XXX. THE ACIDS PRODUCED FROM SUGAR BY A *PENICILLIUM* PARASITIC UPON *ASPERGILLUS NIGER*.

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(Received December 1st, 1933.)

THE mould whose behaviour on sugar solutions forms the subject of the present communication is a *Penicillium* originally isolated from an unsterilised cane sugar nutrient solution. Moulds believed to be of the same species have since been encountered contaminating *Aspergillus* cultures. The same or very similar species have also been found in nature accompanying *Aspergillus niger*. The association of the *Penicillium* with the latter mould is so intimate and its ultimate effect upon the *Aspergillus* so unfavourable that it may fairly be described as parasitic.

Thom [1930] states that members of the *P. rugulosum* series have frequently been found as parasites of other moulds in studies of the *Aspergilli*. Elsewhere Thom and Church [1926] refer a parasite of *A. niger* to the group *P. luteum-purpureogenum* close to *P. rugulosum*.

The present *Penicillium* bears conidia which resemble in size, shape and markings, and nearly in colour, those of *P. rugulosum* Thom. In some other respects, notably in its rapid and intense production of a diffusible red pigment in the mycelium, it resembles rather *P. purpureogenum* Stoll. On certain substrates, e.g. on malt agar, it shows a funiculose phase, forming ropes of red hyphae. On this medium there is often an early growth of pink or red pointed tufts of hyphae. Mature cultures on Czapek agar have herbage of a colour almost matched by "dull blackish green" of Ridgway, Plate XLI, m. 33, with here and there patches of yellow hyphae. The red pigment in the inferior surface and medium already referred to is very characteristic. Cultures have no marked odour. Colonies are not floccose. Sclerotia have not been observed. The penicillus is symmetrically biverticillate. The metulae, usually four in number, measure $11\mu \times 2.5\mu$. The sterigmata $11\mu \times 2.2\mu$. The conidial chains, which may be very long and densely packed, usually remain intact in fluid mounts. The conidia are elliptical ($3.4\mu \times 2.6\mu$), pigmented and have a roughened contour when ripe.

It is not proposed to attempt to identify the organism more closely than this, but to refer to it by the index letters under which it has been kept in culture, as *Penicillium* "R.B."

In its action upon sugar solutions, *Penicillium* "R.B." has been compared with type cultures of the other species named above. It is found to differ sharply from them by giving rise to an acid substance which is almost insoluble in water, but easily soluble in alcohol and other solvents specified later. Colourless crystals of this substance encrust the mycelium and glass in acid conditions. When chalk is present a soluble calcium compound forms in the solution. The explanation offered by Alsberg and Black [1913] for the distribution of mycophenolic acid according to the reaction of the medium probably applies here also.

As regards tolerance towards acidity *Penicillium* "R.B." grows well on carbohydrate media over a large range of p_H and is tolerant of a high initial acidity in the medium. Thus, for example, it will grow well on sugar solutions in the presence of chalk and on similar sugar solutions having an initial p_H as low as 2.5. Nevertheless a certain acidity appears favourable for the germination of the conidia, and in the earlier experiments on chalk-containing media germination was quite erratic, frequently not taking place at all. On acidified media this trouble has never been experienced, and the difficulty with chalk was overcome by allowing germination to begin under acid conditions and then running the desired quantity of sterilised chalk suspension beneath the mould into the liquid. The conidia of many other moulds are known to germinate best in acid conditions [Webb, 1921]. The cultural device just mentioned may therefore have a more general interest.

The mould grows over a wide range of temperatures, with a growth-optimum probably at about 37°. It will grow at room temperature and 40°. Seed tubes kept over a year in cold store remained in perfectly good condition and gave rise to normal flask cultures.

The appearances of the cultures in the two cases where chalk is added after germination and where it is not added are very different. The biochemical changes and the fate of the sugar are also greatly affected as the following general description of the outstanding features at 28° in each case will show.

On chalk-containing sucrose medium, an opaque white mycelium forms comparatively rapidly. The inferior surface rapidly develops a blood-red colour which can be seen to diffuse into the solution. It begins first on those areas immediately over the chalk and can be hastened by disturbing the chalk from time to time. Sometimes the mould spores early, but will often remain free from spores to the end. Its surface then passes through various warm shades of colour—yellow, orange, rusty and red shades. Though the chalk is visibly disappearing there is little sign of any evolution of CO_2 . The solution becomes more or less viscous. After some days white nodular crusts of calcium citrate separate out on the mycelium and the walls of the flask. Small quantities of calcium oxalate can also be demonstrated. After 10 days the solution may be run out and filtered from the calcium citrate and any remaining chalk. The filtrate gives a stronger calcium reaction than is accounted for by the dissolved calcium citrate. If it is acidified with HCl a cloud forms, often tardily, and on warming, coagulation and crystallisation take place. At this stage a characteristic aromatic rather pleasant smell is given off. When precautions are taken to decolorise the filtrate before these operations, these crude crystals are practically colourless.

