THE UNIVERSITY OF MANITOBA

STUDIES ON SELECTED ALKALOIDS IN PHALARIS ARUNDINACEA OF INTEREST IN THE PRODUCTION OF NEW CULTIVARS

by

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CONTENTS

ABSTRACT

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Qualitative and quantitative methods were developed for the determination of the indole alkaloids of reed canarygrass. The tryptamine alkaloids were shown to be under simple genetic control. Seasonal variation of the alkaloid levels, both gramine and the tryptamines, was dramatically affected by the management of the grass.

During one season of grazing the tryptamine containing grass was found to be deleterious to sheep. The sheep exhibited distinct preferences for particular clones of grass, but in the tryptamine free grass area these preferences were influenced by the stage of growth of the grass. In the tryptamine containing grass area the preferences of the sheep remained essentially constant independent of the stage of growth.

INTRODUCTION

INTRODUCTION

Phalaris arundinacea has considerable potential as a forage crop in North America, but has three major problems which need to be overcome. These are poor animal acceptability, possible toxicity, and poor seed production. The related species *Phalaris tuberosa* has also been found to be toxic, and this toxicity has been attributed to the presence of tryptamine alkaloids, of similar nature to those reported present in *Phalaris arundinacea*. For this reason it was initially intended to develop a satisfactory rapid quantitative method for the estimation of tryptamine alkaloids in *Phalaris arundinacea*, and to use this method in improving the species as a forage by reduction of the alkaloid content. A gas chromatographic procedure was already available, but was not considered rapid enough, considering the numbers of samples which would need to be examined. This slowness is caused by the fairly critical clean up technique required, and the inherent slowness of the gas chromatographic analysis. Thin layer chromatographic techniques were developed which separated the alkaloids of interest, and a densitometric method of measurement was produced.

After analysis of a number of clones it was found that some of the clones were free of tryptamine alkaloids, suggesting

that these compounds could readily be eliminated for the synthesis of new cultivars. The research objectives were therefore extended to include studies on the genetic control of the tryptamine alkaloids, and the effect of these alkaloids on the grazing animals. As this species behaves as an allotetraploid it seemed likely that a single dominant gene would be responsible for the control of the alkaloids. This hypothesis is supported by the demonstration of this type of genetic control of other chemical constituents in other species, and is in agreement with the one gene - one enzyme theory. The studies with grazing animals were included mainly because of the lack of direct evidence of the harmfulness of the tryptamine alkaloids in Phalaris arundinacea.

For the studies on genetic control a rapid method was developed for determining whether or not a plant contained tryptamine alkaloids. This was necessitated by the large numbers of plants which need to be examined to determine any genetic ratio reliably. This method involved test tube extraction and color development rather than Soxhlet extraction and thin layer chromatography as had previously been used. The result was an increase in speed from 15 samples daily to over 200 samples daily, a reduction in sample size from 5 g dried grass to about 1-2 g fresh grass, and an improvement in sensitivity. A number of crosses were evaluated using this procedure, and it was possible to evaluate the seedlings at 12 weeks or older. A satisfactory agreement with the single dominant gene for tryptamines hypothesis was obtained.

For the studies on the effect of tryptamines on the grazing animal a design was used which permitted a simultaneous evaluation of palatability differences between clones, as poor palatability is a major problem with this species. Alkaloids have been suggested as a cause of palatability differences in this species, and although the grazing trial was not designed specifically to examine this possibility, some evidence was obtained indicating that alkaloids alone are not responsible for palatability differences. Dramatic differences in palatability were evident in both the plants containing tryptamines and those free of tryptamines. Neither group of animals did particularly well, but those on the tryptamine containing pasture suffered under heat stress whereas the others were essentially normal in this respect.

During two grazing periods the sheep on the tryptamine containing pasture lost weight significantly, whereas the sheep on the tryptamine free pasture showed no significant change in weight. This information, together with the observations on the effect of heat stress, was taken as evidence for the deleterious effects of the tryptamines.

Of interest in evaluating the effect of tryptamine alkaloids on animals was the variation in alkaloid quantity throughout the season. Both tryptamines and gramine were examined, under two management conditions, free growth and regular clipping. Under both conditions a marked rise in alkaloid levels was apparent about mid-season, this being most noticeable in the

plants which were clipped regularly. Leaf material contained more alkaloids than stem material, and this is presumably the reason that the regularly clipped grass produced higher alkaloid levels than the free growth material.

LITERATURE

Introduction

Reed canary grass, Phalaris arundinacea L. is distributed widely in northern temperate regions, and has been introduced into Australia. The grass is widely adapted to a range of moisture and soil conditions and offers many advantages as a forage grass. The major problems with use of this species for forage are its unpalatability and potential toxicity. From the point of view of commercial utilization its high rate of shattering makes seed production difficult.

Agronomic potential

The potential of this species and the related species P. tuberosa L. as forage crops have been adequately reviewed[93,52]. The advantages of P. arundinacea include the ability of the plant and seeds to withstand long periods of total immersion in water, and its ability to continue to grow and tiller on completely waterlogged soil. The plant's drought resistance is considerable and it appears to be indifferent to soil texture, and to pH over the range 4.9 to 8.2. A sward formed on soft mud is soon firm enough to support machinery. The leaves remain green even after seed shedding, and thus can provide a late source of green fodder. The plant is also highly frost resistant, and grows vigourously early in the year. Hay yields and protein content of the hay are usually as good or better than other forage grasses. Once established its growth is

vigorous enough to restrict weeds such as wild barley[99]. The species is moderately pest resistant. It has been shown very resistant to grass hopper attack[32] although frit fly[100], nematode [96,89] and Helminthosporium[26] infections have been reported.

Genetics

The species appears to be mainly tetraploid (2n = 28)[37] but plants of higher ploidy levels have been reported[33,62,61,28]. The presence of diploids[76] is doubtful[62]. The chromosome behaviour at meiosis is reported that of an allotetraploid[53,33] with almost always 14 bivalents forming. In genetic studies it is necessary to consider the possibility of gene duplication. An anthocyanin pigment (Pelargonidin glucoside) from the roots has been shown to be under the control of a single dominant gene with several modifying ones[63]. This is presumed to be the same pigment that had been shown previously to be under a single dominant gene control[24] although it is possible that these pigments represent two examples of single dominant gene control in this species.

Toxicity

During a forage intake study at the University of Manitoba a sheep feeding on P. arundinacea died, and post mortem examination suggested chronic alkaloid poisoning as the cause[6]. This work commenced following cattle deaths attributed to alkaloidal poisoning when cattle were grazed on pastures which included P. arundinacea[9]. Poor weight gains of sheep grazing on reed canary grass pasture have been reported[78]. However

really satisfactory evidence of the toxicity of P. arundinacea is lacking. Most of the evidence arises from the fact that the related species P. tuberosa is toxic, and the alkaloids of this species have been implicated. Similar and identical alkaloids have been found in P. arundinacea[36, 98].

It is possible that other compounds present could be responsible for toxicity. These include transaconitic acid[14] and substances having estrogenic and anti-estrogenic activity[20]. Cyanogenic compounds do not appear to have been reported in P. arundinacea although they have been reported in P. tuberosa. In the latter species they do not seem responsible for the "staggers" syndrome[42]. Other reported compounds which may be of some interest are an anti-inflammatory substance[56] and a seed growth inhibitor[49], isolated from P. arundinacea, and an antibiotic reported in P. minor[29].

Toxicity as related to P. tuberosa

In Australia P. tuberosa has caused the disease "Phalaris staggers" in sheep. Poisoning occurs very rapidly, as early as 4 hours after grazing[46]. The alkaloids of this species have been shown to be toxic and to cause similar, but not identical, symptoms to those of "Phalaris staggers"[47]. The major alkaloids of this species are N,N-dimethyl-5-methoxytryptamine, N,N-dimethyltryptamine and bufotenine[36]. The first two of these alkaloids have been found in P. arundinacea[98] and also 5-methoxy-N-methyltryptamine[97,98], N-methyltryptamine[98], gramine[9], two tetrahydro-β-carbolines[9,*], and hordenine[9,97,98]. The pharmacology of some of these alkaloids is summarised

*Dr. R. C. S. Audette, personal communication

briefly in appendix 5. The major site of action of the alkaloids is the brain and spinal chord, where two distinct effects, convulsive and spastic, are produced[45]. Myeline degeneration of the spinal chord has been observed in cases of Phalaris staggers[41]. The "Phalaris staggers" disease has three sorts of symptoms, peracute in which sudden collapse and death due to heart failure occur, acute neurological disorder and chronic neurological disorders which include CNS lesions and appear over a period of time. The correlation between the action of the dimethyltryptamines and the disease symptoms is reported better for the acute than for the chronic symptoms[43].

There are conflicting reports on alkaloid content and toxicity relationships. Moore et al studied the relations between growing conditions, toxicity and alkaloid levels, and found that conditions which encouraged high alkaloid levels such as high nitrate levels, also induced greater toxicity[65,66,67,68]. Their work indicated that the autumn was the most dangerous time. The dilution of P. tuberosa stands with other species reduced the toxicity[55], indicating that the animals can tolerate the usual levels of alkaloids occurring in this grass. Differences have been found in levels of alkaloids between varieties[73] and during the season[72]. Highest levels of alkaloids were found in the autumn, but the animals which exhibited symptoms of poisoning did so later, in the early spring or summer. Furthermore symptoms of poisoning occurred on varieties containing low levels of alkaloids as well as those containing high levels. Apart from variation in levels within varieties[73]

variation in alkaloids in individual plants has also been reported[79]. There would therefore appear to be room for selection on an individual plant basis for low alkaloids in P. tuberosa.

Blood levels of anticholinesterase have been examined in cases of "Phalaris staggers", and no relationship between their levels and the occurrence of the disease was found[95]. P. minor has also been reported to cause staggers, but this was attributed to organo phosphorus treatment of the grassland[64].

Palatability

Considerable work has been done in examination of palatability of reed canary grass. Much of this has been reviewed by Marten[57]. Palatability is indicated as a heritable character with a high heritability estimate of 90% from a group of 8 selfed plants[18]. Most work indicates that there are real differences between clones for palatability, but there is considerable management/genotype interaction which results in difficulties in dealing with this problem[7,69,70,15,24]. Brown[24] concluded that palatability was due to either additive or recessive genes. The general unpalatability of reed canary grass as a variety has been reported[78,58], and various plant components have been examined to attempt to find the cause of unpalatability. Some evidence has been produced that the tryptamine alkaloids may be responsible for low palatability[15,98] rather than other agronomic features[18,69,70]. This agrees with the conclusion of Brown[24] regarding recessive genes, if considered along with the recent evidence[102] for dominant

genes for tryptamines. However palatability of reed canary grass has been shown to be increased by applied nitrogen[5] a condition which would be expected to cause a rise in alkaloid level. Against this hypothesis is the work of Roe and Mottershead[81] who correlated low palatability with high ploidy level. They also extracted unpalatable components from reed canary grass using solvents which would not be expected to remove the tryptamines from the plant. There is also the evidence that harding grass, although containing tryptamine alkaloids, is not considered unpalatable.

Digestibility

Digestibility of reed canary grass has been studied by several workers. A good correlation was found between digestibility and intake when intake was *ad libitum*, dry matter digestibilities being around 60%[16,17], but in a subsequent analysis of this work a difference between palatable and unpalatable clones for organic matter digestibility was found in only one grazing period[69]. A correlation of 0.69 for *in vitro* dry matter digestibility between parents and top cross progenies has been shown[27]. That high quantities of hemicelluloses in reed canary grass reduce digestibility has been shown[74], and the fact that a large proportion of available carbohydrate in this species is fructosan has also been shown[71]. Other workers have shown that high nitrogen fertilizing increases the protein and digestibility[31,19].

Seed retention and other agronomic characters

As seed production is a problem with this species, some studies have been made on this character. Both the mechanism of seed shattering[22] and the effect of fertilizer and management[88] have been examined. Seed shattering[12], together with other components of seed yield[23] seem to be very variable, and there is opportunity to improve this feature by suitable breeding techniques[26].

Leafiness, leaf width and other agronomic characters have also been shown to be very variable and capable of considerable improvement by breeding[11].

Hybrids

There has been some interest in production of hybrids of *P. arundinacea* with *P. tuberosa*, and it has been suggested that the hexaploid variety of reed canary grass is in fact a *P. arundinacea* by *P. tuberosa* cross[62]. Much of the work of hybrid production has been summarised by Devine[38]. Generally the cross seems to be more productive in the *P. arundinacea* by *P. tuberosa* direction, aided in this respect by the high degree of self sterility of *P. arundinacea*[84,24]. The hexaploid cross may be useful as a winter hardy and seed retaining cultivar[3,4].

MATERIALS AND METHODS

(A) CHEMICAL

Thin layer chromatography

Three systems were developed, as described[101], of which two were preferred, these being the methanol/strong ammonia solution on silica gel and butanol/formic acid/water on cellulose systems.

Development of chromatography systems

Initially not all the compounds later detected were available as reference materials. Those available were: tryptamine, 5-hydroxy-N,N-dimethyltryptamine, N-methyltryptamine, N,N-dimethyltryptamine, gramine, hordenine and 5-hydroxytryptamine which was used initially but rejected later as decomposition appeared to occur rapidly. Ehrlich's Reagent was used as a detecting spray for the tryptamines, and Dragendorff's Reagent for hordenine and gramine (Appendix 1).

Phillipson and Shellard[75] working with Mitragyna alkaloids developed several thin layer solvent systems, which were used on silica gel plates. Certain of these were examined for suitability for Phalaris indoles.

Results

In all cases a running distance of 15 cm was used. The plates used were activated silica gel (Appendix 2).

(1) Neutral solvents:- Ether, chloroform and acetone failed to move the compounds from the origin. Methanol produced some movement, but all the compounds had low Rf values, 0.1 or less.

(2) Basic systems:- Ammonia was tried as a base in mixtures

with methanol. The reference substances were moved from the origin, and partially resolved.

(3) Acidic system:- Methanol containing hydrochloric acid was used. Movement and separation of the reference substances occurred, the order of the compounds being different to that using the ammonia system. The system is potentially of use for identification of compounds, but for routine work it appears to offer no advantage over the methanol/ammonia system. Iron in the silica gel layer also produced a yellow band which may be a nuisance.

The methanol/ammonia system was the preferred system for use on silica gel. As this solvent system contains aqueous ammonia, it seemed unlikely that layer activity would affect resolution of the compounds. A comparison of activated and non-activated layers showed no detectable difference between the two.

The methanol/ammonia system had a disadvantage in that the ammonia evaporated more readily than the methanol, and if several plates were developed consecutively Rf values gradually decreased. Following the suggestion of Bull *et al*, and others[25, 30, 75] silica gel layers were made with various amounts of sodium hydroxide in the water used. Layers incorporating high concentrations of sodium hydroxide (5%) developed surface cracking and, when developed in methanol produced diffuse spots of reference materials with high Rf values (0.95). Low concentrations of sodium hydroxide (0.5%) produced discrete spots with low Rf values (0.30 for dimethyltryptamines and 0.15 for N-methyltryptamines). An intermediate concentration produced diffuse spots, with slightly

higher Rf values (0.40 and 0.20 respectively). As the preferred separation is for discrete spots with Rf values about 0.5 sodium hydroxide impregnated plates were rejected as an improved method.

A further disadvantage of the methanol/ammonia and methanol/hydrochloric acid systems was that they both separated the tryptamines in terms of their degree of methylation at the aliphatic amino nitrogen, but did not separate the 5-methoxy derivatives from their unmethoxylated counterparts. A paper chromatography system was available which separated the tryptamines in terms of their 5-methoxy group, independent of their degree of aliphatic nitrogen substitution, using butanol acetic acid/water 80:3:17 as the running solvent*. This system had the disadvantages that it was lengthy (16 hours running time) and that the spots were diffuse and not well separated. This system was modified first by changing to cellulose layers, and then by using formic acid instead of acetic acid in the running solvent. Use of hydrochloric acid instead of formic acid was possible but offered no improvement in resolution, and introduced the problem of a double solvent front. The final system as published[101] gave a reasonable separation of the methoxylated tryptamines from the unmethoxylated tryptamines and gramine, but this was the limit of its usefulness.

An improvement on the published system would be one using a developing solvent more volatile than butanol/formic acid/water. A number of systems were examined, using methanol, ethanol, propanol, acetone, ether and chloroform, alone and in various mixtures with each other, and with formic acid or

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*Dr. A. B. Simons, personal communication

ammonia. None of the systems tried offered any advantage in separation over the butanol/formic acid/water system, indeed apart from acetone/formic acid/water and acetone/formic acid/methanol no respectable separations were produced.

As the reference tryptamines were separated on silica gel layers in terms of their degree of substitition at the aliphatic nitrogen, and cellulose layers separated them depending on the presence of a 5-methoxy group, it was hoped that a system could be developed that would separate the compounds totally. Such a system would provide a convenient method of identification of compounds, and would serve as a tool in any biosynthetic study which may be undertaken later, and could be used as a basis for a quantitative estimation of alkaloids. Three techniques were tried, use of alumina layers, use of two band layers for two way chromatography, and use of mixtures of silica gel and cellulose.

Alumina is a weaker adsorbent than silica gel[77] and the tryptamines have been separated on an alumina column[97]. However after several experiments alumina was rejected as it did not seem to be possible to improve on the silica gel system with this layer.

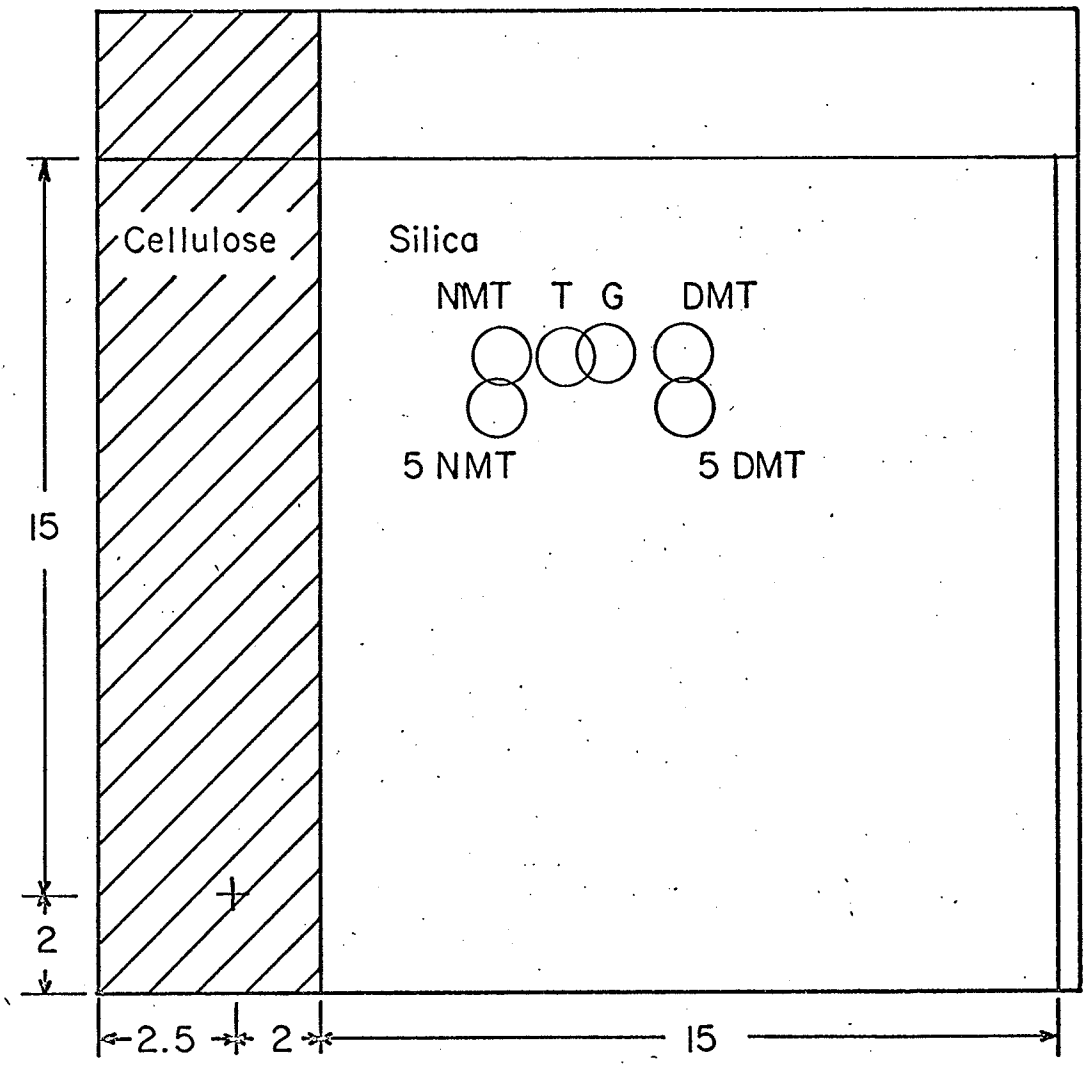
Plates having a narrow band of cellulose along one side, with the remainder of the layer silica gel, were prepared (Appendix 2). The sample was applied to the narrow cellulose band and separated along it first using the aforementioned solvent for cellulose, butanol/formic acid/water 80:5:15. The layers were

then developed in the second direction using the methanol/strong ammonia solution 7:1 solvent for silica gel. Some success was achieved, the separation being as would be expected from the Rf values on each system (Fig. 1). Two practical points are noteworthy with this technique. The first is that on preparing the plates, the depth of each component in the spreader should be the same, or material flows under the partition and makes the division between adsorbents irregular. The second point concerns the choice of which adsorbent is to be used as the narrow band. It is important that the compounds of interest have high Rf values on the narrow band adsorbent when using the wide band running solvent.

Plates were prepared from mixtures of cellulose and silica gel G, and reference compounds run on these using both methanol/strong ammonia solution 7:1, and butanol/formic acid/water 80:5:15. Using the 50:50 mixture it was considered that too much cellulose was present, as the Rf values in the methanol/ammonia solvent were high and separation was impaired--it was not considered feasible to improve this by reduction of the ammonia present. The mixture was then modified to cellulose/silica gel G 40:60, and with this mixture separation in the methanol/strong ammonia solution solvent was satisfactory, and separation in the butanol/formic acid/water solvent partially satisfactory. The main problem with the latter solvent was the reduced Rf values. It was considered that this could be improved by increasing the acid component of the system, and this was in fact found to be the case (Fig. 2).

