BEHAVIOR OF ALKALOIDS IN THREE SUCCESSIVE GENERATIONS OF SELECTED DISEASE RESISTANT MARYLAND TOBACCO LINES

by

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INTRODUCTION

The reduction in yield and quality of tobacco brought about by the occurence of tobacco mosaic disease is well known. Quite as well known is the fact that using resistant varieties and strains is one of the best, if not the best, means of controlling this virus disease. Varieties and strains of Maryland tobacco have been bred for resistance to this disease. In introducing the resistance factor to the Maryland type of tobacco, it is necessary that rigorous selection and intensive experimentation be done not only to obtain mosaic resistant plants but to maintain the original type characteristic in the new strains as well.

The Maryland type of tobacco varies from other types in aroma, color and texture. Characteristically different from other types in its mildness and good burning qualities, it contains a low amount of nicotine. Mildness or strength of a product has most always been associated with alkaloidal content. With the desire to maintain the characteristic mildness and strength of the type in the process of breeding for resistance, it became necessary that evaluations for alkaloidal content be made on the breeding lines.

Recent studies on various alkaloids associated with tobacco and related species suggest genetic as well as environmental implications. To determine the extent of variations in quality and quantity of alkaloids within the breeding lines, analyses were made on three successive generations of the current breeding lines.

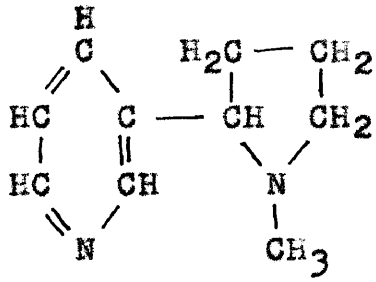
LITERATURE REVIEW

Properties of alkaloids found in the genus Nicotiana

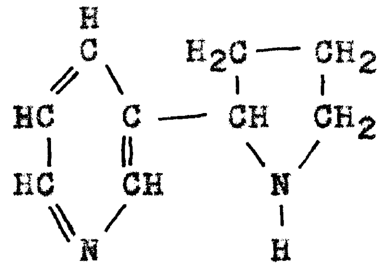
Alkaloids are generally understood to mean complex basic substances, occuring naturally, possessing some physiological reaction and usually derived from pyrrole, pyridine, quinoline, isoquinoline, or similar cyclic nitrogenous nuclei (51). A number of alkaloids are still being discovered and isolated from some species of the genus *Nicotiana*. Definitely known to be associated with *Nicotiana* according to Henry (26) are the following:

1. Nicotoine ------------- $C_8H_{11}N$
2. Nornicotine ----------- $C_9H_{12}N_2$
3. Nicotine -------------- $C_{10}H_{14}N_2$
4. Nicotimine ------------ $C_{10}H_{14}N_2$
5. Anabasine ------------- $C_{10}H_{14}N_2$
6. Iso nicoteine --------- $C_{10}H_{12}N_2$
7. Anatabine ------------- $C_{10}H_{12}N_2$
8. Nicotyrine ------------ $C_{10}H_{10}N_2$
9. Nicotelline ----------- $C_{10}H_8N_2$
10. 2:3′-Dipyridyl --------- $C_{10}H_8N_2$
11. N-Methyl anabasine ---- $C_{11}H_{16}N_2$
12. L-N-Methylanatabine --- $C_{11}H_{14}N_2$

Of these the two most predominant alkaloids in *N. tabacum* are nicotine and nornicotine. Henry (26) and Bonner (5) give the structural formula as:



Nicotine



Nornicotine

The presence of nicotine was first demonstrated by Vauquelin in 1809. Pure nicotine is a colorless oil with a boiling point at 246°C (26). It distills unchanged in a current of steam and is readily soluble in alcohol, ether or light petroleum. When oxidized by $KMnO_4$ or AgO it gives 1-nornicotine. Nornicotine which was first isolated as an alkaloid from *Duboisia hapwoodii* was later identified by Spath in 1936 as nornicotine (51). It is a colorless liquid with a faintly basic odor when pure and boils at 139-140°C (26). Upon oxidation with nitric acid it furnishes nicotinic acid. Both alkaloids form precipitates with silicotungstic acid.

Whether tobacco and its alkaloids are beneficial or harmful to man has long been and still stands as a controversal issue. The insecticidal value of nicotine and nornicotine (31,40) as well as its value as source of nicotinic acid (35) are widely recognized. Although nicotine is no longer of therapeutic interest it is still employed in veterinary medicine (26). Ward (51) cites the claim of O'Shea that to immature smokers nicotine produces a distinct detrimental effect on mental processes and a consistent decline in intellectual ability. On mature people on the other hand, it may be detrimental to some and beneficial to others. While nicotine-free tobacco is shunned by connoisseurs of tobacco products, many do likewise with products of high nicotine content. Garner (21) suggests that free nicotine is correlated with flavor of tobacco and responsible in part at least for the harsh and disagreeable taste. Mildness of a product is accounted for by its low nicotine content (23).

The biosynthesis of the different alkaloids during plant growth and the changes that occur after harvesting are not yet fully understood. Also not definitely established is the role of alkaloids in plants. Ward (51) gives three views on metabolic changes that give rise to alkaloids.

1. As nutritive materials used by the plant in metabolism
2. As protective materials against attacks by plants and animals
3. As end products of metabolism rendered harmless and stored where they are not readily absorbed

Vickery, Pucher, Wakeman and Leavenworth (50) do not regard nicotine as an active metabolite. Frankenburg (20) on the other hand, presents indications that the alkaloids or their transformation products influence the conversions in the leaf, as negative or positive catalysts, as hydrogen donors and acceptors and may have an influence in the oxidation-reduction systems. Dawson (19) has this to say:

> For instance, it may be possible that nicotine originates as a by-product of a number of irreversible and physiologically useless reactions. Or it may be that it is formed from any one of several reasons as a by-product of chemical reactions that do play a role in cell metabo-

> lism. Again, the final steps in the synthesis may involve reactions of use to the cell; or the finished product may itself participate in important activities which are not at present recognized.

In the absence of detectable amounts of nicotine, he found that growth was normal (14).

In a series of classic experiments by Dawson (13-18), he has definitely established the site of nicotine, nornicotine and anabasine synthesis in the plant. Reciprocal grafts of tomato and tobacco proved conclusively that nicotine is synthesized in tobacco roots (14,15). In _N. glauca_ however, he found that excised leaves as well as roots contain and could accumulate anabasine and nicotine suggesting their production in both places independently (16). By way of the xylem vessels, nicotine synthesized in the roots is transported to the leaves where it accumulates (15). New growth on tobacco scion after the graft to a tomato stock was devoid of nicotine (14). This suggests that once nicotine is deposited in the leaf, it is not translocated. When the tobacco leaves are allowed to root however, nicotine accumulation starts again (13). Nicotine synthesis in roots of intact tobacco plants depends on an adequate supply of carbohydrates from the leaves (19). Frankenburg (20) believes that nicotine is synthesized in the root from inorganic salts. When the leaves were detached, accumulation in these organs stopped abruptly. The exact nature of nicotine synthesis is yet to be clarified.

Nornicotine on the other hand presents an altogether different picture. Dawson (17,18) believes that it originates only from nicotine via demethylation. The mechanism of transformation is still unknown but in N. glutinosa, the demethylation was found to take place in the leaves. Bonner (5) suggests the presence of a methyl acceptor in the leaves of high nornicotine strains capable of receiving the methyl group of nicotine. Of the synthesis of the other alkaloids, very much less is known about them.

Genetics of the alkaloids

The three predominant alkaloids nicotine, nornicotine and anabasine are not peculiar to the genus *Nicotiana*. According to Smith and Smith (44), anabasine has been found in a genus of the family *Chenopodiaceae*. Nicotine was isolated from a species belonging to the family *Asclepiadaceae* and nornicotine from some other solanaeceous plants. *Nicotiana* as a rule contain preponderant quantities of one or two alkaloids and extremely small amounts of the others (20). Of the 29 wild species of *Nicotiana* examined by Smith and Smith (44), five apparently contained only nornicotine, four only nicotine, two with mixtures of anabasine and nicotine, and the rest were mixtures of nicotine and nornicotine. The predominant alkaloids in some of the species are as follows:

Species	Main alkaloid	Secondary alkaloid
N. glauca	anabasine	nicotine
N. glutinosa	nornicotine	none
N. longiflora	nicotine	nornicotine
N. sylvestris	nornicotine	nicotine
N. tomentosa	nornicotine	none

Interspecific hybrids between some of them and with N. *tabacum* presented an interesting and complex genetic behavior. The same authors found that genetic factors for anabasine formation are partly dominant over those controlling nicotine in the F_1 of N. *tabacum* x N. *glauca*. Partial dominance of nornicotine over nicotine was noted in N. *glutinosa* x N. *tabacum* and N. *tomentosa* x N. *tabacum*. They further concluded that the predominant alkaloid now may become the secondary in the later generations and vice versa. Results obtained by some Russian investigators on interspecific hybrids carried beyond the first generation are summarized by Smith and Smith (44) as follows:

1. Some segregants were produced that contained only one or the other of the alkaloids involved in the cross, while the remaining segregants contained mixtures of the two in different proportions.
2. A secondary alkaloid that is present in small amounts in one parent may become the chief alkaloid in some individuals of a hybrid or inbred family.
3. No obvious Mendelian ratios were obtained in families segregating for different alkaloids, nor would they be expected, since the interspecific hybrids and extra chromosomal forms all show irregularities at meiosis.

Valleau (47) presented further evidence that nicotine-nornicotine inheritance is genetically controlled. It was possible then to develope strains with high nicotine yield (43) and low nicotine strains (24). Selective breeding now in progress is probably changing the predominance of nicotine (32). It was noticed by Valleau (47) and Markwood (32) that on samples containing very small quantities of total alkaloids, there is increased probability that much of it will be nornicotine.

Variations in alkaloidal content both quantitatively and qualitatively between types, varieties and strains may be attributed in part to genetic differences as influenced by different standards of selection for each one. In selecting and breeding cultivated strains, desirable characteristics such as vigor, size of the plant, and high content of aromatic substances were aimed at. Following the development of these features, Frankenburg (20) believes that specific productivity for alkaloids is automatically increased. He includes Robinson Maryland Medium Broadleaf and Cash Flue Cured varieties in the category of mostly nornicotine tobaccos. According to Bacon (4), cigar type tobaccos generally have a higher alkaloidal content than cigarette types. Chewing tobaccos have higher content than smoking grades (24). Markwood (33) has shown differences between cigarette varieties within that type. Aside from heritable variations, other factors contribute to quantitative differences between types and between plants of the same variety or strain.

Other factors influencing alkaloidal content

The alkaloidal content of an individual plant is controlled by limitations imposed by heredity, environment of the plant, and the treatments and conditions to which the plant, the leaf or the products are exposed (20). Wide yearly variations in nicotine content were noted by Garner (22) and McMurtrey, Bowling, Brown, and Engle (36). The leaf produced during the dry season were small, dark and dull in color, high in nicotine and lacking in elasticity. Moisture relations appear to be the dominant factor in this respect. Plants grown without irrigation or when water was applied late in the growing season had higher nicotine content than plants grown under continous irrigation. This paralleled the effect on nitrogen content. Coupled with the effects of moisture is the availability of nutrients in the soil. Studies made by Woodsmansee, Rapp and McHargue (52), Ward (51), McMurtrey, Bacon and Ready (35) and Garner (22) showed that within certain limits, the kind of fertilizers and the proportion and rates of their application can very well vary the nicotine content of an individual plant. Related to nutrient availability is the effect of spacing. Wider spacing tends to increase nicotine content within certain limits, depending upon the variety (35). Several investigators have reported that nicotine content of isolated leaves can be increased by the application of nicotinic acid (12) and proline (30).

Very well demonstrated by many investigators (4,35, 52) is the fact that topping and suckering tend to increase the alkaloidal content of the mature crop. As a rule, the earlier the topping and the less number of leaves left in the plant, the higher the alkaloidal content. McMurtrey, Bacon and Ready (35) also found that late seeding as well as late transplanting distinctly lowered the yield of nicotine per acre from a crop of N. rustica.

Nicotine content is known to vary not only between the leaves, roots and stem of a plant but also between leaves at different positions on a plant (4,22). The latter is reflected by the stage of maturity of the leaves at the time of harvest. According to Vickery et al (48,49), the general pattern of accumulation begins in seedlings 9-11 days after germination. By transplanting time, differential nature of alkaloid distribution becomes apparent. Beginning at an early stage the percentage of nicotine continues to increase regularly throughout the growing season in all parts of the plant until maturity is reached (39,42). According to Dawson (13) and Mothes (38), in the absence of senescent changes, the total nicotine content per leaf decreases with the increase in height of the leaf position in the stalk. Garner (9) noted however an increase in nicotine content as the leaf got mature. Beyond maturity, a decrease follows. The most rapid rate of overall nicotine accumulation may occur at a time in the life of a plant when growth in terms of dry weight increase has all but ceased (49). The above discussions would serve to explain in part why some investigators (2,9,52) are not consistent as to which group of leaves in a single plant has the highest nicotine content. In a single leaf alone, Bacon (4) and Cicerone and Marocchi (10) showed that there is an increasing concentration of nicotine from center to margin and from base to apex.

During the curing and fermentation of tobacco, Metsapa (37) found that practically all organic matter in the leaf undergoes a change and the complex organic substances break down into simpler forms. Nicotine is also included in this process and it has been shown that among other things, ammonia is one of its breakdown products. This fact was further substantiated by Garner (22). Furthermore, Jeffrey (29) noted a decrease in alkaloidal content in samples that have been in storage for one year. He has reason to believe that a third alkaloid aside from nicotine and nornicotine was present in the samples analyzed.

