THE ISOLATION OF TRIMETHYLAMINE FROM SPORES OF TILLETIA LEVIS, THE STINKING SMUT OF WHEAT*

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Bunt, or stinking smut of wheat, is of common occurrence in most regions where wheat is grown extensively. Infection by the causal fungus takes place while the wheat plant is in the seedling condition; subsequently, the fungous hyphæ permeate the growing plant and eventually invade the young kernels, converting them into masses of dark brown spores. In the process of threshing, many of these infected kernels, or smut balls, are broken open and the spores which they contain are dusted over the surface of the sound grain. Wheat contaminated in this way is slightly dark in color and usually sells at an appreciable discount. The smut spores may also, although not always, impart to the grain a disagreeable fishy odor resembling herring brine. This odor is suggestive of trimethylamine, but, so far as the writers are aware, the isolation of this substance from bunt spores has not been accomplished. It is with this problem that the present paper deals.

The stinking smut disease may be caused by one of two fungi, *Tilletia levis* or *Tiletia tritici*. These two species, although closely related, may be distinguished from one another by the appearance of their spore walls, the spores of *Tilletia levis* being smooth, whereas those of *Tilletia tritici* are reticulate. In the

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experience of one of the authors the production of the fishy odor already referred to is associated only with the spores of *Tilletia levis*. The two species have been grown on a number of wheat varieties, both in the greenhouse and in the field, and the spores of *Tilletia tritici* were invariably free from this odor. The experiments recorded in this paper, although somewhat incomplete, tend to confirm this observation.

Materials

The spores used in the present experiments were collected from wheat grown in 1931 on the plots of the Dominion Rust Research Laboratory at Winnipeg, Canada. One set of plots, comprising the varieties Little Club, Kota, Reward, and Ceres, was grown from seed which had been artificially inoculated with spores of *Tilletia levis*. Another set of plots of the same wheat varieties was grown from seed inoculated with spores of *Tilletia tritici*. Some additional collections of *Tilletia levis* were made from plots of Marquis, Marquillo, and Garnet wheat grown from artificially inoculated seed.

At harvest time a separate collection of infected heads was made from each plot. The smut balls were then removed from the heads and stored in paper bags in the laboratory until the end of February, 1932, when each lot was crushed in a mortar and the spores were sifted through a 200 mesh sieve. The material passing through the sieve consisted almost wholly of smut spores and represented approximately 75 per cent, by weight, of the smut balls.

Determinations were made of the total nitrogen in spores of *Tilletia levis* and *Tilletia tritici* collected on Ceres wheat. The spores of *Tilletia levis* were found to contain 4.51 per cent nitrogen and those of *Tilletia tritici* 4.41 per cent.

Determination of Total Volatile Base and of Ammonia

Preliminary experiments indicated that ammonia is evolved when an aqueous suspension of bunt spores is distilled under reduced pressure in the presence of barium hydroxide. It was therefore apparent that, if trimethylamine were the substance responsible for the characteristic odor of the spores, the amount recoverable from a given weight of spores could not be in excess of that repre-

sented by the difference between the total volatile base and the ammonia present in the distillate. With this consideration in view determinations were made of the total base and of the ammonia obtained on distillation from an equal weight of each collection of spores. The following procedure was adopted.

10 gm. of spores, together with 100 cc. of distilled water and 10 cc. of N sulfuric acid, were placed in a 2 liter Claissen flask. The mixture was heated and kept boiling for 3 minutes, after which it was cooled rapidly under the tap; 150 cc. of ethyl alcohol and 70 cc. of saturated barium hydroxide were added to the flask, and the mixture was distilled at about 60° under reduced pressure. Distillation was continued for 35 minutes from the time boiling began, the distillate being collected in 20 cc. of 0.1 N hydrochloric acid to which had been added 2 drops of methyl red. The total volatile base obtained from the spores was then determined by titration of the distillate with 0.1 N sodium hydroxide.