FIGURE 1: SEPARATION OF REFERENCE COMPOUNDS BY TWO DIMENSIONAL CHROMATOGRAPHY ON BANDED LAYER PLATES

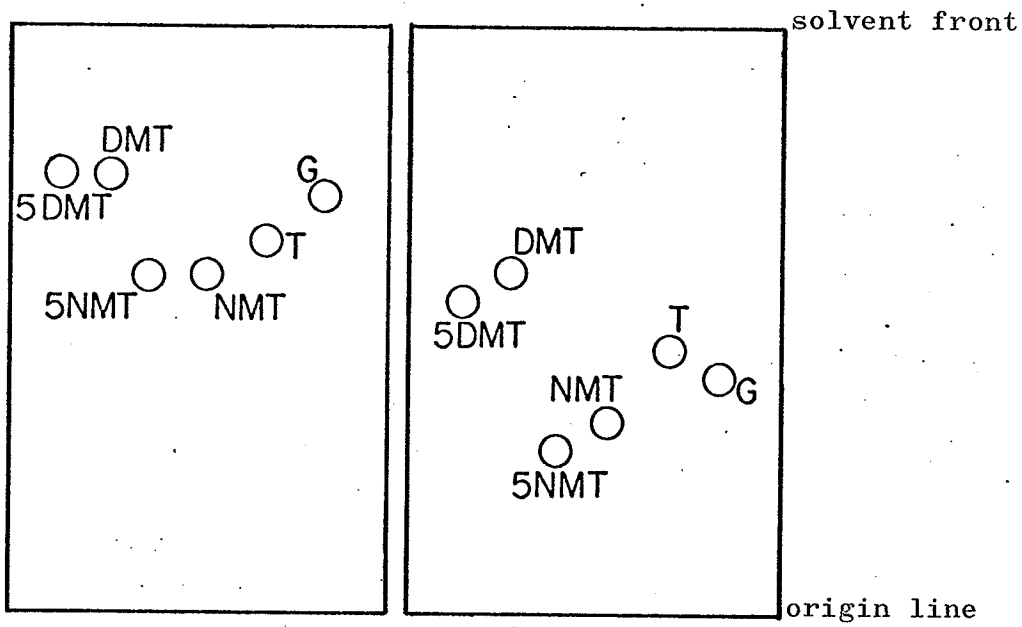
All dimensions are in centimetres

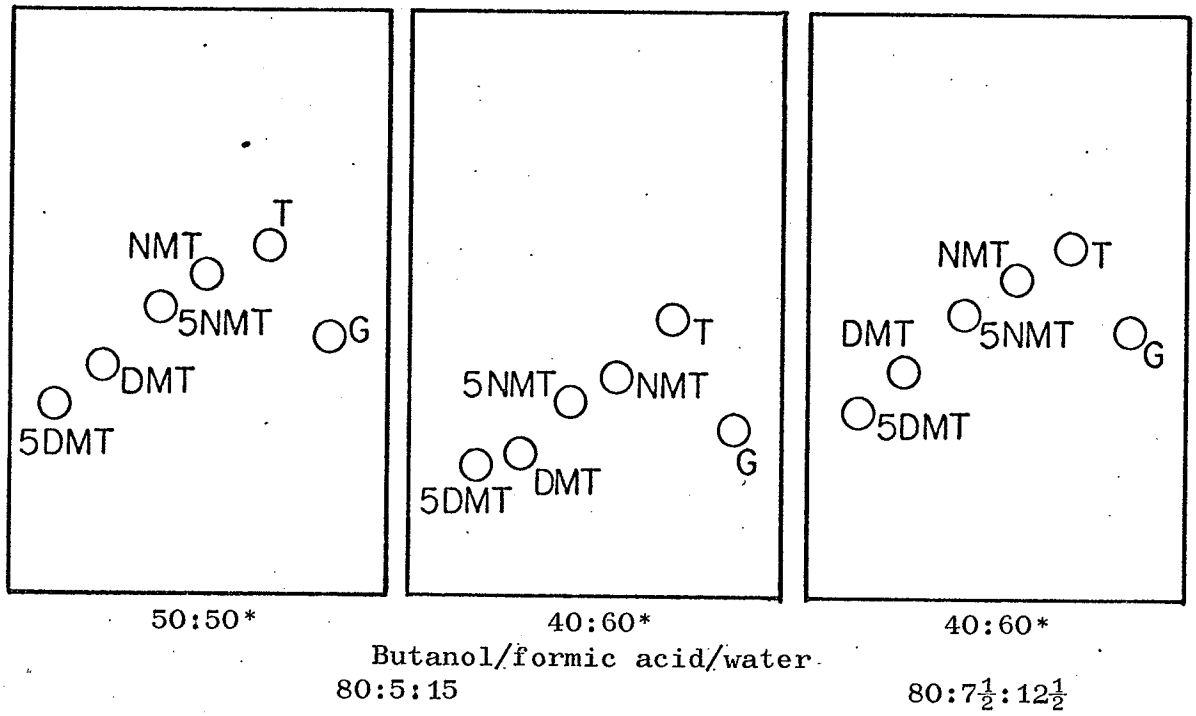


Abbreviations:

G	Gramine
T	Tryptamine
NMT	N-methyltryptamine
DMT	N,N-dimethyltryptamine
5NMT	5-methoxy-N-methyltryptamine
5DMT	N,N-dimethyl-5-methoxytryptamine

FIGURE 2: SEPARATION OF REFERENCE MATERIALS ON MIXTURES OF CELLULOSE AND SILICA GEL





Abbreviations as on figure 1.

*The ratios refer to the w/w proportion of cellulose/silica gel

This mixture was also usable as a two dimensional system, using the usual methanol/strong ammonia solution and butanol/formic acid/water 80:7½:12½ as running solvents.

Spray reagents for chromatography

Three reagents were used routinely[101], these being Ehrlich's, Dragendorff's and Xanthydrol reagents (Appendix 1). Ehrlich's reagent was also useful for quantitative chromatography, as its colours with tryptamines were all similar, and thus only one filter was used for densitometry. The colours produced are tabulated[101]. Smith[85] suggests that a note be made of the rate of appearance of colours and any changes that occurred. This was done where identification was needed, but for most routine purposes the final colour was adequate.

Because of the extra double bond more light absorption would be expected when using p-dimethylaminocinnamaldehyde than when using p-dimethylaminobenzaldehyde (Ehrlich's reagent)[50]. The reagent would thus be expected to be more sensitive. It has been reported as being ten times more sensitive than Ehrlich's reagent but unfortunately it is more reactive and thus less selective. When tried as a thin layer spray using various concentrations of 5-methoxy-N,N-dimethyltryptamine it was found to produce coloured spots more rapidly, but appeared to be no more sensitive than Ehrlich's reagent. As the spot colours differ from those produced using Ehrlich's reagent the compound is of potential use for identification purposes.

Extraction and clean-up procedures

For the first screening procedure a Soxhlet extraction method was used on dried grass[101]. This had several disadvantages, including the length of time required for each sample, and the loss of alkaloids in the later stages. For this reason it was superseded by a cold extraction procedure on fresh material, which was more sensitive, better quantitatively, and much quicker[102].

A quantitative method of extraction and clean-up was developed prior to the method which was used for the first routine screening. Dried grass (20 g) was Soxhlet extracted with 95% ethanol for 16 hours. The alcoholic extract was evaporated to small bulk, about 5-10 ml, and transferred to a separatory funnel using alternately 2N sulphuric acid and chloroform, in 50 ml lots, two lots of each. The mixture was shaken and after settling the chloroform was removed, and washed with 50 ml of 2N sulphuric acid. The acid washings were returned to the bulk of the acid solution, and the chloroform pigment solution was discarded. This stage was repeated twice more using 2 x 50 ml quantities of chloroform each time. The aqueous phase was then saturated with sodium chloride and concentrated ammonia added to raise the pH to 10. This alkaline solution was extracted with 10 x 50 ml quantities of chloroform, and the chlorororm dried over magnesium sulphate. The magnesium sulphate was washed with a further 100 ml of chloroform, and the bulked chloroform solution evaporated to dryness under vacuum. The alkaloidal residue was made to 1 ml with chloroform before thin layer chromatographic examination.

Quantitative extraction of tryptamine hydrochloride at levels of 0.1125 mg/g of grass was only 34% effecient; this value was determined by using thin layer densitometry. Furthermore the efficiency was dependent on the amount of ammonia added, doubling the quantity of ammonia raised the extraction efficiency to 40%. A value of 37% was used as a suitable average. Both of these values compare unfavourably with the 55% efficiency of the method subsequently used for screening in which sodium hydroxide was used as the base.

A critical examination of the procedure revealed that most of the alkaloids were extracted by the Soxhlet procedure within the first 3 hours, and some were still being extracted after 6 hours. Overnight (16 hours) was convenient to use, and no further alkaloids appeared to be recovered. The major loss of alkaloids was in the transfer from the basic aqueous layer to the chloroform, even 10 extractions with chloroform did not totally recover the alkaloids. Some alkaloids were retained by the magnesium sulphate. The pigmented chloroform layer which was removed was almost totally free of alkaloids.

This method, and its various modifications, had many disadvantages. It was a slow process, the extraction efficiency was low, the end product was not as free of contamination as would be liked, and there was risk of breakdown of alkaloids at least at two stages.

Several methods were tried to overcome these disadvantages, before the final procedures[102] were produced.

(1) Modification of the chromatographic system

As the compounds of interest did not move at all when ether was used as developing solvent on silica gel G, it seemed possible that with alkaloidal extracts of Phalaris development of the plate first in ether, and then in the methanol/ammonia system would serve to remove some of the remaining pigments from the region of interest of the chromatogram. This was tried, but no improvement was found compared with plates not developed in ether.

(2) The use of cation exchange resin to purify crude alkaloidal extracts

It seemed possible that under suitable conditions alkaloids could be retained on a cation exchange resin, while pigments were washed through, and could then be removed from the resin in a relatively pure state. It was hoped that this would be easier than partition methods, and also more quantitative.

Bio Rad AG 50W-X2* resin was used, 200-400 mesh. This was washed with 95% ethanol to remove throw, and equilibrated with 95% ethanol overnight. A column 7.5 cm by 1 cm diameter was prepared, and activated by eluting with 95% ethanol containing 1% concentrated hydrochloric acid until the effluent was acid. Excess acid was removed by washing with 95% ethanol until the effluent was chloride free.

Ethanolic extract of an alkaloid containing Phalaris clone, prepared by Soxhlet extraction, was applied to the

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*Dow Chemical

column, which was then washed with 95% ethanol to remove pigments. Elution was with 100 ml of 95% ethanol containing 1% strong ammonia solution. The eluate was evaporated to dryness under vacuum, and dissolved in 0.2 ml chloroform. Of this solution 10, 20 and 40 µl were applied to a thin layer chromatogram.

Several faults were found with this method. The major problem was that the pigments were not satisfactorily removed, and although it was possible to determine the presence of alkaloids from the chromatograms, this was less easy to do than with the partition prepared extracts. However it seemed that larger amounts of alkaloids had been recovered. The ammonia containing eluent caused column shrinkage, and hence poor elution through channelling. A further fault was the apparent poor regeneration of the column.

(3) The use of molecular sieves

It seemed possible that the alkaloids could be separated from the various pigments by use of molecular sieves. This was doubtful from the commencement, as the molecular weights involved were not really large enough, but the idea seemed worth trying. Sephadex LH20*, a cross linked dextran capable of swelling in organic solvents was used. After swelling 5 g for 3 hours in 95% ethanol, a column was packed, diameter 1.3 cm, length 13 cm, void volume 6 ml. Use of an ethanolic grass extract on this column revealed some separation of

*Pharmacia

natural pigments, but the fractions containing the alkaloids were still too pigmented for the system to be of any use for routine work.

(4) Elimination of the acid/base partition steps

After Soxhlet extraction of dried grass, the ethanol was evaporated to small bulk, and water added. The precipitated pigments were centrifuged off, and the aqueous liquid freeze-dried. The product was moderately free of pigments, and gave good yields of alkaloids.

(5) The use of fresh grass

The fresh grass was first frozen to break up the cells, and then triturated with sand and ethanol. The ethanol was filtered off, and concentrated under vacuum to small bulk. The extract was then processed by the acid/base partition process described[101] except that only 1 lot of each of 2N sulphuric acid and chloroform was required to transfer the product to a separatory funnel. Very little grass was needed to obtain enough alkaloids for thin layer chromatography, and consequently less pigments were obtained. Therefore the use of fresh grass represented a big improvement in the procedure.

(6) Elimination of evaporation stages

The processes[102] which were ultimately used for quantitative and qualitative extraction of alkaloids have several aspects which need comment. The samples were frozen at $-30^{o}C$ to break up the cell membranes, and thereby improve

the extraction efficiency. The extraction mixture contained chloroform so that the extracting volume could be large, and also have its volume reduced at the first step of the clean-up procedure. Enough methanol was present to allow the water from the fresh grass to enter the mixture while still retaining a one-phase system, and also to increase the penetration of the mixture into the grass. The ammonia was added so that extractions could be performed under uniformly basic conditions, and also because the free bases are more readily soluble in chloroform than their salts. Two mixtures were used as standards, chloroform/methanol/strong ammonia solution 26:33:1 for the quantitative procedure, and 18:11:1 for the qualitative procedure. The qualitative mixture was designed around a minimum extracting volume of 15 ml, having as much methanol as possible without causing precipitation of sodium sulphate at the stage of addition of sodium hydroxide. In the quantitative mixture a larger volume of extracting solvent was used and as more wash steps were included the precipitation of sodium sulphate was of less importance. However it was very important that the extracting fluid did not become biphasic, and thus more methanol was used.

Preparation of reference materials

The lack of suitable reference materials especially 5-methoxy-N-methyltryptamine, resulted in various attempts to prepare a sample suitable as a quantitative chromatographic reference. This process was taken as far as obtaining a

mixture of 5-methoxy-N-methyltryptamine with some N-methyl tryptamine, and then abandoned as synthetic 5-methoxy-N-methyltryptamine became available.

The extraction procedures used were either scaled up from the quantitative method previously described using dried grass and Soxhlet extraction with 95% ethanol, or involved extraction by maceration of the dried grass with 95% ethanol containing 10 ml/L concentrated hydrochloric acid. In either case the product was purified to a crude base mixture before further separation into components.

A column of silica gel[a] (25 cm long and 1 cm diameter) was prepared by wet packing in methanol. The alkaloidal material from 5 gm of dried grass prepared by the routine screening procedure method was dissolved in chloroform and applied to the column. The column was eluted with methanol/strong ammonia 7:1 and fractions of approximately 2 ml collected after the first pigmented band was eluted. Each fraction was evaporated to dryness, dissolved in 0.2 ml of chloroform and 10 µl used for thin layer examination. Some fractionation was apparent, fractions 9-12 containing the dimethyltryptamines in quantity, while fractions 10-16 contained the N-methyltryptamines.

Unfortunately this type of silica gel was no longer available, and as an alternative a silicic acid column[b] was prepared and tried. This lacked the absorbing power of the silica gel, and everything was eluted with methanol in the first few fractions.

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[a] British Drug Houses silica gel for chromatography
[b] British Drug Houses

Column chromatography was attempted using an alumina column. This had previously been reported as a successful means of purifying 5-methoxy-N-methyltryptamine using chloroform as an eluting solvent[97]. A column of alumina 20 cm x 3 cm diameter (150 g alumina*) was wet packed in chloroform, and the crude bases applied in chloroform. No alkaloids were recovered by the time 500 ml of chloroform had passed through the column. It proved possible to strip the bases from the column with as little as 200 ml of chloroform/methanol 50:50, and therefore a gradient from chloroform to chloroform/methanol was used.

After re-applying the alkaloids to the column it was eluted with chloroform until the first pigment band was leaving the column. The column was then eluted with a linear gradient from chloroform to chloroform/methanol 50:50 in 500 ml. Fractions of 5.3 ml were collected, and analysed using thin layer chromatography. Fractions 22-28 contained compounds considered, on the basis of this analysis, to be β-carbolines, fractions 42-50 contained the dimethyltryptamines, gramine was first detectable around fraction 65, and the N-methyltryptamines were eluted as a very weak band in fractions 80-92.

The total recovery of tryptamines was very low, indicating possible loss due to breakdown on the column. Neither this or the silicic acid column was regarded as particularly successful.

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*British Drug Houses Aluminium oxide for chromatographic adsorption analysis

Crude bases obtained from a bulk extraction were purified by thin layer chromatography using 500 μ silica gel layers and methanol/ammonia solvent. Four bands were removed from the plates after edge spraying and ultra violet light (long wavelength 365 nm) marking of their positions. These bands were classified as:

1. The upper blue band "dimethyltryptamines"
2. The pink "gramine" band
3. The lower strong blue band "monomethyltryptamines"
4. A minor low blue band

Of these band 3 was of most interest. Bands 1 and 3 were extracted with 3 x 100 ml 2N sulphuric acid, and free bases obtained by making alkaline and extracting with 2 x 100 ml of chloroform. Each sample was evaporated to dryness, dissolved in 2 ml chloroform, and quantities of 10 and 20 μl applied to a thin layer chromatography plate for examination.

Using the silica gel, methanol/ammonia system each sample appeared relatively pure, but the cellulose butanol/formic acid/water system resolved each of the bands into two components. These were presumed to be 5-methoxy-N-methyltryptamine and N-methyltryptamine from band 3, and 5-methoxy-NN-dimethyl tryptamine and N,N-dimethyltryptamine from band 1.

The base mixture from band 3 was dissolved in ether and half of it made to 100 ml which was then saturated with dry hydrogen chloride gas to precipitate the hydrochloride. A white precipitate formed, but rapidly became a green oil. This is suggestive of nucleophilic attack by Cl^-. The solution was

immediately evaporated under vacuum, but thin layer chromatographic examination of the residue showed the compound to be almost totally destroyed.

As an alternative purification a picrate was prepared[92]. To a solution of the semi-pure 5-methoxy-N-methyltryptamine in ethanol was added an alcoholic solution of picric acid. Water was added causing precipitation, and the precipitate was washed several times with water. The precipitate was air dried and recrystallized from chloroform. This did not separate the 5-methoxy-N-methyltryptamine from its unmethoxylated counterpart, as could be shown by thin layer chromatography using the cellulose butanol/formic acid/water system.

A small quantity of chromatographically pure 5-methoxy-N-methyltryptamine was obtained by preparative thin layer chromatography of the N-methyltryptamine mixture on the cellulose system. This however did not yield any usable quantity.

Identification of compounds

Most of the compounds mentioned in this thesis have been previously isolated and identified from either Phalaris arundinacea or P. tuberosa. The main method of identification of the compounds used was to compare their behaviour with standard materials. Evidence for identity included thin layer chromatography on three systems, colour reactions, and gas chromatography. The three thin layer systems, and the colour reactions with Ehrlich's reagent and Xanthydrol have been described[101]. Using the silica gel, methanol/strong ammonia

solution chromatography system, with either spray reagent, the presence of unmethoxylated compounds was concealed by the methoxylated derivatives. However when the band containing either the N-methyl or N,N-dimethyl compounds was isolated from the silica system it could be resolved into two compounds on the cellulose, butanol/formic acid/water system. The Rf values and colour reactions of the two components were considered good evidence for the presence of N-methyltryptamine in the 5-methoxy-N-methyltryptamine isolate, and N,N-dimethyl tryptamine in the 5-methoxy-N,N-dimethyltryptamine isolate.

The colours produced by the compounds with Xanthydrol were easily distinguished. An absorption curve of each compound after the Xanthydrol reaction was determined using a recording spectrophotometer*. The absorption curve from crude bases isolated from tryptamine free plants gave a good agreement with a reference curve produced from pure gramine. The absorption spectra from the crude bases extracted from the tryptamine containing clones were what would be expected to be obtained from mixtures of reference compounds (Figs. 3 and 4).

Another identification was by means of gas chromatography. The chromatographic systems were already available, and a crude base extract obtained late in the growing season (Sept. 25) from regularly clipped grass was examined using the diethyleneglycol succinate system [8]. Co-chromatography with N-methyltryptamine, 5-methoxy-N-methyltryptamine, 5-methoxy-N,N-dimethyltryptamine, and 2,9-dimethyl-6-methoxy-1,2,3,4-

*Cary Instruments California Model 15 recording spectrophotometer.

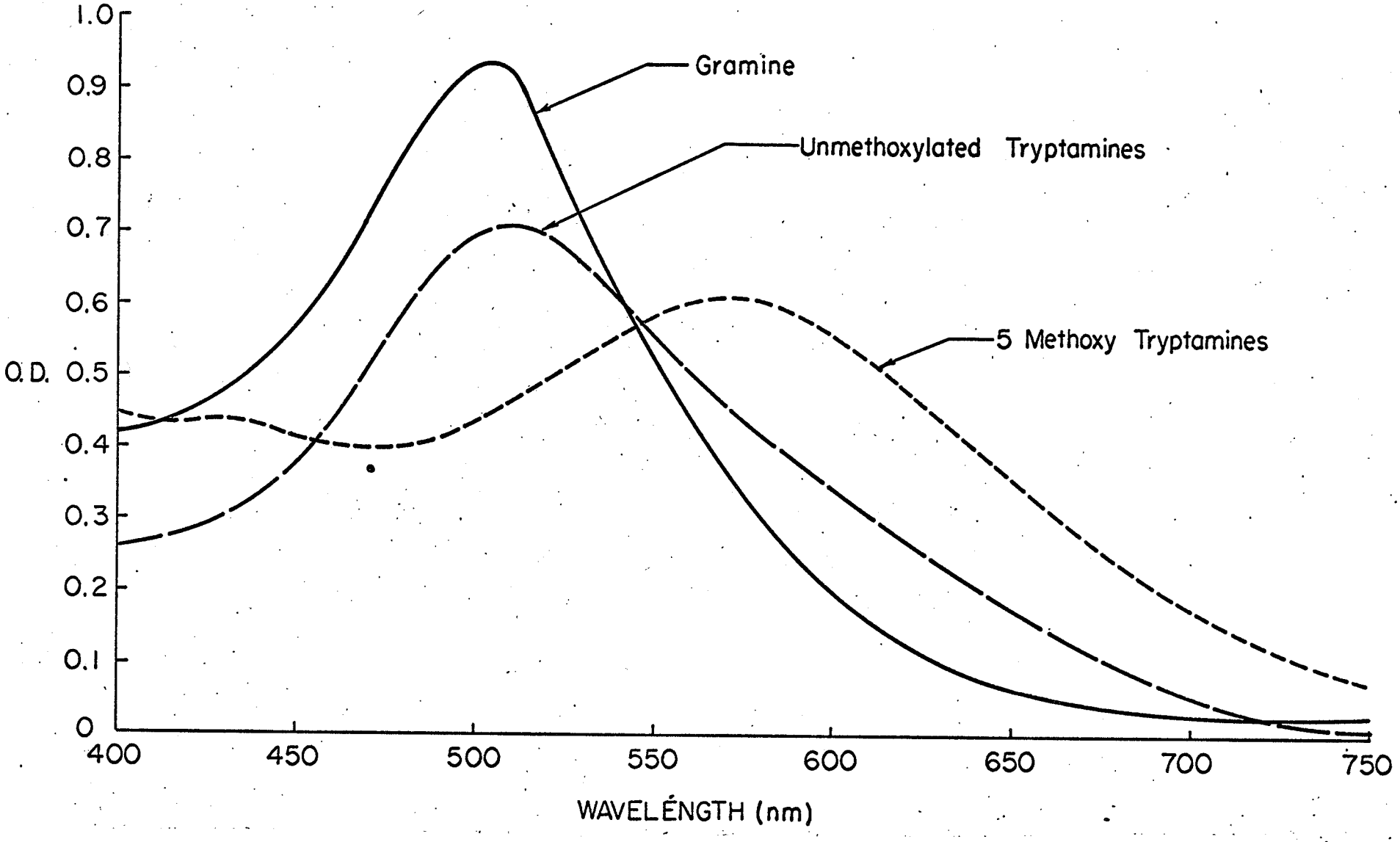

Figure 3: Absorption curves of the xanthydrol reaction with reference materials

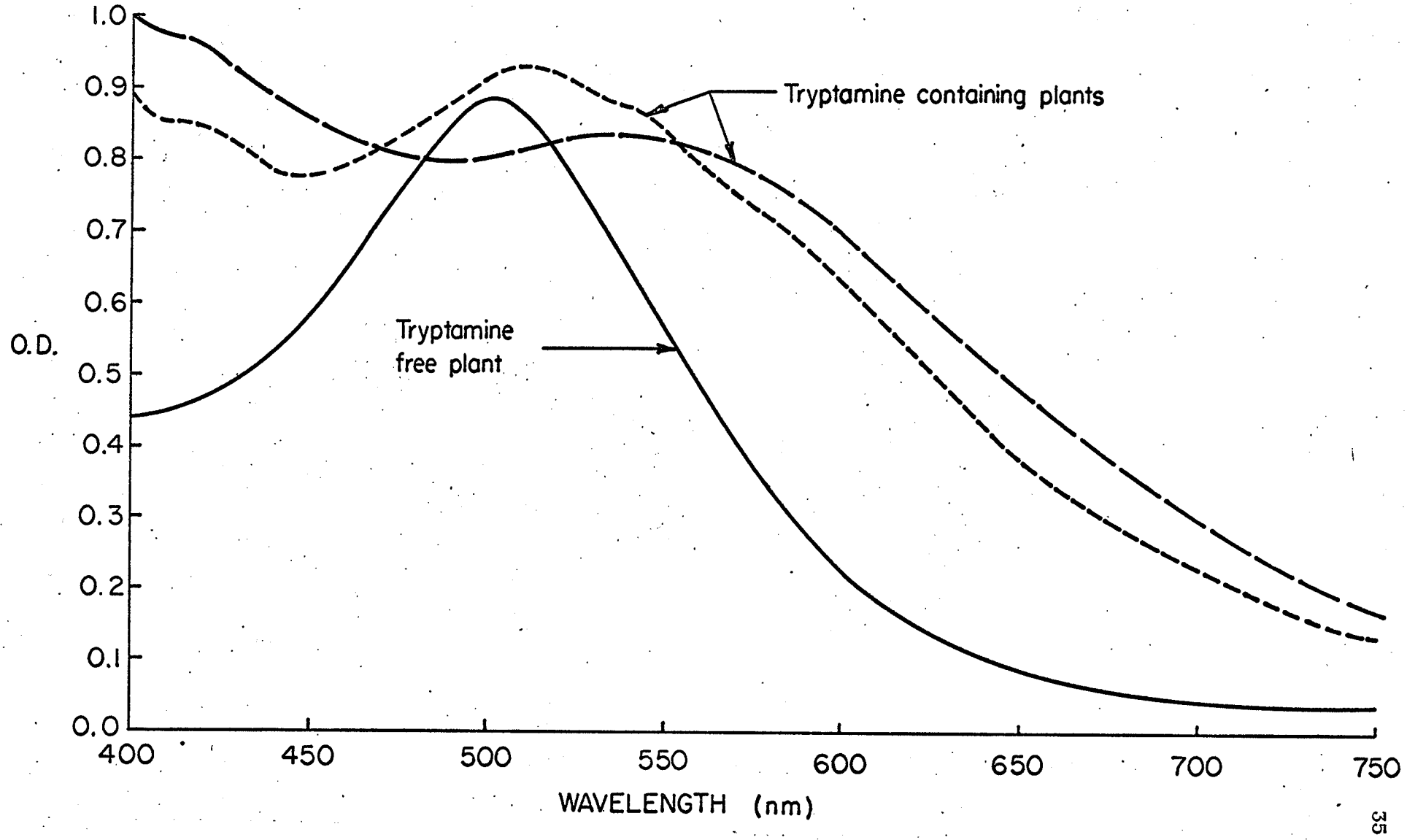

Figure 4: Absorption curves of the xanthydrol reaction with crude base extracts

tetrahydro-β- carboline gave evidence of the occurrence of these compounds. The relative quantities of each compound could also be distinguished (Fig. 5).