Effect of tobacco mosaic virus on tobacco

Tobacco mosaic disease affects all parts of the plant except the seeds. Allard (1) in his article gives a full description of symptoms expressed by the virus. McMurtrey (34) has shown a marked decrease in quality and yield on the mosaic infected crop particularly when the virus has established itself early in the field. Silberschmidt (41) found that injection of mosaic virus raised the percentage of nicotine quite considerably. Virus infection, according to Hills and McKinney (27), causes a marked increase in total nitrogen of susceptible lines and hybrids but a decrease in chlorophyll amount due to a low activity of the enzyme chlorophyllase. They detected no change in resistant varieties.

Resistance to this disease has been found in several species of Nicotiana and a South American variety of tobacco, Ambalema. The latter has practically been abandoned as a source of resistance due to undesirable factors closely linked with the resistance factor. Breeders have focused their attention on N. glutinosa as a very promising source of resistance to tobacco mosaic. Classic experiments by Clausen and Goodspeed (11) showed how the resistance factor was transferred from N. glutinosa (n=12) to N. tabacum (n=24) by way of a fertile amphidiploid N. digluta (n=36). Further studies by Holmes (28) confirmed the findings of Allard (1) and Clausen and Goodspeed (11) that a single dominant gene controls the necrotic type response of N. glutinosa to tobacco mosaic infection. The present mosaic resistant strains of Maryland tobacco owe their resistance to N. glutinosa.

Methods of analysis for alkaloids

Various methods for quantitative and qualitative analysis for alkaloidal content are being used on tobacco samples. A better understanding about tobacco alkaloids at present has rendered some of these methods obsolete while others had to be improved upon to give speedy, more accurate and reproduceable results. One of the less expensive methods requiring a not too complicated apparatus is the one used by Garner, Bacon, Bowling and Brown (23). It works on the principle that gasoline or ether extracts nicotine from plant materials made strongly alkaline. The amount is quantitatively determined by titrating the excess acid that did not react with the nicotine in the extract. In samples containing other alkaloids besides nicotine, Bowen and Barthel (7) found difficulty in completely removing the alkaloids from alkaline plant material by repeated extraction with ether. Jeffrey (29) found that Garner's method could not be taken to give total alkaloids or even the nicotine fraction in samples with mixed alkaloids. When employed on tobacco high in nicotine however, results are comparable with other more accurate methods.

Steam distillation coupled with precipitation of the alkaloids with silicotungstic acid is the basis of recent methods being used for alkaloid analysis. Alkaloids are steam volatile and form insoluble precipitates with silicotungstic acid. This is the method adopted by the A.O.A.C. (3). Several modifications on the procedure and apparatus used were made to reduce the size of sample and the time of distillation. With their distilling apparatus, Bowen and Barthel (8) were able to reduce the sample size to two grams and the distilling time to 30 minutes. Griffith and Jeffrey (25), with their improved distillation apparatus assures completeness of extraction using a sample size as low as 0.5 gram and as short a distilling time as three minutes per sample. The accuracy of steam distillation is a function of the nicotine-nornicotine ratio and the alkalinity of the material when being distilled (6,25). Bowen (6) recommends the use of a strong NaOH + NaCl instead of a slight excess in making the material alkaline. According to Griffith and Jeffrey (25) however, that should be recognized as total steam volatile alkaloids and not nicotine alone since other alkaloids will be present in the distillate. Bowen and Barthel (7) later improved on their method. This involved the determination of total alkaloids first, then the nicotine fraction; the difference between the two is considered as nornicotine. This is based on the assumption that in N. tabacum the other alkaloids if present are present only in negligible amounts.

In a comparison of various methods of alkaloid analysis, Jeffrey (29) showed that the methods, including ultraviolet spectroscope determination, agree quite closely on samples high in nicotine. In samples with mixed alkaloids particularly those high in nornicotine such as the Maryland Medium Broadleaf, the results given by the different methods varied quite considerably.

Paper chromatography is one other method that can be used for alkaloid analysis. The method as applied to tobacco samples is still under development and is far from being able to give a complete picture as yet of the different alkaloids present. Using only cured samples of

Robinson Maryland Medium Broadleaf, Tso and Jeffrey (46) recently separated 24 substances from that sample by solvent extraction and paper chromatography. So far, eight of these have been identified. Given below is the alkaloidal content of the sample.

Alkaloid	Amount in per cent
Nornicotine	1.42
Nicotine	0.47
Anabasine	0.27
Myosmine	0.14
Nicotinic acid	0.08
Oxynicotine	0.05
2,3 dipyridyl	0.006
3-acetyl pyridine	0.003
Unknown "alkaloids"	1.55
Estimated total "alkaloids"	3.99

MATERIALS AND METHODS

Materials used

The standard varieties of Maryland tobacco are Robinson and Wilson. The other lines used in this experiment are from the breeding materials of Dr. E. E. Clayton of the U. S. Department of Agriculture. The identity of the lines are given in table 1. The factor for resistance to ordinary tobacco mosaic was inherited from N. glutinosa, wildfire resistance from N. longiflora and root rot resistance from Burley. The current breeding lines are selections made after several generations of breeding with disease resistance as the primary basis for selection. Evaluations on their performance in the field was also taken into consideration.

To afford a better measure of genetic variability on their alkaloidal content, the performances of the lines were followed for three generations. In the 1952 plantings were included some seedlings of the same generation as the 1950 crop. For this purpose, RWM 16 and RWM 17 were used. This was meant to serve also as a measure for seasonal effect on alkaloidal content. Inoculations of mosaic virus were made on the same strains including Robinson to determine its effect on alkaloidal content.

The plants were grown at the University of Maryland Tobacco Experimental Farm near Upper Marlboro. The soil, loamy fine sand of the Monmouth series, is typical of the

Table 1. Identity of the selected lines.

Line number	Breeding line 1/	Number of plots 1950	1951	1952	Description or kind of resistance
1	Robinson	3	4	3	Standard check
2	Wilson	-	3	2	Check
3	Md. Mo. 2 A	3	4	1	Mosaic
4	RWM 16	2	4	3	Mosaic, wildfire, root rot
5	RWM 17	3	3	3	Mosaic, wildfire, root rot
6	Md. 9-12 B	1	3	3	Mosaic, wildfire, root rot
7	Md. 9-13 A	1	4	3	Mosaic, wildfire, root rot
8	Md. 9-40 B	1	2	3	Mosaic
9	Md. 9-45 A	1	4	2	Mosaic
10	Md. 9-51 A	1	4	3	Mosaic
11	Md. 9-16 E	1	3	3	Mosaic, wildfire
12	Md. Mo. 0-5	-	4	2	Mosaic
13	Bdlf. 97 A 1	-	4	3	Mosaic, wildfire
14	Bdlf. 97 A 2	-	4	3	Mosaic, wildfire
15	Bdlf. RM 0-23	-	3	2	Mosaic, root rot
16	Bdlf. RM 0-25	-	4	3	Mosaic, root rot
17	Bdlf. RWM 0-8	-	3	3	Mosaic, wildfire, root rot
18	Bdlf. RWM 0-10	-	3	3	Mosaic, wildfire, root rot
19	Bdlf. RWM 0-12	-	3	3	Mosaic, wildfire, root rot
1*	Robinson	-	-	3	Standard check
4*	RWM 16	-	-	2	Same generation as the 1950 crop
4	RWM 16	-	-	3	
5*	RWM 17	-	-	1	
5	RWM 17	-	-	1	

1/ Identifying numbers and letters correspond to that of the breeder. All lines are of the Maryland type of tobacco.

* Inoculated with the mosaic virus.

land where a great bulk of the Maryland tobacco is grown. Plots were 15' x 36' in dimension. There were 14 plants to a row, 5 rows to a plot. This gave a planting distance of 36" between rows and 30" within the row.

Rainfall data obtained from records at the experimental farm are presented in table 2.

Cultural operations

Except for the inoculations made on some plots, all the plots received identical treatments. Fertilization was done at the rate of 1000 pounds of 4-8-12 per acre. Other cultural procedures as well as curing and grading were performed according to the best practices followed for growing Maryland type of tobacco in that region. The dates of the various operations are given below.

Operation	1950	1951	1952
Transplanting	June 21-22	June 13-19	June 20-30
Fertilization	(Before or at transplanting time)		
Topping	Sept. 9	Aug. 20	Sept. 20-25
Harvesting	Sept. 25-29	Sept. 1	Sept. 30
Stripping and grading	December	December	December

Preparation of the samples

After the air-cured leaves were stripped from the stalks and graded, random samples were pulled from each grade of each plot. The samples were stemmed, ground in a 60" mesh Wiley mill and stored in sealed tin containers. The moisture content of each ground sample was determined by oven drying a gram sample for 3 hours at 100°C. The per cent moisture was calculated from the loss in weight

Table 2. Biweekly rainfall at the Tobacco Experimental Farm, Marlboro, Maryland during the three summer periods, in inches.

Periods	1950	1951	1952
June 1-14	1.54	4.88	.81
June 15-28	.65	1.02	1.20
June 29-July 13	2.68	1.05	3.17
July 14-27	3.54	1.59	.49
July 28-Aug. 10	.60	2.68	2.40
Aug. 11-24	2.28	.82	2.35
Aug. 25-Sept. 7	.92	1.97	4.32
Sept. 8-21	6.39	.63	.43
Sept. 22-30	1.10	.00	.88
Total	19.70	14.64	16.05

after drying. Alkaloidal content presented in this paper are all on dry weight basis.

For comparison of alkaloidal content between cured and green samples, 2 plants from a plot of the Robinson strain and 2 plants from a plot of line 8 were picked. With leaf number 1 as the lowest leaf in a standing plant, leaf number 1 and every third leaf thereof were picked right after harvest and analyzed for alkaloidal content. The rest, still on the stalk, were allowed to cure in the barn. Since one leaf would not be sufficient for alkaloidal and moisture analysis, leaves 1 and 4 were grouped together as well as 7 & 10, 13 & 16, and 19 & 22. Likewise in the cured leaves, 2 & 3, 5 & 6, 8 & 9, 11 & 12, 14 & 15, 17 & 18, and 20 & 21 were analyzed in groups of two. For comparison, groups 2 & 3, 8 & 9, 14 & 15, and 20 & 21 in the cured leaves were meant to correspond approximately to groups 1 & 4, 7 & 10, 13 & 16, and 19 & 22 respectively in the green samples.

Methods of analysis

Because of the easy accessibility and inexpensiveness of chemicals and equipment used in the gasoline extraction method for nicotine determination, it was tried on the 1950 crop. This method is fully described by Garner, Bacon, Bowling and Brown (23). In this experiment however, petroleum ether was used instead of gasoline. Results obtained by this method did not warrant its use in the next two generations.

The other method used on cured samples was developed by Bowen and Barthel (7). It was modified for use in Griffith and Jeffrey's (25) improved distillation apparatus. (See fig. 1). An electrical heater was installed around each still to prevent frothing and excessive condensation of steam in the distillation chamber. The steam trap is connected to a suction flask by a capillary tube. This setup not only prevents water condensed from steam from entering the distillation chamber but serves also as a safety valve for excessive steam pressure. With a steam pressure of about 3.5 pounds per square inch, the average rate of distillation was 3 minutes per sample. The distillate volume was about 180 cc. The method consists essentially of two parts: the analysis for total alkaloids and the analysis for nicotine. The nornicotine constituent is the difference between the two. The procedure used was as follows:

> Approximately a gram sample in a measuring spoon was introduced into a distilling chamber after 0.5 grams of NaCl had been introduced. Particles of the sample sticking on the spoon were washed into the distillation chamber. Two cubic centimeters of 30 per cent NaOH were added into the chamber. The chamber was closed with a rubber stopper and the stopcock opened to start the distillation. The distillate was received in a beaker containing 3 cc of HCl (1 to 4). After 180 cc of the distillate were collected, an additional 10 cc portion was tested with a drop of 12 per cent silicotungstic acid for completeness of distillation. The distillate was then brought to 200 cc volume, half of which was to be tested for total alkaloids and the

