SCIENTIFIC SECTION, AMERICAN PHARMACEUTICAL ASSOCIATION

ON THE DETECTION OF MOLD IN DRUGS, FOODS, AND SPICES.*

With Special Reference to a Specific Stain.

BY ARNO VIEHOEVER.

It is noteworthy that even in recent books dealing with the microscopy of drugs, foods or spices, the subject of molds and their detection has scarcely, if at all, been discussed.

As commonly understood, the term "mold" refers to certain types of lower vegetable organisms, botanically called "fungi." The best known of these types are the black molds or mucor and the green and yellow molds or Penicillium and Aspergillus species.[1]

While in the manufacturing process of some food products such as Roquefort and Camembert cheeses the presence of certain molds is desired, in most instances the occurrence of molds in foods and drugs is not wanted. Molds usually not only spoil the appearance of the foods and act repellant to the taste, but also change their appearance and their physical and chemical properties.

As long as the products are in their natural state, or nearly so, it is usually comparatively easy to detect either the mold or the change that has taken place through its growth. This is, however, different, when the condition of the products has been changed through grinding or some other manufacturing process, such as cutting, mixing, boiling or roasting. In this condition it will usually be impossible to detect the mold with the naked eye, and depending on the manufacturing process used, it will often be impossible to detect with certainty any change in color or flavor due to the mold growth in the original product.

The difficulty of detecting the mold in the manufactured product has often encouraged and still encourages the utilization of moldy fruits, vegetables, drugs and spices in the manufacture of either prepared food products or powdered drugs and medicinal preparations.

METHODS FOR THE DETECTION OF MOLD.

There are three ways open for the analyst to detect the hidden mold:

1. *The Cultural Method.*—Mold may be grown by exposing parts of the material suspected of infection by mold under favorable conditions of temperature and humidity. If accidental infection is prevented by very careful sterile manipulation in which the adhering mold fragments are removed by flaming the outside of the material one may obtain excellent results with this method, provided the mold hyphae or spores have not been killed, by the manufacturing process or storage. In this method a number of parallel experiments should be undertaken as well as such in which the conditions of cultivating are varied in order to make sure that a number of other molds possibly present will not be suppressed in their growth. It should be remembered that it takes several days to obtain a result

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and that this method only works satisfactorily in the hands of an analyst trained along this line. No accurate information as to the extent of the mold infection can properly be expected with this method.

2. *Microscopic Examination of the Material not Especially Treated or Stained.*—Where the analyst wants a quick result, or where he wants to support his findings obtained with the first method, a direct examination under the microscope often is of value. If the material is not changed too much through the manufacturing process or through storage and decomposition, it may be possible in many cases to detect the presence of mold. It may even be possible to determine approximately the extent of the mold contamination by mounting and examining a certain definite dilution of the suspended material in a Zeiss counting chamber, such as is used for counting blood corpuscles. This method works best only in the hands of a well-trained microscopist familiar with the microscopy of plant products as well as molds. It is used by the Bureau of Chemistry in examining tomato pulp and tomato ketchup.[2]

3. *Microscopic Examination of the Material Especially Treated and Specifically Stained.*—While staining of the microscopic preparation with some of the stains commonly used for vegetable tissues, *e. g.*, methylene blue, methyl violet, etc., will prove of some advantage in the differentiation of plant tissue and mold mycelium, these stains are not specific stains for the cell wall of fungi. van Wisselingh[3] (1898) already pointed out that coloring substances like methylene blue, ruthenium red, brillant blue, and congo red, have no distinct value if one looks for stains to detect chemical substances of definite character. His experiments with another microchemical stain, iodine, followed by sulphuric acid, gave him the desired result, but this stain worked only on material previously treated with concentrated KOH solution. His results will be mentioned later, after a discussion of the work done previously by others on the isolation and identification of the specific substance present in the cell wall of fungi.

OCCURRENCE OF CHITIN IN THE CELL WALL OF FUNGI.