Quantitative determination of alkaloids by thin layer densitometry

Quantities of crude base extract and reference amounts of tryptamine were chromatographed on silica gel thin layer plates in the methanol/strong ammonia solution solvent, and after spraying with Ehrlich's reagent and allowing colour development, to measure the coloured spots produced using a densitometer. The instrument[a] was modified to carry 20 x 20 cm glass plates, and control analyses were performed to determine the optimum method of use. As the spots produced on the plates were blue, a red filter was used for all readings. A slit of 15 mm x 1 mm was used with its long axis across the path of plate travel. The instrument has a number of response modes, numbered 1 to 12; the manual indicates that response mode 1 is a reading of transmittance, mode 5 measures optical density, and that other values are "quasilogarithmic" the log function increasing with the number. To determine which response mode to use 2 thin layer plates each having various quantities of tryptamines on them were scanned using 4 of the modes. The areas of the curves produced by the densitometer were measured using a planimeter[b], and an analysis performed

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[a] Densicord Recording electrophoresis densitometer model 542 Photovolt Corp. 1115 Broadway NY 10 NY USA

[b] Aristo planimeter Hughes Owens Germany

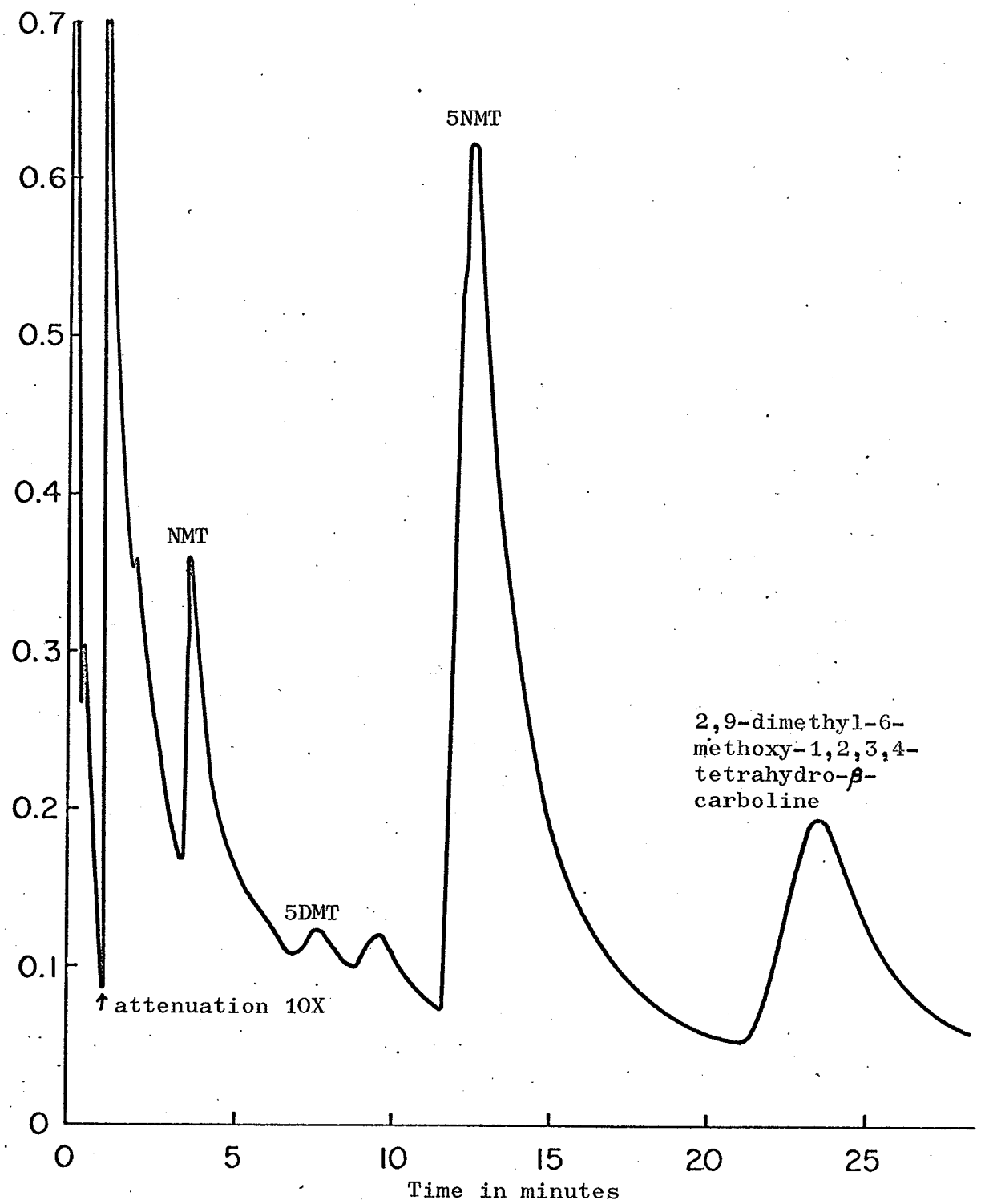


FIGURE 5: G. L. C. OF CRUDE BASES FROM REED CANARYGRASS

Beckman model GC-5 gas chromatograph
Column: 1% diethyleneglycol succinate on Chromosorb W AW-DMCS
Carrier gas: helium Flow rate: 100 ml/minute
Oven temperature: 180°C Inlet temperature: 205°C
Detector temperature: 260°C

comparing the correlation between quantity of reference tryptamine and area of output trace, using each of the 4 instrument modes, and 5 commonly used transformations between quantity and area[83]. The area obtained from one plate using different instrument modes varied from 11 planimeter units for 1 µg of tryptamine to 60 planimeter units for 5 µg of tryptamine. The planimeter was set at 16 units per square cm. The values of r were calculated for each plate and then averaged via a Z transformation to avoid plate to plate differences (Table 1). In comparing the average values from different response modes and different transformations each value of r was treated as arising from 18 observations (Table 1). The average value of r from response mode 5 was significantly larger than that from modes 1 or 12 ($p = 0.05$). The average value of r from transformation 4 was significantly greater than those from transformations 1, 2 or 3. The highest value of r in modes 5 or 9, transformations 4 or 5 was mode 5 transformation 4, and this was used in all later analysis.

Tryptamine was applied at 3 µg per spot and 9 spots per plate, to 6 plates. After running and colour development the densitometry readings at response mode 5 were analysed to evaluate the precision of the method. Analysis of results revealed significant differences between plates, and a coefficient of variation (standard deviation/mean) of 11.5% (Table 2).

The length of time for colour stability was also examined, and 24 hours selected as a suitable time for colour development.

TABLE 1: MEAN VALUES OF THE CORRELATION COEFFICIENT r USING FOUR INSTRUMENT MODES AND FIVE DATA TRANSFORMATIONS

Transformation	Instrument response mode 1	5	9	12	Average
1. Q vs R	0.926	0.977	0.968	0.962	0.962
2. Q vs $\sqrt{R}$	0.912	0.973	0.956	0.964	0.956
3. $\log_{10}Q$ vs R	0.955	0.973	0.970	0.925	0.959
4. $\log_{10}Q$ vs $\sqrt{R}$	0.961	0.982	0.980	0.997	0.978
5. $\sqrt{Q}$ vs R	0.948	0.980	0.963	0.979	0.967
Average	0.945	0.977	0.968	0.964	

Q = quantity

R = planimeter reading

Each individual value considered as deriving from 18 observations.

TABLE 2: ANALYSIS OF VARIANCE TO DETERMINE PRECISION OF DENSITOMETRIC METHOD

Source	df	Sum of squares	Mean square	F
Total	53	4850		
Plates	5	4472	894.40	114**
Error	48	378	7.88	

**Significantly different at the 1% level

Plate means in planimeter units

35.9	21.6	19.1	38.3	17.5	15.0

Overall mean 24.5

Standard deviation 2.8

Coefficient of variation 11.5%

By 72 hours colour had faded to give $\frac{2}{3}$ to $\frac{1}{2}$ the planimeter reading obtained at 24 hours.

For consistency the instrument zero was always set immediately below the spot to be measured and the plate was scanned in the direction of origin to solvent front. For uniformity in colour development the plates were sprayed until appearing uniformly moist, and then allowed to stand for 24 hours in a closed cupboard before scanning.

When used as an assay quantities of unknown and reference samples of tryptamine were run on the same plate. Both the areas were measured and a regression line for $\log_{10}$quantity against square root of the area determined from the tryptamine standards. From this line the quantity of unknown in each spot of sample was found, and after conversion to μg/g of grass, the average quantity determined.

Colorimetric determination of alkaloids by the Xanthydrol method

Samples of alkaloids from a screening procedure were available, derived from variable quantities of grass, about 5 - 10 g fresh weight. These had been previously examined by thin layer chromatography and results were known. The preliminary trial was based on a previously developed method[15,39,*]. To each flask was added 10 ml of 2N hydrochloric acid. A 2 ml aliquot was taken and 2 ml of 0.03% Xanthydrol in glacial acetic acid was added. The samples were heated in boiling water for 20 minutes. The samples known to contain the

*Dr. A. B. Simons, personal communication

tryptamines developed a dark blue colour, while those samples containing gramine turned pink. After cooling 2 ml of 5% sodium bisulphite was added, which caused the blank to lose its yellow colour but appeared to have no effect on the other samples. This colorimetric process was considerably quicker than thin layer chromatographic examination and it was considered useful to improve it so that gramine could be measured quantitatively. The major risk in the qualitative assay is that plants containing no 5-methoxy tryptamines, but containing the other unmethoxylated tryptamines would give a purple colour which could be confused with the pink colour produced by gramine.

This was tested by the same technique as used in the preliminary work, using reference alkaloids, and it was found that the purple colour produced by the unmethoxylated tryptamines could be distinguished from the pink colour produced by gramine.

A colorimetric method for measurement of gramine was developed based on other work[15,59,*] with modifications. The major question was what combination of acid strength and time to use.

Seven acid concentrations and six heating times were considered. Reaction tubes were 150 x 20 mm. In each tube was placed: 0.5 ml gramine solution (125 μg/ml in N/10 hydrochloric acid), 5.0 ml hydrochloric acid of suitable strength and 0.5 ml Xanthydrol reagent (1.25 mg/ml in 95% ethanol). After mixing the tubes were heated in a boiling water bath for varying periods of time, at the end of which they were cooled

*Dr. A. B. Simons, personal communication

under running water, and 0.5 ml of sodium metabisulphite solution added. At this stage the solutions were observed to be slightly cloudy, the ones having the shorter heating time being the most cloudy. The addition of 2.5 ml of 95% ethanol removed the cloudiness, and was therefore added to all tubes (less was actually necessary in the tubes receiving the longer heating). In the stronger acid with moderate or low heating times the tubes were yellowish. The sodium metabisulphite removed this in the tubes having a moderate heating period. The ethanol did so in the remainder. The optical density was measured in a 10 mm cuvette at 500 mμ. The results are expressed in table 3.

An acid strength of 80% concentrated acid and 16 minutes heating time were selected as usable conditions for the assay, mainly because the stronger acid needed more ethanol, and hence more dilution, to clear the yellow color, and also the dilution of acid strength by traces of water in the prepared samples of crude bases would be less in the weaker acid. Under the conditions chosen only 1 ml of ethanol was needed to prevent any troublesome precipitation.

Having established a suitable method for extraction and for analysis[102] two control experiments were performed. The effect that differences in heating time could have on the resultant optical density was investigated, as exact heating is difficult to control.

Five standard curves were prepared using the same reference solution of gramine, differing in times of heating. The

TABLE 3: OPTICAL DENSITIES OF GRAMINE SOLUTION AFTER VARIOUS REACTION CONDITIONS

Blank was distilled water.

Concentrated hydrochloric acid titrated as 11.6N

Time of heating in minutes	Strength of acid added, as % of concentrated hydrochloric acid						
	40	50	60	70	80	90	100
4	.025	.070	.100	.120	.100	.090*	.120*
8	.030	.060	.085	.100	.115	.130	.130*
12	.025	.045	.085	.080	.115	.125	.150*
16	.035	.040	.085	.095	.100	.150	.120
20	.045	.035	.060	.055	.110	.080	.130
24	.030	.050	.060	.065	.090	.105	.110

*These samples were yellow until the ethanol was added

slope of the line log 1000 optical density against log µg varied from 0.65 to 0.76. There were significant differences between lines but the error introduced would be relatively small. For example an optical density of 0.5 would give values of 360 (14 minutes), 400 (16 minutes) and 340 µg (18 minutes) (Fig. 6).

The overall extraction efficiency of the method was also investigated. Aliquots of a reference solution of gramine were carried through a standard quantitative extraction procedure and assayed colorimetrically. A standard curve from the same reference solution was produced at the same time. The proportion of the gramine detected after quantitative extraction decreased as the amount of gramine in the sample being extracted increased. For this reason the line between optical density of extracted sample and µg gramine was preferred as a standard curve, as this avoided the need for complex corrections in later calculations (Table 4 and Fig. 7)

FIGURE 6: EFFECT OF HEATING TIME (MINUTES) ON REGRESSION LINES FOR GRAMINE

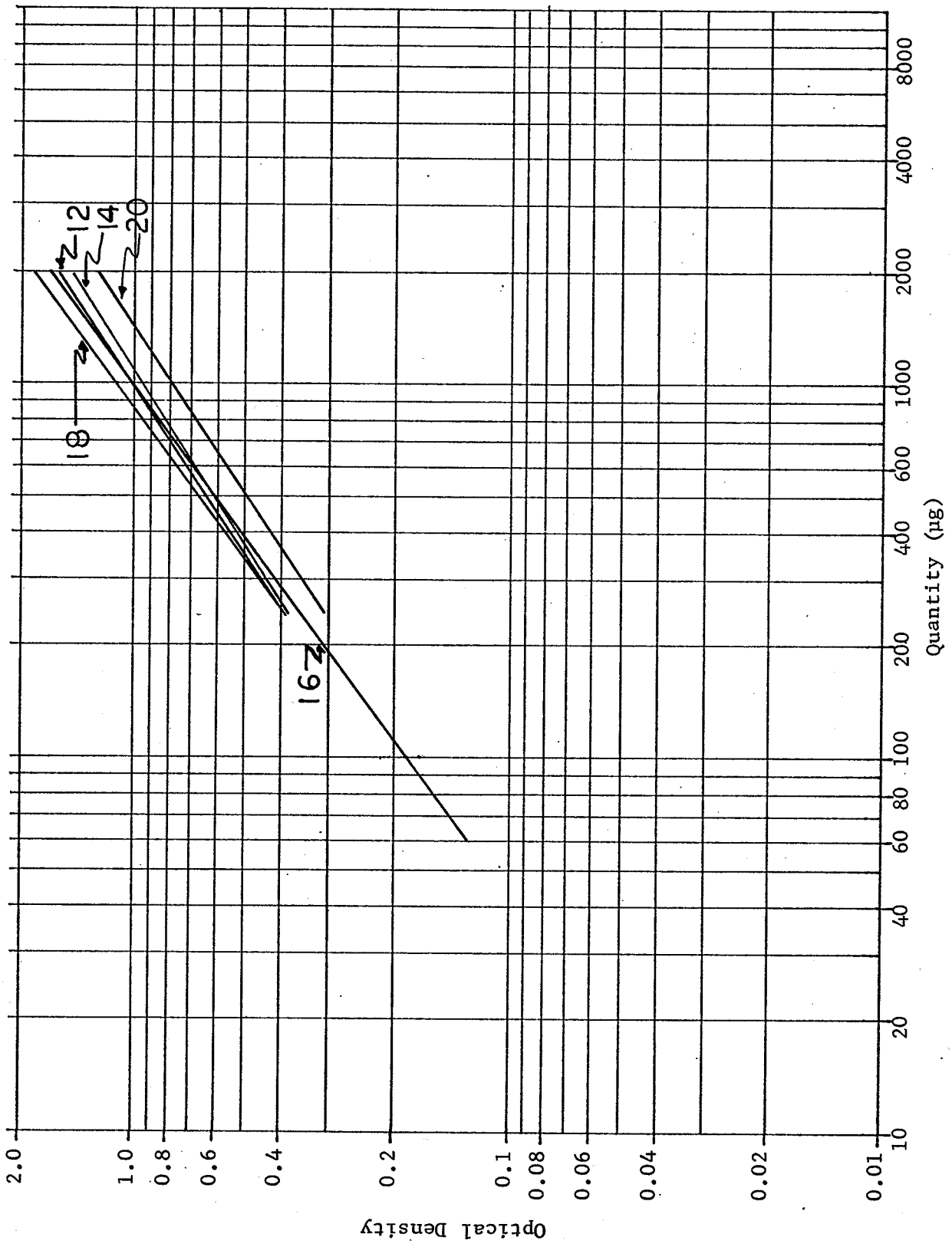
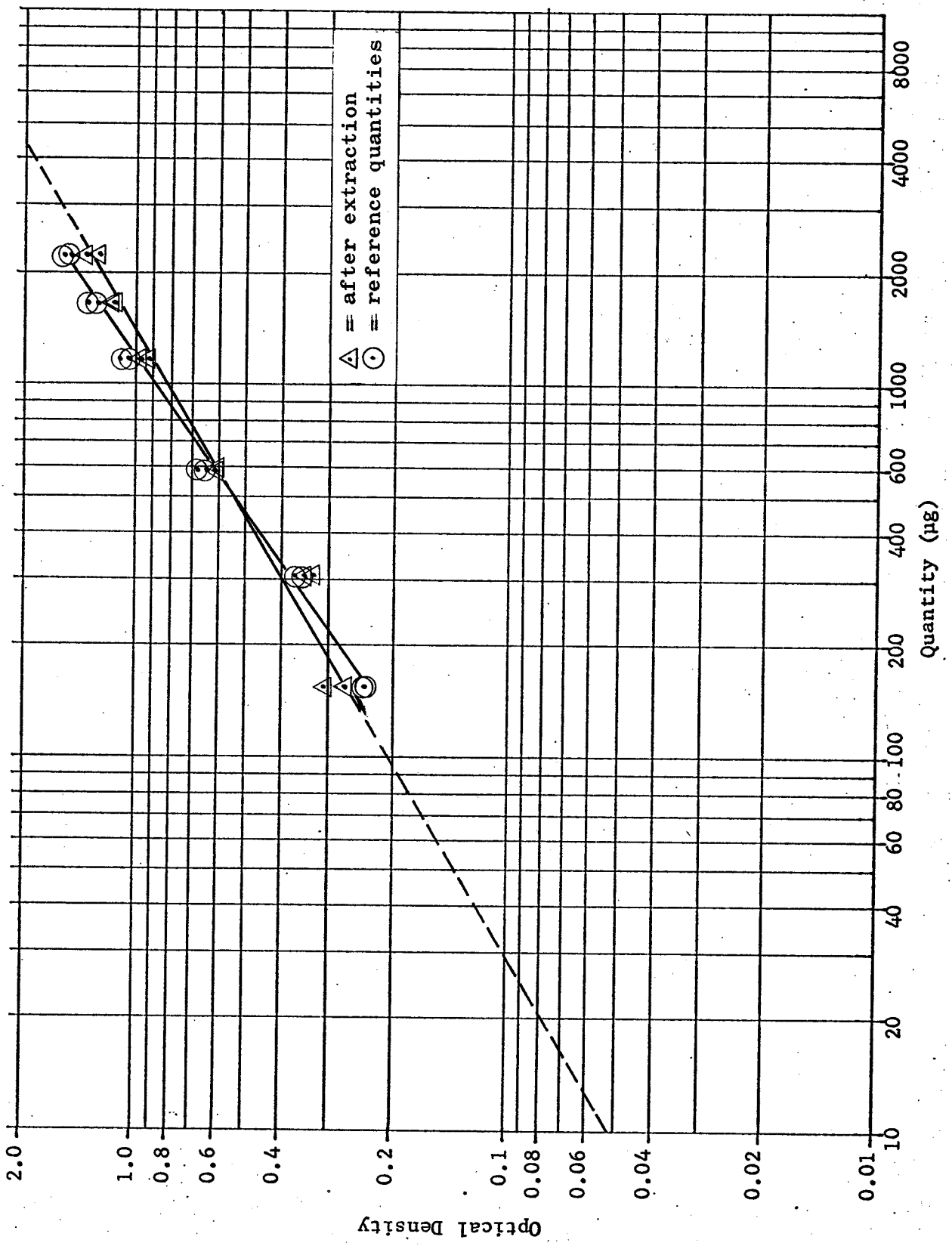


TABLE 4: EXTRACTION EFFICIENCY FOR GRAMINE, FROM O.D. MEASUREMENTS

µg gramine	OD reference	OD after extraction procedure	apparent amount extracted (µg)*	apparent % extracted*
2240	1.52	1.21	1520	68
	1.55	1.37	1825	81
1690	1.32	1.18	1480	84
	1.34	1.15	1420	88
1174	1.13	0.98	1150	98
	1.08	0.96	1100	94
592	0.65	0.60	560	95
	0.68	0.61	580	98
304	0.36	0.35	264	87
	0.38	0.36	275	90
156	0.25	0.27	185	119
	0.25	0.31	222	142

*Calculated from reference sample regression line
log 1000 x OD = 0.7070log µg + 0.8302

FIGURE 7: REGRESSION LINES OF O. D. AGAINST µg GRAMINE



MATERIALS AND METHODS

(B) BIOLOGICAL

Cytological examination of Phalaris arundinacea

Open pollinated seeds were used; these seeds were germinated in a cold room at 0°C for 2 weeks (January 9 - January 23, 1970) and then incubated at room temperature for 5 days. The root tips were removed and stored in ice cold water for 1 day and then fixed for 3 days in a mixture of 95% ethanol/glacial acetic acid 3:1. After fixing, the roots were hydrolysed in 1N hydrochloric at 60°C (4 - 5 minutes found suitable) and placed in Feulgen's stain until suitably stained. The tips were mounted in 1% acetocarmine and examined microscopically.

Five root tips were examined and all appeared to contain 28 chromosomes, supporting the assumption that the variety grown here is tetraploid.

