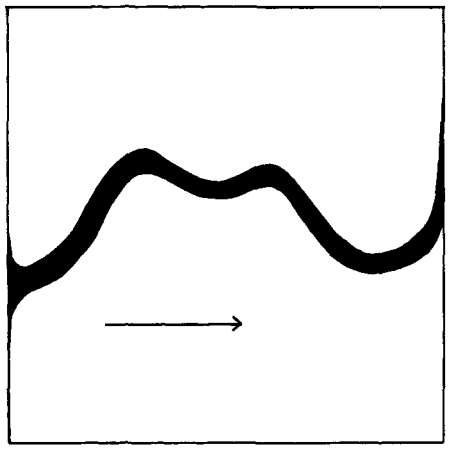

Fig. 1.—Carboxypeptidase in 2.5 *M* NaCl–0.02 *M* Veronal, pH 7.5. Sedimentation is from left to right after 7900 sec. at 59,780 r.p.m. at 25°.

leading to the inference that an electrostatic attraction might be the basis of the polymerization.

We have now observed double boundary formation with carboxypeptidase A at very *high* ionic strengths. This seems to be the first specific example of polymerization of a proteolytic enzyme at these high salt concentrations. Hence the physicochemical basis of this polymerization must differ from that for chymotrypsin and mercuripapain since here an electrostatic repulsion must apparently be overcome before the polymerization occurs.

Carboxypeptidase A has been sedimented in 1 *M* NaCl at concentrations up to 18 mg./ml.[7] A single boundary was found but the concentration dependence of the sedimentation coefficient was positive over the whole range. During a new sedimentation study of the enzyme, in 2 *M* NaCl and up to a concentration of 28 mg./ml., only a single boundary was observed, but at concentrations of enzyme greater than 22 mg./ml. the concentration dependence of the sedimentation coefficient became negative, similar to that which is usually seen. The $s^0_{20,w}$ was 3.3 *S*.

The solubility of the enzyme is a limiting factor in obtaining higher concentrations. Previous investigations of the influence of ionic strength upon its solubility have been extended, and it was found that in 2.5 *M* NaCl a concentration of 44.3 mg./ml. of enzyme could be obtained. Under these conditions the sedimentation pattern exhibits two boundaries (Fig. 1). When this solution is diluted to a concentration of 37.9 mg./ml., two boundaries also appear, but the area under the faster boundary decreases, while that under the slower boundary remains constant. These phenomena are characteristic of a rapidly equilibrating, polymerizing system where the polymer formed consists of more than two monomers.[4,5]

Table I gives the $s_{20,w}$ values, as a function of concentration, for the slow peak, for the fast peak, and for the point dividing the area of the pattern in half. The theoretical prediction[4,5] that the $s^0_{20,w}$ of the slow peak should agree with that of the monomer is borne out, since the value of $s^0_{20,w}$ for the slow peak is 3.3 *S*, in good agreement with the extrapolated $s^0_{20,w}$ in 2 *M* NaCl. Since carboxypeptidase, in contrast to chymotrypsin and papain, exhibits double boundary formation at high ionic strength, electrostatic repulsion must apparently be overcome to allow polymerization. That this is not an effect of enzyme concentration alone is demonstrated by the sedimentation pattern obtained at an enzyme concentration of 28 mg./ml. In 2 *M* NaCl the patterns are symmetric but in 2.5 *M* NaCl marked asymmetry develops.

(7) J. A. Rupley and H. Neurath, *J. Biol. Chem.*, **235**, 609 (1960).

TABLE I

SEDIMENTATION COEFFICIENT OF CARBOXYPEPTIDASE A

(2.5 *M* NaCl–0.02 *M* Veronal, pH 7.5)

Concentration, mg./ml.	$s_{20,w}$, *S* Slow peak	Fast peak	Midpoint
44.3	3.4	7.1	5.0
37.9	3.3	6.9	4.3
27.4	3.3		4.0
20.9	3.3		3.7
13.3	3.3		3.4

Over the concentration range from 2 to 44.3 mg./ml. no concentration-dependent change in optical rotation can be detected in the wave length range 325–600 mμ. The profound change in the relative concentration of the different species present in the solution therefore is not reflected in those parameters conventionally held to be dependent upon conformation.

