109. BIOCHEMISTRY OF THE WOOD-ROTTING FUNGI

2. A STUDY OF THE ACIDS PRODUCED BY *CONIOPHORA CEREBELLA* PERS.

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THE biochemical activities of the lower fungi commonly classed as moulds have been intensively studied and a great deal of information is now available about the metabolism of certain species such as *Aspergillus niger*. Comparatively little attention has been given on the other hand to the metabolic activities of the higher Basidiomycetes which grow in wood. In a previous paper [Birkinshaw & Findlay, 1940] the isolation and identification of aromatic substances produced by *Lentinus lepideus* were described; the present paper deals with the acids produced by wood-rotting fungi and describes an investigation into the acids produced by *Coniophora cerebella*.

Since decay in wood and its prevention are matters of economic importance the decomposition of wood has been investigated in some detail but little attention has been given to the study of substances elaborated by the fungi which decompose it. In recent years the chemistry of decay in wood brought about by certain wood-rotting Basidiomycetes has been studied by a number of workers, notably by Falck in Germany, Lutz in France, Hawley in the United States of America and more recently by W. G. Campbell at the Forest Products Research Laboratory in this country, and the general nature of the changes taking place has been elucidated. Two types of decay may be recognized.

(1) The brown rot type in which cellulose and the associated pentosans are preferentially decomposed but in which the lignin remains virtually unattacked. In this type of decay the primary chemical processes involved resemble simple acid hydrolysis.

(2) The white rot type in which all the constituents of the wood including the lignin are attacked. In this type of decay oxidation in addition to hydrolysis appears to take place.

It has long been known that all wood-destroying fungi render distinctly acid any medium on which they are growing [cf. Curtin, 1927] and that they make satisfactory growth only in media which are acid in reaction. The change in pH of a malt extract solution in which active wood-rotting fungi were growing was followed and it was found that there is almost always an initial increase in acidity. With certain species this initial increased acidity disappears and the medium may finally become less acid than it was before being infected with the fungus.

It was found that the brown rot fungi render the medium more acid than do the white rot fungi and that this acidity tends to persist longer than in the case

of the latter. With most of the white rot fungi tested, the minimum pH was reached 3–6 weeks after infection and thereafter the pH rose again and the acid first formed disappeared. No evidence that this reduction in acidity was due to the production of ammonia could be obtained. It is more probable that the acids first formed are later oxidized, being used after the depletion of the sugars as a source of energy. It is noteworthy that a medium in which any of these white rot fungi have been grown gives a positive test for oxidase.

Rabanus [1939] made similar experiments over somewhat longer periods on the change of pH in solutions of malt in which various wood fungi were growing, but did not correlate the rapid reversal from acid production towards neutrality which occurs with fungi such as *Polystictus versicolor*, with the fact that these are fungi which produce oxidases and bring about a white rot in wood.

This reversal in the direction of the pH change does not occur when the fungi are growing in wood; in this case it is probable that there is an ample supply of nutriment and fresh acid is continually produced. The pH of an extract of pine or spruce wood in which *Merulius lacrymans* had been growing for about one year was found to be about 3.0 while that of an extract of beech wood severely decayed by a white rot fungus, *Polystictus versicolor*, was about 4.5.

Preliminary experiments were made to determine the amount of titratable acidity present in an extract of wood decayed by various fungi which had been found to produce a low pH figure in liquid media. It was found that *Coniophora cerebella* Pers. produced twice as much acid as any other of the species tested and it was therefore decided to investigate fully the nature of the acids which this fungus produces by its action on wood. As far as can be ascertained no careful analytical work has previously been undertaken to identify the acids produced by this or similar fungi. Falck [1912], using Fresenius's methods, made a few qualitative tests and claimed to have identified free malic and succinic and combined oxalic and tartaric acids but failed to find any citric acid. Rabanus [1939] states that *Polyporus vaporarius* undoubtedly produces oxalic acid in addition to other acids.

