[CONTRIBUTION NO. 271 FROM THE DEPARTMENT OF ORGANIC CHEMISTRY AND ENZYMOLOGY, FORDHAM UNIVERSITY]

Investigations on Lignin and Lignification. XIII. Electrophoresis of Native and Enzymatically Liberated Lignins[1]

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The native lignins from oak and white Scots pine wood were analyzed electrophoretically at various stages of purification. The dependence of the electrophoretic mobility of these lignins on *p*H and ionic strength was determined. Oak, maple and cork lignins and commercial indulin were found to present patterns characteristic of a homogeneous compound. Native and enzymatically liberated lignins from white Scots pine gave identical patterns, both characterized by the presence of small secondary components. The application of this method to lignins has definite advantages for determining the purity and homogeneity of the preparations, but is limited in scope, mainly due to the only small differences in electrophoretic mobilities observed between various lignins.

With the extraction of about 3% of the lignin present in woody tissues, a method[2] was found for obtaining lignin which did not appear to be different from lignin as it exists *in situ*. However, the failure of the residual lignin present to be so extracted led some workers[3] in the field to believe that either this native lignin was not identical with the bulk of the lignin or, that the non-extractable lignin was chemically linked with the cellulose in the wood.

It was later demonstrated[4] that at an optional interruption of the decay as much as 23% of the residual lignin present in white Scots pine wood could be obtained through cellulolysis by wood-destroying molds of the "brown rot" type. This enzymatically liberated lignin was found to be chemically identical with the native lignin fraction. Similar studies were conducted on oak, birch, maple[4] and bagasse.[5a,b]

However, significant differences were found between the lignins originating from various woods with regard to elementary composition, methoxyl content, and ultraviolet and infrared absorption spectra.[6]

That lignins are polymers composed of substituted phenylpropane building stones is well established. However, the nature of the linkage between these groups is as yet unknown. Observed variations in lignins obtained from various woods, thus, may arise from two sources: (a) differences in the nature of the linkages binding phenylpropane building stones, and (b) differences in the degree of polymerization, namely, the number of aromatic residues actually present in the lignin molecule. It is also entirely possible that lignin isolated from any one species of wood is not homogeneous, but present as a mixture of closely similar substances. An example of this is to be found in the oak and birch lignins.[5a] The methoxyl content of the native lignin fraction of these woods was found to be about 15%, whereas the lignins isolated from these woods with 72% sulfuric acid,[7] were found to have a methoxyl content of 20%.

As a result, lignins may be regarded as mixtures of compounds all possessing very similar chemical structures but with a notable possibility of certain chemical differences. It is for such mixtures of compounds which may differ both in structure and in molecular weight that the notion of *Gruppenstoffe* was applied,[8] as distinct from isomeric compounds which possess the same molecular weight and small differences in structure or, from homologous series in which compounds have different molecular weights though the same structure.

The individuation and characterization of such substances by classical methods of organic chemistry presents notable difficulties. Thus, in the field of lignin chemistry one has had recourse mainly to elementary composition, methoxyl content and isolation of derivatives. To overcome these limitations, physico-chemical means have been employed, and analyses by means of ultraviolet and infrared absorption spectroscopy have been applied to advantage.[9]

In view of the success of applying electrophoretic methods to the characterization and fractionation of proteins, an investigation as to the possible availability of the method to lignins was undertaken. The usefulness of the moving boundary method of Tiselius to low molecular weight compounds was recently shown.[10] Our results reveal that this technique is quite applicable to lignins.

Experimental

The lignins from white Scots pine, oak, birch, maple, bagasse and cork[11] served as substrates in this study.

Isolation of Native Lignin.—The lignins employed in this investigation were isolated according to the method of Brauns.[2] Air-dry wood, ground to 40 or 60 mesh was extracted at room temperature in a percolator-type extractor[12] with 95% ethyl alcohol until the extract no longer gave the phloroglucinol–hydrochloric acid color test. Upon removal of the alcohol by distillation at reduced pressure, a resinous material remained. This was washed well with water and with ether. The resulting powder was dried, dissolved in dioxane to make a 10% solution, centrifuged, filtered and precipitated into thirty times its volume of ice-cold distilled water. The precipitate was dried, re-

(1) Presented before the Division of Cellulose Chemistry of the American Chemical Society at the autumn meeting held in Atlantic City, N. J., 1952. For Communication No. XI in this series, see THIS JOURNAL, **75**, 305 (1953); for Communication No. XII, see *Proc. Nat. Acad. Sci.*, U. S., **39**, No. 2 (1953).