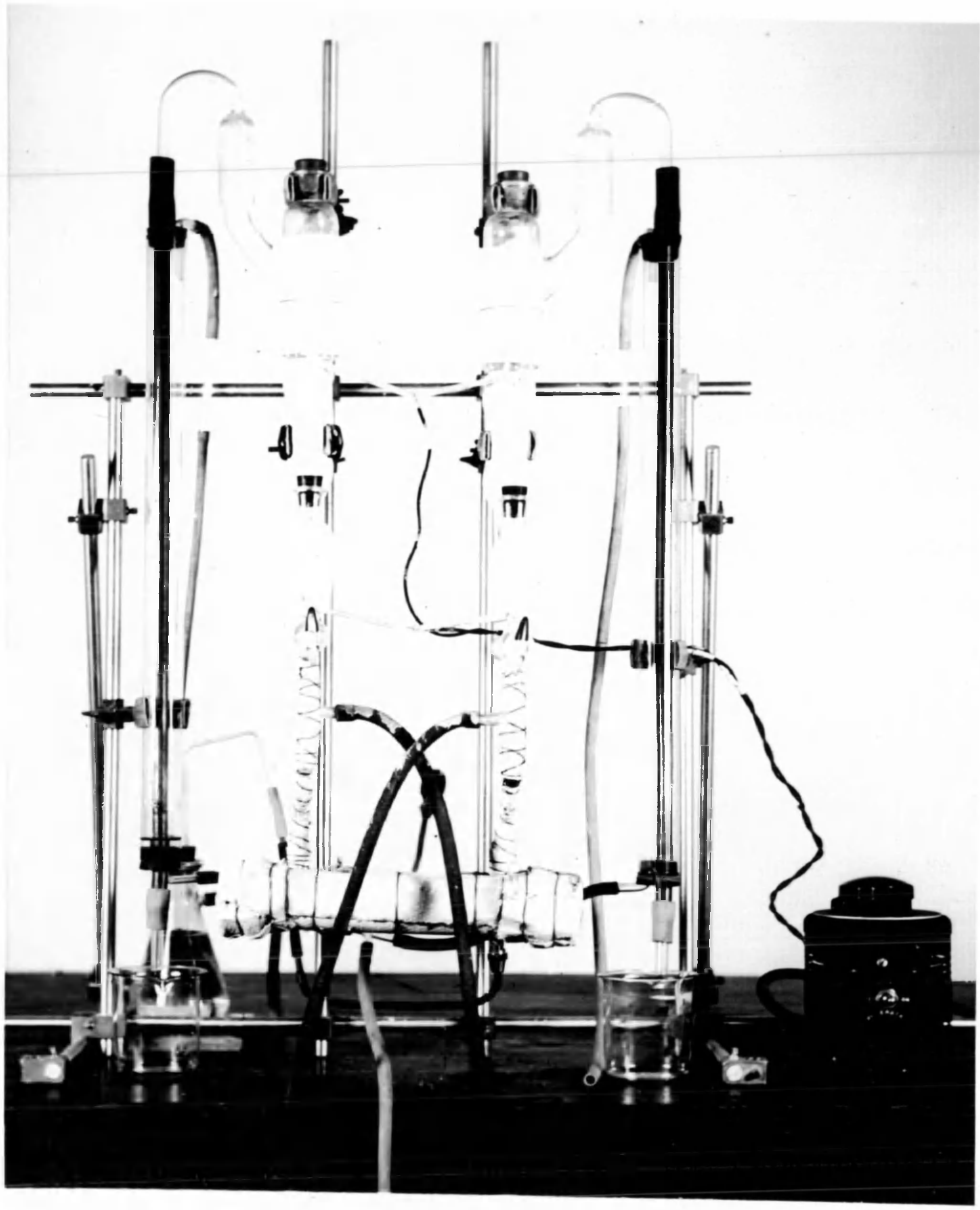


Fig. 1. Steam distillation apparatus for tobacco alkaloid determinations

other half for the nicotine portion.

The alkaloids in the first 100 cc portion were precipitated by the addition of 2 cc of 12 per cent silicotungstic acid and placed on a hot plate till the precipitate settled. It was then cooled to room temperature, tested with a drop of the acid for completeness of precipitation, and placed in a refrigerator at 7°C overnight. The precipitate was filtered the next day through a tared medium porosity Selas crucible; dilute HCl (1 to 2000) was used in making the transfer and washing the precipitate free from silicotungstic acid. The precipitate was ignited in a muffle furnace at 650°C for one hour. After having cooled to room temperature in a dessicator, the crucible was weighed and the weight of oxide A determined.

The other 100 cc portion was evaporated to about 5 cc volume on a hot plate. After cooling to room temperature, 0.5 grams of $NaNO_2$ and 2 cc of 30 per cent CH_3COOH were added to nitrosify the nornicotine so that it would not distill over with the nicotine. The mixture was steam distilled again but instead of using NaOH, a gram of MgO was introduced into the chamber to make the distilland just slightly alkaline. The steps following this were identical to the steps followed in the first 100 cc portion. The oxide obtained here was designated as oxide B.

Oxide B multiplied by the factor 0.1140 gives half of the nicotine portion of the sample. The difference between oxide A and oxide B represents half of the nornicotine portion when multiplied by the factor 0.1042. The per cent total steam volatile alkaloids is the sum of the nicotine and nornicotine content in per cent.

Since this method utilizes the nitrosification of nornicotine as the only basis for separation between nicotine and nornicotine, it follows that of the other steam volatile and silicotungstic acid precipitable alkaloids, those that could be nitrosified would be included as part of the nornicotine fraction while the rest would fall under the nicotine portion.

On green samples, determinations followed the general procedure employed in steam distilling cured or ground samples. The difference lies in the preparation of the sample for distillation. For fresh samples, the procedure was as follows:

> A 30 gram weight of fresh green leaf sample and 150 cc of acetone were mixed in a Waring blendor for 5 minutes. After filtration, the extract was brought to 250 cc volume with acetone. (At this point, the extract may be stored in a refrigerator for as long a period as two weeks.) The extract volume was reduced to 50 cc by vacuum distillation. An aliquot portion of 10 cc was taken and placed in a beaker. Two drops of concentrated H_2SO_4 were added. The acetone was evaporated off on a hot plate. From here on, the procedure followed that of dry samples.

In both methods of analysis, duplicates were run on each sample. Another set was run in cases when duplicates did not check closely enough. The average of the two determinations was taken as the final figure for each sample. The per cent alkaloids for each plot were computed as weighted averages, taking into consideration the proportion of grades in each individual plot. In the case of the ground samples, the amount introduced into the distillation chamber was accurately determined by difference using an analytical balance.

The alkaloid contents of the 1950 crop were obtained from a previous unpublished work (53) of the author of of this paper.

EXPERIMENTAL RESULTS

The four leaf grades are "Farmer's grades" and not standard market grades for Maryland tobacco. In the process of stripping the cured leaves from the stalk, the leaves are sorted into these four grades. The basal leaves of a standing stalk constitute the seconds, the next few leaves are graded as brights, then the next few are called dull brights and the uppermost leaves are designated as dulls. To obtain then more accurate and representative values for individual plots in their alkaloidal content, the distribution of the cured leaf weights in the leaf grades had to be taken into consideration.

<u>Proportion of the different leaf grades in the lines</u>.

Presented in tables 3a, 3b and 3c are the distribution of the cured leaf weights in the leaf grades of each line for each year. The figures for each line are averages of their corresponding number of plots or replicates. In each of the three years, differences between grades were found to be highly significant. However, the trend of distribution was not consistent between the three years (see L.S.D. values in tables 3a, 3b and 3c). In the 1950 crop, almost half of the cured leaves were graded as brights; the dulls made up the smallest portion. The 1951 crop presented an even distribution more or less. In the third crop year, the bulk of the cured leaves

Table 3a. Distribution of cured leaf weights in the leaf grades of the lines grown in 1950.

Lines	Leaf grades				Acre yield
	Seconds	Bright	Dull bright	Dull	
	per cent	per cent	per cent	per cent	pounds
1	22.7	41.6	21.9	13.8	916
3	26.5	35.6	23.3	14.6	1050
4	19.1	42.9	21.4	16.6	1148
5	17.8	51.1	20.6	10.5	1001
6	15.8	45.6	20.9	17.7	1039
7	14.4	43.9	28.8	12.9	1072
8	15.7	51.7	21.4	11.2	979
9	14.7	53.8	21.0	10.5	983
10	15.3	49.2	19.9	15.6	1013
11	23.3	47.8	18.7	10.2	1059
Av.	18.53	46.32	21.79	13.36	1026

The least significant difference between grades at 5% level: 3.617

Table 3b. Distribution of cured leaf weights in the leaf grades of the lines grown in 1951.

Lines	Leaf grades				Acre yield
	Seconds	Bright	Dull bright	Dull	
	per cent	per cent	per cent	per cent	pounds
1	23.7	24.0	25.4	26.9	741
2	23.8	33.1	19.3	23.8	1215
3	20.9	28.9	21.4	28.8	871
4	22.6	28.9	19.9	28.6	1148
5	20.0	28.0	23.6	28.4	1192
6	18.1	33.5	21.7	26.7	1018
7	15.9	35.9	25.0	23.2	1060
8	23.1	22.9	28.0	26.0	922
9	27.0	23.6	22.0	27.4	825
10	21.6	37.4	22.1	18.9	848
11	25.8	27.8	23.1	23.3	932
12	23.0	30.5	23.3	23.2	1005
13	22.1	32.8	17.5	27.6	1035
14	18.7	36.8	19.4	25.1	1129
15	22.8	26.7	20.2	30.3	1144
16	24.1	30.7	20.8	24.4	1146
17	20.4	27.8	19.3	32.5	1228
18	21.6	28.0	21.8	28.6	1110
19	23.8	30.9	19.6	25.7	1131
Av.	22.05	29.91	21.76	26.28	1037

The least significant difference between grades at 5% level: 2.079

Table 3c. Distribution of cured leaf weights in the leaf grades of the lines grown in 1952.

Lines	Leaf grades				Acre yield
	Seconds	Bright	Dull bright	Dull	
	per cent	per cent	per cent	per cent	pounds
1	20.2	25.7	30.4	23.7	932
2	22.6	29.8	24.6	23.0	1285
3	16.7	5.2	45.8	32.3	660
4	20.2	19.9	23.8	36.1	818
5	27.3	15.9	24.7	32.1	866
6	17.5	17.4	25.8	39.3	826
7	20.0	28.0	21.4	30.6	917
8	26.2	18.3	28.8	26.7	893
9	27.5	24.7	21.9	25.9	1082
10	25.8	30.4	19.9	23.9	1055
11	22.8	13.1	26.3	37.8	877
12	22.1	32.5	22.0	23.4	1046
13	22.8	23.2	25.8	28.2	1045
14	25.7	13.3	27.6	33.4	1179
15	16.8	35.7	22.6	24.9	1093
16	23.8	22.9	30.4	22.9	1072
17	19.9	15.1	24.1	40.9	850
18	24.1	17.4	20.1	38.4	817
19	22.8	32.1	23.9	21.2	1047
Av.	22.36	22.14	25.78	29.72	966

The least significant difference between grades at 5% level: 3.972

shifted to the grades constituting the upper leaves. The inconsistencies existed not only between years but also between lines and between replicates. The differences however, were not significant. A great variability between the replicates in their trend of distribution was noted. Although each line showed a characteristic trend, differences between the lines were not significant due to wide variabilities between the replicates. Several factors are involved in the resulting variabilities in trend among which are genetic, environmental (soil and climate), cultural and human factors. The effect or contribution of each factor to the variations in trend are discussed later.

Petroleum ether extraction versus steam distillation.

The ether extraction method for quantitative analysis of nicotine was widely used during the past few years. Although it has largely been replaced by better methods, it is still being used in routine analysis. Several investigators (7,29) have shown the inaccuracy of the ether extraction method particularly on tobacco with mixed alkaloids. It was found quite satisfactory on samples with high nicotine content. Table 4a gives a comparison between this method of analysis and the modified steam distillation process as proposed by Bowen and Barthel (7). On Maryland tobacco, it was found in this experiment that the ether extraction method gave values that represented neither the nicotine, the nornicotine nor the total alkaloid content of the sample. Assuming that all the nicotine was included in the ether extraction method, about 60 per cent of the nornicotine portion was included too. This was in accordance with Jeffrey's (29) statement that approximately half of the nornicotine is included in the ether extraction method. For alkaloid analysis in this experiment then, further use of this method on the succeeding generations was deemed unnecessary.

<u>Alkaloidal content in the cured leaves</u>.

Tables 4a, 4b and 4c show the alkaloidal content of the individual plots of the lines grown in 1950, 1951 and 1952 respectively. It would be noticed that the nicotine, nornicotine and total alkaloids did not vary as much between replicates as it did between the lines within the years. Highly significant differences were obtained between lines within the year in their total alkaloids (see L.S.D. values in tables 4a, 4b and 4c). Lines 1 (standard check), 4, 5, 8 and 9 were the low total alkaloid containing lines during the first year while lines 3, 6, 7, 10 and 11 had relatively high contents. This was true also for the second and third years. In table 4d is summarized the average total alkaloids of each line for the three years, of each line with all three years taken as a whole and of the three years

Table 4a. Alkaloidal content of the cured leaves from plots of lines grown in 1950 using two methods of analysis, expressed as per cent of the dry weight.

Lines	Plot number	Nicotine by ether extraction	Steam distillation			Nornicotine in ether extraction *
			Nicotine	Nornicotine	Total	
		per cent	per cent	per cent	per cent	per cent
1	15	.887	.390	1.021	1.411	48.68
	30	1.297	.764	.864	1.628	61.69
	80	1.047	.359	1.105	1.464	62.26
	Av.	1.077	.504	.997	1.501	57.54
3	10	2.061	1.680	.514	2.194	74.12
	22	2.513	2.096	.593	2.689	70.32
	71	2.583	2.414	.332	2.746	50.90
	Av.	2.386	2.063	.480	2.543	65.11
4	24	2.140	1.880	.424	2.304	61.32
	67	2.167	1.841	.447	2.288	72.93
	Av.	2.154	1.861	.435	2.296	67.13
5	17	1.733	1.640	.388	2.028	23.97
	100	1.126	.945	.364	1.309	49.73
	108	1.842	1.497	.468	1.965	73.72
	Av.	1.567	1.361	.406	1.767	49.11
6	119	2.325	2.008	.522	2.530	41.57
7	120	2.499	2.117	.622	2.739	61.41
8	126	1.001	trace	1.676	1.676	59.73
9	73	1.830	.860	1.398	2.258	69.38
10	74	3.114	2.512	.837	3.349	71.92
11	79	2.612	2.175	.644	2.819	67.86

* Assumed that all the nicotine is included in the ether extraction method.

The least significant differences between the lines on total alkaloids at 5% level:

Lines with three replicates	.539
Lines with two replicates	.660
Lines with one replicate	.934

Table 4b. **Alkaloidal content of cured leaves from plots of lines grown in 1951**, expressed as per cent of the dry weight.