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RECEIVED SEPTEMBER 20, 1963

On the Nature of the Free-Radical Moiety in Lignin[1,2]

Sir:

Recent reports[3,4] of the existence of a stable free radical in lignin and wood pulp have prompted us to investigate the nature of this species by electron paramagnetic resonance spectrometry. The results of a study of the paramagnetism of a number of well defined lignin preparations strongly support the existence of a semiquinone-type radical entity, coexistent with a diamagnetic quinhydrone moiety.

All solid lignin preparations tested showed significant unpaired spin content when measured in a 100-kc. modulation Varian e.p.r. spectrometer (Table I).

TABLE I

FREE-RADICAL CONTENT OF VARIOUS LIGNIN PREPARATIONS[a]

Sample	Spins/gram	Estimated mol. wt.	Spins/mole
Brauns native spruce	0.5×10^{17}	1,000[b]	5×10^{19}
Bjorkman spruce	1.0×10^{17}	11,000[c]	1.1×10^{21}
Klason spruce	0.4×10^{17}	5,000[b]	1.5×10^{20}
Klason redwood	0.9×10^{17}		
Decayed western hemlock wood meal	0.9×10^{17}		
Kraft yellow pine	3.0×10^{17}	5,000[b]	1.5×10^{21}
Kraft-treated native spruce	4.0×10^{17}		
Calcium ligninsulfonic acid	1.5×10^{17}	10,000[b]	3.0×10^{21}
Indulin AT	3.0×10^{17}		

[a] Spin concentrations estimated by comparison with solid diphenylpicrylhydrazyl. Number of radicals was assumed to be proportional to signal height times signal width squared. [b] F. E. Brauns, "The Chemistry of Lignin," Academic Press, New York, N. Y., 1952, p. 192. [c] F. E. Brauns and D. A. Brauns, "The Chemistry of Lignin. Supplemental Volume," Academic Press, New York, N. Y., 1960, p. 179.

(1) Presented at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept., 1963.

(2) This work was supported in part by the United States Atomic Energy Commission (Grant No. AT(11-1)908) and by the Petroleum Research Fund (Grant No. 970-A4).

(3) R. W. Rex, *Nature*, **188**, 1185 (1960).

(4) T. N. Kleinert and J. R. Morton, *Nature*, **196**, 334 (1962).

Native, Bjorkman, and Klason preparations had the lowest spin concentration; alkali and fungal preparations showed a 5–10-fold greater free-radical content. Inasmuch as the latter specimens have undergone some demethylation during their preparation, it is possible that *ortho*-quinoid or quinonemethide groups have been formed.[5] These could accommodate a stable free radical to a greater extent than the native and acid preparations.

When certain lignin samples were converted to their sodium salts (by the addition of absolute ethanol to an aqueous alkaline solution of lignin), a nearly 100-fold increase in spin concentration was observed (native spruce,[6a] yellow pine Kraft, and Indulin AT,[6b] Table II). When these salts were acidified, the recovered products had regained their original low spin content. This behavior was not significantly altered when the salt formation process was carried out under nitrogen. Klason (sulfuric acid) samples did not yield recoverable sodium salts.

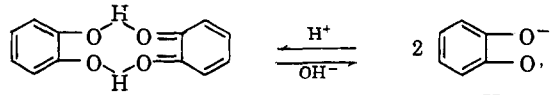
TABLE II

FREE-RADICAL CONTENT OF LIGNIN DERIVATIVES[a]

Sample	Untreated	Sodium salt	Acidified salt	$NaBH_4$ reduced	Na salt of $NaBH_4$ reduced
Brauns native spruce	0.5	50	0.5	1.1	8.4
Bjorkman spruce	1.0	15			
Kraft yellow pine	3.0	100–300	3.0	1.3	22
Kraft-treated native spruce	4.0	70			
Indulin AT	3.0	72			

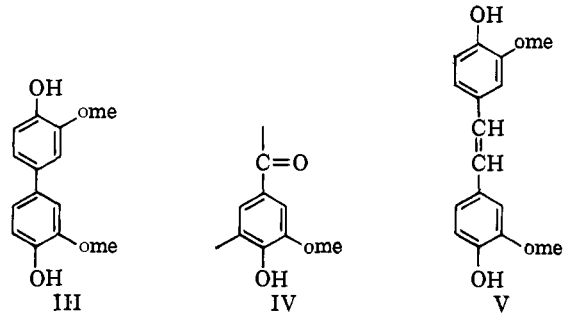
[a] All values multiplied times 10^{17}. Spin concentrations determined as in Table I.

