tobacco peroxidase, occur in the fresh as well as in the recently fermented Florida tobacco leaf.[1] The former enzym is, however, much more sensitive to heat than the latter, being killed at from 65° to 66° C. (149° to 151° F.), while the latter is killed only at from 87° to 88° C. (188.6° to 190.4° F.).

Dried tobacco leaf (not cured) was finely pulverized, and 1 gram of the powder left with 20 grams of water for one hour at the ordinary temperature. A part of the filtrate was heated for three minutes to 55° C. (131° F.). To about 2 cc. of this liquid were then added a few drops of tincture of guaiac,[2] whereupon a blue coloration appeared in a few minutes, exactly as in the control case. A second portion was now heated to 65° C. (149° F.) for three minutes, and the test applied after cooling, but only a slight trace of a blue color was noticed after ten minutes. Evidently most of the tobacco oxidase was destroyed at that temperature.

The killing temperature of the tobacco peroxidase was determined in a similar manner. However, here reaction is still obtained with great intensity after the solution is heated for three minutes to 80° C. (176° F.), but very feebly after heating for one minute to 87° C. (188° F.).

Another reaction for oxidizing enzyms is the so-called indophenol reaction, consisting in the production of a blue color when an alkaline solution of α naphthol with paraphenyleudiamine is acted upon by an oxidase. This reaction must, however, quickly set in and with great intensity, otherwise no reliable conclusion can be drawn. Cured and fermented tobacco from Florida did not show this reaction in a marked manner,[3] but it set in at once upon the addition of a little peroxide of hydrogen. The latter alone will not produce this result in the absence of an oxidizing enzym.

In the manner above mentioned the writer's investigations have shown that dark tobacco two years old, from Quincy, Fla., yielded no reaction for tobacco oxidase, but still a moderate one for tobacco peroxidase, while a sample of light-colored tobacco four years old from the same source yielded not the slightest reaction either for the oxidase or the peroxidase. Evidently these enzyms themselves are gradually changed. From these observations it may be inferred that the cold sweat, or after-fermentation, might thus proceed for about two years and end by the gradual dying off of the oxidase and peroxidase.

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[1] French savants were the first to call attention to this difference between the oxidizing enzyms. The names oxidase and peroxidase, proposed by a French savant, are not specific names, but group names. There may exist among various oxidases and peroxidases as many differences as there are among protein bodies. Hence it is entirely unjustifiable, at this stage of our knowledge, to introduce one specific name for all peroxidases, as one author has done.

[2] In all these cases freshly prepared guaiac tincture (1:50) was employed, as old guaiac tincture is unreliable and with peroxide of hydrogen alone will sometimes yield a greenish coloration.

[3] Only a slow and weak reaction was thus developed.

When the fresh leaf of the tobacco is rapidly dried at about 60° C. (140° F.) and then moistened again and kept in a moist atmosphere, the veins and their finest ramifications turn brown in about half an hour, while the mesophyll and epidermal cells remain green even after a week. Further investigations on this point will be made later. In the fresh leaf, both oxidizing enzyms, the oxidase and the peroxidase occur in the ribs and veins as well as in the parenchyma, the indications being that they are more abundant in the ribs than in the parenchyma. The bundle sheath and sieve tissue give the most intense reaction on the oxidase, while the reaction on the peroxidase sets in quickly and with about uniform intensity in all the cellular tissues. The growing point and youngest leaves contain an especially large quantity of the oxidase. A section through the stalk shows oxidase only in the sieve tissue and bast parenchyma, while peroxidase also is contained in the pith.[1] Both enzyms are found in the root, the former more in the central and the latter in the peripheral parts and also in the flower. The stigma of the pistil and the stigmatic fluid also show strong reaction upon oxidase.

The two oxidizing enzyms are also contained in the young tobacco plants. Several dozen of these, measuring on an average not more than 3 to 4 cm. from the tip of the root to the plumula, were rubbed in a mortar with a little water and some sand. The filtrate gave a very intense reaction for oxidase,[2] and after this was destroyed by warming to 70° C. an intense reaction for peroxidase also.