Lines	Plot number	Steam distillation: Nicotine	Steam distillation: Nornicotine	Steam distillation: Total
		per cent	per cent	per cent
1	10	.321	.669	.990
	50	.042	.671	.713
	77	.179	.983	1.162
	86	.422	.945	1.367
	Av.	.241	.817	1.058
2	7	2.045	.476	2.521
	49	1.147	.384	1.531
	67	1.647	.408	2.055
	Av.	1.613	.423	2.036
3	16	1.533	.327	1.860
	42	1.124	.353	1.477
	65	1.500	.404	1.904
	97	1.469	.412	1.881
	Av.	1.407	.374	1.781
4	17	1.022	.181	1.203
	54	.913	.253	1.166
	104	.847	.255	1.102
	61	1.486	.374	1.860
	Av.	1.067	.266	1.333
5	12	1.071	.354	1.425
	36	1.440	.416	1.856
	75	1.380	.295	1.675
	Av.	1.297	.355	1.652
6	1	2.815	.600	3.415
	41	2.058	.637	2.695
	89	1.753	.600	2.353
	Av.	2.209	.612	2.821
7	18	1.862	.398	2.260
	33	2.033	.686	2.719
	94	1.590	.434	2.024
	102	1.797	.585	2.383
	Av.	1.821	.526	2.347
8	38	.165	1.023	1.188
	69	.136	1.137	1.273
	Av.	.151	1.080	1.231

Table 4b. Alkaloidal content of cured leaves from plots of lines grown in 1951, expressed as per cent of the dry weight. (Cont'd)

Lines	Plot number	Steam distillation		
		Nicotine	Nornicotine	Total
		per cent	per cent	per cent
9	22	.098	.555	.653
	60	.140	.618	.758
	66	.377	.665	1.042
	95	.094	.476	.570
	Av.	.177	.579	.756
10	25	1.658	.423	2.081
	35	1.248	.464	1.712
	88	1.536	.530	2.066
	106	1.576	.588	2.164
	Av.	1.505	.501	2.006
11	6	2.700	.696	3.396
	37	1.667	.362	2.029
	74	1.864	.500	2.364
	Av.	2.077	.519	2.596
12	20	1.329	.421	1.750
	34	1.334	.602	1.936
	83	1.506	.543	2.049
	101	1.351	.534	1.885
	Av.	1.380	.525	1.905
13	3	.966	.345	1.311
	46	1.273	.584	1.857
	80	1.403	.539	1.942
	98	1.057	.458	1.515
	Av.	1.175	.482	1.657
14	8	1.012	.380	1.392
	43	.969	.429	1.398
	63	1.390	.576	1.966
	96	1.139	.516	1.655
	Av.	1.128	.475	1.603
15	13	1.773	.346	2.119
	47	2.342	.510	2.852
	64	2.547	.727	3.274
	Av.	2.221	.527	2.748
16	28	1.476	.434	1.910
	52	2.251	.842	3.093
	70	1.781	.506	2.287
	99	1.538	.596	2.134
	Av.	1.762	.594	2.356

Table 4b. Alkaloidal content of cured leaves from plots of lines grown in 1951, expressed as per cent of the dry weight. (Cont'd)

Lines	Plot number	Steam distillation		
		Nicotine	Nornicotine	Total
		per cent	per cent	per cent
17	5	.879	.266	1.145
	48	.817	.227	1.044
	87	.981	.336	1.317
	Av.	.892	.277	1.169
18	30	.885	.179	1.064
	53	1.267	.419	1.686
	71	1.361	.421	1.782
	Av.	1.171	.340	1.511
19	26	1.316	.232	1.548
	58	1.096	.310	1.406
	84	1.061	.265	1.326
	Av.	1.158	.269	1.427

The least significant differences between the lines on total alkaloids at 5% level:

Lines with four replicates	.467
Lines with three replicates	.539
Lines with two replicates	.660

Table 4c. Alkaloidal content of cured leaves from plots of lines grown in 1952, expressed as per cent of the dry weight.

Lines	number	Steam distillation		
		Nicotine	Nornicotine	Total
		per cent	per cent	per cent
1	13	.152	.568	.720
	112	.047	.452	.499
	113	.031	.458	.489
	Av.	.077	.493	.570
2	12	1.636	.430	2.066
	46	1.331	.452	1.783
	Av.	1.484	.441	1.925
3	64	.847	.317	1.164
4	65	.856	.123	.979
	99	.912	.211	1.123
	106	.607	.147	.754
	Av.	.792	.160	.952
5	69	.924	.247	1.171
	101	.643	.104	.747
	113	1.183	.281	1.464
	Av.	.917	.210	1.127
6	70	1.152	.368	1.520
	102	1.170	.378	1.548
	112	1.505	.260	1.765
	Av.	1.276	.335	1.611
7	68	1.122	.309	1.431
	97	.599	.530	1.129
	105	.748	.256	1.004
	Av.	.823	.365	1.188
8	79	.021	.515	.536
	100	.031	.738	.769
	107	.040	.767	.807
	Av.	.032	.673	.705
9	58	.180	.530	.710
	98	.011	.444	.455
	Av.	.096	.487	.583
10	62	1.867	.610	2.477
	103	1.679	.296	1.975
	111	1.331	.272	1.603
	Av.	1.626	.393	2.019

Table 4c. Alkaloidal content of cured leaves from plots of lines grown in 1952, expressed as per cent of the dry weight. (Cont'd)

Lines	Plot number	Steam distillation		
		Nicotine	Nornicotine	Total
		per cent	per cent	per cent
11	80	1.453	.537	1.990
	104	1.227	.378	1.605
	110	1.531	.341	1.872
	Av.	1.404	.419	1.823
12	85	1.048	.361	1.409
	109	1.310	.198	1.508
	Av.	1.179	.280	1.459
13	4	1.387	.381	1.768
	51	1.351	.411	1.762
	108	.770	.260	1.030
	Av.	1.169	.351	1.520
14	2	1.585	.250	1.835
	54	1.264	.383	1.647
	96	1.002	.301	1.303
	Av.	1.284	.311	1.595
15	3	1.847	.615	2.462
	67	1.091	.129	1.220
	Av.	1.469	.372	1.841
16	5	1.460	.237	1.697
	89	1.322	.342	1.664
	111	.885	.310	1.195
	Av.	1.222	.296	1.518
17	8	1.126	.175	1.301
	31	.778	.208	.986
	83	.326	.173	.499
	Av.	.743	.185	.928
18	10	.834	.238	1.072
	52	.682	.234	.916
	78	.568	.110	.678
	Av.	.695	.194	.889

Table 4c. Alkaloidal content of cured leaves from plots of lines grown in 1952, expressed as per cent of the dry weight. (Cont'd)

Lines	Plot number	Steam distillation		
		Nicotine	Nornicotine	Total
		per cent	per cent	per cent
19	9	.884	.195	1.079
	56	.871	.218	1.089
	81	.292	.171	.463
	Av.	.682	.195	.877

The least significant differences between the lines on total alkaloids at 5% level:

Lines with three replicates	.539
Lines with two replicates	.660
Lines with one replicate	.934

Table 4d. Summary table on total alkaloids of the lines, expressed as per cent of the dry weight.

	1950		1951		1952		Average	
Lines	Per cent	*	Per cent	*	Per cent	*	Per cent	*
1	1.501	3	1.058	4	.570	3	1.044	10
2	--	-	2.036	3	1.925	2	1.991	5
3	2.543	3	1.781	4	1.164	1	1.989	8
4	2.296	2	1.333	4	.952	3	1.420	9
5	1.767	3	1.652	3	1.127	3	1.516	9
6	2.530	1	2.821	3	1.611	3	2.261	7
7	2.739	1	2.347	4	1.188	3	1.961	8
8	1.676	1	1.231	2	.705	3	1.042	6
9	2.258	1	.756	4	.583	2	.921	7
10	3.349	1	2.006	4	2.019	3	2.178	8
11	2.819	1	2.596	3	1.823	3	2.296	7
12	--	-	1.905	4	1.459	2	1.756	6
13	--	-	1.657	4	1.520	3	1.598	7
14	--	-	1.603	4	1.595	3	1.599	7
15	--	-	2.748	3	1.841	2	2.383	5
16	--	-	2.356	4	1.518	3	1.997	7
17	--	-	1.169	3	.928	3	1.049	6
18	--	-	1.511	3	.889	3	1.200	6
19	--	-	1.427	3	.877	3	1.152	6
Total	--	17	--	66	--	51	--	134
Av.	2.200	--	1.781	--	1.269	--	--	--

*Number of replicates

The least significant differences between the lines on the average total alkaloids at 5% level:

Lines with ten replicates	.296
Lines with nine replicates	.308
Lines with eight replicates	.330
Lines with seven replicates	.352
Lines with six replicates	.382
Lines with five replicates	.417

The least significant differences between the years on the average total alkaloids at 5% level:

Lines with 66 replicates	.117
Lines with 51 replicates	.131
Lines with 17 replicates	.227

considering all lines. Comparatively speaking, the high alkaloid containing lines and the low alkaloid containing lines retained their respective alkaloid yielding property. The total alkaloid differences between the lines were found to be highly significant. (See L.S.D. values in table 4d.) The alkaloid contents of the 19 lines are graphically represented in figures 2 and 3. It can be observed that the two standard Maryland varieties (lines 1 and 2) were significantly different from each other in alkaloidal content both qualitatively and quantitatively. In total alkaloids, lines 8, 9, 17, 18 and 19 resembled line 1. Qualitatively however, only lines 8 and 9 closely resembled line 1 while all the rest of the lines had a high proportion of nicotine to total alkaloids similar to line 2.

Considering all the plots in each year, highly significant differences were obtained between the total alkaloid averages of the three growing seasons (see L.S.D. values in table 4d). The 1950 season produced tobacco much higher in alkaloid content than the other two years; crop year 1952 produced the lowest. To remove genetic variability as a contributing factor to yearly variations, plants of the same generation were grown in 1950 and 1952. The results are presented in table 5. Although the analysis of pooled variance showed only a significance at 30 per cent level, it can be seen that in both lines, their 1950 crop had consistently higher total

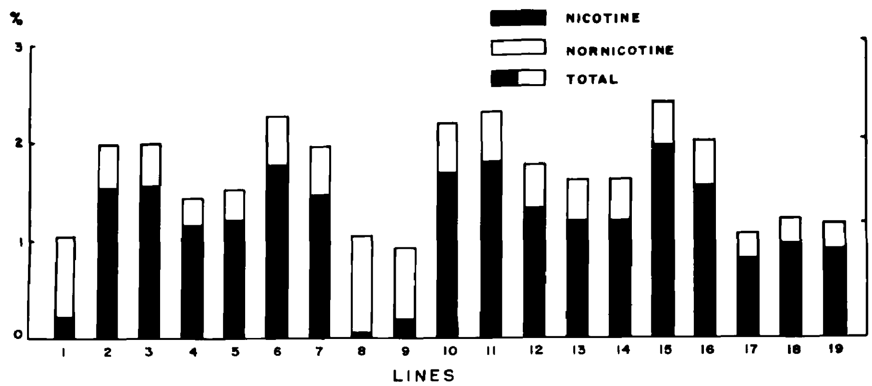


FIG. 2. ALKALOIDAL CONTENT --- THREE YEAR AVERAGE

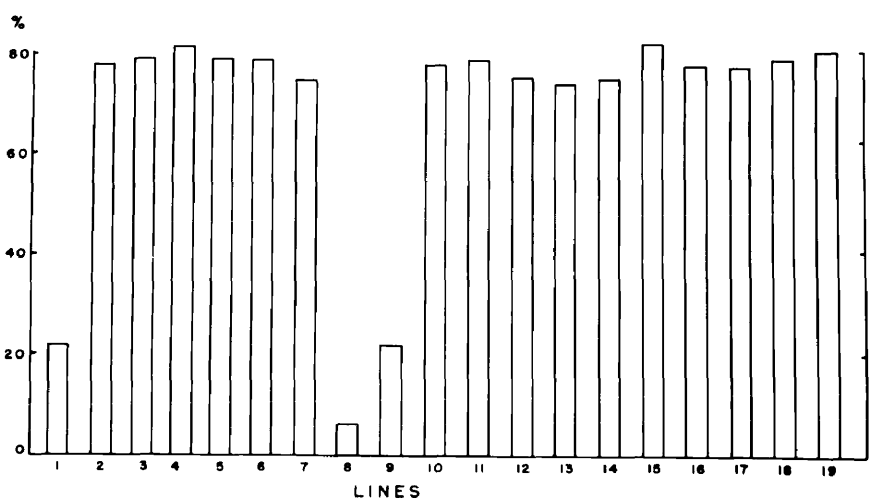


FIG. 3. PROPORTION OF NICOTINE TO TOTAL ALKALOIDS --- THREE YEAR AVERAGE

Table 5. Alkaloidal content of plants from the same generation grown at different seasons, expressed as per cent of the dry weight.

Line	Season	Plot number	Steam distillation: Nicotine	Steam distillation: Nornicotine	Steam distillation: Total	Proportion of nicotine to total
			per cent	per cent	per cent	per cent
4	1950	24	1.880	.424	2.304	81.60
		67	1.841	.447	2.288	80.46
		Av.	1.861	.435	2.296	81.03
	1952	7	1.885	.216	2.101	89.72
		48	1.714	.223	1.937	88.49
		77	1.492	.178	1.670	89.34
		Av.	1.697	.206	1.903	89.18
5	1950	17	1.640	.388	2.028	80.87
		100	.945	.364	1.309	72.19
		108	1.497	.468	1.965	76.18
		Av.	1.361	.407	1.768	76.98
	1952	19	1.080	.256	1.336	80.84
		47	1.067	.227	1.294	82.46
		Av.	1.074	.241	1.315	81.65
Av.	1950	--	1.561	.418	1.979	78.26
	1952	--	1.448	.220	1.668	86.17

Significant difference at 30% level only between average total alkaloids of the two years.

