CXXIV. RESEARCHES ON THE FERMENTATION OF DRIED TOBACCO.

II. THE ENZYMIC PRODUCTION OF VOLATILE PRODUCTS FROM NICOTINE UNDER THE INFLUENCE OF TOBACCO-LEAF EXTRACTS.

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INTRODUCTION.

THE decomposition of nicotine during the so-called fermentation process which takes place in the tobacco leaf is one of the most important reactions concerned in this complicated process, as a result of which the raw or dried tobacco becomes converted, in the fermentation stores, into the valuable product of commerce. According to the statements in the literature, decomposition of nicotine also occurs in green tobacco before drying. It is our intention to study the processes taking place during the fermentation, especially to find out the enzymic constituents of the cells of the tobacco leaf which take part in the fermentation. In this connection the literature is lacking in systematic investigations, although individual biochemists express the supposition that oxidases play a part in the process [Loew, 1899, 1900, 1901; Jensen, 1908]. On the other hand, many investigators to-day suggest that the process of fermentation here concerned depends upon bacterial action [Schmidt, 1924; Behrens, 1896, 1901; König, 1900; Suchsland, 1892].

The question is still open and has to be investigated whether or not bacteria, either directly or indirectly, limit the actual fermentation process.

We prepared, as will be shown below, expressed juices and macerates from tobacco leaves under the usual conditions of sterility and examined their effect on nicotine.

We found as a result that definite effects take place, and that volatile nitrogen-containing bases are produced, which can be estimated after distillation.

From their effect on silk peptone (increase of amino-nitrogen) it was ascertained that these juices are able to produce a peptolytic effect. This observation is obviously in agreement with the fact, known in the literature, that protein disappears during the fermentation process. The following researches demonstrate these results.

The problem as to what products are formed from the nicotine on its

decomposition is important. In a preliminary investigation we attempted to find whether, after allowing the nicotine to be acted upon for 2 hours at 37° by tobacco extracts, demonstrable quantities of acid are produced.

It was found that the nicotine solution used as substrate had after approximate neutralisation a p_H value of 4.79. The expressed juices, also approximately neutralised, had a p_H value of 5.74. This gives by calculation for a mixture of equal volumes a p_H of 5.05, whereas the electrometric determination gave for this value the figure 5.29. Hence the mixture has become slightly alkaline during the process. Therefore there can be no question of the production of any considerable amount of acid. On the other hand, a preliminary qualitative test showed that we have to deal with the production of carbon dioxide. We also found that the solutions obtained in the decomposition of the nicotine had a remarkably strong odour of pyridine and methylamine. With KI—HgI₂ reagent we obtained the familiar white precipitate which is given by solutions of pyridine and which is soluble in excess of the precipitating solution (see previous paper). In all probability the nicotine here undergoes a decomposition process with the formation of pyridine, methylamine and carbon dioxide as end products.

Hence the process must be interpreted as the effect of an enzyme, of the class of oxidases, on the nicotine.

In how far this is to be ascribed to the action of a specific oxidase will be shown by later experiments.

Since the solutions, kept in the thermostat for 2 hours at 37°, showed no change in appearance under the conditions we employed, there can be no possibility of bacterial action.

EXPERIMENTAL.

In the quantitative determination of the decomposition products of nicotine, produced by the enzyme action of macerates and expressed juices of dried and fermented tobacco leaves, the volatile non-nicotine nitrogen which is produced under their influence during 2 hours at 37° at neutral reaction was estimated by the method described in the previous article. The materials necessary for this were of the following description.

As substrate we made use of a solution of nicotine (Nikotin, pur, Merck) in water, of which the titre towards sodium alizarinsulphonate as indicator was determined. For the calculation, the figure thus obtained has to be multiplied by two, since only one nitrogen atom can be titrated, whilst in the decomposition of the nicotine, both N atoms of the nicotine molecule become converted into volatile non-nicotine nitrogen.

The enzyme extracts were prepared as follows:

(a) Expressing the juice from freshly gathered and finely chopped tobacco leaves (obtained from the Agricultural School at Mikveh Israel, near Jaffa)¹ and diluting it ten times.

¹ For which we have to thank the Director, Mr Krause.

(b) Preparing extracts from finely powdered dried green tobacco leaves of the same variety as in (a); the drying took place in a vacuum drying oven at about 30°.

(c) Making macerates from slowly dried powdered tobacco leaves, of a brown colour, as they were supplied from the stores of Messrs Lubliner at Rosh-Pinah in Upper Galilee¹.

