showing the aromatic aminoketones to be bases with ionization constants of the same order as that of aniline.

7. Acetophenonoxime and benzophenonoxime are bases, the former has $k_b^{27^\circ} = 1.16 \times 10^{-10}$ while the latter has $k_b^{24^\circ} = 2.0 \times 10^{-11}$. The Beckmann rearrangements of these oximes obey the criterion for the non-reversible intramolecular rearrangements.

8. A colorimetric method is developed by means of which the ionization constant of sufficiently soluble acids and bases may be determined with a mean error of 2%.

URBANA, ILLINOIS.

THE ENZYMES OF THE TOBACCO PLANT.

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There are a great many changes taking place in the tobacco plant throughout its growth, as well as during the curing and fermentation periods. Certain chemical compounds are formed and others are broken down to form new products. It is the result of these which give the color, texture, aroma, etc., to the finished product. Yet all these changes can be easily stopped by subjecting the tobacco to unfavorable conditions during the curing, and a whole crop can easily become worthless. Again, a crop may undergo some of these changes under favorable conditions and still one of its desirable qualities may be lacking. The causes of these changes have been studied by several scientists and numerous treatises have been published in regard to this subject.

The theory that bacteria are largely instrumental in bringing about the many changes of the different stages of the curing process, has been advanced by Suchsland[1] and other scientists.[2] Some scientists have even advanced the idea that the bacteria produced on the aromatic Vuelta Abajo (a variety of tobacco noted for its aroma) could be transferred to some of our American varieties and that the fermentative processes initiated by this would develop an aroma equal to that of the Cuban tobacco. If this was the case, then all the differences in the various types of tobacco due to soil, climate and localities would have been broken down, and all that would be necessary to grow a fine crop of tobacco would be to inoculate our tobacco with some of these aroma-producing bacteria.

Recently, however, Dr. Loew,[3] formerly of the United States Department of Agriculture, has proven that the fermentation and curing of tobacco is not caused by bacteria, nor is the aroma of tobacco due to the

[1] *Ber. bot. Ges.*, 9, 79–81 (1891).

[2] O. Loew disproves this theory in Report No. 59, Div. of Veg. Physiology and Pathology, U. S. Dept. of Agriculture.

[3] Reports Nos. 59 and 65, Div. of Veg. Physiology and Pathology, U. S. Dept. of Agr.

action of specific bacteria. He also showed that the fermenting leaves are destructive of bacterial life. Furthermore, Dr. Loew, among others, has demonstrated, in a series of experiments, that these changes were due to the action of soluble ferments or enzymes found in the plant while growing. These enzymes resemble chemical compounds, not living organisms like bacteria or molds, which under proper conditions cause many chemical changes in the substances with which they come into contact. A familiar example is diastase, the enzyme of malt, which will change two thousand parts of starch into sugar for each part of itself. In the fermentation of tobacco leaf, according to Dr. Loew, the main changes are caused by two oxidizing enzymes alone. These have the power of taking oxygen from the air and uniting it with the various compounds in the leaf, causing the splitting up of existing chemical substances and the creation of new products. Dr. Loew worked on the oxidases, peroxidases, catalase, diastase, the proteolytic and the cellulose-dissolving enzymes contained in the different varieties of tobacco.

These enzymes are very highly complex protein forms and are present in the protoplasm of the cell. Under favorable conditions they can do their work but they are very easily destroyed either by too much heat or by too rapid a loss of their moisture. If a freshly cut, matured leaf be killed all at once by heat, the curing and fermentation will not go on, consequently the dried leaf will have a greenish color, but if the leaf is allowed to dry slowly and the temperature and moisture conditions in the drying room are kept within certain limits, these enzymes will separate themselves from the protoplasm and act on their respective compounds, thereby producing in the cured leaf the brown color characteristic of a well-cured tobacco.

Von Jensen[1] has shown that tobacco fermentation is not prevented by treating the leaf with corrosive sublimate, formaldehyde, or chloroform, and that fermentation is promoted by weighting down the mass and by fermenting in large bulks. Fermentation was found impossible in small quantities of tobacco even though air was forced through or they were inoculated with fermenting leaf. The effects of fermentation either in part or in their entirety were brought about by heating with steam from 10 minutes to 2 hours at a temperature from 90°–100°. Wet tobacco was brought to an active state of fermentation even when the bulk was small. Fermentation was impossible in tobacco which had been treated with steam, as well as in leaf which had already gone through the process. Forcing oxygen through the tobacco to be fermented did not promote fermentation. Therefore, he draws the following conclusions, that fermentation is not to be considered as a "microbiological" or "enzymatical" process, but warmth and moisture are essential to its beginning.

[1] *Chem. Ztg. Report*, 1908, s. 541.

Hirmke[1] carried out many extensive experiments in regard to the fermentation of tobacco and he considers tobacco fermentation an individual case of natural occurrence of nature, as the self-heating in the curing of hay and the like.

Boekhout[2] and De Vries claim to have proven that tobacco fermentation and self-heating of hay are analogous occurrences, *viz.*, processes of oxidation which result from its union with the oxygen of the air, producing as one of the products carbon dioxide; and, furthermore, that the iron which is contained in the tobacco probably takes the part of a "catalytic" agent during these changes.

In this connection it may be of interest to note that the curing of hay may also be due to the action of certain enzymes. Jacobson[3] worked on the seeds of alfalfa for certain enzymes and found that peroxidases, emulsin, amylase, protease and rennin were present in the seed. As a result of his work it may be possible that these and other enzymes may also play an important part in the curing of alfalfa and other hays. Further work, of course, must be done on the green and cured plant before any definit conclusions can be reached.

A treatise by H. Vernhout[4] appeared in 1899 in which a microbe closely related to *Bacillus subtilis* is described as having been obtained from fermenting tobacco leaves in Java. To this the author ascribed the phenomena of tobacco fermentation. However, Dr. Loew proved that bacteria cannot produce fermentation and multiply on tobacco leaves with only 25% of water. Dr. Loew seems to think that some of the enzymes, for instance the oxidases and peroxidases, may disappear under unfavorable conditions, while catalase, the third enzyme, may still persist.

