STUDIES ON THE FERMENTATION OF TOBACCO[1]

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INTRODUCTION

After curing, cigar-leaf types of tobacco in particular are allowed to undergo one or more fairly definite periods of fermentation or "sweating." This process is characterized chiefly by an exchange of gases, the generation of heat, and a modification of the flavor and aroma of the leaf. The aging process in tobacco is not in all cases clearly distinguishable from fermentation, except that the rate of activity in the latter is more rapid and results in an appreciable liberation and accumulation of heat and gases. Although the subject of fermentation has received considerable attention in the past no satisfactory technic for measuring improvement in quality has been devised, and estimates of the progress of fermentation are dependent largely upon the opinion of those experienced in judging tobaccos.

As there is little exact knowledge concerning the nature of the process of fermentation, comprehensive investigations from several different angles will be necessary to establish the facts. The present investigations, in which the Dewar-flask method was used, are primarily concerned with the possible relationship between micro-organisms and the changes involved in the fermentation of cigar-leaf types of tobacco.

EARLIER STUDIES

The chemical changes occurring in tobacco during fermentation have been given particular attention by several investigators, but need not be reviewed here. It should be recalled, however, that tobacco fermentation is generally believed to be an oxidation process, and the close relation of air to the results secured has been generally recognized. Analyses show significant decreases in nitrogen compounds, including nicotine, and other organic substances, accounting for a loss of solid matter sometimes exceeding 5 percent, during fermentation. The total loss of weight, including that of free water, may be considerably higher during the process. On the other hand there is a marked liberation of ammonia and carbon dioxide gases as a consequence of this activity. The improvement of the aroma, flavor, burn, and other qualities is to be regarded as of major importance even though not chemically determinable. Separating the essential and desirable changes from incidental changes constitutes one of the chief difficulties of the chemical aspects of the problem.

The previous investigations which are of most interest in relation to the results secured in the present investigations are those dealing with the possible relationship of enzymes and micro-organisms to the fermentation process. There have been certain claims (*17*) [2] and

[1] Received for publication Mar. 12, 1934; issued August 1934.

[2] Reference is made by number (italic) to Literature Cited, p. 159.

there is some evidence for the contention that, given the proper conditions, fermentative changes may occur in the absence of any enzymic or microbial activity, but this theory has not received much support. The problem seems rather to involve the relative importance of the enzymic and the microbial activities during normal fermentation, even though these may be regarded only as agencies speeding up the rate of oxidation and other chemical changes.

As early as 1858 an analogy between tobacco fermentation and alcoholic fermentation was implied by Koller (*10*), who added yeast with the purpose of increasing the rate of tobacco fermentation. Later, tobacco fermentation was compared with that of silage (*4*), "brown" hay (*1*), manures (*19*), etc. The bacterial theory of tobacco fermentation was most definitely brought forward in 1891 by Suchsland (*21*), who isolated bacteria from sweating tobacco, prepared pure cultures, and inoculated these back into tobacco. His claim that the flavor and odor of a specific type of tobacco could be developed in another type through the use of bacterial cultures has not, however, been substantiated. Miciol (*15*), Dâvalos (*3*), Vernhout (*22*), Behrens (*1*), Koning (*11*), Jörgensen (*9*), and others soon afterward isolated organisms from tobacco and in general supported Suchsland's hypothesis. These workers showed that a variety of organisms were present, in what were considered large numbers, i.e., as high as 112,500 bacteria and 12,500 fungus spores on 100 cm^2 of freshly fermented leaf.

Suchsland's bacterial theory soon fell into disrepute under a vigorous though not convincing attack by Loew (*12*) in 1899. Loew not only claimed that bacteria were not present in sufficient numbers to influence fermentation, but that sufficient moisture was not normally present for their development, and that even if sufficient moisture were present, bacteria do not find tobacco a congenial medium for growth.

An even stronger argument against the bacterial hypothesis, however, was the enzymic theory of tobacco fermentation developed by Loew and treated in further detail in two succeeding papers (*13*, *14*). This theory ascribes tobacco fermentation to oxidizing enzymes normally present in all living material, as oxidase, peroxidase, and catalase, the latter being present in dried, cured, and fermented tobacco, even after several years. The chief role in fermentation was first ascribed to peroxidase, an enzyme readily identified by its reaction with tincture of guaiacum in the presence of hydrogen peroxide. Loew's theory has since been supported mainly by Boeckhout and Ott de Vries (*2*) and Jensen (*5*), but very little new evidence to support or controvert the enzyme theory has appeared in the literature. Jensen (*6*) in 1915 used the Dewar-flask method of study and concluded that fermentation of leaf tobacco cannot be inhibited through the addition of chemicals detrimental to micro-organisms. The data presented are not clear on this point, however, and the variability of the temperatures in the incubator used was such as to render the data of doubtful value. On the other hand, Jensen suggests the existence of two types of fermentation, namely, one which proceeds at a moisture content of 20 percent or below and another which requires a higher moisture content. Recent investigations in Russia, discussed in considerable detail by Smirnov (*20*), evidently are in general agreement with Jensen's conclusions.

It is evident from the literature that the determination of the nature of tobacco fermentation is complicated by the problem of what constitutes true fermentation and by the variation in the practical requirements of different types of tobacco.

Studies of a related nature have been conducted by many investigators on the spontaneous generation of heat in hay, straw, silage, manures, etc. The purely chemical, enzymic, and microbial explanations have all had stanch supporters, but it is interesting to note that recent investigations support the microbial theory (*16, 18*), at least under temperature and moisture conditions under which organisms will multiply, and even discount the cooperation of enzymes (*16*).

FIGURE 1.—A simple method for preparing tobacco samples for fermentation studies with pure cultures. The sterilized tobacco in the cellophane roll (*A*) may be inoculated at any desired points by inserting the syringe (*B*) through the cellophane before the roll is placed in the wide-mouth Dewar flask (*C*).

MATERIALS AND METHODS

At first the present studies were conducted with the ordinary narrow-mouth 1-quart thermos bottles. Later, wide-mouth flasks were secured which were easier to fill and permitted of handling the tobacco under fairly satisfactory aseptic and pure-culture conditions when desired. This was accomplished by first placing the tobacco in moisture-proof cellophane containers, sterilizing it with heat, and inoculating it with water suspensions of cultures of organisms by means of a Luer syringe inserted through the cellophane at one or more points. The cellophane containers were at first made the desired shape and size before being filled with tobacco; inoculations were then made by injections at a large number of points (fig. 1).

However, more even distribution of inoculum could be secured by preparing larger sealed cellophane bags in which the tobacco was held

loosely and into which the inoculum was injected with a syringe and mixed with the tobacco by turning and agitating it. The tobacco was then pressed into one end of the bag, which was rolled into form to fit the wide-mouth Dewar flask (fig. 2).

It was found that the tobacco could be adequately sterilized in these containers without apparent physical injury to the leaf or any appreciable change in moisture content by placing the roll or bag in a sealed copper container which was placed in an ordinary steamer. Forty-five minutes of steaming, during which the tobacco reached a temperature of 80° C., was sufficient to prevent subsequent thermogenesis, and plating out showed that no organisms were present. In practice,

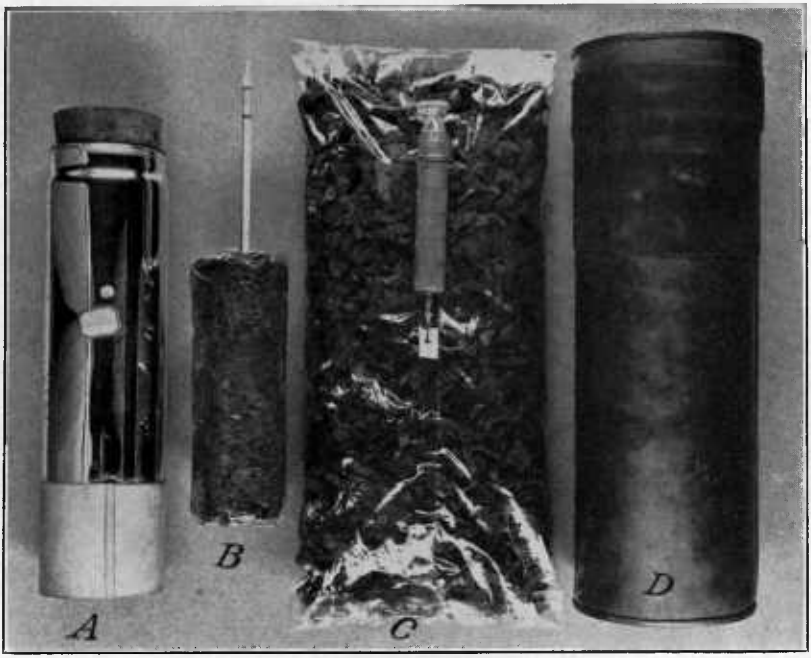

FIGURE 2.—The cellophane-bag (*A*, *B*, *C*) method permits of a uniform application of inoculum under aseptic conditions; the tobacco in the cellophane bag is sterilized in a copper, rubber-tube-sealed container (*D*), without undergoing any change in percentage of moisture.

however, the steaming was allowed to continue for 1 hour, during which time the tobacco reached a temperature of at least 85°. At these temperatures peroxidase was destroyed. In other tests peroxidase was found to be destroyed in water extract if heated for 10 minutes at 83°, and Loew (*12*) reports that the enzyme is destroyed at 87° in 3 minutes.