Crossing and selfing procedures

Emasculation did not seem to be a feasable proposition in this species, if a suitable number of progeny were to be produced. In any case the species is reported moderately self sterile[84]. Selfing was performed by covering the heads with waxed paper bags (19.5 x 4.0 cm) from the time of about $\frac{1}{3}$ emergence. Under these conditions many of the heads were broken off by the wind, and quite a few of the heads managed to work their way out of the bags. Of the remainder many failed to set any seed at all and the highest number of progeny obtained from one head was 20. Crossing was performed by covering the heads with larger bags (19.5 x 7.5 cm) and having a head of the pollen parent within the bag. The

bag was stapled to a stake to reduce breakages, and this made it necessary to move the bag up the stake daily due to the very large rate of stem elongation. It was attempted to keep the cut stem of the pollen parent in a vial of water, but because of the elongation of the seed parent stalk this proved impractical. A few heads were lost due to breakage and due to the bags coming off but the method was far more successful than the use of unsupported bags.

Progeny management

Very erratic germination occurred if the seeds were sown straight after harvest. For this reason the seed heads were stored at 4 ^{o}C for 5 weeks before planting out in sterilised soil. The heads were broken up, and everything was planted in the trays to reduce loss of seeds during winnowing. By 12 weeks the seedlings were ready to harvest, and were especially large where only low numbers of seeds germinated and the seedlings had plenty of space for growth.

Sheep grazing experiment

Two fields of selected clones were planted by hand over a period of time around the end of May 1970. Rainfall was frequent at this time, and the sprigs became established readily. Four replicate areas of each clone were planted, each area being 5 feet square and containing 25 sprigs spaced in 1 foot centres (Figures 8 and 9). There were 30 different clones used in each field, and the overall arrangement was such that the experiment could be used as four separate blocks,

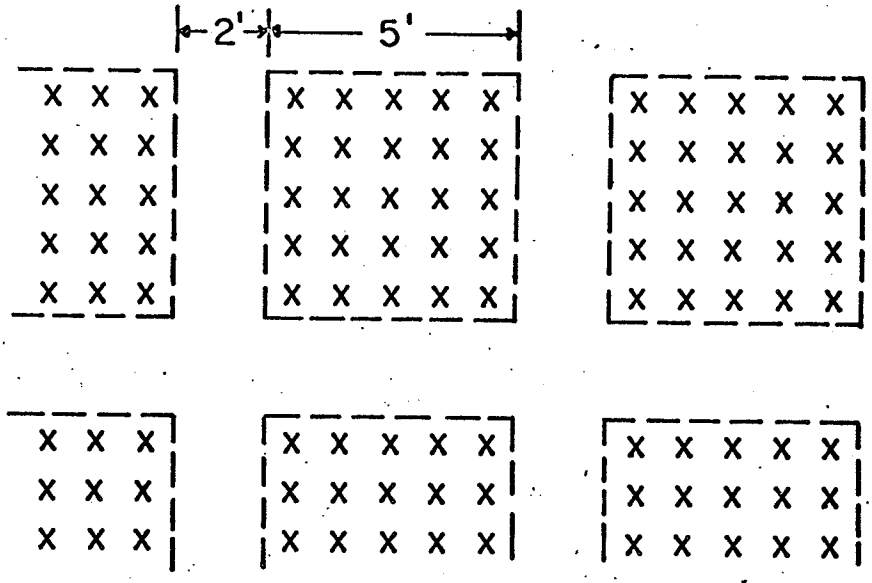


FIGURE 8: INDIVIDUAL PLANT AREA LAYOUT

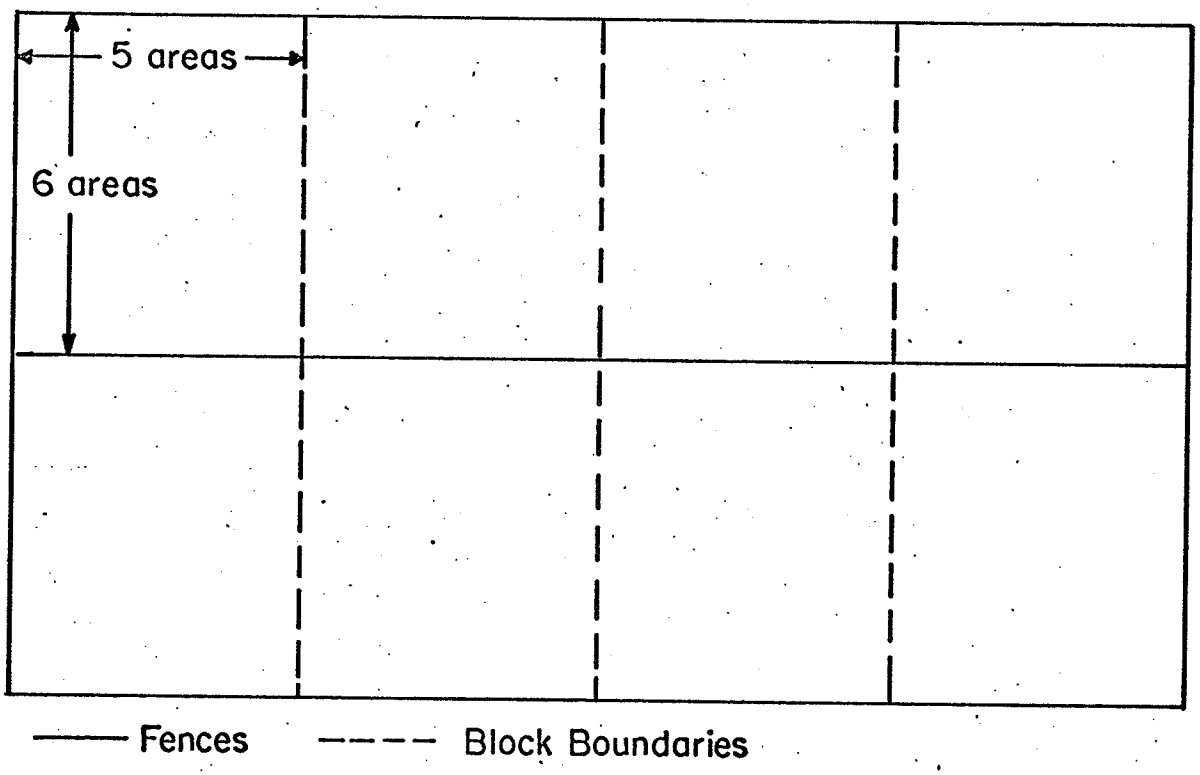


FIGURE 9: FIELD PLAN

but this was not done in the first year of growth as the amount of forage produced was not sufficient. After the first period of grazing the remaining grass was cut down. The field was fertilized at the rate of 120 lb NH_4NO_3 per acre and then irrigated.

Water bins for the sheep were at one end of the field, and were not moved during the trial period.

Forage consumption was estimated on a scale of 0 to 5 every 3 days for the first grazing trial and every 2 days for the second trial. A rating of 0 was given to plants hardly consumed, and 5 to those plants very much consumed. For analysis the data were transformed to $\sqrt{x + \frac{1}{2}}$ where x = value given, and each field analysed as a factorial experiment of randomised complete block design (30 plants x 7 dates or 30 plants x 6 dates for first growth and regrowth data respectively).

Digestibility

In vitro dry matter digestibility was determined by first digesting grass samples with buffered rumen fluid for 48 hours and then digesting with pepsin/hydrochloric acid for 24 hours. The material not passing through a filter pad was dried and considered as non-digested matter[90].

In vivo dry matter digestibility was determined by measuring feed dry matter intake and faeces dry weight over a period of 7 days. The forage was very coarse at the time of this trial, and was chopped with a corn chopper to reduce the amount of selection possible by the sheep. Faeces were

collected twice daily, and the sheep fed (3 lb fresh forage/ animal) and weigh backs collected daily. Crude dry matter digestibility was calculated as:

$$\frac{\text{feed - weighback - faeces}}{\text{feed - weighback}}$$

All values being on a dry matter per day basis.

The preliminary feeding period for this experiment was conducted in outside pens for 10 days. As digestibility was determined using individual indoor stalls, this did not represent a true preliminary feeding period.

RESULTS

The majority of important results are included in publication form at the end of this thesis, as are methods finally used. Some other results were not considered suitable for publication or were published in abbreviated form, and these results are included here.

Densicord results

By densitometry the final extract of crude bases was found to have quantities of N-methyltryptamines varying from 0.72 to 6.57 µg/ml expressed as tryptamine hydrochloride. Allowing for a 37% extraction efficiency and considering the extraction quantities used, the average value of 2.25 µg/ml represents 300 µg/g dry matter.

Results from initial screening of Ottawa Synthetic F

Ottawa synthetic F has now been licensed as the variety "Grove".* The material examined and reported on consisted of the S_0 generation, and the female parent of each clone was known. Only four parental clones were used in the derivation of this new variety, and it is of interest to theorise about the nature of these plants. Direct examination is not possible as the original plants were accidently destroyed.* From a consideration of the parental material some prediction may be made on the behavior of later generations of the variety. The data from which these predictions were made are presented in Table 5, all clones previously designated as "trace"[101] are now classified as tryptamine containing.

*Dr. R. M. MacVicar, personal communication

Two assumptions were made for the purpose of this analysis. These are:

1. That there is, in Phalaris arundinacea, a single dominant gene for tryptamines.
2. That under field conditions the amount of selfing is negligible, but outcrossing to the other lines is random.

As none of the lines of progeny were all T+, none of the parental lines could have been homozygous dominant for tryptamines. Thus crosses between heterozygous or homozygous recessive plants are the only possibility. It is also obviously impossible for all four parents to have been homozygous recessive. In order to be able to evaluate the ratio of T+ to T- plants using a χ^2 analysis the numbers of plants from each parent were adjusted to the lowest total, and summed (Table 6).

The expected ratios for parents ranging from all four heterozygous, to three homozygous recessive and one heterozygous were calculated, based on assumptions 1 and 2. From the predicted numbers and the adjusted total (Table 6) a χ^2 value was found (Table 7).

The best fit was the case with two heterozygous and two homozygous recessive parents. This information may be used to estimate the composition of this variety in generations subsequent to the S_o. This was found to be one homozygous dominant: six heterozygous:nine homozygous recessive, if the Hardy-Weinberg[2] law applies for this character.

TABLE 5: DISTRIBUTION OF TRYPTAMINE CONTAINING PLANTS IN OTTAWA SYNTHETIC F, SECREGATED INTO GROUPS ACCORDING TO THE FEMALE PARENT

All plants previously designated as "trace" are recorded as positive.

Parent (Ottawa nos.)	T+	T-	Total
173	20	26	46
193	20	26	46
237	20	24	44
291	22	18	40
Total	82	94	176

TABLE 6: ADJUSTED DISTRIBUTION OF TRYPTAMINE CONTAINING PLANTS IN OTTAWA SYNTHETIC F

Parent (Ottawa nos.)	T+	T-	Total
173	17.39	22.61	40.00
193	17.39	22.61	40.00
237	18.18	21.82	40.00
291	22.00	18.00	40.00
Total	74.96	85.04	160.00

TABLE 7 : COMPARISON OF PREDICTED RATIO WITH THAT OBSERVED IN OTTAWA SYNTHETIC F

Parents	Predicted ratio (+:-)	Predicted numbers out of 160 (+:-)	χ^2 1df[a]
3(+-)x1(--)	5:3	100 : 60	16.72**
2(+-)x2(--)	11:13	73.33: 86.66	0.0669
1(+-)x3(--)	1:3	40 :120	40.74**

[a]Observed values 74.96:85.04
**Significantly different at the 1% level

TABLE 8 : ANALYSIS FOR FIT TO PREDICTED RATIOS OF PROGENY NUMBERS FROM EACH PARENT OF OTTAWA SYNTHETIC F

Parent (Ottawa nos.)	χ^2 values 7:5 ratio	1:2 ratio	Sample size
173	4.172*	2.134	46
193	4.172*	2.134	46
237	3.006	2.905	44
291	0.1819	8.458**	40

*Significantly different at the 5% level
**Significantly different at the 1% level

It was also possible to decide the nature of each parent. For example a heterozygous plant crossed to two homozygous recessive and one heterozygous, assuming no selfing, would give a positive:negative ratio of 7:5, whereas a homozygous recessive plant crossed to two heterozygous and one homozygous recessive would give a 1:2 ratio. For this purpose a χ^2 analysis was performed on the data from the progenies of each parent (Table 8). From this data it was assumed that Ottawa clones 173 and 193 were homozygous recessive, 237 and 291 were heterozygous. This information is of interest in the search for a homozygous dominant plant, which can only occur in the progeny of one of the heterozygous parents.

Results from initial screening of Ottawa Synthetic C

In this case the female parents of individual clones were not known. All that was known was that the plants were derived from four parental clones. A theoretical evaluation was of interest, as these were the plants which were used for determination of the genetic control of tryptamines.

The data from which predictions were made are presented in table 9. The predicted ratios from various possible parental combinations which produced the lowest χ^2 values are also presented therein.

From table 9 it appears that the most likely parents of the plants designated Ottawa synthetic C were three heterozygous and one homozygous recessive. These would be expected to produce progeny in the ratio one homozygous

TABLE 9: COMPARISON OF PREDICTED RATIOS WITH THOSE FOUND IN OTTAWA SYNTHETIC C

Parents	Predicted ratio (+:-)	Predicted numbers out of 177 (+:-)	χ^2_{1df} [a]
1(++)x3(--)	1:1	88.50:88.50	4.75*
2(++)x2(--)	5:1	147.50:29.50	80.55**
4(+-)	3:1	132.75:44.25	26.67**
3(+-)x1(--)	5:3	110.63:66.37	1.403
2(+-)x2(--)	11:13	81.12:95.88	10.89**

[a] Observed values 103:74
*Significantly different at the 5% level
**Significantly different at the 1% level

dominant:four heterozygous:three homozygous recessive in the S_o generation, and nine homozygous dominant:thirty heterozygous:twenty-five homozygous recessive in subsequent generations.

Genetic control experiment

The majority of results from crosses involving at least one tryptamine positive parent are presented in publication form[102]. The remainder are presented in tables 10 and 11.

Individual results from seasonal variation examinations

The average results from a number of clones are presented in publication form[102]. Individual results from the gramine determination are presented graphically (Figures 10 and 11). Individual results from the tryptamine determination are not presented as the method used was an estimate of quantity present, and the inaccuracies of this method are too great for any real value to be obtained from individual results.

Determination of gramine on a small scale

A number of plants were grown from seed and analysed for gramine content using a small scale version of the method described[102]. The plants were examined twice, at 3 months and at 11 months, after regrowth, using the upper three leaves on two stems, averaging about 2 - 3 g fresh weight. The mean and standard deviation for the first analysis (56 plants) was 231 and 152.5, and for the second analysis 331 and 154.4, all values in µg/g fresh weight. The overall correlation coefficient between the first and second observations was

TABLE 10: CLASSIFICATION OF PROGENY FROM VARIOUS SELFED PLANTS

Parent numbers	Parent type	Progeny numbers	
		+	-
2	-	0	13
7	-	0	10
14	-	0	8
32	-	0	11
40	-	0	20
42	-	0	8
44	-	1	5
47	-	0	7
49	-	0	13
82	-	1	4
23	+	3	2
25	+	6	2
54	+	1	4
141	+	5	2
254	+	3	1
342	+	3	1
383	+	4	5
371	+	16	0

+ refers to tryptamine alkaloid containing plants
- refers to tryptamine alkaloid free plants

TABLE 11: CLASSIFICATION OF PROGENY FROM VARIOUS CROSSED PLANTS

Parent numbers	Parent type	Progeny numbers	
		+	-
25 x 26	+ x -	29	11*
25 x 40	+ x -	25	15
18 x 19	- x -	0	40
18 x 34	- x -	0	40
18 x 35	- x -	0	40
19 x 34	- x -	0	40
34 x 18	- x -	0	7
34 x 19	- x -	0	10
34 x 35	- x -	0	13
35 x 18	- x -	1	16
35 x 19	- x -	0	13
35 x 34	- x -	0	40
35 x 36	- x -	1	25
36 x 19	- x -	0	40
50 x 34	- x -	0	19
67 x 50	- x -	0	7
68 x 67	- x -	0	16
68 x 84	- x -	0	14
82 x 67	- x -	0	40
84 x 67	- x -	0	40

*Significantly different from 1:1 ratio (χ^2 5% 1df)
+ refers to tryptamine alkaloid containing plants
- refers to tryptamine alkaloid free plants

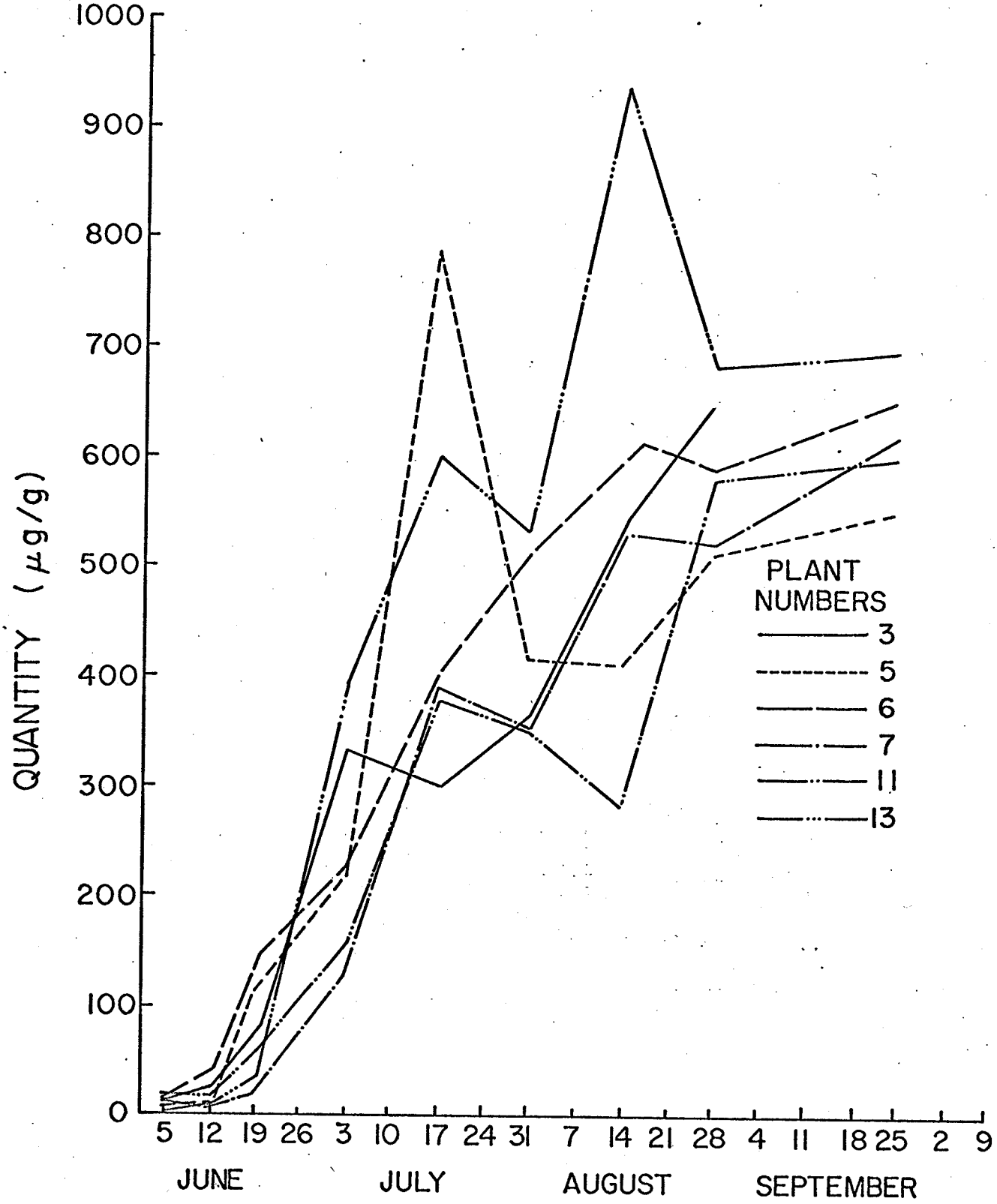


FIGURE 10: SEASONAL VARIATION OF GRAMINE IN INDIVIDUAL PLANTS UNDER A REGULAR CLIPPING MANAGEMENT

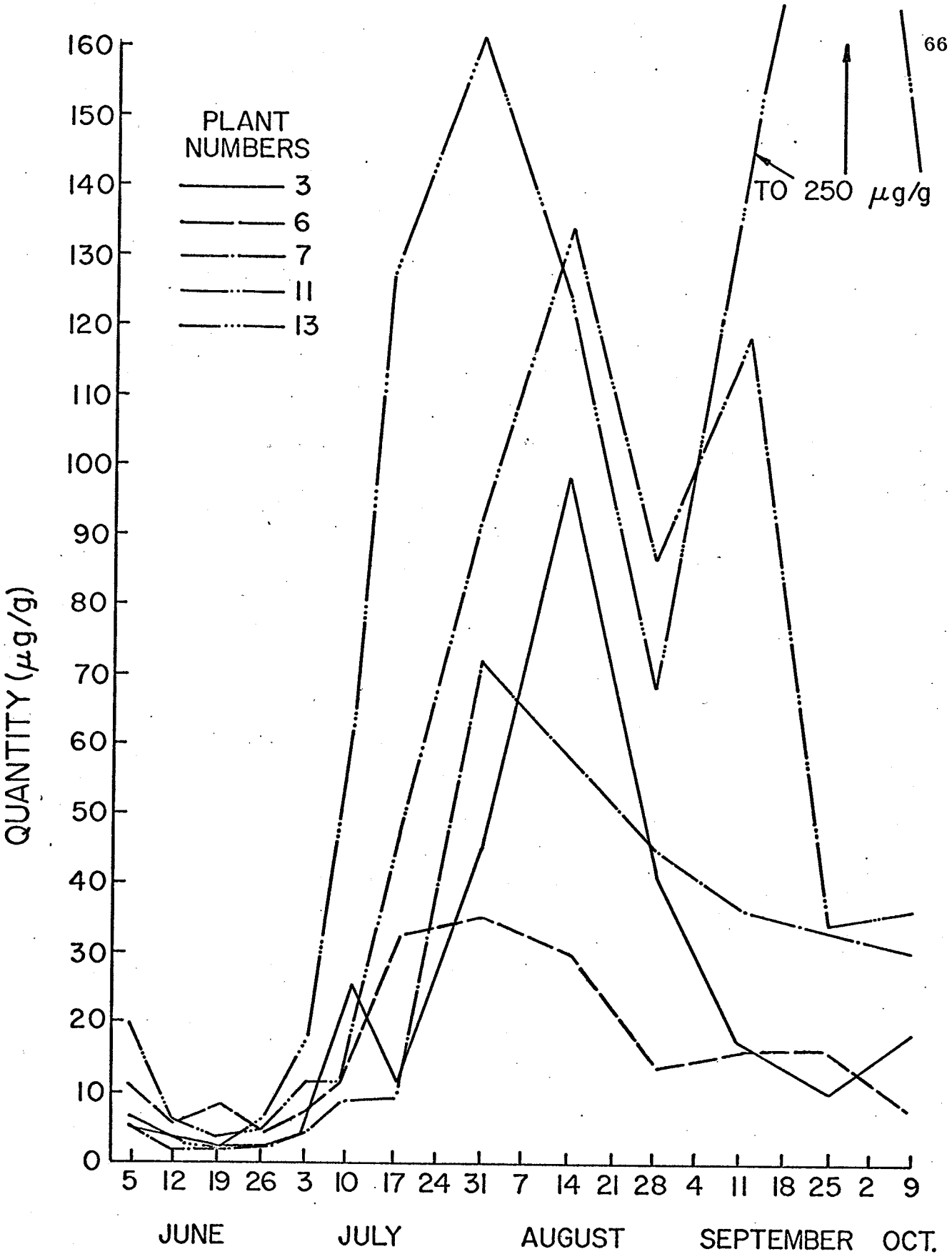


FIGURE 11: SEASONAL VARIATION OF GRAMINE IN INDIVIDUAL PLANTS UNDER FREE GROWTH MANAGEMENT

0.174, which was not significant at the 5% level.

Analysis of reed canary grass samples from other sources

Apart from the clones of Ottawa synthetics C and F which were examined for tryptamine alkaloids, some selections from other sources were also examined. The results are presented in table 12.

