SEP 11 1965

FEB 8 1965

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BIOLOGICAL POLYMERS RELATED TO CATECHOL. UPR AND IR STUDIES
OF MELANIN, TANNIN, LIGNIN, HUMIC ACID AND HYDROXYQUINONES^a

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ABSTRACT

This paper was submitted for publication in the open literature at least 6 months prior to the issuance date of this Microcard. Since the U.S.A.E.C. has no evidence that it has been published, the paper is being distributed in Microcard form as a preprint.

Melanin, lignin, tannin and humic acid have been shown to exhibit similar paramagnetic properties. All of these biological polymers are stable organic free radicals. On conversion to the solid sodium salts, the polymers show a greatly enhanced radical content; this reaction is reversible. On the basis of studies of model compounds, hydroxyquinone and quinhydrone structures are proposed to account for these properties.

INTRODUCTION

Recent experiments in this laboratory with humic acid (1,2,3) and lignin (4) have shown some remarkable similarities in their paramagnetic properties. The extension of these studies to two other naturally-occurring macromolecules, melanin and tannin, confirm our assumptions that these four substances contain radical species which are closely

-
- a. This work was supported in part by an American Cancer Society Institutional Grant and by a grant from the United States Atomic Energy Commission (AT(11-1)908).
 - b. On sabbatical leave at the Forest Products Laboratory, Madison, Wisconsin, until August, 1963.
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RELEASED FOR ANNOUNCEMENT
IN NUCLEAR SCIENCE ABSTRACTS

FEB 11 1965

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related structurally. The paramagnetism probably arises from a charge-transfer complex based on a phenolic-quinoid system as well as from an ordinary semiquinone.

Since 1960, a number of investigators have shown that four biological macromolecules contain stable organic free radicals. These are melanin (5,6,7), tannin (8), lignin (8,9) and humic acid (9). All of these substances are known to be involved in one or more of the following processes: wound tissue formation in plants, disease resistance mechanisms in plants, decaying plant tissue and epithelial and other types of pigmentation. Thus, their structures, properties and metabolism are of special interest to a large number of physical, biological and medical scientists. All substances are presumed to arise from the oxidative polymerization of a simple catechol-derived phenol in biological tissues, by way of the following sequences of reactions:

Melanin: Mason et al. (5)

Lignin: Freudenberg (10)

Condensed Tannins: Hathway (11)

Humic Acid: Flaig (12)

Previous investigators have proposed models to account for the existence of stable organic free radicals in some of these polymers. Evidence from this Laboratory (1,2,4) strongly supported the hypothesis that lignin and humic acid both contain semiquinone radical species coexistent with diamagnetic quinhydrone moieties. Pullman and Pullman (7) and Longuet-Higgins (13) have suggested that the paramagnetism of melanin is due to thermal excitation of electrons from a delocalized molecular orbital band to an unfilled molecular orbital band, somewhat analogous to a semiconductor. Mason *et al.*, (5), on the other hand, favors a semiquinone polymer model for melanin. Although orthoquinoid structures have been suggested for condensed tannin (11), these did not include proposals for free radical species.

To evaluate these ideas and to extend our previous EPR studies on the nature of the radical species in biologically-occurring polymers, we have compared the spin content and spectral properties of selected samples of lignin, humic acid, melanin and tannin, as well as a number of model compounds.

EXPERIMENTAL

Spectra

Paramagnetic resonance spectra were obtained with a Varian 100 kilocycle modulation spectrometer. Spin concentrations were estimated by comparison with solid diphenylpicrylhydrazyl. Number of radicals was assumed to be proportional to signal height times signal width squared.

Samples

Squid melanin was obtained from the sacs of Loligo opalescens, acquired from Dahl Company, 2017 Second St., Berkeley, California, by mechanical separation from an aqueous suspension.

DOPA melanin was prepared from dihydroxyphenylalanine and tyrosinase according to the procedure of Mason (5).

Humic acid (Podzol B hydrolyzed) was prepared from a sample acquired from A. Burges (1).

