.13
			Gt	5	0.38	0.29; 0.28; 0.29; 0.29	0.18; 0.19; 0.19; 0.18
			Hg	5	0.18	0.14; 0.13; 0.14; 0.12	0.07; 0.06; 0.06; 0.06
			Ht	5	0.35	0.28; 0.27; 0.27; 0.28	0.22; 0.21; 0.22; 0.21

Table VIII is continued on the following page; the symbols used are explained at the end of the table.

TABLE VIII (Contd.)

Season	Period between treatment and collection (wks.)	Treatment	Group	No. of plants in group	B.P. Assay Total Alkaloids %	Thin Layer Method	
						Hyoscyamine %	Hyoscine %
1962	2	Co	Ig	5	0.13	0.08; 0.07; 0.07; 0.08	0.07; 0.07; 0.07; 0.07
			It	5	-	0.08; 0.08; 0.09; 0.10	0.11; 0.12; 0.13; 0.12
		T	Jg	5	0.17	0.11; 0.11; 0.12; 0.11	0.07; 0.07; 0.07; 0.07
			Jt	5	-	0.05; 0.05; 0.06; 0.05	0.08; 0.08; 0.07; 0.07
1963	2	Co	Kg	5	0.17	0.14; 0.13; 0.13; 0.14	0.07; 0.07; 0.07; 0.07
			Kt	5	0.19	0.06; 0.06; 0.05; 0.05	0.07; 0.07; 0.07; 0.06
			Lg	5	0.25	0.13; 0.14; 0.14; 0.13	0.14; 0.14; 0.15; 0.14
			Lt	5	0.21	0.07; 0.07; 0.07; 0.06	0.10; 0.12; 0.10; 0.10

Table VIII is continued on the following page; the symbols used are explained at the end of the table.

TABLE VIII (Contd.)

Season	Period between treatment and collection (wks.)	Treatment	Group	No. of plants in group	B.P. Assay Total Alkaloids %	Thin Layer Method	
						Hyoscyamine %	Hyoscine %
1963	2	T	Mg	5	0.26	0.10; 0.10; 0.10; 0.10	0.09; 0.10; 0.10; 0.10
			Mt	5	0.17	0.08; 0.07; 0.08; 0.08	0.12; 0.12; 0.13; 0.12
			Ng	5	0.25	0.09; 0.09; 0.09; 0.09	0.08; 0.08; 0.08; 0.08
			Nt	5	0.18	0.10; 0.10; 0.10; 0.11	0.08; 0.07; 0.08; 0.07
	3	Co	Og	5	0.40	0.30; 0.29; 0.30; 0.28	0.21; 0.20 0.21; 0.20
			Ot	5	0.19	0.11; 0.11; 0.11; 0.10	0.10; 0.09; 0.09; 0.09
			Pg	5	0.41	0.23; 0.23; 0.23; 0.22	0.19; 0.19; 0.18; 0.19
			Pt	5	0.25	0.10; 0.09; 0.09; 0.11	0.10; 0.11; 0.10; 0.10

Table VIII is continued on the following page; the symbols used are explained at the end of the table.

TABLE VIII (Contd.)

Season	Period between treatment and collection (wks.)	Treatment	Group	No. of plants in group	B.P. Assay Total Alkaloids %	Thin Layer Method	
						Hyoscyamine %	Hyoscine %
1963	3	T	Qg	5	0.32	0.19; 0.19; 0.19; 0.19	0.15; 0.15; 0.15; 0.15
			Qt	5	0.22	0.12; 0.11; 0.11; 0.11	0.09; 0.10; 0.10; 0.10
			Rg	5	0.30	0.15; 0.15; 0.14; 0.15	0.12; 0.13; 0.13; 0.12
			Rt	5	0.16	0.06; 0.07; 0.07; 0.07	0.08; 0.07; 0.07; 0.08

Co = control plants
T = 1 treatment gibberellic acid (100 µg.)
T_2 = 2 treatments gibberellic acid (each of 100 µg.)
g = garden plants
t = tank plants.

the same groups already compared with regard to plant yields (Table VII).

Considering first the results obtained for total alkaloids by the B.P. assay the findings of earlier workers[32] are confirmed, a decrease being generally observed for treated plants, tank and garden alike, although with the two-week garden groups for both season 1962 and 1963, slight increases in total alkaloid content were noted.