The plants from Beaverlodge were clones from world-wide sources*. No general agreement between source and tryptamine content was obvious. The history of the Purdue clones was not known, and the Saskatoon clones were supplied as seed.

Reliability of the new[102] classification method

A duplicate analysis was performed on 263 plants which had previously been classified as tryptamine alkaloid free (Table 12). Only one of these was found to contain tryptamines on the second analysis.

Sheep grazing

Two results of interest, which were only observations and not measurements, were obtained during the sheep grazing trials. During the first grazing period the sheep on the tryptamine alkaloid containing pasture were quite severely purged. It at first seemed possible that this could be the result of parasites, but examination of fecal samples revealed that only two of the animals had detectable parasites, and these were at such a low level as to be considered unimportant by a veterinarian. The second observation was made during the period between first and second grazing, when it seemed that

*Dr. S. G. Bonin, personal communication

TABLE 12: COMPOSITION OF SELECTIONS

OF P. ARUNDINACEA FROM VARIOUS SOURCES

Source	Tryptamine containing		Tryptamine free	
	number	%	number	%
Beaverlodge (Alta)	7	9%	68	91%
Lafayette (Ind)	11	69%	5	31%
Saskatoon (Sask) seed retaining line	58	18%	267	82%
Saskatoon (Sask) yellow seeded line	7	64%	4	36%
Ottawa (Ont) synthetic C	103	58%	74	42%
Ottawa (Ont) synthetic F	82	47%	94	53%

the sheep on the tryptamine alkaloid containing forage were consuming considerably more water than the other group of sheep.

Apparently more pasture was consumed of the tryptamine alkaloid containing field than of the tryptamine alkaloid free field. This observation however may be erroneous as there were less plants available in the former field.

In vitro dry matter digestibility

The plants examined were about 6 weeks old, and were greenhouse regrowths. All plants were subsequently used in the palatability experiment. The in vitro analysis was replicated, (3 replicates), but there was only one plant of each type. There were significant differences between plants within each group, the groups being tryptamine alkaloid containing and tryptamine alkaloid free. The tryptamine alkaloid containing group (14 plants) averaged 62.29% digestibility which was not significantly higher than the average of 61.50% from the tryptamine alkaloid free group (8 plants).

Within clone variation of gramine

Individual shoots of one clone were cut (20 shoots June 2) and assayed for gramine content using a small scale version of the published quantitative method[102]. A mean value of 179 μg gramine/g of fresh grass was obtained with a mean shoot weight of 1.28 g and a standard deviation of 209 μg/g. Values ranged from a minimum of 34 μg/g to a maximum of 1642 μg/g.

Where possible in the routine screening procedure 200 g of grass was used. This could be considered as 200/1.28

(= 156) shoots, the standard deviation of the mean would thus be $209/\sqrt{156} = 16.8\ \mu g/g$. Thus the coefficient of variation at the commencement of the seasonal variation determination was estimated as about 10%.

Other compounds in tryptamine free plants

Regular cutting produced very high levels of gramine in the late season. At this time the quantities of tryptamines in the tryptamine containing plants treated similarly were also very high[102]. To examine for any trace of tryptamines samples of crude base from regularly clipped grass (cut Sept. 25) were examined by thin layer chromatography (silica gel, methanol/strong ammonia solution system). Apart from an extremely large gramine spot, spots were also observed in the N-methyl and N,N-dimethyltryptamine regions. These did not however give the characteristic colours of either the 5-methoxytryptamines or their unmethoxylated counterparts when treated with the xanthydrol spray, but gave colours more typical of gramine. Traces of compounds were also detected in the β-carboline region, whose colour reactions were similar to those of the reported β-carbolines. With Ehrlich's reagent the substances in the tryptamine regions barely reacted.

DISCUSSION

Thin layer chromatography

Although the silica gel plates with the methanol/concentrated ammonia solution running solvent were reasonably satisfactory, the same cannot be said for the cellulose system, as this was time consuming, and some streaking still occurred. There are several other potential means of improving the systems. The use of an organic amine in the running solvent for the silica gel layers may overcome the problem of ammonia volatility. A combination of cellulose powder with silica gel was moderately successful for total separation of the compounds of interest. Possibly this could be improved upon by using ion exchange cellulose, and thus obtaining with one adsorbent both types of separation which have been found to occur. A further alternative would be to use kieselguhr instead of cellulose as the adsorbent in the partition chromatography step[86]. There is also the possibility of improvement of the detecting spray, but considering the very high sensitivity and the clear background produced using Ehrlich's reagent, this does not seem to be a particularly good prospect. Ninhydrin glacial acetic acid[85] may be a suitable reagent for additional confirmation of identity.

Extraction and purification procedures

The use of the cold extraction procedure with fresh frozen grass[102] represented a marked improvement over the

Soxhlet extraction of dried grass that had been used previously[101]. The advantages were threefold, better yields of alkaloids, less pigmentation, and considerable reduction in time due largely to the removal of evaporation stages. For the qualitative process no evaporation stages were used at all, the bases being extracted from the last chloroform layer into hydrochloric acid. Possibly this could be incorporated into a quantitative procedure, with a subsequent reduction in time and an improvement in product quality as the hydrochloric acid layer was always colourless. In any process where extraction into acid was undesirable as a final step initial extraction with a solvent containing acetone rather than methanol might offer some advantage. Soxhlet extraction of dried material with acetone rather than ethanol was found to produce a less pigmented product.

Preparation of reference materials

Due to supplies of reference materials becoming available the purification of reference materials was not completed. The use of suitable silica gel columns, either wet packed or using the dry column technique, seems to be a feasible method of separating the compounds in terms of their basic strength. The problem of satisfactory separation of the 5-methoxy derivatives from their unmethoxylated counterparts is less easy to solve. Crystallization of the picrate did not seem to separate the components, nor is there any reason to suppose that such techniques as precipitation with Dragendorff's reagent and subsequent regeneration of the bases with sodium carbonate[35] would produce any better results. Column chromatography on cellulose

would probably be the best approach. Wilkinson[97] succeeded in obtaining pure 5-methoxy-N-methyltryptamine by the use of an alumina column, which in this laboratory was not found to be particularly successful. Culvenor et al separated a mixture of the dimethyltryptamines from other bases by use of a counter-current system between 0.2N hydrochloric acid and chloroform, but were compelled to use partition chromatography, either glass powder/phosphate buffer or paper, to separate one from the other[36].

Identification of compounds

Evidence for the identity of the compounds reported in this thesis derives from a number of sources. Most of the compounds mentioned have been previously isolated from Phalaris arundinacea and characterised by other workers. Thus hordenine[9,97], gramine[9,36], 5-methoxy-N-methyltryptamine[97], 2,9-dimethyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline[9] and 6-methoxy-2-methyl-1,2,3,4-tetrohydro-β-carboline* have all been reported. The presence of both 5-methoxy-N,N-dimethyltryptamine and its unmethoxylated counterpart in Phalaris tuberosa[36] suggests the possibility of the unmethoxylated components in P. arundinacea. Barnes et al[15] reported dimethyltryptamines in reed canary grass, but the chromatography system used at that time did not separate dimethyl from N-methyltryptamine. It did, however, give evidence for the presence of unmethoxylated compounds in this species. In a subsequent publication[98] gramine, hordenine and all four of the tryptamines (N-methyl, N,N-dimethyl

*Dr. R. C. S. Audette, personal communication

and their 5-methoxy derivatives) were separated by use of gas chromatography and their identity confirmed by use of mass spectroscopy. Thus all the compounds mentioned in this thesis have been identified in the literature as deriving from reed canary grass. For the purposes of this research the identification of the tryptamines and gramine by thin layer chromatography and colour reactions seemed to be adequate. The observed absorption maxima for the xanthydrol derivatives of the tryptamines occurred at lower wavelengths to those reported elsewhere and gramine has been reported as not forming a coloured product with xanthydrol[59]. However the different reaction conditions used may account for these differing results. The identification of the β-carbolines was less satisfactory, particularly in view of the occurrence of substances which behave similarly on thin layer chromatography in plants essentially free of tryptamines. The identification of the β-carbolines may not be critical, as there is evidence that under some circumstances the tryptamines may be converted in the animal to β-carbolines[60]. The occurrence of β-carbolines as their tetrahydro derivatives in association with tryptamines is quite common. Examples include Shepherdia sp. (Elaeagnaceae)[10], Desmodium sp. (Loganiaceae)[13], and Gymnacranthera sp. (Myristicaceae)[54]. For positive identification and measurement of the β-carbolines, gas chromatography has distinct advantages over thin layer chromatography.

The unknown compounds produced in the late season tryptamine free grass, which had thin layer chromatographic behaviour

similar to the tryptamines but gramine type colour reactions with the xanthydrol reagent remain unidentified. Both xanthydrol and Ehrlich's reagent react with the nucleus[40,91], and the colour produced appears to depend on side chain length and other nucleus substituents. Thus these unknown compounds possibly are similar to gramine. However the thin layer separation is in terms of basic strength, and it seems difficult to imagine a simple 'gramine' which is a weaker base than gramine itself without having larger groups than methyl substituted at the aliphatic nitrogen. Methyl groups are the most common natural derivatives of -OH or $-NH_2$ groups. Further work would be required to identify these compounds, the above being little more than conjecture.

Quantitative thin layer chromatography

The first problem on any quantitative thin layer system is the production of a uniform spot of material at the origin. Fortunately using the silica gel methanol/strong ammonia solution system this problem was readily overcome by application of the bases to the plate in chloroform or chloroform/methanol mixtures. These solvents did not move the compounds to any extent on the silica gel layers, and thus an extremely small area of bases was obtained. Uniform colour development did not seem to be a problem with this system, and variations in this and layer thickness were largely accounted for by use of reference standards on the plate. Undoubtedly the most accurate results were obtained when the reference quantities bracketed the quantities of unknown. The unavailability of

suitable compounds giving exactly the same colour as the samples being measured, and the occurrence of a mixture in the major spot being measured, undoubtedly contributed to inaccuracies in the method.

An excellent review of the problems involved when using this technique is available[83].

The colorimetric method of assay

The method used worked quite well for gramine determinations and gave reasonable absorption curves with mixtures of tryptamines in low concentrations. When the tryptamines were in higher concentration the reaction appeared to be more vigorous with the accompanying gramine, resulting in a shift in the absorption curve to lower wavelengths. The results obtained with gramine in its higher concentrations were not considered as reliable as those obtained in the more moderate concentrations.

Both of these faults could probably be remedied by use of suitable dilutions to keep the sample concentration within a fixed range (about 200 - 600 μg gramine would be suitable), and by increasing the amount of xanthydrol reagent added to the reaction mixture.

Crossing and selfing procedures

Due mainly to rapid stem elongation there were considerable problems with breakage of the heads in the wind. Temperatures above 60^{o}F have been reported as detrimental to

flowering in this species*, and may possibly be harmful to seed production. Any modification of technique should be made with this consideration in mind. No emasculation of florets was done as the species is reported moderately self sterile[76,84]. Evidence was obtained in the genetic study that some self pollination did occur. It will be easy to check the degree of self and cross pollination occurring in the field by the use of a homozygous tryptamine containing plant as a tester.

Cytology

Reed canary grass from northern regions appears to be predominately tetrapoid (2n = 28) with hexaploid strains reported arising from more southerly regions[62]. Diploids have also been reported[76]. The hexaploid varieties have been reported as more unpalatable than the tetraploids[81]. The cytological examination revealed that the open pollinated progeny of one of the plants of Ottawa synthetic F were tetraploid. Thus it is likely that this variety is of the tetraploid type.

Sheep grazing experiment

The design used for the sheep grazing experiment proved quite successful as a means of evaluating palatability differences between clones. Only one seasons data were obtained, and this was from the year of planting. As a consequence of the recent planting many of the areas were not completely filled in, and as this was more so in the tryptamine alkaloid containing pasture than in the other it resulted in higher

*Dr. S. Bonin, personal communication

grazing pressure in the former area. For this reason the results from each field are not really comparable, and the contrast apparent between the great range of palatability in the tryptamine alkaloid containing field and the more general uniformity of the tryptamine alkaloid free field should be regarded with some reservation.

During this first year of grazing the sheep water bins were maintained at one end of the field, which may have had the effect of increasing grazing pressure at that end. In the future it will be possible to graze this pasture as individual blocks, in which case the association of the water supply with one block only will be removed.

Analysis of results from Ottawa Synthetics C and F

The analysis of these results was based on the assumption of a single dominant gene for tryptamine alkaloids. The data presented[102] provided evidence in favour of this assumption. This condition was not unexpected as there are many examples of dominant genes for chemical components reported in the literature. Suitable examples are: a single dominant gene for red root tips in Phalaris arundinacea[63], dominant genes for alkaloids in lupinus spp.[80], and the genetic control of plant pigments[94]. The analysis of results was necessitated by the loss of the parental clones, but by calculation the nature of these parental clones could be deduced. The information is useful in the search for a homozygous dominant tryptamine alkaloid containing plant. Such a plant would have three uses, these being; a) to provide additional evidence for the

genetic control system proposed; b) to provide an easy means of production of a pure tryptamine alkaloid containing pasture; and c) to provide a convenient tester for any evaluation of degree of outcrossing in reed canary grass.

Genetic control

The results presented[102] provided evidence for a single dominant gene controlling the presence of tryptamine alkaloids. This conclusion should not be regarded as absolute, since the data were derived from only parents and F_1 progeny, and the occurrence of a homozygous dominant was not found among the parents used in the crosses. Phalaris arundinacea has been shown to behave as an allotetraploid[53,33]. It is possible for both sets of chromosomes to carry alleles for tryptamine alkaloids, and because of the limited parentage of the plants used in the analysis this possibility has not been discarded. However from the results obtained it seems that within the synthetics examined there is only one gene for tryptamine alkaloids.

Plant material other than the Ottawa Synthetics

There was considerable variation in the number of plants containing tryptamine alkaloids in the various lines examined, varying from 9% to 69%. Without knowing the history of these selections and synthetics it is not possible to estimate the general proportion of tryptamine alkaloid containing plants to be expected. Obviously the gene for these alkaloids is fairly widespread in the material being used by plant breeders

in North America.

An interesting observation was that one source of material (from Purdue University, Lafayette, Ind.) provided, in the tryptamine alkaloid containing pasture, both extremes of palatability. Furthermore these extremes were very distinctly separated from the general grouping of other plants, predominantly derived from the Ottawa synthetics. This emphasises the need to examine material from diverse sources in searching for material of varying palatability.

Variation in gramine content

Estimation of the genetic variation for gramine content is made difficult by the considerable variation in gramine level from date to date, from shoot to shoot, and with varying management. As gramine may also be harmful it may be desirable to select and breed for low yielding lines, and this will be rendered more difficult by the variations in level which occur in the plant.

Reliability of the qualitative method

Re-examination of a large number of samples indicated that the method basically was sound. There is a possibility of error however if a plant containing low levels of tryptamines, which are mostly unmethoxylated, is examined. At moderate concentrations the purple colour with these compounds is easily distinguished from the pink produced by gramine, but at low levels these could be confused. Fortunately all those

plants examined by thin layer seemed to have the 5-methoxy derivatives present as a large if not dominant proportion of the tryptamines.

The interrelation of reed canary grass with the grazing animal

Two main interactions are of interest, those of palatability and toxicity. Palatability is probably the most important problem with this species now, as toxicity, assuming it is attributable to the tryptamines, is an easily eliminated component. Poor palatability has been attributed to one of the tryptamines[18] in which case at least some of this problem can also be easily resolved. However the results in this thesis show that there is considerable variation in palatability in tryptamine free plants, and that this feature is not constant in any one plant. There is a lack of information as to what components in this grass could be responsible for the palatability variation in the tryptamine free plants. It is quite possible that the change in palatability of the tryptamine alkaloid free plants during the season may be further complicated by an interaction with soil fertility, as the ability to produce succulent growth after fertilising could cause an unpalatable plant to appear palatable. This may have been the cause of the change in rank of the plants during the grazing experiment.

The actual mechanism of toxicity of the alkaloids in P. arundinacea is probably via stimulation of the sympathetic nervous system. The observations of high respiration rates under heat stress, high water consumption and purging of the

animals agree with this theory.

CONCLUSIONS

Tryptamine alkaloids in Phalaris arundinacea are under the control of a single dominant gene and may thus be readily eliminated from commercial varieties. Preliminary evidence indicates that these alkaloids may be an important cause of toxicity, and with knowledge of their genetic control it is now possible to test this hypothesis with relative ease.

The indole alkaloid gramine undergoes marked changes with season and management, and may in itself be toxic. This alkaloid appeared in all the tryptamine alkaloid free plants examined.

No determinations were made on hordenine levels although it is possible that this compound also contributes to toxicity.

Low palatability is probably the most important problem to deal with in this grass, and an extreme range of palatability types were found. The change in rank for palatability over two grazing periods in the tryptamine alkaloid free plants presents considerable problems in any attempt to determine the genetic control of, or chemical causes for, unpalatability.

APPENDICES

APPENDIX 1: REAGENTS

Ehrlich's reagent

p-Dimethylaminobenzaldehyde (0.7 g) was dissolved in 150 ml concentrated hydrochloric acid and 100 ml water was added[86]. The reagent was stored for at least one week before use.

Dragendorff's reagent

Solution A:- Bismuth subnitrate (1.7 g) in 100 ml acetic acid/water 4:1.

Solution B:- Potassium iodide (40 g) in 100 ml water.

For use 50 ml of A were mixed with 22 ml of B.

This differed from the recommended solution[21] in the final mixture:

(a) The proportion of B was doubled.

(b) No extra water was added.

Note: It was found feasible to overspray Ehrlich's with Dragendorff's.

Sulphuric acid 2N

Concentrated sulphuric acid (95.5% H_2SO_4 S.G. 1.84) was diluted with water to contain 98 g (53 ml) per litre.

Ammonia concentrated

Fisher reagent 28-30% NH_3, S.G. 0.90.

Hydrochloric acid concentrated

Canadian Industries Ltd. 37% HCl S.G. 1.19

p-Dimethylaminocinnamaldehyde reagent

p-Dimethylaminocinnamaldehyde (1.0 g) was dissolved in 50 ml of 6N hydrochloric acid, and 100 ml of 95% ethanol added[7].

Xanthydrol reagent for chromatography

Xanthydrol (0.1 g) was dissolved in 100 ml of ethanol 95%/ concentrated hydrochloric acid 95:5, and used on the day of preparation[15].

APPENDIX 2: THIN LAYER PLATES

Quantities are for 5 20x20 cm plates.

Thickness was 250 μ.

Silica gel: Silica gel G (Merck)[a] (20 g) was triturated with 40 ml water. Initially the plates were air dried and then activated at 105°C for 1 hour. Later the activation step was ommitted.

Cellulose: Cellulose powder MN300 (Machery, Nagel)[b] (10 g) and 60 ml water were thoroughly mixed in a high speed blender. The plates were air dried.

Alumina: Aluminium oxide G (Merck)[a] Neutral (10% aqueous slurry pH7.5) (40 g) was triturated with 50 ml of water. The plates were air dried.

Banded layer: Cellulose powder (2.5 g) water (15 ml) and silica gel (20 g), water (40 ml). Narrow band width 4.5 cm. The plates were air dried.

Cellulose/Silica gel G 50:50: Cellulose (7.5 g) (water required 45 ml), silica gel (7.5 g) (water required 15 ml), total water 60 ml.

Cellulose/Silica gel G 40:60: Cellulose (6 g) (water required 36 ml), silica gel (9 g) (water required 18 ml), total water 54 ml.

---

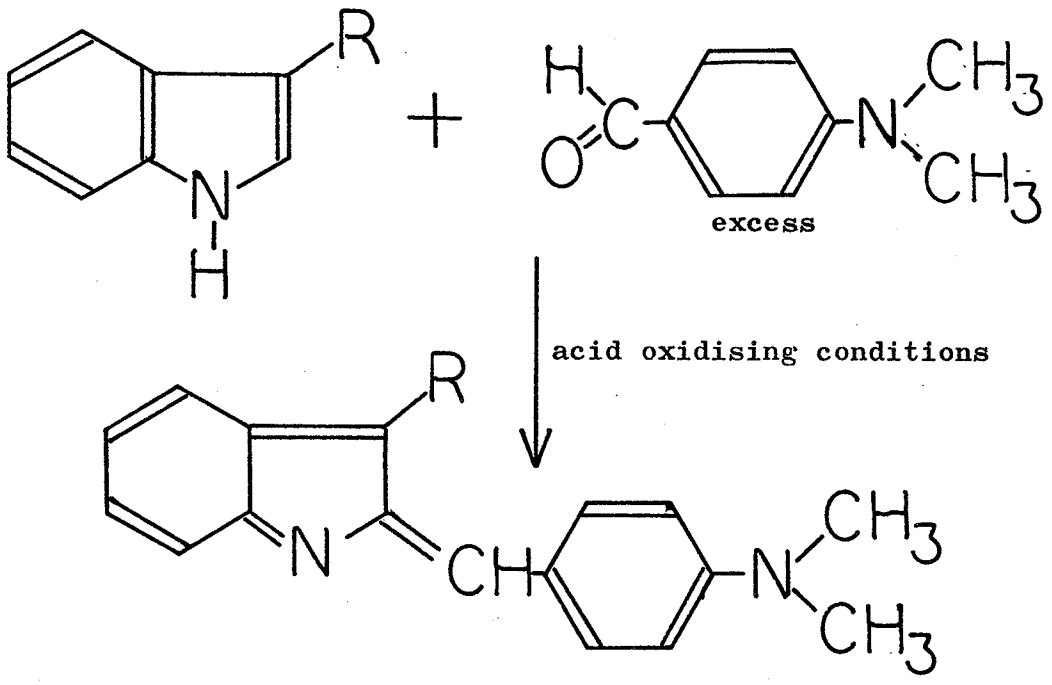
[a] E. Merck Ag. Darmstadt Germany. Canlab suppliers.
[b] Machery, Nagel and Co. Duren Germany. Canlab suppliers.

APPENDIX 3: APPARATUS

Because of the large numbers of samples to be dealt with it was necessary that efficient and rapid apparatus was used. Repeating delivery pipettes were used for adding fluids, and an adjustable syringe was used for sampling. Separations were performed in test tubes using a glass tube connected to a suction line to remove appropriate layers. A trap in the system served to recover chloroform. Stirring was accomplished by use of a glass rod with the end flattened out, which was moved up and down within the tube.

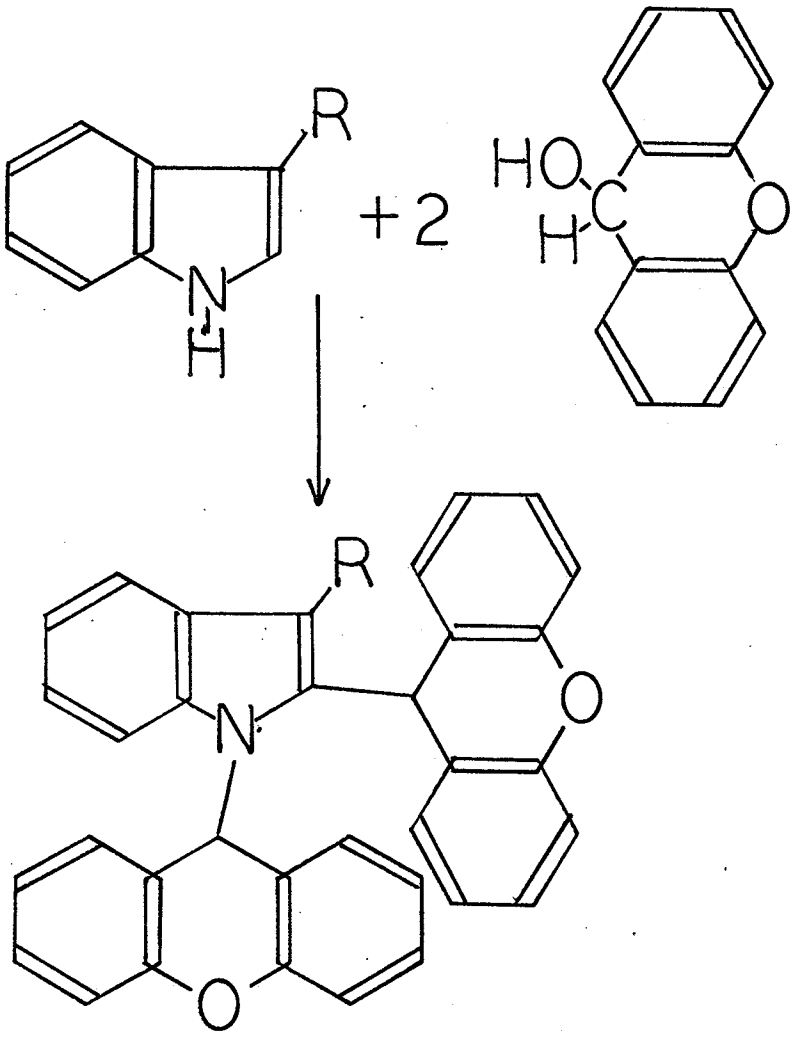
APPENDIX 4: THE CHEMICAL REACTIONS INVOLVED IN COLOUR FORMATION WITH THE INDOLES

The Ehrlich reaction appears to be[91]:



However the need for the reagent to age before a satisfactory colour is produced indicates that some intermediate compound may have to be produced first.