(d) Preparing macerates from finely cut cured tobacco as used by Messrs Maspero in their cigarette manufactory.

The macerates were prepared by allowing 10 g. of the tobacco together with ten times the quantity of water to stand at room temperature for 6 hours and then filtering.

I. EXPERIMENTS WITH THE EXPRESSED JUICES.

1. *Peptolytic ferment.*

In order to detect effects due to peptolytic ferments 5 cc. of the juice after neutralisation were treated with 10 cc. peptone and 5 cc. disodium hydrogen phosphate as buffer.

The amino N titration was carried out by Sørensen's method and the following values were obtained:

$$t_0 = 13.85, \quad t_2 = 14.7, \quad t_4 = 14.8.$$

The control test showed no change.

It is thus evident that a progressive, although slow, hydrolysis of the peptone occurs.

2. *Nicotine-splitting ferment.*

(a) The following solution was used:

5 cc. nicotine (= 6.1 cc. $N/10$ H_2SO_4) + 5 cc. expressed juice + 6.1 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine showed a titre of 26 cc. $N/10$ H_2SO_4 corresponding to the content of volatile non-nicotine nitrogen.

(b) In this case the following mixture was used:

5 cc. nicotine (= 16.2 cc. $N/10$ H_2SO_4) + 20 cc. expressed juice (made neutral to litmus)
+ 16.2 cc. $N/10$ H_2SO_4 (for neutralisation).

The solution was made up to 100 cc. and 20 cc. of it taken for titration.

Calculated quantity of nicotine = 3.24 cc. $N/10$ H_2SO_4 . Non-nicotine N in the filtrate from the nicotine precipitate = 2.6 cc. $N/10$ H_2SO_4 .

(c) 5 cc. expressed juice contain a quantity of

nicotine + non-nicotine N corresponding to 0.28 cc. $N/10$ H_2SO_4 .

Hence, even if the total alkalinity of the juice should be considered as corresponding to non-nicotine nitrogen, there are still in the distillates, after precipitating the nicotine, non-nicotine bases originating from the nicotine corresponding to (2.6 - 0.28) cc. = 2.32 cc. $N/10$ H_2SO_4 .

¹ We have to acknowledge our obligations to the Directors of the firm of Lubliner, and especially Dr Zlocisti, for their valuable assistance.

The extent to which the process goes on may be calculated as follows:

We commenced with a quantity of nicotine corresponding to titre value of 3.24 cc. $N/10$ H_2SO_4 . This means a total N content corresponding to 6.48 cc. $N/10$ H_2SO_4 .

From this there resulted nitrogen products, in the form of volatile bases, corresponding to a titre of 2.32 cc. $N/10$ H_2SO_4 .

The process therefore occurs to the extent of 35 %.

II. EXPERIMENTS WITH THE MACERATES.

3. Nicotine-splitting ferment in macerates from dried, brown, tobacco.

The following solution was used:

5 cc. nicotine (= 15 cc. $N/10$ HCl) + 5 cc. macerate + 15 cc. $N/10$ H_2SO_4 (for neutralisation).

The mixture was made up to 50 cc. and 20 cc. of this taken for the experiment.

Calculated quantity of nicotine...	...	...	...	= 6	cc. $N/10$ H_2SO_4
Non-nicotine N in the distillate after precipitating the nicotine				= 1.9	cc. $N/10$ H_2SO_4

2 cc. of the maceration liquid contain:

(Nicotine + non-nicotine) N	...	...	...	= 0.53	cc. $N/10$ H_2SO_4
∴ Volatile non-nicotine N arising from nicotine...	...	...	...	= 1.37	cc. $N/10$ H_2SO_4

From a solution of nicotine corresponding to a titre of 6 cc. $N/10$ H_2SO_4 and therefore containing total N equivalent to 12 cc. $N/10$ H_2SO_4 , there was thus produced a quantity of non-nicotine bases equivalent to 1.37 cc. $N/10$ H_2SO_4 ; hence decomposition must have occurred to the extent of 11.4 %.

4. Comparative investigation of the nicotine-splitting ferments in the macerates obtained from unfermented, dry, green and brown tobacco, and from dried, cured tobacco.

(a) Dried green tobacco leaves (unfermented).

10 g. of dry green tobacco leaf were treated for 6 hours at room temperature with 100 cc. water and then filtered.

20 cc. of the macerate contained:

Nicotine N = 1.5 cc. $N/10$ H_2SO_4 ; non-nicotine N = 1.3 cc. $N/10$ H_2SO_4 .