Muck Humic acid is a product of Fluka Company, Bucks, S.G., Switzerland.

Catechol humic acid was made by the oxidation of catechol in the presence of four amino acids, by K. B. Roy, Department of Macromolecules, IACS, Calcutta, India.

Catechin polymer was prepared by dissolving 100 mg. of d-catechin in water, adding 4 mg. of tyrosinase and stirring in the presence of air for four hours. The precipitate resulting from the evaporation of this solution was dried at room temperature under vacuum.

Lignin samples were obtained from a variety of sources, as previously described (4).

Gambir tannin was obtained from the methyl ethyl ketone extract of the commercial product known as "Gambir", which is a dried aqueous extract of the leaves and twigs of Uncaria gambir (Hunter), Roxburgh, (Fam. Rubiaceae) marketed by S. B. Penick and Company, New York.

Alkannin and quinizarin were supplied by Aldrich Chemical Company, Milwaukee, Wisconsin. Alizarin was obtained from California Corporation for Biochemical Research. Alkannin was recrystallized from chloroform-petroleum ether, alizarin from absolute ethanol.

Sodium Salts

These were prepared as previously outlined (3), by precipitating the salt from an aqueous NaOH solution with absolute ethanol.

Infrared Absorption Spectra

All spectra were determined with a Beckman IR-4 spectrophotometer on KBr pellets of the samples.

RESULTS

Free Acids

All samples showed an appreciable free radical content when measured in the solid state by EPR spectrometry (Table I). The radical species was remarkably stable with respect to time and chemical treatment. Both humic acid (3) and melanin were stable under prolonged acid hydrolysis; both yielded increased spin contents after the hydrolysis. Squid melanin was particularly resistant to acid and alkaline reagents; treatment with concentrated sulfuric acid appeared to dehydrate it and simultaneously to raise its spin content by a factor of 10 (See Table I). Unlike air-dried DOPA melanin, it did not dissolve in base.

TABLE I

Spin Concentrations of Solid Samples^(*)

I. Biological Polymers

<u>Sample</u>	<u>Acid Spins/gram</u>	<u>Sodium Salt Spins/gram</u>
Melanin (squid)	1.9×10^{18}	No salt
oven dried	2.1×10^{19}	"
treated with cold H_2SO_4	1.9×10^{19}	"
treated with hot H_2SO_4	3.5×10^{19}	"
oven dried, treated with NaOH	1.1×10^{19}	"
Melanin (DOPA)	2×10^{18}	1.0×10^{20}
oven dried	5×10^{18}	No salt
Tannin		
Gambir	0.7×10^{16}	3.5×10^{18}
Catechin polymer	0.3×10^{18}	3.1×10^{19}
Humic Acid		
Podzol (3)	0.2×10^{18}	1.0×10^{19}
Muck (3)	0.6×10^{18}	1.6×10^{19}
Catechol (synthetic)	2.3×10^{18}	5.4×10^{19}
Lignin		
Brauns native spruce (4)	0.5×10^{17}	5.0×10^{18}
Bjorkman spruce (4)	1.0×10^{17}	1.5×10^{18}
Kraft yellow pine (4)	3.0×10^{17}	$1.0-3.0 \times 10^{19}$

TABLE I (Continued)

II. Quinones

<u>Sample</u>	<u>Acid Spins/gram</u>	<u>Sodium Salt Spins/gram</u>
Quinhydrone	not detectable	2.2×10^{19}
✓ Alizarin	2×10^{16}	4.5×10^{20}
Alkannin	2.8×10^{17}	9.3×10^{18}
✓ Quinizarin	2.8×10^{17}	3.4×10^{19}

⊗ Estimated by comparison to a solid sample of diphenylpicrylhydrazyl using signal height times width squared as a measure of paramagnetism.

DOPA melanin, when prepared in vitro from dihydroxyphenylalanine and dried at room temperature under vacuum, was quite soluble in 1 N base. However, after it was dried at 110°C for six hours, its solubility in base was markedly reduced, and we could not form a sodium salt from this material.

