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CHEMICAL INVESTIGATIONS OF THE TOBACCO PLANT

IV. THE EFFECT OF THE CURING PROCESS ON THE ORGANIC ACIDS OF TOBACCO LEAVES

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1. INTRODUCTION

The investigations of this laboratory have been concerned for some time with the description and measurement of certain of the chemical changes that take place in the leaves of the tobacco plant when these are detached and allowed to dry with maximal exposure to the air. The process is referred to in the technology of the tobacco industry as that of curing, but from the standpoint of plant physiology the term katabolism perhaps more accurately describes what occurs. No attempt has been made to study any of these changes exhaustively; the system is far too complex to admit of this, and our knowledge of the composition of leaf material is much too limited. The effort has therefore been largely expended upon the study of certain groups of related substances in the hope that facts might be obtained that would reveal the general tendencies of the process. It was further hoped that relationships might be suggested between the behavior of the different groups from which inferences could be drawn respecting the chemical mechanisms involved. The results of one of these studies have been published as Bulletin 324 from this Station (18). In this bulletin was described the behavior of the protein and of the various soluble forms of nitrogen during the curing of tobacco leaves under commercial conditions. The experimental data were obtained from the analysis of five identical samples of leaves picked on the same day from Connecticut shade-grown tobacco plants. Each lot weighed 50 kilos as picked. One of these was extracted with boiling water and analyzed at once, the others were placed in the curing sheds in the customary way. A sample was withdrawn at the yellow stage of curing, one at the initial phase of the brown stage, and one at the time the leaves had become fully cured. The last sample was later subjected to fermentation along with the main crop from the same field. Extracts from each sample were prepared in the same way and the final results were expressed in terms of the actual weight of each component for which analytical data had been obtained. With the analysis of the fresh leaves as a control it was therefore possible to calculate the magnitude of the changes that occurred. This method of expression of the data is especially useful in cases in which the fundamental datum, the dry or fresh weight, or the total nitrogen, usually employed in expressing data on a percentage basis, undergoes alteration during the course of the experiment as was the case for all three of these factors in the experiment under discussion.

NOTE: The chemical investigations of tobacco herein described were carried out as part of a general project under the title "Cell Chemistry," by the Department of Biochemistry of the Connecticut Agricultural Experiment Station, New Haven, Conn. The Department has enjoyed the benefit of close coöperation from the Tobacco Substation. The expenses were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

The organic acids present in the extracts from three of these samples of tobacco leaves have now been investigated in detail and the results are described in the present bulletin. In a preliminary study of the organic acids of the tobacco plant, published as Bulletin 323, 1931, from this Station (17), we have already discussed the general problems presented by the metabolism of the organic acids in green leaves, and have reviewed in some detail the literature relating to the organic acids of the tobacco plant. The methods employed in the past for the study of organic acids in tobacco were described and the modifications we have introduced into the classical methods of organic acid investigation were given. In the course of the present study a number of sources of error in the earlier methods were detected and further improvements in details of the identification of the individual acids have been made; these are discussed in the following pages.

2. ISOLATION OF THE ORGANIC ACID FRACTION

The preparation of the water extracts from the fresh and cured tobacco leaves has been described in Bulletin 324 (18) to which reference should be made for details. The extracts that represented the fresh leaves, the leaves that had been cured until the yellow stage was reached (12 days), and the leaves that had been fully cured (51 days), viz. Lots E, A, and C of that bulletin, were worked up for the present study. The quantities of extract remaining from the previous investigation corresponded to 43.88 kilos of fresh leaves for Lot E, to 45.16 kilos of fresh leaves for Lot A (cured 12 days), and to 46.02 kilos for Lot C (cured 51 days) and each occupied approximately 7 liters. The data obtained from the analysis of these fractions are expressed herein on a uniform basis of 50 kilos of original fresh leaves.

The procedure adopted for the preparation of the organic acid fraction was as follows: Two volumes of alcohol (93 per cent) were slowly added to the extract with constant stirring; the precipitate of inorganic salts, gums, pectins, etc., was allowed to settle, the clear fluid was decanted and the sludge was centrifuged. The solid material was suspended in about 12 liters of water, was warmed on a steam bath until no more material seemed to pass into solution, and was then cooled and treated with two volumes of alcohol. The precipitate was removed as before and washed twice successively by shaking in the centrifuge bottles with 65 per cent alcohol.

The mother liquors and washings were combined and hot saturated barium hydroxide solution was slowly added, with careful stirring, until the precipitate assumed a yellow color and the fluid was alkaline to phenolphthalein; a volume of alcohol equal to that of the barium hydroxide used was then added and the precipitate was allowed to settle overnight. The clear fluid was decanted, the

precipitate was centrifuged off, washed twice with 65 per cent alcohol, and was then suspended in about 8 liters of water; sulfuric acid was added to produce a reaction of pH 1.0 (thymol blue paper) and the mixture was heated, with constant stirring, in a steam jacketed glass-lined kettle until the barium sulfate had become granular. The precipitate was allowed to settle, the clear fluid was decanted, and the barium sulfate was washed several times by decantation before being centrifuged. The main fluid and washings were then concentrated *in vacuo* to about 5 liters, two volumes of alcohol were added, and the precipitation with barium hydroxide was repeated. The acid solution obtained after decomposition of the second barium hydroxide precipitate was concentrated to 3 liters; it is designated in the following as the barium salts fraction.

It will be noted that the step designed to remove phosphoric acid, described in Bulletin 323, was omitted in the present instance.

The ether soluble organic acids contained in the barium salts fraction were removed in a continuous ether extractor of the Hagemann type similar to that described in Bulletin 323 (17). It accommodated a charge of about 3000 cc. of aqueous fluid and each charge was extracted for about 600 hours. An aqueous phase separated from the ether in the boiling flask as extraction proceeded and this was removed from time to time. After extraction was complete the ether was distilled, and the aqueous solutions were collected and evaporated to a sirup *in vacuo*. Absolute alcohol was repeatedly added and removed by distillation *in vacuo* until the water had been driven off, and the acids were then esterified by the alcohol vapor distillation method of Phelps and Phelps (13) using hydrogen chloride as catalyst. The esters were dissolved in about 3 volumes of ether and the solution was shaken with small portions of 20 per cent sodium carbonate solution (see Section 7, p. 679) until the aqueous phase remained alkaline. The ether solution was then washed with successive 25 cc. portions of water until the aqueous phase was neutral.

The alkaline and water washings of the esters were combined and acidified with sulfuric acid to pH 1 whereupon a small precipitate separated. This was removed and the filtrate was concentrated and extracted with ether in a continuous apparatus as before. The ether extract was dehydrated, esterified, washed with sodium carbonate and water, and added to the main lot of esters which had meanwhile been dried over anhydrous sodium sulfate. The alkaline washings were acidified and extracted several times in a separatory funnel with ether. The ether solution so obtained constitutes the "phenol" fraction, the acid aqueous solution remaining constitutes the "unesterifiable" fraction.

The different steps in this procedure may perhaps be more readily followed from Diagram 1 in which data from an actual experiment are included in order to give an idea of the magnitudes involved.

DIAGRAM 1

EXTRACT FROM PARTIALLY CURED LEAVES (LOT A)
DATA CALCULATED ON A 50 KILO BASIS
(ACTUAL 45.16 KILOS)
VOLUME 7660 CC.

Total N	189.4	gm.
Nicotine N	18.45	"
Nitrate N	54.13	"
Amino N	38.17	"
Ammonia N	21.94	"
Amide N	14.84	"
Organic solids	2026	"
Inorganic solids	977	"

+2 volumes alcohol

Alcohol precipitate
Organic solids... 216 gm.
Inorganic solids.. 1005 "
Total N..... 14.32 "

Filtrate (24 liters)
+excess hot saturated $Ba(OH)_2$ solution and an equivalent volume alcohol

Barium salts 1
$+H_2SO_4$ to pH 1

Filtrate A

$BaSO_4$

Organic acids (5 liters)
+2 volumes alcohol
+excess $Ba(OH)_2$ solution and an equivalent volume alcohol

Barium salts 2
$+H_2SO_4$ to pH 1

Filtrate B
Combined with Filtrate A

$BaSO_4$

Organic acids
Concentrated to 3 liters

Total N	35.66	gm.
Nitrate N	7.69	"
NH_3+nicotine N	4.66	"
Organic solids	907.1	"
Inorganic solids	40.5	"

Extracted with ether 600 hours.

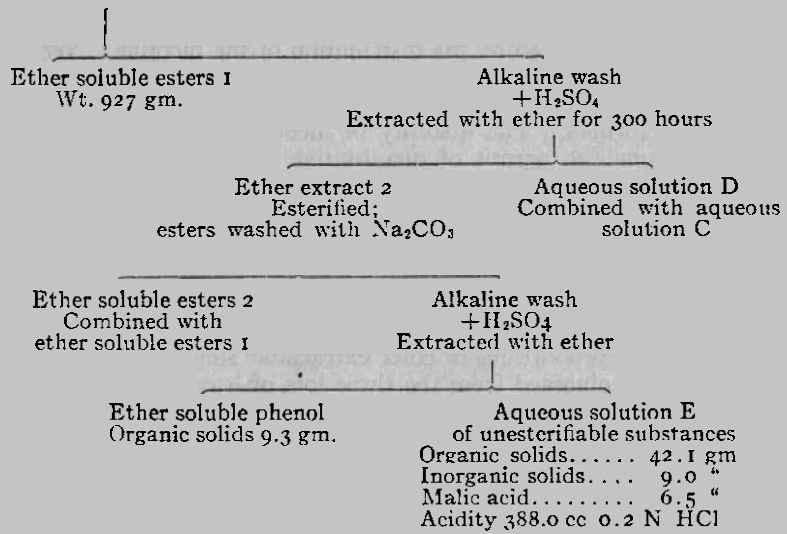
Ether extract 1
Wt. 585 gm.
Acidity 46,885 cc. 0.2 N HCl
Esterified; esters washed with Na_2CO_3

Aqueous solution C

Total N	28.08	gm.
Nitrate N	2.44	"
NH_3+nicotine N	4.36	"
Amino N	16.10	"
Total carbohydrate	16.9	"
Fermentable carbohydrate	6.0	"
Organic solids	310.5	"
Inorganic solids	34.1	"

Insoluble

Total N	0.68	gm.
Organic solids	13.1	"
Inorganic solids	0.12	"



Filtrates A and B were combined, freed from barium and from alcohol, and preserved for future investigation of the nitrogenous constituents. Aqueous solutions C and D were combined and preserved for investigation of the nitrogenous substances that are precipitated by barium hydroxide and alcohol.