The neutral distillate was acidified with a few drops of 0.1 N hydrochloric acid, and the alcohol and most of the water were removed from it by distillation. The residue was made up to 100 cc. The ammonia in portions of this solution was then determined by Nessler's method. That this method can be employed for the determination of ammonia in the presence of trimethylamine has been shown by Vickery and Pucher.[1]

The results of the determinations are summarized in Table I, each value being the mean of two experiments. Only small collections of *Tilletia levis* spores were made from Marquis, Marquillo, and Garnet wheat, consequently the quantity of these spores used for each determination had to be reduced to duplicate 5 gm. portions. For purposes of comparison similar determinations were made with spores of *Ustilago tritici*, the loose smut of wheat; these results have also been included in Table I. In the calculation of the data no allowance was made for the moisture content of the spores. However, spores of *Tilletia levis* and *Tilletia tritici* collected on Ceres wheat were dried to constant weight over concentrated sulfuric acid in a vacuum desiccator; the loss in weight was found to be 4.6 and 4.7 per cent respectively. It is probable, therefore, that, after having been stored in the laboratory for several months, all of the spore collections had reached

[1] Vickery, H. B., and Pucher, G. W., *J. Biol. Chem.*, **83**, 1 (1929).

moisture equilibrium, and a recalculation of the results on a moisture-free basis would not appreciably affect any conclusions which might be drawn from them.

The data indicate that two collections of smut spores belonging to the same species, and similar in appearance, but grown on different varieties of wheat, may yield widely different amounts of ammonia. It is therefore apparent that the host plant plays an important rôle in determining the chemical composition of the smut spores which develop upon it. There is also some indication that spores which develop on high quality wheats, rich in protein,

TABLE I

Volatile Bases Obtained from Spores of Tilletia levis, Tilletia tritici, and Ustilago tritici

Wheat variety	Smut	Weight of spores	Total volatile base N	Ammonia N	Difference
		gm.	*mg.*	*mg.*	*mg.*
Little Club	*Tilletia levis*	10	5.22	4.44	0.78
" "	" *tritici*	10	4.72	4.58	0.14
Kota	" *levis*	10	5.96	5.24	0.72
"	" *tritici*	10	5.01	4.85	0.16
Reward	" *levis*	10	12.20	11.74	0.46
"	" *tritici*	10	11.74	11.88	
Ceres	" *levis*	10	8.43	7.73	0.70
"	" *tritici*	10	7.66	7.11	0.55
Marquis	" *levis*	5	5.71	5.20	0.51
Marquillo	" "	5	7.19	6.74	0.45
Garnet	" "	5	3.74	3.54	0.20
Several	*Ustilago tritici*	5	2.02	1.92	0.10

have a higher ammonia content than do those that develop on low quality wheat.

The spores of *Tilletia levis* contain significantly greater amounts of total volatile base than those of *Tilletia tritici* growing on the same wheat variety. Furthermore, the difference between total base and ammonia tends to be higher in spores of *Tilletia levis* than in those of *Tilletia tritici*. It will be shown later that the spores of *Tilletia levis* actually contain small quantities of trimethylamine, the substance responsible for the characteristic odor of bunt spores. If the difference between total base and ammonia be considered as a rough measure of the amount of trimethylamine

present in the spores, it would appear probable that the spores of *Tilletia tritici* contain very little, if any, of this substance. Judged by the chemical evidence presented in Table I, the only collection of *Tilletia tritici* spores which might be expected to contain trimethylamine is that grown on the variety Ceres. The distillate collected from 10 gm. of these spores was evaporated to dryness and extracted with chloroform. The chloroform extract was then dried, and a few drops of 0.1 N sodium hydroxide were added to it, but no odor of trimethylamine could be detected.

The *Tilletia tritici* spores collected on Little Club, Kota, and Reward wheat gave very small differences between the total volatile base and the ammonia, the values being of about the same order of magnitude as that obtained for *Ustilago tritici*, the loose smut of wheat, the spores of which lack any fishy odor. The experimental evidence available, therefore, seems to support the observation already mentioned, that the spores of *Tilletia levis* alone liberate the characteristic odor usually attributed to bunt spores.

It has been shown by several workers that *Tilletia tritici* comprises several physiologic forms which differ from one another in their parasitic capabilities. The possibility exists that certain of these forms may be able to produce trimethylamine, whereas others may not. The spores of *Tilletia tritici* used in this investigation were considered to belong to a single physiologic form. However, if the hypothesis just suggested is correct, other forms of this fungus may occur, the spores of which contain trimethylamine.