A limited amount of aeration was made available to the tobacco by various means. In the case of the narrow-mouth bottles a pliable perforated lead tube extended to the bottom of the flask. In the case of the cellophane containers, aeration was only provided by puncturing the cellophane at several points with the syringe or a hot needle and by not sealing the mouth of the flasks tightly. No other provisions for aeration were made, but since, as a rule, the tobacco was only loosely packed and the experiments continued for only 10 days, it is safe to

say that the amount of oxygen available was equal to that in normal fermentation in large boxes or bulks. At least, preliminary trials with air aspirated through the bottles did not appreciably increase thermogenesis, whereas the replacement of the air with nitrogen, followed by sealing, greatly reduced the thermogenic power.

The tobacco used in the tests was largely of the local variety known as Havana No. 142, grown on the Wisconsin Experiment Station farm. This tobacco contained approximately 30 percent moisture, which is close to normal for Wisconsin tobacco in the bale soon after stripping. The leaves were first stemmed and cut into strips about one-fourth inch in width; this made the pack more uniform and easier to handle. One hundred and fifty grams (about 5½ ounces) of this

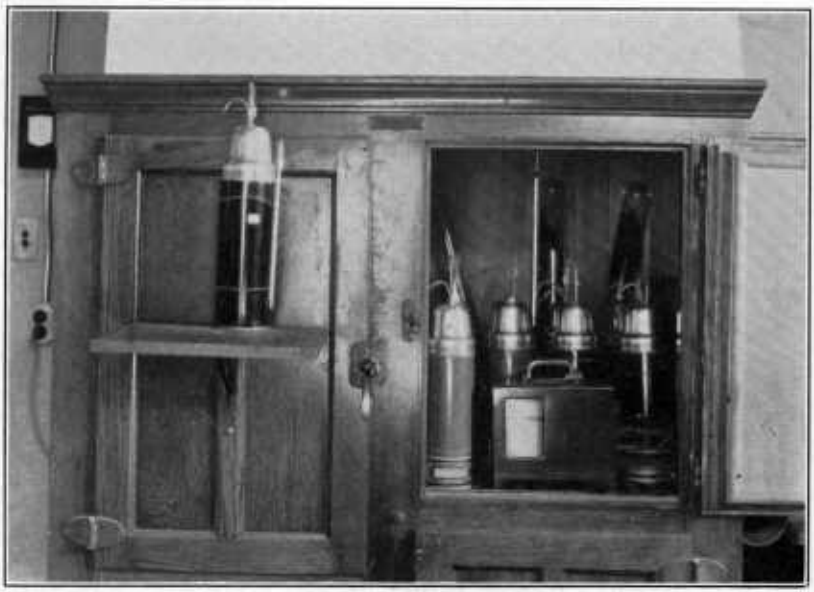

FIGURE 3.—One of the constant-temperature incubators and the thermos bottles used in the earlier experiments.

tobacco was usually used in the 1-quart thermos bottles. The moisture content was usually raised to 35 percent or more by atomizing the tobacco with the desired quantity of distilled water. In most instances the chemicals were applied with the water.

As soon as the bottles were filled they were placed in automatically regulated constant-temperature chambers. Most of the experiments were run in a large 30° C. incubator in which the temperature normally varied less than 1° (fig. 3). More significant results would no doubt have been secured in some cases by incubating at a somewhat lower temperature. Readings were taken at 24-hour intervals (i.e., at 9 a.m.), usually over a period of 10 days. The temperature increases shown in the tables were then based on the average of the incubator readings subtracted from the average of the flask readings over the 10-day period. The maximum increases, which usually occurred about the fifth day, were considerably higher than the average. In most cases the incubator temperature recorded was

that of a thermometer inserted in a corresponding empty thermos bottle.

After the completion of the test in the thermos bottles, rough estimations of ammonia and carbon dioxide were usually made by drawing air from the aeration tube over concentrated hydrochloric acid and through limewater. These estimations of gaseous products are to be largely regarded as confirmatory information as to the direction and degree of activity exhibited. Exact quantitative determinations, while desirable, did not appear to be justified in the present connection, and would probably not have influenced the conclusions reached. The tobacco was then withdrawn from the flask, and a random sample of approximately one-half gram, or 10 square inches of leaf was introduced into about 5 cc of sterile water, agitated, and allowed to stand for about 1 hour before being plated out in nutrient agar or potato agar. Notes were also made at this time on the color of the

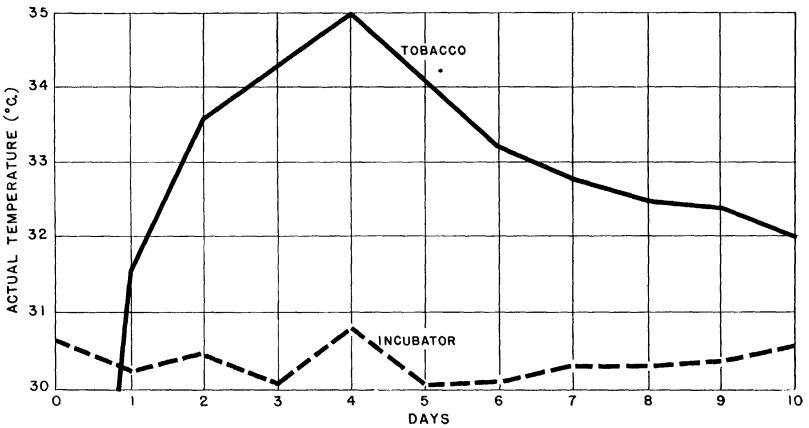

FIGURE 4.—Thermogenic behavior of tobacco in a thermos bottle held for 10 days at an incubator temperature of between 30° and 31° C.

water extract, and the odor, as well as on tests for peroxidase with tincture of guaiacum and hydrogen peroxide. The entire lot of tobacco was again weighed and then placed in an oven for determination of dry weight.

EXPERIMENTAL RESULTS

In the present investigation about 350 separate fermentation trials were made in Dewar flasks. The presentation of all the data secured seems unwarranted, and consequently only portions will be used to illustrate the conclusions drawn. The trials were rarely run in duplicate, preference being given to repeated successive trials, which were feasible since practically all environmental factors were under control and the data were based on average temperature readings. Control flasks, i.e., flasks containing untreated tobacco, were run, however, in connection with practically all trials.

The typical thermal behavior of such a control may be noted from the graph shown in figure 4. The tobacco being placed in the flask at room temperature and the flask then placed in an incubator averaging 30.5° C. (87° F.), the temperature rose very rapidly during the

first 24 hours as a consequence of both the heat from without and the thermogenesis from within, which in the presence of sufficient moisture usually gets under way the first day. The temperature normally continues on the upgrade in the flasks for 3 to 5 days, after which time it usually declines gradually. This decline is in part due to a reduction of the potential heat-producing constituents of the tobacco, since if this same lot of tobacco is removed and replaced in the flask with the original moisture content, the temperature curve is lowered and shortened. However, a gradual reduction in available oxygen and an accumulation of gases such as ammonia and carbon dioxide injurious to thermogenesis may also play a role in this decline.

EFFECT OF MOISTURE

The percentage of moisture in Wisconsin tobacco at the time it is baled on the farm averages approximately 30 percent,[3] the variation being generally between 25 and 40 percent. This tobacco may be fermented in several different ways. If the leaf is sorted, it is packed tightly in boxes or placed in bulks, where it goes through one or more sweats before being packed in boxes. If the leaf is stemming tobacco, the bales are stacked in large bulks, where they undergo fermentation. Following a period of aging, usually accompanied by natural drying, this leaf is moistened heavily before being stemmed, and the strips are again fermented in large bulks at a moisture content of about 40 percent. The results secured under the various methods of handling stemming tobacco may naturally be expected to be quite different, and the results in this type as a whole are apparently not comparable with those obtained in tobacco types stored at moisture contents of 20 percent or below, where little or no thermogenic activity occurs.