On acidified sucrose medium the young mycelium is more frail and translucent and has a dangerous tendency to become "water-logged" and become submerged. Herrick and May [1928] record a similar observation with a variety of *P. purpurogenum* under certain conditions. *Penicillium* "R.B." usually remains free from spores and shows more generous folding but produces no red colour. The solution is colourless, and in the early days becomes opalescent. It clears later, and small bunches of colourless crystals are found hanging from the mycelium or fallen to the bottom or adhering to the sides of the flask. From these may be obtained by purification and recrystallisation a substance which appears to be identical with "glauconic acid I" of Wijkman [1931].

The solution gives no detectable reaction for citric acid. The titratable acidity after the removal of the crystals is negligible. The crystals themselves show the same solubilities and behaviour as those derived from the calcium compound in the chalk cultures. If the inferior surface of the mycelium is touched with alkali, the bright red colour characteristic of the chalk-grown mycelium appears.

The substantial suppression under acid conditions of any accumulation of citric acid—an acid whose formation is known to be favoured by acid reactions in the case of other moulds [Currie, 1917; Butkewitsch, 1924]—and its accumulation when chalk is present is noteworthy. If oxalic acid is formed at all, it is only in traces. The presence of gluconic acid is not excluded.

EXPERIMENTAL.

Seed tubes for the inoculation of flask cultures have been grown on Czapek agar; malt-extract agar; and bread. Of the different temperatures used, 20°, 28° and 37°, 28° is probably the best for maintaining characteristics during sub-culture. 28° is also a satisfactory temperature for the incubation of flask cultures on sugar media.

(a) *Typical fermentation with Penicillium "R.B." on chalk-containing medium.*

Currie's solution, sucrose 150 g., NH_4NO_3 2.5 g., KH_2PO_4 1.0 g., MgSO_4 , $7\text{H}_2\text{O}$ 0.25 g., HCl to p_{H} 3.4–3.5, water to 1000 ml. was used as a basis for culture solution. 250 ml. Erlenmeyer flasks containing 50 ml. of the solution were used. The initial p_{H} of the medium ensured sterility after 15–20 minutes' steaming at atmospheric pressure.

Five flasks inoculated from a bread culture of *Penicillium* "R.B." grown at 28° were incubated at 30–31°. After 16 hours' germination conidia had formed a delicate surface veil. 10 ml. of a sterilised chalk suspension were then run into each flask by pipette and the cultures returned to the incubator. After 10 days the cultures were sterilised and the liquors filtered. Washings from the mould and calcium citrate and the residual chalk were added to the filtrate. Analysis of a small sample of the filtrate gave a total dissolved calcium of 0.823 g. Rather more than the equivalent of hydrochloric acid was then added, and the fluid became turbid. On boiling, a quantity of crude crystals weighing 1.8 g. was deposited. This is 4.8 % of the original sugar in solution.

Numerous flask experiments on this medium have been made at various temperatures. The water-soluble calcium compound is produced more freely at higher temperatures, 28°–37°, than at room temperature, and 28° was chosen. Other experiments on the same medium were made to find what differences resulted from adding the chalk suspension successively later and later after the germination of spores. Addition 12–18 hrs. after the beginning of incubation at 28° was found to give satisfactory types of culture. Other tests were made to detect differences due to agitating the chalk during incubation, without any decisive result—probably owing to the small depth of the solution. Among early fermentations many were designed to overcome the difficulty of irregular germination already mentioned.

The crude insoluble acid from most of these small tests was saved and bulked for purification later.

The solid calcium compounds were dissolved in excess of HCl, filtered and re-neutralised in the cold. A small precipitate, not wholly soluble on addition of acetic acid, formed. This was removed, and from it were obtained characteristic crystals of calcium oxalate. The calcium citrate in the solution was precipitated by boiling, filtered off and washed with boiling water. The dry weight of calcium citrate was 6.4 g. equivalent to 4.7 g. of citric acid; i.e. 12.5 % of the original sugar in solution.

Residual sugar was not estimated.

(b) *Typical fermentation with Penicillium "R.B." on acid medium.*

To examine the behaviour of *Penicillium* "R.B." in absence of chalk a similar medium of the following composition was prepared and steamed for 20 mins. at atmospheric pressure: sucrose 150 g., NH_4NO_3 2.25 g., KH_2PO_4 0.875 g., $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.225 g., HCl 4.5 ml. of $N/5$, water to 1000 ml. Flasks, quantities of liquid and incubation temperature as before.

The mycelium had a slight peppering of olive-green spores interspersed with tufts of pale sulphur-yellow hyphae; it became only pale yellow on the under surface with traces of red at a few points on the rim in contact with the glass. After 11 days when the cultures were run out numerous small colourless crystals covered the under surface and the bottom of the flask.

Fermentation liquor and loose crystals were run out into a beaker, the bulk of those adhering to the mycelium were detached in running water and added to those in the beaker and all washed by decantation. They were filtered, washed with cold water and dried at 100° . The average weight crystallised from each flask was 0.125 g., 1.66 % of original sugar supplied. Melting-point of the crude crystals insoluble in water was between 193 and 197° . The average weight of dry mycelium from each flask was 0.28 g. The increase in the titratable acidity of the liquor was negligible. Qualitative tests for oxalic acid and citric acid were both negative. As before the sugar of the medium was not exhausted.