Significant difference at 2% level between average proportion of nicotine to total alkaloids of the two years.

alkaloids than their 1952 crops. The low "t" value may be accounted for by the wide variability among the variates.

To determine whether there is any relation between yield and total alkaloids, a correlation test was ran on plots of line 1. As shown in table 6, a correlation coefficient of -.23 was the result. Although not significant, there seem to be a slight indication that a high cured leaf yielding plant would tend to have low total alkaloid content and vice versa.

Proportion of nicotine to total alkaloids.

The proportion of nicotine to total alkaloids for each plot are shown in table 7. Variabilities could be observed between replicates within the lines in each of the three years. Comparing between lines within each year, the "F" value obtained was highly significant. Considering all three years, the differences between lines were highly significant too. Figure 3 is a graphic representation of the average proportion of nicotine to total alkaloids for each line. In this respect, the nineteen lines may be grouped into two. One group would consist of the mostly nornicotine lines under which would fall lines 1, 8 and 9. The rest of the other lines would constitute the group with nicotine as the predominant alkaloid. This grouping could be applied to any of the three years indicating a strong dependence of the lines upon genetic constitution in this respect.

Table 6. The relation between yield, total alkaloids and proportion of nicotine to total alkaloids in line 1, total alkaloids expressed as per cent of the dry weight.

Year	Plot number	Total alkaloids	Proportion of nicotine to total	Acre yield
		per cent	per cent	pounds
1950	15	1.411	27.64	950
	30	1.628	46.93	976
	80	1.464	24.52	823
1951	10	.990	32.42	747
	50	.713	5.89	778
	77	1.162	15.41	740
	86	1.367	30.87	605
1952	13	.720	21.11	808
	112	.499	9.42	1122
	113	.489	6.34	908

Correlation between total alkaloids and yield:

$$r = -.23$$

Correlation between total alkaloids and proportion of nicotine to total alkaloids:

$$r = +.82$$

Correlation between yield and proportion of nicotine to total alkaloids:

$$r = -.14$$

Table 7. Proportion of nicotine to total alkaloids.

Lines	Replicates	Crop year 1950	1951	1952	Average	Total replicates
		per cent	per cent	per cent	per cent	
1	1	27.64	32.42	21.11		
	2	46.93	5.89	9.42		
	3	24.52	15.41	6.34		
	4	--	30.87	--		
	Av.	33.03	21.15	12.29	22.06	10
2	1		81.12	79.19		
	2		74.92	74.64		
	3		80.15	--		
	Av.	--	78.73	76.92	78.00	5
3	1	76.57	82.42	72.77		
	2	77.95	76.10	--		
	3	87.91	78.78	--		
	4	--	78.97	--		
	Av.	80.81	79.07	72.77	78.93	8
4	1	81.60	84.95	87.44		
	2	80.46	78.30	81.21		
	3	--	76.86	80.50		
	4	--	79.89	--		
	Av.	81.03	80.00	83.05	81.25	9
5	1	80.87	75.16	78.91		
	2	72.19	77.59	86.08		
	3	76.18	82.39	80.81		
	Av.	76.41	78.38	81.93	78.91	9
6	1	79.37	82.43	75.79		
	2	--	76.36	75.58		
	3	--	74.50	85.27		
	Av.	79.37	77.76	78.88	78.47	7
7	1	77.29	82.39	78.41		
	2	--	74.77	53.06		
	3	--	78.56	74.50		
	4	--	75.41	--		
	Av.	77.29	77.78	68.66	74.55	8
8	1	0.00	13.89	3.92		
	2	--	10.68	4.03		
	3	--	--	4.96		
	Av.	0.00	12.29	4.30	6.25	6

Table 7. Proportion of nicotine to total alkaloids. (Cont'd)

Lines	Replicates	Crop year 1950	1951	1952	Average	Total replicates
		per cent	per cent	per cent	per cent	
9	1	38.09	15.01	25.35		
	2	--	18.47	2.42		
	3	--	36.18	--		
	4	--	16.49	--		
	Av.	38.09	21.54	13.89	21.72	7
10	1	75.01	79.67	75.37		
	2	--	72.89	85.01		
	3	--	74.35	83.03		
	4	--	72.83	--		
	Av.	75.01	74.94	81.14	77.27	8
11	1	77.15	79.51	73.02		
	2	--	82.16	76.45		
	3	--	78.85	81.78		
	Av.	77.15	80.17	77.08	78.42	7
12	1		75.94	74.38		
	2		68.90	86.87		
	3		73.50	--		
	4		71.67	--		
	Av.	--	72.50	80.63	75.04	6
13	1		73.68	78.45		
	2		68.55	76.67		
	3		72.25	74.76		
	4		69.77	--		
	Av.	--	71.06	76.63	73.45	7
14	1		72.70	86.38		
	2		69.31	76.75		
	3		70.70	76.90		
	4		68.82	--		
	Av.	--	70.38	80.01	74.51	7
15	1		83.67	75.02		
	2		82.12	89.43		
	3		77.79	--		
	Av.	--	81.19	82.23	81.61	5
16	1		77.28	86.03		
	2		72.78	79.45		
	3		77.87	74.06		
	4		72.07	--		
	Av.	--	75.00	79.85	77.08	7

Table 7. Proportion of nicotine to total alkaloids. (Cont'd)

Lines	Replicates	Crop year 1950	1951	1952	Average	Total replicates
		per cent	per cent	per cent	per cent	
17	1		76.77	86.55		
	2		78,26	78.90		
	3		74.49	65.33		
	Av.	--	76.51	76.93	76.72	6
18	1		83.18	77.80		
	2		75.15	74.45		
	3		76.37	83.78		
	Av.	--	78.23	78.68	78.46	6
19	1		85.01	81.93		
	2		77.95	79.98		
	3		80.02	63.07		
	Av.	--	80.99	74.99	77.99	6
Total replicates		17	66	51	--	134
Average		63.59	68.09	67.52	--	--

The least significant differences between lines within the year and between years within the line at 5% level:

Lines or years with four replicates	8.79
Lines or years with three replicates	10.15
Lines or years with two replicates	12.43
Lines or years with one replicate	17.58

The least significant differences between lines considering all three years at 5% level:

Lines with ten replicates	5.56
Lines with nine replicates	5.86
Lines with eight replicates	6.21
Lines with seven replicates	6.64
Lines with six replicates	7.18
Lines with five replicates	7.86

The least significant differences between years considering all lines at 5% level:

Year with 66 replicates	2.16
Year with 51 replicates	2.46
Year with 17 replicates	4.26

Yearly differences were found to be significant at 5 per cent level not only between years within the line but also between years disregarding line differences (see L.S.D. values in table 7). Line 1 exhibited the greatest yearly variation. It showed a decreasing proportion in the succeeding generations. On the whole, the 1950 season produced a crop containing a lower ratio of nicotine to total alkaloids than the 1951 or 1952 crop seasons. As an indication of yearly variation devoid of genetic complications, an analysis of pooled variance was made on plants of the same generation grown in 1950 and 1952. A "t" value significant at 2 per cent level was obtained indicating that the 1952 season effected a significant increase in the nicotine-nornicotine ratio over the 1950 season.

A correlation test between total alkaloids and proportion of nicotine to total alkaloids in line 1 gave a highly significant correlation coefficient of +.82 as shown in table 6. This means that plants with high total alkaloid content tend to have higher nicotine-nornicotine ratio than plants of the same generation with low total alkaloid content. A negative correlation though not significant was found between the ratio and yield. This seemed logical due to the fact that a negative correlation between yield and total alkaloids plus a positive correlation between ratio and total alkaloids would result in a negative correlation between ratio and yield.

Distribution of alkaloids in the leaf grades.

Remembering the relative positions of the leaf grades in a plant, one may observe in tables 8a, 8b and 8c a certain trend of distribution common to most of the lines in each of the three years. It appears that for total alkaloids as well as nicotine and nornicotine, there was an increasing concentration from the basal leaves towards the middle leaves. From the middle to the upper leaves, a decreasing concentration was presented in a majority of the lines. The deviations from the so-called general trend, however, were not common to the same lines at different years. This may imply that there were no genetic differences between these lines as regards their trend of alkaloid distribution in the leaf grades, the differences existing only as differential responses to varying environmental factors and cultural practices. Figure 4 illustrates the inconsistencies in trend of three lines grown during the three successive years.

Disregarding line variations and comparing the average trend of each year, yearly differences are noticeable. The 1951 and 1952 seasons produced crops with trends conforming to the general trend of alkaloid distribution in the leaf grades. The seconds of the 1951 crop had the highest concentration of total alkaloids while the dull bright of the 1952 crop had it. On the

Table 8a. Distribution of alkaloids in the leaf grades of the 1950 crop, content expressed as per cent of the dry weight.

Lines	Grades*	Steam distillation Nicotine	Nornicotine	Total
		per cent	per cent	per cent
1	S	.352	.796	1.148
	B	.543	1.107	1.650
	DB	.439	1.061	1.500
	D	.728	.732	1.460
3	S	1.759	.480	2.239
	B	2.217	.437	2.654
	DB	2.103	.511	2.614
	D	2.225	.475	2.700
4	S	1.474	.473	1.947
	B	2.142	.423	2.565
	DB	1.686	.494	2.180
	D	1.746	.370	2.116
5	S	1.285	.409	1.694
	B	1.510	.406	1.916
	DB	1.253	.451	1.704
	D	1.024	.306	1.330
6	S	1.674	.383	2.057
	B	2.356	.464	2.820
	DB	1.681	.423	2.104
	D	1.798	.913	2.711
7	S	1.799	.577	2.376
	B	2.104	.581	2.595
	DB	2.289	.707	2.996
	D	2.435	.623	3.058
8	S	trace	1.313	1.313
	B	trace	1.945	1.945
	DB	trace	1.491	1.491
	D	trace	1.230	1.230
9	S	.234	.804	1.038
	B	1.170	1.193	2.363
	DB	.550	1.939	2.489
	D	.766	2.205	2.971
10	S	1.999	.624	2.623
	B	2.665	.967	3.632
	DB	2.414	.620	3.034
	D	2.661	.913	3.574

Table 8a. Distribution of alkaloids in the leaf grades of the 1950 crop, content expressed as per cent of the dry weight.

Lines	Grades*	Steam distillation: Nicotine	Nornicotine	Total
		per cent	per cent	per cent
11	S	1.588	.508	2.096
	B	2.145	.654	2.799
	DB	2.666	.737	3.403
	D	2.757	.734	3.491
Av.	S	1.216	.637	1.853
	B	1.676	.818	2.494
	DB	1.508	.843	2.352
	D	1.614	.850	2.464

* S - Seconds
B - Bright
DB - Dull bright
D - Dull

The least significant difference for average total alkaloids between grades at 5% level: .310

Table 8b. **Distribution of alkaloids in the leaf grades of the 1951 crop,** content expressed as per cent of the dry weight.

Lines	Grades*	Steam distillation Nicotine	Nornicotine	Total
		per cent	per cent	per cent
1	S	.170	.993	1.163
	B	.326	.917	1.243
	DB	.234	.769	1.003
	D	.251	.640	.891
2	S	1.601	.382	1.983
	B	1.755	.440	2.195
	DB	1.586	.469	2.055
	D	1.479	.401	1.880
3	S	1.061	.383	1.444
	B	1.508	.448	1.956
	DB	1.544	.389	1.933
	D	1.436	.385	1.821
4	S	1.091	.217	1.308
	B	.946	.284	1.220
	DB	1.153	.312	1.465
	D	1.090	.284	1.374
5	S	1.062	.347	1.409
	B	1.358	.349	1.707
	DB	1.383	.435	1.818
	D	1.325	.294	1.619
6	S	2.337	.597	2.934
	B	2.231	.723	2.954
	DB	2.236	.602	2.838
	D	2.037	.493	2.530
7	S	1.657	.471	2.128
	B	1.819	.502	2.321
	DB	1.905	.558	2.463
	D	1.883	.582	2.465
8	S	.121	1.214	1.335
	B	.110	1.122	1.232
	DB	.142	1.088	1.230
	D	.240	.915	1.165
9	S	.246	.740	.986
	B	.168	.630	.798
	DB	.141	.459	.600
	D	.167	.489	.656

Table 8b. Distribution of alkaloids in the leaf grades of the 1951 crop, content expressed as per cent of the dry weight. (Cont'd)

Lines	Grades *	Steam distillation Nicotine per cent	Nornicotine per cent	Total per cent
10	S	1.416	.410	1.826
	B	1.516	.517	2.033
	DB	1.617	.587	2.204
	D	1.465	.470	1.935
11	S	1.662	.401	2.063
	B	2.150	.544	2.694
	DB	2.336	.574	2.910
	D	2.227	.593	2.820
12	S	1.211	.451	1.662
	B	1.371	.571	1.942
	DB	1.516	.513	2.029
	D	1.402	.544	1.946
13	S	1.015	.393	1.408
	B	1.111	.476	1.587
	DB	1.208	.511	1.719
	D	1.325	.524	1.849
14	S	.829	.336	1.165
	B	1.176	.577	1.753
	DB	1.168	.457	1.625
	D	1.160	.455	1.615
15	S	2.073	.527	2.600
	B	2.359	.658	3.017
	DB	2.367	.450	2.817
	D	2.122	.461	2.583
16	S	1.617	.557	2.174
	B	1.794	.670	2.464
	DB	1.994	.682	2.676
	D	1.671	.485	2.156
17	S	.808	.256	1.062
	B	.772	.305	1.077
	DB	1.098	.362	1.460
	D	.922	.219	1.141
18	S	.999	.287	1.286
	B	1.166	.312	1.478
	DB	1.276	.335	1.611
	D	1.181	.404	1.585

Table 8b. Distribution of alkaloids in the leaf grades of the 1951 crop, content expressed as per cent of the dry weight. (Cont'd)

Lines	Grades*	Steam distillation Nicotine	Nornicotine	Total
		per cent	per cent	per cent
19	S	.946	.215	1.161
	B	1.146	.299	1.445
	DB	1.261	.292	1.553
	D	1.278	.262	1.540
Av.	S	1.154	.483	1.637
	B	1.304	.544	1.848
	DB	1.377	.518	1.895
	D	1.298	.468	1.767

* S - Seconds
B - Bright
DB - Dull bright
D - Dull

The least significant difference for average total alkaloids between grades at 5% level: .106

Table 8c. Distribution of alkaloids in the leaf grades of the 1952 crop, content expressed as per cent of the dry weight.