As has been pointed out before, Dr. Loew worked on several of these enzymes in the different varieties of tobacco, and proved the presence of diastase, oxidase, peroxidase and a proteolytic enzyme in several of them. He did not get a test for a cellulose-dissolving enzyme. Kastle[5] and Shedd have also found oxidases in the green burley tobacco. It is well known what an important role enzymes play in every organism and this is especially the case in the curing and fermentation of tobacco.

Since very little work has been done on the occurrence of enzymes in two of our most typical and widely different types of Kentucky tobaccos, namely the Burley, from the Burley region, and the dark types grown in Western Kentucky, it was thought that these would be good samples upon which a study could be made to see if any differences could be noted as to enzymes which they might contain.

[1] *Fachl. Mitteil. K. K. "Oesterr Tabakregie,"* 1908, Heft 2.

[2] *Chem. Ztg. Report,* 1910, s. 1; *Zentralbl. Baketeriol.,* 1909 (II).

[3] THIS JOURNAL, 34 (1912).

[4] "Onderzoek over Bakterien by de Fermentatie der Tabak," *Batavia,* 1899.

[5] *Am. Chem. J.,* 26 (1901).

As a representative sample of the Burley region, plants were selected from the White Burley variety and from the dark region, plants were selected from the Yellow Pryor variety.

Enzymes of the Tobacco Plant.

In testing for the presence of the various enzymes, samples were taken from the following parts of the tobacco plant:

A.—*Seed:* 1911 and 1912 Burley; 1911 Torian Yellow. As no Yellow Pryor seed was at hand during these experiments, it was decided to substitute the Torian Yellow which resembles it. No 1912 seed of this was used, as the sample was received too late to be included in the work.

B.—*Green Burley Leaf:* Seedling or transplanting stage; one week before topping; topping stage; one week after topping; two weeks after topping, and at maturity.

C.—*Cured Leaf:* Burley and Yellow Pryor. Samples of 1912 crop cured with green color (probably due to cutting before maturity was reached). Burley and Yellow Pryor. Samples of 1912 crop cured with proper color. These samples were dried at 40° for the experiment.

D.—*Soil Sample:* Taken from 1–6 inch deep. Virgin soil taken from the soil at the back of the Agricultural Building, along the fence. Hardly any vegetation on soil when sample was taken with exception of some blue grass. Soil was frozen when samples were taken.[1]

All of the above examples were ground up with fine white sand (1 part sand to 2 parts sample) in a mortar. The mid-ribs and stems of leaf were not used.

Different amounts of samples were used in the various experiments, as follows: 0.5 gram of cured leaf and seed, 1 gram green leaf and 2 grams of soil. Bottles of 75 cc. capacity were used in the work on invertase, diastase, inulase and emulsin, and test tubes of about 37 cc. capacity for the other experiments.

Definitions of terms used:

Water Control.—This was used as a check. Everything was added as in the other bottles or test tubes with the exception of sample. The water controls were not used in some of the experiments.

Active Control.—This was used to see if any changes would take place without the substrate being added.

Boiled Control.—In this case, the sample was first boiled for 5 minutes or more so as to destroy the enzymes and see whether any changes would take place if these were absent. The substrate and everything else were supplied to this.

Active Extract.—Here the unboiled sample was used as in the active control, but substrate, etc., were also added. These were made in duplicate in each experiment.

Toluene was used as the preservative in all the digestions.

[1] It was thought that it would be of interest to try a sample of soil at the same time for these various ferments and the results obtained are included in the tables under the different enzymes.

During the first experiments, but not including those on the oxidase tests, duplicates were made by having the bottles tightly stoppered and others with a piece of cotton in the neck. No differences were noted in the results. The plan was then followed by keeping the bottles or tubes tightly stoppered during the digestion in all experiments except in those on the oxidizing enzymes, so as to prevent any loss of the preservative.

Invertase.

The following experiments were carried out to determin if this enzyme was present in the plant at different stages of its growth:

Active Control.—0.5 gram of unboiled sample + 50 cc. H_2O + 0.5 cc. toluene.

Boiled Control.—0.5 gram of boiled sample + 50 cc. H_2O (made up to this after boiling the plant material) + 0.5 gram cane sugar + 0.5 cc. toluene.

Active Extract.—0.5 gram unboiled sample + 50 cc. H_2O + 0.5 gram cane sugar + 0.5 cc. toluene.

Let stand in incubator at 37° C. for 22 hours before testing with Fehling's solution.

Tests were made as follows: To 2 cc. of a standard Fehling's solution (1 cc. = 0.005 gram glucose) was added 10 cc. H_2O. On boilng added 10 cc. of the solution to be tested. The solution was decanted and was free from the plant material. This was added slowly and boiled for 2 or 3 minutes and set aside.

The results obtained in this series were as follows:

TABLE I[1].—INVERTASE REACTIONS WITH FEHLING'S SOLUTION.

Samples.	Active control.	Boiled control.	Active extract.	Active extract.
1911 Burley seed	+	++	+++	+++
1912 Burley seed	+	++	+++	+++
Burley leaf from seedling (transplanting stage)	+	++	+++	+++
Burley leaf cured with green color	o	+	++++	++++
Burley leaf cured with proper color	+	+	++++	++++
Burley leaf cured with proper color	++	++	++++	++++
1911 dark seed (Torian Yellow)	++	+	+++	+++
Yellow Pryor cured with green color	++	++	++++	++++
Yellow Pryor cured with green color	++	++	++++	++++
Yellow Pryor cured proper color	++	++	+++	+++
Soil, 24 hours digestion	o	o	++	++
Soil, 90 hours digestion	o	+++	+++	+++

[1] o indicates no reduction.
+ indicates trace reduction.
++ indicates small reduction.
+++ indicates good reduction, but not complete.
++++ indicates complete reduction.

There was not much difference in the results obtained on the 1911 and 1912 Burley seed, neither was there any difference noted between the samples cured properly and those cured with a green color. The above tests clearly indicate the presence of invertase.