However, in this work the main aim was to discover if the ratio of hyoscyamine:hyoscine had altered due to gibberellic acid treatment. It is unfortunate that the results obtained by the B.P. assay method for total alkaloids and those determined by summating the amounts of individual alkaloids calculated by the thin layer chromatographic method do not entirely agree, (Table IX) there being approximation in only about half of the determinations made. However, in garden plants, with only one exception (2 week group, 1962), the expected decrease in total alkaloids due to gibberellic acid treatment was again shown. The results for the tank-grown plants were inconsistent there being apparent increases in total alkaloids for 2 week plants (1963 season), 4 week plants and 8 week plants, but reduction in total alkaloid in the other groups examined.

TABLE IX

Effect of Gibberellic Acid on Plant Constituents.

Mean Results of Analyses.

Season	Period between treatment and collection (wks.)	Treatment	Group	No. of plants in group	B.P. Assay Total Alkaloids %	Thin Layer Method		
						Hyoscyamine %	Hyoscine %	Total Alkaloids %
1962	4	C_o	(A+B)g	10	0.29	0.13	0.13	0.26
			(A+B)t	10	0.28	0.09	0.09	0.18
		T	(C+D)g	10	0.16	0.13	0.10	0.23
			(C+D)t	10	0.21	0.10	0.10	0.20
	8	C_o	(E+F)g	10	0.51	0.24	0.14	0.38
			(E+F)t	10	0.37	0.21	0.20	0.41
		T_2	(G+H)g	10	0.34	0.19	0.10	0.29
			(G+H)t	10	0.37	0.28	0.20	0.48
	2	C_o	Ig	5	0.13	0.07	0.07	0.14
			It	5	-	0.09	0.12	0.21
		T	Jg	5	0.17	0.11	0.07	0.18
			Jt	5	-	0.05	0.08	0.13
1963	2	C_o	(K+L)g	10	0.21	0.13	0.11	0.24
			(K+L)t	10	0.20	0.06	0.08	0.14
		T	(M+N)g	10	0.25	0.10	0.09	0.19
			(M+N)t	10	0.17	0.09	0.10	0.19
	3	C_o	(O+P)g	10	0.41	0.26	0.20	0.46
			(O+P)t	10	0.22	0.10	0.10	0.20
		T	(Q+R)g	10	0.31	0.17	0.14	0.31
			(Q+R)t	10	0.19	0.08	0.09	0.17

Table X, recording some individual plant analyses by partition column chromatography is included.

TABLE X

Individual Plant Analyses by Partition Column Chromatography.

Season	Period between treatment and collection (wks.)	Treatment	*Plant	Hyoscyamine %	Hyoscine %
1962	8	Co	Eg(2)	0.39; 0.36	0.16; 0.16
			Eg(5)	0.45; 0.46	0.03; 0.02
			Fg(1)	0.35; 0.38	0.15; 0.15
			Fg(3)	0.37; 0.38	0.11; 0.11
			Ft(1)	0.23; 0.24	0.19; 0.20
			Ft(2)	0.22; 0.23	0.11; 0.12
			Ft(5)	0.27; 0.27	0.16; 0.16
		T_2	Gt(1)	0.21; 0.21	0.12; 0.12
			Gt(2)	0.22; 0.20	0.15; 0.14
			Gt(5)	0.29; 0.27	0.13; 0.13

* The letters have the same meaning as in previous tables, the figures in brackets denote the row from which the individual plant was taken.

The sums of the individual alkaloids found by this method have good correlation with the average value for total alkaloids determined for the corresponding group of five plants by the B.P. Assay.

Ontogenetic Considerations.

Table XI records the summary of information on hyoscyamine/ hyoscine ratios in the main experiments during 1962 and 1963.

TABLE XI

Summary of Information on Hyoscyamine/Hyoscine Ratios in the Main Experiments 1962 - 1963.

Age (wks.)	Garden-grown plants		Vermiculite-grown plants	
	Untreated	Treated	Untreated	Treated
2	1:1	3:2	3:4	5:8
	2:1	1:1	1:1	2:3
	1:1	1:1	2:3	5:4
3	3:2	4:3	5:4	1:1
	11:9	5:4	1:1	1:1
4	5:4	4:3	1:1	2:3
	3:4	7:5	1:1	1:1
8	9:5	2:1	1:1	3:2
	7:4	2:1	1:1	9:7

In any season the total alkaloids increase with increasing age of plants.