Three fractions of interest in connection with the investigation of the organic acids were obtained. The bulk of these substances are included in the ether soluble esters 1 and 2. In addition there are the small "phenol" fraction, and the unesterifiable material represented by aqueous solution E. This last fraction was freed from chlorides and sulfates and analyzed for malic and citric acids according to the methods described in Section 7. The difference between the sum of these acids and the organic solids represents organic acids of unknown nature, but of types that are not readily, or are only partially, esterified.

3. THE COMPOSITION OF THE BARIUM HYDROXIDE-ALCOHOL PRECIPITATE

The organic acid fraction obtained from tobacco leaf extracts by precipitation with barium hydroxide and alcohol contains a complex mixture of substances other than organic acids of the usual type. Much of this material undoubtedly formed barium compounds insoluble in 65 per cent alcohol. Evidence that little of it owes

its presence to failure to wash the precipitate of barium compounds adequately is furnished by the distribution of the nicotine. When 3 cc. of the concentrated solution of the fraction were diluted, acidified, and treated with silicotungstic acid a barely detectable turbidity was formed. The quantity of nicotine present was manifestly too small to permit of quantitative estimation without the expenditure of an excessive amount of the fraction.

The presence of a considerable amount of nitrate in the barium salts fraction is readily explicable; barium nitrate has a very limited solubility in aqueous alcohol, the figure given by Seidell (15) being 1.85 per cent in 57 per cent alcohol, and the solubility rapidly decreases as the concentration of the alcohol is increased.

The relative proportions of ether extractable solids in the barium salts fractions obtained from the three lots of leaves are shown in Table 1.

TABLE 1. THE DISTRIBUTION OF ETHER EXTRACTABLE AND OTHER ORGANIC SOLIDS IN THE BARIUM SALTS FRACTIONS

	E Fresh leaf gm.	A Cured 12 days gm.	C Cured 51 days gm.	E Fresh leaf %	A Cured 12 days %	C Cured 51 days %
Organic solids of leaf extract.....	1981	2026	1820	100	100	100
Organic solids of barium salts fraction.	882	907	915	44.5	44.7	50.2
Organic solids of ether extract.....	669	585	577	33.8	28.8	31.8
Organic solids not extracted by ether..	259	311	262	13.1	15.3	14.4

The ether extractable solids amounted to 76 per cent of the solids of barium salts fraction from the fresh leaf, to 64.4 per cent in the case of the 12 day cured leaf and to 63.4 per cent in the case of the fully cured leaf.

Little information has been obtained regarding the chemical nature of the substances that are not extracted by ether from these solutions, but it is clear that at least two well defined chemical groups can be distinguished; these are acidic nitrogenous compounds, and reducing substances a part of which certainly represents fermentable carbohydrates.

The nitrogenous compounds are especially interesting. The order of magnitude of the quantities involved can be best appreciated from the data in Table 2. The figures show the total nitrogen of the barium salts fractions exclusive of the nitrate and ammo-

TABLE 2. THE DISTRIBUTION OF THE NITROGEN IN THE BARIUM SALTS FRACTIONS AFTER REMOVAL OF THE ORGANIC ACIDS WITH ETHER

	E gm.	A gm.	C gm.	E %	A %	C %	E %	A %	C %
Main extract from leaves before removal of organic acid fraction.									
Total N.....	105.1	189.4	173.0	100	100	100			
Amino N.....	11.9	38.2	37.9	11.3	20.2	21.9			
Amide N.....	3.4	24.6	27.4	3.2	13.0	15.8			
Barium salts fraction									
Total N.....	8.8	21.3	23.4	8.4	11.3	13.5	100	100	100
Amino N.....	3.0	14.7	16.6	2.9	7.8	9.6	34.1	69.0	71.0
Undetermined N	5.8	6.6	6.8	5.5	3.5	3.9	65.9	31.0	29.0

nia nitrogen, and of the quantities of nitrogen lost by adsorption on the precipitates of barium sulfate. It will be noted that a relatively large part of the nitrogen is present in forms that are not determinable by the ordinary indirect methods of analysis and that the quantity of this nitrogen in the barium salts fraction changed but little during curing.

Much of the amino nitrogen of this fraction probably represents aspartic and glutamic acid. These substances form barium salts that are insoluble in 65 per cent alcohol and, as curing progressed, there was doubtless a marked increase in the amounts of these acids present owing to hydrolysis of the protein.

The reducing substances in the barium salts fraction were determined by the method of Hagedorn and Jensen (7). A description of the modification of this method necessary when dealing with extracts from tobacco is to be given in a forthcoming publication (19). Table 3 shows the quantities of reducing substances before and after yeast fermentation in the three extracts; the figures are calculated arbitrarily in terms of glucose. The reducing substances in this fraction evidently make up a very small part of the reducing substances in the fresh leaf extract, and it is probably significant

TABLE 3. REDUCING CARBOHYDRATES OF THE BARIUM SALTS FRACTION AFTER REMOVAL OF ORGANIC ACIDS WITH ETHER

	E gm.	A gm.	C gm.	E %	A %	C %	E %	A %	C %
Original extract									
Total reducing sugars as glucose..	348	139	65	100	100	100			
Barium salts fraction									
Total reducing sugars.....	14.5	16.9	12.1	4.2	12.1	18.6	100	100	100
Fermentable....	4.7	6.0	4.2	....	....	....	32.4	35.5	34.7
Non-fermentable	9.8	10.9	8.0	....	....	....	67.6	64.5	65.3

that the quantity changes but little during curing although the quantity of reducing substances in the original extracts diminished enormously. The behavior suggests that the reducing substances, both of the yeast fermentable and unfermentable types, in the barium salts fraction are distinctly more stable than are the much larger quantities of reducing substances in the original extracts.

Little can be said regarding the nature of the balance of the solids in the barium salts fraction. Of the 259 gm. of solids in the fresh leaf fraction not extractable by ether approximately 90 gm. may be assumed to be nitrogenous, if the average nitrogen content of these substances is assumed to be 10 per cent; in addition there are approximately 15 gm. of reducing substances calculated as glucose. Thus the fraction contains about 150 gm. of substances concerning the chemical nature of which we know nothing; this is 17 per cent of the organic solids precipitated by barium hydroxide and alcohol from the fresh leaf extract.

4. THE ORGANIC ACIDS OF TOBACCO LEAVES

THE ACIDS OF FRESH TOBACCO LEAVES

A general description of the procedure whereby the esters of the organic acids of each extract were obtained has already been given. Owing to an accident whereby nearly the whole of the ester fraction derived from the fresh leaf extract was lost a detailed analysis could not be carried out. However, samples of the original extract, and of the barium salts fraction, had been preserved, and indirect determinations of malic and citric acid[1] were conducted on these. It was shown in the preliminary investigation (17) that such indirect determinations give results in close agreement with those derived from distillation of the esters and this conclusion is supported by data to be presented below.

The samples of extract and of barium salts fraction were acidified and extracted with ether in a continuous extraction apparatus. Malic acid was determined in the extract by the polarimetric method of Dunbar and Bacon (4) and citric acid by the pentabromoacetone method of Hartmann and Hillig (8, 9). The total acidity was determined by electrometric titration (for details see forthcoming paper) and from this the number of gram equivalents of organic acid present could be calculated. The data are shown in Table 4.

TABLE 4. THE MALIC AND CITRIC ACID CONTENT OF THE EXTRACT FROM 50 KILOS OF FRESH TOBACCO LEAVES

	Original extract		Barium salts fraction	
	gm.	equiv.	gm.	equiv.
Total organic acids.....	...	10.6	....	9.0
Malic acid.....	394	5.9	391	5.84
Citric acid.....	...	....	39.8	0.62

[1] Details of these methods are given in Section 7, p. 677.

It is evident that 1.6 equivalents, or 15 per cent of the ether extractable acid, are not precipitated in the barium salts fraction. This material probably represents monobasic acids. The remaining 85 per cent of the ether extractable acid consists of polybasic acids, and 6.46 equivalents, or 61 per cent of the total acidity, can be accounted for as malic and citric acids. The balance of 24 per cent of the total acidity is made up in part of polybasic acids that can be esterified and distilled. but the most of it consists of acidic substances of as yet unknown nature. A comprehensive investigation of the organic acids of fresh tobacco leaves is now being carried out.

THE ACIDS OF TOBACCO LEAVES CURED FOR 12 DAYS

Aliquot parts of the original extract, and of the barium salts fraction, derived from the sample of tobacco leaves that had been cured for 12 days, or until they had reached the yellow stage, were analyzed for malic and citric acids. The results are shown in Table 5. Only 1.2 equivalents, or approximately 11 per cent of the total acidity, escaped precipitation in the barium salts fraction. Malic and citric acid together account for 81.5 per cent of the total ether extractable acidity and only 7.5 per cent belongs to acids precipitable as barium salts but of unknown nature.

TABLE 5. THE MALIC AND CITRIC ACID CONTENT OF THE EXTRACT FROM 12 DAY CURED TOBACCO LEAVES WHICH, WHEN FRESH, WEIGHED 50 KILOS

	Original extract		Barium salts fraction	
	gm.	equiv.	gm.	equiv.
Total organic acids.....	....	11.6	....	9.87
Malic acid.....	407	6.07	404	6.03
Citric acid.....	....	....	191	2.99

The most striking feature of these data is the relatively enormous increase in citric acid that occurred. Comparison with Table 4 shows that nearly five times as much of this acid was present after the leaves had been cured 12 days. The amount of malic acid, however, remained unchanged.