The results obtained on the soil are interesting, but cannot be explained as wholly due to an enzyme but rather to something else contained in the soil in the nature of a weak acid, alkali or salt action.

Diastase.

The following experiments were carried out to determin if there was any diastase present in the tobacco plant:

Active Control.—0.5 gram unboiled sample + 50 cc. H_2O + 0.5 cc. toluene.

Boiled Control.—0.5 gram boiled sample + 40 cc. 1% starch solution + 10 cc. H_2O + 0.5 cc. toluene.

Active Extract.—0.5 gram unboiled sample + 40 cc. 1% starch solution + 10 cc. H_2O + 0.5 cc. toluene.

Digested in incubator at 37° for 16 hours.

The starch used was 1% potato starch solution and unfiltered. Prepared by mixing the starch with water into a thin paste and pouring into boiling water, cooling and making to mark.

After the digestion the extracts were tested with Fehling's solution, as in case of invertase. Tests with iodine solution were made in some cases to see whether any starch remained in the solutions after the digestion period. These were made by adding a drop of the iodine to 2 cc. of the solution diluted with an equal volume of water.

The results obtained in this series, using both Fehling's solution and iodine, are given in Tables II and III.

TABLE II.[1]—DIASTASE REACTIONS WITH FEHLING'S SOLUTION.

Samples.	Active control.	Boiled control.	Active extract.	Active extract.
1911 Burley seed	o	o	++	++
1912 Burley seed	o	o	++	++
Burley leaf from seedling (transplanting stage)	+	o	++	++
Burley leaf, cured with green color	++	+	++++	++++
Burley leaf, cured with proper color	++	++	+++	+++
1911 dark seed (Torain Yellow)	o	o	++	++
Yellow Pryor, cured with green color	++	++	+++	+++
Yellow Pryor, cured with proper color	++	+	+++	+++
Soil, 24 hours digestion	o	o	o	o
Soil, 90 hours digestion	o	o	++	++
Soil, 144 hours digestion	o	o	++	++

[1] o indicates no reduction.
+ indicates trace reduction.
++ indicates small reduction.
+++ indicates good reduction, but not complete.
++++ indicates complete reduction.

TABLE III.—DIASTASE.

Tests of solutions with iodine at end of the digestion period.

Samples.	Active control.	Boiled control.	Active extract.	Active extract.
Burley leaf from seedling (transplanting stage)	No blue	Blue	Bluish violet	Bluish violet
Burley leaf, cured with green color	No blue	Blue	No blue	No blue
Burley leaf, cured with proper color	No blue	Blue	Purple	Purple
Yellow Pryor, cured with green color	No blue	Blue	No blue	No blue
Yellow Pryor, cured with proper color	No blue	Blue	No blue	No blue
Soil	No blue	Blue	Blue	Blue

An attempt was made to estimate the reducing sugars in the active extracts of the green cured Burley, the properly cured Burley and the green cured Yellow Pryor samples, using Fehling's solution, but on account of the color imparted by the solutions and the difficulty with which the copper oxide settled, this could not be done with any degree of accuracy. From the above tests this enzyme seems to be present to a greater or less extent in both varieties of tobacco and also in the Burley during the different stages of its growth. A fair test was also obtained in the soil.

Lipase.

The following experiments were made to determin if there was a fat-splitting enzyme present in the tobacco plant:

SERIES A.

Water Control.—10 cc. H_2O + 0.26 cc. ethyl butyrate + 0.1 cc. toluene.

Active Control.—0.5 gram unboiled sample + 10 cc. H_2O + 0.1 cc. toluene.

Boiled Control.—0.5 gram boiled sample + 10 cc. H_2O + 0.26 cc. ethyl butyrate + 0.1 cc. toluene.

Active Extract.—0.5 gram unboiled sample + 10 cc. H_2O + 0.26 cc. ethyl butyrate + 0.1 cc. toluene.

Heated to 40° for 5 minutes before adding the ethyl butyrate.

Duplicate sets of the above were digested in incubator at 37° for different periods of time. At the end of the digestion period the solutions were titrated with $N/20$ potassium hydroxide, using phenolphthalein as an indicator.

SERIES B.

Water Control.—10 cc. H_2O + 0.1 cc. toluene + 0.3 cc. olive oil.

Active Control.—0.5 gram unboiled sample + 10 cc. H_2O + 0.1 cc. toluene.

Boiled Control.—0.5 gram boiled sample + 10 cc. H_2O + 0.3 cc. olive oil + 0.1 cc. toluene.

Active Extract.—0.5 gram unboiled sample + 10 cc. H_2O + 0.3 cc. olive oil + 0.1 cc. toluene.

Titrated Series B as follows: Used 85% alcohol solution which had been neutralized with $N/20$ potassium hydroxide, using phenolphthalein as indicator. Added 20 cc. of the above alcohol to each solution and let stand for some time, shaking frequently. Titrated with $N/20$ potassium hydroxide, using the above indicator. The cured leaf samples were filtered, but on account of the color of these solutions it was very difficult to titrate them, as the end point was not distinct.

The results obtained in these two series are given in Table IV:

TABLE IV.—LIPASE; TITRATION WITH $N/20$ POTASSIUM HYDROXIDE.[1]