The xanthydrol reaction appears to be[40]:



APPENDIX 5: THE PHARMACOLOGY OF SOME OF THE ALKALOIDS OF REED CANARY GRASS

The compounds occurring in reed canarygrass have a number of actions, with considerable interactions. Hordenine has been used as a myocardial stimulant, and in small doses as an intestinal relaxant. In larger doses it is an intestinal stimulant [87]. Because of the disubstituted nitrogen hordenine would be expected to be more of a β than an α adrenergic effector [48]. Thus an increase in heart force and dilation of skeletal muscle blood vessels could occur. This is not to say that there would be no α adrenergic effects, and peripheral vasoconstriction could also occur.

Indole compounds are reported to be 5-hydroxytryptamine (5HT) antagonists [48]. The compounds mentioned were gramine, harmine and tryptamine derivatives. They are believed to interact with 5HT receptors at smooth muscle or nervous tissue sites. Unfortunately 5HT has extremely diverse and often unpredictable effects in the body, and thus antagonism of its effects can also produce many effects. Among the effects of 5HT are a stimulation of afferent nerves in the respiratory system, causing a short lived increase in respiratory volume and variable effects on respiration rate, a stimulation of lower intestinal activity, and central effects [48]. 5HT, adrenalin and norepinephrine are deaminated by monoamine

oxidase. Hallucinogenic activity has been found for N,N-dimethyltryptamine and tetrahydroharmine[82,51], the latter possibly acting through monoamine oxidase inhibition. It has been shown that indolic monoamines bind to receptor sites in the brain intact, if their breakdown to indole aldehydes is inhibited by monoamine oxidase inhibitors[1]. The carbolines reported in reed canary grass (see appendix 7) would be expected to have monoamine oxidase inhibitory activity[51]. There is also a possibility of formation of β-carbolines in the animal from tryptamines[34,60].

The dimethyltryptamines, and gramine, have been shown to cause tachycardia when given intravenously to sheep at rates of 5 mg/Kg for the tryptamines and 10 mg/Kg for gramine[47]. Oral administration, or parenteral administration at low doses (0.1 mg/Kg) of the tryptamines produced signs of nervous disturbance. There is also evidence that tissues may become sensitised to normal biological amines, by the tryptamine compounds, and death can occur some time after ingestion of the alkaloids brought about by a rise in levels of adrenalin[44]. The major sites responsible for the toxic action of the dimethyl tryptamines appears to be the brain and spinal chord[45]. Myeline degeneration of the spinal chord has been reported in cases of phalaris staggers[41].

APPENDIX 6: CROP SCIENCE 11: 121-122. 1971

VARIATION IN THE CONTENT OF TRYPTAMINES IN CLONES OF *PHALARIS ARUNDINACEA* L.[1]

D. L. Woods and K. W. Clark[2]

ABSTRACT

A number of clones of reed canarygrass (*Phalaris arundinacea* L.) from two synthetic cultivars were examined by thin layer chromatography for the presence of substances reacting with Ehrlich's reagent. Approximately half of the clones examined appeared to be free of substances giving the blue color considered indicative of tryptamines, but in these tryptamine-free clones a substance giving the pink color indicative of gramine was detected.

Additional key words: Alkaloids in forage, Reed canarygrass.

TRYPTAMINES have been reported present in both reed canarygrass (*Phalaris arundinacea* L.) and hardinggrass (*P. tuberosa* L.) and have been considered responsible for the toxic effects of hardinggrass (1, 2, 3). Since reed canarygrass is used for forage purposes and is potentially of considerable value, an examination was made of the tryptamines in individual clones, with a view to breeding a cultivar with a reduced content of tryptamines.

In order to perform this examination a thin layer chromatographic system was developed which separated reference tryptamines into groups depending on their degree of substitution at the aliphatic amine nitrogen. This has previously been reported as a basis for separation (4).

Experimental Procedures

Material. The cultivars 'Ottawa Synthetic C' and 'Ottawa Synthetic F' arose as progeny from open-pollinated selections of reed canarygrass of worldwide origin. Selection of the parental clones was for late-maturing lines which would be leafy and respond well to high fertility levels. The clones were planted as sprigs at the beginning of June 1968, and by the time of harvest in September 1968 had tillered profusely, but had not headed out. The cut grass was stored in a cold room at approximately —36C until needed. Before use the material was dried at 27C and powdered in a Wiley mill through a 2-mm screen.

Extraction. Powdered grass, 5 g, was Soxhlet-extracted overnight with 95% ethanol and the liquid was taken to dryness under vacuum. The residue was dissolved by shaking alternately with 50 ml of 2*N* sulphuric acid and 50 ml chloroform (two lots of each). The pigmented chloroform was discarded and the aqueous layer saturated with sodium chloride, and then made to above pH 11 with 40% w/v sodium hydroxide (15 ml sufficient). The aqueous layer was then extracted for alkaloids with 50 ml chloroform. The solution was evaporated to dryness, the residue was made to 1 ml, and 10 μl were used for thin layer chromatographic examination.

Thin layer chromatography. Thin layer plates were silica gel G (Merck) or cellulose (MN300 Mackery Nagel), wet thickness 250 μ, air dried. Length of run was 15 cm. Spray reagents were: Ehrlich's reagent—0.7 g p-dimethylaminobenzaldehyde dissolved in 150 ml concentrated hydrochloric acid, made to 250 ml with distilled water, which was used for the screening procedure (this spray reagent should be at least a week old before use); Xanthydrol—0.1 g xanthydrol in 100 ml of 95% ethanol: concentrated hydrochloric acid 95:5 freshly prepared. In both cases the colors were allowed to develop without heating.

Solvent systems: Methanol:strong ammonia solution (29% NH_3) 7:1 was used on silica gel plates for screening. Methanol: concentrated hydrochloric acid 9:1 was used on silica gel plates. Butanol:formic acid:water 16:1:3 was used on cellulose plates. R_f's of standards and color reactions are tabulated (Table 1).

Results and Discussion

Many of the clones examined were without compounds giving the blue color indicative of tryptamines with Ehrlich's reagent. The clones which possessed these compounds produced one major spot on the silica plate in the methanol:ammonia system used for the screening. This spot gave the same color reaction as and co-chromatographed with 5-methoxy-N-methyltryptamine. Another spot was also present in variable quantities which gave the same color as and co-chromatographed with N,N-dimethyl-5-methoxytryptamine. As the screening system did not separate the unmethoxylated compounds from the 5-methoxy compounds,

Table 1. R_f values and colors produced by reference compounds.

Compound	R_f value: NH_3 solvent on silica	HCl solvent on silica	Butanol solvent on cellulose	Color: Ehrlich's reagent	Color: Xanthydrol reagent
Gramine*	.38	.65	.75	(Slowly) pink	Pink
Bufotenine	.45	.60	.45	Blue-purple	Blue
Tryptamine (T)	.35	.85	.75	Blue-grey	Purple
N, N-Dimethyl T*	.50	.60	.75	Blue-grey	Purple
N, N-Dimethyl-5-methoxy T*	.50	.60	.65	Royal blue	Blue
N-Methyl T*	.25	.75	.75	Blue-grey	Purple
5-Methoxy-N-methyl T*	.25	.75	.65	Royal blue	Blue
2, 9-Dimethyl-6-methoxy-1, 2, 3, 4 tetrahydro-β-carboline	.55	.55	.65	Blue-grey (poor reaction) becoming brown	Purple
6-Methoxy-2-methyl-1, 2, 3, 4 tetrahydro-β-carboline	.55	.65	.65	Blue-grey (poor reaction) becoming brown	Purple

* Not distinguishable by any of these characteristics from the compounds found in Phalaris arundinacea samples, and co-chromatographing with them.

[1] Contribution No. 233, Department of Plant Science, University of Manitoba, Winnipeg 19, Manitoba, Canada. Received March 5, 1970.

[2] Graduate Student and Professor, respectively, University of Manitoba, Winnipeg, Manitoba, Canada.

it was not possible to conclude that the unmethoxylated compounds were absent. However, the spots were usually of the royal blue color characteristic of the 5-methoxy compounds, and it was therefore concluded that the compounds usually present included the 5-methoxy derivatives.

Further analysis of a bulked lot of the grass samples previously examined indicated that as many as four tryptamines might be present. These were the 5-methoxy-N-methylated tryptamines previously mentioned and also their unmethoxylated counterparts. This conclusion is based on partial purification of compounds by preparative thin layer chromatography using the screening system, examination of the partially purified products on alternative thin layer systems, and use of the alternative xanthydrol spray reagent. This spray reagent was preferred to Ehrlich's reagent for this purpose because the color difference between the methoxy compounds and their unmethoxylated counterparts was more marked. Small scale isolation of pure compounds in sufficient quantities to confirm the structures was not successful.

Gramine was easily detected in clones not containing tryptamines and appeared to be present in all of these. The chromatographic system and spray reagent used for the screening procedure did not resolve the mixture well enough for any conclusions to be reached regarding its presence in the tryptamine-containing clones.

The identification of some of these compounds is supported by the work of others, although they have used bulked material. Gramine has been reported (1, 5, 6), as has 5-methoxy-N-methyltryptamine (2, 5), in *P. arundinacea*. The dimethylated tryptamines have so far only been reported in *P. tuberosa* (1), and N-methyltryptamine has not previously been reported in either species. Previous work with the same material as used for this report did not show the presence of any of the tryptamines (5) but did show the presence of a β-carboline.

In nearly all cases the difference between the presence or absence of tryptamines was marked, but occasionally clones having tryptamines present in only small quantities were found. These were recorded as "trace" quantities (Table 2). Examination of clonal material at different times of the year and also grown under greenhouse conditions did not reveal any changes in the clones from tryptamine-containing to tryptamine free, or vice-versa.

The thin layer system was found to be extremely sensitive, the lower limit for tryptamine hydrochloride being between 0.04 and 0.08 μg per spot, in a mixture with a grass extract. Spots of this intensity would have been recorded as "trace." Quantities were usually far above this level, averaging about 9 μg per spot, expressed as tryptamine hydrochloride[3]. Thus, the usual quantities of tryptamines present exceed by a factor of 100 the lower limit of detection by this technique.

The quantity of 9 μg per spot represents approximately 120 μg g dried grass expressed as tryptamine hydrochloride, allowing for an extraction efficiency of 37%[3]. The actual amount present may be considerably above this value, as it has been suggested that in *P. tuberosa* there is a reduction in the quantity of these alkaloids with drying (1). No information is available on the oral dose of the 5-methoxy-N-methyltryptamine for toxicity, but in sheep N,N-dimethyl-5-methoxytryptamine and dimethyltryptamine have been shown to be toxic at 5-mg/kg in intravenous injection, and N,N-dimethyl-5-methoxytryptamine has been shown to be rapidly absorbed from the gastrointestinal tract (3).

Table 2. Tryptamines in two synthetic cultivars of *Phalaris arundinacea* L.

Tryptamine content	Ottawa Synthetic C		Ottawa Synthetic F	
	Number of plants	Percentage	Number of plants	Percentage
None	92	49.2	94	53.4
Trace	21	11.2	10	5.7
Positive	74	39.6	72	40.9

Recent trials using *P. tuberosa* showed maximum toxicity as the measurable tryptamines declined (7), possibly indicating that a metabolic product of the tryptamine is more important in toxicity problems than the actual tryptamines. This theory may be tested in *P. arundinacea* by the use of tryptamine-containing and tryptamine-free lines. For breeding purposes the presence of clones lacking tryptamines is of considerable value, and the distinct separation into positive and negative groups suggests a simple genetic control mechanism which would be easily exploited. This control mechanism is being investigated.

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The authors thank Miss Talat Ali for performing the routine analysis of the individual clones.

The authors also thank Dr. R. M. MacVicar of the Canadian Department of Agricultural Research Station, Ottawa, for supplying the clones of the two cultivars examined, and Dr. H. M. Vijayanagar of the School of Pharmacy, University of Manitoba, for providing samples of 5-methoxy-N-methyltryptamine, 2,9-dimethyl-6-methoxy-1,2,3,4-tetrahydro-β-carboline and 6-methoxy-2-methyl-1,2,3,4-tetrahydro-β-carboline.

Financial support from the Canadian Department of Agriculture is gratefully acknowledged.

Literature Cited

1. Culvenor, C. C. J., R. Dal Bon, and L. W. Smith. 1964. Occurrence of indolealkylamine alkaloids in *Phalaris tuberosa* and *arundinacea*. Australian J. Chem. 17:1301-1304.
2. Wilkinson, S. 1958. 5-methoxy-N-methyltryptamine: A new indole alkaloid from *Phalaris arundinacea* L. J. Chem. Soc. 2079-2081.
3. Gallagher, C. H., J. H. Koch, R. M. Moore, and J. D. Steel. 1961. Toxicity of *Phalaris tuberosa* for sheep. Nature 204: 542-545.
4. Chalmers, A. H., C. C. J. Culvenor, and L. W. Smith. 1965. Characterization of Pyrrolizidine alkaloids by gas, thin layer, and paper chromatography. J. Chromatog. 20:270-277.
5. Audette, R. C. S., J. Bolan, H. M. Vijayanagar, R. Bilous, and K. W. Clark. 1969. Phytochemical investigation of Manitoba plants. II. A gas-liquid chromatographic screening technique for the identification of the alkaloids of *Phalaris* species. J. Chromatog. 43:295-302.
6. ———, H. M. Vijayanagar, J. Bolan, and K. W. Clark. 1970. Phytochemical investigation of Manitoba plants. I. A new indole alkaloid and associated alkaloids from *Phalaris arundinacea*. Can. J. Chem. 48:149-155.
7. Oram, R. N. 1970. Genetic and environmental control of the amount and composition of toxins in *Phalaris tuberosa* L. Int. Grassland Congr., Proc. 11th (Queensland, Australia) p. 785-788.

[3] D. Woods. Unpublished data.

APPENDIX 7

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GENETIC CONTROL AND SEASONAL VARIATION OF SOME ALKALOIDS IN REED CANARYGRASS

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ABSTRACT

In unclipped reed canarygrass, gramine content rose to a maximum at about seed shedding. With regular clipping the rise was more rapid and continued into the Fall. This difference was partially explained by the dilution effect of low levels of gramine in the stems in unclipped grass samples.

The content of tryptamine alkaloids followed the same general pattern as did gramine. The presence of tryptamine alkaloids in this reed canarygrass was controlled by a single dominant gene.

* * * * * * * * *

Le Controle genetique et la variation saisoniere de certains alcaloides de l'alpiste roseau

Lorsque l'alpiste roseau ne fut pas tondu le contenu en gramine augmenta et atteignit son maximum à peu près lorsque le grain commenca à s'égrenner. Lorsque l'herbe fut tondue regulierement, l'augmentation fut plus rapide et se continua jusqu'à l'automne. Cette difference fut expliquée en partie, par un effet de dilution dû au niveau très bas de la gramine dans les tiges de l'herbe non tondue.

Le contenue en alcaloides du type tryptamine evolua en general de facon semblable à celui de la gramine. La presence d'alcaloides du type tryptamine dans l'alpiste roseau est controlee par un seul gène dominant.

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INTRODUCTION

Reed canarygrass (Phalaris arundinacea L.) has considerable potential as a forage crop (5). There is a possible problem however, in that the alkaloids present in this grass may cause poisoning (3, 4, 6). Three types of alkaloids: gramine, hordenine and tryptamine analogues have been found in reed canarygrass. Evidence for the identity of these alkaloids has been previously presented (1, 2, 3), and also it has been suggested that alkaloids of the tryptamine group are the cause of toxicity in the related species P. tuberosa L. (4). It has been shown that not all plants contain the tryptamine group of alkaloids, but that all those plants which were free of the tryptamine group contained gramine (3).

This publication deals with the genetic control of the tryptamine group of alkaloids, and the seasonal variation of both gramine and the tryptamine group under two conditions of management. A much improved method for the detection of tryptamines in individual plants is described.

MATERIALS AND METHODS

Plant material

The plants used consisted of members of the S_o generation of the cultivar "Grove" (previously Ottawa synthetic F), and members of the S_o generation of Ottawa synthetic C. Each plant was divided into two clones which were planted adjacent to each other in the nursery. At the commencement of the seasonal variation study the clones had been established for two years and each clone occupied an area of approximately 4 sq. feet.

Two management conditions were used in the study of seasonal variation of alkaloids. One clone of each parental plant was cut regularly to a height of 6 inches, and the clippings used for the assay. The other clone was allowed to grow freely, and the grass growing above a height

of 6" sampled regularly. Care was taken in the latter case to avoid including plant material which had regrown from previously cut areas of the plant

Selfed seed was obtained by bagging individual heads. Crossed seed was obtained by bagging heads with a flowering stalk of the pollen parent at the beginning of anthesis. Keeping the stalk of the pollen parent in a vial of water proved impractical due to the rapid extension of the stalk of the female parent.

After harvest the seeds were stored at 4.4°C for 5 weeks, and then planted in beds of sterilised soil. The seedlings were harvested when 12 weeks old.

Quantitative extraction procedure

The grass sample, fresh weight about 200 g, was chopped into pieces about 1-2 cm long, and stored until needed at -30°C, the minimum storage time being 48 hours. From this frozen material a representative sample of 5 or 10 g was taken, and extracted by standing overnight (16 hours) with 100 ml of a mixture of chloroform, methanol and concentrated ammonia (26:33:1). Samples of 20 ml were taken from this, and extracted in large test tubes (20 x 175 mm) by the following procedure:

(1) Sulphuric acid, 10 ml of 2N was added and the tubes stirred.

(2) After settling the pigmented chloroform layer was removed to waste.

(3) Chloroform 10 ml was added, and after settling this was also removed to waste.

(4) Sodium hydroxide 2 ml of 40% w/v was added, followed by chloroform 10 ml, and the mixture stirred.

(5) After settling, the aqueous layer was discarded.

(6) Water 10 ml was added, the mixture stirred, and after settling the aqueous layer was removed to waste.

(7) Step (6) was repeated.

(8) The chloroform layer containing the extracted bases was evaporated under vacuum until all the chloroform had been removed (some water remained but did not interfere with later steps).

Colorimetric assay for gramine

To the bases obtained by the above method was added 5 ml of 9.3N hydrochloric acid and 0.5 ml of 1.25 mg/ml Xanthydrol in 95% ethanol. The mixtures were transferred to test tubes (18 x 150 mm) and heated in a boiling water bath for 16 minutes. After cooling in cold water for 2 minutes, 0.5 ml of 12.5% w/v sodium metabisulfite and 1 ml of 95% ethanol was added. This mixture was filtered (Whatmann No. 1) and measured, using distilled water as a reference. The optical density was measured at 500 nm, using either a Spectronic 20 Spectrophotometer[1] or a Cary 15 recording spectrophotometer[2] and 1 cm cells. Duplicate samples of crude bases were measured.

Calculation of results

The observed sample optical density (OD) was compared with a calibration curve to determine the amount of gramine in the sample. The calibration curve was a least squares fit to reference samples carried through the quantitative extraction procedure, and therefore took account of extraction efficiency. The line was given by:

$$\log 1000 \times OD = 0.7070 \log \mu g + 0.8302$$

---

[1] Bausch and Lomb. New York

[2] Cary Instruments. California

With each batch of samples a reference quantity of gramine was also colorimetrically assayed, and variations in the color development corrected by multiplying observed sample values by a factor of: reference quantity of gramine/apparent quantity from OD measurements. This value lay between 0.87 and 1.08 in all cases, except the last two dates when a value of 1.48 was obtained.

Detection of tryptamines by thin layer chromatography

The crude bases obtained from the quantitative extraction procedure were dissolved in 1 ml of methanol/chloroform 1:1, and 5 or 10 µl applied to a thin layer plate. After development and spraying, the plates were photographed, and the color slides used to compare the results over a period of time. Reference quantities of tryptamine and N,N-dimethyl-5-methoxytryptamine were similarly treated, and used as a basis for quantitative estimations. The estimations of concentrations of individual tryptamines were represented as a smooth curve on a graph of concentration versus date (Fig. 2).

The chromatography systems were: methanol/strong ammonia solution on silica gel G layers, and butanol/formic acid/water on cellulose layers, as previously reported (8).

Extraction for qualitative detection of tryptamines

The procedure used was basically a shortened version of the quantitative scheme, with some slight modifications. Grass seedlings were dug at 12 weeks and the roots were washed carefully to ensure that only one seedling was used for each extraction, and to ensure that seedlings were not accidentally subdivided. The whole seedling was pushed into a large test tube and frozen. Each sample was extracted overnight (16 hours)

with 15 ml of a mixture of chloroform/methanol/strong ammonia solution 18:11:1. The extracted grass was removed, and the alkaloids purified by a procedure identical to steps (1)-(6) of the quantitative procedure, and omitting step (3).

The chloroform was then filtered to remove traces of soil introduced by the roots, which otherwise interfered with the final colour produced. The chloroform was then extracted with 5 ml of 9.3N hydrochloric acid, and the chloroform layer discarded. Xanthydrol reagent (0.5 ml) was added to the acid layer and, after mixing, the samples were heated in a water bath. The samples could be readily classified as blue to green -- tryptamine containing, and yellow to pink -- tryptamine free (the pink colour was due to gramine).

RESULTS

Seasonal variation of gramine

When the spaced plants in the field were allowed to grow freely the quantity of gramine reached a maximum value of 80.5 µg/g fresh weight about August 14. This is the average from 4 genotypes, which ranged from 30 to 134 µg/g. Under regular clipping a continual rise in gramine content was observed, the average from 5 genotypes being 605 µg/g on September 25, with individual results ranging from 550 to 685 µg/g. The dry matter content of the samples rose during the season, the rise being considerably more in the case of the unclipped grass. This results in an even greater difference between the gramine content of the plants under the two management conditions if plotted on a dry matter basis (Fig. 1).

In the late season (September 25) leaf tissue from free growth material represented about 25% of the fresh weight of the samples, and contributed about 90% of the gramine detected.

One plant was rejected from the analysis of unclipped grass because it failed to head out in the same way as the others, and remained in an intermediate vegetative condition with many leaves and few heads. This plant reached a gramine level of 160 µg/g on July 31, and remained at around this level for the remainder of the season.

Seasonal variation of tryptamines

The variation in tryptamine content during the season was similar to the variation of gramine. At the beginning of the season only one strong spot was revealed using the silica gel system, corresponding to 5-methoxy-N-methyltryptamine (5MeONMeT). Chromatography on cellulose revealed that some N-methyltryptamine (NMeT) was present. As the season progressed the amount of 5MeONMeT rose dramatically in the continually cut grass, to an estimated 200 µg/g of fresh grass. Furthermore a marked increase in other components running in the tryptamine (T), dimethyl-tryptamine (DMeT) and β-carboline (β-C) regions was observed using the silica gel system. The cellulose system revealed that the components lacking the 5-methoxy derivative were increasing at about the same rate as the 5MeONMeT. The cellulose system also revealed the presence of other as yet unidentified compounds of high R_f value which increased slowly during the season. When the grass was allowed to grow freely the 5MeONMeT level at first declined to a minimum at June 26, then rose to a maximum at around July 31. Again the level of the compounds lacking the 5-methoxy group paralleled the 5MeONMeT. The free growth grass produced at best a faint trace of compounds in the T or DMT regions, but the compounds in the β-C region rose steadily throughout the season (Fig. 2).