In large bulks or under high pressure, tobacco may evidently go through a normal fermentation process at a relatively low moisture content (22 to 28 percent). In thermos bottles with only 150 g of tobacco, loosely packed, no significant thermogenic activity was obtained at a moisture content of below 30 percent. As the moisture content is increased above 30 percent, the thermogenic power was found to increase proportionately (table 1). The rate of this increase was distinctly raised by increasing the pressure, i.e., placing 300 g of tobacco in the same volume (fig. 5). However, significant results by the thermos-bottle method are dependent upon having a moisture percentage of 35 to 40, which, though comparable with the amount of moisture present during the practical process of fermenting stemming tobacco, is not comparable with the usual percentage of moisture in sorted tobacco. It seems reasonable to assume, however, that within certain limits higher moisture contents in the thermos bottles for a time at least only serve to hasten activity of the same character as proceeds more slowly at lower moisture contents. In order that the generation of heat may be measurable in the thermos bottles, it must naturally accumulate at a more rapid rate than that at which it is lost through the insulating power of the flask. Furthermore, moisture is actually liberated during normal fermentation, as is suggested by the term "sweating", which no doubt renders the leaf and the environing air increasingly favorable for additional forms of activity. Taking

[3] The moisture percentages as given throughout this paper are computed on the basis of total weight.

all factors into consideration, limited generalizations with respect to the similarity of fermentation activity at ranges of from 25 to 40 percent are permissible. The situation in tobacco stored at moisture contents below approximately 20 percent may be expected to be of

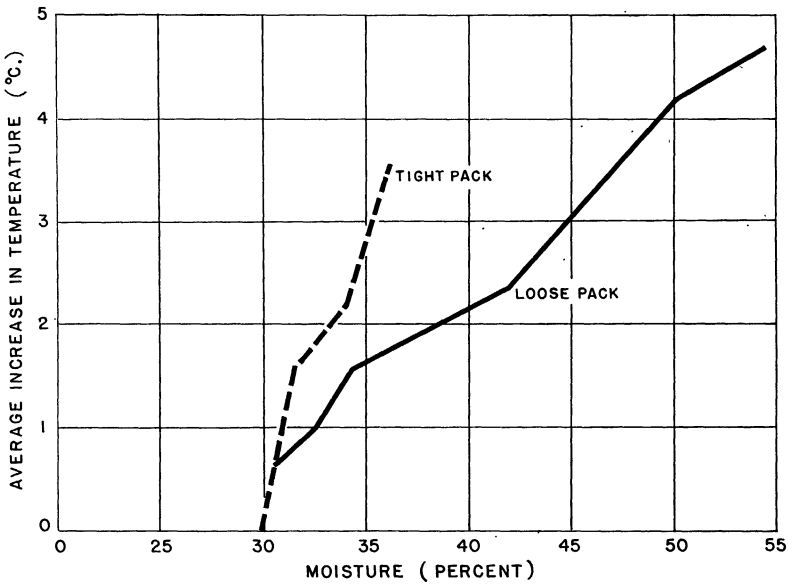

FIGURE 5.—Relation of percentage of moisture and tightness of packing to the thermogenic activity of tobacco in thermos bottles held at an average temperature of 30.2° C.

quite another nature, as are those in tobacco maintained at excessively high moisture contents.

TABLE 1.—*Relation of percentage of moisture in tobacco to its fermentation in Dewar flasks* [1]

Moisture (percent)	Average temperature			Ammonia	Carbon dioxide	Odor	Color of extract
	Incubator	Flask	Increase				
	° *C.*	° *C.*	° *C.*				
30.4	30.3	31.0	0.7	+	0	Raw	+
32.9	30.3	31.3	1.0	++	+	do	++
34.2	30.3	31.9	1.6	++	+	Mild	++
37.9	30.3	32.9	2.6	+++	++	do	+++
41.5	30.3	32.7	2.4	+++	+++	Good	+++
50.0	30.3	34.8	4.5	+++	+++	Strong	+++
54.3	30.3	35.0	4.7	+++	+++	do	+++

[1] In this and succeeding tables, plus signs indicate relative amounts, i.e., + small, ++ medium, +++ large.

EFFECT OF TEMPERATURE

Under favorable conditions cigar-leaf tobacco is ready for normal fermentation as soon as it is properly cured. In practice, some months may elapse between the completion of the curing and the beginning of fermentation. While this interim is partly a result of the time required for marketing, sorting, packing, and storage, fer-

mentation does not normally get under way until favorable natural or artificial temperatures are presented.

Little is definitely known about the most favorable temperature for fermentation, or regarding the lowest or highest temperatures at which it occurs. The solution of this problem presents many difficulties. An investigation of the problem by the Dewar-flask method suggests, however, many points of interest. The results of a series of trials are shown in table 2. The various temperature control chambers used could not be maintained regularly at a variation of less than 1° C.; consequently small temperature increases at the extremes were difficult to detect. Evidently, however, little or no activity occurred at temperatures below 10° C. (50° F.). At about 16° C. (61° F.) fairly evident thermogenesis began, reaching a maximum at 20° C. (68° F.) in this series. In other trials the indications were that the maximum of heat production lies closer to 25° C. (77° F.) (fig. 6). At any rate, spontaneous generation of heat begins

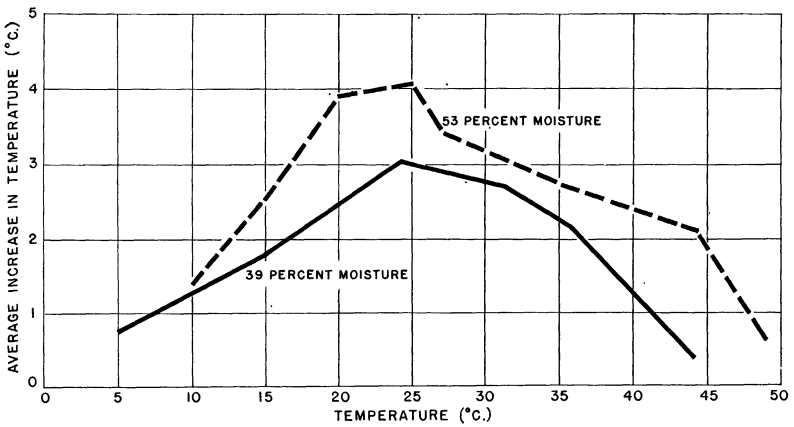

FIGURE 6.—Relation of temperature of incubation to the thermogenic activity of tobacco at two different moisture contents.

to drop off at 30° C. (86° F.) and is apparently entirely eliminated before a temperature of 50° C. (122° F.) is reached. Temperatures as high as 50° C. are rarely, if ever, reached in fermenting boxes of leaf tobacco, though in bulk fermentation considerably higher temperatures may be attained (*23*), whereas in stemming tobacco the bulk may be allowed to reach 60° C. (140° F.). For cigar wrapper and binder tobacco, however, it is now generally believed that temperatures above 50° C. are injurious to the quality of the leaf. Normally the bulk is therefore turned or the leaf transferred to boxes before this temperature is reached. The curve of the temperature rise in such a bulk is typically such as is shown in figure 7, in which it will be seen that following a rapid rise of temperature, the rate is diminished considerably beyond 43° C. (110° F.) and, in the fourth, fifth, and sixth rebulkings, drops off at increasingly lower temperatures, the primary reason perhaps being the lowered moisture content. In practice, bulks are now usually turned only once or twice before being boxed.

TABLE 2.—*Relation of temperature to the fermentation of tobacco in Dewar flasks* [1]

Moisture (percent)	Average temperature			Ammonia	Carbon dioxide	Odor	Color of extract	Peroxidase
	Incubator	Flask	Increase					
	° C.	° C.	° C.					
39.3	9.8	9.3	[2] −0.5	0	0	Raw	++	++
38.3	16.5	17.8	1.3	++	++	do	++	+++
39.1	20.0	23.7	3.7	++	+++	Good	+++	+++
37.8	25.4	28.0	2.6	+++	+++	Strong	+++	+++
37.0	27.0	28.4	1.4	+++	+++	do	+++	+++
37.3	36.6	38.4	1.8	+++	+++	do	+++	+++
37.0	44.1	44.9	.8	+	0	Mild	+++	+++
38.0	48.5	48.3	[2] −.2	0	0	do	+++	+

[1] See footnote, table 1. [2] Decrease.