As long ago as 1811 Braconnot[4] had found the presence of a substance different from cellulose in the cell walls of fungi. He isolated from a number of fungi, including mushrooms and mucor septicus, a product similar to cellulose but containing nitrogen, and called it fungin. Fremy[5] (1859) isolated a substance which was, like cellulose, not soluble in copperoxide-ammonia and called it metacellulose. De Bary[6] (1866) called it Pilzzellulose, since the substance isolated from molds and mushrooms was insoluble in copperoxide-ammonia and, unlike cellulose, did not give a blue color with iodine and sulphuric acid. Tschirch[7] (1889) p. 191, recommended the name "mycin" in analogy with lignin and suberin. Gilson[8] (1894) isolated from the common mushroom (*Agaricus campestris*), as well as from ergot, a substance which he called mycosin and which treated with hydrochloric acid gave glucosamin. Winterstein[9] (1894) also isolated glucosamin at the same time from other mushrooms (*Boletus edulis*) or fungi imperfecti (*Botrytis cinerea*). In a later publication Gilson[10] (1895) pointed out that the mycosin isolated by him from fungi was identical with the chitosan isolated by Hoppe-Seyler[11] (1894) from animal tissue. Winterstein[12] (1895) had already, a few months previous to this announcement of Gilson, isolated chitosan from a number of mush-

rooms, mostly edible, and the opinion of Gilson was thus confirmed. Iwanoff[13] (1901) also isolated from *Boletus edulis*, *Aspergillus niger* and ergot the substance which gave all the reactions of the substance found in certain animal tissue, especially of insects, and called chitin. The composition of chitin has often been studied and quite recently by Brach[14] (1912) who reports that complex groups of monoacetylglucosamines form the smallest building stones of chitin ($C_{32}H_{54}N_4O_{21}$). This on treatment with KOH is split into chitosan and part of the acetic acid groups present. The constitution of chitin however is not entirely cleared up.

van Wisselingh[8] (1898) was the first to support the chemical findings of others by microchemical tests. He used the method of Hoppe-Seyler and Gilson, heating the material with KOH to 180° C. and after removal of the KOH colored it with iodin followed by H_2SO_4 in a dilute form, thus staining the chitin converted to chitosan or mycosin. Gilson had previously found the reagent to work better in great dilution.

van Wisselingh tested a great number of fungi at different stages of their development for the presence of chitin and found it generally with only a very few exceptions (the Peronosporaceae and Saprolegniaceae) throughout the group of fungi. He found it in the common molds and confirmed thus the findings of the chemists. Ilkewitsch[15] (1908) on the basis of inadequate experiments came to the conclusion that the fungi contained neither chitin nor cellulose, but a substance closely related, which he called mycetin. Wester[16] (1909) confirmed the results of van Wisselingh, pointing out the errors of Ilkewitsch. Wester extended the search for chitin and found it in a number of new species of fungi.

Viehoever[17] (1912) repeated the experiments and confirmed the presence of chitin in the cell walls of a number of fungi, mentioning especially *Sporodinia grandis* and *Aspergillus glaucus*. Cihlar (see Vouk,[18] 1915) found chitin in the cell walls of a number of fungi belonging to the three main groups, Phycomycetes, Ascomycetes and Basidiomycetes, including common molds and mushrooms.

APPLICATION OF THE CHITIN TEST.

Recently in examining drug products suspected of containing mold, *e. g.*, ground nutmegs, a patent medicine containing a great number of drugs in suspension, and flour containing ergot, it occurred to the writer that the chitin reaction might be applicable to detect the presence of mold or ergot. The experiments were wholly successful and were extended to other drugs like areca nuts and gentian root, spices like ginger and capsicum, and to food products such as moldy lemons, cherries, strawberries, peaches, tomatoes, water-damaged coffee beans, etc.

Further experiments are being carried on in the Bureau of Chemistry to determine not only whether or not all the molds occurring in food or drug products can be detected with this stain, but also if all stages of development of the different molds can be stained.

So far no tissues of higher organized plants have been found to contain chitin. It is believed that the general application of this chitin reaction in the analysis of food and drug products will enable the micro-analyst to detect much more easily the presence, location and nature of a mold. The analyst probably will obtain

more accurate information concerning the amount of mold present. (The writer has already made a number of experiments to determine ergot quantitatively in flour and believes that the results are promising.) He will not have to depend on living mold and will not have to fear a complete destruction of the mold cells through the manufacturing process, since dead mold hyphae and mold cell fragments will naturally show the stain as well.