Coniophora cerebella Pers. is a common fungus which attacks many different kinds of wood causing a brown rot in which the decayed wood becomes much darkened and tends to split along the grain. The superficial mycelium which is at first yellowish in colour later becomes a very dark brown. It is a frequent cause of decay of flooring timbers in buildings and is especially common when floor boards have been laid directly on to damp concrete. It is noteworthy that this fungus requires a higher moisture content in wood for its optimum growth than most wood-rotting fungi. It seems probable that the production of large amounts of organic acids may be correlated with its ability to grow in wood which contains only a very limited supply of air. Under these semi-anaerobic conditions an accumulation of acids occurs. When sawdust instead of solid wood is used as a culture medium much less acid is produced. This may be due to the better aeration present in the sawdust.

For the purpose of the present investigation *Coniophora cerebella* was grown on blocks of Scots pine sapwood. Samples of the infected wood were examined after 2, 4 and 6 months' incubation. In the method finally adopted the acids were extracted from this wood by means of cold water, the extract being then concentrated *in vacuo*. The distillate contained small amounts of formic and acetic acids. In the concentrated liquor polysaccharides were precipitated by addition of alcohol. The alcohol was then removed and the concentrated aqueous solution was exhaustively extracted with ether. This removed about 50 % of the total titratable acidity. The ether-soluble acid fraction contained a considerable

amount of citric acid which was isolated as methyl citrate and accounted for about one-third of the total acidity of the original aqueous extract for the longer periods of incubation. The acids not extracted by ether were converted into barium salts and separated by precipitation with alcohol. An account of these is reserved for a further communication.

In order to obtain information as to the acids present in sound wood, healthy Scots pine sapwood chips were subjected to similar treatment. Volatile acids were found and formic acid was actually determined in the mixture. From the ether-soluble acids, however, no citric acid could be isolated as the trimethyl ester, and it seems reasonable to conclude that it is not present in sound wood. In order to obtain quantitative figures for the amount of citric acid present after decay, determinations of citric acid by the pentabromoacetone method [Lampitt & Rooke, 1936] were undertaken on the aqueous extracts. These values were in each case higher than the amount of citric acid obtained by isolation as methyl citrate, as would be expected, but the determination on the extract from sound wood gave a small positive citric acid value, whereas in the isolation experiment none could be detected.

Although healthy wood may possibly contain small amounts of citric acid which has hitherto escaped detection, the more probable explanation is that some substance is present in sound wood which is not citric acid but which yields pentabromoacetone under the conditions of the citric acid determination. Whatever the explanation of this divergence it is quite obvious even from the determinations of citric acid that the growth of the fungus on the wood does actually give rise to citric acid, which is therefore a true metabolic product. So far as we are aware the production of citric acid by species of wood-destroying fungi has not hitherto been recorded.

It is noteworthy that although the rate of decay as indicated by the loss in weight of the wood is steady throughout the 6 months' incubation period, yet the titratable acidity has reached a maximum in 2 months and the citric acid yield is constant after 4 months. This may represent the establishment of a balance between production and decomposition of acid. It is possible that further increase of acidity beyond this level would render the pH of the medium too low for the fungus. If this were the case much higher yields of citric acid might be attained by allowing the decay to proceed in the presence of some substance which could buffer or neutralize the acid formed. Further work is contemplated to investigate this aspect of the problem.

EXPERIMENTAL

In the preliminary experiments designed to afford a general survey of the acid production by various species of wood-destroying fungi on liquid media the pH of sample flasks containing 30 ml. of Kepler's malt solution was measured at various periods after inoculation. By testing separate flasks at each interval instead of withdrawing material from single large flasks, the volume of nutrient liquid available for the growth of the fungi was not reduced in the later stages of the experiment, but of course slight variation between the growths in different flasks was inevitable and the results do not lie on perfectly smooth curves. In earlier experiments the pH was determined colorimetrically with a Hellige comparator but in later experiments the glass electrode was used. The results are collected in Table 1.

The points to be noted in these preliminary experiments are:

(1) All the fungi tested show an initial lowering of pH, but whereas the brown rots maintain the pH at a low value, with the white rots the pH passes

through a minimum value and then rises again, in some cases to the original value or higher.

(2) The depression in pH produced by the brown rots is considerable, the greatest being shown by *Coniophora cerebella* where a pH of 2.0 is attained after 10 weeks.

