On Chagi

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The phenolic material of chaga (*Poria obliqua*) sporophores is examined by degradations, methylation studies, and spectroscopic techniques, and is a polymer formed by oxidative polymerisation, *in situ*, of lignin breakdown products translocated from the host timber. The polymer sub-units are shown to be typical of the phenolics produced by white rot of hardwoods.

"CHAGI" is the phenolic material obtained by hot-water infusion of the sterile fruit-bodies of the Siberian chaga fungus, *Poria obliqua* (Pers.) Bres. [≡ *Inonotus obliquus* (Fr.) Pil. *f. sterilis* (Van.) Bond], which form dark brown or black clinker-like lumps, yellowish-brown within, upon birch.[1] The infusion is used in folk-medicine and more serious, but still dubious, medicinal claims have been made. Several of the triterpenoids typical of Polyporaceae have been described from this fungus: lanosterol, obliquol [2] (probably ≡ inotodiol [3]), and other triterpene alcohols and acids.[3] Characterisation of the phenolic material is less clear-cut. Shivrina and co-workers [4] found that prolonged acid digestion of chagi afforded small amounts of monomeric phenols including *p*-hydroxybenzoic, vanillic, and syringic acids, sinapic aldehyde, and some methoxycinnamic acids. They consider chagi to be a humin-like material synthesised by the fungus from breakdown products of the host lignin. This description interested us since in *Polyporus hispidus* we had described the *in vivo* formation of a lignin-like polymer from the pigment hispidin,[5] in which at least part of the molecule derives from the host lignin.[6] Through Dr. B. K. Bakshi of the Forestry Commission we obtained samples of Scottish chaga for examination.

The chagi pigment was obtained by percolation of the crushed chaga with wet steam; this product is clearly polymeric and covers a wide range of molecular weights. A small proportion can be dissolved in ether, rather more in dioxan or methanol, about half can be redissolved by boiling with water, and nearly all dissolves in aqueous alkali. The pigment is precipitated from water by dilute acid or ammonium sulphate, etc., and gives a very dark precipitate with ferric chloride. Some fractionation can be achieved by selective extraction with organic solvents, by chromatography on polyamide powders, or by counter-current distribution, but there is no sharp distinction between the fractions, which seem to differ partly in the proportion of free phenolic groups and partly in the degree of polymerisation. For most of our work we used the water-soluble material, reprecipitated by cautious acidification. An excess of acid seems to cause further polymerisation.

The crude pigment contains more oxygen, and less methoxyl, than typical lignins (MeO, 6·3% in our samples, 5·8% in Russian samples,[4] compared with 14—15% in lignins). Like humic acids, it gives an appreciable semiquinone-type electron spin resonance (e.s.r.) signal, but it contains relatively few quinonoid groups; the colour is mainly due to non-specific absorption (see below) and is only slightly affected by borohydride or dithionite. We found that various types of hydrolytic degradation (*e.g.*, heating with 50% phosphoric acid under reflux, with hydrazine, with alkali, with ethanol in sealed tubes, etc.) always gave very small yields of degradation products, but methods applicable to lignins were rather more successful. For example, nitrobenzene oxidation gave a *ca.* 25% yield of identifiable products, mainly *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde with the corresponding acids and guiacol. Similarly, degradation with sodium amalgam gave *ca.* 10% of a mixture of acids in

[1] K. St. G. Cartwright and W. P. K. Findlay, "Decay of Timber and its Prevention," H.M.S.O., London, 1958, p. 146.
[2] R. S. Ludwiczak and U. Wrzeciono, *Roczniki Chem.*, 1960, **34**, 77, 1627, and 1701.
[3] E. V. Loviagina and A. N. Shivrina, *Biokhimya*, 1962, **27**, 794.
[4] E. V. Loviagina, E. G. Platonova, and A. N. Shivrina, *Biokhimya*, 1958, **24**, 59; 1960, **25**, 640; *Doklady Akad. Nauk S.S.S.R.*, 1960, **132**, 1444.
[5] J. D. Bu'Lock, P. R. Leeming, and H. G. Smith, *Experientia*, 1961, **17**, 553; *J. Chem. Soc.*, 1962, 2085.
[6] J. D. Bu'Lock and B. Kaye, unpublished observations.