Lines	Grades*	Nicotine per cent	Nornicotine per cent	Total per cent
1	S	.047	.708	.755
	B	.160	.531	.691
	DB	.096	.410	.506
	D	.055	.327	.382
2	S	1.680	.506	2.186
	B	1.655	.450	2.105
	DB	1.356	.434	1.790
	D	1.194	.357	1.551
3	S	.842	.383	1.225
	B	1.020	.456	1.476
	DB	.941	.323	1.264
	D	.686	.252	.938
4	S	.727	.144	.871
	B	.892	.185	1.077
	DB	.826	.195	1.021
	D	.688	.139	.827
5	S	.924	.160	1.084
	B	.892	.214	1.106
	DB	.958	.278	1.236
	D	.928	.206	1.134
6	S	1.342	.319	1.661
	B	1.494	.360	1.854
	DB	1.317	.353	1.670
	D	1.136	.321	1.457
7	S	.845	.397	1.242
	B	.888	.452	1.340
	DB	.803	.333	1.136
	D	.763	.292	1.055
8	S	.002	.746	.748
	B	.016	.676	.692
	DB	.031	.510	.541
	D	.060	.605	.665
9	S	.120	.504	.624
	B	.166	.569	.735
	DB	.016	.498	.514
	D	.074	.409	.483

Table 8c. Distribution of alkaloids in the leaf grades of the 1952 crop, content expressed as per cent of the dry weight. (Cont'd)

Lines	Grades*	Steam distillation Nicotine per cent	Nornicotine per cent	Total per cent
10	S	1.698	.454	2.152
	B	1.836	.393	2.229
	DB	1.544	.401	1.945
	D	1.320	.328	1.648
11	S	1.129	.328	1.457
	B	1.361	.407	1.768
	DB	1.539	.460	1.999
	D	1.514	.459	1.973
12	S	1.082	.333	1.415
	B	1.452	.276	1.728
	DB	1.121	.234	1.355
	D	.942	.280	1.222
13	S	1.126	.331	1.457
	B	1.223	.346	1.569
	DB	1.048	.327	1.375
	D	1.224	.338	1.562
14	S	1.143	.190	1.333
	B	1.458	.266	1.724
	DB	1.360	.340	1.700
	D	1.148	.369	1.517
15	S	1.629	.254	1.883
	B	1.620	.425	2.045
	DB	1.471	.387	1.858
	D	1.097	.333	1.430
16	S	1.273	.361	1.634
	B	1.268	.273	1.541
	DB	1.210	.304	1.514
	D	1.187	.282	1.469
17	S	.650	.202	.852
	B	.776	.163	.939
	DB	.626	.152	.778
	D	.823	.201	1.024
18	S	.764	.217	.981
	B	.664	.199	.863
	DB	.637	.200	.837
	D	.696	.177	.873

Table 8c. Distribution of alkaloids in the leaf grades of the 1952 crop, content expressed as per cent of the dry weight. (Cont'd)

Lines	Grades*	Steam distillation Nicotine	Nornicotine	Total
		per cent	per cent	per cent
19	S	.700	.201	.901
	B	.788	.204	.992
	DB	.608	.183	.791
	D	.641	.183	.824
Av.	S	.933	.355	1.287
	B	1.033	.360	1.393
	DB	.921	.333	1.254
	D	.851	.308	1.160

* S - Seconds
B - Bright
DB - Dull bright
D - Dull

The least significant difference for average total alkaloids between grades at 5% level: .094

Table 8d. Summary table for total alkaloid distribution in the leaf grades of the lines, three year data combined, expressed as per cent of the dry weight.

Lines	Leaf grades			
	Seconds	Bright	Dull bright	Dull
	per cent	per cent	per cent	per cent
1	1.022	1.195	1.003	.911
2	2.085	2.150	1.923	1.716
3	1.636	2.027	1.937	1.820
4	1.375	1.621	1.555	1.439
5	1.396	1.576	1.586	1.361
6	2.217	2.543	2.204	2.233
7	1.915	2.085	2.198	2.193
8	1.132	1.290	1.087	1.020
9	.883	1.199	1.201	1.370
10	2.200	2.631	2.391	2.386
11	1.872	2.420	2.771	2.761
12	1.539	1.835	1.692	1.584
13	1.433	1.578	1.547	1.706
14	1.249	1.739	1.663	1.566
15	2.282	2.531	2.338	2.007
16	1.904	2.003	2.095	1.813
17	.957	1.008	1.119	1.083
18	1.134	1.176	1.224	1.229
19	1.031	1.219	1.172	1.182
Av.	1.540	1.780	1.721	1.652

The least significant difference between grades at 5% level considering all lines: .092

Not significant between grades within the line.

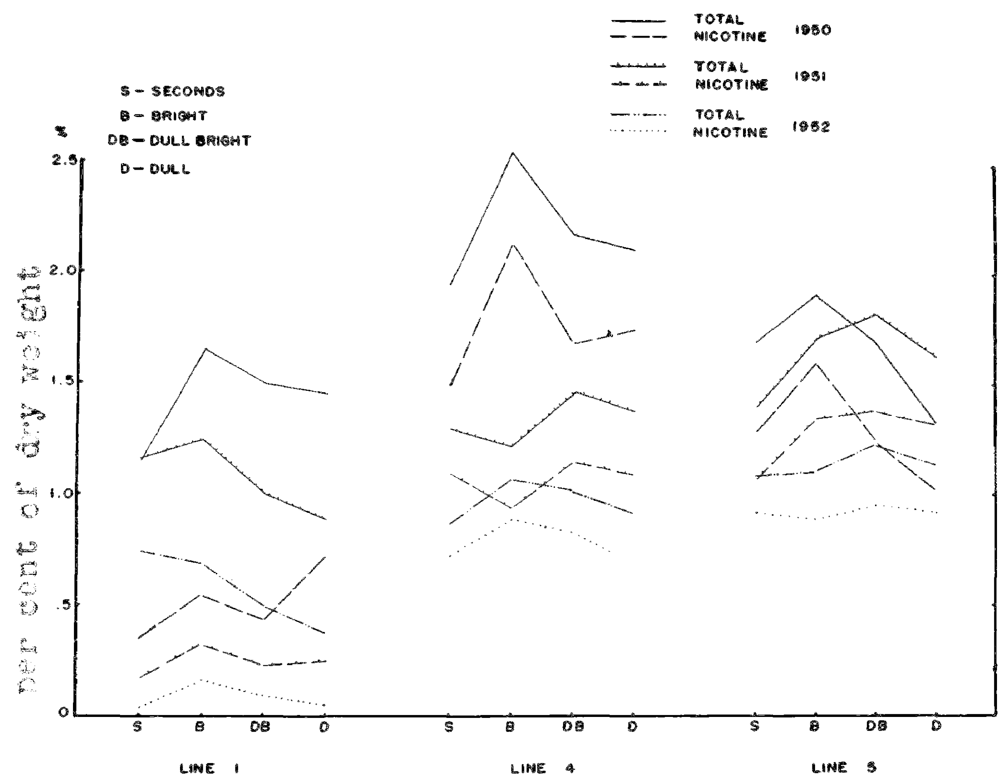


FIG. 4. DISTRIBUTION OF ALKALOIDS IN THE LEAF GRADES OF LINES GROWN FOR THREE SUCCESSIVE YEARS

other hand, the 1950 crop presented a slightly different trend. An increasing trend showed up but the difference was not significant as indicated by the L.S.D. value in table 8a. Yearly differences may be attributed to a combination of environmental variations, variations in farm practices (planting, topping, harvesting, curing and grading) and sampling.

Proportion of nicotine to total alkaloids in the leaf grades.

The trend in the leaf grades tended to follow the same general trend taken by the individual alkaloids in the individual lines for each year which were illustrated in the preceding tables and figure. The difference lies in their dulls. It may be seen in tables 9a and 9b that the proportion of nicotine to total alkaloids increased in the dulls. This particular characteristic was found significant in the 1951 crop. The same is true for the overall grade average (see L.S.D. values in tables 9a and 9b). Topping, more than anything else, may have brought about the increased concentration of nicotine in the upper leaves. Differences between the grades within the crop years 1950 and 1952 were not significant but they showed also the same characteristic trend. This is due probably to a wide variation in the variates. This may also account for differences between grades within the line being insignificant.

Behavior of alkaloids in the leaves of individual plants as affected by drying and curing.

Table 9a. Proportion of nicotine to total alkaloids in the leaf grades.

Lines	Crop year	Leaf grades: Seconds per cent	Bright per cent	Dull bright per cent	Dull per cent
1	'50	30.66	32.91	29.27	49.86
	'51	14.62	26.23	23.33	28.17
	'52	6.23	23.15	18.97	14.40
	Av.	17.17	27.43	23.52	30.81
2	'51	80.74	79.95	77.18	78.67
	'52	76.85	78.62	75.75	76.98
	Av.	78.80	79.29	76.47	77.83
3	'50	78.56	83.53	80.45	82.41
	'51	73.48	77.10	79.88	78.86
	'52	68.73	69.11	74.45	73.13
	Av.	73.59	76.58	78.26	78.13
4	'50	75.71	83.51	77.34	82.51
	'51	83.41	77.54	78.70	79.33
	'52	83.47	82.82	80.90	83.19
	Av.	80.86	81.29	78.98	81.68
5	'50	75.86	78.81	73.53	76.99
	'51	75.37	79.55	76.07	81.84
	'52	85.24	80.65	77.51	81.83
	Av.	78.82	79.67	75.70	80.22
6	'50	81.38	83.55	79.90	66.32
	'51	79.65	75.52	78.79	80.51
	'52	80.79	80.58	78.86	77.97
	Av.	80.61	79.88	79.18	74.93
7	'50	75.72	77.61	76.40	79.63
	'51	77.87	78.37	77.34	76.39
	'52	68.04	66.27	70.69	72.32
	Av.	73.88	74.08	74.81	76.11
8	'50	0.00	0.00	0.00	0.00
	'51	9.06	8.93	11.54	20.60
	'52	.27	2.31	5.73	9.02
	Av.	3.11	3.75	5.76	9.87
9	'50	22.54	49.52	22.10	25.78
	'51	24.95	21.05	23.50	25.46
	'52	19.23	22.59	3.11	15.32
	Av.	22.24	31.05	16.24	22.19

Table 9a. Proportion of nicotine to total alkaloids in the leaf grades. (Cont'd)

Lines	Crop year	Leaf grades			
		Seconds	Bright	Dull bright	Dull
		per cent	per cent	per cent	per cent
10	'50	76.21	73.38	79.56	74.45
	'51	77.55	74.57	73.37	75.71
	'52	78.90	82.37	79.38	80.10
	Av.	77.55	76.77	77.44	76.75
11	'50	75.76	76.63	78.34	78.97
	'51	80.56	79.80	80.27	78.97
	'52	77.49	76.98	76.99	76.74
	Av.	77.94	77.80	78.53	78.23
12	'51	72.86	70.60	74.72	72.05
	'52	76.47	84.03	82.73	77.09
	Av.	74.67	77.32	78.73	74.57
13	'51	72.09	69.94	70.27	71.66
	'52	77.28	77.95	76.22	78.36
	Av.	74.69	73.95	73.25	75.01
14	'51	71.16	67.08	71.88	71.83
	'52	85.75	84.57	80.00	75.68
	Av.	78.46	75.83	75.94	73.76
15	'51	79.73	78.19	84.03	82.15
	'52	86.51	79.22	79.17	76.71
	Av.	83.12	78.71	81.60	79.43
16	'51	74.38	72.81	74.51	77.50
	'52	77.91	82.28	79.92	80.80
	Av.	76.15	77.55	77.22	79.15
17	'51	76.08	71.68	75.21	80.81
	'52	76.29	82.64	80.46	80.37
	Av.	76.19	77.16	77.84	80.59
18	'51	77.68	78.89	79.21	74.51
	'52	77.88	76.94	76.11	79.73
	Av.	77.78	77.92	77.66	77.12
19	'51	81.48	79.31	81.20	82.99
	'52	77.69	79.44	76.86	77.79
	Av.	79.59	79.38	79.03	80.39

Table 9a. Proportion of nicotine to total alkaloids in the leaf grades. (Cont'd)

Lines	Crop year	Leaf grades			
		Seconds	Bright	Dull bright	Dull
		per cent	per cent	per cent	per cent
Av.*	'50	59.24	63.95	59.71	61.69
	'51	67.51	66.66	67.95	69.37
	'52	67.42	69.08	67.04	67.76

* Ten replicates for the 1950 averages and nineteen replicates for the 1951 and 1952 averages.