A colorless clear solution of the tobacco peroxidase can be obtained in the following manner: A number of fresh tobacco leaves are well crushed in a mortar, with the addition of sand and some dilute alcohol of 30 per cent. This mixture is pressed and the turbid liquid directly mixed with three times its bulk of strong alcohol. After standing two hours the mixture is thrown upon a filter and the filter contents, after being washed with some alcohol, extracted with about four to six times its bulk of water at the ordinary temperature, heated for a minute to 70° C. (158° F.), and filtered. This clear, colorless filtrate gives no indication of the oxidase, but a very intense reaction for peroxidase. When this solution is compared with that of the juice of fresh tobacco leaves it is easy to decide what result is caused by the oxidase alone.

To determine whether the tobacco oxidase bears more resemblance to the tyrosinase or the laccase a few drops of freshly expressed juice of normal tobacco leaf were added to 2 cc. of a cold saturated tyrosin solution, but even after four hours no characteristic darkening of the

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[1] In order to observe the localization of the peroxidase small pieces of the tissue are treated with strong, but not absolute, alcohol for three minutes at 70° C. (158° F.). Thus the oxidase is killed and can not interfere with the tests for the peroxidase.

[2] The indophenol reaction did not turn out satisfactorily, only a weak violet-blue color resulting. On the addition of hydrogen peroxide, however, an intense blue reaction was at once obtained.

mixture was observed. In this regard, therefore, that oxidase would resemble laccase more than it would tyrosinase.[1]

To extract oxidases from fermented or cured tobacco as completely as possible it is necessary to thoroughly pulverize the samples and to let the water act for some time at from 20° to 30° C. (68° to 86° F.) before filtering. After complete drying, the samples can be easily pulverized very fine. The following experiment proves that when the tissue is not pulverized the peroxidase is but very imperfectly extracted, the passage through the cellular walls being quite slow. Fermented tobacco leaves were three times soaked in water and the brown liquid pressed out, the first soaking lasting half an hour and the second and third soakings five minutes each. Although the sample was thus nearly exhausted, it nevertheless yielded, when left with some alcohol of 30 per cent for one day, a light-colored liquid with a very intense reaction for peroxidase.

It may safely be assumed that in the majority of instances the oxidase will prove the more energetic of the two oxidizing enzyms. For example, its action upon pyrocatequol and hydroquinone is much more energetic than that of the peroxidase. On the other hand, however, the former succumbs much more quickly to noxious influences, e. g., the action of alcohol or rising temperature.

The fact that the peroxidase forms guaiac blue from guaiaconic acid with the aid of hydrogen peroxide only does not indicate that its oxidizing action in every case depends upon the presence of the latter.[2] The peroxidase can, on the contrary, also exert oxidizing action upon various compounds without the assistance of hydrogen peroxide. Thus, para-amidophenol is gradually changed by it to a dark brown substance.

Hydroquinone in dilute solution gradually assumes a reddish color in the presence of the peroxidase, but in its absence there is scarcely a trace of coloration within twenty-four hours.

Pyrocatechol is scarcely attacked by the peroxidase within twenty-four hours, but on a further addition of a little hydrogen peroxide it turns to a dark brown in five minutes. Hydrogen peroxide added alone does not act thus.

Pyrogallol is slowly attacked by the peroxidase and turns brown in twenty-four hours. The oxidase acts also in this case much more energetically than the peroxidase.

Tannin solution shows in twenty-four hours a yellow color in the presence of the peroxidase, but in the control solution merely a slight

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[1] The peroxidase of tobacco on the other hand bears resemblance to the peroxidase of pus described by Linossier.

[2] The hydrogen peroxide is decomposed by enzyms into water and oxygen, but this oxygen in *status nascens* is charged with more chemical energy than the free oxygen of the air, i. e., the two atoms constituting the molecule are for a time in a more energetic motion than in the latter case, hence the action of the oxidizing enzyms is facilitated by this nascent oxygen.

trace of coloration is perceptible. The addition of a little hydrogen peroxide to both will increase the difference of coloration still more.