which were identified *p*-hydroxybenzoic, vanillic, and syringic together with guiacylpropionic (hydroferulic) and syringylpropionic (hydrosinapic) acids. These degradations confirm the conclusion [4] of Loviagina and co-workers that the monomer units of chagi are C_6C_1 and C_6C_3 phenolics very similar to those of hardwood lignins, *i.e.*, of the lignin of the birch trees upon which chaga grows. Our material appears to contain vanillyl, syringyl, and *p*-hydroxyphenyl residues in the approximate ratio 8 : 3 : 1, but in the Russian material syringyl groups usually predominate.

The u.v. spectrum of chagi is discussed subsequently. The i.r. spectrum (Nujol mull) is dominated by broad bands due to H-bonded OH, carboxyl and other carbonyl group, aromatic nuclei, and phenolic and ether groups, the principal bands being *ca.* 3200, 1690—1700, 1600, 1500, 1230, and 1120 cm.$^{-1}$. The most striking difference from the spectrum of typical lignins is the strong absorption in the carbonyl region; most of this absorption is due to carboxyl, shifting to 1710—1720 cm.$^{-1}$ with diazomethane and to 1580 cm.$^{-1}$ in the Na salt. There are no strong bands between 1600 and 1690 cm.$^{-1}$, from which we conclude that few of the carboxyl groups are of the αβ-unsaturated (cinnamic) type. The absorption in the carbonyl region is removed by treatment with lithium aluminium hydride but is not greatly affected by sodium borohydride.

Elementary analysis and methoxyl content approximate an empirical formula $[C_2H_2O]_n$ or, per methoxyl group, $C_{22}H_{18}O_9$; birch lignin approximates to $[C_{2\cdot5}H_{2\cdot7}O]_n$, or per methoxyl group, $C_{10}H_{11}O_4$. In comparison, therefore, chagi is more highly oxygenated, and contains less of this oxygen in methoxyl groups. Chagi can be methylated in various ways, and the analytical data for the products provide further information. We suppose that diazomethane reacts with carboxyl groups and with the more reactive phenolic groups, and that the methyl ester groups thus formed are susceptible to alkali. With dimethyl sulphate and alkali, however, some further, more H-bonded, phenolic groups are etherified but carboxyl groups are unaffected and can subsequently be methylated with diazomethane. The analytical data for the products of various successive treatments are fairly self-consistent (Table), and the i.r. spectra of these products accords with expectations.

Treatment	OMe (%)
None	6·3—6·5
Aqueous alkali	6·6—6·7
Diazomethane	19·9—22·1
(i) Aqueous alkali, (ii) diazomethane	21·5—22·8
(i) Diazomethane, (ii) aqueous alkali	12·2—12·8
Dimethyl sulphate and alkali	18·2—19·8
(i) Diazomethane, (ii) dimethyl sulphate and alkali	19·0
(i) Dimethyl sulphate and alkali, (ii) diazomethane	25·6—26·8

If for convenience we take 480 as the molecular unit of chagi, which contains one methoxyl group, the data in the Table require that this unit also contains 1·0 reactive phenolic hydroxyl, about 1·1 less reactive (H-bonded) phenolic hydroxyl, and about 1·3 carboxyl groups. A similar proportion of acidic groups is revealed by electrometric back-titration of an alkaline solution of chagi; the shape of the titration curve indicates, per original methoxyl group, about 1·1 acidic phenolic groups and about 1·4 carboxyl groups. The pK_a of these two kinds of acidic grouping are *ca.* 9·5—10·0 and 4·5, respectively, the latter value being consistent with the deductions from i.r. data.