(2) F. E. Brauns, THIS JOURNAL, **61**, 2120 (1939).

(3) W. J. Wald, P. F. Ritchie and C. B. Purves, *ibid.*, **69**, 1371 (1947).

(4) W. J. Schubert and F. F. Nord, *ibid.*, **72**, 977, 3835 (1950).

(5) (a) S. F. Kudzin and F. F. Nord, *ibid.*, **73**, 4619 (1951); (b) G. de Stevens and F. F. Nord, *ibid.*, **73**, 4622 (1951); **75**, 305 (1953).

(6) S. F. Kudzin, R. M. DeBaun and F. F. Nord, *ibid.*, **73**, 4615 (1951).

(7) E. C. Sherrard and E. E. Harris, *Ind. Eng. Chem.*, **24**, 103 (1923).

(8) H. Staudinger, "Makromolekulare Chemie und Biologie," Wepf and Co., Basle, 1947.

(9) E. J. Jones, Jr., *TAPPI*, **32**, 167, 311 (1949).

(10) R. M. Bock and R. A. Alberty, *J. Biol. Chem.*, **193**, 435 (1951).

(11) R. M. DeBaun and F. F. Nord, THIS JOURNAL, **73**, 1358 (1951).

(12) F. F. Nord and W. J. Schubert, *Holzforschung*, **5**, 1 (1951).

dissolved in dioxane, centrifuged, filtered and precipitated into thirty times its volume of ether. This procedure was repeated until a constant methoxyl value was obtained. In the case of oak native lignin, the above outlined procedure was repeated eight times. The preparation obtained after each purification step was subjected to electrophoretic analysis.

Isolation of Enzymatically Liberated Lignin.—The ground woody tissue, freed of its native lignin fraction, was decayed with various "brown rot" fungi, which preferentially attack cellulose. After an optional period of decay of each wood, the mycelia of the mold were removed from the wood by immersion into an excess of water. The wood was then collected, dried, and the liberated lignin extracted and purified according to the procedure outlined above.

Measurements.—Thirty to 50 mg. of lignin were dissolved in a small amount of 0.5 *N* alkali and equilibrated overnight by dialysis against a buffer containing 0.05 *M* sodium glycinate and varying concentrations of NaCl or KCl. The *p*H of the buffer was adjusted by additions of hydrochloric acid. Only a negligible amount of lignin was found to diffuse through the Visking membrane tubing and no change in the lignin by this treatment was revealed on ultraviolet absorption analysis. A Perkin–Elmer apparatus Model 38 served for carrying out the electrophoretic analyses. As the above lignin solutions are strongly colored, the transmitted light being red whereas the buffer is, of course, colorless, the photographic registration presented some difficulties. To minimize the differences in light absorption of the lignin solutions and the buffer in the two electrophoresis cell channels, a red Corning glass filter was inserted in the optical system and high speed pan type 428 Du Pont film served for the recording of the patterns. The convenient concentration range was limited to between 0.2 and 0.5% of lignin, the solutions being too dark at higher concentrations. The high index of refraction of lignin[13] accounted for the well-defined patterns obtained at low concentrations.

A. Electrophoretic Study of Oak and White Scots Pine Lignins

Results.—Preliminary measurements have indicated that both the lignin isolated from white Scots pine and oak woods are electrophoretically inhomogeneous. Therefore, we first studied the effect of repeated precipitation of lignin from ether solutions on the electrophoretic patterns. In Fig. 1 are presented the patterns obtained on the oak lignin after four and eight precipitations, and the patterns of the white Scots pine native lignin after five precipitations. It can easily be recognized that the oak lignin appears electrophoretically homogeneous, whereas the patterns obtained with the white Scots pine lignin are characteristic of a multicomponent system. Whereas occasionally two well defined peaks appeared, the inhomogeneity mostly revealed itself only in the existence of trailing boundaries. The inhomogeneous white Scots pine lignin contains components of both higher and lower mobility than the main components.

The oak lignin was electrophoretically analyzed after every reprecipitation. Under identical conditions (*p*H 10, KCl 0.1 *M*, sodium glycinate-HCl 0.05 *M*), the electrophoretic mobility was not affected by the purification to any significant degree. The mobility of the ascending boundary was found to be always slightly higher than that of the descending boundary, the respective values being -10.7 ± 0.3 and $-10.0 \pm 0.3 \times 10^{-5}$ cm.^{2}/v. sec. The main reason for the relatively large scattering of

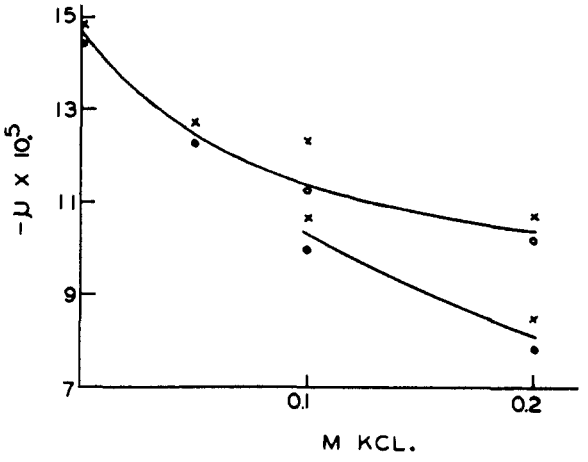


Fig. 2.—Influence of salts concentrations on the mobility of lignins: buffer, indicated concentrations of KCl plus 0.05 *M* sodium glycinate-HCl, *p*H 10; upper curve, white Scots pine lignin; lower curve, oak lignin; ×, ascending boundary; O, descending boundary.

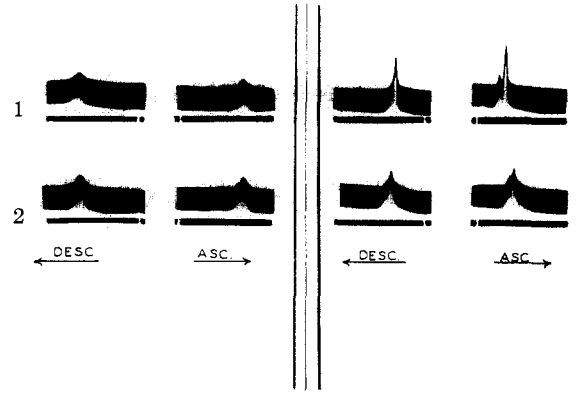


Fig. 1.—Electrophoretic patterns of oak and white Scots pine lignins: buffer, KCl 0.1 *M*, sodium glycinate-HCl 0.05 *M*: 1, oak lignin after 4 precipitations, *p*H 10; 2, oak lignin after 8 precipitations, *p*H 10; 3, white Scots pine lignin after 5 precipitations, *p*H 9.65; 4, white Scots pine lignin after 5 precipitations, *p*H 10.4.

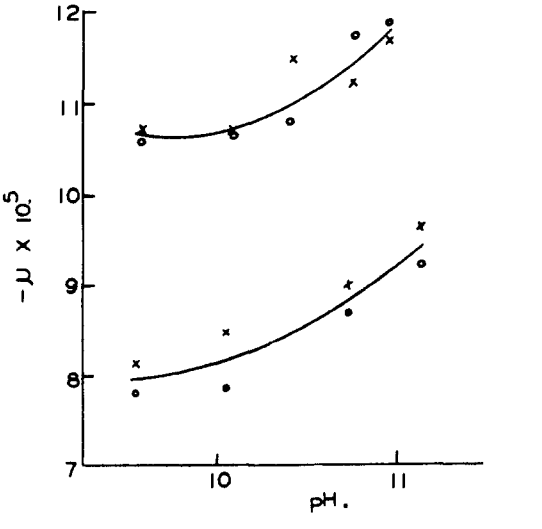


Fig. 3.—Influence of *p*H on the mobility of lignins: upper curve, white Scots pine lignin; lower curve, oak lignin; ×, ascending boundary; O, descending boundary.

(13) F. E. Brauns, "Chemistry of Lignin," Academic Press, Inc., New York, N. Y., 1952, p. 215.

the individual values is the difficulty of handling the strongly alkaline solutions of lignin and the buffers.

In Fig. 2 are recorded the rather large effects of ionic strength on the mobility of lignins. These measurements were carried out in the presence of 0.05 *M* glycine, *p*H 10. The mobility of lignins increases significantly with the lowering of the ionic strength. Under identical conditions the mobility of the oak lignin is persistently lower than that of the white Scots pine lignin.

In Fig. 3 is illustrated the effect of changing the *p*H at constant ionic strength. With increasing *p*H, the mobility of both lignins also increases. These measurements could not be extended below *p*H 8 as the lignins become insoluble and precipitate out of the solution. It is significant that even in the close neighborhood of this precipitation point both lignins still migrate with a considerable mobility, much higher than a protein near its isoelectric point.

Discussion.—In the electrophoretic analysis of oak and white Scots pine native lignins, great similarities with the behavior of proteins were observed. Notably, in the case of purified oak lignin a symmetrical boundary, characteristic of an electrophoretically homogeneous compound is observable, while the white Scots pine lignin to the contrary, presents a more complicated pattern such as is encountered in electrophoretically inhomogeneous proteins. The influence of *p*H and ionic strength is also similar, at least in a qualitative way. Thus, the increase in ionic strength decreases considerably the electrophoretic mobilities of the lignins. An analogous situation is also encountered in proteins. With increase in *p*H, the mobility of the lignins mounts similarly to that of proteins on the alkaline side of their isoelectric point.

The behavior of the lignins from oak and white Scots pine differ in two significant respects. After four or five precipitations, oak lignin becomes electrophoretically homogeneous, but not the white Scots pine lignin. Such purification, however, is generally considered as sufficient to ensure a constancy in elementary composition and methoxyl content, as has been frequently demonstrated. Also, under comparable conditions, the oak lignin possesses a lower mobility than that of white Scots pine lignin. From the analytical data listed in Table I it can be recognized that the differences in electrophoretic behavior are a reflection of the difference in the chemical composition of the two lignins.

TABLE I

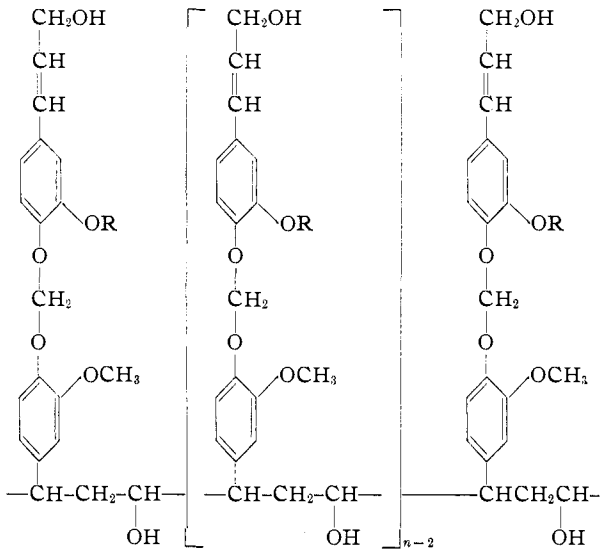
COMPARISON OF THE CHEMICAL COMPOSITION OF THE NATIVE LIGNINS FROM WHITE SCOTS PINE AND OAK

	White Scots pine native lignin	Oak native lignin
C, %	64.0	58.6
H, %	6.3	5.3
OCH_3, %	14.5	14.8
No. of phenolic groups per lignin unit weight[6]	1	3

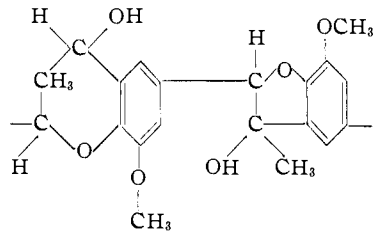
The mobility of the lignins is relatively large over the whole *p*H range of their solubility. In the case of proteins the origin of the charge causing migration is due to the ionization of a number of different groups, such as carboxyl, amino or guanidino groups. It is known that phenolic hydroxyl groups also contribute to the total charge of a protein, as they dissociate in the *p*H range 10–12. In the case of lignin, we may consider the dissociation of phenolic groups as the main source of charge, together with enolizable carbonyl groups, present in some lignins.

Although a number of suggestions have been advanced for the structure of lignin,[14] establishment of a valid constitutional formula for this substance is still far from realized.

It was considered[15] that the building stones of lignin are linked together by a methylene dioxy bridge and also through C_3 and C_1 of the propane side chain



According to an early speculation[16] the various types of building stones occur irregularly in lignin. Nevertheless, it was claimed that lignin is formed through the condensation of coniferyl alcohol groups with the formation of benzofuran or/and benzopyran rings



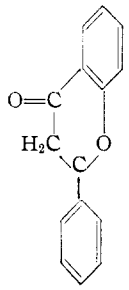
Recent chemical and physical studies from this Laboratory gave support to the consideration that a flavanone type moiety can reasonably be considered to be present in bagasse lignin.[5b]

The discrepancies encountered on attempting to interpret the physicochemical properties of lignin are even more striking. For example, there are reported in the literature values for the molecular

(14) F. E. Brauns, "Chemistry of Lignin," pp. 621–672.

(15) K. Kürschner, *Zellstoff-Faser*, **32**, 19 (1935).

(16) K. Freudenberg, "Tannin, Cellulose, Lignin," Springer Verlag, Berlin, 1933.



flavanone

weight of lignin ranging from 250 to the colloidal dimensions of 12,000.[17]

It can, therefore, be concluded that the status of the phenolic groups and ketonic groups in lignin is not at all well defined.

In Table II are recorded the dissociation constants of certain phenolic compounds. From these data it becomes obvious that the introduction of aldehyde groups or other substituents changes the dissociation constant of phenolic hydroxyls by over 100-fold. Moreover, oxidation studies carried out in this Laboratory have shown that the native lignin from bagasse, white Scots pine and birch contains not only guaiacyl building stones, but also *p*-hydroxyphenyl building stones. This is based on the finding that *p*-hydroxybenzaldehyde was identified as a degradation product of these lignins.[18] Furthermore, the lignins from oak, birch, maple and bagasse also give rise to syringaldehyde upon oxidation. Consequently, the evidence establishes the presence in lignin of phenolic building stones which exhibit different degrees of substitution. In view of this finding and also of the fact that the introduction of substituents alters the dissociation constant of phenolic hydroxyl groups, no rational explanation of the dissociation of lignins is as yet possible. This might also have some bearing on the difficulties encountered in explaining their base binding capacity[19] and is certainly the reason for the observed higher mobility of the white Scots pine lignin as compared to the oak lignin, although the latter has a higher phenolic hydroxyl content. Thus not only the number but also the position and the substitution on the aromatic ring of the phenolic groups has to be considered.

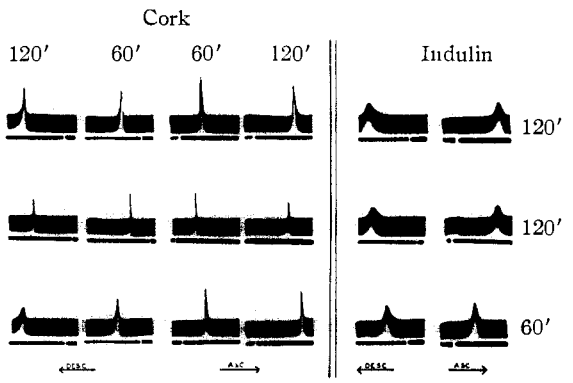


Fig. 4.—Electrophoretic patterns of fractionated cork lignins[11] and of indulin: *p*H 10.7, NaCl 0.2 *M*, sodium glycinate-HCl 0.05 *M*.

TABLE II

COMPARISON OF THE DISSOCIATION CONSTANTS OF VARIOUS PHENOLIC COMPOUNDS[a]

Compound	Dissociation constant
Phenol	1.2×10^{-10}
p-Hydroxybenzaldehyde	2.2×10^{-9}
m-Hydroxybenzaldehyde	1.0×10^{-8}
Catechol	3.5×10^{-10}
Protocatechualdehyde	2.8×10^{-8}
Resorcinol	3.7×10^{-10}
Guaiacol	1.2×10^{-10}
o-Cresol	6.3×10^{-11}
m-Cresol	9.8×10^{-11}
p-Cresol	6.7×10^{-11}
Benzaldehyde	5.0×10^{-13}

[a] Compiled from data recorded in Landolt-Börnstein, "Physikalisch Chemische Tabellen," Springer, Berlin, 1923–1936.

B. Application of Electrophoresis to Various Lignins

In the preceding section it was shown that purified oak lignin possesses an electrophoretic pattern characteristic of a homogeneous compound, whereas the white Scots pine lignin is electrophoretically heterogeneous. As further examples of electrophoretically homogeneous lignins the electrophoretic patterns of cork lignin and of commercial indulin purified by fractional precipitation are presented in Fig. 4. Since the electrophoretic pattern of white Scots pine native lignin was found to be inhomogeneous it was of particular interest to compare it with the pattern of enzymatically liberated lignin from that wood. These patterns are recorded in Fig. 5. A comparison with the patterns of preparations of native lignins (Fig. 1) shows that both are identical. Of the other lignins investigated, maple lignin is electrophoretically homogeneous while the bagasse native lignin presents only slight inhomogeneity.

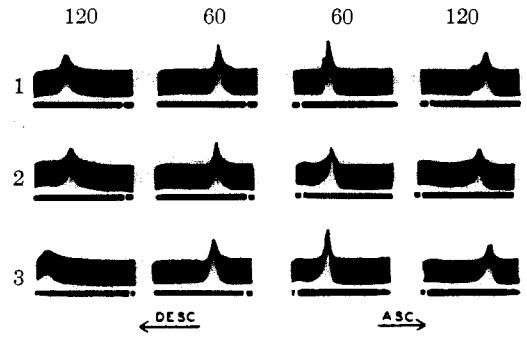


Fig. 5.—Electrophoretic patterns of lignins: *p*H 10.7, NaCl 0.2 *M*, sodium glycinate-HCl 0.05 *M*: 1, white Scots pine enzymatically liberated lignin: 2, mixed samples of white Scots pine native and enzymatically liberated lignin; 3, mixed sample of native lignins from oak and cork.

The results of these electrophoretic analyses are summarized in Table III. Only the mobilities of the main boundaries for the lignins possessing secondary smaller boundaries are presented. It is

(17) F. E. Brauns, "Chemistry of Lignin," p. 192.

(18) G. de Stevens and F. F. Nord, *Proc. Nat. Acad. Sci.*, U. S., **39**, No. 2 (1953).

(19) L. Kalb, F. Kucher and O. Toursel, *Cellulosechem.*, **12**, 1 (1921).

evident that under similar conditions of *p*H and of buffer composition all of the lignins possess comparable mobilities. The differences observed are, however, significant, and this is illustrated in Fig. 5 (line 3) in which the diagrams of a mixture of oak native and cork lignins are reproduced. Two well defined peaks are to be recognized, the mobilities being characteristic of the respective components.

TABLE III

ELECTROPHORETIC MOBILITY OF VARIOUS LIGNINS IN GLYCINE–NaCl BUFFER, *p*H 10.7

Lignin samples	Mobility, cm.²/v. sec. × 10⁵ Ascending	Descending
Indulin	− 9.9	− 9.7
White Scots pine	−10.2	−10.1
Bagasse	− 9.8	− 9.1
Maple	− 9.3	− 8.3
Oak	− 8.7	− 8.2
Cork	− 9.5	− 8.9

In view of the complexity of the lignin molecule, and the possibility of variations in structure, it was noteworthy to find that the majority of lignins do give patterns characteristic of electrophoretically homogeneous compounds, the most significant exception being the white Scots pine lignin. It was of particular interest to observe here that this lignin, whether native or enzymatically liberated presents identical patterns and, on mixing the samples, the electrophoretic patterns did not change. Therefore, it must be assumed that this lignin is a mixture of electrophoretically distinct lignins, the ease of extraction of the components being the same both before and after enzymatic decay. As a result it is justifiable to assume that the electrophoretic analysis reflects the relative concentration of the components in the total lignin of the woody tissue.

The electrophoretic analyses of our various lignin samples have thus yielded results which could not be obtained, as yet by any other method. As in the case of proteins, electrophoresis supplies us with valuable information on the purity and homogeneity of the lignin preparations. The main limitations to a wider application of this method to lignin lies in the comparatively close mobility of all the samples thus far investigated.

Acknowledgment.—The white Scots pine wood used in this investigation was obtained through the courtesy of Dr. L. C. Swain of the Department of Forestry of the University, Durham, N. H.; the indulin from Dr. F. J. Ball of the West Virginia Pulp and Paper Co., Charleston, S. C. The samples of oak, birch and maple were obtained from the Composition Materials Co., New York, N. Y. We wish to thank Godchaux Sugars, Inc., New Orleans, La., for a supply of bagasse. The mold cultures were obtained through the courtesy of Dr. W. J. Robbins of the New York Botanical Garden.

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[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

The Synthesis of 4-Methyl-, 5-Methyl-, 6-Methyl- and 4,6-Dimethyltryptophans

BY H. R. SNYDER, HARRY R. BEILFUSS AND JOHN K. WILLIAMS

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Procedures are described for the synthesis of 4-methyl-, 5-methyl-, 6-methyl- and 4,6-dimethyl-DL-tryptophans by the Fischer cyclization of the properly substituted phenylhydrazones of γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde.

All the syntheses reported in this paper make use of the general procedure established by Moe and Warner[1,2] for the preparation of DL-tryptophan. The reaction sequence, which has also been used[3] for the synthesis of DL-5-fluorotryptophan, is given by formulas I–VI.

The monomethylated amino acids under consideration have been previously prepared by alkylation with the corresponding gramines[4,5] or from the alkyl indole-3-aldehyde *via* the well-known hydantoin synthesis.[6] The present research was directed toward a synthetic route adaptable to larger scale preparations and one in which the starting materials would be more readily available. In general, the appropriate toluidine or xylidine (I) was diazotized and the diazonium salt was reduced to the hydrazine (II) with stannous chloride. The reaction of II with γ,γ-dicarbethoxy-γ-acetamidobutyraldehyde[1] (III) provided the hydrazone (IV) in excellent yields. This intermediate was used for cyclization without purification. The diethyl alkylskatylacetamidomalonate (V) was obtained by cyclization of IV in boiling aqueous sulfuric acid. The highest yields of VI were obtained when the hydrolyses and decarboxylation were carried out in three steps by a suitable modification of the procedures of Snyder and Smith[7] and of Rydon.[4]

The synthesis of DL-5-methyltryptophan utilized *p*-toluidine as the raw material, and the reaction sequence outlined above presented no difficulty. Although it has been reported[8] that the reactive position in a *m*-substituted arylhydrazone, under

(1) O. A. Moe and D. T. Warner, THIS JOURNAL, **70**, 2763 (1948).
(2) O. A. Moe and D. T. Warner, *ibid.*, **70**, 2765 (1948).
(3) H. Rinderknecht and C. Niemann, *ibid.*, **72**, 2296 (1950).
(4) H. N. Rydon, *J. Chem. Soc.*, 705 (1948).
(5) M. E. Jackman and S. Archer, THIS JOURNAL, **68**, 2105 (1946).
(6) W. Robson, *J. Biol. Chem.*, **62**, 495 (1924).
(7) H. R. Snyder and C. W. Smith, THIS JOURNAL, **66**, 350 (1944).
(8) C. Hollins, "Synthesis of Nitrogen Ring Compounds," Ernest Benn, Ltd., London, 1924, p. 96.