Inasmuch as a semiconductor-type model has been proposed for melanin, we felt it would be instructive to examine the temperature-dependence of the EPR signal intensity in this material. The results are plotted in Figure 1. It is apparent that both squid and DOPA melanin obey an ordinary Curie Law $1/T$ type relationship fairly well, indicating a temperature-independent unpaired spin concentration. This would seem to rule out an orbital band picture of the paramagnetism.

Sodium Salts

With the exception of squid melanin, all macromolecules showed a 10 to 100 fold increase in spin content (as measured by EPR spectrometry), when converted to the solid sodium salts (Table I). These changes were reversible; when the free acids were regenerated from the salts, the spin contents returned to their original values. No significant change in spin content was observed between salts formed under aerobic conditions and salts formed under nitrogen, indicating the apparent absence of air oxidation. However, partial oxidation of sensitive phenols such as catechol and hydroquinone derivatives could take place in alkaline medium, even under the "anaerobic conditions" of our studies. The products of oxidation would be quinhydrones, or semiquinone radical salts. When catechol and hydroquinone were converted rapidly to salts under nitrogen, the products showed spin concentrations of the order of 10^{17} spins/gram (or 10^{19} spins/mole), indicating that 0.01% of the molecule had been oxidized by a residual amount of oxygen in the solutions or atmosphere. This should be compared to the radical contents of the biological polymers and hydroxyquinones, which are in the range 10^{19} spins/gram (10^{21} - 10^{23} spins/mole). Thus, the magnitude of the spin content of the sodium salts of biological polymers and hydroxyquinones supports the assumption that simple phenolic oxidation is not a major factor in formation of these radicals.

It should be also noted that resorcinol derivatives are not readily oxidized to radical salts under these conditions, as has been observed in our laboratory as well as elsewhere (14, 15).

Infrared Absorption Spectra

The major absorption bands of a number of samples of melanin, lignin, tannin, humic acid and quinones are shown in Table II. All samples exhibit almost identical bands in the OH region (2.90μ). A band at 6.4μ

TABLE II

Principal Infrared Absorption Bands

I. Biological Polymers

<u>Salts</u>		<u>Bands in μ</u>		
Kraft Lignin	2.90		6.3	
DOPA melanin	2.90		6.3	
Humic Acid	2.90	6.1	6.3	6.4 (weak)
Bjorkman Lignin	2.90	6.1	6.2	
Catechin Polymer	2.90		6.3	6.4 (weak)
<u>Acids</u>				
Kraft Lignin	2.90	5.9	6.1	
DOPA Melanin	2.90	5.9	6.1 (shoulder)	
Humic Acid	2.90	5.9	6.1	
Squid Melanin	2.90	5.9	6.1	
Bjorkman Lignin	2.95	5.9	6.1 (sh.)	6.3

II. Quinones

<u>Salts</u>				
Quinhydrone	2.95 (low)	6.2	6.3 (sh.)	
Quinizarin	2.95	6.1	6.3 (sh.)	6.4
Alkannin	2.90		6.3	6.4
<u>Acids</u>				
Quinhydrone	2.95 } 3.1 }	6.15	6.3 (medium)	
Quinizarin (1,4-dihydroxyanthraquinone)	-----	6.15	6.3	6.4 (weak)
Alkannin	2.95	6.1 (infl.)	6.25	6.4 (low)

appears for the salts of catechin polymer (IIIa) and for alkannin and quinizarin. Matsunaga (16) has reported a similar absorption for the sodium salt of the quinhydrone of biphenquinone; Chandross (17) and others have assigned this band to the phenoxy radical ion. High absorption in the 6.3μ region is shown by most of the salts, a property noted by Elofsen (18) when he examined the sodium salts of coal, peat and artificial humic acids. These could arise from carboxylate or phenoxide ions. The fact that all biological samples, except Bjorkman lignin (which has a very low carboxyl content) show a disappearance of the 5.9μ band in the sodium salts with the emergence of a 6.3μ band, would tend to confirm this supposition. A high absorption at 6.1μ is characteristic of H-bonded quinones or unsaturated carbonyls and is shared by all samples.