In the late season, leaf tissue from the unclipped grass contained quite high levels of tryptamines, whilst the stem material was almost tryptamine free.

Genetic control

From 10 tryptamine free parents which were selfed a total of 101 progeny were obtained, of which 2 were tryptamine containing, the rest being tryptamine free. From 7 tryptamine containing plants which were selfed the 42 progeny consisted of 25 tryptamine containing plants and 17 tryptamine free plants. One tryptamine containing plant produced on selfing 16 tryptamine containing progeny and no tryptamine free progeny.

From 18 crosses involving only tryptamine free parents a total of 462 progeny were obtained, two of which contained tryptamines, the remainder being tryptamine free.

The numbers and classification of progeny produced from crosses involving tryptamine containing parents are tabulated.

(TABLE I HERE)

DISCUSSION

The presence of tryptamines was evidently under the control of a single dominant gene. Two of the progeny of the selfed plants apparently did not fit within this scheme, but the authors considered that these plants were due to accidental outcrossing or seed transfer in the greenhouse. Precautions were taken to attempt to avoid these problems when the crossed seeds were produced and grown. Within the progeny of the crossed plants two extremes may be distinguished, those which fit the expected ratio for a single dominant gene control, and those which deviate from this ratio. Those groups of progeny which deviate from the expected ratio do so in favour of the ratio which would be expected if the female parent had selfed. This is what is considered to have happened in these cases. The two T+ progeny from the crosses involving both T- parents were assumed to be accidentals.

The data presented derive from only F_1 progeny, and are therefore incomplete for a completely satisfactory determination of genetic control. The expected proportion of homozygous dominants for alkaloids among the positive parental clones used in the crosses was 1 out of 5 (D. Woods, unpublished data), and as only 5 positive clones produced progeny, the failure to detect a homozygous positive was not surprising. That a dominant gene is involved in the control of tryptamines is without doubt, and this information is of considerable value for production of tryptamine free cultivars. These cultivars would be of considerable value, as tryptamines are potentially harmful to grazing animals (4), and may also be partly responsible for unpalatability in this species (7).

Rapid vegetative growth appeared to be the criterion for high alkaloid production, either of gramine or of the tryptamines. Some of the difference between alkaloid content of the regularly clipped and the free growth grass could be accounted for by the dilution effect of the stem tissue, but even so, the leaves of the clipped plants were found to have about four times the amount of gramine that is present in the leaves of the free growth material. This relationship was also shown by the behaviour of one plant which developed an intermediate vegetative form, and also had an intermediate level of gramine.

The marked variation in levels of gramine throughout the season, and the considerable influences of management must be considered when attempts are made to reduce gramine content by breeding. As tryptamines are readily eliminated the variation in level is less important, but should be considered in the grazing of established swards.

ACKNOWLEDGEMENTS

The authors thank Dr. R. M. MacVicar of Canada Department of Agriculture, Research Station, Ottawa, for supplying the clones of the parental cultivars. Financial support from the Canada Department of Agriculture is gratefully acknowledged.

- 10 -

REFERENCES

1. Audette, R. C. S., Bolan, J., Vijayanagar, H. M., Bilous, R., and Clark, K. W. 1969. Phytochemical investigation of Manitoba plants. II. A gas-liquid chromatographic screening technique for the identification of the alkaloids of Phalaris species. J. Chromatog. 43: 295-302.

2. Audette, R. C. S., Vijayanagar, H. M., Bolan, J., and Clark, K. W. 1970. Phytochemical investigation of Manitoba plants. I. A new indole alkaloid and associated alkaloids from Phalaris arundinacea. Can. J. Chem. 48: 149-155.

3. Culvenor, C. C. J., Dal Bon, R., and Smith, L. W. 1964. Occurrence of indolealkylamine alkaloids in Phalaris tuberosa and arundinacea. Aust. J. Chem. 17: 1301-1304.

4. Gallagher, C. H., Koch, J. H., Moore, R. M., and Steel, J. D. 1964. Toxicity of Phalaris tuberosa for sheep. Nature 204: 542-545.

5. Vose, P. B. 1959. The agronomic potentialities and problems of the canary grasses, Phalaris arundinacea L. and Phalaris tuberosa L. Herbage Abstracts 29: 1-7.

6. Wilkinson, S. 1958. 5-methoxy-N-methyltryptamine: A new indole alkaloid from Phalaris arundinacea L. J. Chem. Soc. 2079-2081.

7. Williams, M., Barnes, R. F., and Cassady, J. M. 1971. Characterization of alkaloids in palatable and unpalatable clones of Phalaris arundinacea L. Crop Science (in press).

8. Woods, D. L. and Clark, K. W. 1971. Variation in the content of tryptamines in clones of Phalaris arundinacea L. Crop Science 11: 121-122.

Table 1. Classification of progeny from various crosses

Parent numbers †	Parent types ‡	Progeny numbers		X^2 test	5% §
		+	-	1:1	3:1
51 x 36	+ x -	12	9		
52 x 35	+ x -	18	12		
52 x 36	+ x -	22	18		*
52 x 68	+ x -	32	8	*	
83 x 82	+ x -	23	5	*	
83 x 66	+ x +	27	13	*	
34 x 51	- x +	3	9		*
36 x 51	- x +	5	10		*
36 x 52	- x +	4	12		*
50 x 51	- x +	2	12	*	*
50 x 66	- x +	16	24		*
67 x 51	- x +	5	15	*	*
67 x 66	- x +	19	21		*
68 x 52	- x +	25	15		*
68 x 83	- x +	13	27	*	*
82 x 66	- x +	23	17		*
82 x 83	- x +	21	19		*

† All tryptamine free parents were also involved in at least one - x - cross

‡ + refers to tryptamine containing, - to tryptamine free

§ * indicates significant difference at 5% level from the numbers predicted at the indicated rates

FIGURE CAPTIONS

Figure 1. Content of gramine on a fresh weight basis in reed canarygrass under two management conditions.

Figure 2. Content of N-methyltryptamines and β-carbolines on a freshweight basis in reed canarygrass under two management conditions.

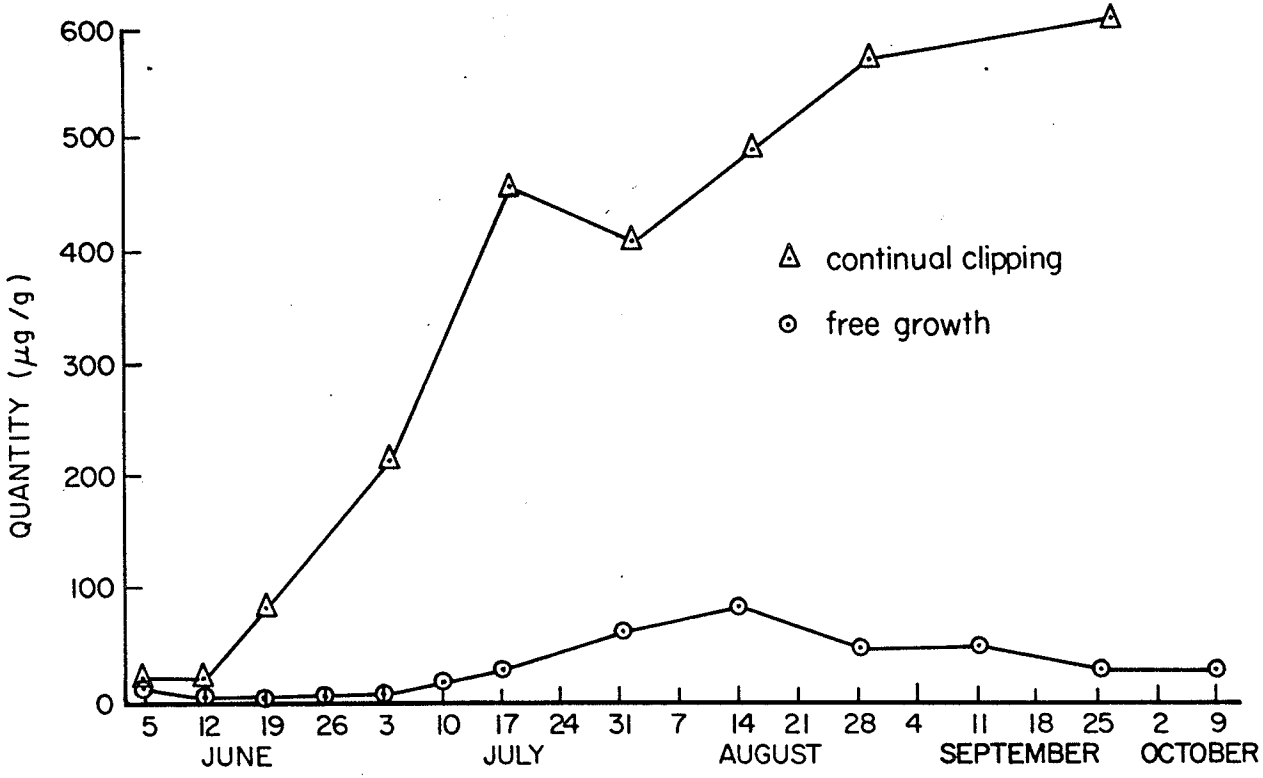
600
500
400
300
200
100
0
QUANTITY (µg/g)
continual clipping
free growth
5
12
19
26
3
10
17
24
31
7
14
21
28
4
11
18
25
2
9
JUNE
JULY
AUGUST
SEPTEMBER
OCTOBER

Figure 1.

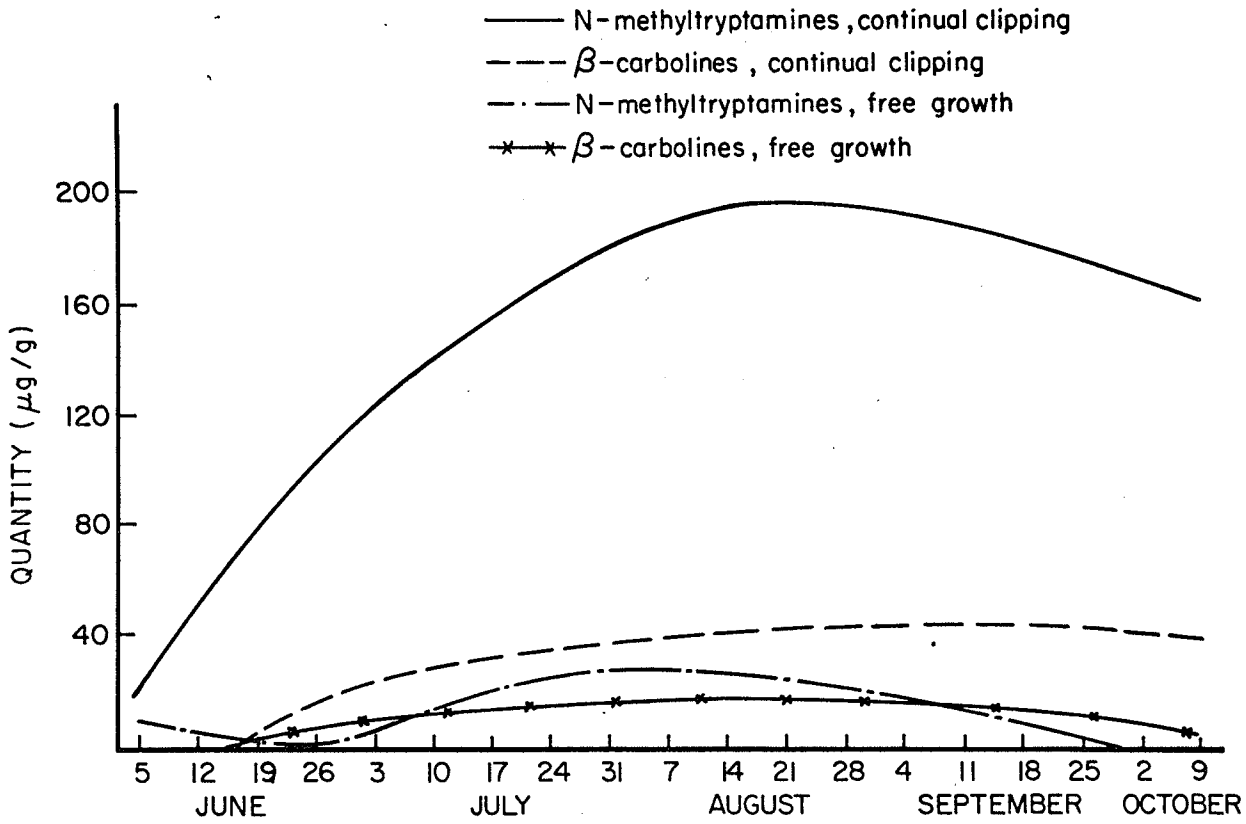
N-methyltryptamines, continual clipping
β-carbolines, continual clipping
N-methyltryptamines, free growth
β-carbolines, free growth
QUANTITY (μg/g)
200
160
120
80
40
5 12 19 26 3 10 17 24 31 7 14 21 28 4 11 18 25 2 9
JUNE
JULY
AUGUST
SEPTEMBER
OCTOBER

Figure 2.

APPENDIX 8

EVALUATION OF REED CANARY GRASS PASTURE USING SHEEP

INTRODUCTION

For use as pasture reed canary grass Phalaris arundinacea L. has two important limitations. These are potential toxicity and low palatability. Animal poisoning has not yet been conclusively attributed to reed canary grass, but this species was present in the pasture grazed by cattle which subsequently showed alkaloid type lesions of the liver (1). Poor milk yield and poor weight gains in cattle have also been reported (1), as have poor weight gains in sheep (2). Reed canary grass may also be considered potentially harmful due to the presence of tryptamine alkaloids (1, 3, 4, 5), some of which have been held responsible for the toxicity of the related forage harding grass P. tuberosa L. (6, 7). There has recently been some doubt as to whether the tryptamines are responsible for poisoning, as sheep grazing harding grass died at a time when the grass did not contain its maximum level of tryptamines (8).

Poor palatability of reed canary grass has been reported both on a varietal basis (9, 10) and on an individual plant basis (11, 12, 13) and the variation in palatability has in some cases been attributed to alkaloid content, both tryptamines (4) and possibly gramine (14, 15) which also occurs in this species.

The occurrence of clones free of tryptamine alkaloids has been reported (5) and the genetic control of tryptamines, and seasonal variation of both tryptamines and gramine reported (16).

This report deals with an examination of sheep performance on two types of pasture, one free of tryptamine alkaloids and one containing them. In vivo digestability of the two types of pasture was also determined, and an evaluation made of relative palatability of individual plants.

MATERIALS AND METHODS

The clones of reed canary grass used were selected from various sources, many from the cultivars Ottawa synthetic C and F (cf. 5). Plants were evaluated according to whether or not they contained tryptamine alkaloids and thirty clones of each type were used. The clones were subdivided and planted out at the end of May 1970. A field of each type was planted, each field consisted of each of the 30 clones of one type replicated four times. Each replicate was a square 5' by 5' and was made up of up to 25 sprigs at 1' centres. A 2' space was allowed between areas.

Seven lambs were allowed to free graze each pasture for two periods of time. The first period was from August 5 to 25, and the second period was from September 17 to 22. After the first period of grazing the grass was cut down, fertilised at a rate of 60 lb N/acre, and irrigated. During the grazing trial an evaluation of the consumption of each clone was made, every 3 days for the first period and every 2 days for the second period. Each area was given a score from 0 to 5, 0 being untouched, 5 being total consumption. The results from this were analysed as a factorial experiment using a randomised complete block design, with 30 plants, 4 replicates

and 7 or 6 dates for the first and second period respectively. All data was first transformed to $\sqrt{x + \frac{1}{2}}$ where x was the score given.

Between grazing trials the sheep were kept penned and fed on chopped reed canarygrass with the appropriate tryptamine content. This feed was obtained from an area of mature clones growing on clay subsoil, each clone of which had previously been evaluated for tryptamines (5). After 10 days of consumption of this forage in open pens, 4 of the sheep were moved inside for a 7 day period to evaluate dry matter digestibility of each forage type by the faeces collection method. Samples of each type of forage that was used in this trial were also subjected to proximate analysis. The forage at this time was mature and contained a large amount of stem material.

RESULTS

In the group of plants containing tryptamine alkaloids an extreme range of palatability was observed, varying from essentially untouched at the end of each grazing period to total consumption within 5 days on the first grazing period, and total consumption within 1 day on the regrowth grazing period. The order of rank of the plants was essentially the same for both trials although a few plants did deviate appreciably. A good correlation ($r = 0.8586$) was obtained between the mean transformed scores from each grazing period (Table I).

In the group of plants free of tryptamine alkaloids the differences in palatability were not as extreme, and the correlation between the mean transformed score for each grazing

period was essentially zero ($r = 0.0296$) (Table II).

During both grazing periods the sheep on the tryptamine containing plants lost weight significantly ($P < 0.05$). The sheep on the tryptamine free plants gained weight slightly during the first grazing period and lost weight slightly during the second period, neither being significant. The difference between the groups was significant ($P < 0.05$) for the first period, but not so during the second grazing period.

The sheep which had been grazing on the tryptamine containing pastures also exhibited abnormally high respiration rates under conditions of heat stress.

The *in vivo* dry matter digestibilities were found to average (4 sheep, 7 days) 43% for the tryptamine containing forage and 48% for the tryptamine free forage, these values not being significantly different ($P < 0.05$). Average intake values were 0.43 and 0.48 Kg dry matter per sheep per day respectively.

Proximate analysis of the feed of each type of forage revealed low protein and high ash (5.38% crude protein = N x 6.25 12.99% ash average). The low protein value was presumably due to the high proportion of stem material in the forage, and the fact that the grass was growing on clay subsoil.

DISCUSSION

The differing behaviour of the two types of pastures in terms of palatability seems to support the suggestions that tryptamine alkaloids may be at least in part responsible for palatability differences. However the fact that there were

significant differences between clones free of tryptamine alkaloids indicates that these alkaloids are certainly not the entire cause for poor palatability.

The poor weight gains from the sheep in general may be at least partially due to general low palatability and low protein in the forage. The loss in weight of the sheep on the tryptamine alkaloid containing pasture would at first seem to indicate some detrimental effect of these alkaloids, but this observation may be confounded by the interaction with the extremely low palatability clones in this pasture. The lack of pasture replication, short grazing periods, small number of animals, and particularly the lack of control of grazing pressure are great weaknesses in this grazing trial. The weight gain data should therefore only be regarded as an indication of possible harmfulness of the tryptamine alkaloid containing pasture. The extremely high rate of respiration under heat stress observed in the sheep which had been grazing the tryptamine alkaloid containing pasture is a far better indication of the harmful effect of this pasture type.

For breeding a highly palatable tryptamine alkaloid free line the major problem appears to be to separate the palatability component from the alkaloid component, if indeed this is possible. The presence of highly palatable clones in the tryptamine alkaloid containing group would indicate that this is indeed possible, and production of tryptamine free plants has been shown to be quite simple (16).

It has been shown in this publication and elsewhere (13) that management and time of evaluation (17) can affect the palata-

bility ratings quite dramatically. There is evidence (13) that palatability is a heritable trait if measured consistently under the same management conditions. Thus, provided satisfactory methods of forage palatability evaluation can be evolved it seems feasible to produce highly palatable tryptamine free varieties of reed canary grass.

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TABLE I: PALATABILITY EVALUATION OF 30 REED CANARYGRASS ALL CONTAINING TRYPTAMINE ALKALOIDS

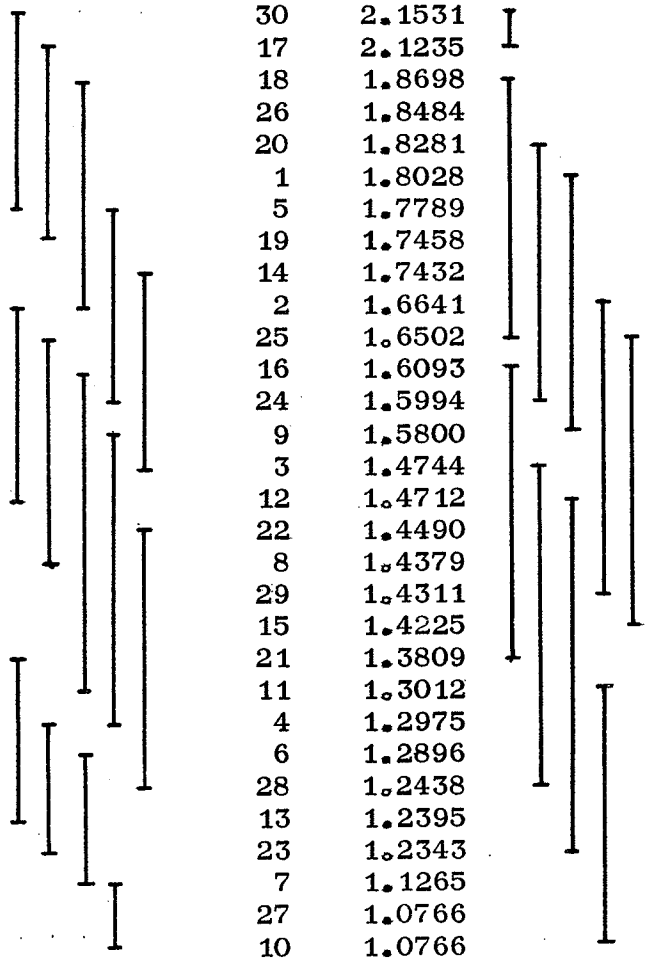
Rank	1st. grazing period			2nd. grazing period		
	plant number	average score	Duncans test 5%	plant number	average score	Duncans test 5%
1	28	2.2092		10	2.3453	
2	8	2.1794		9	2.3452	
3	9	2.1760		8	2.3452	
4	10	2.1424		4	2.3452	
5	2	2.0703		2	2.3452	
6	22	2.0368		19	2.2695	
7	17	2.0212		15	2.1366	
8	26	2.0001		24	2.1207	
9	12	1.8796		17	2.0433	
10	30	1.8393		23	1.9553	
11	15	1.8254		14	1.9499	
12	4	1.7861		22	1.9489	
13	11	1.7528		12	1.9058	
14	19	1.7217		28	1.8912	
15	13	1.7158		20	1.8899	
16	16	1.7102		11	1.8811	
17	27	1.6628		16	1.8026	
18	24	1.5597		18	1.7899	
19	23	1.5551		27	1.7519	
20	1	1.5535		1	1.6745	
21	18	1.5154		26	1.6423	
22	14	1.4961		30	1.6268	
23	21	1.4890		13	1.5685	
24	20	1.3998		25	1.4711	
25	29	1.3788		21	1.3789	
26	25	1.3400		29	1.3650	
27	7	0.9174		7	0.7071	
28	5	0.8435		6	0.7071	
29	6	0.7761		5	0.7071	
30	3	0.7256		3	0.7071	

$S\bar{x}$ 1st. grazing period 0.0536 F = 59.80
$S\bar{x}$ 2nd. grazing period 0.0528 F = 91.42

TABLE II: PALATABILITY EVALUATION OF 30 REED CANARYGRASS

ALL FREE OF TRYPTAMINE ALKALOIDS

Rank	1st. grazing period			2nd. grazing period		
	plant number	average score	Duncans test 5%	plant number	average score	Duncans test 5%
1	15	1.8614		30	2.1531	
2	6	1.8497		17	2.1235	
3	11	1.7756		18	1.8698	
4	16	1.7747		26	1.8484	
5	21	1.7449		20	1.8281	
6	18	1.7216		1	1.8028	
7	2	1.6613		5	1.7789	
8	9	1.6569		19	1.7458	
9	3	1.6301		14	1.7432	
10	10	1.6033		2	1.6641	
11	12	1.5032		25	1.6502	
12	23	1.4760		16	1.6093	
13	30	1.4656		24	1.5994	
14	29	1.4436		9	1.5800	
15	24	1.4347		3	1.4744	
16	22	1.4186		12	1.4712	
17	17	1.3724		22	1.4490	
18	4	1.3515		8	1.4379	
19	20	1.2917		29	1.4311	
20	7	1.2907		15	1.4225	
21	26	1.2843		21	1.3809	
22	14	1.2835		11	1.3012	
23	28	1.2521		4	1.2975	
24	25	1.1986		6	1.2896	
25	8	1.1829		28	1.2438	
26	1	1.0862		13	1.2395	
27	19	1.0815		23	1.2343	
28	5	1.0364		7	1.1265	
29	27	0.8674		27	1.0766	
30	13	0.8596		10	1.0766	



$S\bar{x}$ 1st. grazing period 0.0631 F = 19.36
$S\bar{x}$ 2nd. grazing period 0.0712 F = 16.31

TABLE III: AVERAGE SHEEP GAINS

Kg ± standard error

	Sheep on tryptamine containing forage	Sheep on tryptamine free forage
Grazing period 1	- 2.33 ± 0.59	+ 0.35 ± 0.36
Penned period	- 0.31 ± 0.28	- 0.43 ± 0.57
Grazing period 2	- 1.54 ± 0.24	- 0.41 ± 0.43

TABLE IV: SHEEP STANDING RESPIRATION RATES

Breaths per minute $\pm$ standard error

	Sheep on tryptamine containing forage	Sheep on tryptamine free forage
Day 1*	57 ± 4	52 ± 5
Day 2*	$98 \pm 13^{@}$	60 ± 3

*Conditions were:
Day 1 73°F relative humidity 86% cloudy
Day 2 78°F relative humidity 55% sunny

@Significantly different ($P < 0.05$) from all other observations

REFERENCES

1. Audette, R. C. S., Vijayanagar, H. M., Bolan, J., and Clark, K. W. 1970. Phytochemical investigation of Manitoba plants. I. A new indole alkaloid and associated alkaloids from Phalaris arundinacea. Can. J. Chem. 48: 149-155.