The least significant difference between grades within the year 1951 at 5% level: 1.541

Not significant between grades within the years 1950 and 1952.

Table 9b. Summary table on proportion of nicotine to total alkaloids in the leaf grades, three year data combined.

Lines	Leaf grades: Seconds	Bright	Dull bright	Dull
	per cent	per cent	per cent	per cent
1	17.17	27.43	23.52	30.81
2	78.80	79.29	76.47	77.83
3	73.59	76.58	78.26	78.13
4	80.86	81.29	78.98	81.68
5	78.82	79.67	75.70	80.22
6	80.61	79.88	79.18	74.93
7	73.88	74.08	74.81	76.11
8	3.11	3.75	5.76	9.87
9	22.24	31.05	16.24	22.19
10	77.55	76.77	77.44	76.75
11	77.94	77.80	78.53	78.23
12	74.67	77.32	78.73	74.57
13	74.69	73.95	73.25	75.01
14	78.46	75.83	75.94	73.76
15	83.12	78.71	81.60	79.43
16	76.15	77.55	77.22	79.15
17	76.19	77.16	77.84	80.59
18	77.78	77.92	77.66	77.12
19	79.59	79.38	79.03	80.39
Av.	67.64	68.71	67.69	68.78

Least significant difference between grades at 5% level: 1.064

Not significant between grades within the line.

To obtain a better trend of distribution of alkaloids in a plant and to find out the effects of drying and curing on the alkaloids, leaves were picked from individual plants at certain stages and analyzed. The leaves were so picked out such that comparisons between the green leaves, leaves dried at 100°C for three hours and cured leaves would be possible. The results are presented in tables 10a, 10b, 10c and 10d and graphically represented in figures 5, 6, 7 and 8. It is obvious that drying decidedly reduced the nicotine content, total alkaloid content and to a certain extent the nornicotine portion. This was true for all four plants. Curing also reduced greatly the total alkaloids and the nicotine portion but the nornicotine concentration was more or less maintained. The peculiar behavior of nornicotine coupled with the decrease in nicotine and total alkaloids suggests strongly the fact that certain transformations of alkaloids are effected by drying and curing.

Total alkaloids in the green and cured leaves from both plants of line 1 did not show any smooth trend as seen in figure 5. Line 8 presented a much smoother trend of alkaloid distribution. Except in cured leaves, the line graphs show that the nicotine trends followed closely the total alkaloid trends having the same pattern. The two trends are wider apart at the lower leaves and tend to intersect at the upper leaves. This may mean that

Table 10a. Distribution of alkaloids in the leaves of plant A from line 1, expressed as per cent of the dry weight.

Condition	Leaf number	Steam distillation: Nicotine	Steam distillation: Nornicotine	Steam distillation: Total	Proportion of nicotine to total
		per cent	per cent	per cent	per cent
Green	1- 4	1.039	1.453	2.492	41.7
	7-10	1.767	1.188	2.955	59.8
	13-16	1.822	.720	2.542	71.7
	19-22	2.118	.387	2.505	84.6
	Av.	1.687	.937	2.624	64.3
Oven dried	1- 4	1.018	.801	1.819	56.0
	7-10	1.230	.649	1.919	64.1
	13-16	1.395	.574	1.969	70.8
	19-22	1.711	.457	2.168	78.9
	Av.	1.339	.620	1.959	68.4
Cured	2- 3	.412	.697	1.109	37.2
	5- 6	.301	1.193	1.494	20.1
	8- 9	.252	1.266	1.518	16.6
	11-12	.150	1.341	1.491	10.1
	14-15	.052	1.090	1.142	4.6
	17-18	.464	.798	1.262	36.8
	20-21	.600	.700	1.300	46.2
	Av.*	.329	.938	1.267	26.0
Effect of drying		-.348	-.317	-.665	+4.1
Effect of curing		-1.358	+.001	-1.357	-38.3

* Average of leaves 2-3, 8-9, 14-15 and 20-21 only.

Table 10b. Distribution of alkaloids in the leaves of plant B from line 1, expressed as per cent of the dry weight.

Condition	Leaf number	Steam distillation: Nicotine	Steam distillation: Nornicotine	Steam distillation: Total	Proportion of nicotine to total
		per cent	per cent	per cent	per cent
Green	1- 4	.976	1.409	2.385	40.9
	7-10	1.342	.841	2.183	61.5
	13-16	1.589	.716	2.305	68.9
	19-22	1.734	.490	2.224	78.0
	Av.	1.433	.864	2.297	62.4
Oven dried	1- 4	.752	.968	1.727	43.5
	7-10	1.027	.606	1.633	62.9
	13-16	1.054	.579	1.680	62.7
	19-22	1.365	.273	1.638	83.3
	Av.	1.050	.607	1.657	63.4
Cured	2- 3	.185	1.118	1.303	14.2
	5- 6	.065	1.181	1.246	5.2
	8- 9	.078	1.137	1.215	6.4
	11-12	.033	.750	.783	4.2
	14-15	.053	.768	.821	6.5
	17-18	.034	.845	.879	3.9
	20-21	.069	.718	.787	8.8
	Av.*	.096	.935	1.031	9.3
Effect of drying		-.383	-.257	-.640	+1.0
Effect of curing		-1.337	+.071	-1.266	-53.1

* Average of leaves 2-3, 8-9, 14-15 and 20-21 only.

Table 10c. Distribution of alkaloids in the leaves of plant A from line 8, **expressed as per cent of the dry weight.**

Condition	Leaf number	Steam distillation: Nicotine	Steam distillation: Nornicotine	Steam distillation: Total	Proportion of nicotine to total
		per cent	per cent	per cent	per cent
Green	1- 4	.899	1.058	1.957	45.9
	7-10	1.112	.671	1.783	62.4
	13-16	.634	.435	1.069	59.3
	19-22	.915	.339	1.254	73.0
	Av.	.890	.626	1.516	58.7
Oven dried	1- 4	.424	.812	1.236	34.3
	7-10	.577	.510	1.087	53.1
	13-16	.378	.328	.706	53.5
	19-22	.496	.235	.721	68.8
	Av.	.469	.471	.940	50.0
Cured	2- 3	.093	1.052	1.145	8.1
	5- 6	.148	1.002	1.150	12.9
	8- 9	.057	.699	.756	7.5
	11-12	.033	.564	.597	5.5
	14-15	.035	.543	.578	6.1
	17-18	.034	.320	.354	9.6
	20-21	.033	.298	.331	10.0
	Av.*	.054	.648	.702	7.7
Effect of drying		-.421	-.155	-.576	-8.7
Effect of curing		-.836	+.022	-.814	-51.0

* Average of leaves 2-3, 8-9, 14-15 and 20-21 only.

Table 10d. Distribution of alkaloids in the leaves of plant B from line 8, expressed as per cent of the dry weight.

Condition	Leaf number	Steam distillation: Nicotine	Steam distillation: Nornicotine	Steam distillation: Total	Proportion of nicotine to total
		per cent	per cent	per cent	per cent
Green	1- 4	.501	.829	1.330	37.7
	7-10	.461	.606	1.067	43.2
	13-16	.487	.294	.781	52.3
	19-22	.595	.320	.915	65.0
	Av.	.511	.512	1.023	50.0
Oven dried	1- 4	.316	.706	1.022	30.9
	7-10	.347	.457	.804	43.2
	13-16	.271	.298	.569	47.7
	19-22	.424	.227	.651	58.0
	Av.	.340	.422	.762	44.6
Cured	2- 3	trace	.587	.587	0.0
	5- 6	trace	.476	.476	0.0
	8- 9	trace	.324	.324	0.0
	11-12	trace	.231	.231	0.0
	14-15	trace	.180	.180	0.0
	17-18	trace	.120	.120	0.0
	20-21	trace	.130	.130	0.0
	Av.*	trace	.305	.305	0.0
Effect of drying		-.171	-.090	-.261	-5.4
Effect of curing		-.511	-.207	-.718	-50.0

* Average of leaves 2-3, 8-9, 14-15 and 20-21 only.

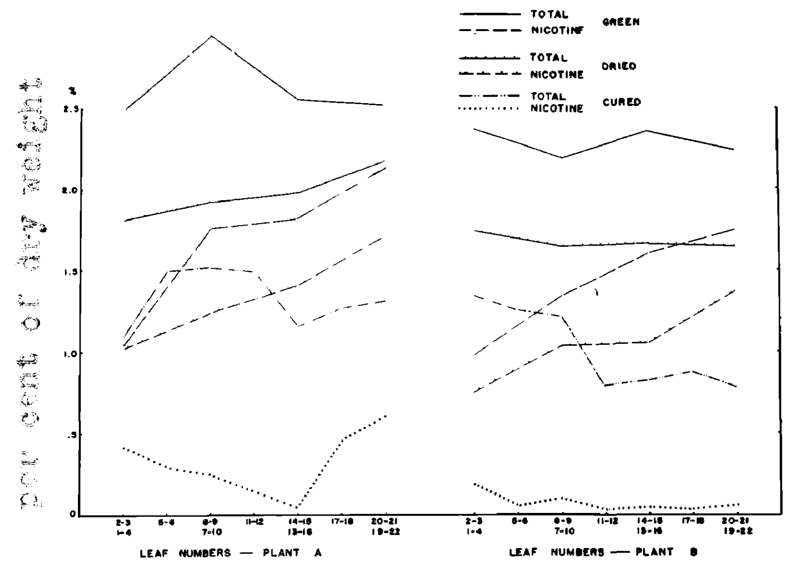


Fig. 5. DISTRIBUTION OF ALKALOIDS IN LINE I

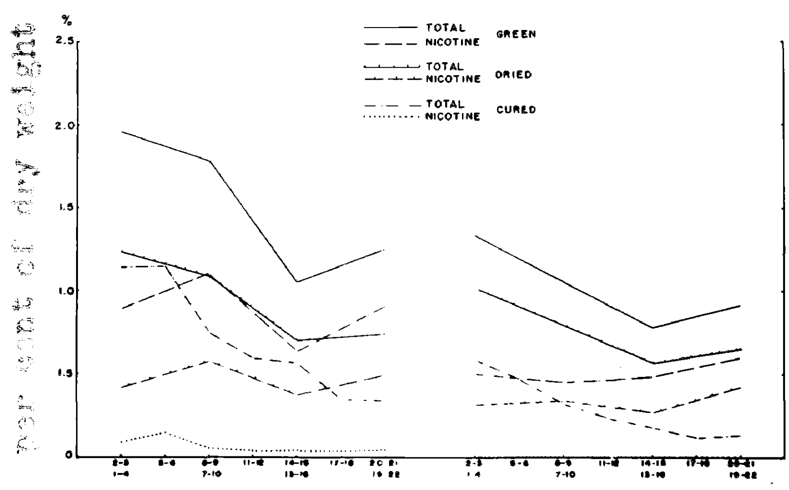


Fig. 6. DISTRIBUTION OF ALKALOIDS IN LINE 8

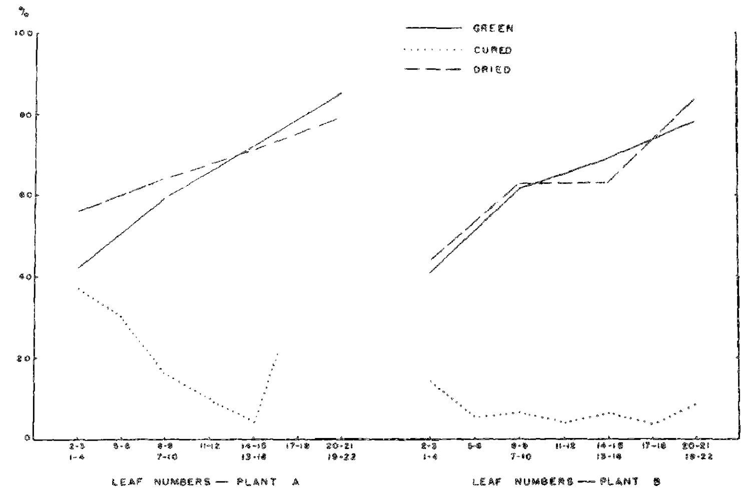


Fig. 7. PROPORTION OF NICOTINE TO TOTAL ALKALOIDS IN LINE 1

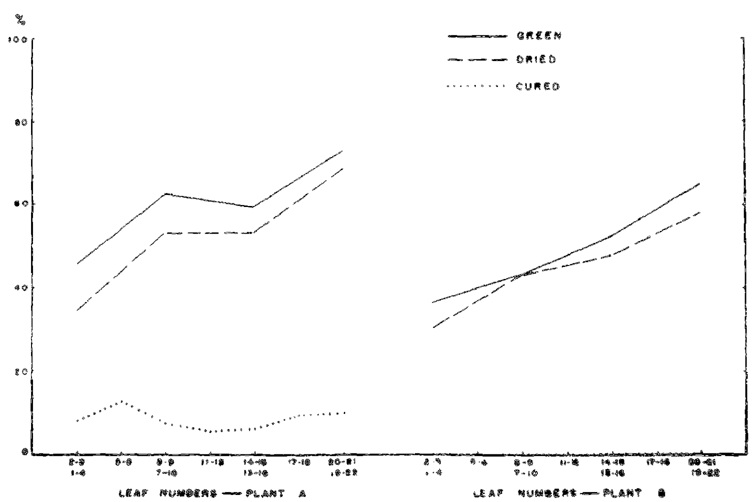


Fig. 8. PROPORTION OF NICOTINE TO TOTAL ALKALOIDS IN LINE 8

the higher the leaf is in a plant, the higher is the proportion of nicotine to total alkaloids. In other words, the younger the leaf the higher the proportion of nicotine to total alkaloids. Figures 7 and 8 illustrate this better. Curing apparently alters this relationship. It may account for the wide variation in the trends found in tables 8a, 8b and 8c.