Samples.	Substrate.	Time. Hrs.	H_2O control. Cc. $N/20$ KOH.	Active control. Cc. $N/20$ KOH.	Boiled control. Cc. $N/20$ KOH.	Active extract. Cc. $N/20$ KOH.	Active extract. Cc. $N/20$ KOH.
1911 Burley seed.....	Olive oil	45	0.30	4.13	2.22	4.75	5.20
	Ethyl butyrate	27	..	0.05	0.15	0.10	...
	Ethyl butyrate	75	0.05	2.63	0.52	1.60	1.73
1912 Burley seed.....	Olive oil	45	0.30	7.15	2.80	6.90	6.80
	Ethyl butyrate	27	..	0.05	0.20	0.25	...
	Ethyl butyrate	75	0.05	4.07	0.55	3.88	4.28
Burley leaf from seedling (transplanting stage).....	Olive oil	45	0.25	2.35	2.45	3.25	3.45
	Ethyl butyrate	75	0.05	1.15	0.55	2.55	2.40
Burley leaf, cured with green color.....	Olive oil	45	0.30	11.35	7.70	12.30	11.25
	Ethyl butyrate	27	..	0.05	2.70	3.00	3.00
Burley leaf, cured with proper color.....	Olive oil	45	0.30	10.55	8.10	10.95	11.15
	Ethyl butyrate	27	..	0.05	2.80	3.20	3.20
1911 dark seed (Torian Yellow).....	Olive oil	45	0.30	4.45	2.55	4.90	4.65
	Ethyl butyrate	27	..	0.05	0.15	0.20	...
	Ethyl butyrate	75	0.05	2.75	0.45	1.77	1.80
Yellow Pryor, cured with green color.....	Olive oil	45	0.30	11.05	10.95	10.90	13.60
	Ethyl butyrate	27	..	0.05	2.25	2.60	2.60
Yellow Pryor, cured with proper color.....	Olive oil	45	0.40	19.40	14.20	19.80	18.50
Soil.....	Olive oil	72	0.20	0.20	0.30	0.40	0.30
	Olive oil	95	0.40	0.50	0.50	0.50	0.50

A strong odor of butyric acid was detected in the active extracts of the Burley seedling but none could be detected in the active and boiled controls of the same experiment.

The above results seem to indicate that there has been a small lipolytic action in some of the experiments.

[1] 0.26 cc. ethyl butyrate at 30° requires 39.70 cc. $N/20$ KOH for complete hydrolysis. 0.30 cc. of neutral olive oil requires 21.36 cc. $N/20$ potassium hydroxide to saponify it.

Oxidases.

The following experiments were made to determin if there is an oxidase present in the tobacco plant, using both guaiacum and phenolphthalein as reagents.

Since some discussion has arisen as to whether the guaiacum test for the oxidizing enzymes is entirely reliable in all cases, it was thought desirable to have some confirmatory test. Some workers in this field have claimed that other substances in the plant besides the enzyme will sometimes blue guaiacum. Loew, on the other hand, believes that guaiacum is a reliable reagent for this purpose.

The other reagent which has been used in this work is phenolphthalein, which was proposed by Kastle and Shedd and has since been found satisfactory by several workers as a very sensitive and reliable reagent for the oxidizing ferments. In this connection it might be of interest to note that Loew[1] states that if nitrites should be present in the plant, they would blue guaiacum and probably be mistaken for the oxidizing ferment. Tests have been made in this work using 1 cc. of guaiacum or phthalin and adding 10 cc. of sodium nitrite solution containing 10 parts per million of nitrite N. No blue or red color was obtained at once, only a trace after 1 hour and a shade deeper after 24 hours' standing at room temperature. This seems to prove that both reagents are reliable in the presence of nitrites in amounts which probably exceed any that would be found in plants.

Series B.

Water Control.—10 cc. H_2O + 1 cc. phthalin.

Boiled Control.—1 gram boiled sample + 10 cc. H_2O + 1 cc. phthalin.

Active Extract.—1 gram unboiled sample + 10 cc. H_2O + 1 cc. phthalin. Let stand for 1 hour and then made alkalin by adding 5 cc. $N/20$ potassium hydroxide.

Series C.

This series was the same as B except the solutions to be tested were allowed to stand over night for a period of from 16 to 20 hours before making alkalin with $N/20$ KOH.

Series A.

Water Control.—10 cc. H_2O + 1 cc. guaiacum.

Boiled Control.—1 gram boiled sample + 10 cc. H_2O + 1 cc. guaiacum.

Active Extract.—1 gram unboiled sample + 10 cc. H_2O + 1 cc. guaiacum.

The tests obtained with guaiacum were noted just after adding it and again after it had stood in the solution for some time. The guaiacum used was a fresh solution of 20% tincture of guaiacum which had been digested for several days in alcohol.

[1] Div. of Veg. Physiology and Pathology, U. S. Dept. Agr., *Report* No. 65, p. 55.

The results obtained in Series A, B and C are given in the following table:

TABLE V.[1]—OXIDASES: REACTIONS.

Samples.	Time.	Reagent.	Water control.	Boiled control.	Active extract.	Active extract.
1911 Burley seed.....	At once	Guaiacum	o	o	o	o
	At once	Guaiacum	o	o	o	o
	45 hrs.	Guaiacum	o	o	++	++
	1 hr.	Phthalin	++	++	++	++
	16–20 hrs.	Phthalin	++	++	+	+
	45 hrs.	Phthalin	++	++	++	
1912 Burley seed.....	At once	Guaiacum	o	o	o	o
	At once	Guaiacum	o	o	o	o
	45 hrs.	Guaiacum	o	o	+	+
	1 hr.	Phthalin	++	++	++	++
	20 hrs.	Phthalin	++	++	+	+
	45 hrs.	Phthalin	++	++	++	++
Burley leaf from seedling (transplanting stage)....	At once	Guaiacum	o	o	+++	+++
	1 hr.	Phthalin	+	o	o	o
	16–20 hrs.	Phthalin	++	++	+++	+++
Burley leaf from plant one week before topping....	At once	Guaiacum	o	+	+++	+++
	1 hr.	Phthalin	+	+	++	++
	16–20 hrs.	Phthalin	+	+	+++	+++
Burley leaf from plant at topping stage.....	At once	Guaiacum	o	+	+++	+++
	1 hr.	Phthalin	o	o	o	o
	16–20 hrs.	Phthalin	++	+	+++	+++
Burley leaf from plant one week after topping.....	At once	Guaiacum	o	+	+++	+++
	1 hr.	Phthalin	+	+	++	++
	16 hrs.	Phthalin	+	o	++	++
Burley leaf from plant 2 weeks after topping.....	At once	Guaiacum	o	+	++	++
	1 hr.	Phthalin	o	o	o	o
	16 hrs.	Phthalin	++	+	++	++
Burley leaf from lower leaves of plant 2 weeks after topping. This leaf was devoid of chlorophyll.....	At once	Guaiacum	o	o	++	++
	16 hrs.	Phthalin	++	+	++	++
Burley leaf, cured with green color.....	At once	Guaiacum	o	o	+	+
	At once	Guaiacum	o	o	++	++
	1 hr.	Phthalin	+	o	o	o
	16–20 hrs.	Phthalin	++	o	o	o
	16–20 hrs.	Phthalin	++	o	o	o
Burley leaf, cured with proper color.....	At once	Guaiacum	o	o	++	++
	At once	Guaiacum	o	o	++	++
	16–20 hrs.	Phthalin	+	o	o	o
	16–20 hrs.	Phthalin	++	o	o	o

TABLE V (*continued*).