2. Rayment, A. F. 1970. The evaluation of hay and pasture mixtures on Newfoundland peat soils. Canadian Society Agronomy meeting.

3. Wilkinson, S. 1958. 5-Methoxy-N-methyltryptamine: A new indole alkaloid from Phalaris arundinacea L. J. Chem. Soc. 2079-2081.

4. Williams, M., Barnes, R. F., and Cassady, J. M. 1971. Characterization of alkaloids in palatable and unpalatable clones of Phalaris arundinacea L. Crop Science (in press)

5. Woods, D. L. and Clark, K. W. 1970. Variation in the content of tryptamines in clones of Phalaris arundinacea L. Crop Science 11: 121-122.

6. Gallagher, C. H., Koch, J. H., Moore, R. M., and Steel, J. D. 1964. Toxicity of Phalaris tuberosa for sheep. Nature 204: 542-545.

7. Gallagher, C. H., Koch, J. H., and Hoffmann, H. 1966. Poisoning by grass. New Scientist 31: 412-414.

8. Oram, R. N. 1970. Genetic and environmental control of the amount and composition of toxins in Phalaris tuberosa L. Proc. XI Int. Grassland congress 785-788.

9. Vose, P. B. 1959. The agronomic potentialities and problems of the canary grasses, Phalaris arundinacea L. and Phalaris tuberosa L. Herbage Abstracts 29: 1-7

10. Marten, G. C. 1969. Measurement and significance of forage palatability. Proceedings of the National Conference on forage quality evaluation and utilization

11. Brown, J. A. M. 1961. Evaluation of certain morphological and chemical characteristics in relation to palatability in reed canary grass (Phalaris arundinacea L.). Dissertation Abstracts 22: 373.

12. Roe, R. and Mottershead, B. E. 1962. Palatability of Phalaris arundinacea L. Nature 193: 255-256.

13. Asay, K. H., Carlson, I. T., and Wilsie, C. O. 1968. Genetic variability in forage yield, crude protein percentage, and palatability in reed canary grass Phalaris arundinacea. Crop Science 8: 568-571

14. Culvenor, C. C. J., Dal Bon, R., and Smith, L. W. 1964. Occurrence of indolealkylamine alkaloids in Phalaris tuberosa and arundinacea. Aust. J. Chem. 17: 1301-1304.

15. Moore, R. M., Williams, J. D., and Chia, J. 1967. Factors affecting concentrations of dimethylated indolealkamines in Phalaris tuberosa. Aust. J. Biol. Sci. 20: 1131-1140.

16. Woods, D. L., and Clark, K. W. 1971. Genetic control and seasonal variation of some alkaloids in reed canary grass. Submitted for publication.

17. Barnes, R. F., Nyquist, W. E., and Pickett, R. C. 1970. Variation in acceptability and covariation with agronomic characters in Phalaris arundinacea L. Proc. XI Int. Grassland Congress 202-206.

BIBLIOGRAPHY

1. Alivisatos, S. G. A., Papaphilis, A. D., Ungar, F., and Seth, P. K. 1970. Chemical nature of binding of serotonin in the central nervous system. Nature 226: 455-456.

2. Allard, R. W. 1964. Principles of plant breeding. John Wiley and Sons Inc. 485pp.

3. Allison, D. C. 1961. Cytogenetic studies of advanced generations of interspecific hybrids between Phalaris arundinacea L. and Phalaris tuberosa var stenoptera (Hack) Hitch. Dissertation Abstracts 21: 1715.

4. Allison, D. C., and Starling, J. J. 1963. Cytogenetic studies of the BC_1 and BC_2 generations from interspecific hybrids between Phalaris arundinacea and P. tuberosa. Crop Science 3: 154-157.

5. Andrews, O. N. Jr., and Hoveland, C. S. 1965. Apparent palatability of reed canarygrass and harding grass as affected by nitrogen. Agronomy Journal 57: 315-316.

6. Anonymous. 1968. 14th. Annual progress report on agricultural research and experimentation. University of Manitoba.

7. Asay, K. H., Carlson, I. T., and Wilsie, C. O. 1968. Genetic variability in forage yield, crude protein percentage, and palatability in reed canarygrass Phalaris arundinacea. Crop Science 8: 568-571.

8. Audette, R. C. S., Bolan, J., Vijayanagar, H. M., Bilous, R., and Clark, K. 1969. Phytochemical investigation of Manitoba plants. II. A gas liquid chromatographic screening technique for the identification of the alkaloids of Phalaris species. Journal of Chromatography 43: 295-302.

9. Audette, R. C. S., Vijayanagar, H. M., Clark, K. W., and Bolan, J. 1970. Phytochemical investigation of Manitoba plants. I. A new indole alkaloid and associated alkaloids from Phalaris arundinacea. Canadian Journal of Chemistry 48: 149-155.

10. Ayer, W. A., and Browne, L. M. 1970. Alkaloids of Shepherdia argentea and Shepherdia canadensis. Canadian Journal of Chemistry 48: 1980-1984.

11. Baltensperger, A. A., and Kalton, R. R. 1958. Variability in reed canary grass, Phalaris arundinacea L. I. Agronomic characteristics. Agronomy Journal 50: 659-663.

12. Baltensperger, A. A., and Kalton, R. R. 1959. Variability in reed canary grass, Phalaris arundinacea. II. Seed shattering. Agronomy Journal 51: 37-38.

13. Banerjee, P. K., and Ghosal, S. 1969. Simple indole bases of Desmodium gangeticum (Leguminosae). Australian Journal of Chemistry 22: 275-277.

14. Barau, R. and Stout, P. R. 1965. Trans-aconitic acid in range grasses in early spring. Science 150: 766-767.

15. Barnes, R. F., Marten, G. C., and Simons, A. B. 1969. Indole alkaloid derivatives in Phalaris arundinacea L. of varying palatability. American Society of Agronomy annual meeting, in Agronomy Abstracts: p. 57.

16. Barnes, R. F., and Mott, G. O. 1970. Evaluations of selected clones of Phalaris arundinacea L. I. In vivo digestability and intake. Agronomy Journal 62: 719-722.

17. Barnes, R. F., Mott, G. O., O'Donovan, P. B., Plumlee, M. P., and Packett, L. V. 1963. In vivo evaluation of selected reed canary grsss clones. Journal of Animal Science Abstracts 22: 1133-1134.

18. Barnes, R. F., Nyquist, W. E., and Pickett, R. C. 1970. Variation in acceptability and covariation with agronomic characters in Phalaris arundinacea L. Proceedings of the XI International Grassland Congress, Australia: 202-206.

19. Barth, K. M., Vandernoot, G. W., and Cason, J. L. 1959. A comparison of the nutritive value of alfalfa hay with brome grass and reed canary grass at various levels of fertilisation. Journal of Nutrition 68: 383-391.

20. Biely, J. and Kitts, W. D. 1964. The antiestrogenic activity of certain legumes and grasses. Canadian Journal of Animal Science 44: 297-302.

21. Bobbit, J. M. 1963. Thin layer chromatography. Reinhold, New York: 208pp.

22. Bonin, S. G., and Goplen, B. P. 1963. A histological study of seed shattering in reed canary grass (Phalaris arundinacea). Canadian Journal of Plant Science 43: 200-205.

23. Bonin, S. G., and Goplen, B. P. 1966. Heritability of seed yield components and some visually evaluated characters in reed canarygrass. Canadian Journal of Plant Science 46: 51-58.

24. Brown, J. A. M. 1961. Evaluation of certain morphological and chemical characteristics in relation to palatability in reed canary grass (Phalaris arundinacea L.). Dissertation Abstracts 22: 373.

25. Bull, L. B., Culvenor, C. C. J., and Dick, A. T. 1968. The Pyrrolizidine Alkaloids. North Holland Research Monographs, Frontiers of Biology, volume 9: 293pp.

26. Carlson, I. T. 1966. Clonal and topcross evaluation of selections of reed canarygrass. Proceedings of the Xth. International Grassland Congress, Finland: 637-641.

27. Carlson, I. T., Asay, K. H., Wedin, W. F., and Vetter, R. L. 1969. Genetic variability in in vitro dry matter digestibility of fall-saved reed canarygrass Phalaris arundinacea L. Crop Science 9: 162-164.

28. Carnaham, H. L, and Hill H. D. 1956. Cytogenetics of progenies of 35 chromosome reed canarygrass (Phalaris arundinacea L.). Agronomy Journal 48: 356-359

29. Celayeta, F. D. 1960. Action of the tissues of various plants on the growth of Sphacelia segetum. Farmacognosia 20: 91-101. Chemical Abstracts 55 (1961): 5647b.

30. Chalmers, A, H., Culvenor, C. C. J., and Smith, L. W. 1965. Characterization of pyrrolizidine alkaloids by gas, thin layer and paper chromatography. Journal of Chromatography 20: 270-277.

31. Chapulpa, W. V., Cason, J. L., and Baumgardt, B. R. 1961. Nutritive value of reed canarygrass as hay when grown with various nitrogen levels. Journal of Dairy Science 44: 874-878

32. Chu, I-wu, and Knutson, H. 1970. Preference of eight grasshopper species among eleven species of cultivated grasses. Journal of the Kansas Entomological Society 43: 20-31. Biological Abstracts 51 (1970): 63570.

33. Cialzeta, C. 1968. Cytogenetic studies on Phalaris arundinacea L. var Picta L. Obtainment of a new form. Boletin de Genetica del Instituto de Fitotecnia Castelar 5: 7-12.

34. Cohen, G., and Collins, M. 1970. Alkaloids from catecholamines in adrenal tissue: possible role in alcoholism. Science 167: 1749-1751.

35. Cromwell, B. T. 1955. "The alkaloids" in Modern methods of plant analysis. Editors Paech, K., and Tracey, M. V. Volume IV. Springer Verlag: 373-374.

36. Culvenor, C. C. J., Dalbon, R., and Smith, L. W. 1964. Occurrence of indolealkylamine alkaloids in Phalaris tuberosa and arundinacea. Australian Journal of Chemistry 17: 1301-1304.

37. Darlington, C. D., and Wylie, A. P. 1955. Chromosome atlas of flowering plants. George Allen and Unwin Ltd., London. 519pp.

38. Devine, T. E., and Starling, J. L. 1969. Impediments to hybridization of Phalaris arundinacea and P. tuberosa. Crop Science 9: 140-143.

39. Dickmann, S. R., and Crockett, A. L. 1956. Reactions of Xanthydrol. IV. Determination of tryptaphan in blood plasma and in protein. Journal of Biological Chemistry 220: 957-965.

40. Dickman, S. R., and Westcott, W. L. 1954. Reactions of Xanthydrol. I. Amino acids. Journal of Biological Chemistry 210: 481-489.

41. Gaggino, O. P., and Carillo, B. J. 1965. Phalaris staggers IV. Its occurrence in dairy cows. Idia 206: 31-32. Biological Abstracts 47 (1966): 63352.

42. Gaggino, O. P., Carillo, B. J., and Frontera, A. R. 1965. Phalaris staggers III. The forage plant Phalaris tuberosa L. as a cyanogenetic agent. Idia 206: 27-30. Biological Abstracts 47 (1966): 63351.

43. Gallagher, C. H., Koch, J. H., and Hoffman, H. 1966. Disease of sheep due to ingestion of Phalaris tuberosa. Australian Vetinary Journal 42: 279-286. Biological Abstracts 49 (1968): 78584.

44. Gallagher, C. H., Koch, J. H., and Hoffmann, H. 1966. Poisoning by grass. New Scientist 31: 412-414.

45. Gallagher, C. H., Koch, J. H., and Hoffmann, H. 1967. Electromyographic studies on sheep injected with N,N-dimethylated tryptamine alkaloids of Phalaris tuberosa. International Journal of Neuropharmacology 6: 223-228. Biological Abstracts 49 (1968): 2684.

46. Gallagher, C. H., Koch, J. H., and Hoffmann, H. 1967. Deaths of ruminants grazing Phalaris tuberosa in Australia. Australian Veterinary Journal 43: 495-500. Biological Abstracts 49 (1968): 67776.

47. Gallagher, C. H., Koch, J. H., Moore, R. M., and Steel, J. D. 1964. Toxicity of Phalaris tuberosa for sheep. Nature 204: 542-545.

48. Goodman, L. S., and Gilman, A. The Pharmacological basis of therapeutics. 4th. edition, 1794pp. Macmillan 1970.

49. Grant, E. A., and Sallans, W. G. 1964. Influence of plant extracts on germination and growth of eight forage species. Journal of the British Grassland Society 19: 191-197.

50. Harley-Mason, J., and Archer, A. A. P. G. 1958. Use of p-Dimethylaminocinnamaldehyde as a spray reagent for indole derivatives on paper chromatograms. Biochemical Journal 69: 60P.

51. Ho, B. T., McIsaac, W. M., and Tansey, L. W. 1969. Inhibitors of monoamine oxidase IV. 6 (or 8) substituted tetrahydro-β-carbolines and their 9-methyl analogues. Journal of Pharmaceutical Sciences 58: 998-1001.

52. Hughes, H. D., Heath, M. E., and Metcalfe, D. S. 1962. Forages. 2nd. edition, Iowa State University Press. 707pp.

53. Jenkin, T. J., and Sethi, B. L. 1932. Phalaris arundinacea, P. tuberosa, their F_1 hybrids and hybrid derivatives. Journal of Genetics 26: 1-35.

54. Johns, S. R., Lamberton, J. A., Occolowitz, J. L. 1967. 1,5-Dimethoxy-3-(dimethylaminomethyl) indole. The major alkaloid from Gymnacranthera paniculata (A. DC.) Warb var Zippeliana (Miq.) J. Sinclair (Family Myristicaceae).

Australian Journal of Chemistry 20: 1737-1742.

55. Lee, H. J., Kuchel, R. E., and Trowbridge, R. F. 1956. The etiology of Phalaris staggers in sheep. II. The toxicity to sheep of three types of pasture containing Phalaris tuberosa. Australian Journal of Agricultural Research 7: 333-344. Chemical Abstracts 50: 14900f. (1956)

56. Majnarich, J. 1969. Anti-inflammatory and hypocholesterolemic agents from Phalaris arundinacea. U. S. patent 3,420-935. Chemical Abstracts 71 (1969): 105203n.

57. Marten, G. C. 1969. Measurement and significance of forage palatability. Proceedings of the National Conference on Forage Quality Evaluation and Utilization. D1 – D55. Nebraska Centre for Continuing Education (1970).

58. Marten, G. C., and Donker, J. D. 1968. Determinants of pasture value of Phalaris arundinacea L. versus Bromus inermis leyss. Agronomy Journal 60: 703-705.

59. McComb, E. A., Androulidakis, N., and Rendig, V. V. 1969. Paper chromatographic detection and colorimetric determination of some 5-O-substituted tryptamines (3-(2-aminoethyl)indoles), utilizing the formation of xanthylium salts. Journal of Chromatography 40: 125-129.

60. McIsaac, W. M. 1961. Formation of 1-methyl-6-methoxy-1,2,3,4-tetrahydro-2-carboline under physiological conditions. Biochimica et Biophysica Acta 52: 607-609.

61. McWilliam, J. R. 1962. Interspecific hybridization in Phalaris: hybrids between Phalaris tuberosa and the hexaploid race of Phalaris arundinacea. Australian Journal of Agricultural Research 13: 585-598.

62. McWilliam, J. R., and Neal-Smith, C. A. 1962. Tetraploid and hexaploid chromosome races of Phalaris arundinacea L. Australian Journal of Agricultural Research 13: 1-9.

63. McWilliam, J. R., and Shepherd, C. J. 1964. The nature and genetic control of a red anthocyanin pigment in the root merristems of Phalaris (Gramineae). Australian Journal of Biological Sciences 17: 601-608.

64. Mendel, V. E., Crenshaw, G. L., Daker, N. F., and Muniz, R. 1969. Staggers in pastured cattle. Journal of the American Veterinary Medical Association 154: 769-772. Biological Abstracts 50 (1969): 98381.

65. Moore, R. M., Arnold, G. W., Hutchings, R. J., and Chapman, H. W. 1961. Poisoning of Merino sheep on Phalaris tuberosa pastures. Australian Journal of Science 24: 88-89. Biological Abstracts 37 (1962): 23100.

66. Moore, R. M., and Hutchings, R. J. 1967. Mortalities among sheep grazing Phalaris tuberosa. Australian Journal of Experimental Agriculture and Animal Husbandry 7: 17-21. Biological Abstracts 48: 69975. (1967)

67. Moore, R. M., Williams, J. D., and Chia, J. 1966. Effect of environmental factors on alkaloids in Phalaris tuberosa. Proceedings of the Xth. International Grassland Congress, Finland: 524-527.

68. Moore, R. M., Williams, J. D., and Chia, J. 1967. Factors affecting concentrations of dimethylated indolealkamines in Phalaris tuberosa. Australian Journal of Biological Sciences 20: 1131-1140.

69. O'Donovan, P. B., Barnes, R. F., Plumlee, M. P., and Mott, G. O. 1967. Ad libitum intake and digestibility of selected reed canarygrass (Phalaris arundinacea) clones as measured by the fecal index method. Journal of Animal Science 26: 1144-1152.

70. O'Donovan, P. B., Barnes, R. F., Plumlee, M. P., Packett, L. V., and Mott, G. O. 1967. Soluble and structural components of alfalfa (Medicago sativa L.) and selected reed canarygrass (Phalaris arundinacea L.) clones. Agronomy Journal 59: 478-481.

71. Okajima, H., and Smith, D. 1964. Available carbohydrate fractions in the stem bases and seed of timothy, smooth bromegrass, and several other northern grasses. Crop Science 4: 317-320.

72. Oram, R. N. 1970. Genetic and envircnomental control of the amount and composition of toxins in Phalaris tuberosa L. Proceedings of the XIth. International Grasslands Congress, Australia: 785-788.

73. Oram, R. N., and Williams, J. D. 1967. Variation in concentration and composition of toxic alkaloids among strains of Phalaris tuberosa L. Nature 213: 946-947.

74. Packett, L. V., Plumlee, M. L., Barnes, R., and Mott, G. O. 1965. Influence of hemicellulose A and B on cellulose digestion, volatile fatty acid production, and forage nutritive evaluation. Journal of Nutrition 85: 89-101.

75. Phillipson, J. D., and Shellard, E. J. 1966. The correlation between the steriochemistry of some indole and oxindole alkaloids and their behaviour on thin layer chromatograms. Journal of Chromatography 24: 84-92.

76. Poehlman, J. M. 1959. Breeding Field Crops. Holt, New York. 427pp.

77. Randerath, K. 1966. Thin layer chromatography. 2nd. edition. Academic Press. 285pp.

78. Rayment, A. F. 1970. The evaluation of hay and pasture mixtures on Newfoundland peat soils. Canadian Society Agronomy meeting, Ottawa.

79. Rendig, V. V., Welch, R. M., and McComb, E. A. 1970. Variation in indolalkylamine content of individual Phalaris aquatica L. plants. Crop Science 10: 682-683.

80. Robinson, T. 1968. The biochemistry of alkaloids. Springer Verlag, New York. 149pp.

81. Roe, R., Mottershead, B. E. 1962. Palatability of Phalaris arundinacea L. Nature 193: 255-256.

82. Schultes, R. E. 1969. Hallucinogens of plant origin. Science 163: 245-254.

83. Shellard, E. J. (editor). 1968. Quantitative paper and thin layer chromatography Academic Press, London. 140pp.

84. Smith, D. C. 1944. Pollination and seed formation in grasses. Journal of Agricultural Research 68: 79-95.

85. Smith, I. 1960. Chromatographic and electrophoretic techniques. Volume I. Chromatography. Interscience Publishers Incorporated, New York. 617pp.

86. Stahl, E. 1969. Thin layer chromatography - a laboratory handbook. 2nd. edition. Springer Verlag, Academic Press Incorporated. 1041pp.

87. Stecher, P. G., Finkel, M. J., Siegmund, O. H., and Szafranski, B. M. 1960. The Merck Index of Chemicals and Drugs. 7th. edition. Merck and Company, Incorporated, U. S. A. 1642pp.

88. Thompson, J. R. 1965. Yield and seed quality of Phalaris arundinacea L. under certain fertiliser and management practices. Dissertation Abstracts 25: 4898.

89. Thorne, G. 1969. Hypsoperine ottersoni sp. n. (Nemata, Heteroderidae) infesting canarygrass, Phalaris arundinacea (L) reed in Wisconsin. Proceedings of the Helminthological Society of Washington 36: 98-102.

90. Tilley, J. M. A., and Terry, R. A. 1963. A two-stage technique for the in vitro digestion of forage crops. Journal of the British Grassland Society 18: 104-111.

91. Treibs, A. and Henmann, E. 1955. Pyrrole dyes IV. The Ehrlich reaction of pyrroles and indoles. Hoppe Seylers Zeitschrift fur Physiologische Chemie 299: 168-185. Chemical Abstracts 50: 943i.

92. Vogel, A. I. 1966. Practical organic chemistry. 3rd. edition. Wiley, New York. 1188pp.

93. Vose, P. B. 1959. The agronomic potentialities and problems of the canarygrasses Phalaris arundinacea L. and Phalaris tuberosa L. Herbage Abstracts 29: 1-7

94. Wagner, R. P., and Mitchell, H. K. 1955. Genetics and metabolism. 2nd. edition. John Wiley and Sons Incorporated, New York. 673pp.

95. Walker, D. J. 1959. Blood acetylcholineesterase in Phalaris staggers. Nature 184, supplement 18: 1411.

96. Webber, A. J., and Baker, K. R. 1967. Biology of the pseudo root knot nematode Hypsoperine ottersoni. Phytopathology 57: 723-728. Biological Abstracts 50 (1969) 10323.

97. Wilkinson, S. 1958. 5-Methoxy-N-methyltryptamine: A new indole alkaloid from Phalaris arundinacea L. Journal of the Chemical Society: 2079-2081.

98. Williams, M., Barnes, R. F., and Cassady, J. M. 1970. Characterization of alkaloids in palatable and unpalatable clones of Phalaris arundinacea L. Crop Science 11 (in press).

99. Wilson, D. B. 1967. Growth of Hordeum jubatum under various soil conditions and degrees of plant competition. Canadian Journal of Plant Science 47: 405-412.

100. Wolf, D. D. 1967. Yield reductions in reed canarygrass caused by frit fly infestation. Crop Science 7: 239-240.

101. Woods, D. L., and Clark, K. W. 1971. Variation in the content of tryptamines in clones of Phalaris arundinacea L. Crop Science 11: 121-122.

102. Woods, D. L., and Clark, K. W. Genetic control and seasonal variation of some alkaloids in reed canarygrass. Submitted for publication.

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