With plants grown under normal field (garden) conditions, in very young plants the proportions of hyoscyamine and hyoscine are approximately the same but as the plants become older the relative proportions alter and hyoscyamine predominates.

With plants grown under artificial conditions in a vermiculaponic system the proportions of hyoscyamine and hyoscine remain approximately the same. This observation on the relative increased proportion of hyoscine in the aerial parts of plants fed on nutrient solutions is similar to the observation of Evans and Griffin[75] who reported an increase

of hyoscine in the total root alkaloids of Datura innoxia grown under artificial conditions. (In concordance with these workers' findings, except for I and J groups, which were adversely affected by weather conditions, all tank plants, treated and untreated, showed much greater root development than the corresponding garden plants. This is probably due primarily to the nature of the growing medium vermiculite, which is very light and loose, and permits unrestricted growth of roots.)

The treatment with gibberellic acid does not appear to alter this known ontogenetic production of hyoscyamine and hyoscine in Datura stramonium whether grown under field conditions or in an artificial medium.

EFFECT OF FERTILISER TREATMENT ON Datura stramonium var. tatula.

The previous results having indicated some deficiency in alkaloids in plants grown on vermiculite as compared with similar plants grown under field conditions led to work in which groups of plants were grown in John Innes Compost No. 2 and also in vermiculite using different nutrient solutions, and various effects were noted.

Effect on Morphology.

Table XII records the effect of the different fertiliser treatments on plant height; a great difference was noted between plants grown on John Innes Compost and those grown in vermiculite, although between the latter groups no great difference was observed.

TABLE XII

Effect of various Fertiliser Treatments on Plant Height.

Group	"Fertiliser"	No. of plants in group	Plant Height (Cm.)
1	N,P,K 200 ppm.	10	50 - 69.5 - 79
2	N 300 ppm. P,K 200 ppm.	10	50 - 65.2 - 90
3	K 100 ppm. N,P 200 ppm.	10	57 - 73.3 - 91
4	John Innes Compost No. 2	10	29 - 42.3 - 60

The vermiculite-grown plants looked much healthier than those

grown on compost, their leaves being larger and darker in colour. Towards the end of the growing season, the vermiculite-grown plants still looked strong and vigorous, whereas the plants grown on John Innes Compost, at the time of uprooting, had far fewer bud formations and their lower leaves were beginning to wither, many being yellow-coloured. Thus it would appear that the ready availability of nutrients in the vermiculaponic system not only increases the size of plants in a given time but also increased the growing period of Datura stramonium.

Effect on Dry Plant Yields.

Table XIII records the weights of aerial parts, roots, and stems greater than 5 mm. found for the different groups. Again, the difference between plants grown in John Innes Compost and the vermiculite-grown plants was found to be very large, the vermiculite-grown plants having a much greater dry plant yield for all parts. Varying the ratios of N, P and K, within the ranges shown, did not greatly alter the yield.

TABLE XIII

Effect of various Fertiliser Treatments on Plant Yield. (Dry Weights).

Group	"Fertiliser"	No. of plants in group	Weight (g.)		
			Aerial parts	Roots	Stems > 5 mm.
1	N,P,K 200 ppm.	10	7.75-11.11-15.52	8.41-13.10-22.57	4.34-7.60- 9.60
2	N 300 ppm. P,K 200 ppm.	10	8.52-13.57-19.02	5.44-11.87-19.00	3.09-7.53-13.69
3	K 100 ppm. N,P 200 ppm.	10	9.00-11.68-15.21	6.63-10.94-20.02	4.30-6.67- 8.97
4	John Innes Compost No. 2	10	2.05- 4.81- 8.10	0.54- 2.92- 8.85	0 -0.95- 3.40

Effect on Plant Constituents.

Table XIV records the effect of the various fertiliser treatments on D. stramonium plants.

TABLE XIV

Effect of Various Fertiliser Treatments on Plant Constituents.