All these tests were made at from 18° to 20° C. (64.4° to 68° F.). A number of other compounds were also tested, such as arbutin, guaiacol, and toluidine, but no decisive reaction was obtained within twenty-four hours at the ordinary temperature.

There may exist great differences in the amount of tobacco oxidase and tobacco peroxidase produced in different varieties of the tobacco plant and under different conditions. The quantity of each may even differ in the upper leaves fully exposed to the sun and the lower leaves growing mostly in the shade. There may also be formed compounds in certain varieties of tobacco that will more quickly destroy the enzyms during curing, or fermentation, than in other varieties. Thus considerable difference was noticed in comparing a sample of tobacco from Connecticut with one from Florida. In the fermentation of the former the tobacco peroxidase was almost completely destroyed, while in that of the latter a considerable part was still intact. Moreover, neither the fermented nor the cured Connecticut leaf contained any tobacco oxidase, although it was found in a greenhouse specimen of the fresh leaf.

It is interesting to note that the best way of bringing the oxidizing enzym to the fullest action possible is that practiced in the curing of the Perique tobacco.[1] The rolls, or twists, of the tobacco leaves are subjected to a pressure of about 7,000 pounds per square foot to bring the juice from the interior of the cells to the surface. After twenty-four hours the tobacco is taken out and aired a few minutes, which causes a darkening to set in. In this way the juice is reabsorbed by the tissues, whereupon the pressure is again applied. This operation is repeated daily for ten consecutive days, and at longer intervals thereafter. A very dark product is thus obtained, but it is not strong, as the oxidation of the nicotine has been carried very far.

One of the most interesting features of the sweating of tobacco is the destruction of a part of the nicotine, this part yielding up its nitrogen probably as ammonia, which is indeed a product of the sweating. It was, of course, of importance to prove that the oxidizing enzyms contained in the tobacco leaf can decompose nicotine, and for this purpose 50 grams of cured tobacco from Connecticut which had not yet been subjected to fermentation and showed a strong reaction for peroxidase, but none for oxidase, was thoroughly moistened with water. After two hours 250 cc. of alcohol of 50 per cent was added and the mixture allowed to stand for two days. The liquid obtained by pressing was now mixed with one and a half times its volume of absolute alcohol and the brown-colored precipitate washed upon the filter with some alcohol. After pressing between filter paper, the precipitate, containing a large proportion of the peroxidase, was dissolved

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[1] Farmers' Bull. No. 60, U. S. Dept. of Agr.

in 20 cc. of water and 0.5 gram of nicotine tartrate added. This mixture was digested for two days at from 50° to 60° C. (122° to 140° F.) in a large flask, holding about 500 cc. of air, to enable oxidation to go on. An addition of a small amount of thymol prevented bacterial growth. A small U tube, holding 10 cc. of dilute chemically pure sulphuric acid of 0.2 per cent, was attached to the flask. The examination of this acid after two days with Nessler's reagent indicated that ammonia was present, but the colorimetric comparison showed that the amount was hardly more than 0.1 milligram. However, the amount of ammonia formed but not volatilized was much larger, as indicated by the strong reaction obtained after the addition of a little potassium carbonate to the mixture and warming for a short time in order to liberate the ammonia in the form of carbonate from other less volatile salts.[1] Thus there can be no doubt that the tobacco peroxidase can attack nicotine with formation of ammonia, but this process is exceedingly slow. Indeed, the sweating, lasting fully eight weeks, can diminish the nicotine content on an average only by about one-third.[2]

What the products of destruction of nicotine are besides ammonia can be determined only when the purified enzyms and a pure nicotine salt serve in large quantities for the experiment. It may be mentioned, however, that the writer has examined in vain an aqueous extract of fermented tobacco for nicotyrin and nicotinic acid—known oxidation products of nicotine.