Chagi contains more hydrogen than would be expected for a polymer based simply on hydroxybenzoic acid derivatives, and the acidic functional groups account for no more than half the oxygen content. Since treatment with alkali does not significantly alter either the methoxyl content or the content of groups reactive to diazomethane (Table), the remaining oxygens are not present in ester groups. Equally, the i.r. data rule out any large proportion of aldehyde or ketone functions. The most fully-methylated product (Table) still shows appreciable OH absorption around 3400 cm.$^{-1}$ in the i.r. and this is ascribed to aliphatic hydroxyl groups. The presence of some aliphatic side-chains, which this implies, would also explain the hydrogen content of the chagi. In addition, we observed that in the breakdown of chagi by reductive cleavage with sodium amalgam the total alkali-soluble product was readily, and reversibly, oxidised in air. Such quinol- or catechol-like behaviour is absent in chagi itself, and this suggests that a further part of the oxygen content is in aryl benzyl ether groups, which on reductive fission would generate new phenolic centres.

The ultraviolet–visible absorption spectrum of chagi shows strong, but superficially non-specific, absorption ($E^{1\%}_{1\,cm.} \simeq 200$ at 270 mμ, 100 at 325 mμ), with a slight inflexion at *ca.* 275—285 mμ. This spectrum is intensified at longer wavelengths when alkali is added. Analysis of the difference spectrum, using the methods developed by Aulin-Erdtman [7] allows an estimate of the content of various ionisable chromophores. By this method, the total number of phenolic groups ionised up to pH 12 is found to be *ca.* 0·9—1·05 per original OMe group (which agrees with the number of groups etherified by diazomethane). The most conspicuous feature of the Δε curve is a broad maximum at 350—360 mμ, but on analysis the curve is found to result from *ca.* 5% *p*-hydroxycinnamoyl chromophores, *ca.* 15% *p*-hydroxybenzoyl chromophores, and *ca.* 80% *p*-hydroxybenzoate and *p*-hydroxyphenyl chromophores. The predominance of the hydroxybenzoate chromophore is significant and accords with other data.

In the breakdown of lignin by white-rot fungi [8-10] the initial attack, by extracellular enzymes, involves the aliphatic side-chains of the phenylpropane units. Units may be split off as arylglycerol, cinnamoyl, or arylpyruvic acid derivatives, but these mainly undergo

[7] G. Aulin-Erdtman, *Svensk kem. Tidskr.*, 1958, **70**, 145; *Svensk Papperstidn.*, 1953, **56**, 287.
[8] M. E. K. Henderson, *Nature*, 1955, **175**, 634.
[9] T. Fukuzumi, *Bull. Agric. Chem. Soc. Japan*, 1960, **24**, 728.
[10] H. Ishikawa, W. J. Schubert, and F. F. Nord, *Arch. Biochem. Biophys.*, 1963, **100**, 131, and 140.

further oxidation to benzoic acids. At the same time methoxyaryl groups can be demethylated to form free phenols.[10] Such processes would account completely for the range of functional groups, and the type of sub-units, found in chagi. Similar processes also affect the still-polymeric lignin residues—loss of methoxyl, increase in hydroxyl, and appearance of carboxyl—and indeed the i.r. spectrum of a decayed lignin [9] is very similar to that of chagi. The essential difference between chagi and a highly-decayed lignin lies in their origin—to reach the developing sporophore where chagi accumulates, the monomeric lignin breakdown products must be transported, as such, by the invasive mycelium of the fungus, and then at the site of their accumulation they must undergo repolymerisation.

From the properties of chagi we conclude that this final polymerisation is of the type effected by oxidative coupling of the phenolic units, so that the polymer is mainly linked by C–C and C–O–C bonds and is very resistant to hydrolytic breakdown. The case of *Poria obliqua* is thus very similar to that of *Polyporus hispidus*, with the difference that in *P. hispidus* at least part of the lignin breakdown products is somewhat modified by conversion into the C_{13} unit of hispidin.[5,6]

EXPERIMENTAL

Extraction.—The sporophore material was broken to a coarse powder and percolated with wet steam. The extract was cooled, filtered to remove solids, and carefully brought to pH 2·5 with acid. The gelatinous precipitate was centrifuged down and dried in a vacuum oven (yield 10—15%). Batches of this material were purified as required by dissolving in hot water and reprecipitation at pH 2·5. (Typical analysis: C, 56·7; H, 4·8; OMe, 6·3%; N and ash negligible.)