Group	"Fertiliser"	No. of plants in group	Total Alkaloids %		Hyoscyamine %	Hyoscine %	Ratio Hyoscyamine/ hyoscine (approx.)
			BP Assay	T.L.C			
1	N,P,K 200 ppm.	10	0.31	0.27	0.09	0.18	1:2
2	N 300 ppm. P,K 200 ppm.	10	0.41	0.34	0.14	0.20	2:3
3	K 100 ppm. N,P 200 ppm.	10	0.34	0.32	0.10	0.22	1:2
4	John Innes Compost No. 2	10	0.17	0.18	0.08	0.10	4:5

Considering the average values for total alkaloids the results of analyses carried out by both the British Pharmacopoeial assay ard a thin layer chromatographic method, showed that increased nitrogen content in the nutrient solution caused increased alkaloid content in plants grown under soil-less conditions and this increase was due mainly to increased hyoscyamine content. However it was noted again that plants grown under

artificial conditions had a higher proportion of hyoscine than is normally expected; in this particular instance the values having been calculated on plants left for a much longer growing period (14 weeks after hardening off) than plants in the main experiments described earlier. All artificially grown groups had a higher alkaloidal content than the group grown in John Innes Compost No. 2.

Diurnal Effect on Alkaloidal Content.

Table XV, a more detailed table than Table XIV records the alkaloid content obtained for the groups, half of which were uprooted in the morning, the remainder in the afternoon. However, no firm conclusions of diurnal variability of total alkaloidal content in these groups could be drawn, since two sets of results showed no difference between the morning and afternoon collections while two showed that some increase had occurred by leaving the plants until the afternoon. (Table XV).

TABLE XV

Effect of Fertiliser Treatment and Time of Collection on Plant Constituents.

Group		No. of plants in group.	Total Alkaloids % (B.P. Assay)	Thin Layer				
				Total Alkaloids %	Hyoscyamine %		Hyoscine %	
					Individual values	Mean	Individual values	Mean
N,P,K 200 ppm.	M	5	0.27	0.27	0.11; 0.11; 0.11; 0.11	0.11	0.16; 0.16; 0.16; 0.17	0.16
	A	5	0.36	0.28	0.07; 0.08; 0.08; 0.08	0.08	0.20; 0.20; 0.20; 0.20	0.20
N 300, P,K 200 ppm.	M	5	0.42	0.33	0.13; 0.14; 0,14; 0.14	0.14	0.19; 0.19; 0.19; 0.19	0.19
	A	5	0.41	0.35	0.13; 0.14 0.13; 0.13	0.13	0.22; 0.22; 0.22; 0.21	0.22
K 100, N,P 200 ppm.	M	5	0.29	0.22	0.06; 0.05 0.06; 0.06	0.06	0.17; 0.16; 0.17; 0.16	0.16
	A	5	0.39	0.41	0.13; 0.14; 0.14; 0.14	0.14	0.27; 0.27; 0.27; 0.27	0.27
John Innes Compost No. 2	M	5	0.17	0.13	0.05; 0.05; 0.05; 0.05	0.05	0.08; 0.09; 0.09; 0.08	0.08
	A	5	0.17	0.20	0.10; 0.10; 0.10; 0.10	0.10	0.10; 0.10; 0.10; 0.10	0.11

M = morning uprooting

A = afternoon uprooting

SUMMARY

SUMMARY

This thesis incorporates results of some cultivation studies on plants of Datura stramonium var. tatula and in particular records some observations of the effects of gibberellic acid on these growing plants.

In cultivation studies variation of soil type and condition may lead to anomalous results therefore some of the plants used in this work were grown in a controlled hydroponic system supported in vermiculite. Vermiculaponic culture has been described for a variety of crops but no published information was available on its use for the growth of experimental plants; other plants were grown under field conditions and some in boxes of soil. Very healthy plants were obtained on the artificial medium and whereas young plants of corresponding age were similar in size when comparing garden-grown with those raised on vermiculite, the latter eventually gave a greater yield, the plants being taller and showing much more extensive root development. Similarly, in a small-scale experiment, plants grown in any one of three different nutrient solutions, supported in vermiculite, showed much better development than corresponding plants grown on John Innes compost. Whereas plants grown under field conditions tended to show the usual signs of chlorosis after gibberellic acid treatment, this effect was not observed in plants fed with nutrient solutions.