For the purpose of the test a solution was made up containing:

10 cc. macerate as above + 5 cc. nicotine (= 3 cc. $N/10$ H_2SO_4)
+ 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine contained non-nicotine N corresponding to 1.9 cc. $N/10$ H_2SO_4 .

The balance-sheet of the decomposition of the nicotine is therefore as follows:

	cc. $N/10$ H_2SO_4
Nicotine in the macerate	0.75
Nicotine in the substrate	3.00
Calculated total nicotine	3.75
Non-nicotine N in the macerate	0.65
Non-nicotine N in the substrate + macerate	1.9
Non-nicotine N of substrate, by difference	1.25

Since from a solution of nicotine, equivalent to 3.75 cc. $N/10$ H_2SO_4 , which therefore actually contains nicotine N equivalent to 7.5 cc. $N/10$ H_2SO_4 , there was produced non-nicotine N corresponding to 1.25 cc. $N/10$ H_2SO_4 , the process has gone on to the extent of 16.7 %.

(b) *Dried brown tobacco leaf* (unfermented).

10 g. tobacco were treated with 100 cc. water and then filtered.

20 cc. of macerate contained:

Nicotine N = 1.2 cc. $N/10$ H_2SO_4 ; non-nicotine N = 1.8 cc. $N/10$ H_2SO_4 .

For the test the following solution was used:

10 cc. macerate as above + 5 cc. nicotine (= 3 cc. $N/10$ H_2SO_4)
+ 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate, after precipitation of the nicotine, contained non-nicotine N = 3.0 cc. $N/10$ H_2SO_4 .

Thus we have:

			cc. $N/10$ H_2SO_4
Nicotine in the macerate	...	...	= 0.6
Nicotine in the substrate	...	...	= 3.0
Total nicotine	...	...	= 3.6
Non-nicotine N in macerate + substrate			= 3.0
Non-nicotine N in the macerate	...		= 0.9
Non-nicotine N in the substrate	...		= 2.1

Thus, in a solution containing nicotine showing a titre for N of 3.6 cc. $N/10$ H_2SO_4 and therefore actually corresponding to a total N content equivalent to 7.2 cc. there was produced non-nicotine N corresponding to 2.1 cc. $N/10$ H_2SO_4 ; hence the process has gone on to the extent of 29 %.

(c) *Fermented tobacco*.

10 g. of this tobacco were treated for 6 hours at room temperature with 100 cc. water and then filtered.

20 cc. of the macerate contained:

Nicotine N = 2.2 cc. $N/10$ H_2SO_4 ; non-nicotine N = 2.6 cc. $N/10$ H_2SO_4 .

The following solution was made up for the test:

10 cc. macerate as above + 5 cc. nicotine (= 3 cc. $N/10$ H_2SO_4)
+ 3 cc. $N/10$ H_2SO_4 (for neutralisation).

The distillate after precipitation of the nicotine was found to contain non-nicotine N corresponding to 2.0 cc. $N/10$ H_2SO_4 .

The data for the nicotine splitting are:

			cc. $N/10$ H_2SO_4
Nicotine in the macerate	...	...	= 1.1
Nicotine in the substrate	...	...	= 3.0
Total nicotine	...	...	= 4.1
Non-nicotine N in macerate + substrate			= 2.0
Non-nicotine N in macerate	...	...	= 1.3
Non-nicotine N in the substrate	...	...	= 0.7

Since a solution containing nicotine giving a titre for N of 4.1 cc. $N/10$ H_2SO_4 and therefore actually containing total N equivalent to 8.2 cc. gave rise to non-nicotine N corresponding to 0.7 cc. $N/10$ H_2SO_4 , the process has gone on to the extent of $8\frac{1}{2}$ %.

SUMMARY AND DISCUSSION OF RESULTS.

From the experiments described in II. 4. (a)–(c), which were carried out under similar conditions, it appears that the fermentative effect is greater in the case of unfermented tobacco leaves allowed to dry naturally than in the case of tobacco obtained by drying rapidly. Of course the comparison is not absolute, since the latter may still contain not inconsiderable amounts of water. The effectiveness of an equal amount of fermented tobacco in comparison is remarkably slight.

Thus it seems that the greater part of the activity disappears during the fermentation process. These phenomena remind us very strongly of the results which in the meantime have been observed in this Institute in connection with the alteration in polypeptide-splitting activity during the germination process.

Here also the stability of the enzyme system becomes much reduced.

It will be our object in further investigations to study more definitely the identity of the products of decomposition of the nicotine, as also the rôle of the oxidase effect occurring during the process of fermentation.

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