Nitrobenzene Oxidation.—Chagi (12 g.) in 2N-sodium hydroxide (720 ml.) mixed with nitrobenzene (96 ml.) was heated in a stirred autoclave (165°, 2·5 hr.); the nitrobenzene was removed with steam and the mixture was acidified with aqueous hydrochloric acid, left for 24 hr., and filtered. The filtrate was continuously extracted with ether for 24 hr. and the ethereal extract fractionated by successive extractions with 20% sodium bisulphite, 8% sodium hydrogen carbonate, and 5% sodium hydroxide. Aldehydes from the sodium bisulphite extract (2·5 g.) were separated by partition chromatography between acid-washed wet "Celite 535" and 2-methylheptane–benzene–water mixtures; a sample of crude aldehydes (150 mg.) thus afforded crystalline vanillin (90 mg.), syringaldehyde (35 mg.), and *p*-hydroxybenzaldehyde (15 mg.). Acids from the sodium hydrogen carbonate extract (0·9 g.) were chromatographed between silica gel loaded with 2M-potassium dihydrogen phosphate and n-butanol–chloroform mixtures; crude acids (150 mg.) gave crystalline syringic acid (36 mg.), vanillic acid (98mg.), and *p*-hydroxybenzoic acid (12 mg.). Paper chromatography of the phenolic (sodium hydroxide-soluble) fraction (0·1 g.) revealed guiacol as the main component.

Reduction with Sodium Amalgam.—Chagi (2·5 g.) in 10% aqueous sodium hydroxide (100 ml.) was heated under reflux in a stream of nitrogen with 3% sodium amalgam (350 g. in several lots over 48 hr.). The solution (clear yellow, turning dark brown on admission of air) was cooled, acidified, and the supernatant extracted with ether. Acidic material recovered from the ether extract by washing with sodium hydrogen carbonate was shown by paper chromatography (using n-butanol–acetic acid and n-butanol–aqueous ammonia) to contain *p*-hydroxybenzoic, vanillic, syringic, guiacylpropionic (hydroferulic), and syringylpropionic (hydrosinapic) acids together with at least ten minor unidentified components.

Methylation.—(a) *Dimethyl sulphate.* The material for methylation (1 g.) was dissolved in 2% aqueous sodium hydroxide (100 ml.), an equal volume of acetone was added, and dimethyl sulphate (25 ml.) and 10% aqueous sodium hydroxide (40 ml.) stirred in over 5—6 hr., at 25 or 100°. Water was then added and after standing overnight the solid product was filtered off (analytical data given in the Table).

(*b*) *Diazomethane.* The material for methylation (1 g.), dissolved in methanol (100 ml.), and was treated with a solution of diazomethane [from *N*-nitrosomethylurea (10 g.)] in ether (100 ml.), for 24 hr. at 25° with occasional agitation; water (100 ml.) was added, the methanol and ether distilled off under reduced pressure, and the solid product filtered off (analytical data given in the Table).

Difference Spectra.—Buffer, pH 12, contained boric acid (6·2 g.) in 0·1N-sodium hydroxide (to 1 l.); buffer, pH 6, contained 0·2N-potassium dihydrogen phosphate (495 ml.) and 0·1N-sodium hydroxide (113 ml.) in water (to 2 l.). The chagi was made up as a 0·1—0·2% solution in pH 12 buffer; fresh solution (2·0 ml.) was diluted to 25 ml. with pH 12 buffer, and a further 2·0 ml., together with 0·1N-sulphuric acid (2·0 ml.) was diluted to 24 ml. with pH 6 buffer.

Titration.—Chagi (0·75 g.) in N-sodium hydroxide (25 ml.) was titrated with N-hydrochloric acid with vigorous stirring, allowing any precipitate to redissolve before each pH reading. The pH curve was not affected by allowing the alkaline solution to stand before titration.

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