Judging from the results obtained with the thermos bottles, fermentation apparently does not proceed much below 18° C. (65° F.)

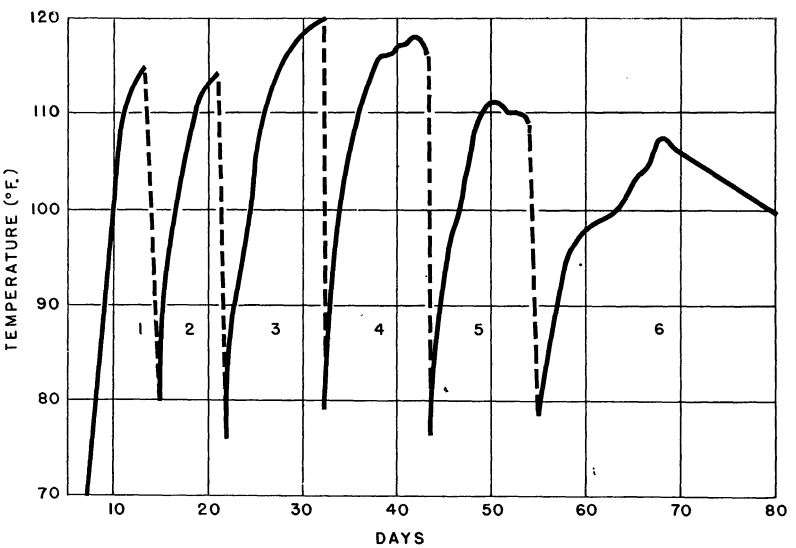

FIGURE 7.—Generation of heat in a large bulk of cigar-leaf tobacco following repeated rebulking.

or above 45° C. (113° F.). Several trials with tobacco "incubated" in a moist condition at temperatures between 45°–55° C. (113°–131° F.) for 20 days yielded little or no evidence of normal fermentative changes. On the other hand, there is some chemical evidence that heating tobacco directly may bring about changes resembling certain of those due to true fermentation, although evidently such heating cannot replace fermentation. Apparently, it is not the actual temperature reached in fermentation which is significant, but the actual increase in temperature above the surrounding atmosphere, since this increase represents the intensity of the process. Taking all facts into consideration, it will be seen that problems of considerable practical importance arise in this connection. Apparently fermenting rooms should preferably be maintained at approximately 22° C. (72° F.), and temperatures occurring in the fermenting leaf above

45° C. are less likely to be effective than are lower temperatures. A 30-degree increase in temperature between 75° and 105° F. should, for example, be considerably more effective than a 30-degree rise in temperature between 100° and 130° F. Hence, bulkings which result in excessive accumulation of heat may actually retard true fermentative changes.

EFFECT OF HEAT AND ANTISEPTICS

If tobacco is heated to a sufficiently high temperature in dry or moist heat, both the microbial flora and the enzymes present are destroyed. If such heated tobacco is placed in a thermos bottle in a moist condition, heat generation will still occur, though after some delay. This is true microbial thermogenesis, resulting from reinfestation with micro-organisms. If, however, the technic is modified so as to insure the introduction of heat-sterile tobacco into the container and the maintenance of aseptic conditions, thermogenesis is completely prevented, and the leaf is essentially preserved and unchanged (table 3). However, it is evidently not possible by the use of heat to destroy the microbial flora without destroying or injuring the plant enzymes or other constituents of the plant cells which may be concerned with normal fermentation, since the thermal inactivation points of most forms of living matter often too closely overlap.

It seemed quite conceivable, however, that certain chemicals, especially those of an antiseptic nature, might exert a selective action between microbial activity and the activity inherent in plant cells. Johnson and Murwin (*8*), for example, found in testing various disinfectants for tobacco seed, that while corrosive sublimate under certain conditions completely checked germination, silver nitrate under similar conditions was not injurious to the germination of the seed, a function evidently dependent upon enzymic activity. Both chemicals, however, were about equally destructive to micro-organisms. Chloroform, toluene, and acetone are usually conceded to be effective germicides, but with the additional property of not being particularly destructive to enzymes or harmful to their activity at low concentrations.

TABLE 3.—*Effect of heat applied to moist tobacco in air-tight copper containers on subsequent behavior in Dewar flasks when reinfestation with micro-organisms is prevented* [1]

Heat treatment of 150 g of tobacco	Average temperature increase	Ammonia	Carbon dioxide	Peroxidase	Bacteria	Fungi
	° C.					
None	4.3	+++	++	+++	+++	++
20 minutes, to 45° C.	2.8	+++	++	+++	++	++
30 minutes, to 67 ° C.	2.0	+	++	+	+	+
40 minutes, to 74° C.	.2	+	0	0	+	+
45 minutes, to 80° C.	[2] −.1	0	0	0	0	0
60 minutes, to 85° C.	[2] −.2	0	0	0	0	0

[1] See footnote 1 table 1. [2] Decrease.

According to Loew (*14*), many salts and bases are not harmful to catalase activity, and some may be beneficial. Mercuric chloride however, was found to be distinctly harmful, as was formaldehyde in high concentrations (1 to 5). Chloroform was not found to be

destructive. While the data on this subject are not entirely clear in differentiating between destruction and inactivation, nevertheless the report by Jensen (*5*) that mercuric chloride, formol (formaldehyde) and chloroform do not prevent tobacco fermentation has been regarded by some as strong evidence in support of Loew's theory of enzymic fermentation, and good evidence against the microbial theory.

The results of the experiments here reported, in which the thermos-bottle method was used, have been quite contradictory to those of Jensen. Not only do mercuric chloride and chloroform effectively prevent thermogenesis, but acetone, toluene, beta-napthol, and other antiseptics at low concentrations also prevent thermogenic activity (table 4). The data secured with formaldehyde were less certain. Under the conditions of the experiments, both enzymes and microbes were apparently inactivated but not necessarily destroyed by many antiseptics. Chemicals other than antiseptics, such as ethylene chlorhydrin, acids, and bases, were tested with the purpose of discovering possible stimulatory action on thermogenic activity, but no conclusive evidence in this direction was secured.

TABLE 4.—*Effect of antiseptics and other chemicals on tobacco fermentation in Dewar flasks* [1]

Treatment of 150 g tobacco	Moisture	Average temperature			Ammonia	Carbon dioxide	Odor	Peroxidase	Staphylococci to the square inch [2]
		Incubator	Flask	Increase					
	Per cent	*° C.*	*° C.*	*° C.*					*Number*
None	37.0	30.3	33.7	3.4	+++	+++	Mild	+++	100,000
Acetone, 5 cc	37.0	30.3	30.7	.4	0	0	raw	+++	120
Toluene, 5 cc	37.0	30.3	30.7	.4	0	0	do	+++	0
Chloroform, 1 cc	37.8	30.3	30.5	.2	+	+	do	+++	
Formalin, 2 cc	51.1	30.0	31.7	1.7	+++	++	do	+++	500
Mercuric chloride, 1 g	37.1	30.5	30.8	.3	0	0	do	++	0
Silver nitrate, 1 g	37.1	30.2	31.7	1.5	+	+++	do	+++	0
Silver nitrate, 3 g	41.3	30.2	31.6	1.4	+	+++	do	+++	0
Ethylene chlorhydrin, 1 cc	43.8	27.7	30.3	2.6	+++	+++	Strong	+++	20,000
None	43.8	27.7	32.4	4.7	+++	+++	do	++	50,000
Potassium hydroxide, 2.5 g	38.5	30.5	33.4	2.9	+++	+++	do	++	100,000
Oxalic acid, 0.5 g	41.0	30.3	34.4	4.1	+++	++	do	+++	10.000

[1] See footnote 1, table 1. [2] Estimated.

The results secured with silver nitrate are of particular interest as indicative of a differential effect on microbial and enzymic activity. Under the conditions of the experiments, silver nitrate reduces thermogenesis to about one-half that of the untreated controls. The chemical, however, is quite as effective as the other antiseptics used in reducing microbial activity, as indicated by the freedom of the treated tobacco from surface fungus growth and from the results of plating out. Varying the quantity of silver nitrate from 0.25 to 4.0 g to 150 g of tobacco did not appear to affect the type of result secured. While the bacteria are evidently killed by the silver nitrate, many fungus spores may survive, raising some doubt as to the efficiency of the silver nitrate in checking fungus activity. The proof of their elimination by silver nitrate therefore must be sought by other means. This may evidently be accomplished by first destroying both the enzymes and the microbes in the tobacco by heat. Such tobacco will soon develop strong thermogenic activity as a consequence of micro-

bial development resulting from natural or artificial reinfestation. If, however, such heated tobacco is in addition treated with silver nitrate, no thermogenesis follows (table 5). It seems logical to conclude, therefore, that silver nitrate effectively checks microbial activity and that the thermogenesis occurring in unheated tobacco with silver nitrate is not due to microbial action, but is evidently due to some constituents (presumably enzymes) of the tobacco leaf itself, which are not harmfully affected by silver nitrate. The evidence suggests, therefore, that thermogenesis in tobacco may be brought about by enzymes alone, by enzymes in combination with micro-organisms, or by micro-organisms alone.