The effects of the growth-promoting gibberellins have been extensively studied on a wide variety of plants, including several of medicinal importance. In the latter group, studies have been made on plant growth and also on the production of important chemical constituents; some work has been done on alkaloid-yielding plants, including Datura species, but the results reported to date give information on total

alkaloids only. In this present work an attempt was made to determine the effects of gibberellic acid treatment on the hyoscyamine/hyoscine ratio in the aerial parts of D. stramonium var. tatula at different stages of growth. On plants grown under both field and hydroponic conditions, the well known effect of gibberellic acid on stem elongation was further confirmed, most treated plants being taller than corresponding untreated controls; in general there was also an increased weight of aerial parts produced, although this was not so for some groups of garden-grown plants. The effect of treatment on root growth (dry weight) was variable and, since no conclusions can be drawn from the results obtained, no clarification of earlier controversial findings was achieved.

The separate assay of hyoscyamine and hyoscine in small samples of plant material presented considerable difficulty, the published method utilising partition chromatography being found to be unreliable. Attempts were made to use a method based on area measurements of the alkaloids separated on paper chromatograms and also to devise a method utilising similar measurements of areas obtained on thin layer chromatograms. The latter method was found to be reliable using pure solutions of the alkaloids but some of the results on plant samples may be suspect since in certain cases the sum of the amounts of the two major alkaloids (hyoscyamine and hyoscine) were at considerable variance with the amount of total alkaloid as determined by the assay method described in the British Pharmacopoeia.

While it was quite clear that the total amount of alkaloid in the aerial parts was reduced following gibberellic acid treatment, thus confirming published results, only a tentative conclusion can be proposed with regard to the hyoscyamine/hyoscine ratio. For garden-grown plants,

both untreated and treated, the total alkaloid increased but the proportion of hyoscine decreased as the plants matured. Thus gibberellic acid does not appear to exert any effect on the previously described ontogenetic production of these alkaloids. On the whole, the plants grown in vermiculite tended to be somewhat poorer in alkaloid content than the corresponding garden plants and the hyoscine appeared to form a higher proportion of the total alkaloid, particularly in the control plants; this proportion did not seem to decrease to any great extent as the plants matured. This relatively higher proportion of hyoscine in the aerial parts of plants fed by nutrient solutions, as compared with similar plants grown on soil, is of interest in that it confirms a similar report on the proportions of root alkaloids in Datura innoxia grown under different conditions.

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APPENDIX

A P P E N D I X

CONSTRUCTION OF TANK

A wooden tank, measuring 8' long, 4' wide and 1' deep (plate 6) and waterproofed by coating the inside with bituminous paint was erected on the roof of The Royal College of Science and Technology, Glasgow. The tank was supported on legs high enough to allow a container of approximately 30 litres capacity to be positioned beneath its lower edge, and it was set to give a $\frac{1}{2}$" fall over its length towards an outlet on the bottom of the lower edge. This outlet was covered internally with fine wire mesh and fitted externally with a black iron tap. (It is important that metal fittings be constructed only of black iron or stainless steel, in order to avoid the possibility of release, at toxic level, of ions of other metals into the nutrient solution). A black iron tube, 4' 2" in length, closed at both ends, but pierced with small holes 1" apart, was attached across the width at the higher end of the tank to serve as an inlet spray for the nutrient solution which was fed from a 30 litre aspirator bottle.

Washed granite chips ($\frac{1}{2}$") were spread to a depth of 1" over the base of the tank, carefully and evenly covered with a 1" layer of damp, well-washed horticultural sand and then with 8 cwt. of Natkruit vermiculite to give an 8" layer. This material was saturated with water (admitted through the inlet spray), the water was drained off and replaced by triple strength nutrient solution which was drained off after 18 hours, leaving the vermiculite damp and ready to receive the hardened off plants.

While the tank was positioned on the roof of the building to take advantage of the maximum available sunlight, this resulted in it being

PLATE 6

DIAGRAM OF VERMICULAPONIC TANK.