The crude esters obtained as described in Section 2 were separated into three subfractions a) ether soluble esters, b) phenol fraction, c) water soluble products. The relative proportions of these fractions are shown in Table 8. The esters were distilled in the apparatus described in Bulletin 323 and the fractions secured are shown in Table 6. The proportions of malic and citric esters in the different fractions were determined respectively from the rotation in absolute alcohol solution and from the weight of pentabromoacetone. The melting points of derivatives of the esters are given in the last column of the table. A detailed description of the technique for the preparation of these derivatives is given in Sec-

tion 7. Aliquot parts of each fraction were saponified by barium hydroxide, the reagent was removed and the total solids of the solution were determined; the remainder of the solution was then subjected to fractional crystallization for the isolation of the individual acids.

The esters of Fraction 1, when treated with hydrazine hydrate, gave a small quantity of oxalic dihydrazide of m.p. 242-243°.. The

TABLE 6. DISTILLATION OF ESTERS OF ORGANIC ACIDS DERIVED FROM TOBACCO LEAVES CURED FOR 12 DAYS, AND COMPOSITION OF FRACTIONS

Weight of crude esters = 927 gm.
Pressure 4-7 mm.

Fraction No.	Boiling point °C	Weight of distillate gm.	Composition of fraction: Diethyl malate %	Triethyl citrate %	Diethyl oxalate %	Unknown esters %	Remarks
1	47–56	12.32	6.94	0.0	13.7	79.36	Oxalic dihydrazide m. p. 242-243°. Succinic benzylidene dihydrazide m.p. 233-234° Fumaric benzylidene dihydrazide m.p. 206°
2-3-4	96–102	564.94	100	0.0	0.0	0.00	Crystalline malic dihydrazide m.p. 179-180° $[\alpha]_D = -10.79°$
5	102–125	15.07	88.7	7.93	0.00	3.37	Malic dihydrazide m.p. 178-179°
6	130–136	173.0	?	91.5	0.0	8.5	Citric trihydrazide m.p. 104-106°. Citric benzylidene trihydrazide m.p. 194-195° 5.59 gm. in 25 cc. alcohol in 2 dm. tube: $\alpha = -0.17°$
7	136–140	80.3	?	88.9	0.0	11.1	Citric trihydrazide and benzylidene trihydrazide isolated. 5.59 gm. in 25 cc. alcohol in 2 dm. tube: $\alpha = -0.45°$
Residue 8		76.3	?	25.2	0.0	74.8	

Total esters recovered by distillation = 845.6 gm. or 91.4%
Distillation residue = 76.3 gm.
Total recovery = 921.9 gm. or 99.5%

identity of this was established by the determination of the melting point of a mixture with authentic material. The optical activity

of the esters in alcohol corresponded to the presence of 0.61 gm. of malic acid, but no malic dihydrazide could be isolated and, consequently, the presence of this acid in the fraction is not certain. After saponification the free acids were subjected to fractional crystallization. Fumaric acid separated readily in practically pure form; the succeeding crops of crystals were mixtures of lower and lower melting points until final crops of the correct melting point (185 to 186°) of succinic acid were obtained. This material, when condensed with p-toluidene, yielded pure succinic di-p-toluide (m. p. 254 to 255°). When a solution of the crystals was treated with acid permanganate, however, reduction occurred, and the solution furthermore gave a precipitate on treatment with mercurous nitrate. These tests indicated that the final crops, although melting at the correct temperature for succinic acid and showing no depression of melting point when mixed with authentic succinic acid, were still contaminated with fumaric acid. A specimen was therefore oxidized with permanganate until the fumaric acid had been destroyed and the silver salt of the remaining acid was prepared; a yield of 80 to 85 per cent was obtained. Moreover the consumption of permanganate during the oxidation corresponded to the presence of approximately 18 per cent of fumaric acid. The behavior indicates that these two acids form a eutectic mixture that is indistinguishable in melting point from succinic acid. Obviously therefore other criteria must be employed to determine the composition of the crops of crystals that separate from such a mixture.

The final mother liquor was sirupy and contained approximately half of the solids of the fraction. No information regarding the identity of the components of this mixture of acids was secured; small amounts of both fumaric and succinic acid were undoubtedly present. The aqueous solution was strongly acid to Congo red, and gave a voluminous precipitate with silver nitrate in concentrated solution; the silver salts dissolved on dilution. The solution absorbed bromine in the cold and yielded a crystalline sparingly soluble barium salt. Attempts to isolate maleic acid or its anilide gave inconclusive results. The barium salt gave an acid that melted at 125 to 130° and absorbed bromine in the cold. Maleic acid melts at 130°. The condensation product with aniline melted at 180 to 182° but too little was obtained to permit of purification. The maleic acid derivative melts at 209 to 211°.

A summary of the data obtained from the examination of fraction 1 is shown in Table 7.

Fractions 2, 3, and 4 consisted of pure *l*-diethyl malate. The specific rotations were $[\alpha]_D^{21} = -10.79°$, $-10.80°$, and $-10.77°$ respectively. The dihydrazides melted at 179 to 180°.

Fraction 5 was a small intermediate fraction that consisted largely of malic ester together with a small proportion of triethyl citrate and a little acid of unknown nature. Fractions 6 and 7 con-

tained the bulk of the triethyl citrate contaminated with approximately 10 per cent of esters of other acids. Both fractions possessed a small levo rotation, that of Fraction 7 being slightly greater than that of Fraction 6. Malic ester could not be detected in these fractions. The citric ester content was secured by determination

TABLE 7. THE COMPOSITION OF FRACTION 1 OF THE DISTILLED ESTERS FROM TOBACCO LEAVES CURED 12 DAYS

Weight of esters = 12.32 gm.
Weight of free acids = 7.56 gm.

Acid	gm.	
Oxalic	1.04	Oxalic dihydrazide m.p. 242-243°
Malic (?)	0.61	Calculated from optical activity
Fumaric	0.95	m.p. 282-283°
Succinic	1.12	m.p. 187-188°; ditoluide m.p 255-256°
Unknown	3.85	

of the yield of pentabromoacetone. Fumaric acid was not detected after saponification of the esters.

Fraction 8, the distillation residue, was about one-quarter citric ester mixed with esters of unknown composition. This fraction will be more fully discussed below.

The phenol fraction obtained from the alkaline washings of the esters by extraction with ether after acidification consisted of 9.32 gm. of a thick, dark brown, viscous sirup which deposited no crystals even after long standing in a vacuum desiccator.

The acid aqueous solution that remained after removal of the phenol was freed from inorganic ions. It is designated as the "unesterifiable fraction" since it contained those acids which either failed to esterify at all under the conditions employed, or were only partially esterified, together with any small quantities of acids the esters of which had been saponified during the process of purifying the main ester fraction. It contained 42.1 gm. of organic solids of which 6.51 gm. were malic acid as determined from the rotation after the addition of uranium acetate; citric acid could not be detected. The proportion of the total acids of the leaf in this fraction was materially less than was found in the similar fraction reported in Bulletin 323 (17); the technique of esterification employed in the present experiments was, however, much more thorough, and the purification of the ester fraction was accomplished with sodium carbonate instead of sodium hydroxide (See Section 7, p. 677).

Table 8 shows a summary of the composition of the ether extractable organic acids derived from the 12 day cured tobacco leaves. In assembling these data a number of assumptions have been made; these are explained in the foot-notes. Indirect analyses that may be compared with these figures are contained in Table 5.

TABLE 8. THE ACIDIC SUBSTANCES EXTRACTED BY ETHER FROM THE BARIUM SALTS FRACTION DERIVED FROM TOBACCO LEAVES THAT HAD BEEN CURED FOR 12 DAYS

The quantities are equivalent to 50 kilos of fresh leaf.

	gm.
Malic acid[1]	408.3
Citric acid	174.1
Oxalic acid	1.0
Succinic acid	1.1
Fumaric acid	2.4
Total identified acids	586.9
Unknown acids from esters[2]	53.0
Phenol fraction[3]	9.32
Unesterifiable fraction	35.6

[1] Includes 10 gm. of malic acid from ester fractions 1 and 5, the identity of which is in some doubt as the data depend on optical rotation only.

[2] Arbitrary factors, i.e. those for malic and citric acids, employed in calculating the weight of the acids from that of the esters: also includes the residue from distillation.

[3] The weight is probably high since the material may have contained ester groups.

THE ACIDS OF FULLY CURED TOBACCO LEAVES

Aliquot parts of the barium salts fraction and of the original extract derived from the fully cured tobacco leaves were analyzed for malic and citric acid with the results shown in Table 9. 1.63 equivalents of acid, or 15.4 per cent of the total acidity, were not precipitated by barium hydroxide and alcohol. The agreement in order of magnitude of this figure with the similar figures obtained from the fresh leaf, and from the leaf cured for 12 days, indicates that this part of the total acidity of the leaf tissue remained substantially constant throughout the process of curing. The malic and citric acids together account for 79.4 per cent of the total acidity and for 94 per cent of the acidity of the barium salts fraction.

The esters were separated into ether soluble, phenol, and unesterifiable fractions in the usual way. The ether soluble esters subjected to distillation weighed 885 gm. and the fractions obtained are shown in detail in Table 10.

The optical activity of Fractions 1 and 2 indicated that these contained considerable malic acid; aliquot parts were removed for the preparation of the hydrazides and the remaining esters were com-

TABLE 9. THE MALIC AND CITRIC ACID CONTENT OF THE EXTRACT FROM FULLY CURED TOBACCO LEAVES WHICH, WHEN FRESH, WEIGHED 50 KILOS

	Original extract		Barium salts fraction	
	gm.	equiv.	gm.	equiv.
Total organic acids	....	10.6	....	8.97
Malic acid	351	....	323	4.82
Citric acid	....	....	230	3.60

bined and saponified. The malic acid content, as determined from the rotation of the esters, was 14.67 gm., as determined from the rotation of the free acid after addition of uranium acetate, was 13.72 gm. Crystallization of the free acid yielded a small proportion of fumaric acid, but no succinic acid could be detected. A summary of the composition of these fractions is shown in Table 11. It will be noted that the unknown acids of this low-boiling fraction amount

TABLE 10. DISTILLATION OF ESTERS OF ORGANIC ACIDS DERIVED FROM FULLY CURED TOBACCO LEAVES, AND COMPOSITION OF FRACTIONS

Weight of crude esters = 885 gm.
Pressure 4-7 mm.