TABLE 5.—*Relation of silver nitrate to fermentative activity of tobacco in Dewar flasks* [1]

Treatment of tobacco, 150 g	Silver nitrate used	Artificial inoculation	Average temperature increase	Peroxidase	Bacteria	Fungi
	Grams		°C.			
Not sterilized	0	None	3.7	+++	+++	+
	1	do	1.5	+++	0	+
Sterilized	0	do	4.6	0	+	+++
	1	do	[2] −.1	0	0	0
	1	*Aspergillus flavus*	0	0	0	+
	1	*Aspergillus ochraceus*	.3	0	0	+
	1	*Aspergillus terreus*	0	0	0	+
	0	do	4.1	0	0	+++

[1] See footnote 1, table 1. [2] Decrease.

PRODUCTION OF AMMONIA

The most characteristic odor in rooms where large amounts of actively fermenting tobacco in bulks are being processed, is that of ammonia gas. According to Loew (*13*), this ammonia results from the destruction of the nitrogenous compounds by oxidizing enzymes and is not a product of putrefaction. The data secured in the present experiments repeatedly indicate the formation of large amounts of ammonia in the absence of oxidizing enzymes in the case of heated peroxidase-free tobacco. Ammonia production seems in all cases to be closely correlated with microbial activity and with thermogenesis. However, the determinations of ammonia here reported are only estimations made on the basis of the amount of fuming produced when air from the aeration tube was drawn over concentrated hydrochloric acid. More accurate determinations of correlation between enzymic activity and ammonia production must be made before it can be definitely concluded that peroxidase, catalase, or other enzymes are not also instrumental in the formation of ammonia in fermenting tobacco.

Ammonia production is only faintly discernible at low moisture percentages, where thermogenesis is not evident, but rises rapidly in direct proportion to the increase in moisture. At such low temperatures as 12° C. (54° F.) or below, ammonia production either is not determinable by the method used or is entirely absent even though slight thermogenesis has occurred. Its production then gradually rises up to about 25° C. (77° F.), and falls off again gradually to 44° C. (111° F.), with zero production again at 48° C. (118° F.) (table 2).

Mercuric chloride, acetone, and toluene may entirely prevent ammonia production, although measurable quantities developed in the presence of chloroform, beta-napthol, and Ceresan, in cases where thermogenesis was practically nil. Silver nitrate apparently checks ammonia production more effectively than it does thermogenesis. Other chemical agents used of a nonantiseptic nature had no depressing effect on the formation of ammonia.

PRODUCTION OF CARBON DIOXIDE

The formation of carbon dioxide is a result of oxidation and should therefore be closely correlated with the fermentation process. The data secured on this subject are again only estimations based upon drawing the atmosphere from the thermos bottles through limewater. As with the formation of ammonia, carbon dioxide formation is fairly closely correlated with thermogenesis, although it may often apparently be absent under conditions which permit some formation of ammonia. Its production is completely checked at low percentages of moisture and at the extremes of temperature. Most antiseptics usually completely prevent its formation, but silver nitrate usually reduced but did not check its production.

OTHER FERMENTATIVE CHANGES

The desired results of fermentation are those which bring about the best flavor and aroma in tobacco. While the term "aroma" is sometimes used in referring to leaf tobacco, it should preferably be reserved for cigars or manufactured tobacco and particularly for the smoking quality. The term "odor" will therefore generally be used for the changes which occur during fermentation. Chemically, there may be no particular relation between the odor and the aroma, although in practice a desirable odor is believed to bring about a desirable aroma.

The lack of any standard of measurement of odor, either qualitative or quantitative, makes it a difficult problem to attack. The ability of some persons to judge odor by the sense of smell is, to be sure, often remarkably developed, and, following much and varied experience, may be, for all practical purposes, sufficiently reliable for fine distinctions. In the present experiments it has been necessary to confine the estimations to rather wide differences in odor. The designation "strong" odor may be quite unsatisfactory, however, since it probably more correctly refers to the odor of ammonia, which may actually mask other odors of greater significance. The terms "raw" and "none", "mild" and "good" are more reliable. "Sweet" or "pleasant" odors were rather unusual types, secured at the higher temperatures, and may not have been of a true fermentative nature.

No fermentative odors are produced in the thermos bottles in the period of 10 days unless the moisture content is above about 32 percent, even though some heat production and some gases may be evident. At a 35-percent moisture content, however, a fair development of odor occurs at 30° C. (86° F.) in 10 days and apparently increases rapidly in direct proportion to the moisture present, up to a 45-percent moisture content or more, in which case a very strong but undesirable odor occurs in considerably less than 10 days. With a moisture content of 35 to 40 percent, no apparent fermentative odor develops below 18°, in 10 days. At temperatures beyond 20°, the

characteristic odor develops rapidly, reaching the maximum probably between 25° and 35°. Beyond 40°, the strength of the odor is strikingly decreased, and a sweet and more pleasant odor, sometimes perfumelike, results at temperatures up to 48°. At still higher temperatures it is doubtful whether any true fermentative odors are discernible.

Such antiseptics as were found to prevent active thermogenesis (mercuric chloride, chloroform, acetone, toluene, beta-napthol, etc.) also prevented the formation of any desirable or undesirable fermentative odors. Such chemicals as potassium hydroxide, oxalic acid, sodium carbonate, and ethyl chlorhydrin were without particular effect. Silver nitrate, while it permitted some thermogenic activity, checked the formation of fermentative odors quite effectively. Heating the tobacco to 80° C., or above, is in itself productive of some change in odor which may be confused with the odor of fermentation. However, it seemed apparent from a limited number of trials that the destruction of peroxidase by heat does not prevent the subsequent production of a fairly typical fermentation odor, when proper conditions for microbial activity are again brought about. Under such conditions there appears, however, to be more likelihood of certain fungi or other undesirable organisms developing excessively, with the resultant production of "wild" fermentative changes.

The color of a water extract of tobacco is of some value in comparing the relative degree to which the fermentative changes have progressed, although of little value in comparing different lots of tobacco with each other. As the fermentative activity increases, or the time is prolonged, the extract becomes increasingly darker in color; this condition is generally closely correlated with thermogenesis.

ENZYMIC RELATIONS

Although the presence of a number of different enzymes may be demonstrated in cured tobacco, it has not yet been conclusively shown that any particular enzyme, or these enzymes as a group, are responsible for fermentation in whole or in part. For the present, therefore, it may be satisfactory to select one of the easily determinable enzymes in cured tobacco as a general criterion of probable relation of enzymes to the experimental results secured. Peroxidase, as determined with tincture of guaiacum in the presence of hydrogen peroxide, was therefore chosen for this purpose. Unfortunately, this test does not distinguish between the presence of peroxidase in the active and in the inactive state; consequently, since it is normally present in tobacco, the significance of the present tests is largely limited to cases in which this enzyme is destroyed and hence eliminated as a factor in fermentation.

Peroxidase, as compared with living matter in general, is fairly resistant to unfavorable conditions. None of the antiseptics at the strengths used destroyed the peroxidase reaction with guaiacum. Since certain of these same antiseptics prevented thermogenesis, it must be assumed either that peroxidase is not connected with thermogenesis or that the enzyme was rendered inactive by the antiseptic. The latter assumption is quite logical.

In water extract from cured tobacco leaves peroxidase is destroyed at approximately 83° C., at a 10-minute exposure. When 150 g lots

of moist tobacco are placed in a dry-air oven for 24 hours, it normally required a temperature of 75° to 80° to destroy the peroxidase. In the moist condition peroxidase withstood a temperature of 49° for 10 days, though it was decidedly weakened by this treatment. Exposure to a temperature of 54° for 10 days in the moist condition destroyed peroxidase completely. It is therefore very unlikely that any thermogenesis resulting from peroxidase can occur above 50° C. (122° F.).