A = vermiculite

B = sand

C = granite chips

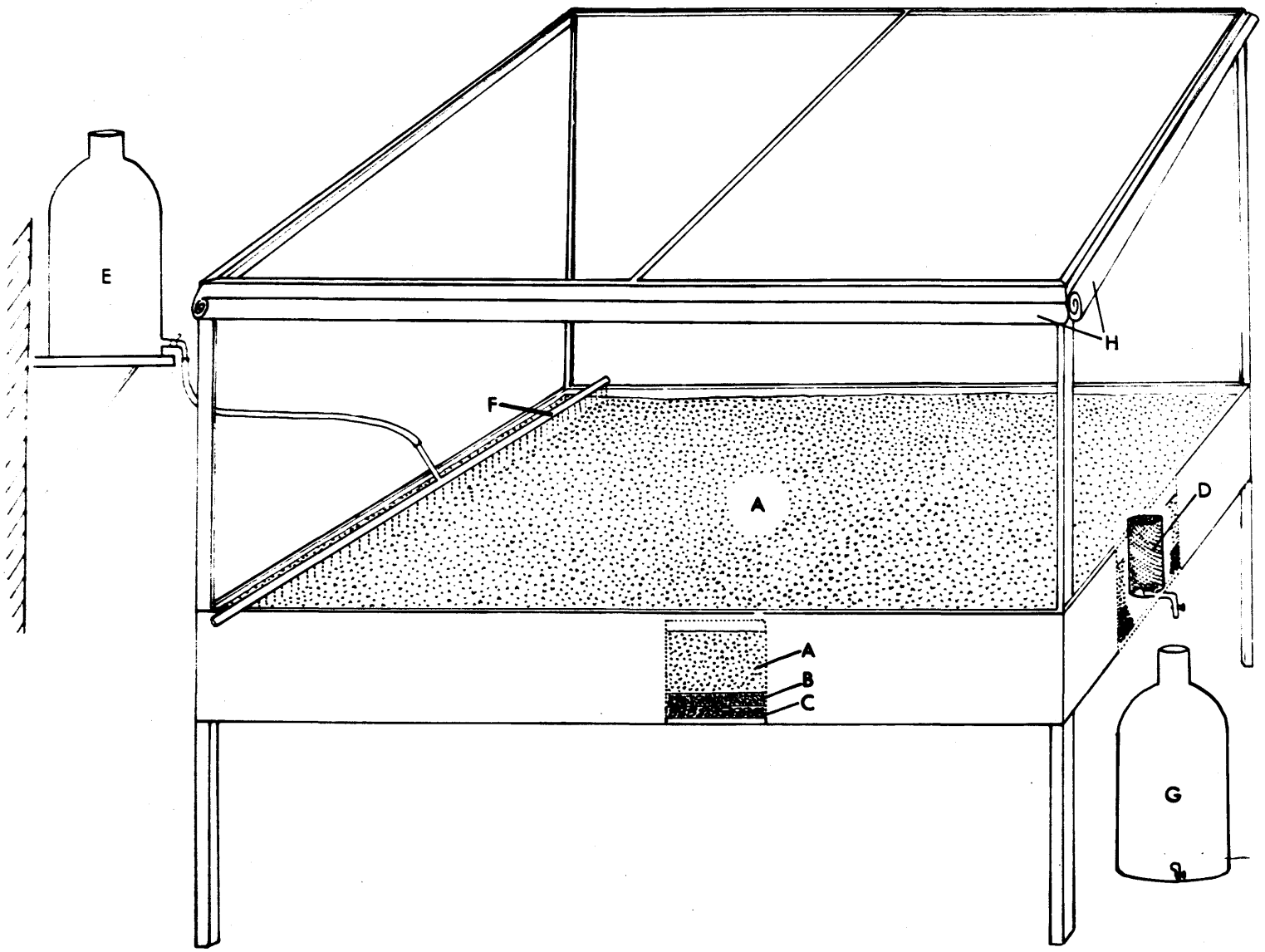
D = wire mesh protecting outlet

E = aspirator containing nutrient solution

F = spray

G = aspirator containing partially exhausted nutrient solution

H = protective terylene sheeting


E
H
F
A
D
A
B
C
G

rather exposed, and it was necessary to erect a 6' high wind-break. Further protection to the plants against unfavourable weather conditions was provided by attaching a sloping roof and side panels, both made of terylene sheeting, to a wooden framework built above the tank. The side panels could be rolled up and the roof removed during fine weather. It was found, however, that the terylene sheeting became badly torn during a particularly high wind. Plastic sheeting, reinforced with wire mesh was tried, but gave little improvement.