Samples.	Time.	Reagent.	Water control.	Boiled control.	Active extract.	Active extract.
1911 dark seed (Torian Yellow)	At once	Guaiacum	o	o	o	o
	At once	Guaiacum	o	o	o	o
	45 hrs.	Guaiacum	o	+	++	++
	1 hr.	Phthalin	++	++	++	++
	16–20 hrs.	Phthalin	++	++	+	+
	45 hrs.	Phthalin	++	++	+++	
Yellow Pryor, cured with green color	At once	Guaiacum	o	o	+++	++
	At once	Guaiacum	o	o	++	++
	1 hr.	Phthalin	++	o	o	o
	16–20 hrs.	Phthalin	++	o	o	o
	16–20 hrs.	Phthalin	++	o	o	o
Yellow Pryor, cured with proper color	At once	Guaiacum	o	o	o	o
	16–20 hrs.	Phthalin	++	o	o	o
Soil	At once	Guaiacum	o	++	+++	+++
	16–20 hrs.	Phthalin	++	++	+++	+++

[1] o indicates no blue or red color.
+ indicates faint blue or red color.
++ indicates distinct blue or red color.
+++ indicates deep blue or red color.

The solutions of Burley cured with proper color and Burley cured with green color and Yellow Pryor cured with green color had a reddish brown color due to the tobacco, which it was thought, at the time, might have interfered with the characteristic color of phenolphthalein in the solution, but after making alkalin and obtaining no test, one drop of 1% phenolphthalein solution was added, which gave a good red coloration, showing that the color would not have been obscured by the natural color of the solution had oxidases been present in the leaf.

The seed solutions were turbid but not colored, so that the test would not have been interfered with in this experiment. This was further verified at the time by adding a drop of phenolphthalein.

The guaiacum tests, after standing over night, became deeper in color in the active extracts. The active control and boiled control remained just about the same as they were one hour after standing. Of the three samples, namely, those of the properly cured Burley, green cured Burley and green cured Yellow Pryor, the best guaiacum test was obtained in the green cured samples.

From our observations it is evident that oxidases are present in the tobacco leaf at all stages of its growth. They gradually increase in amount from the transplanting or seedling stage until the topping stage, where the maximum tests were obtained. From this stage the oxidases seem to gradually decrease until, in the cured leaf, they practically disappear. In the cured leaf, however, a slight test was obtained with guaiacum but not with phthalin.

Another interesting fact that was observed was that while the red color obtained with phthalin was clear and distinct until the topping stage was reached, from this period the color was not a distinct red but more of a brownish red, showing that some further change was taking place in the plant at these stages of its growth, which interfere with the tests for oxidases. This can probably be accounted for on the assumption that the plant in the green state, from the topping stage to maturity, is gradually developing the characteristic brown color found in the cured plant. That the oxidases are taking some part in this gradual change of color in the leaf is also doubtless true, as has been asserted by some scientists, expecially Dr. Loew.

The above facts are further proven by some attempts which were made to estimate the amount of oxidase reaction in the plant at different stages of its growth. The plan was followed by comparing the color obtained in the phthalin tests with the color imparted by a standard solution of phenolphthalein in the Dubosc colorimeter. In these trials the solution of the active extracts and controls after filtering and washing were made to a definit volume and compared with 2 cc. of 0.01% phenolphthalein solution contained in the same volume. The amount of color obtained in the water controls were subtracted in each case. The results were as follows:

Leaf from plant one week before topping:

Boiled Control.—Color not comparable with standard.

Active Extract.—(From one gram of fresh leaf.) Phthalin oxidized, *i. e.*, 0.000268 gram phenolphthalein.

Duplicate of Above.—Phthalin oxidized, *i. e.*, 0.000262 gram phenolphthalein.

Leaf from plant at topping stage:

Boiled Control.—Color not comparable with standard.

Active Extract.—(From one gram of fresh leaf.) Phthalin oxidized, *i. e.*, 0.000368 gram phenolphthalein.

Duplicate of Above.—Phthalin oxidized, *i. e.*, 0.000325 gram phenolphthalein.

Leaf from plant one week after topping:

Color could not be compared on account of red-brown color of solution.

Leaf from plant two weeks after topping:

Both in leaves with chlorophyll and in those devoid of chlorophyll, colors could not be compared on account of red-brown color.

Leaf from plant three weeks after topping:

Leaves devoid of chlorophyll.

Boiled Control.—Color not comparable with standard.

Active Extract.—(From one gram of fresh leaf.) Phthalin oxidized, *i. e.*, 0.000012 gram phenolphthalein.

Duplicate of Above.—Phthalin oxidized, *i. e.*, 0.000013 gram phenolphthalein.

The colors in this case were not strictly comparable on account of the active extract having a red-brown color. These readings were approximately correct.

Distribution of the Oxidases in the Green Plant.—In order to further prove the presence of oxidases in the tobacco plant and to locate in which parts of the plant they were present, a green tobacco plant about 17″ high and about at the topping stage was selected. The plant had not been topped and the flower head was just about to bloom. The plant was cut 4″ from the ground and suspended by means of an iron clamp and stand into a small open glass containing a solution of phthalin. and left this way for 26 hours. The solution of phthalin was prepared as follows:

0.064 gram phenolphthalein.
18.00 cc. H_2O.
2.00 cc. 0.1 *N* KOH.