It is furthermore believed that the stain can be used with advantage in the detection of infection of plants by fungi. The author in fact, used it to prove that caraway fruits which were suspected of being attacked by a fungus, were indeed infected, some very slightly, others quite markedly. The appearance and staining properties of the fungus were very similar to those of ergot, occurring especially on rye. The test was furthermore successfully used to show the infection of wheat grains with smut spores (*Tilletia foetens* (B. C.) Trel.).

The detection of fungi causing skin and other diseases of animal tissue (see Kolle and Wassermann,[19] 1913) may be facilitated if the test is applied to the infected tissue. The reaction may be of help in toxicological cases in deciding if moldy food or mushrooms might have been consumed and caused the intoxication.

OCCURRENCE OF CHITIN IN LICHENS, MYXOMYCETES AND BACTERIA.

Chitin has been found in a number of forms of lichens, which was to be expected since fungi are a part of lichens. This fact may be of use in determining the origin and source of certain barks. *Rhamnus* barks are, for example, characterized by the presence of lichens on the outer surface. Since lichens occur only on the stem bark, that is, on places exposed to light, the reaction might be of value in deciding whether or not a bark is obtained from the root or stem, for example, in the case of pomegranate bark.

van Wisselingh found chitin in the Myxomycete called "Club Root Cabbage" (*Plasmodiophora brassicae*) and succeeded also in staining this slime mold by treating the infected tissue of cabbage.

Chitin has also been found in bacteria, which fact supports the opinion of systematic botanists like Arthur Meyer[20] (1912) that bacteria are closely related to fungi. Ivanoff[13] (1901) obtained chitin from *Bacillus anthracis* and two other bacteria species, and although van Wisselingh (1898[3]–1916[21]), Garbowski[22] (1907) and Wester[16] (1909) could not detect it microchemically, Viehoever[17] (1912) succeeded in locating the chitin microchemically in a number of species of bacteria. The stain, in this case, is sometimes only visible with difficulty. The bacterial membranes moreover are usually not so resistant to KOH as the membrane of mold cells and the treatment has to be modified somewhat.

NATURE OF MOLDS FOUND IN FOODS AND DRUGS.

Those who are interested in knowing what molds have been found in our daily foods may find much information collected in the books of Kossowicz[23] (1911), Stevens[24] (1913) and Conn[25] (1912). A number of recent articles on the subject of fungi affecting food products may be found in the Journal of Agricultural Research, including a paper by Thom and Shaw[26] (1915) dealing with the molds occurring in butter, etc., and one dealing with those occurring in strawberries (Stevens[27] 1916). As far as the writer is aware very little attention has been paid thus far to molds occurring in crude drugs or drug preparations. However,

reference may be made here to the work on the molds causing different kinds of rot of the Ginseng root. (Rosenbaum and Zinnsmeister,[28] 1915, Rosenbaum[29] 1915.) The molds found thus far in our work on drugs and spices and isolated by the Microbiological Laboratory of the Bureau of Chemistry consisted of different species of *Mucor*, *Penicillium* and *Aspergillus*. Of special interest may be that caraway seed and cumin seed were found to be infected by a fungus transforming the fruits to ergot-like sclerotia.

DESCRIPTION OF METHOD.

The test as mentioned above relies on the transformation of chitin to chitosan which is stained in a way specific to chitosan.

TRANSFORMATION OF CHITIN TO CHITOSAN.