Fraction No.	Boiling point °C	Weight of distillate gm.	Composition of fraction: Diethyl malate %	Triethyl citrate %	Unknown esters %	Remarks
1	50–75	27.4	38.33	....	61.67	Malic dihydrazide m.p. 177-178° Malic benzylidene dihydrazide m.p. 173° Fumaric benzylidene dihydrazide m.p. 209°
2	75–98	4.58	91.14		8.86	Malic dihydrazide m.p. 178-179°
3	97–98	425.0	100		0.00	$[\alpha]_D^{25} = -10.7°$ Malic dihydrazide m.p. 179-180°
4	123–132	6.78	14.33	72.03	13.64	
5	132–133	170.5	?	91.56	8.44	Citric trihydrazide m.p. 103-106° 5.51 gm. in 25 cc. alcohol in 2 dm. tube: $\alpha = -0.10°$
6	133–136	125.8	?	94.84	5.16	Citric trihydrazide m.p. 103-105° Citric benzylidene trihydrazide m.p. 193-194°. 5.42 gm. in 25 cc. alcohol in 2 dm. tube: $\alpha = -0.45°$
Residue 7		73.1	?	25.17	74.83	

Total esters recovered by distillation = 760.0 gm. or 86%
Distillation residue = 73.1 gm.
Total recovery = 833.0 gm. or 94%

TABLE II. THE COMPOSITION OF FRACTIONS 1 AND 2 OF THE DISTILLED ESTERS FROM FULLY CURED TOBACCO LEAVES

Weight of esters = 31.98 gm.
Weight of free acids = 18.96 gm.

Acid	gm.
Oxalic	0.0
Malic	14.67
Fumaric	1.94
Succinic	0.0
Unknown	2.25

to only 12 per cent of the fraction in contrast to 50 per cent in the case of the leaves cured for 12 days.

Fraction 3 was pure diethyl malate of specific rotation $[\alpha]_D^{25} = -10.7°$.

Fraction 4 was a small intermediate fraction that consisted mostly of triethyl citrate and Fractions 5 and 6 consisted almost entirely of this ester. Aliquot parts of Fractions 4, 5 and 6 were combined and saponified with barium hydroxide; alcohol was then added to make 20 per cent by weight, and the fluid was cooled and centrifuged. The precipitate of barium citrate was decomposed with sulfuric acid, the reagents were removed, and the solution was subjected to crystallization. No difficulty was experienced in obtaining well crystallized anhydrous citric acid of melting point 151 to 152°, and the mother liquor from the crystals, after being dried to constant weight, yielded a solid that melted at 148 to 152°, and showed no depression of melting point on being mixed with pure citric acid; the barium citrate was therefore practically pure. The quantity obtained was equivalent to 191.3 gm. whereas analysis of the fractions by the pentabromoacetone method indicated that 195 gm. of citric acid were present.

The filtrate from the barium citrate was freed from reagents and subjected to fractional crystallization. No fumaric acid was detected and the solution yielded a sirup which contained 24.2 gm. of solids and yielded no crystalline material even on long standing. It contained little or no citric acid according to the results of tests with Denigès reagent.

Ester fractions 5 and 6 were weakly levorotatory, the rotation of 6 being greater than that of 5. The presence of malic acid could not be detected.

Fraction 7, the residue from the distillation, was saponified and analyzed for citric acid, approximately 25 per cent being found. It therefore resembled the residue from the distillation of the esters from the leaves cured for 12 days. The balance consisted of esters of high boiling points together with small amounts of dark colored decomposition products.

Equal weights of the residues from the distillation of the esters of the 12 day and the fully cured leaves were mixed and saponified

with barium hydroxide, and the barium salts were precipitated by the addition of two volumes of alcohol. The precipitate was removed, decomposed with sulfuric acid, freed from inorganic ions and treated with norite until colorless. On evaporation a small quantity of pure fumaric acid melting at 280° was deposited, but the mother liquor failed to yield further crystalline material. The acids present were manifestly very soluble and the solution was strongly levorotatory. It was therefore analyzed for malic and citric acid. The results are shown in Table 12 expressed in terms of 100 kilos of original fresh leaf. If equal distribution of the acids be assumed, the data, on division by 2, become comparable to the data in the previous tables.

The isolation of pentabromoacetone is positive evidence of the presence of citric acid in this distillation residue, but the observation of a levo rotation equivalent to the presence of 39 per cent of malic acid rendered the positive identification of this acid necessary. Accordingly, after the removal of the fumaric acid, the mother

TABLE 12. RESIDUES FROM THE DISTILLATION OF THE ESTERS DERIVED FROM TOBACCO LEAVES CURED FOR 12 DAYS AND 51 DAYS

Each lot represented the material derived from 50 kilos of fresh leaf and the figures given represent 100 kilos of original fresh leaf.

	gm.
Residues	149.4
Total solids after saponification	101.6
Acids precipitated by barium hydroxide and alcohol	93.7
Citric acid	20.2
Malic acid	36.6
Fumaric acid	2.53
Unknown acids	28.4

liquor was diluted with water and the citric acid was precipitated by the addition of barium hydroxide. The barium salts were filtered off, decomposed, and the free acid was subjected to crystallization. Crystals of anhydrous citric acid were readily obtained. The filtrate from the barium citrate was freed from reagents, concentrated to a sirup *in vacuo* and finally to a hygroscopic solid in a vacuum desiccator. The optical rotation indicated that this material consisted of malic acid to the extent of 53 per cent. Portions of it were condensed with p-nitrobenzyl bromide according to the directions of Lyman and Reid (11), and also with aniline according to Mulliken (12). The nitrobenzyl esters and anilides obtained were obviously mixtures, but, by repeated fractional crystallization, a specimen of the p-nitrobenzyl ester of malic acid of melting point 122 to 123° was secured. A specimen of the same compound, derived from authentic malic acid prepared from tobacco, melted at 124 to 126° and a mixture of the two melted at the same temperature. These melting points are 4° lower than those of Lyman

and Reid, the most probable explanation being a slight racemization of our material.

Analysis of our specimen of the p-nitrobenzyl ester gave C, 53.49; H, 4.85; N, 7.15: theory for $C_{18}H_{16}N_2O_9$, C, 53.44; H, 3.99; N, 6.93 per cent. This analysis completes the proof of the presence of malic acid in the residues from the distillation of the esters.

The anilides were more difficult to purify than the nitrobenzyl esters but a preparation the melting point of which remained constant after three successive crystallizations was ultimately secured. Under the microscope the crystals were undistinguishable from those of authentic malanilide prepared from malic acid obtained from the Eastman Kodak Co. The melting point was 193 to 193.5° uncorrected. Authentic malanilide melted at 196 to 198°, and a mixture of the two melted at 193 to 194°. Analysis gave C, 67.57; H, 5.91; N, 9.85: theory for $C_{16}H_{16}N_2O_3$, C, 67.57; H, 5.68; N, 9.86 per cent. There is therefore no doubt that our preparation was malanilide; the somewhat low melting point may have been due to a partial racemization during the various steps of the isolation.

The presence of malic acid among the esters of boiling point higher than that of citric ester accounts for the slight optical activity of the ester fractions 5 and 6 from the 12 day cured leaf and the fully cured leaf. It will be recalled that, in each case, the activity of the sixth fraction was somewhat greater than that of the fifth. The data suggest that malic acid is present in the form of an ester of high boiling point, a portion of which distilled into these fractions.

The optical activity of the distillation residues themselves is also probably largely due to the presence of this material although the presence of other optically active acids is not excluded by the evidence at hand.

The chemical nature of this ester of malic acid is uncertain. There is a possibility that it is simply the half ester, or it may be a self ester of malic acid in which the hydroxyl group of one molecule of the acid is combined with the carboxyl of another. Franzen and Ostertag (6) encountered an analogous behavior when investigating the acids from *Echiveria secunda glauca*. A fraction that distilled above the boiling point of citric ester yielded *l*-malic dihydrazide and, after saponification, silver malate. They postulated the presence of diethyl ester of malylmalic anhydride in this fraction, but do not account for the hydrolysis which must have preceded the formation of the simple dihydrazide they isolated. Further investigation of the behavior of malic acid on esterification and distillation will be required before the presence of malic acid in these fractions of high boiling point is understood.

The phenol fraction derived from the cured tobacco leaf weighed 5.06 gm. and consisted of a dark and viscous oil. No crystalline material separated from it even on long standing.

The unesterifiable fraction contained 70.9 gm. of solids of which 23 gm. consisted of malic acid. The greater quantity of malic acid in this fraction than in the analogous fraction from the 12 day cured leaf is probably connected with the more extensive washing the esters received during their preparation. The balance of 47.9 gm. of solids consisted of acids of unknown nature or partial esters of these.

TABLE 13. THE ACIDIC SUBSTANCES EXTRACTED BY ETHER FROM THE BARIUM SALTS FRACTION DERIVED FROM FULLY CURED TOBACCO LEAVES

The quantities are equivalent to 50 kilos of fresh leaf.

	gm.
Malic acid[1]	333.8
Citric acid	207.9
Oxalic acid	trace
Succinic acid	0.0
Fumaric acid[2]	3.2
Total identified acids	544.9
Unknown acids from esters[3]	54.2
Phenol fraction	5.1
Unesterifiable fraction	47.9

[1] Includes 14.67 gm. from distilled ester fractions 1 and 3 calculated from rotation and identified as hydrazides: also 23 gm. from unesterifiable fraction calculated from rotation.

[2] Includes 1.4 gm. from distillation residue.

[3] Calculated from weight of solids after saponification of various fractions and, in part, by use of arbitrary factors for the conversion of the weight of the unknown esters to weight of acid.

The analytical data secured from the examination of the acidic substances derived from the fully cured tobacco leaf are summarized in Table 13. Indirect analyses of the malic and citric acid content of this fraction with which these figures should be compared are shown in Table 9.