DISCUSSION

The results of this investigation demonstrate that the four classes of naturally-occurring macromolecules exhibit rather similar properties. These may be summarized as follows:

1. All are stable free radicals.
2. All base-soluble compounds can be reversibly converted to sodium salts, with an attendant 10 to 100 fold increase in unpaired spin content.
3. All samples show absorption bands in the infrared absorption spectra on conversion to their sodium salts which are consistent with hydroxyquinone structures.

It would be useful to accommodate these results to structures which are also consistent with the known structural features of the macromolecules. In earlier communications (1,2,3,4) we had proposed a mixed system containing an ordinary semiquinone radical species and a diamagnetic quinhydrone species

for the acid forms of lignin and humic acid. Upon basification, the quinhydrone would be converted to the radical anion salt (*); such a change would be readily detected by EPR spectrometry (Figure 2) and indeed it was (1,2,3,4).

In the light of the present results, we can propose an alternative model system which contains both quinoid and hydroquinoid moieties in the same molecule. Such a system can be considered as an internal charge-transfer complex, and could exhibit some paramagnetism even in the neutral or "acid" form, as does quinizarin (VII) and related compounds, and perhaps also biphenyls (VIII) related to tannin and lignin, although we have no data on these.

(*) The radical anion, which is in equilibrium with the diamagnetic dianion, is probably stabilized by adsorption on the surface of the solid sodium salt of quinhydrone (19). Thus, its concentration is never more than a few mole percent of the total quinhydrone content, and varies considerably with reaction conditions.

Both VIIa and VIIIa would be stabilized by basification, with a net enhancement of the paramagnetism (Table I). For N-containing polymers, such as melanin and humic acid, additional charge-transfer species could arise, as illustrated by 1,4-bis(dimethylamino)-anthraquinone (IX) or a species such as X.

In the solid state, intermolecular charge-transfer complexes would be expected to predominate instead of biradicals such as VIIa through Xa. Thus, monoradical species such as XIa should be present and stabilized by base.

Our studies have shown the dominant species in the solid to be the doublet state monoradical (such as XIa); experiments to establish the existence of some triplet state character (biradicals such as VIIa through Xa) were not conclusive.

Parenthetically, it should be mentioned that Russell (20) has recently shown that radicals may be generated with strong base from compounds containing donor and acceptor functions (such as hydroxyl and carbonyl); Yang (21) has demonstrated that internal charge-transfer polymers can be made with N-donor groups.

Therefore, it appears that the necessary condition for radical generation in base probably exists in all four macromolecules: namely, the presence of a donor-acceptor system with a readily ionizable hydrogen. In all cases, this can be satisfied with a quinone-hydroquinone species in the same molecule. Additionally, nitrogen may also be a donor, as well as hydroxyl; protonation of the nitrogen atom should reduce the charge-transfer effect.

We suggest that any structures proposed for melanin, tannin, lignin or humic acid should include a quinone-hydroquinone system.