Fifteen cc. of this solution were put in the glass in which the plant was suspended. After 26 hours, 12 cc. of the solution were absorbed by the plant and the cut end was just above the liquid, so that it would probably have absorbed more had it been present.

Cross sections were then made of the various parts of the plant and the test for oxidases was made as follows: A drop of 3% potassium hydroxide was placed on the cross sections and then examined under the microscope.

I. Cross section of stalk about an inch from bottom: This gave a deep red all over the surface of cross section, with the best test in cambium layer. Showed test in epidermal hairs.

II. Cross sections of stalk just below the flower head: These gave a red color. Here the test did not show in the woody part. It gave a slight test in the epidermal cells of the cambium layer, and a good test in the bast cells. No test was obtained here in the epidermal hairs.

III. Central top flower just coming in bloom: A cross section of this part gave the most prominent red test in the calyx, varying off to yellowish pink in the placenta, not as deep in color as in calyx. Shading was deepest along fibro-vascular bundles.

IV. Cross section of mid-rib or stem of the leaf (first leaf from bottom): This showed deep red in fibro-vascular bundles (central part of mid-rib), with a slight greenish pink towards the mesophyll or epidermal cells. Cross section of a vein showed same results as mid-rib.

V. Cross section of mid-rib of same leaf at tip of leaf: This showed same results. No color could be seen in the downy hairs of the leaf. These are transparent and secrete the gummy substance of the leaf.

VI. Cross section of the base of mid-rib of top leaf: Obtained a good test in central part of mid-rib or fibro-vascular bundles and in the fibro-vascular bundles which lead to the blade, one on each side of the mid-rib. No red color in other parts of the cross section could be seen.

VII. Cross section of the tip of the mid-rib of top leaf: Obtained delicate test in same regions as in VI.

VIII. Longitudinal section of mid-rib of leaf: This showed a deep red color running along the central part or fibro-vascular bundles of the mid-rib.

IX. Cross section of veins and longitudinal section of leaf: In this case obtained the deep pink color wherever these small veins in the leaf were cut across, with a tinge of yellowish pink in parts of the leaf.

X. Longitudinal section of leaf: No test obtained except where the very minute veins were cut across and these gave a delicate pink.

Emulsin.

Experiments to determin if there was a glucoside-splitting ferment in the tobacco were made as follows:

Active Control.—0.5 gram unboiled sample + 50 cc. H_2O + 0.5 cc. toluene.

Boiled Control.—0.50 gram boiled sample + 50 cc. H_2O + 0.5 cc. toluene + 0.5 gram amygdalin.

Active Extract.—0.5 gram unboiled sample + 50 cc. H_2O + 0.5 gram amygdalin + 0.5 cc. toluene.

Let stand in incubator at 37° C. for 45 hours.

Tested the above for reducing sugars with 2 cc. of Fehling's solution as in case of invertase.

Examined the solutions to see whether any benzaldehyde odor could be detected.

Tested solutions for hydrocyanic acid as follows:

5 cc. of solution were used and to this added 2 drops of 10% $FeCl_3$ and 1 drop of 10% $FeSO_4 + 7H_2O$. Made alkalin with NaOH heated and then made acid with dilute HCl.

The results obtained in this series are given in the following tables:

TABLE VI[1].—EMULSIN: REACTIONS WITH FEHLING'S SOLUTION.

Sample.	H_2O control.	Active control.	Boiled control.	Active extract.	Active extract.
1911 Burley seed	o	+	o	++	++
1912 Burley seed	o	o	o	+++	+++
Burley leaf, transplanting stage	o	+	o	+++	+++
Burley leaf, cured with green color	o	++	++	+++	+++
	o	++	+	+++	+++
Burley leaf, cured with proper color	o	++	+	+++	+++
	o	++	+	+++	+++
1911 dark seed (Torian Yellow)	o	+	o	++	++
Yellow Pryor, cured with green color	o	++	++	+++	+++
	o	++	++	+++	+++
Yellow Pryor, cured with proper color	o	++	++	+++	+++
Soil, 26 hours digestion	o	o	o	+	+
Soil, 90 hours digestion	o	o	o	o	o

[1] o indicates no reduction.
+ indicates trace reduction.
++ indicates small reduction.
+++ indicates good reduction.

TABLE VII.[1]—EMULSIN: TESTS FOR HYDROCYANIC ACID.

Samples.	Active control.	Boiled control.	Active extract.	Active extract.
1911 Burley seed	o	o	o	o
1912 Burley seed	o	o	+++	+++
Burley leaf from seedling (transplanting stage)	o	o	o	o
Burley leaf, cured with green color	o	o	o	o
	o	o	o	o
Burley leaf, cured with proper color	o	o	+++	+++
1911 dark seed (Torian Yellow)	o	o	o	o
Yellow Pryor, cured with green color	o	o	+++	+++
Yellow Pryor, cured with proper color	o	o	+	++
Soil	o	o	o	o

[1] o indicates no blue color.
+ indicates faint blue color.
++ indicates fair blue color.
+++ indicates good blue color and precipitate.

TABLE VIII.[1]—EMULSIN: TESTS FOR BENZALDEHYDE.

Samples.	Water control.	Active control.	Boiled control.	Active extract.	Active extract.
1911 Burley seed	o	o	o	++	++
1912 Burley seed	o	o	o	+++	+++
Burley leaf from seedling (transplanting stage)	o	o	o	+	+
Burley leaf, cured with green color	o	o	o	+++	+++
	o	o	o	+++	+++
Burley leaf, cured with proper color	o	o	o	+++	+++
	o	o	o	+++	+++
1911 dark seed (Torian Yellow)	o	o	o	++	++
Yellow Pryor, cured with green color	o	o	o	+++	+++
	o	o	o	+++	+++
Yellow Pryor, cured with proper color	o	++	o	++++	++++
Soil	o	o	o	o	o

[1] o indicates no odor of benzaldehyde.
+ indicates faint odor of benzaldehyde.
++ indicates distinct odor of benzaldehyde.
+++ indicates good odor of benzaldehyde.
++++ indicates strong odor of benzaldehyde.