EXAMINATION OF THE RESIDUES FOR THE PRESENCE OF ACIDS

The extracted leaf residues remaining after treatment of the leaves with boiling water may be expected to contain oxalic acid combined as the insoluble calcium salt. Other insoluble combinations of organic acids may also be present and, in order to obtain a complete picture of the organic acids of the leaves, it is therefore necessary to study the residues as well as the water extract. In the course of this study observations were made upon the volatility of oxalic acid and of its esters which showed that accurate determinations of this acid can be made only by special methods. These methods together with the results of their application to the material under investigation will be presented in a later section. In the present section the results of the preliminary study are given. It must be emphasized that these are only of qualitative significance with respect to oxalic acid.

Samples of 2 kilos each of the residues from the three lots of leaves were separately heated with 1 N sulfuric acid for 3 hours, were then filtered and pressed, and the press cakes were washed with water. The acid extracts were treated with an excess of barium hydroxide and then with 2 volumes of alcohol. The precipitates were decomposed and the solutions were combined and extracted with ether in the continuous extraction apparatus. The acids were then esterified and the esters were separated into ether soluble, phenol and unesterifiable fractions in the usual way.

The data obtained from the examination of these fractions is shown in Table 14 in which the quantities represent 150 kilos of original fresh leaf material. Only 75.7 gm. of acid were obtained and of this only 16.6 gm. or 22 per cent were identified. The quantities of malic and citric acid found are essentially negligible and show that the extraction with hot water of the leaf material had been extremely efficient. Furthermore it is clear that only very small amounts of these acids are present in insoluble combinations in the fresh leaves.

TABLE 14. ACIDS DERIVED FROM EQUAL QUANTITIES OF THE RESIDUES AFTER HOT WATER EXTRACTION OF FRESH, 12 DAY CURED, AND FULLY CURED TOBACCO LEAVES

The figures are equivalent to a weight of 150 kilos of fresh leaves.

	gm.
Malic acid[1]	11.5
Citric acid	4.8
Oxalic acid[2]	0.3
Total identified acids	16.6
Unknown acids from esters	14.7
Phenol fraction	4.2
Unesterifiable fraction	40.2

[1] Calculated from optical activity of esters and identified as malic dihydrazide.

[2] Identified as oxalic dihydrazide.

The extremely small quantity of oxalic acid found drew our attention to the losses of this acid that occur during the usual process of preparation and analysis of the ester fraction, and gave rise to the study of the behavior of oxalic acid described in Section 5.

5. THE DETERMINATION OF OXALIC ACID

THE VOLATILITY OF OXALIC ACID AND OF ITS ESTER

A general survey of the procedure for the preparation of the esters of organic acids from leaf material suggested that losses of oxalic acid might occur during the process of dehydrating the ether extract previous to esterification. Accordingly 50 gm. of oxalic acid dihydrate were dissolved in 500 cc. of water, and the solution was concentrated to a sirup *in vacuo* and dehydrated by adding 300 cc. of alcohol, distilling this, and repeating the last operation twice

more. The oxalic ester in the alcoholic distillate was saponified by boiling with alkali, and oxalic acid was determined by precipitation as calcium oxalate; the equivalent of 16.07 gm. of the dihydrate were found. The residue in the flask was likewise saponified and analyzed, 23.05 gm. of oxalic acid being found. During the operations, therefore, 21.8 per cent of the oxalic acid taken was lost in the form of volatile acid or ester that was removed by the vacuum pump.

In a second experiment 20 gm. of oxalic acid were treated in the same way, but the distillate was received into an excess of alkali. In this case 11.97 gm. or 58.8 per cent of the oxalic acid were found in the receiver while 8.38 gm. or 41.2 per cent remained in the distillation flask. It is clear therefore that the dehydration and esterification of oxalic acid cannot be quantitatively conducted under the customary conditions. In view of this the data for oxalic acid in tobacco extracts described above, and also those given in Bulletin 323 (17), have no quantitative significance.

Fortunately, however, it is relatively easy to determine oxalic acid by other methods. The esterification procedure is necessary in order to provide incontrovertible evidence of the presence of the other organic acids. Under the extremely thorough conditions adopted in the experiments described above it is probable that nearly all the oxalic acid was volatilized and lost; this acid therefore provides only a minor source of error in the weight of the esters obtained and its presence may be disregarded.

THE DETERMINATION OF OXALIC ACID IN ETHER EXTRACTS OF DRIED LEAVES

In view of the certainty of loss of oxalic acid during the processes involved in the preparation of the esters attention was turned to the development of a method that could be applied directly to samples of dried leaf tissue. Preliminary investigation showed that the whole of the oxalic acid could be removed by ether extraction provided the sample were previously treated with sufficient sulfuric acid to bring about a reaction of approximately pH 1. The conditions for its extraction are therefore similar to those employed in the determination of nitric acid in leaf tissue by ether extraction by Pucher, Vickery and Wakeman (14) and reference may be made to their paper for a detailed description of the technique.

A 2.00 gm. sample of dry leaf tissue is adjusted to pH 1.0 with sulfuric acid and extracted with alcohol-free ether continuously for 18 to 24 hours. The ether solution is treated with 25 cc. of water and 2 cc. of 5 N sodium hydroxide, the ether is evaporated, and the aqueous solution is made to 100 cc. A 25 cc. portion of this solution is acidified to Congo red with 0.5 N hydrochloric acid, the precipitate is allowed to flocculate, is filtered on asbestos in a Gooch

crucible and washed with a little water. A drop of methyl red is added to the clear filtrate, ammonium hydroxide is added to a faint alkaline reaction, and 2 to 3 cc. of glacial acetic acid are added followed by 5 cc. of 10 per cent calcium chloride dihydrate solution. The mixture is allowed to stand at room temperature for two hours, the calcium oxalate is filtered on asbestos in a Gooch crucible and washed with very dilute ammonia. The crucible and its contents are transferred to a 100 cc. beaker, 5 cc. of 50 per cent sulfuric acid and 20 cc. of water are added, and the solution is heated to boiling and titrated with N/50 permanganate. The oxalic acid is calculated from the relation 1 cc. of N/50 permanganate = 0.9 mg. anhydrous or 1.26 mg. hydrated oxalic acid.

If the directions are followed with strict attention to details citric acid is not precipitated and reprecipitation of the calcium oxalate is not necessary. Data given in Table 15 illustrate the accuracy of the method as applied to mixtures of oxalic and citric acids. The proportions of citric acid were chosen so as to exceed those to be expected in extracts from tobacco leaves. The data are given in terms of the anhydrous acids.

TABLE 15. THE DETERMINATION OF OXALIC ACID IN THE PRESENCE OF CITRIC ACID

Oxalic acid taken mg.	Citric acid taken mg.	Oxalic acid found mg.
13.5	0	13.7
13.5	0	13.6
13.5	0	13.5
13.5	32.0	13.6
13.5	32.0	13.7
13.5	32.0	13.7
13.5	64.0	14.0

As a demonstration of the identity of the oxalic acid derived from tobacco, two 2 gm. samples were extracted with ether, the aqueous solutions were combined, and the oxalic acid was precipitated from the whole solution under conditions analogous to those described. The calcium oxalate, after being dried, weighed 0.1638 gm. and yielded 0.0634 gm. of calcium oxide instead of the theoretical 0.0621 gm.

THE FORM OF COMBINATION OF OXALIC ACID IN TOBACCO LEAVES

The evidence that oxalic acid is present in tobacco leaves almost entirely as calcium oxalate is necessarily indirect but seems almost conclusive. Ten gm. samples each of fresh and of cured leaf tissue were exhaustively extracted by boiling water. The tissue was analyzed both before and after the treatment, and the extracts, after being concentrated in vacuo, were likewise examined for oxalic acid. The data are collected in Table 16 and show that from 80 to 90 per cent of the oxalic acid remains insoluble in boiling water.

Even more interesting is the demonstration that the small proportion of oxalic acid that was removed in the water extract was almost entirely lost during the concentration; traces only were found unless the solution was made faintly alkaline before this operation was conducted.

TABLE 16. THE OXALIC ACID CONTENT OF 10 GM. OF TOBACCO LEAF BEFORE AND AFTER EXTRACTION WITH BOILING WATER

	Before extraction	After extraction	Extract	Insoluble
	mg.	mg.	mg.	%
Fresh leaf	191	156	1.4	81.5
Fresh leaf	191	156	1.4	81.5
Cured leaf	242	224	trace	92.8
Cured leaf	242	224	19.1[1]	92.8

[1] The extract was adjusted to pH 8.0 before concentration.

Another striking illustration of the magnitude of the loss of oxalic acid that may occur through volatilization during the concentration of aqueous solutions was obtained in the course of preparing extracts from three 100 kilo samples of fresh leaves. The extracts were made by boiling the leaves three successive times with water acidified to pH 3 in order to retain the nicotine. The residues were pressed and ground between each extraction and the total volume of extract obtained in each case amounted to about 120 liters; this was concentrated *in vacuo* to 5 liters, treated with 2 volumes of alcohol and the filtrate from the precipitate was again concentrated to 5 liters. The oxalic acid data are shown in Table 17.

TABLE 17. THE LOSS OF OXALIC ACID ENCOUNTERED IN THE PREPARATION OF ACID AQUEOUS EXTRACTS (pH 3) FROM 100 KILO SAMPLES OF FRESH TOBACCO LEAVES

The figures are grams of oxalic acid per 100 kilos of tissue.

	Original leaf	Extracted residue	Extract	Loss
	gm.	gm.	gm.	
I	249	149	0	40.2
II	308	190	0	38.3
III	279	146	0	47.5

The acidulated water was a much more effective extracting agent for the oxalic acid than is water itself, but the whole of the oxalic acid rendered soluble disappeared during the vacuum concentrations. Oxalic acid is obviously volatile with water vapor and analytical data obtained on even faintly acid solutions that have been concentrated may be grossly erroneous.

The volatility of oxalic acid from boiling aqueous solutions has also been recently observed by Howard (10) in connection with an investigation of the industrial hazard involved in working with hot solutions of this acid.

THE BEHAVIOR OF OXALIC ACID DURING CURING OF TOBACCO

The oxalic acid content of dried subsamples of the fresh leaf, the 12 day cured, and the fully cured tobacco leaf described above was determined by the method given on p. 668. The data are given in Table 18 and show that the oxalic acid changed very little if at all during the operation of curing.