It had been hoped to analyse the nutrient solution drained from the tank in order to ascertain the quantity of each chemical absorbed by the plants, but dilution of the nutrient solution by rain in the adverse weather conditions experienced made this impracticable. The cover, described above, was found to give very poor protection when the rain was driven by wind. An answer to this problem was found by fixing to the framework a number of hinged shutters made of reinforced plastic set in wooden frames. With these in position the tank assumed the character of a cold frame, but this was not done until late in the 1963 season, therefore the plants grown in the tank and used in this work can be regarded as having been grown under open conditions equating with those obtained for the field-grown plants.

The tank, as finally protected, could be used for cultivation studies in which it was desired to periodically analyse the nutrient solutions in the tank in order to determine their change in composition during plant growth.

NUTRIENT SOLUTIONS FOR PLANTS GROWN IN VERMICULITE

The composition of nutrient solution used in the present work was selected taking account of the formula for a nutrient solution successfully used for growing Nicotiana spp.[81] but also of the fact that the proportions 10:10:10 for N:P:K are reported[70] to be the most effective in increasing alkaloid production in Datura stramonium.

The fertiliser grade compounds, which were to provide the major elements were analysed to determine their percentage purity and then the appropriate amounts, (Table XVI), were calculated to give the desired parts per million of the necessary elements. Particularly with the trace elements, it is usually important to take into account the quantities present in the water to be used for making the solution, but with the high degree of purity of Glasgow tap water, this was unnecessary in the present work. For convenience in making up the nutrient solution, stock solution of the compounds giving minor elements were used except for the ferrous sulphate which quickly oxidises in solution to give ferric ion, and this is not utilised by growing plants.

Before use, the pH of the nutrient solution was adjusted to 4.6.

TABLE XVI

Composition of full strength Nutrient Solution used in Season 1962.

Major elements	Compound used	*Wt. in 30 litres of soln.	p.p.m.
Ca N	Calcium nitrate	27.58 g.	155.2 108.5
K P	Potassium dihydrogen phosphate	13.19 g.	126.4 100.1
K N	Potassium nitrate	5.84 g.	73.6 26.6
Ca P	Super phosphate	12.19 g.	64.6 99.9
N	Ammonia sulphate	9.19 g.	65.0
Mg	Magnesium sulphate	31.92 g.	98.7
Minor elements			
Mn	$Mn\ SO_4$	46.14 mg.	0.5
B	$H_3\ BO_3$	87.0 mg.	0.5
Fe	$Fe\ SO_4\ 7H_2O$	120.0 mg.	0.8
Zn	$Zn\ SO_4\ 7H_2O$	6.6 mg.	0.05
Cu	$Cu\ SO_4\ 5H_2O$	2.4 mg.	0.02
Mo	$Mo\ O_3$	2.1 mg.	0.05

* The weights quoted for compounds used to give the major elements apply only to the particular samples of fertiliser grade chemicals used in

this work; each sample was first assayed to estimate its percentage purity before calculating the weight required to give the necessary parts per million.

The 1962 solution (Table XVI) contained 200 parts per million each of N, P and K. For certain experiments in 1963 two other strengths of solution were also used:-

1. For N:P:K 300:200:200 p.p.m. 16.929 g. of ammonium bicarbonate were added per 30 litres of a solution otherwise as above.
2. For N:P:K 200:200:100 p.p.m. the potassium dihydrogen phosphate was reduced to 2.76 g. while 5.85 ml. of syrupy phosphoric acid was added per 30 litres to maintain the P level.

In each case the final pH was adjusted to 4.6.

MODIFIED DRAGENDORFF'S REAGENT

Bismuth subnitrate (0.85 g.) was added to a mixture of water (40 ml.) and acetic acid (10 ml.) in a 250 ml. flask. Potassium iodide (25 g.) was dissolved in water (50 ml.) and this was added to the flask and the mixture shaken until the bismuth subnitrate had completely dissolved. The resultant solution (5 ml.) diluted with glacial acetic acid (10 ml.) was made up to 50 ml. with distilled water and this solution used for spraying.