Table 1. pH changes during growth on Kepler's malt solution

Period of incubation weeks	Brown rot fungi						
	pH by comparator			pH by glass electrode			
	<i>Merulius lacrymans</i>	* <i>M. lacrymans</i>	<i>Daedalea quercina</i>	<i>Coniophora cerebella</i>	<i>Lenzites trabea</i>	<i>Fomes pinicola</i>	
0	6.8	6.4	5.8	7.4	5.6	5.6	
1	6.6	6.1	4.65	7.0	4.7	4.05	
1½	—	—	—	6.15	4.1	3.05	
2	5.35	5.15	3.3	2.6	4.0	2.85	
3	3.0	3.6	—	2.25	—	—	
3½	—	—	2.8	—	3.5	2.7	
4	2.9	2.3	—	2.25	—	—	
5	—	—	2.8	—	3.1	2.6	
6	2.0	2.2	—	2.2	—	—	
7	—	—	—	—	3.05	2.55	
8	—	—	2.4	2.35	—	—	
9	—	—	—	—	3.1	2.65	
10	—	—	—	2.0	—	—	
11	2.6	3.0	—	—	3.15	2.55	
12	—	—	2.8	2.1	—	—	

Period of incubation weeks	White rot fungi							
	pH by comparator				pH by glass electrode			
	<i>Polystictus versicolor</i>	* <i>P. versicolor</i>	<i>Stereum hirsutum</i>	<i>Fomes annosus</i>	<i>P. versicolor</i>	<i>Trametes rubescens</i>	<i>Ganoderma applanatum</i>	<i>Fomes ignarius</i>
0	6.8	6.4	5.8	5.8	7.4	7.4	5.6	5.6
1	5.45	5.3	4.6	5.3	6.8	6.9	5.55	5.5
1½	—	—	—	—	6.25	6.5	4.65	5.15
2	4.2	4.2	3.5	3.9	4.4	6.15	4.25	(5.4)
3	4.2	4.2	—	—	4.35	4.95	—	—
3½	—	—	4.0	3.8	—	—	4.35	4.05
4	4.2	4.0	—	—	5.45	5.25	—	—
5	—	—	4.0	3.8	—	—	4.3	4.25
6	5.8	6.0	—	—	6.55	5.95	—	—
7	—	—	—	—	—	—	(4.05)	4.6
8	—	—	4.2	4.6	7.15	5.7	—	—
9	—	—	—	—	—	—	5.25	4.25
10	—	—	—	—	6.65	5.55	—	—
11	6.7	7.2	—	—	—	—	7.25	4.5
12	—	—	5.0	5.6	7.3	5.65	—	—

Columns marked with an asterisk are for 5% malt solution, the rest are for 2% solution.

In view of its behaviour not only on liquid media, but also on wood, where twice as much acid was produced as was given by other species tested, the fungus *C. cerebella* was selected for a closer study and for the purpose of identifying the acid metabolic products.

Organism. A culture of *C. cerebella* Pers. originally isolated by Prof. R. Falck at Hann-Münden was used. This strain, which has been called the "*Ida-weiche* form", has been extensively employed as a test fungus in toxicity tests of wood preservatives (British Standards Specification No. 838 of 1939).

Method of culture

The method used for cultivating the fungus was exactly similar to that already described for the examination of *Lentinus lepideus* [Birkinshaw & Findlay, 1940]. Small blocks of Scots pine sapwood sterilized by autoclaving for 30 min. were introduced into Kolle flasks in which the fungus was growing on malt agar. They were supported on low glass rests so that they came into contact only with mycelium thereby minimizing any diffusion of materials from the agar into the blocks. The flasks containing the samples were kept in an incubator at 22° and batches of samples were removed after 2, 4 and 6 months' incubation.

Method of examination of infected wood

The partly decayed wood was extracted and the water-soluble products were investigated after periods of incubation of 2, 4 and 6 months' in order to ascertain the changes in nature and amount of products as incubation progressed. The treatment of batch I was rather of an exploratory nature; the technique was modified for succeeding batches in the light of experience gained from batch I.

Treatment of batch I (2 months' incubation)

100 blocks of the infected wood (total moist weight 998 g.) were cut into chips and treated with 7 l. of cold distilled water. The container was evacuated until all the chips sank below the surface when air was readmitted. After 20 hr. the aqueous solution was removed and a second extraction was performed with 5 l. of water. After 24 hr. the extract was removed and the wood was pulped in a manual mincer. The pulp was pressed in a tincture press and extracted with a further 5 l. of water, the pulp being again pressed after extraction. Titration of 5 ml. samples of the extracts showed that the third extract had only about 1/7 of the acidity of the first extract. Further extraction was not considered profitable.

The total volume of the combined extracts, pressings and wash water was now 18,020 ml., the acidity by titration being equivalent to 182.4 ml. of *N*.

After removing a reference sample of 1/20 of the volume the remaining liquor was concentrated *in vacuo*. The distillate was collected and titrated for volatile acids. The total titratable acidity of the combined distillates was 118.8 ml. of *N*/10.

When cooled to 0° the dark brown concentrate deposited a dark-coloured sediment (2.2 g.; ppt. I) which was separated by filtration. The liquor was then neutralized to litmus by the addition of 85 ml. of 2*N* NH_4OH . A solution containing 30 g. of $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ was then added and the mixture left for 12 hr. in the cold. A slight precipitate was obtained; this was separated by filtration (0.39 g.; ppt. II).

The filtrate and washings (600 ml.) were treated with 2500 ml. of absolute alcohol to make an 80% alcohol solution. The bulky precipitate was separated at the centrifuge, washed and dried (26.67 g.; ppt. III).

Concentration of the alcoholic liquor and reprecipitation gave only small amounts of sticky precipitates which were not further investigated.

Examination of the fractions obtained

Volatile acids. The combined distillates obtained on evaporation had been neutralized and required 118.8 ml. of *N*/10 NaOH . The solution was evaporated almost to dryness and the acids were liberated by means of phosphoric acid and redistilled *in vacuo*, in order to eliminate CO_2 which might have been present

in the first distillation. The titration of the distillate was now 89.8 ml. of $N/10$ NaOH.

The sodium salts were evaporated to dryness and a portion (0.1 g.) was converted into phenylphenacyl esters by heating with *p*-phenylphenacyl bromide (0.3 g.) in 6 ml. of 60 % alcohol under reflux for 90 min. The crystals obtained on cooling had m.p. 91–95°, raised by recrystallization to 107–108° and by vacuum sublimation to 109–110°. It showed no depression in m.p. when mixed with authentic *p*-phenylphenacyl acetate of m.p. 110–111°. (Found: C, 75.63; H, 5.80 %. Calc. for $C_{16}H_{14}O_3$, C, 75.58; H, 5.55 %.) The substance is therefore *p*-phenylphenacyl acetate and indicates the presence of acetic acid in the volatile acids. A further portion of the sodium salts of the volatile acids was found to give a heavy precipitate of metallic silver when treated with silver nitrate solution, indicating the probable presence of considerable amounts of formic acid. A definite identification of formic acid was obtained from the second batch.

Precipitate I. The product was found to contain material extractable by ether, the extract amounting to about 10 % of the total weight. This might be resinous material from the wood.

The main portion appeared to be a salt. It was acid to litmus, gave a strong potassium flame and had a sulphated ash value of 54.3 %. It was thought that it might be potassium hydrogen tartrate. However, by qualitative tests (Fenton's reaction, Feigl's fluorescein reaction [Feigl *et al.* 1936]), tartaric acid could not be detected. Further, a determination of tartaric acid by oxidation with periodic acid gave an apparent potassium hydrogen tartrate content of only 15.3 %. As this test is not specific even the low figure obtained may have been due to other substances. There is thus no definite evidence of the presence of tartaric acid in the precipitate and if present at all the amount must be relatively small. As this ppt. I was found to contain considerable amounts of iron—no doubt derived from the mincer or press—it was not further examined. In working up further batches care was taken to avoid these sources of contamination.

Precipitate II. Identification of oxalic acid. A portion of this precipitate (0.34 g.) was acidified with 2.5 ml. of 2*N* HCl and extracted with ether in a continuous extractor. The total extract (0.15 g.) was sublimed *in vacuo* and gave a crystalline sublimate of m.p. 189°. Freshly sublimed oxalic acid had m.p. 190° and there was no depression on mixing. Hence oxalic acid is definitely present in this precipitate but the total amount formed is relatively small since the total weight of ppt. II, which is presumably crude calcium oxalate, is only 0.39 g.

Precipitate III. (Wt. 26.67 g.) This, the largest fraction, was subjected to a more detailed examination. Various quantitative and qualitative tests were performed on the aqueous solution in order to obtain some idea of the nature of the product.

(1) *Sulphated ash content.* 32.15 %. Assuming that the only cation present is Ca this gives a Ca content of 9.46 %. Actually the ash was found to contain iron.

(2) *Estimations of sugar as glucose.* The apparent glucose content of the precipitate by the Shaffer-Hartmann method was 14.15 %. Another portion of the precipitate was hydrolysed by heating 0.2039 g. in 10 ml. of water with 20 ml. of N H_2SO_4 for 1 hr. at 100°. The apparent glucose content after hydrolysis (corrected for dilution) was 33.63 %. Thus the substance evidently contains polysaccharides or polyuronic acids. A qualitative test for polysaccharides was made by dissolving a little of the precipitate in dilute HCl and treating with 4 vol. of alcohol. A considerable amount of flocculent precipitate indicated the presence of polysaccharides.

(3) *Estimation of aldehyde groups* (Willstätter and Schudel). The precipitate (0.2492 g.) was dissolved in 100 ml. of water and treated with 40 ml. of *N*/10 iodine and 50 ml. of *N*/10 NaOH. After 15 min. 10 ml. of 2*N* H₂SO₄ were added and the solution was titrated with *N*/10 thiosulphate. The iodine deficiency (8.73 ml.) corresponded with CHO content of 0.0127 g. or a glucose content of 31.5%.

(4) *Test with 2:4-dinitrophenylhydrazine*. An aqueous solution of the precipitate gave a precipitation with dinitrophenylhydrazine hydrochloride in 2*N* HCl after 4 hr. which gradually increased in amount over several days. No crystalline product could be obtained from this.

(5) *Test for hexuronic acids*. The precipitate was dissolved in water and an equal volume of conc. HCl was added together with a little naphthoresorcinol. On warming, a greenish colour appeared at 70°, steel grey at 80°. Amyl alcohol extracted a deep blue colour with green fluorescence. In ether a purple colour was obtained. The test indicates the presence of a uronic acid grouping.

(6) *Attempt to crystallize the Ca salts*. A portion of the precipitate was dissolved in water, filtered and the filtrate was allowed to evaporate slowly in a desiccator. The substance dried down eventually to a horny mass showing no trace of crystals.

(7) *Esterification of the acids*. The precipitate (5 g.) was dissolved in water and filtered from a small amount of insoluble matter. The dark brown solution was treated with a slight excess of oxalic acid and after removal of the calcium oxalate by filtration the solution was evaporated to dryness *in vacuo* (3.94 g.). The solid was treated with methyl alcohol, in which a part dissolved and was methylated with a solution of diazomethane in ether. It was then filtered from the insoluble portion (1.52 g.) and the solution was evaporated. The sticky residue weighed 2.40 g. Of this residue 0.05 g. was subjected to sublimation in high vacuum. A crystalline sublimate m.p. 72–75° was obtained. The remainder was extracted three times with about 50 ml. of ether under reflux. The extract, 0.60 g., was refluxed with light petroleum (b.p. 50–60°). This gave crystals on cooling of m.p. 77.5–78° unchanged after recrystallization. Further extractions and evaporation of the mother liquors gave further crops of crystals (0.25 g. in all) of slightly lower m.p. There was no depression in m.p. when the crystals were mixed with authentic methyl citrate of the same m.p. (Found: C, 46.72, 46.40; H, 6.14, 5.96; OCH₃, 39.4, 39.2%. Calc. for C₉H₁₄O₇ (with 3OCH₃), C, 46.15; H, 6.02; OCH₃, 39.8%.)

For further confirmation the amide was prepared. The crystals (0.1 g.), m.p. 76–77°, were treated with 0.5 ml. of aqueous ammonia saturated at 0°. The ester dissolved, and on keeping crystals were obtained which, when recrystallized from water, had m.p. 206–207°. Pure citramide had m.p. 207–208°. There was no depression on mixing. (Found: C, 38.58, 38.50; H, 5.73, 5.88; N, 21.3, 20.9%. Calc. for C₆H₁₁O₄N₃, C, 38.09; H, 5.86; N, 22.21%.)

The methyl ester fraction soluble in methyl alcohol but not extracted by ether was treated according to Goebel [1935] for the preparation of the hepta-acetyl methyl ester of aldobionic acid. The ester (0.74 g.) was heated at 50° for 12 hr. under reflux with 5 ml. of acetic anhydride containing 0.5 g. of anhydrous ZnCl₂. The acetic anhydride was removed *in vacuo* and the residue was taken up in chloroform, washed 5 times with ice water, separated, dried with calcium chloride and filtered. It was then evaporated *in vacuo* and the residue was taken up in alcohol and chilled. No crystals could be obtained.

Treatment of batch II. (4 months' incubation)

As already stated the procedure employed in the examination of batch II was modified in the light of experience gained with batch I. The modified procedure was designed (1) to avoid contamination with iron, (2) to separate the polysaccharides before precipitation of the salts of the acids, (3) to obtain the barium instead of the calcium salts, since the former might be more readily separated by a process of fractional precipitation.

The second batch of decayed wood had a total moist weight of 853.5 g. It was cut into chips and extracted with cold water, the extractions being repeated until the titration value of the extract had fallen from 0.97 ml. of *N*/10 NaOH per 5 ml. (1st extraction) to 0.09 ml. (4th extraction). Mincing and pressing were avoided in order to prevent metallic contamination. The total volume of the extract was 18,660 ml. and the total acid titration value 167.8 ml. of *N*. One-twentieth of the mixed extracts was retained for reference and was preserved by the addition of a little toluene; the remainder was evaporated *in vacuo*, the distillate being retained for examination of the volatile acids.

The aqueous extract was evaporated to 300 ml. and the precipitate which formed was filtered off (1.14 g.). The solution was then treated with absolute alcohol sufficient to make 90 % by volume in order to remove the polysaccharides. The precipitate was separated by centrifuging (8.50 g.). The alcoholic solution was evaporated *in vacuo* to about 150 ml. During evaporation a small amount of sticky material separated. The aqueous solution of 150 ml. remaining after evaporation of all the alcohol and some of the water was then extracted in a continuous extractor with ether for 72 hr. After this period the amount of material still being extracted by ether was negligible. The extract, after removal of ether, weighed 12.43 g. This was dissolved in methyl alcohol. There was a small amount of insoluble matter (0.05 g.).

The aqueous solution which had been extracted with ether was treated with a filtered solution of 8.1 g. of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$ in hot water, the amount required to neutralize the solution having been ascertained by titration of a small sample. The baryta was added slowly to the solution, which was mechanically stirred during the addition. The precipitate obtained was separated by centrifuging, washed and dried (0.88 g.).

The solution (370 ml.) was treated with an equal volume of absolute alcohol and the precipitate was again removed by centrifuging (7.21 g.). The alcohol was then made up to 80 % and the precipitate was separated (3.68 g.).

Batch II. Examination of the fractions obtained

Volatile acids. The total titration value of the distillate was 53.47 ml. *N*/10 NaOH. The neutralized solution was evaporated to dryness and the acids were redistilled after addition of 1 g. of phosphoric acid to the salts. The titration value of the distillate was now 38.27 ml. *N*/10. The neutralized solution was again evaporated and made up to 50 ml. A portion (5 ml.) showed a formic acid content of 9.73 mg.; thus the titratable acidity due to formic acid would correspond with 55.3 % of the total acidity.

The remaining solution (45 ml.) of the salts of volatile acid was distilled with aqueous phosphoric acid *in vacuo*. PbCO_3 (1 g.) was added to the distillate which was then warmed and filtered. The filtrate was evaporated to 2.5 ml. and chilled. Crystals were obtained (0.13 g.) in the form of long needles characteristic of lead formate. By sulphated ash the Pb content of the crystals was

69.9, 69.7 %. Calc. for $\text{Pb}(\text{HCOO})_2$, 69.7 %. Thus the presence of formic acid in the distillate is definitely established.

All the residues and washings were collected, acidified with phosphoric acid and distilled. The distillate was titrated, and the neutralized liquid after concentration was treated with the calculated amount of *p*-phenylphenacyl bromide. Sufficient alcohol was added to maintain the bromide in solution and the mixture was refluxed for 1.5 hr. The products were fractionated from alcohol-water. Crystals were obtained having m.p. 108–109° which showed no depression in m.p. when mixed with authentic phenylphenacyl acetate. The presence of acetic acid in the volatile acids is thus again confirmed.

Treatment of acids extracted by ether. The total acids extracted by ether (12.43 g.) were dissolved in 65 ml. of methyl alcohol, the small amount of insoluble matter was separated by filtration and the solution was treated with diazomethane in ether until the evolution of nitrogen ceased. The product was evaporated to dryness and extracted with ether. The ether-soluble portion (9.79 g.), after removal of solvent, consisted of a mass of crystals in a syrupy matrix. This was spread on a porous plate. The syrup was slowly absorbed, leaving 4.21 g. of crude crystals. After two recrystallizations from light petroleum (b.p. 40–50°) the crystalline material showed the correct m.p. 77.5–78° for methyl citrate.

The substance absorbed by the porous plate was recovered by extraction and dried. It had acid equivalent on saponification of 121.8. Attempts to prepare from it a crystalline hydrazide, phenylhydrazide or amide failed.

Batch III. (6 months' incubation)

The treatment of batch III (moist weight 1128 g.) was substantially the same as for batch II and need not therefore be described in detail. The titration values, etc. of the total and volatile acids are recorded below:

Aqueous extract

Total volume of aqueous extract	...	19,550 ml.
Titration value		240.5 ml. <i>N</i> NaOH

Volatile acids

Titration value of distillate		50.23 ml. <i>N</i> /10
After redistillation		28.96 ml. <i>N</i> /10
% of acidity due to formic acid	...	26.8 %

Two separations of the polysaccharide fraction were undertaken, as in work on the previous batch this fraction had not been completely eliminated, before proceeding with the precipitation of the barium salts.

The evaporated aqueous solution (300 ml.) was treated with 9 vol. of alcohol and the polysaccharide precipitate separated (12.80 g.). After removal of alcohol the aqueous solution was exhaustively extracted with ether. The total weight of the ether extract was 16.4 g. The extracted solution was then again treated with 9 vol. of alcohol and the precipitated polysaccharides removed (2.75 g.). Further addition of alcohol up to 95 % caused no further precipitation. The alcohol was removed and the solution (now less than 100 ml.) was neutralized with baryta (9.11 g. of $\text{Ba}(\text{OH})_2 \cdot 8\text{H}_2\text{O}$) and the Ba salts were fractionally precipitated with alcohol.

Isolation of citric acid as the methyl ester from the acids extracted by ether. The method was identical with that already described for batch II except that one-

quarter of the total acids extracted by ether was used instead of the whole amount. The amount isolated showed no increase over batch II.

Examination of sound wood

The treatment of the sound wood followed the method described for batches II and III of the decayed wood.

Chips of Scots pine sapwood (743 g.) which had been steam distilled for several hours were extracted three times with cold water as follows: 3.5 l. for 60 hr., 3.2 l. for 48 hr., 3 l. for 48 hr. The titration fell from 0.225 ml. of *N*/10 NaOH per 5 ml. for the 1st extraction to 0.053 ml. for the 3rd extraction. The total titration value of the mixed extracts was 25.9 ml. of *N* NaOH. The extract was distilled *in vacuo*, and gave an acid distillate of titration value 37.0 ml. of *N*/10. On redistillation of the distillate the titration was 24.74 ml. of *N*/10. The formic acid content was 69.0 mg. equivalent to 60.7 % of the total acidity of the volatile acid fraction. The evaporated aqueous solution containing the non-volatile acids was made up to 90 % alcohol and gave a polysaccharide fraction of 3.58 g. The solution was evaporated and deposited a little brownish oil. This was separated by filtration and the clear liquid was exhaustively extracted with ether. The whole of the extract was esterified with diazomethane and the solvent was evaporated. The product was extracted with ether, and gave an ether-soluble portion of 2.78 g. and an insoluble residue of 0.03 g. There was no crystallization in the syrup remaining after removal of the ether. A small portion of the syrup sublimed *in vacuo* gave no crystals but only an oil which distilled at 100°. In a further attempt to obtain crystals of methyl citrate the ether-soluble portion was extracted with light petroleum (b.p. 40–50°). The extract (1.40 g.) was still an uncrystallizable oil. Hence it may be concluded that citric acid is not present in sound wood. By a second precipitation with alcohol of the acid fraction not extracted by ether a further small amount (0.83 g.) of polysaccharides was obtained. The acids remaining after removal of the alcohol had a titration value of only 5.5 ml. of *N*/10 and gave 0.12 g. of barium salts. Thus practically the whole of the material obtained as barium salts from batches II and III of decayed wood may be regarded as metabolic products of the fungus.

Determination of citric acid

Citric acid was determined by the pentabromoacetone method as described by Lampitt & Rooke [1936] in samples of unevaporated aqueous extract of each of the three batches of decayed wood and also in a similar extract from sound wood. These figures are not of great accuracy since the amounts found in 100 ml. (the volume used for the determination) are near the lower limit of the method but they are interesting for comparison with the amounts of citric acid actually isolated as methyl citrate. The values obtained are given in Table 2, the amounts of citric acid being expressed in each case as the weight of anhydrous acid obtainable from 100 blocks of wood (estimated dry weight 793 g.). The dry weight of decayed wood after extraction is given for comparison in order to show the loss in weight owing to decay. Total and volatile acidities are also shown.

It is of interest to note that the control estimation of citric acid in sound wood gives a definite although small positive value, yet no citric acid could be isolated as methyl citrate. The reason for this anomaly is obscure; possible explanations have already been discussed.

Table 2. *Data obtained on wood extracts*

Wood sample	Dry wt. of wood after extraction g.	% loss in wt.	Total acid (ml. N) extracted by H ₂ O	Volatile acid (ml. N)	% of volatile acidity due to formic acid	Anhydrous citric acid			
						Determined by pentabromo-acetone method		Isolated as Me ₃ citrate	
						Wt. g.	% of total acidity	Wt. g.	% of total acidity
Control	783	1.31	30.2	3.04*	60.7	0.44	22.8	0	0
Batch I (2 months)	669	15.64	182.4	9.46	40.2†	1.19	10.2	1.18	10.1
Batch II (4 months)	546	31.15	167.8	4.03	55.3	4.95	46.1	3.69	34.4
Batch III (6 months)	445	43.88	185.0	2.35	26.8	4.98	42.1	3.68	31.1

All yields are based on 100 blocks of wood of estimated dry weight 793 g. before inoculation.

* This value is almost certainly low owing to the material having been subjected to steam distillation prior to extraction.

† Known to be a low value.

SUMMARY

From a comparison of various wood-destroying fungi grown on aqueous malt extract and on wood *Coniophora cerebella* was selected as the best acid producer. The acidic products formed by growth of *C. cerebella* on Scots pine sapwood were therefore investigated after incubation periods of 2, 4 and 6 months. The acids identified were formic, acetic, traces of oxalic acid and relatively large amounts of citric acid. The last-named acid was isolated as the methyl ester. It was obtained in amounts equivalent to about one-third of the total titratable acidity after 4 and 6 months' growth. Other acids of higher molecular weight were also present. Qualitative tests indicated the presence of hexuronic acids in this fraction.

Examination of sound wood revealed the presence of volatile acids including formic acid in amounts comparable with those isolated from decayed wood. The volatile acids are not therefore to be regarded as metabolic products of the fungus. Citric acid, on the other hand, is considered to be a true metabolic product of the growth of *C. cerebella* on wood.

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