The data in the preceding sections imply that oxalic acid is a relatively unimportant constituent of the tobacco leaf at any stage of curing, traces only being detected in the ester fractions subjected to distillation. The observations of the behavior of oxalic acid reported in the present section furnish the explanation of our

TABLE 18. THE OXALIC ACID CONTENT OF FRESH, 12 DAY CURED AND FULLY CURED TOBACCO LEAF

The figures are grams per 50 kilos of original fresh leaf.

	Oxalic acid	Dry material
	gm.	%
Fresh leaf	131.5	1.98
12 day cured leaf	122.6	2.12
Fully cured leaf	119.1	2.17

failure to detect significant amounts of this acid in the specimens of tobacco described in Bulletin 323 and in the present work. As has long been known oxalic acid occurs in important amounts in tobacco leaves but the ester distillation method is entirely unsuitable for the analysis of mixtures that contain this acid. Fortunately, however, that part of the oxalic acid which is extracted from the leaves at pH 3 by hot water is practically entirely volatilized during the subsequent concentration so that the ester distillation method can be relied on to furnish accurate information regarding the other acids present. The traces of oxalic acid that remain do not provide an important source of error in such data as the total acidity or the weights of the esters.

6. THE METABOLISM OF THE ORGANIC ACIDS DURING THE CURING OF TOBACCO

The data for the malic, citric and oxalic acid content and for the total acidity of the extracts from partially cured and fully cured tobacco leaves are brought together in Table 19. The difference between the figure for acidity of the water extract and that for the acidity of the barium salts fraction furnishes an approximate measure of the acids that form barium salts soluble in 60 per cent alcohol. It is assumed for the present that these consist mainly of monobasic acids. The figures for the equivalents of unknown acids were obtained by subtracting the sum of the equivalents of citric and malic acid from the acidity of the barium salts fraction; the factor 0.67 was used to calculate the weight of these acids from the esters, that

is they are arbitrarily calculated as malic acid. The figures for

TABLE 19. THE ORGANIC ACIDS OF TOBACCO LEAVES
The figures are grams per 50 kilos of original fresh weight and gram equivalents on the same basis.

	Fresh leaves		Cured 12 days		Fully cured	
	gm.	equiv.	gm.	equiv.	gm.	equiv.
Organic acids of water extract		10.6		11.06		10.6
Organic acids of barium salts fraction		9.0		9.87		8.97
Unknown monobasic acids (?)		1.6		1.29		1.6
Malic acid	391	5.84	404	6.03	323	4.82
Citric acid	39.8	0.62	191	2.99	230	3.60
Unknown polybasic acids	170	2.54	50	0.85	37	0.55
Oxalic acid	131.3	2.92	122.6	2.72	119.1	2.66

oxalic acid were obtained by analysis of samples of the dry leaf tissue and it is assumed that no oxalic acid was present either in the concentrated water extract or the barium salts fraction.

The accuracy with which the total acidity of the extract can be determined is probably of the order of 10 per cent. The figures indicate, therefore, that the total acidity underwent no significant change during the curing process. Constancy of the acidity implies constancy of the basicity of the leaves. The reaction of the water extract of the fresh leaf was pH 5.4, that from the 12 day cured pH 5.6 and that from the fully cured pH 5.55.

The acidity of the barium salts fraction likewise remained constant within the limits of error of the titration method employed.

The data for malic and citric acids in Table 19 were obtained by indirect methods of analysis. That these methods are trustworthy is shown by the data for the results of the distillation of the esters given in Table 20. Unfortunately the complete data from the partially and the fully cured leaves only are available.

The agreement of the figures for malic and citric acids in Table 20 with those in Table 19 is extremely close. The figure for malic acid from the 12 day cured leaf shown in Table 20 depends on the weight of ester fractions more than 97 per cent of which possessed a specific rotation of $[\alpha]_D^{24} = -10.7$ to $-10.8°$ and was therefore pure. In the case of the fully cured leaf 91 per cent of the malic acid was isolated in an equal state of purity. The remainder of the malic acid in each case was obtained by observations of the optical rotation of various fractions, and from each of these fractions a derivative of malic acid was isolated in pure form.

The figures for citric acid depend upon the weight of pentabromoacetone obtained from the different fractions in which the ester of this acid was found. Moreover pure crystalline citric acid was secured from some of the ester fractions as evidence of their purity, and, in all cases, derivatives of citric acid were prepared in order to be certain of the identity. Consequently there is every reason to believe that the figures obtained for malic acid by the

optical rotation in the presence of uranium acetate, and for citric acid by the pentabromoacetone method, given in Table 19 are essentially correct. The following discussion of the metabolic changes that occurred during curing is therefore founded on these figures.

TABLE 20. THE ORGANIC ACIDS FROM 12 DAY AND FROM FULLY CURED TOBACCO LEAVES AS DETERMINED FROM THE RESULTS OF DISTILLATION OF THE ESTERS

The figures show the quantities found in 50 kilos of original fresh leaf.

	Cured 12 days	Fully cured
	gm.	gm.
Malic acid	408.3	333.8
Citric acid	174.1	207.9
Succinic acid	1.1	0.0
Fumaric acid	2.4	3.2
Total identified acids	585.9	544.9
Unknown acids	53.0	54.2
Phenol fraction	9.3	5.1
Unesterifiable fraction	35.6	47.9

The quantity of malic acid did not change during the first 12 days of curing but subsequently diminished by about 16 per cent. This change is greater than the order of magnitude of the error of the measurement, and it seems clear that malic acid is involved, although to only a limited extent, in the katabolic processes that occur late in the curing operation.

The quantity of citric acid in the leaf tissue increased enormously; at the expiration of 12 days nearly five times as much of this acid was present as was found in the fresh leaf, and, at the end of the curing period, the quantity had further increased to 5.8 times the original amount. The actual quantities involved show that the synthesis of citric acid was one of the major changes in the composition of the tissue.

The period during which the most active citric acid synthesis took place was also that in which a number of other major changes occurred, and it is of interest to attempt to correlate the phenomena. It is shown in Bulletin 324 (18) that, during the first 12 day interval, 51.2 gm. of nitrogen originally combined in the protein of the leaf became soluble in hot water. Of this nitrogen 26.3 gm. appeared in the extract as amino nitrogen and 21.2 as amide nitrogen. It may be assumed that the amide nitrogen arose from the oxidative deamination of amino acids produced by protein hydrolysis and, consequently, the carbon chains of these amino acids became available for the synthesis of nitrogen-free compounds. If it be assumed that the average nitrogen content of these amino acids was 10 per cent, about 200 gm. of deamination products were thus made available. Within this period 134 gm. of citric acid were

formed, and it would therefore appear possible that the citric acid may have arisen as a by-product of the deamination of amino acids. On chemical grounds, however, this is hardly likely. Very few of the products of protein hydrolysis are so constituted as to form probable precursors of citric acid. Glycine and alanine alone on deamination might be assumed to yield products that could be easily elaborated into citric acid, and these two amino acids as a rule make up a relatively small part of the products of hydrolysis of most proteins. It seems improbable therefore that the decomposition products of the protein played an important part in the formation of the citric acid.

The other component of the tissue which underwent an extensive contemporaneous change in quantity is the carbohydrate. Unfortunately our data for the carbohydrate content of the extracts of these lots of tobacco leaves include only measurements of the total reducing power. However, the change in the reducing power during the 12 day interval of curing indicated a loss of 209 gm. of glucose if it is permissible to calculate the reducing power in terms of this sugar. Recent studies of extracts from other lots of fresh tobacco leaves have shown that about 60 per cent of the total reducing power is due to the presence of yeast fermentable carbohydrate. Consequently the contemporaneous disappearance of the equivalent of 209 gm. of reducing sugar and the formation of 134 gm. of citric acid indicates as a reasonable possibility that the citric acid had its origin from this carbohydrate. Further evidence is supplied by the observation that, in the interval of 39 days required to complete the curing process, 34 gm. more of citric acid were formed and 74 gm. of apparent carbohydrate vanished.

This view is also reasonable from the strictly chemical point of view. It has long been known that carbohydrates can be converted into citric acid by molds and the recent experiments of Chrzaszcz and Tiukow (2) and of Bernhauer and Siebenäuger (1) are of significance in this connection.

Although the view that the citric acid formed during curing originates from carbohydrates has much to recommend it, an equally strong argument can be advanced for an entirely different origin of this acid. It will be recalled that the total acidity of the extract, and also of the barium salts fraction, underwent no substantial change during curing. Reference to Table 19 shows that 0.62 equivalents of citric acid were present in the fresh leaf and 2.99 equivalents in the 12 day cured leaf—an increase of 2.37 equivalents. In the same period the quantity of unknown acids decreased from 2.54 equivalents to 0.75 equivalents, that is by 1.79 equivalents. These last figures were obtained by difference and are consequently less trustworthy than are the measurements of the acidity of the extract. Within the limits of error of the method used, therefore, the order of magnitude of the quantity of citric acid that appeared

is the same as the order of magnitude of the unknown acids that disappeared. Consequently it is possible to regard the synthesis of citric acid as a conversion of unknown, but acidic precursors, into citric acid. The substantiation of this view involves the isolation and identification of a considerable proportion of the unknown acids of the fresh leaf. If it can be shown that these are substances that might be expected to be converted into citric acid by natural processes this view of the origin of the citric acid will receive material support. If, however, these acids turn out to be largely substances of a totally different type the idea of carbohydrate origin for the citric acid will become the more probable. For the present we do not feel that a choice can properly be made between these two views.

The rapid diminution of the quantity of unknown acids is itself an interesting example of the katabolic processes that occurred in the early stage of curing. If these substances were not converted to citric acid the question of their fate remains open. One possibility may be suggested. During the first 12 days, as has already been mentioned, 21.2 gm. of amide nitrogen made its appearance. Although definite proof has not been obtained that this amide nitrogen originated from the deamination of amino acids that in turn arose from the hydrolysis of the protein, the quantities involved are such that this is highly probable. It may be assumed that the ammonia so formed condensed with some suitable carbon compound with the formation of asparagine. 21.2 gm. of amide nitrogen are equivalent to 200 gm. of asparagine, and the conversion of a carboxyl into an amide group involves very little change in the weight of the product. Although the probable weight of the unknown acids that disappeared is not sufficient to account for the weight of the asparagine that was formed it is possible that some of these acids played a part in this process.

