Table II

Incidence of tail and grasp reflexes in 50 normal rats resembling those seen after Morphine or Reserpine

Weight in g	Sex	No.	Tail		Grasp reflex
			down	up	
25–54	M	6	—	—	4
25–54	F	2	—	—	—
55–99	M	3	—	—	—
55–99	F	5	—	—	—
100–249	M	10	—	1	—
100–249	F	4	—	1	1
250–580	M	15	4	—	—
250–580	F	5	—	—	—

We are grateful to CIBA Laboratories for supplying Reserpine.

J. COLE and D. P. DEARNALEY

University Laboratory of Physiology and University Department of Biochemistry, Oxford, October 9, 1959.

Zusammenfassung

Charakteristisch verschiedene Schwanzstellungen zeigen Albino-Ratten und -Mäuse als Morphium- bzw. Reserpinreaktion. Verstärkte Schwanzreaktionen erhält man bei der Maus nach Morphium, bei der Ratte nach Reserpin. Methylphenidat erweist sich bei der Ratte antagonistisch gegen Reserpin, während im Mäuseversuch nach Reserpin injiziertes Morphium kompetitiv zu wirken scheint.

DISPUTANDUM

Lignin and the Formation of Wood

The paper by F. F. NORD and W. J. SCHUBERT on the title subject in Experientia *15*, 245 (1959) requires several amendments and supplementary comments. For the sake of brevity, the discussion here has been restricted to the grossest inadequacies and discrepancies.

The best preparation of wood lignin available at present is BJÖRKMAN's milled wood lignin (MWL)[1]. MWL prepared from spruce wood contains 0·3 phenolic hydroxyl groups per C_6–C_3 unit, whereas the minute fraction of ligninlike material which is extractable from sawdust according to F. BRAUNS contains twice this amount of phenolic hydroxyl[2,3a]. Here the latter preparation was extracted from wood using acetone in preference to alcohol and then separated from lignans and other admixtures by countercurrent distribution. NORD and SCHUBERT claim that 'enzymically liberated lignin' is identical with BRAUNS' 'native lignin'. This implies therefore that it is not iden-itcal with the main part of *true* wood lignin or protolignin. Thus, for instance, for Scots pine, values of 815 and 695 were found by NORD *et al.*[4] for the molecular weights of native and enzymically liberated lignin respectively. Spruce MWL on the other hand, although probably partially degraded by milling, has a molecular weight of 11,000[1], in the same range as that of FREUDENBERG's biosynthetic lignin[3b].

BROWN and NEISH[5] were the first to show that radioactive shikimic acid introduced into plants is incorporated into lignin and that radioactive vanillin etc. is formed on oxidation of such lignin. Independently, EBERHARDT and NORD[6a] confirmed this in more elaborate experiments. The role of shikimic acid in lignin formation is just the same as in all other cases of biosynthesis of C_6–C_3 substances. Various American authors have shown that shikimic acid is transformed into prephenic acid and thence into phenylpyruvic acid or cinnamic acid or phenylalanine. As FREUDENBERG has shown, labelled phenylalanine is transformed by spruce saplings either in the needles or in the cambium or en route from the needles to the cambium into coniferin, which was isolated in radioactive form from cambial sap[7].

In conifers coniferin and in other plants corresponding glucosides in addition assume the cardinal position in lignin formation. These are the immediate starting materials for lignification, as has been demonstrated by FREUDENBERG *et al.*[8] in the case of spruce lignin using radioactive coniferin.

NORD, SCHUBERT and ACERBO[9] have shown that radioactive *p*-hydroxyphenylpyruvic acid is incorporated into sugar cane lignin and that such lignin forms radioactive aldehydes on oxidative degradation. This important observation does not indicate however that *p*-hydroxyphenylpyruvic acid is the only or even an essential starting material for lignin formation, as NORD and SCHUBERT suggest. In an analogous experiment carried out with labelled ferulic acid, FREUDENBERG[10] found that lignin is formed which yields radioactive ketones on ethanolysis according to HIBBERT. The correct interpretation of this data is that phenylpyruvic acid or cinnamic acid or phenylalanine is oxidized on the way leading to coniferin in the 4-position and in the 3-position, which is also methylated. The acids are reduced at the carboxyl group, are converted into unsaturated compounds and finally glucosidized. The absolute sequence of these operations has not yet been definitely established[11]; *p*-hydroxyphenylpyruvic acid and ferulic acid may be essential intermediates in these transformations or may merely be transformed independently in side reactions into coniferin and other glucosides. The role of *p*-hydroxyphenylpyruvic acid is rather uncertain, as it has been shown that its incorporation into conifer lignin proceeds rather unsatisfactorily[11,12].

[1] A. BJÖRKMAN, Svensk Papperstidning *59*, 477 (1956).

[2] G. AULIN-ERDTMAN and L. HEGBOM, Svensk Papperstidning *61*, 187 (1958).

[3] a) K. FREUDENBERG, in *Biochemistry of Wood*, Proc. 4th Int. Congr. Biochem., Vol. 2, p. 125 (1959). – b) K. FREUDENBERG and K. DALL, Angew. Chem. *42*, 606 (1955).

[4] G. DESTEVENS and F. F. NORD, Fortschr. chem. Forsch. *3*, 95 (1954).

[5] S. A. BROWN and A. C. NEISH, Nature *175*, 688 (1955).

[6] a) G. EBERHARDT and F. F. NORD, Arch. Biochem. Biophys. *55*, 578 (1955). – b) G. EBERHARDT and W. J. SCHUBERT, J. Amer. chem. Soc. *78*, 2835 (1956).

[7] K. FREUDENBERG and F. NIEDERCORN, Chem. Ber. *91*, 591 (1958).

[8] K. FREUDENBERG, H. REZNIK, W. FUCHS, and M. REICHERT, Naturwiss. *42*, 29 (1955).

[9] F. F. NORD, W. J. SCHUBERT, and S. N. ACERBO, J. Amer. chem. Soc. *79*, 251 (1957); *80*, 1990 (1958).

[10] K. FREUDENBERG, Angew. Chemie *68*, 92, 511 (1956). – S. A. BROWN and A. C. NEISH, Canad. J. Biochem. Physiol. *34*, 769 (1956).

[11] S. A. BROWN, D. WRIGHT, and A. C. NEISH, Canad. J. Biochem. Physiol. *37*, 25 (1959).

[12] G. BILLEK, in *Biochemistry of Wood*, 4th Int. