trophoretic mobility, we conclude that hydrolysis occurs at about the same point in the sequence of nucleotides for both protein-free 23*S* RNA and 23*S* RNA in the 50*S* ribosome. The rate constant for the hydrolysis of this site in the 50*S* ribosome is over twice the rate for the site in protein-free 23*S* RNA. Therefore this region of RNA has a different conformation in the 50*S* ribosome than in the protein-free 23*S* RNA.

Hydrolysis of the 30*S* ribosome gave four RNA fragments with designations and molecular weights as follows (Fig. 1, curves e and f): 30A (210,000), 30B (130,000), 30C (50,000), and 30D (28,000). Hydrolysis of the protein-free 16*S* RNA gives nine fragments: 16A (450,000), 16B (320,000), 16C (290,000), 16D (225,000), 16E (195,000), 16F (160,000), 16G (120,000), 16H (53,000), and 16I (18,000). The presence of these bands was confirmed by staining gels with methylene blue. Because we lacked adequate low-molecular-weight markers, these values are not of sufficient accuracy to exclude the possibility that 30A = 16D, 30B = 16F or 16G, 30C = 16H, and 30D = 16I. However, fragments 16A, 16B, and 16C are not present in the fragments from 30*S*. This shows that sites of hydrolysis exist in protein-free 16*S* RNA which do not exist in the 30*S* ribosome. In the 30*S* ribosome these RNA sites are either sterically shielded by the ribosomal protein, or the interaction of proteins with 16*S* RNA changes the conformation of the RNA to eliminate these sites. We found no obvious relations between the molecular weights of fragments from the 30*S* ribosome or the 16*S* RNA. The 30*S* ribosome and 16*S* RNA also resulted in more fragments, respectively, than 50*S* and 23*S*. The hydrolysis reactions of 30*S* and 16*S* are probably more complicated than those of 50*S* and 23*S*.

The kinetics of degradation of 30*S* ribosomes and 16*S* RNA was examined by plotting ln C_0/C against time. For 16*S* RNA, initial points fit a straight line confirming pseudo first-order kinetics with k' (16*S*) equal to 3.3×10^{-2} min^{-1} (Fig. 2, line labeled 16*S*). The 30*S* ribosomes required much higher concentrations of ribonuclease to produce significant hydrolysis at 0°C than did 16*S* rRNA. The data for 30*S* gave curved lines which became asymptotic to a constant ordinate value suggesting enzyme inhibition. We estimate that k'(30*S*) is about 1/20 of k'(16*S*). Also, k'(23*S*)/k'(16*S*) is about 2, which suggests that the conformations of 23*S* and 16*S* RNA's differ.

Our results are consistent with those of Cahn *et al.* (*9*) who also observed fragments 50A and 50B from 50*S* ribosomes. They showed that 50*S* ribosomes containing these fragments have little or no activity in a cell-free amino acid incorporation system. Our rate constants indicate that the region of 23*S* RNA which is hydrolyzed in the 50*S* ribosome has a special conformation. This region is much more accessible to ribonuclease than is the same region in deproteinized RNA. In view of the inactivation observed by Cahn *et al.*, this region of RNA may have an important role in protein synthesis.

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Polymeric Structure of Spruce Lignin

Abstract. *From the Flory-Stockmayer theory and molecular weights of successive fractions of lignin obtained by extraction of spruce wood with a mixture of water, dioxane, and hydrochloric acid and by sulfonation, the average length of the primary chains was estimated to be 18 phenylpropane units. From data on the acid-catalyzed cleavage of model compounds in various solvents the degrees of cross-linking in four extraction processes were calculated. The average degree of cross-linking of intact lignin was found to be 0.277; that is, 5 out of every 18 phenylpropane units bear cross-linking benzyl ether groups. Of the five, three are also etherified at their phenolic ends (B groups), and two are not (X groups).*

In the sulfite pulping of wood, the only reactions of lignin positively known to have a bearing on the mechanism of delignification occur at the benzyl positions of its constituent phenylpropane units (*1*). We have accumulated experimental evidence that in other processes of delignification in acidified solvents, both aqueous and nonaqueous, the significant delignifying reactions also occur at the benzyl positions (*2*). In one key instance—delignification of softwoods by means of acidified 2,2-dialkoxypropane (*3*)—only reactions at the benzyl positions occur with sufficient speed and to an adequate degree to account for the experimental results (*4*). We have now examined the implications of these results in terms of currently accepted theories of polymer cross-linking (*5*) and have found that the extraction of lignin from wood can be treated as the depolymerization of a three-dimensional gel composed of relatively short linear (or slightly branched) polymer chains joined by frequent cross-linking benzyl ether groups.

These conclusions are distinct from those of Szabo and Goring (*6*), who assumed that delignification is the reverse of polymerizing a mixture of di- and trifunctional monomers. They further assumed that the lignins of the cell wall and the middle lamella have somewhat different structures and, hence, different patterns of delignification. By modifying the appropriate well-known mathematics (*5*), Szabo and Goring were able to generate theoretical curves of lignin concentration in secondary wall and middle lamella versus the total

extent of delignification in kraft pulping, and their calculated curves agreed qualitatively with known experimental results. They also calculated the variations of the molecular weights of soluble fractions as a function of extent of delignification and obtained good quantitative agreement with experiments for both kraft and sulfite pulping.

The chemical implications of the Szabo and Goring treatment are that all the ether bonds in lignin are of comparable reactivity, so that bond scission can occur with equal probability at di- or trifunctionally linked groups. In contrast, our approach assigns the specific role of cross-linking units to the benzyl ether groups, generally known in organic chemistry to be more reactive than any of the other ether linkages found in lignin.

If we assume that an average segment of the intact softwood lignin macromolecule can be approximately represented by the concept offered by Freudenberg in 1965 (*7*) (Fig. 1), we can simplify its representation by reducing it to a form expressed in terms of mono-, di-, and trifunctionally linked phenylpropane units (Fig. 2a). This illustrates how all trifunctionally linked units, except numbers 10 and 15, are the bearers of benzyl ether links. There are five benzyl ether–bearing groups among the 18 phenylpropane units shown in the formulation; and, of the five, two have free phenolic groups and are usually designated (*1*) as X. The symbol X′ is used here to distinguish groups in which the benzylic hydroxyl groups are etherified. The other three of the five cross-linking units are etherified at both the phenolic and benzylic hydroxyl positions, and are usually designated as B.

When all the benzyl ether links are broken, the fragments formed (Fig. 2b) are all linear, that is, linked bifunctionally; and the previous exceptions, numbers 10 and 15, are now merely the bearers of short branches. Thus, on paper, breaking all the benzyl ether bonds of this structure reduces it entirely to linear, or slightly branched, fragments. This is the sort of process that would convert a cross-linked polymer into a soluble, linear form. Since the products are polymeric, delignification is better treated mathematically as the reverse of cross-linking preformed chains, rather than as the reverse of polymerizing di- and trifunctional monomers.

According to the theories of Flory and Stockmayer (*5*), at any given degree of cross-linking, the weight fraction (w_s) and the weight average degree of polymerization ($\bar{x}'_w$) of the material in solution are given by

$$\bar{x}'_w = \frac{\bar{y}'_w \{1 + \rho w_s [1 + 2\rho(1 - w_s)]\}}{1 - \rho w_s [1 + 2\rho(1 - w_s)] (y'_w - 1)} \quad (1)$$

$$w_s = \sum_{y=1}^{\infty} w_y [1 - \rho(1 - w_s)]^y \quad (2)$$

where $\bar{y}'_w$ is the weight average degree of polymerization of the primary linear chains, and ρ is the average degree of cross-linking in the total system, including sol and gel, expressed as the fraction of trifunctionally linked units in all the monomer units. In the specific case of delignification, w_s is the yield of dissolved lignin expressed as a decimal fraction of the total lignin originally present. The distribution of primary chain lengths is unspecified in these equations. However, by making calculations based on published experimental data (*8*), we found that a negligible error is introduced by taking these chains to be monodisperse. Equations 1 and 2 were accordingly reduced to

$$\bar{x}'_w = \frac{y\{1 + \rho w_s [1 + 2\rho(1 - w_s)]\}}{1 - (y - 1)\{\rho w_s [1 + 2\rho(1 - w_s)]\}} \quad (3)$$

$$w_s = [1 - \rho(1 - w_s)]^y \quad (4)$$

From Eq. 4, w_s and ρ bear a fixed relation to each other, depending only on the value of y. Therefore, if y, a constant, is assigned a value, ρ can be eliminated from Eq. 3 and the relationship of $\bar{x}'_w$ to w_s can be calculated. This operation was performed for all integral values of y, from 10 to 50, and the resulting curves were tested for best fit to a set of data derived from experiments on delignification of spruce wood by sulfonation (*9*), and by the procedure commonly called "dioxane lignin extraction" (*10*). Both processes are reactions in acidified solvents, and the similarity of their mechanisms is suggested by the near concordance of the experimental points (Fig. 3a). The best fitting theoretical curves were those computed for $y = 18$ and 19 (*11*).

To test these results experimentally we made two assumptions: (i) that the proportion of phenylpropane units bearing cross-linked benzyl ether groups in intact lignin is 5/18 ($\rho = 0.277$), as indicated by the Freudenberg picture

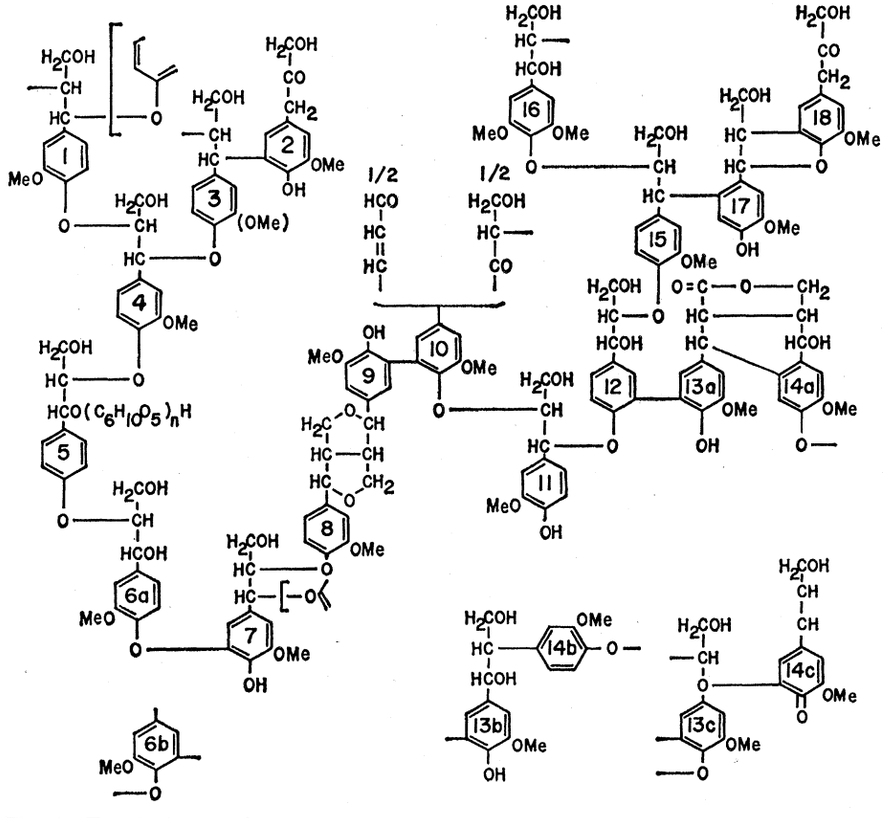

Fig. 1. Freudenberg's picture (1965) of an average 18-unit segment of the softwood lignin macromolecule (*7*).

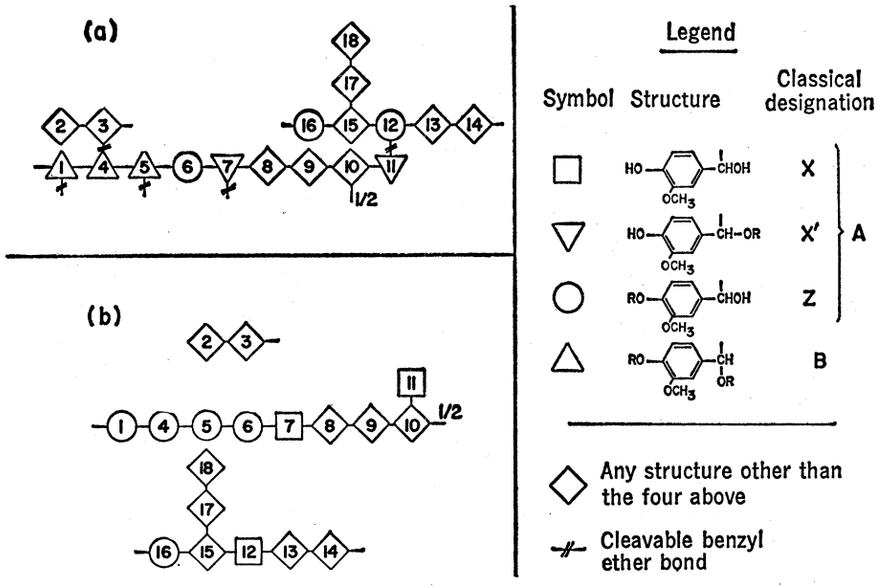

Fig. 2. (a) A simplified version of the Freudenberg structure proposed for lignin, indicating the five locations of cross-links at the sites of benzyl ether groups. Two are X' groups and three are B groups. (b) The result of cleaving the benzyl ether groups; formation of linear or slightly branched fragments.

(Figs. 1 and 2); and (ii) that benzyl ether groups in appropriately constructed model compounds in solution would cleave to approximately the same extent as the equivalent groups in lignin treated under precisely the same conditions. When a model for the X' group (*12*) was reacted under conditions used to extract mixtures of lignin with water, acid, and dioxane, of alcohol, acid, and dioxane, and of 2,2-dimethoxypropane, acid, and dioxane, respectively, roughly 80 percent of the benzyl ether group was removed in each instance. A model for the B group (*13*) lost 47 percent of its benzyl ether group in the water, acid, dioxane system, 58 percent in the alcohol, acid, dioxane system, and 80 percent in the 2,2-dimethoxypropane, acid, dioxane system under equivalent conditions. Since there are two X' groups and three B groups in every 18 units of the Freudenberg formula, the total cleavage of benzyl ether groups is 60, 67, and 80 percent, respectively (*14*). Thus the value of ρ at the end of each type of extraction was readily calculated. The corresponding yields of dissolved lignin, w_s, were experimentally determined as 0.171, 0.321, and 0.68 (*2, 3*). Figure 3b shows how these three points fit the theoretical curve for $y = 18$, calculated from Eq. 4. The fourth point represents the condition in which extraction occurs without cleavage of benzyl ether bonds (the classical method of Brauns, percolation of neutral ethanol through wood meal) for which ρ is 0.277 and which gives a yield (w_s) of 0.007 to 0.015 of purified lignin (*15*). The fit of all four points appears to justify the assumptions made. The fit for $y = 19$ was not quite as good.

Thus, from these results, intact coniferous lignin behaves as a network polymer composed of basic chains containing, on the average, about 18 phenylpropane units, five of which are cross-linking benzyl ether groups. Of the latter, two are of the type here designated X', and three are of the type classically symbolized as B. The agreement of these results with the Freudenberg picture of the lignin macromolecule appears to confirm the general validity of the latter, for, in effect, it correctly predicts yields and molecular weights of dissolved fractions.

Cross-linking has not been measured before, but, with respect to the length of primary chains, it may be significant that, as Sarkanen has pointed out (*16*), the isolation of veratric acid from methylated wood (*17*) in a yield corresponding to 0.055 free guaiacyl units per methoxyl suggests that they have an average degree of polymerization of no more than 18. He has also drawn attention to the results of nitration (*18*) in which the yield of dinitroguaiacol obtained from spruce wood was equivalent to one free guaiacyl group per 15 phenylpropane units in the lignin.

It is doubtless also significant that the present interpretation may account for the well-known feature of sulfite delignification (*19*): that, in sulfonation under neutral conditions (formation of stage I lignosulfonic acid), sulfur is taken up, but no appreciable proportion of lignin is dissolved. In terms of our model this involves the

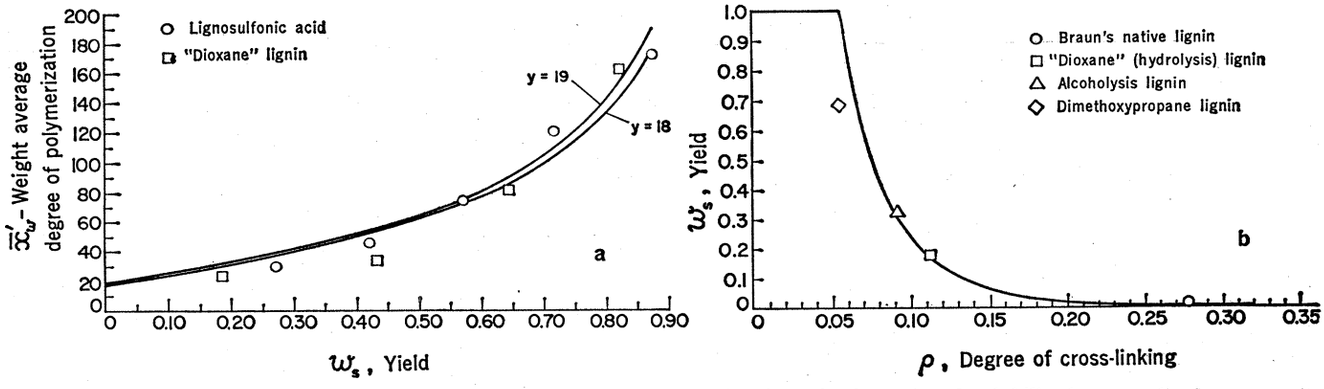

Fig. 3. (a) Experimental values (*9, 10*) of the weight-average degree of polymerization, $\bar{x}'_w$ of solubilized spruce lignin, versus its yield, w_s, expressed as a decimal fraction, and the curves calculated from theory for a basic chain length, y, of 18 and 19. (b) The fit of the experimentally determined points relating w_s to ρ, the degree of cross-linking (ratio of trifunctional units to total monomeric units), to the curve calculated from theory for a basic chain length of 18.

reduction of ρ to no less than 0.166, or 60 percent of its initial value, and less than 5 percent of the lignin becomes soluble (Fig. 3b). Stage II, the dissolution stage, requires the cleavage of B groups, and is achieved by acid hydrolysis, with or without subsequent sulfonation.

Contrary to our previous work (*3*), our new model appears to obviate the need for considering the breaking of lignin-carbohydrate bonds as a necessary step in solubilizing lignin. Furthermore, like the Szabo and Goring model (*6*), it indicates how the increasing molecular weight of soluble lignin during delignification is a natural consequence of the statistical course of depolymerization and does not require invoking a hypothesis of "condensation" of small molecules.

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8. In view of its yield, Brauns' native lignin comprises mainly primary chains which are not cross-linked (see Fig. 3a). The molecular weight distribution of this lignin was estimated from data of C. L. Hess [*Tappi* **35**, 312 (1952)], as $M_w/M_n = 1.04$. Applying this distribution factor to some of the calculations described in this report made no significant difference in the results.
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11. Standard deviations ($\pm$) for $y = 15$ to 20 inclusive, were 25.2, 19.9, 15.2, 12.2, 12.1, 15.0, 19.8, and 25.3, respectively.
12. 1-Ethoxy-1-(3′-methoxy-4′-hydroxyphenyl)-2-(2″-methoxyphenoxy)-3-hydroxypropane.
13. 1-Ethoxy-1-(3′,4′-dimethoxyphenyl)-2-(2″-methoxyphenoxy)-3-hydroxypropane.
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Lysopine and Octopine Promote Crown-Gall Tumor Growth in vivo

Abstract. *Growth of tumors induced on primary leaves of bean plants by* Agrobacterium tumefaciens *was increased by the addition of lysopine and octopine. A detectable response was observed when as little as 1 microgram of these compounds was added per leaf, and the mean volume of the tumors was increased two- to threefold when greater amounts were applied. The specificity of the response and the unique association of these compounds with the tumors suggest that endogenous lysopine and octopine contribute to the growth characteristics of these tumors.*

Crown-gall tumors produce octopine [*N*-α-(1-carboxyethyl)-L-arginine] (*1*) and lysopine [*N*-α-(1-carboxyethyl)-L-lysine] (*2*). Octopine has not been detected in normal plant tissues (*3*) and, while lysopine is a normal constituent of plants, its concentration in these tumors is about 24 times greater than in nontumorous tissues (*4*). No physiological function for these compounds related to the tumorous characteristics of these tissues, however, has been reported. Our results show that small amounts of octopine and lysopine can promote the growth of crown-gall tumors.

The growth of individual crown-gall tumors induced by *Agrobacterium tumefaciens* (strain B6) on primary pinto bean leaves is less than maximum when fewer than 60 to 80 tumors are formed on each leaf (*5*). A bioassay for tumor growth based on this observation was devised and used to demonstrate the presence of tumor growth promotion by extracts of tumorous leaves and the absence of such activity in extracts of control leaves (*6*). A modification of this bioassay was used to show the promotion of tumor growth by lysopine and octopine. The primary leaves of 7-day-old pinto bean plants were inoculated with cells of strain B6 sufficient to initiate about ten tumors per leaf. Substances tested for promotion of tumor growth were spread over each leaf (0.1 ml per leaf) on the 3rd day after inoculating the bacteria, and at 6 days after infection the diameter of tumors was measured at ×30 magnification with an ocular micrometer in a dissecting microscope. Each sample was tested on 14 to 16 leaves, and a maximum of three tumors was selected and measured on each leaf following a prescribed pattern designed to assure a random choice of tumors in each sample of leaves. Coded solutions were used in some experiments to assure that these procedures were not prejudicial to the results. Only the tumors with a nearly circular perimeter were measured, since this shape was found to be maintained during the 3-day period of growth and the measurements of such tumors were more readily made and interpreted volumetrically. The results obtained with this bioassay were similar to those obtained when the daily growth of several specific tumors was followed in the previous bioassay (*6*). The validity of the modified assay is further attested to by the degree of specificity evident in the limited number of compounds that gave this growth response and the proportionality found between the concentration of octopine or lysopine and the resulting amount of tumor growth. A more complete description of the bioassay will be published elsewhere.

Table 1 presents results from a single tumor growth experiment in which lysopine and octopine (*7*) showed growth-promoting activity. A spherical model was estimated to closely approximate the volume occupied by the tumors because they were selected to be circular in a plane parallel to the leaf surface, and such tumors are nearly circular in cross sections perpendicular to the leaf surface. The mean volume of tumors at day 6, estimated from the