The results in Tables VI, VII and VIII seem to indicate that there is a glucoside-splitting ferment present in the tobacco plant at all stages of its growth. In the test on the leaf of the plant at the transplanting stage, given in Table VI, the solution was again tested after standing five days and the reduction was then complete.

Inulase.

The following experiment was made to determin if inulase was present: The inulin solution was prepared by dissolving the inulin in hot water,

cooling, making to mark and taking aliquots equivalent to the amounts used in the work.

Active Control.—0.50 gram unboiled sample + 50 cc. H_2O + 0.5 cc. toluene.

Boiled Control.—0.5 gram boiled sample + 0.10 gram inulin + 50 cc. H_2O + 0.5 cc. toluene.

Active Extract.—0.5 gram unboiled sample + 0.10 gram inulin + 50 cc. H_2O + 0.5 cc. toluene.

Let stand in incubator at 37° for 70 hours.

After the digestion the solutions were tested with 2 cc. of Fehling's solution as in the case of invertase.

In the case of the cured leaf the solutions were filtered so as to get a fairly clear solution.

The results seemed to indicate that this enzyme is not present to any great extent in the plant although a rather good test was obtained in the 1911 Burley seed and slight tests in the growing plant.

Proteolytic Enzymes.

Experiments were made to determin if there was a proteolytic enzyme present in the plant. The method used was that proposed by Meunier as given in Euler's General Chemistry of the Enzymes.[1] This method takes into account the fact that since hydrochloric acid combines with protein during digestion, the diminution in the amount of free acid expresses the extent of the action.

Unless there is a decided proteolytic action, this method is not very satisfactory and had time permitted probably a better method could have been substituted. In most of the work on the cured tobacco leaf where titrations were involved, the results have not been satisfactory on account of the natural color of the solutions interfering with the color changes of the indicator.

This series was prepared as follows:

Active Control.—0.5 gram unboiled sample + 50 cc. H_2O + 0.4 cc. concentrated HCl + 0.5 cc. toluene.

Boiled Control.—0.5 gram boiled sample + 50 cc. H_2O + 0.4 cc. concentrated HCl + 1 gram casein + 0.5 cc. toluene.

Active Extract.—0.5 gram unboiled sample + 50 cc. H_2O + 0.4 cc. concentrated HCl + 1 gram casein + 0.5 cc. toluene.

Commercial casein that had been prepared with rennet was used.

Before the digestion the bottles were thoroughly shaken and the contents allowed to settle. The initial acidity of each solution was then determined

[1] Euler-Pope, 1912: Wiley & Sons, page 298.

by withdrawing aliquot portions of 2 cc., adding 50 cc. of water, and titrating with 0.1 *N* potassium hydroxide, using Congo red as indicator. (Congo red used was 1% alcoholic solution prepared with 30% alcohol.)

The solutions were digested in the incubator at 37° and at intervals titrations were made on 2 cc. aliquot portions as above. The bottles were kept tightly stoppered during the digestion.

The end point in the titrations was not distinct, as there was a disadvantage in titrating from acid to neutral, and besides the color of the tobacco interfered to an appreciable extent. The color in acid solutions was a dirty bluish violet.

The digestions were only made of the leaf of the green cured Burley and Pryor and properly cured Burley samples and after a period of 144 hours' digestion, the results obtained were not conclusive in proving the presence of this enzyme, although the titrations indicated a slight proteolytic action.

Reductases.

Some experiments have been made to determin if there is a reductase present in the tobacco.

For this work a methylene blue solution was used which contained 1 cc. of a saturated 95% alcohol solution, diluted, to 40 cc. with water.

The tests were made as follows:

Water Control.—Methylene blue + 0.25 cc. toluene + water.

Boiled Control.—1 gram boiled sample + methylene blue + 0.25 cc. toluene + water.

Active Extracts.—1 gram unboiled sample + methylene blue + 0.25 cc. toluene + water.

Test tubes of 37 cc. capacity were used for the above tests and after preparing them they were filled with water, so that when rubber stoppers were inserted all the air was excluded. They were then heated at 37° for 22 hours in the incubator.

Where it was possible, the diminution in the amount of blue color in the boiled and unboiled samples as compared with the water control was noted after the digestions were made. In most of the samples, however, no blue color comparable with the water control could be obtained before the digestion was commenced, since the green samples of tobacco gave a blue-green color and the cured samples a dark green. By using the same amounts of methylene blue, however, very good comparisons could always be made in the color of the solutions of the boiled and unboiled samples, and this fact should be borne in mind in arriving at any definit conclusion in regard to the results given in Table IX:

TABLE IX.—REDUCTASE: COLOR OF SOLUTION AT END OF DIGESTION.

Samples.	Water control.	Boiled control.	Active extract.	Active extract.
1911 Burley seed.....	Blue.	Light blue color.	No blue color.	No blue color.
1912 Burley seed.....	Blue.	Faint trace blue color.	No blue color.	No blue color.
Burley leaf from seedling (transplanting stage)...	Blue.	Deep green-blue. On standing became deeper in color.	Greenish blue but much less than in boiled control. On standing became faint greenish blue color.	
Burley leaf, cured with green color.....	Blue.	Deep green.	Light brownish green, not quite as deep as active extract of green Yellow Pryor.	
Burley leaf, cured with proper color.....	Blue.	Deep green color.	Not quite as deep green as boiled control.	
1911 dark seed (Torian Yellow.....	Blue.	Light blue color.	No blue color.	No blue color.
Yellow Pryor, cured with green color.....	Blue.	Deep green.	Light brownish green.	Light brownish green.
Yellow Pryor, cured with proper color.....	The dark natural color of the solution prevented the tests from being made on this sample.			

Conclusions.