REFERENCES

1. Steelink, C. and Tollin, G., *Biochim. Biophys. Acta*, 59, 25 (1962).
2. Tollin, G., Reid, T. and Steelink, C., *Biochim. Biophys. Acta*, 66, 444 (1963).
3. Steelink, C., *Geochim. Cosmochim. Acta.*, 28, 1615 (1964).
4. Steelink, C., Reid, T. and Tollin, G., *J. Am. Chem. Soc.*, 85, 4048 (1963).
5. Mason, H.S., Ingram, D.J.E. and Allen, B., *Arch. Biochem. Biophys.*, 86, 225 (1960).
6. Leach, K. and Edwards, M.L., *Nature*, 190, 1005 (1961).
7. Pullman, A. and Pullman, B., *Biochim. Biophys. Acta*, 54, 384 (1961).
8. Rex, R.W., *Nature*, 188, 1185 (1960).
9. Kleinert, T.N. and Morton, J.R., *Nature*, 196, 334 (1962).
10. Freudenberg, K., "Fourth International Congress of Biochemistry", Volume II, Pergamon Press, N.Y., 1959, p. 121.
11. Hathway, D.E., *Biochem. J.*, 70, 34 (1958).
12. Flaig, W., *Suomen Kemistilehti A*, 33, 229 (1960).
13. Longuet-Higgins, H.C., *Arch. Biochem. Biophys.*, 86, 213 (1960).
14. Musso, H., Figge, K. and Becker, D., *Chem. Ber.*, 94, 1107 (1961).
15. Stone, T.J. and Waters, W.A., *J. Chem. Soc.*, 4302 (1964).
16. Matsunaga, Y., *Can. J. Chem.*, 38, 1172 (1960).
17. Chandross, E.A. and Kreilick, R., *J. Am. Chem. Soc.*, 86, 118 (1964).
18. Elofson, R.M., *Can. J. Chem.*, 35, 926 (1957).
19. Bijl, D., Kainer, H. and Rose-Innes, A.C., *Nature*, 174, 830 (1954).
20. Russell, G.A. and Strom, E.T., *J. Am. Chem. Soc.*, 86, 744 (1964), and previous papers, especially *ibid*, 84, 4156 (1962).
21. Yang, N.C. and Gaoni, Y., *J. Am. Chem. Soc.*, 86, 5023 (1964).

REFERENCES

1. Steelink, C. and Tollin, G., *Biochim. Biophys. Acta*, 59, 25 (1962).
2. Tollin, G., Reid, T. and Steelink, C., *Biochim. Biophys. Acta*, 66, 444 (1963).
3. Steelink, C., *Geochim. Cosmochim. Acta.*, 28, 1615 (1964).
4. Steelink, C., Reid, T. and Tollin, G., *J. Am. Chem. Soc.*, 85, 4048 (1963).
5. Mason, H.S., Ingram, D.J.E. and Allen, B., *Arch. Biochem. Biophys.*, 86, 225 (1960).
6. Leach, K. and Edwards, M.L., *Nature*, 190, 1005 (1961).
7. Pullman, A. and Pullman, B., *Biochim. Biophys. Acta*, 54, 384 (1961).
8. Rex, R.W., *Nature*, 188, 1185 (1960).
9. Kleinert, T.N. and Morton, J.R., *Nature*, 196, 334 (1962).
10. Freudenberg, K., "Fourth International Congress of Biochemistry", Volume II, Pergamon Press, N.Y., 1959, p. 121.
11. Hathway, D.E., *Biochem. J.*, 70, 34 (1958).
12. Flaig, W., *Suomen Kemistilehti A*, 33, 229 (1960).
13. Longuet-Higgins, H.C., *Arch. Biochem. Biophys.*, 86, 213 (1960).
14. Musso, H., Figge, K. and Becker, D., *Chem. Ber.*, 94, 1107 (1961).
15. Stone, T.J. and Waters, W.A., *J. Chem. Soc.*, 4302 (1964).
16. Matsunaga, Y., *Can. J. Chem.*, 38, 1172 (1960).
17. Chandross, F.A. and Kreilick, R., *J. Am. Chem. Soc.*, 86, 118 (1964).
18. Flosson, R.M., *Can. J. Chem.*, 35, 926 (1957).
19. Bijl, D., Kainer, H. and Rose-Innes, A.C., *Nature*, 174, 830 (1954).
20. Russell, G.A. and Strom, F.T., *J. Am. Chem. Soc.*, 86, 744 (1964), and previous papers, especially *ibid*, 84, 4156 (1962).
21. Yang, N.C. and Gaoni, Y., *J. Am. Chem. Soc.*, 86, 5023 (1964).

FIG. 1

FIG. 2