The behavior of enzymes and micro-organisms toward heat and antiseptics, as well as toward environmental conditions in general, is such that it becomes difficult to satisfactorily separate their activity in this manner in tobacco fermentation. Tobacco catalase, however, appears to be an exception. The optimum temperature for the activity of tobacco catalase as measured by the evolution of oxygen gas

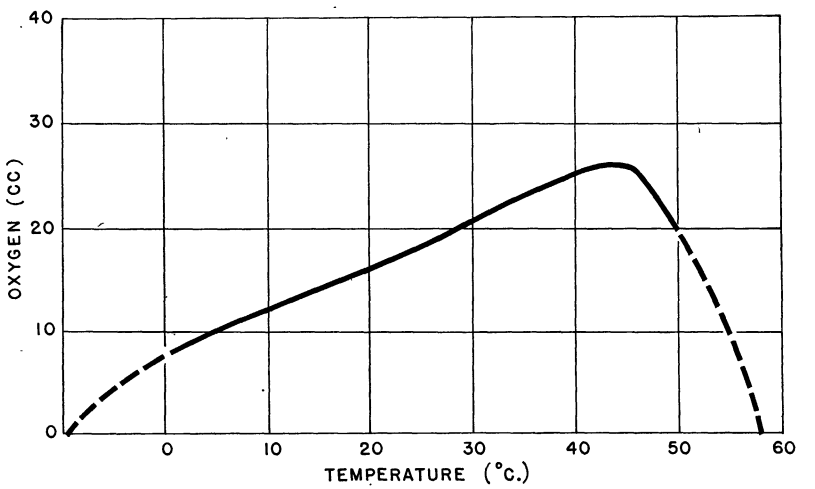

FIGURE 8.—Relation of temperature to the activity of catalase as measured by the evolution of oxygen from hydrogen peroxide.

with hydrogen peroxide was found to be close to 45° C., with the maximum at perhaps 58° and the minimum below 0° (fig. 8). According to determinations of others, the activity of catalase between 10° and 40° is more or less constant, and inactivation occurs at 55°. While the higher temperatures for catalase activity correlate roughly with all the fermentative changes and their decline at the upper limits, this correlation is no less evident for microbial activity.

On the other hand, there seems to be very little correlation between catalase activity and fermentative changes at the lower end of the temperature scale. If catalase is, for instance, approximately as active at 15° C. as at 25°, thermogenesis and other changes in tobacco should be quite comparable at these temperatures; whereas they are as a matter of fact strikingly different and more closely correlated with microbial behavior at such temperatures (fig. 9.).

Evidently it is necessary, however, to develop other forms of technic to distinguish enzymic from microbial activity in the case of active thermogenesis in tobacco. Theoretically, the destruction of all enzymes present by adequate heating, followed by the reintro-

duction of enzymes which may be concerned, together with the maintenance of aseptic conditions, should furnish conclusive proof of their connection. However, such evidence may, to be sure, lack definiteness except in the case of positive results. Using the cellophane-bag method for securing aseptic conditions, experiments of the above nature were performed in the following manner: One thousand grams of cured tobacco was extracted with 2,000 cc of water and yielded 1,300 cc of extract. This extract was filtered through a bacteria-proof porcelain filter, into approximately 50 cc lots. The filtered extract was stored for several days and remained sterile, as was shown by plating out from the separate lots. Unfiltered control extract soon became clouded with growth of organisms. These extracts yielded strong peroxidase reactions at dilutions as high as

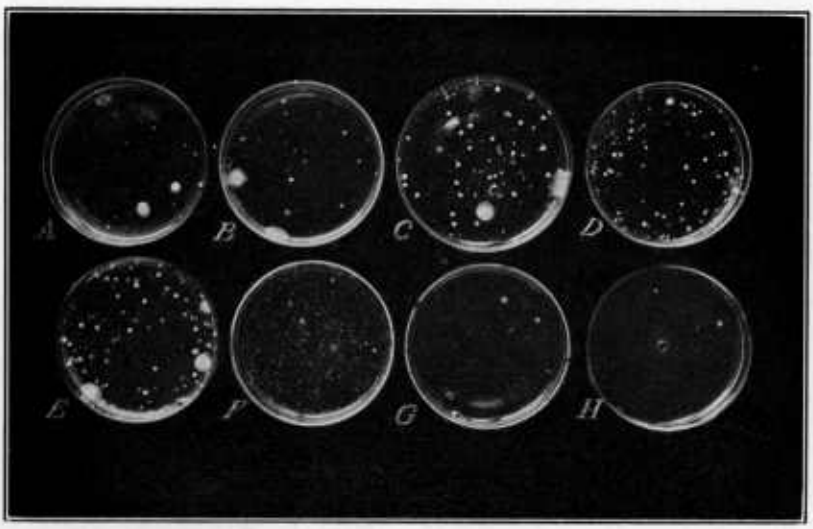

FIGURE 9.—Relation of temperature of incubation of tobacco in Dewar flasks for the 10 days to the development of micro-organisms; *A*, 9.4° C.; *B*, 14.8°; *C*, 19.9°; *D*, 24.4°; *E*, 27.5°; *F*, 30.7°; *G*, 35.5°; H, 40.2°.

1 to 1,000. One-hundred-and-fifty-gram lots of sterilized, peroxidase-free tobacco in cellophane bags were then treated uniformly with these extracts in varying amounts ranging from 40 to 80 cc, the tobacco in these lots being thoroughly wetted by the larger amounts of extract applied. The bags were then rolled and introduced into the thermos bottles. Adequate controls were also prepared. In some cases contamination with organisms occurred as a consequence of providing for aeration, and the usual thermogenic activity resulted. However, in cases where contamination did not occur, no thermogenic activity developed, ammonia and CO_2 were hardly perceptible, and no changes in aroma were evident (table 6). Peroxidase and other enzymes or leaf constituents which are water-soluble were present, as was shown by tests at the end of the experiments, but the quantity of enzymes was evidently below normal. However, it is not unlikely that certain other conditions or activators necessary for enzymic activity were lacking; the results, being negative, are not regarded as conclusive. The method, however, offers possibilities of further development.

TABLE 6.—*Effect of applying varying amounts of filter-sterile extract containing peroxidase to sterile tobacco in Dewar flasks* [1]

Treatment of 150 g of tobacco	Filter-sterile tobacco extract added	Moisture	Average temperature increase	Ammonia	Carbon dioxide	Peroxidase	Fungi
	cc	*Percent*	° *C.*				
Sterilized	40	36.3	[2] −0.1	+	0	0	0
	60	43.2	.1	+	0	+	0
	80	46.0	[2] −.1	+	+	+	0
	[3] 60	43.8	4.1	+++	+++	+	++
Not sterilized	0	43.1	0	+	0	0	0
	0	35.0	3.3	+++	+++	+++	+

[1] See footnote 1, table 1. [2] Decrease. [3] Accidental contamination.

MICROBIAL RELATIONS

Many difficulties also lie in the way of furnishing conclusive evidence of any essential relation between micro-organisms and the fermentation of tobacco, and until the limits of fermentation are more clearly defined than at present, generalizations on this subject are premature. For those types of tobacco in which normal fermentation may be said to proceed at percentages of moisture that entirely preclude the development of micro-organisms, negative proof of microbial relationship should be quite simple. On the other hand, in the fermentation of tobacco in which the activity of micro-organisms may be readily demonstrated, it still remains to be shown that they play an essential rather than an incidental role. Such proof may be rendered difficult for several reasons, particularly the possibility that microbial activity under the conditions of the experiment may replace in whole or in part other agencies which may normally be responsible for fermentation.

The existence of a variety of micro-organisms in relatively high numbers in stored and fermenting tobacco has been repeatedly shown by different investigators. That certain species may find tobacco a favorable substratum for growth under certain conditions is amply demonstrated by the occurrence of such maladies as black rot, musts, and molds. The regularity and frequency with which still other species, which evidently cause no injury, occur suggest that these may exert some influence on fermenting and stored tobacco, although this influence may be admittedly harmful if allowed to progress too far.