From the above experiments it appears that invertase, diastase, emulsin and reductases are present in appreciable amounts in the tobacco seed and in the leaf in all stages of its growth and after curing. Lipase, inulase and a proteolytic enzyme also seem to be present in small amounts, although in some cases the results are doubtful. The tests for enzymes in the soil were negative with one or two exceptions. Oxidases were found in the green leaf in all stages of its growth, gradually decreasing in amount from the topping stage to maturity, but no definit tests were obtained for this enzyme in the cured leaf. A fairly good test, however, was obtained with guaiacum in the leaf cured with a green color, but here, as well as in the other cured samples, no tests were obtained with phthalin. This may be due either to the fact that the enzyme has been used up in the cured leaf for oxidation purposes or that some interfering color prevented a positive test from being obtained.

The fact that the quantitative tests for the amount of oxidase reaction show the smallest amount in the leaf at about the matured stage, taken in connection with the fact that the active extracts of the cured leaf which gave no oxidase test with phthalin, developed a deep red color on the addition of a drop of phenolphthalein leads to the conclusion that the enzyme has been used up in the curing process and is practically absent in the cured sample. The above is in harmony with Loew's work on the oxidases previously referred to, since in several samples he obtained no test, and in others only a slight test for oxidase in the different varieties of cured tobacco which he used in his work.

Now when we consider that during the curing and fermentation periods there is a great loss in weight, for example during the fermentation alone there is a loss of as high as 15% and about one-fourth of this is solid matter, it would be of interest to know what are the results of these changes. During the fermentation period certain gases are developed through the decomposition of certain products and ammonia is one of these gases, which is easily detected in the fermentation room.

Practically all the starch disappears during the early part of the curing process and sugar is formed as a new product. This is accomplished by diastase and hence we see what an important part this enzyme plays in the curing. Again, the sugars disappear and are changed into other substances, probably by oxidation. These changes may be due to the oxidizing enzymes as pointed out by Dr. Loew. The fact that such a good test was obtained for invertase leads to the conclusion that cane sugar may be present and this may be stored in the root and afterwards translocated to the leaf and other parts of the plant during photosynthesis and growth, as Brown and Morris have found to be the case in some plant.

The protein contents of the leaf decrease considerably during the

ripening of the plant; also during the curing and fermentation periods. This is accomplished by the proteolytic enzymes. It has also been shown that amino compounds are formed during these processes and these are probably the products of the action of the same enzyme.

The nitrates decrease and there is also a decrease in the nicotine content of the leaves. The former may be due to the reductases. Some enzymes perhaps have a role in the decrease of the resin and gums in fermentation. If this is true, such enzymes are very necessary, for it is believed that the aroma of a tobacco is partly due to the products formed from the gums and resins after these are broken down. The aroma may partly be due to the breaking up of the glucosides in the tobacco by the glucoside ferment forming an aromatic substance. It is of interest to note, in this connection, that some experiments have been made in this work using emulsin to prove the presence of a glucoside in the green and cured leaf which have not been mentioned. The results proved that there was a small amount of glucoside present. Furthermore, there is no doubt but that positive tests were obtained for a glucoside-splitting ferment.

If there is a large amount of fat or protein present in the leaf, these will create products during combustion which will injure the flavor and aroma of the tobacco. It is due to lipase and the proteolytic enzymes that these objectionable compounds are largely done away with, provided, however, that they have favorable conditions under which to accomplish their work.

The part played by the oxidases in the curing and fermentation of tobacco has already been mentioned.

It has also been shown that during the process of smoking an ethereal oil is formed from certain products of the sweat and from this may be due a portion of the flavor.

Citric, malic and oxalic acids are present in the cured leaf, although in smaller quantities than in the green leaf. Part of the citric and malic acids are perhaps transformed in the fermentation to acetic and butyric acids.

Thus we see that there are many highly complex chemical changes taking place in the plant during its growth and these continue in the curing and fermentation periods. That the enzymes play a very important part in these changes cannot be denied.

The tobacco crop in this respect is somewhat unique as compared with the ordinary farm crops, since the latter when they are matured only need a certain amount of care and labor before they are ready for the market, whereas when the former reaches maturity the crop is at one of its critical stages and requires the utmost care and attention in the curing and fermentation in order to make the finished product have the highest market value. This is the chief reason why tobacco, although it may be con-

sidered a luxury, must, of necessity, from the amount of expense and attention given to the production, always command the highest price of any of the general farm crops.

In conclusion, the writers desire to express their appreciation to Dr. Joseph H. Kastle for suggesting this line of work and for assistance rendered during its preparation.

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NOTE.[1]

The Use of Benzoic Acid as a Standard Material.—The extended use of benzoic acid as a calorimetric standard, and its value as an acidimetric standard, makes it appear desirable to call attention to the fact that the substance is slightly hygroscopic. This was partially recognized by Morey[2] in speaking of the advantage of fusion as preventing "large surface effects."

Definit data on this point were first obtained when comparing, by means of titration with a standard alkali, the samples B and C described below. The following samples were then examined: Sample A was especially prepared to be used as a standard for comparison by starting with the purest material obtainable commercially, and successively crystallizing from alcohol and from water, subliming *in vacuo*, again crystallizing from water and again subliming twice *in vacuo* over fused calcium chloride. The object of the double sublimation at the end was the removal of the last trace of water. Sample B was some of the material purified by Morey[3] and kept for more than a year in a glass-stoppered bottle. Sample C was a commercial product of good quality, but shown to contain a small amount of impurity by the ash left upon ignition.

The acidity of the samples having been determined, a portion of each was carefully fused and the acidity again determined. The average results follow, taking the average value obtained for A, as 100%.

Sample.	Number of determinations.	Average. Per cent.
A	7	100.00
A fused	5	100.00
B	6	99.93
B fused	9	100.01
C	6	99.95
C fused	5	99.96

The results obtained with each sample were very concordant. One series, the results obtained upon sample A before fusion, gave 100.01, 100.02, 99.98, 100.00, 100.00, 99.98, 99.99. This fairly represents the agreement between results from any one sample.

[1] Published by permission of the Director, Bureau of Standards.

[2] THIS JOURNAL, 34, 1027 (1912).

[3] *Loc. cit.*