The writer has now fully established the presence of oxidizing enzyms in the tobacco leaf.[3] That such enzyms can exert a powerful action upon certain compounds, leading even to the formation of carbonic acid, is known.[4] Oxidations produce heat, hence it can safely be inferred that the so-called tobacco *fermentation* consists in the activity of oxidases, while the *curing* of tobacco consists in the combined work of oxidases, diastase, and peptase. As the use of the term "fermentation" might lead in this case to an entirely erroneous conception, the writer proposes "oxidizing enzymosis" or "oxidizing enzymation" as correct scientific designations.

There has already been mentioned an interesting case of oxidase action in a technical branch, viz, the preparation of the Japanese lac. Furthermore, in the manufacture of the natural indigo bacteria are not concerned (Molisch), but simply an oxidizing enzym (Bréaudat).

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[1] The oxidase might have exerted a more powerful action on the nicotine than the peroxidase.

[2] A control experiment was made with a colorless peroxidase solution (p. 29) upon highly diluted free nicotine at the ordinary temperature, in order to observe whether a brown solution is produced by a change of the nicotine, but the mixture remained colorless after one day under these conditions. However, it may be mentioned that nicotine, when exposed a long time to air and light, will turn brown.

[3] He has already pointed out (p. 15) that there is sufficient access of air possible to enable oxidation in the tobacco piles.

[4] The further inference is certainly justified that certain basic compounds might thus give up their nitrogen in the form of ammonia.

Another instance where an oxidizing enzym plays a part in a technical branch is the "fermentation" of the olive, which is practiced in certain parts of Italy. It is believed that by this operation an oil of superior quality is obtained and that the yield by pressure is larger, but this has not been confirmed. It has been shown by Tolomei[1] that this olive "fermentation" is due to the action of an oxidizing enzym, to which also is due the fact that olive oil is bleachable by sunlight.

When the freshly gathered olives are kept in sacks their temperature gradually rises far above that of the rooms, oxygen is absorbed, and carbonic, acetic, and sebacic acids, and small quantities of the higher volatile fatty acids are formed. This process goes on to a larger extent when a temperature of 35° C. (95° F.) is reached. These changes do not occur if the olives are kept in nitrogen or carbonic acid gas; neither do they occur when the olives have been heated to 75° C. (167° F.) for forty-five minutes. For obvious reasons the spontaneous rising of temperature is noticed only when a large number of olives are kept together. Tolomei showed that the oxidase extracted with water and purified by a repeated precipitation with alcohol produces guaiac blue from guaiac tincture, forms purpurogallol from pyrogallol without the aid of peroxide of hydrogen, quinhydrone from hydroquinone, and a brown substance from gallic acid. He calls this oxidizing enzym olease. As unripe olives do not contain this olease, the oil pressed from them will not bleach upon exposure to the sunlight, but will do so after being shaken with an aqueous extract of the ripe olives. On the other hand, olive oil will sooner acquire rancidity in the presence of the olease than when free from it.

Finally, still another case may be pointed out where oxidases might possibly play a part—that is, in the so-called fermentation of the cacao beans, by which a bitter principle is destroyed.

SUMMARY.

(1) The so-called tobacco fermentation is not caused by bacteria.

(2) The amount of water present in normally fermenting tobacco leaves is insufficient to bring nourishment for the microbes from the interior of the cells to the surface of the leaves. It barely suffices for imbibition of the organic matter.

(3) There exists no bacterial coating on the fermenting tobacco leaves under normal conditions, but some spores may occur.

(4) In the so-called petuning of tobacco an immense number of bacteria may be transferred to the leaves. These bacteria, however, are not essential for the fermentation, but on the contrary, may prove noxious as soon as a small surplus of water enables them to further develop.

(5) Suchsland's theory that the aroma of tobacco is caused by specific bacteria is incorrect.

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[1] Atti della Reale Accademia dei Lincei, 1896, p. 122.

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