A large and reversible change in spin concentration, mediated by change in pH alone, is a characteristic of quinhydrone-type systems,[7] as indicated by the following reaction.



I II

Other semiquinone radical-ion precursors which could form quinhydrones and which would still be consistent with well established lignin formulations are III, IV, and V.



III IV V

Further support for a quinhydrone model comes from reduction experiments. Sodium borohydride, which reduces carbonyl and quinoid groups, has very little effect on the original content of the lignin, perhaps because of a balancing off of radical formation and destruction. However, it does decrease the ability of the macromolecule to form radical-ions in base (Table II), a property which is consistent with the loss of quinoid character.

Using the convention of lignin chemists, we have estimated that the semiquinone radical-ion concentration (after conversion to the salt) in native lignin is 0.0017 radical/OCH_3; for Kraft lignin the range is 0.003–0.01 radical/OCH_3. These data can also be expressed in terms of spins/molecule. For native lignin, the value is 0.01; for Kraft lignin, a range of 0.08 to 0.25 is calculated. These yields are in the range expected in view of studies with model compounds.[7]

Adler and Marton[8] have detected as little as 0.01 carbonyl/OCH_3 by the use of ultraviolet spectrophotometry. Our results indicate a quinoid carbonyl content considerably below this value and also demonstrate the usefulness of e.p.r. spectrometry for detecting minor contributions to the structure of this macromolecule.

It appears, then, that lignin and various lignin preparations have low concentrations of organic free radicals, which are apparently stabilized by delocalization of the unpaired electron or shielded by the macromolecular network. A large number of new radical centers can be created by basification, indicating the presence of a diamagnetic quinhydrone species. The exact nature of this quinhydrone, plus structural information about related features of the lignin macromolecule, may be elucidated by causing specific chemical changes to take place and monitoring these changes by e.p.r. spectrometry. Such studies are now under way in this Laboratory.

(5) (a) C. B. Purves, N. Levitan, and N. S. Thompson, *Paper Pulp Mag.* (Canada), **56**, 117 (1955); (b) E. Adler, "Fourth International Congress of Biochemistry," Vol. II, Pergamon Press, New York, N. Y., 1959, p. 137; (c) K. Freudenberg, *ibid.*, p. 121.

(6) (a) Native spruce lignin, when subjected to Kraft pulping conditions, gave a product which exhibited the same paramagnetic properties as a commercial Kraft lignin; (b) kindly provided by the West Virginia Pulp and Paper Co., Charleston, S. C.

(7) Y. Matsunaga, *Can. J. Chem.*, **38**, 1172 (1960).

(8) E. Adler and J. Marton, *Acta Chem. Scand.*, **15**, 370 (1961).

(9) Alfred P. Sloan Foundation Fellow.

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TED REID
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RECEIVED OCTOBER 4, 1963

Hydrogen Exchange at Methine and C-10 Positions in Chlorophyll[1]

Sir:

In previous reports we have shown that both chlorophylls *a* and *b* will exchange at least one hydrogen atom with methanol in carbon tetrachloride solution,[2] and, from n.m.r. measurements, that the δ hydrogen exchanges with an excess of methanol in 48 hr.[3] These observations are correct, but in the course of further work, we became aware that our conclusions about the relative lability of the δ and C-10 (I) protons required re-examination. Since there is considerable current interest in the possible role in photosynthesis of exchangeable hydrogen in chlorophyll, we believe it desirable to communicate our most recent results at this time.

The conclusion that the δ position is more labile than the C-10 was based on n.m.r. measurements in $CDCl_3$ solutions very carefully freed from the CH_3OD used for the exchange. At the time this work was performed we were not aware of the remarkable effects of concentration and solvent polarity on the n.m.r. spectra of chlorophyll solutions.[4] It is now known that in non-

(1) This work performed under the auspices of the U. S. Atomic Energy Commission.

(2) J. J. Katz, M. R. Thomas, H. L. Crespi, and H. H. Strain, *J. Am. Chem. Soc.*, **83**, 4180 (1961).

(3) J. J. Katz, M. R. Thomas, and H. H. Strain, *ibid.*, **84**, 3587 (1962).

(4) G. L. Closs, J. J. Katz, F. C. Pennington, M. R. Thomas, and H. H. Strain, *ibid.*, **85**, 3809 (1963).