Effect of mosaic infection on alkaloidal content.

Shown in table 11 are the alkaloidal contents of three lines, some plots of which were inoculated with tobacco mosaic virus and some were not. Of the three lines, line 1 is mosaic susceptible. The other two are resistant lines. Considering the average of all inoculated plots and the average of all non-inoculated plots, the difference in their alkaloidal content was not significant. Taking only the susceptible line, however, inoculation significally increased the total alkaloid content and to a smaller degree, the proportion of nicotine to total alkaloids. On resistant lines, inoculations had no effect on alkaloidal content.

Table 11. Effect of tobacco mosaic virus inoculation on alkaloidal content of cured leaves, content expressed as per cent of the dry weight.

Lines	Treatment	Replicates	Steam distillation Nicotine	Nornicotine	Total	Proportion of nicotine to total
			per cent	per cent	per cent	per cent
1	Inoculated	1	.685	.422	1.107	61.87
		2	.364	.680	1.044	34.87
		3	.243	.624	.867	28.03
		Av.	.431	.575	1.006	41.59
	Uninoculated	1	.152	.568	.720	21.11
		2	.047	.452	.499	9.42
		3	.031	.458	.489	6.34
		Av.	.077	.493	.570	12.29
4	Inoculated	1	1.440	.229	1.669	86.28
		2	1.836	.386	2.222	82.63
		Av.	1.638	.307	1.945	84.22
	Uninoculated	1	1.985	.316	2.301	86.26
		2	1.714	.323	2.037	84.14
		3	1.492	.178	1.670	89.34
		Av.	1.730	.272	2.002	86.41
5	Inoculated	1	1.080	.406	1.486	72.68
	Uninoculated	1	1.067	.227	1.294	82.46
Inoculated		6	.941	.458	1.399	61.60
Uninoculated		7	.927	.360	1.287	54.15

No significant difference between inoculated and not inocu- on total alkaloids and proportion of nicotine to total if all plots are taken into consideration.

Significant difference at 5% level only between total alkaloids of inoculated and uninoculated plots of line 1. (No significant difference in lines 4 and 5)

Significant difference at 8% level only between proportion of nicotine to total alkaloids of inoculated and uninoculated plots of line 1 only. (No significant difference in lines 4 and 5)

DISCUSSION OF RESULTS

The relative inadequacy and inaccuracy of the present methods of alkaloid determinations on tobacco render genetic studies on alkaloid inheritance very complicated particularly on lines with mixed alkaloids. The nicotine, nornicotine portion and total alkaloids as determined by steam distillation and silicotungstic acid precipitation may not give accurately the true contents of a given sample. For example, Tso and Jeffrey (46) found by solvent extraction and paper chromatography that only 50 per cent of the total alkaloids in the Robinson Maryland Medium Broadleaf was nicotine and nornicotine. The other half was composed of 22 other substances 16 of which were unidentified. A critical comparison between these two methods of analysis may establish some relationship that may be of use in routine analysis.

As was mentioned in the previous pages and also by many other investigators, a combination of various factors may affect the alkaloidal content of a sample in one way or another. Notable among these are heredity, soil and climate, farm practices and some existing abnormal conditions such as disease infection and pest infestation.

Genetic constitution.

The significant differences between the lines in their total alkaloids may be attributed largely to differences in genetic constitution. This is further supported by the fact that in the two succeeding seasons, most of the differences between the lines were maintained. Significant differences between the lines existed even on lines coming from the same original cross. Because selection was based not on alkaloidal content but on other factors directly related to market value, this result was expected.

The effect of differences in genetic constitution is more pronounced in the qualitative analysis of the alkaloids present. The nicotine-nornicotine ratio was more or less maintained by all the lines during the three crop years. Two of the lines (lines 8 and 9) selected for mosaic resistance from the original cross of Maryland Medium Broadleaf and N. glutinosa were predominantly nornicotine. The fact that both parents in the original cross were predominantly nornicotine justifies the resulting alkaloid ratio in the selections. Other selections from the same original cross resulting in a high nicotine-nornicotine ratio may be explained on the theory advanced by Smith and Smith (44). They said that a secondary alkaloid present in small amounts in one parent may become the chief alkaloid in some individuals of a hybrid or inbred family. Of the selections that have N. longiflora or Burley in their parentage, all their ratios were high. This was expected because both Burley and N. longiflora are predominantly nicotine in alkaloidal content. According to Valleau (47), the nicotine-nornicotine ratio is a function of the inherent capacity of a plant to transform alkaloids from one form to another. Bonner (5) suggests that the demethylation of nicotine to form nornicotine may be governed by the presence or absence of a methyl acceptor capable of receiving the methyl group of nicotine. Very little yet is known about this mechanism except that Dawson (17,18) found evidences to show that this mechanism is located in the leaf in the case of _N. glutinosa_.

Soil, climate and time of planting.

Wide variations between replicates of a line within the year indicate soil variability effects more than any other factor. This is more evident on quantity of total alkaloids rather than on quality. Quality changes in this case may be a secondary effect of quantity changes. A significant positive correlation coefficient was found to exist between total alkaloids and the nicotine-nor-nicotine ratio. (See table 6.) Plants within the same plots were observed to vary also. Micro-climate and soil differences may have played a part in the existing plant variations.

The effect of rainfall or moisture coupled with the time of topping and harvesting on alkaloidal content may be one of the main reasons for the significant yearly differences particularly on total alkaloids. The low nicotine content of the 1952 crop may be attributed in part to late transplanting and low moisture content at transplanting time. This supports the contention of McMurtrey, Bacon and Ready (35) that late seeding and transplanting tend to lower the yield of "nicotine" per acre. McMurtrey, Bowling, Brown and Engle (36) claim that leaves produced in a dry season are high in "nicotine". Contrary to their claim, a relatively high alkaloidal content showed up in the 1950 crop despite an earlier transplanting and much wetter season than the 1952 crop. One reason that may be offered is the unusually heavy rain that fell on the 1950 crop at the period between topping and harvesting. This may have accelerated the production of alkaloids in the roots and increased the rate of translocation to the leaves. It may also have resulted in a decreased activity of the alkaloid breakdown mechanism. The time of topping and harvesting may have a lot to do also with this.

Although a positive correlation was found to exist between total alkaloid content and the nicotine-nornicotine ratio, this was not the case when the yearly averages of the 1950 and 1951 crops shown in tables 4d and 7c are compared. One reason that may be offered is that the high moisture content of the 1950 crop at harvest time due to a heavy rain just before harvest may have accelerated the demethylation of nicotine into nornicotine. The ratio was decreased but the total alkaloid content was more or less maintained. Another explanation would be the possibility that the positive correlation between total alkaloids and ratio may be peculiar only to certain lines. Lack of replicates in the other lines that were independent of genetic variability prevented such correlation tests meaning anything.

Topping and harvesting.

The effect of topping on the total alkaloid content and the nicotine-nornicotine ratio was mentioned in the preceeding paragraph. Topping definitely increases alkaloid content and the increase in ratio usually follows. It did increase the ratio in the dulls in majority of the lines. Of more significance is the time lapse between topping and harvesting, ordinarily referred to as early or late topping. This is altogether different from high or low topping which has reference to the number of leaves left in the plant. What determines the earliness or lateness of topping is the farmer's judgment on relative maturity in conjunction with convenience of operations. The low total alkaloid content of the 1952 crop compared to the other two years may be explained by the short time lapse between topping and harvesting in the 1952 crop. The effect of late topping is very well shown in the trend of distribution of the alkaloids in the different leaf grades. As shown by the differences between the average yearly trend, it appears that early topping caused an increase of total alkaloids in the dulls as was the case in the 1950 crop. Late topping of the crop in 1952 caused a decreasing total alkaloid trend. Although the 1951 crop was topped early, a decreasing trend showed up. This, however, may be explained by the occurence of a dry spell which happened at that period between topping and harvesting.

In almost all cases, the seconds had total alkaloid contents and ratios of nicotine to total alkaloids lower than the brights or dull brights. This is due to the fact that the basal leaves are more mature than the upper leaves. Actually, they are overmature and partly cured. Thus, transformation of alkaloids to some other forms may have taken place much earlier and longer in the basal leaves than in the middle or upper leaves. The notable decrease in content and ratio in the dull brights may be due to their relative immaturity with reference to the lower leaves. A crop is usually harvested when their middle leaves which form the greatest part of the crop are at full maturity. Consequently, they have the highest concentration of alkaloids. Even though some of the upper leaves have attained their full size, they have not yet received their full share of the alkaloids from the roots. The increased content and ratio in the dulls were assumed to be the result of early topping. The above discussions are based on the assumption that alkaloids are not translocated in the plant after it has entered the leaves.

Curing and drying.

Curing and drying without doubt reduced greatly the nicotine and total alkaloid content of the leaves. While drying reduced the nornicotine portion, curing resulted in a maintained level of nornicotine concentration. In fact, a slight increase was noted in some cases. (See tables 10a, 10b, 10c and 10d.) This gives strong evidence to Dawson's (17,18) belief that nornicotine is a demethylation and breakdown product of nicotine. There was no appreciable increase in nornicotine concentration in proportion to nicotine decrease due perhaps to a further breakdown of nornicotine into some other forms either non-steam volatile or not precipitated by silicotungstic acid. There is very little known yet about alkaloid transformations and breakdown products during the curing process.

Grading and sampling.

Random errors in sampling and grading may account for the erratic trend of the alkaloids in the leaf grades within a plot and to a certain extent the variabilities between plots within the line. Grading introduces a human factor that is subject to error. In the first place, there is no clear cut definition for each grade. Not only is position in a stalk taken into consideration but the color and size as well. This explains the shift in the bulk of cured leaf weights from one grade to another in the plots. Between the three years however, the shift was due more likely to the earliness of topping rather than grading errors.

Earlier topping would tend to shift the bulk of the cured leaves toward the upper leaves or dull brights and dulls.

Since plant variations exist, sampling errors would do likewise on account of the random sampling of "hands" from each grade. In the process of stripping and grading, leaves in one "hand" of a grade may not come from the same plants that leaves in another "hand" of another grade came from. A true trend cannot be presented then if these two hands were picked out as samples for a particular plot. To have an idea as to the true trend, leaves from individual plants of two lines were analyzed. These are graphically represented in figures 5, 6, 7 and 8. It would be noticed that variations existed even between plants from the same plot.

Effect of tobacco mosaic virus inoculation.

Results obtained in this experiment confirmed the findings of Silberschmidt (41) that tobacco mosaic infection caused an increased nicotine content in the infected leaves. In this experiment, the total alkaloids as well as the proportion of nicotine to total alkaloids was increased significantly by virus inoculation on susceptible lines. The reason for the increase in total alkaloids may be attributed to the possible decrease in total dry matter of infected leaves without much loss in alkaloids. It may also be possible that the virus did not affect the normal synthesis of the alkaloids in the roots and also its translocation to the infected leaves. As regards the increase in the nicotine-nornicotine ratio, the presence of the virus may have impaired the mechanism for alkaloid transformation and breakdown.

SUMMARY AND CONCLUSIONS

1. This study was made on selected disease resistant Maryland tobacco strains to determine their variability in alkaloidal content over a three year period representing three generations. It was intended to show also which of the lines approached the standard Maryland variety as regards alkaloidal content both qualitatively and quantitatively.
2. The petroleum ether extraction method for nicotine analysis was found inaccurate on tobacco lines with high nornicotine content. The results included about half of the nornicotine present as nicotine.
3. Of the seventeen selected disease resistant breeding lines, only lines 8 and 9 approached Robinson Maryland Medium Broadleaf (line 1) in quality and quantity of alkaloid. The other fifteen resembled the Wilson (line 2) strain of Maryland tobacco in proportion of nicotine to total alkaloids at least.
4. A wide genetic variability between the 19 lines existed despite the fact that some of them came from the same original cross. The process of selection taking into consideration only the factors directly related to market value may account for this. The nineteen lines with respect to alkaloidal content are homozygous as indicated by the maintained line differences through the three generations.

5. Soil variability and probably micro-climate differences may account for plot variabilities within the line.
6. Significant yearly differences between the lines may be accounted for by a combination of factors most important of which are rainfall or soil moisture, time of seeding and planting, the time of topping and harvesting. The 1950 crop had the highest total alkaloid content but had the lowest nicotine-nornicotine ratio. The 1952 crop had the lowest total alkaloid content but had a relatively high alkaloid ratio.
7. Seconds are generally low in alkaloid content and nicotine-nornicotine ratio on account of their relative over maturity. The decrease of total alkaloid content in the upper leaves may be the result of immaturity. An increase however, is the consequence of early topping.
8. A significant positive correlation was obtained between total alkaloids and nicotine-nornicotine ratio. Although not significant, a negative correlation seemed to exist between yield and total alkaloids.
9. Curing and drying definitely decreased total alkaloids and the proportion of nicotine to total alkaloids. It implies the occurence of a transformation of alkaloids from nicotine to nornicotine and a further breakdown of alkaloids into products

not sensitive to the method of analysis.

10. Mosaic virus inoculation definitely increased the total alkaloid content as well as the nicotine to total alkaloid proportion of reactive or susceptible lines only. There was no noticeable effect on resistant lines.

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