The quantity of oxalic acid in the tobacco leaves diminished so little during curing that it may be inferred that this substance enters to only a minor degree into the katabolic processes. Oxalic acid is regarded by Ruhland as of fundamental significance in the general metabolism of many plants being called forth in response to the formation of ammonia as a part of the mechanism to provide for the maintenance of the reaction of the tissue within the proper limits. Although we have evidence in the case of the tobacco leaves under discussion that an abundance of ammonia was formed (See Bulletin 324 (18)) there was no corresponding increase in the quantity of oxalic acid.

It is evident from the data given in Tables 19 and 20 that the three chief acids of tobacco leaves can be determined by indirect methods with a considerable degree of accuracy. The results of the ester distillation method serve as confirmatory evidence with respect to the quantities of these acids present as well as to their chemical nature. This method has an additional advantage in that

by its use evidence may be secured of the presence of other acidic substances, these may be separated from each other and, in many cases, identified.

The discussion in the preceding pages has been limited to data secured by examination of water extracts prepared from tobacco leaves and to the barium salts fraction derived from these extracts. With the exception of oxalic acid, which is determined by a special method, insoluble acidic substances have been left out of account. It is desirable for many reasons to be able to determine the total quantity of acidic substances in the leaf tissue, and an electrometric titration method to be described in a forthcoming publication has therefore been developed whereby this can be accomplished. In brief this method consists in extraction of the acids by means of ether from a small sample of the dried tissue that previously has been acidified to pH 1 with sulfuric acid. The extract is then made alkaline, the ether is removed, and the quantity of acid required to titrate a portion of the solution between the limits pH 7.8 and 2.6 is determined with the aid of the quinhydrone electrode. One half of the oxalic acid and about 90 per cent each of the malic acid and citric acid are titrated under these conditions. In order to arrive at the total acidity of the tissue, it remains therefore to determine the oxalic acid in another portion of the solution and to add one half an equivalent of the amount found to the result of the titration. The assumption is made that the unknown acids are titrated to approximately the same extent as the malic and citric acid and, if this is true, the method has an overall accuracy of approximately 90 per cent.

The results of measurements of the total acidity due to organic acids of the three samples of tobacco leaves under discussion are shown in Table 21. The figures in the third row show the total

TABLE 21. THE ORGANIC ACIDS OF FRESH LEAVES, 12 DAY CURED AND FULLY CURED LEAVES OF TOBACCO AS DETERMINED BY ELECTROMETRIC TITRATION

The quantities are gram equivalents in 50 kilos of original fresh leaf.

	Fresh leaf	Cured 12 days	Fully cured
Total organic acids	14.6	14.3	13.6
Oxalic acid	2.92	2.72	2.66
Acidity—oxalic acid	11.7	11.6	11.0
Identified acids	9.36	11.72	11.02
Proportion of total organic acidity as identified acids in per cent	64.1	82.1	81.5

acidity of the tissue exclusive of that due to oxalic acid. They are slightly higher than the figures for total acidity of the water extract shown in Table 19 and suggest that the leaf residues that remain after extraction with hot water contain minor quantities of insoluble acidic substances. This will be further investigated later; for the present purposes it is sufficient to point out that such acids make up

a relatively insignificant part of the total acidity of the samples herein described.

The last row of figures in the table shows the relative proportions of the total acidity of the samples present in the form of malic, citric, and oxalic acid. The traces of fumaric and succinic acids identified have been left out of account in making the calculation since they do not affect the order of magnitude. These figures emphasize, and perhaps more sharply than do any others we have presented, the marked simplification of the organic acid picture that takes place during curing. Less than 65 per cent of the organic acids of the fresh leaf have been identified, more than 80 per cent of the acids of the cured leaf consists of the three acids mentioned.

These figures have considerable interest from the practical point of view. The tobacco technologist is particularly concerned with the acids of cured tobacco. It is clear that determinations of the three chief acids, and of the total acidity, by the methods outlined in the present bulletin will furnish him with a nearly complete picture of the acids of any particular specimen.

7. EXPERIMENTAL METHODS

THE DETERMINATION OF MALIC ACID

Malic acid can be determined in the absence of other optically active substances from the rotation of the uranyl complex according to the method outlined by Dunbar and Bacon (4). Samples (usually 25 cc.) of the hot water extract from the leaf tissue, or of the barium salts fraction, were prepared for this determination by adjusting the reaction to pH 1 (thymol blue paper) with sulfuric acid, and extracting with alcohol-free ether in a small continuous extraction apparatus (17, p. 197). The ether extract was treated with 25 cc. of water, the ether was evaporated, and the aqueous solution was diluted to 100 cc. From 5 to 20 cc. of this solution, depending on the malic acid content, were transferred to a 25 cc. flask and a drop of phenolphthalein solution was added; 2.5 N sodium hydroxide was added until the reaction was alkaline and the solution was then acidified with 0.5 cc. of glacial acetic acid; 3 gm. of powdered uranium acetate and a little norite were added, the flask was shaken for 30 to 60 minutes, and the solution was made to volume with saturated uranium acetate solution. After being mixed the solution was filtered into a 2 dm. polarimeter tube and the rotation was observed. The quantity of malic acid in the 25 cc. of solution secured from the aliquot part of the original solution taken is calculated from the relationships

$$\text{gm. malic acid} = V^\circ \frac{0.036}{4}$$

$$\text{gm. malic acid} = \frac{circular^\circ}{.3468} \cdot \frac{0.036}{4}$$

where V° is the reading of a Ventske sugar scale polarimeter or *circular*° is the rotation in circular degrees.

The malic acid content of samples of esters can be calculated from the specific rotation of pure *l*-diethyl malate $[\alpha]_D^{25} = -10.8°$, or, alternatively, a sample of the ester may be saponified with sodium hydroxide, neutralized and treated as already described.

THE DETERMINATION OF CITRIC ACID

Citric acid, when treated with bromine and potassium permanganate, is converted into pentabromoacetone. This substance is very sparingly soluble in water and can be easily prepared for weighing. The procedure for the oxidation has been standardized by Hartmann and Hillig (8, 9) who have also given factors whereby the weight of the product can be converted with considerable accuracy to the weight of citric acid.

To determine citric acid a suitable aliquot part (from 10 to 50 cc.) of the solution prepared by ether extraction of the organic acids, as described under malic acid, is treated with 5 to 10 cc. of 50 per cent (volume) sulfuric acid and 10 cc. of saturated bromine water. After being allowed to stand 10 minutes the solution is filtered with gentle suction through asbestos in a Gooch crucible and the precipitate is washed with 20 to 25 cc. of water in small portions. The filtrate is treated in a 250 cc. beaker with 10 cc. of N potassium bromide, heated in a water bath at 45 to 48° for 10 minutes and then treated with 40 cc. of 1.5 N potassium permanganate added all at once. The mixture is stirred and, if the color is discharged, more permanganate is *at once* added until the color remains. The beaker is then allowed to stand at room temperature for 10 minutes. The contents are chilled to 10° in an ice bath and ice-cold ferrous sulfate solution (30 gm. hydrated ferrous sulfate, 100 cc. water, 1 cc. concentrated sulfuric acid) is added until the manganese dioxide is dissolved. The beaker is then placed in an ice-box overnight. The solution is filtered on a Gooch crucible and the volume of the filtrate is recorded. The pentabromoacetone on the filter is washed three times with 20 cc. ice-cold 1 per cent (volume) sulfuric acid, and three times with 20 cc. of ice water, is dried to constant weight *in vacuo* over sulfuric acid, and weighed. The pentabromoacetone is then dissolved from the crucible by drawing through it successively three 20 cc. portions of alcohol and three 20 cc. portions of ether. The crucible is again dried and weighed. The citric acid is calculated from the equation

$$[0.424\,P + 0.017\,S]\,1.05 = \text{mg. citric acid}$$

when P is the weight of the pentabromoacetone and S the volume of the filtrate before the product was washed. The factor 1.05 is empirical and allows for the failure of the reaction to give a 100 per cent yield. Success with this method requires a strict adherence to the details of the procedure.

Citric acid is determined in samples of esters by saponification with sodium hydroxide followed by determination of the yield of pentabromoacetone. For example 0.3 to 0.5 gm. of ester are boiled under reflux with 10 cc. of alcohol and 3 cc. of 2.5 N sodium hydroxide for 1.5 hours. The solution is neutralized to phenolphthalein with sulfuric acid, evaporated to dryness, the residue is dissolved in 40 cc. of water and treated as just described.

THE EFFECT OF ALKALI ON *l*-DIETHYL MALATE

The procedure for the purification of the crude esters of the organic acids requires that the ether solution of these be shaken with dilute aqueous alkali in order to remove the hydrochloric acid, and also to remove the phenol fraction and the unesterified material. It was noted that large losses of ethyl malate may occur if sodium hydroxide is used for this purpose. Data are given in Table 22 which show the extent of this loss. In each case a weighed quantity of the ester was dissolved in 100 cc. of ether and shaken in a separatory funnel with 10 cc. of alkali for three minutes. The aqueous layer was then withdrawn and the residual malic acid was determined, after saponification, by means of the optical rotation in the presence of uranium acetate.

TABLE 22. THE EFFECT OF ALKALI AND OF WATER ON AN ETHER SOLUTION OF *l*-DIETHYL MALATE

	Ester taken gm.	Ester lost gm.	Loss %
Sodium hydroxide 5 N	2.177	1.134	51.0
Sodium hydroxide 5 N	5.540	2.63	47.4
Sodium carbonate 20%	2.179	0.0702	3.22
Sodium bicarbonate 7%	2.183	0.0894	4.09
Water	2.181	0.0932	4.27

A small loss of ethyl malate occurs even on washing with water, but this loss is not increased if sodium carbonate or bicarbonate is used. On the other hand as much as half the ester may be destroyed if 5 N sodium hydroxide is employed. This reagent is therefore inadmissible when washing ether solutions of esters and its use has been rigorously avoided in the experimental work described in the present bulletin.