Many other small flask fermentations in absence of chalk have been carried out, varying the nutrients in solution, the temperature of incubation, concentration of sugar, p_{H} of solution and other factors. Many of these produced crude crystals. The yields reported are not large, but other experiments in progress indicate the possibility of considerably increasing the yield.

One solution which failed to yield any crystals was the Czapek-Dox solution. In this case, the cultures spored early and the fluid developed the red colour. The sugar in this solution is glucose, but that it was not the change from sucrose to glucose which caused the difference was proved by other tests.

Purification of the water-insoluble crystals. A quantity of crude crystals obtained as described above was dissolved in an excess of N NaOH . The solution was clarified with norite and filtered. The filtrate was acidified with strong HCl and an almost pure white product obtained, giving, after washing and drying at 100° , over 37 g. The melting-point of this material was not sharp, however, but ranged from 196 to 199° .

A part of this was submitted to recrystallisation from glacial acetic acid. This gave excellent crystals, the presumably purest specimens of which melted at 201 – 202° .

Properties. The substance is practically insoluble in cold water and only slightly soluble in hot water. It is insoluble in light petroleum. It dissolves in alcohol, acetone, ether, benzene, chloroform, pyridine and glacial acetic acid, from which it crystallises with ease. It dissolves in sodium carbonate solution with the evolution of CO_2 , only gradually in dilute solutions of NaOH and may be reprecipitated with acid as described. It may also be recovered crystalline by adding water to a solution in alcohol and warming. It does not reduce Fehling's solution or ammoniacal silver nitrate. The bromine absorption test is negative, and it gives no coloration with FeCl_3 . In concentrated H_2SO_4 it blackens on heating. It burns with a smoky flame.

Titration. The substance was dissolved in ethyl alcohol and titrated with $N/10$ NaOH in presence of phenolphthalein. In the cold there is no very definite

end-point; the alkali is only neutralised slowly. More soda is taken up on heating.

			Equivalent
Cold	0.0774 g. required	5.7 ml. <i>N</i> /10 NaOH	—
Hot	0.0774 g. required	8.55 „ „	90.5
	0.1842 g. required	20.3 „ „	90.7
	0.1591 g. required	18.2 „ „	87.4

(Theoretical for $C_{18}H_{20}O_7$ titrating as a tetrabasic acid, 87.0.)

A search of the literature at the time failed to disclose any compound with the above properties produced by a mould. Before an opportunity occurred for a fuller chemical investigation of the new acid, Wijkman's work on the production of "glauconic acid I and II" by a *P. glaucum* appeared [Wijkman, 1931].

Her experiments were made on a sucrose solution containing very small quantities of mineral salts and without chalk present. In these circumstances she obtained insoluble acids crystallising out during the fermentation and from the crude mixture separated two pure substances, the properties of one of which, glauconic acid I, agree well with those of the acid produced by *Penicillium* "R.B." Glauconic acid I is given the formula $C_{18}H_{20}O_7$, and has a melting-point of 202°.

A thorough chemical examination of the metabolic products of the *Penicillium* "R.B." fermentation has still to be made, but the properties of the insoluble acid already described have since been compared with those described by Wijkman.

Mol. wt. 386, 380; micro-analysis (Schoeller) C, 62.07; H, 5.75 %; calculated for $C_{18}H_{20}O_7$; mol. wt. 348; C, 62.04; H, 5.79 %.

By acting on the acid dissolved in pyridine with acetic anhydride a crystalline derivative has been prepared whose melting-point closely approaches the value 175° given for the corresponding derivative of glauconic acid I. Micro-analysis C, 61.52; H, 5.64 %; calculated for mono-acetyl derivative C, 61.61; H, 5.68 %.

Heated above its melting-point, the original acid gives off a very fragrant vapour as the substance decomposes, as does glauconic acid I.

A substance corresponding to glauconic acid II has not been recovered, but the mother-liquors from the recrystallisation have not been investigated, so that the presence of this cannot be excluded.

The experiments already described were all made with sucrose. Fermentations were made upon 15 % solutions of glucose and fructose and in all cases crystals of the same appearance, giving the same qualitative tests as those obtained with sucrose, resulted. When chalk is present citric acid is also formed in quantities of the same order as with sucrose. Glycerol has also been tried, and the striking feature of the glycerol fermentation is that the solution instead of remaining colourless becomes a deep yellow.

SUMMARY.

A *Penicillium* parasitic on *Aspergillus niger* is described. Its action upon sucrose solutions has been examined.

In the presence of chalk, considerable quantities of calcium citrate and small quantities of calcium oxalate are formed; also the soluble calcium salt of an acid insoluble in water, but soluble in alcohol.

In acid solutions the insoluble acid is formed encrusting the mould and separating on the vessel, but detectable quantities of citric and oxalic acids are not formed.

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The insoluble acid also appears when sucrose is replaced in the medium by glucose or fructose. Citric acid is also formed from glucose and fructose when chalk is present.

The insoluble acid is probably to be identified with "glauconic acid I" of Wijkman, originally found in cultures of a *P. glaucum*.

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