Isolation of Trimethylamine

The acidified *Tilletia levis* distillates not required for ammonia determinations were collected in a flask to which a few drops of toluene had been added. The total distillate, representing 130 gm. of spores, was evaporated under reduced pressure. The residue was taken up with a little water, made alkaline with 20 cc. of concentrated sodium hydroxide, and distilled into 10 cc. of N hydrochloric acid. This procedure removed the methyl red used in titrating the total volatile base. The distillate was then evaporated to dryness under reduced pressure. The residue was transferred with a little water to a small beaker, was evaporated on the steam bath, and finally dried in a vacuum desiccator over con-

centrated sulfuric acid. A few sticks of sodium hydroxide were placed in the desiccator to remove traces of hydrochloric acid present in the residue.

The dried residue was extracted with hot chloroform. This extract was filtered, evaporated, dried in a vacuum desiccator, and weighed. The dried material was dissolved again in hot chloroform, filtered, evaporated, and redissolved in absolute alcohol. A small excess over the calculated quantity of a concentrated chloroplatinic acid solution was added to the alcoholic solution. The yellow precipitate produced was filtered off, washed with absolute alcohol, and redissolved in water. The aqueous solution was filtered, evaporated, and cooled. The orange-colored crystals which separated were filtered off, and redissolved in water. Final crystallization was effected by the addition of absolute alcohol to this solution. The mother liquor was concentrated, and two further small crops of crystals were obtained.

Micro analyses[2] of the first crop of crystals gave the following results: C 13.93, 13.92; H 3.82, 4.15 per cent. These values are in reasonably close agreement with those required by theory for trimethylamine chloroplatinate, which are C 13.63, H 3.82 per cent.

The difference between the total volatile base and the ammonia present in the distillate collected from the 130 gm. of spores was equivalent to 9.4 mg. of nitrogen. If ammonia and trimethylamine were the only two substances present, this weight of nitrogen would correspond to 40 mg. of trimethylamine. It is probable, however, that a closer approximation to the trimethylamine content of the spores is given by the weight of the dried hydrochloride obtained from the first extraction with chloroform, which was 25.4 mg., corresponding to 15.7 mg. of trimethylamine. The weight of crystalline trimethylamine chloroplatinate obtained was 21.1 mg., which represents 4.7 mg. of trimethylamine. Consequently 100 gm. of *Tilletia levis* spores might be expected to contain between 3.6 and 12 mg. of trimethylamine.

It should be recalled that the spores of *Tilletia levis* used in these experiments had been kept in the laboratory for several months before being used. When freshly collected they had a distinct fishy odor but, by the time the experiments were begun, this

[2] Analysis by Research Service Laboratories, New York.

odor had practically disappeared. This change indicated that the spores might be sufficiently alkaline in reaction to liberate trimethylamine. The hydrogen ion concentration of the different lots of spores was therefore determined, and is given in Table II. The spores of *Tilletia levis*, with the exception of those grown on the variety Ceres, are slightly more alkaline than those of *Tilletia tritici*. All of the collections of spores, however, have reactions sufficiently near the neutral point to permit the gradual liberation of both trimethylamine and ammonia. Had freshly collected spores of *Tilletia levis* been available for the isolation of trimethylamine it is probable that a higher yield of this substance would have been obtained.

TABLE II

Reactions of Water Suspensions of Wheat Smut Spores as Determined by Quinhydrone Electrode

Wheat variety	Smut	Reaction
		pH
Little Club	*Tilletia levis*	6.23
" "	" *tritici*	6.11
Kota	" *levis*	6.20
"	" *tritici*	6.08
Reward	" *levis*	6.25
"	" *tritici*	6.15
Ceres	" *levis*	6.28
"	" *tritici*	6.28
Several	*Ustilago* "	5.96

SUMMARY

The amount of total volatile base and ammonia obtainable from a collection of *Tilletia levis* or *Tilletia tritici* spores depends upon the wheat variety on which the spores have developed. The ammonia content of the spores varied from 54 to 143 mg. per 100 gm. of spores.

Distillates from *Tilletia levis* spores have a higher content of total volatile base and show greater differences between total volatile base and ammonia than do those from *Tilletia tritici* spores.

Freshly collected spores of *Tilletia levis* emit an odor resembling that of herring brine. Trimethylamine was isolated from these spores, and it is considered to be the substance responsible for

their odor. The trimethylamine content of these spores was probably between 3.6 and 12 mg. per 100 gm. of spores.

Spores of *Tilletia tritici*, even when freshly collected, did not emit the odor of trimethylamine and none of this substance could be detected in distillates obtained from these spores.