In the present investigations over 350 platings have been made from the Dewar-flask-fermented lots, and from tobacco from various other sources, including widely separated tobacco-growing districts. The regularity of occurrence and the numbers of certain species of organisms were in some instances very striking. On the other hand, the relative infrequency of the occurrence of the known injurious forms, such as *Aspergillus niger* (7) and *Oospora nicotianae* in these plates suggests the limited degree of infestation necessary to produce subsequent extensive changes in tobacco under conditions favorable for the development of the particular species concerned. Two large, white-colony forms of bacteria of undetermined species were commonly present, but these were not regarded as significant for the double reason that no marked increase in numbers occurred during

fermentation and because they were evidently unable to induce thermogenic activity in tobacco when introduced in pure culture. On the basis of almost universal association with stored and fermenting cigar tobacco, a tiny coccus form seemed worthy of more consideration (fig. 10). This organism has regularly been secured on nutrient agar from fermenting tobacco from widely separated sources (table 7), often in numbers indicating 300,000 to 500,000 organisms to the square inch of leaf surface. Preliminary studies on this organism in

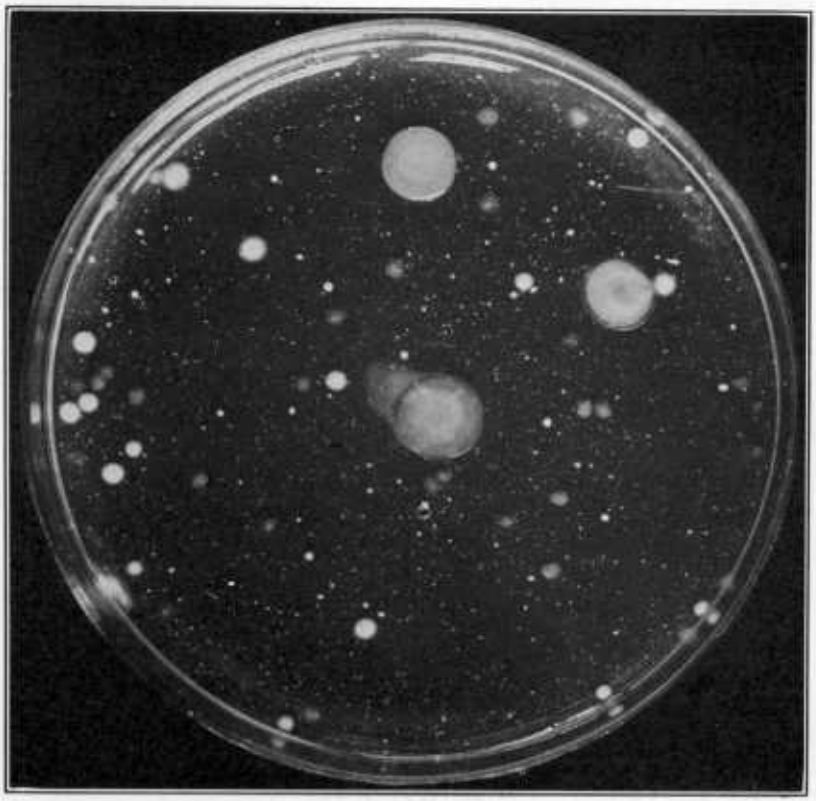

FIGURE 10.—Large numbers of a *Staphylococcus* species secured in dilution plates from fermenting tobacco, and the relative size of these colonies as compared with three colonies of large bacteria.

cooperation with the Wisconsin Department of Bacteriology indicate that it belongs to the *Staphylococcus* group. While the regularity of occurrence and the numerousness of this organism in fermenting tobacco suggest some relation to the fermentation process, it also failed to induce thermogenic activity in tobacco under the conditions of the experiments.

The fungi encountered in stored and fermenting tobacco are normally fairly common species in nature, though little is known about what significance varieties or strains of these fungi may have in the present connection. *Aspergillus flavus* Lk., *A. terreus* Thom, and *Penicillium brevicaule* Sacc. were commonly found in fermenting cigar-leaf tobacco and were used in connection with the present

studies. *A. ochraceus* Wilhelm, a form growing abundantly on a sample of flue-cured tobacco, was included in the studies, as was *A. niger* Tiegh., the causal organism of black rot of fermenting tobacco. These species were either determined or verified by Charles Thom, of the Bureau of Chemistry and Soils, United States Department of Agriculture, from cultures submitted. In contrast with the bacteria, these fungi were found to be very active thermogenically on tobacco and consequently should be given more consideration as possible factors in fermentation, whereas previous students of the microbial hypothesis considered the bacteria of major consequence.

TABLE 7.—*Fermentation results in Dewar flasks with tobacco from various cigar-tobacco growing districts* [1]

Source of tobacco and year grown	Variety	Moisture	Average temperature			Ammonia	Carbon dioxide	Odor	Estimated cocci to square inch	
			Incubator	Flask	Increase				In flasks	Control
		Percent	° *C.*	° *C.*	° *C.*				*Number*	*Number*
Ohio (1931)	Dutch	39.0	23.9	26.9	3.0	+++	+++	Strong	100,000	50,000
Do	Zimmer	38.3	23.9	26.4	2.5	+++	+++	do	150,000	80,000
Connecticut (1930)	Havana seed.	38.5	23.9	25.2	1.3	+	+	Mild	100,000	1,000
Connecticut (1931)	do	35.0	23.9	26.6	2.7	+++	+++	Good	100,000	200
Massachusetts (1931)	do	38.0	23.9	26.1	2.2	+++	+++	do	80,000	0
Canada (1929)	do	44.2	23.9	27.2	3.3	++	+++	do	100,000	3,000
Wisconsin southern (1931)	do	27.3	23.9	23.9	0	+	0	Raw	30	0
Wisconsin northern (1931)	do	33.0	23.9	26.7	2.8	++	+	Slight	100,000	30
Pennsylvania (1931)	do	41.2	23.9	29.0	5.1	+++	+++	Strong	150,000	40,000

[1] See footnote 1, table 1.

TABLE 8.—*Effect of pure cultures of various organisms on sterilized tobacco in Dewar flasks* [1]

Inoculum	Moisture	Average temperature increase	Ammonia	Carbon dioxide	Peroxidase	Color of extract
	Percent	° *C.*				
Aspergillus niger	37.0	4.2	++	+	0	++
Aspergillus ochraceus	36.1	3.6	+++	+	0	+
Aspergillus terreus	37.7	6.1	+++	++	0	+++
Aspergillus flavus	37.8	2.8	+++	+	0	+++
Penicillium brevicaule	40.0	2.7	+++	+++	0	+++
Penicillium baiiolum	37.5	.3	++	0	0	+
Oospora sp	39.1	.6	++	0	0	+
Bacterium sp. 1	38.7	[2] −.2	0	0	0	+
Bacterium sp. 2	40.1	.1	0	0	0	+
Staphylococcus sp	39.0	.1	0	0	0	+
Not inoculated; control	38.1	0	+	0	0	+
Not sterilized; control	39.8	5.1	+++	+++	+++	++

[1] See footnote 1, table 1. [2] Decrease.

By using the cellophane bags in the Dewar flasks, it was possible to first eliminate all possible agencies of fermentation by heating the tobacco for the required length of time. The above-mentioned organisms carried in pure culture could then be subsequently introduced for comparative studies. The bacteria, as has been noted, caused no appreciable thermogenic activity in tobacco, although the *Staphylococcus* form developed in large numbers. Neither did the

bacteria affect any other measurable changes in the leaf under the conditions of the tests. Certain fungi, however, not only usually induced a striking generation of heat, and a resultant production of ammonia and carbon dioxide, but developed odors and other characteristics fairly typical of fermenting tobacco (table 8). Other fungi evidently were capable of little or no influence of a fermentative nature. Detailed studies are required, however, before satisfactory conclusions can be drawn in regard to the comparative activity and actual influence of these and other fungi on the fermentation process under both experimental and commercial conditions.

DISCUSSION OF RESULTS

As understood in the present investigation, the fermentation of cigar-leaf tobacco is an oxidation process resulting in the formation of carbon dioxide and the generation of heat, together with a general improvement in the desired qualities of the tobacco, usually accompanied by the production of ammonia. The influence on quality may be of various kinds, but in general the process eventually removes a "green" or raw taste and odor and develops an aroma. The immediate result may often be the formation of such amounts of ammonia and other products as to render the leaf excessively strong, and the benefits of subsequent storage and aging are in part at least due to the gradual loss of such objectionable substances.