The fact that the chitin reaction is so little known and used is undoubtedly due to the inconvenient procedure as originally recommended by van Wisselingh or as modified by Wester. van Wisselingh in a recent manuscript[30] (1915) still recommends the heating in closed glass tubes to 160° C. in a concentrated or 50 percent KOH in oil bath or in a hot air oven. Wester[16] (1909) recommended an especially constructed oil bath in which the material, imbedded in 60 percent KOH and enclosed in tubes of definite size were heated to 160° C. Viehoever[17] (1912) heated the material in 50 percent KOH in small glass tubes in an autoclave under 6 atmospheres pressure (about 164° C.) for a short while using at first closed glass tubes and later open glass tubes and pressures of 2–3 atmospheres (115–120° C.). Experiments were also carried on in which the material was allowed to stand in 50 percent KOH without heating (Viehoever[31] 1913) with the result that Wester's general conclusions were confirmed, indicating that the action of KOH takes place in the cold as well as at high temperatures, but that it takes months at room temperature to complete the transformation of chitin to chitosan. V. Vouk[18] (1915) recommends the heating of the fungi material in boiling saturated KOH for 20 to 30 minutes.

The writer believes that a heating of the material to almost boiling with 40 to 50 percent KOH or NaOH for 40 to 60 minutes will give satisfactory results in most cases and may be conveniently done on an electric plate in a flask, the opening of which is covered with a funnel.

After centrifuging, if necessary, the excess of KOH is decanted. The material, if it is practicable is pressed out with a glass rod to remove as much KOH as possible. The material is then washed with alcohol or glycerin (about 50 percent) depending on the nature of the product. (Glycerin proved to be very useful in the work with bacteria.)

STAINING OF CHITOSAN.

After removing the last traces of KOH by using more dilute alcohol or glycerin (about 25 percent), and possibly neutralizing at the end with 1 percent sulfuric acid the material is treated with a solution of iodine-potassium iodide (2 iodine, 1 potassium iodide, 200 Cc. water.) The excess of iodine solution is then replaced with dilute sulfuric acid, preferably 1 percent. In the presence of chitin or chitosan a distinct red to violet color is detected.

SPECIAL REMARKS.

If the treatment with KOH is not unduly extended the plant tissue is not very much destroyed and the stain brings out the mold mycelium very distinctly.

In case the color should not be distinct or even be covered by another color in the hyphae, the untreated material may, according to van Wisselingh and Wester, first be heated with glycerin to 300° and then treated with KOH.

In case the material should contain large amounts of starch, which may give a color somewhat similar to that of chitosan, the starch can be hydrolyzed with freshly prepared diastase from malt or with taka-diastase or can be differentiated from the mold with the polarization microscope through its ability to refract the light.

Since alkali carbonates do not seem to effect the transformation of the cell substance, chitin, as well as hydroxides, it is essential that the solution of alkali hydroxides used should not contain considerable amounts of carbonates. After treatment with KOH it is often advisable to carry on the test with part of the material transferred to a watch glass or object slide. It is important that the free KOH be completely removed since otherwise the iodine solution will be discolored and prevent the stain from developing.

The material must be actually stained with iodine solution before replacing the iodine with dilute sulphuric acid. For purposes of preservation the preparation is best kept in dilute glycerine (1 plus 1). The stain becomes gradually weaker and disappears after about 24 to 48 hours.

Other tests have been recommended for the precipitation and detection of chitosan Löwy[32] (1910), van Wisselingh, [30] (1915). Those already tried appear to be not as sensitive and satisfactory as the iodine-sulphuric acid reaction. Experiments are being continued to find a stain which will not only be as sensitive and specific as the iodine-sulphuric acid stain but will also give a permanent coloration or precipitation.

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CHEMICAL MONOGRAPHS AND NEW CHEMICALS IN U. S. P. IX.*

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One of the principal features of the new Pharmacopoeia is the addition of 34 chemicals. More would have been added, especially of the newer chemicals, were it not for the general principle followed in the Revision that "No substance shall be introduced if controlled by patent rights." Nevertheless, the Sub-Committee on Scope recommended the addition of several patented or synthetic chemicals. When permission was asked from the manufacturers to include these chemicals in U. S. P., this was in most cases refused. A sure proof that manufacturers are not overanxious to officialize their products, or do not care to enlighten pharmacist or physician. I became fully convinced of this after a conversation with an agent of a manufacturer, who openly told me that he wanted physicians to remember the short, euphonious, therapeutic name of his product, but not the long chemical term! Truly not the right kind of spirit in our enlightened, but commercial age!

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