PREPARATION OF HYDRAZIDES OF ORGANIC ACIDS AND THEIR BENZYLIDENE DERIVATIVES

The identification of the different organic acids depends upon the isolation of a suitable derivative, the melting point, behavior, and composition of which are characteristic. Among the most easily accessible compounds are the hydrazides, and the benzylidene derivatives of these, and much use has been made of them by Franzen and his collaborators. The preparation of these derivatives in

good yield, and in characteristic crystalline form, requires considerable attention to the purity of the ester and to the conditions under which the condensation is conducted. Furthermore we have found in a number of cases that the melting points given in the literature are at variance with those of our carefully purified specimens. It therefore seems desirable to describe the preparation of some of these derivatives in detail.

The procedure given by Franzen for the synthesis of hydrazides is, in general, satisfactory. A sample of 0.5 to 0.7 gm. of ester is dissolved in 5 to 10 cc. of absolute alcohol and is treated with 1 cc. of 42 per cent solution of hydrazine hydrate (Eastman). If diethyl oxalate is present an immediate precipitation takes place which is complete in 10 to 15 minutes. The crystals are filtered off and the mother liquor is allowed to stand in the ice-box overnight. Any crystalline solid that has separated is removed, 10 cc. of water are added to the filtrate, which is then evaporated *in vacuo* to remove alcohol, and the aqueous solution is diluted to 25 cc. and acidified to Congo red with 2 N hydrochloric acid. The solution is filtered and treated drop by drop with benzaldehyde with vigorous shaking until a *faint* odor of the reagent remains. It is important to avoid adding an excess. About 20 cc. of ether are added and, after shaking, the solid is filtered off. The ether assists in preventing the formation of an oily product. The derivative is washed successively with water, 60 per cent alcohol, and ether, is dried and ground in a mortar with ether, and is recrystallized from boiling 60 per cent alcohol; the product is again triturated with ether and recrystallized. It is not worth while to attempt to obtain second crops from these alcoholic mother liquors and, with relatively pure compounds yields of from 75 to 90 per cent are secured from the final recrystallization.

MALIC DIHYDRAZIDE

Franzen has described this compound as separating in characteristic white nodules without definite crystalline form. The slightly impure malic ester usually obtained by fractional distillation does in fact yield a nodular dihydrazide, but pure specimens yield material crystallized in prisms, though small nodules are sometimes seen along with these. All of our main malic ester fractions yielded crystalline dihydrazides of melting point slightly higher than that given by Franzen. The specimens did not sinter below 177 to 178°.

BENZYLIDENE MALIC DIHYDRAZIDE

Prepared from pure malic dihydrazide this compound separates in shining plates with notched edges of melting point 171 to 172°.

The second crops are usually rosettes of blunt needles or prisms of the same melting point. Franzen records somewhat lower melting points and does not describe the appearance of his preparations.

CITRIC TRIHYDRAZIDE

Turner and Hartman (16) describe citric trihydrazide as occurring in two different forms depending on the conditions; one is hydrated and the other anhydrous. Crystallization is difficult to induce without resorting to seeding. Mr. E. K. Nelson of the Bureau of Chemistry kindly provided us with specimens of his hydrated and anhydrous compounds and, after employing these to induce crystallization, no difficulty was experienced in obtaining the crystalline hydrated compound melting between 104 and 107°. The anhydrous compound usually separated in a form that began to contract at 106 to 108°, and gave a clear oil and gaseous decomposition products at 162 to 163°. This point is characteristic of the anhydrous citric trihydrazide. Nelson's preparation gave a mixture of oil and solid at 151 to 152° and clear oil with gas at 162 to 163°.

BENZYLIDENE CITRIC TRIHYDRAZIDE

Franzen and Helwert (5) describe this compound as yellow flakes that sinter at 175° and melt at 227°. Curtius and Sauvin (3) obtained long colorless needles melting at 227°. The compound in our hands separated in two forms depending on the concentration of the solution and the rate of cooling. The more usual form was that of long colorless hair-like needles that matted into a felt on the filter and through which the filtrate passed with difficulty. The other form consisted of colorless angular prisms with rough edges. The compounds both melted at 200 to 202° corrected (196 to 197° on long stem thermometer uncorrected). This does not correspond at all with Curtius and Sauvin's compound, which is described as melting at 227°, nor with Franzen and Helwert's which sintered at 175°. The ester fractions we employed for the preparation of this derivative were shown by analysis by the pentabromoacetone method to be at least 90 per cent pure. When less pure fractions were employed mixtures of hydrazides that were very difficult to purify were secured.

BENZYLIDENE FUMARIC DIHYDRAZIDE

This compound has not hitherto been recorded in the literature. It was obtained from the mother liquors after the insoluble hydrazides had been removed. On recrystallization it separated in long prisms that sintered at 206° and melted to a clear oil at 207 to 209°.

Analysis gave C, 67.72; H, 5.71; N, 17.72: theory for $C_{18}H_6O_2N_4$, C, 67.51; H, 5.00; N, 17.50 per cent.

The melting points of the derivatives described are collected in Table 23.

TABLE 23. MELTING POINTS OF HYDRAZIDES AND THEIR BENZYLIDENE DERIVATIVES

		Long thermometer °C		Anschütz thermometer °C	Literature °C
l-Malic dihydrazide		178–179			176–179
Benzylidene *l*-malic dihydrazide	sinters	168–169	sinters	172–173	163–165
	oil	171–172	oil	178–179	
	clear	174–175	clear	181–182	
Benzylidene citric trihydrazide	sinters	192–193	sinters	199	sinters 175
	turbid oil	193–194	oil	200–201.5	melts 227
	clear	196–197			
Benzylidene fumaric dihydrazide	sinters	207			
	oil	207.5–208			

8. SUMMARY

The results of this investigation of the organic acids of tobacco leaves, and of the effect of the curing process on these, largely confirm the results of the preliminary investigation published as Bulletin 323 (17) from this Station. Some of the newer results, however, are at variance with the conclusions of that bulletin. This is due to many improvements that have been introduced into the technique, and to a wider experience with the problems presented. All that can be hoped for is that a closer approximation to the truth has now been obtained.

The present data have been obtained from three samples of mature Connecticut shade-grown tobacco leaf, each weighing 50 kilos, that were picked on the same day. One of these was immediately extracted with boiling water and the extract prepared for analysis, the other two were submitted to the curing process, one for 12 days, or until the leaves had become yellow, the other for 51 days, or until curing was complete. The analytical data are expressed in terms of absolute weight of each component in the original 50 kilos of fresh leaf material; in this way the changes that occurred are more readily apparent than would be the case if the results were expressed on a percentage basis.

The general method of analysis described in Bulletin 323 was employed. A faintly acid hot water extract of the tissue was prepared, and the substances that form barium salts insoluble in approximately 60 per cent alcohol were removed. The organic acids in this precipitate were recovered by ether extraction, were esterified and the esters distilled. Identification of the different acids was effected by the preparation of suitable derivatives. Analyses for citric and malic acids were also carried out by indirect methods, and it is shown that the ester method closely confirms the results of the indirect methods. The results of these may therefore be accepted as being thoroughly reliable.

The determination of oxalic acid in tobacco leaves presents a special problem. It is shown that very extensive losses of oxalic acid occur during the ordinary procedures for the preparation of water extracts from tobacco leaf tissue inasmuch as this acid is moderately volatile with water or alcohol vapor. No reliance can therefore be placed on analytical figures secured after such extracts have been concentrated unless precautions are taken to recover the oxalic acid from the distillate. In view of this a method for the determination of oxalic acid in dried leaf tissue has been developed, in which the oxalic acid is removed from the acidified tissue by ether and is determined as calcium oxalate.

The efficiency of the barium salts precipitation method for the isolation of the organic acids has been confirmed. It is shown that from 85 to 90 per cent of the titrable acidity of a water extract of tobacco leaves is found in the barium salts fraction. The complexity of this fraction is, however, further emphasized. It contains a considerable quantity of nitrogen, a part of which presumably belongs to dicarboxylic amino acids, but much of the nitrogen is in unknown forms. A relatively small proportion of the reducing substances of the tissue is found in it. A part of this reducing substance consists of fermentable carbohydrate. In addition evidence was found for the presence of a large quantity of non-nitrogenous organic solids of entirely unknown chemical relationships.

During the curing process the quantity of malic acid in the tissues diminishes. The change is, however, of a minor nature, only about 16 per cent of the original amount of this acid being utilized in the katabolic processes. On the other hand citric acid increases nearly six-fold in the same time. The origin of the citric acid thus synthesized is discussed, two possibilities, the carbohydrate, or the unknown acids of the leaves, being suggested; a protein origin of the synthesized citric acid is improbable. Oxalic acid changes very little during curing. Evidence is presented to show this acid occurs largely in the form of insoluble oxalates; this may account for its failure to share to any important extent in the metabolism.

The total acidity, the acidity in the form presumably of monobasic acids, and the acidity in the form of acids precipitable as barium

salts by 60 per cent alcohol all changed very little, if at all, during curing. On the other hand the quantity of acids of unknown composition diminished greatly, their place being taken by citric acid. The curing process thus results in a very extensive simplification of the organic acid picture. Only 64 per cent of the acidity of the fresh leaves can be accounted for in terms of known acids, chiefly malic, citric, and oxalic. More than 81 per cent of the acidity consists of these three acids after only 12 days of curing and a similar high proportion was identified in the fully cured leaf.

The present investigation was not successful in extending our qualitative knowledge of the acids of tobacco leaf. Minor amounts of succinic and fumaric acids were encountered and the possibility that maleic acid may be present was established. It was found that both malic and citric acids are present in the residue from the distillation of the esters even after the temperature has been carried far beyond the boiling point of triethyl citrate. A similar observation was made with respect to malic acid by Franzen but no definite explanation has yet been obtained. Small but appreciable quantities of malic and citric acids were also found in the so-called unesterifiable fraction. These probably represent partially esterified material, and their small amount furnishes evidence of the efficiency of the esterification procedure we have adopted.

Detailed directions are given for the determination of the three main acids of tobacco leaves and also for the preparation of derivatives whereby these acids can be positively identified.

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