The oxidation process may no doubt be brought about in different ways or at least at markedly different rates, depending upon the environmental conditions. The results secured in the present studies have not eliminated any of the hypotheses held at present as to the nature of the causative agent in fermentation but have rather added some support to both the enzymic and microbial hypotheses. The latter hypothesis has been in danger of being totally discarded from consideration without all the evidence being taken into consideration. Loew's (*12*, *13*) conclusions, for instance, are particularly weak with respect to microbial behavior in tobacco, though his evidence for "oxidizing enzymation" is convincing. Evidently the chief basis for denying any essential relationship between fermentation and microbial activity is the low moisture content present in tobacco types which are "reordered" or redried before being placed in storage. Flue-cured tobacco, in particular, is much more susceptible to fungus development than is cigar tobacco, and unless stored in a condition relatively dry as compared with storage conditions for cigar-leaf tobacco, usually becomes overgrown with fungi to a damaging extent. However, neither the results nor benefits of fermentation as contrasted with those of aging are clearly defined in redried tobacco, and it is quite conceivable that a slow oxidation, entirely independent of microorganisms, may proceed in such tobaccos. On the other hand, millions of pounds of stemming tobacco and cigar tobacco are regularly stored or processed in such a manner as to render microbial development certain. It is not unlikely that this procedure has some essential relation to the desired results. Whether this procedure is to be regarded as of the nature of fermentation or special processing may be open to question.

While it has been claimed by Jensen (*6*) and by Smirnov (*20*), on the basis of trials with antiseptics, that fermentation may proceed

in the absence of microbial activity, the data presented are not convincing on this point. Evidently the selective influence of various antiseptics on enzymes and microbes under different circumstances needs more intensive study. Silver nitrate appears to possess this differential property more completely than any of the other antiseptics used, and, on the basis of the results secured with this chemical, it seems evident that fermentation may proceed in the absence of microbial activity. On the other hand, fermentative changes of an apparently normal character may evidently be brought about by various fungi in tobacco heated sufficiently to destroy all other forms of activity. Bacteria, which have previously been generally believed to be concerned in this process, were not found to be capable of developing thermogenic activity.

SUMMARY

Microbial and enzymic factors which may be concerned with the fermentation of cigar-leaf tobacco were investigated by the Dewar-flask method. Modifications of technic were used which permitted of operations under aseptic conditions and inoculations with pure cultures of micro-organisms.

With 150 g of tobacco, average temperature increases in Dewar flasks over a 10-day period ran as high as 5.6° C. In general, the generation of heat (thermogenesis) was directly proportional to the percentage of moisture present, a minimum of about 30 percent being necessary to secure measurable activity under the conditions of the experiments.

The highest thermogenic increases occurred at incubator temperatures of about 20° to 25° C., very little if any activity developing at temperatures below 10° C. (50° F.) or above 45° C. (113° F.). This experimental maximum temperature is considerably lower than the temperature often allowed in practice by the bulk-fermentation method, and suggests the possibility of obtaining better results at more moderate temperatures.

Chloroform, mercuric chloride, acetone, toluene, or beta-napthol may almost completely check thermogenesis. These antiseptics check microbial activity, and although they do not destroy peroxidase and other enzymes, they apparently cause inactivation. Silver nitrate, on the other hand, reduces thermogenesis under similar conditions to only about one-half that which normally obtains. It is believed that silver nitrate prevents microbial activity without being harmful to the action of enzymes under the conditions of the experiments.

Heating the tobacco to a sufficiently high temperature will completely check thermogenesis or any other expression fermentation, provided aseptic conditions are maintained. Treating such tobacco aseptically with porcelain-filtered extract from unheated tobacco containing peroxidase and other enzymes failed to induce thermogenesis.

Three species of bacteria commonly occurring on tobacco failed to induce thermogenic activity in heated tobacco, but several fungi isolated from tobacco were very efficient in this respect, yielding results thermogenically and otherwise comparable to normal fermentation.

The evidence appears to justify the conclusion that micro-organisms, especially fungi, may play a role in the fermentation or in the

processing of certain types of cigar-leaf tobacco, although such organisms may not necessarily be essential to fermentation.

LITERATURE CITED

(1) BEHRENS, J.
1896. DIE BEZIEHUNGEN DER MIKROORGANISMEN ZUM TABAKSBAU UND ZUR TABAKFABRIKATION. Centbl. Bakt. [etc.] (II) 2: 514–527, 540–545.

(2) BOECKHOUT, F. W. J., and VRIES, J. J. OTT DE
1909. ÜBER TABAKSFERMENTATION. Centbl. Bakt. [etc.] (II) 24: 496–511.

(3) DÁVALOS, J. U.
1892. NOTA SOBRE LA FERMENTACIÓN DEL TABACO. Cronica Medico-Quirurgica Habana no. 15. [Abstract in Centbl. Bakt. [etc.] (I) 13: 390–392, 1893.]

(4) FESCA, M.
1888. UEBER KULTUR, BEHANDLUNG UND ZUSAMMENSETZUNG JAPANISCHER TABAKE. Landw. Jahrb. 17: [329]–272.

(5) JENSEN, H.
1908. UEBER DIE NATUR DER TABAKFERMENTATION. Centbl. Bakt. [etc.] (II) 21: 469–483, illus.

(6) ———
1915. PROEVEN OVER TABAKSFERMENTATION IN "DEWAR'SCHE VATEN". Proefsta.. Vorstenland. Tabak [Dutch East Indies], Meded. 12: 22–38.

(7) JOHNSON, J.
1914. BLACK ROT, SHED BURN, AND STEM ROT OF TOBACCO. Wis. Agr. Expt. Sta. Research Bull. 32, pp. [63]–86, illus.

(8) ——— and MURWIN, H. F.
1925. EXPERIMENTS ON THE CONTROL OF WILDFIRE OF TOBACCO. Wis. Agr. Expt. Sta. Research Bull. 62, 34 pp., illus.

(9) JÖRGENSEN, A. P. C.
1925. MICRO-ORGANISMS AND FERMENTATION. Ed. 5, reset, revised throughout ..., 467 pp., illus. London.

(10) KOLLER, J. B. C.
1858. DER TABAK IN NATURWISSENSCHAFTLICHER, LANDWIRTSCHAFTLICHER U. TECHNISCHER BEZIEHUNG ... 134 pp. Augsburg.

(11) KONING, C. J.
1900. DER TABAK; STUDIEN ÜBER SEINE KULTUR UND BIOLOGIE. 86 pp., illus. Amsterdam and Leipzig.

(12) LOEW, O.
1899. CURING AND FERMENTATION OF CIGAR LEAF TOBACCO. U.S. Dept. Agr. Rept. 59, 34 pp.

(13) ———
1900. PHYSIOLOGICAL STUDIES OF CONNECTICUT LEAF TOBACCO. U.S. Dept. Agr. Rept. 65, 57 pp.

(14) ———
1901. CATALASE, A NEW ENZYME OF GENERAL OCCURRENCE, WITH SPECIAL REFERENCE TO THE TOBACCO PLANT. U.S. Dept. Agr. Rept. 68, 47 pp., illus.

(15) MICIOL.
1891. NOTE SUR LES VÉGÉTATIONS QUI SE DÉVELOPPENT PENDANT LA FERMENTATION DU TABAC. Mem. Manfr. État, Tabacs—Allumettes 2(2): [182]–191.

(16) MIEHE, H.
1931. THE PRESENT STATUS OF THE PROBLEM OF THE SPONTANEOUS HEATING OF HAY. Fifth Internatl. Bot. Cong., Cambridge, Rept. Proc. 1930: 45–57.

(17) NESSLER, J.
1867. DER TABAK, SEINE BESTANDTHEILE UND SEINE BEHANDLUNG. 151 pp. Mannheim.

(18) NORMAN, A. G.
1930. THE BIOLOGICAL DECOMPOSITION OF PLANT MATERIALS. PART III. PHYSIOLOGICAL STUDIES ON SOME CELLULOSE-DECOMPOSING FUNGI. Ann. Appl. Biol. 17: 575–613, illus.

(19) SCHLOESING, T. H.
1888–89. SUR LA FERMENTATION EN MASSES DU TABAC POUR POUDRE. Mem. Manfr. État, Tabacs—Allumettes 1: [514]–552, 1888; 2: 119–136, 192–210, 1889.
(20) SMIRNOV, A. I.
1933. THE PHYSIOLOGICO-BIOCHEMICAL PRINCIPLES OF TOBACCO CURING AND FERMENTATION. 507 pp., illus. Krasnodar. (Tabakovedenie t. 3).
(21) SUCHSLAND, E.
1891. UEBER TABAKSFERMENTATION. Ber. Deut. Bot. Gesell. 9: 79–81.
(22) VERNHOUT, J. H.
1899. RAPPORT OVER HET BACTERIOLOGISCH VAN GEFERMENTIERDE TABAK. Ber. Lands Plantentuin 34. [Abstract in Centbl. Bakt. [etc.] (II) 4: 778–779.]
(23) WHITNEY, M., and MEANS, T. H.
1899. TEMPERATURE CHANGES IN FERMENTING PILES OF CIGAR-LEAF TOBACCO. U.S. Dept. Agr. Rept. 60, 28 pp., illus.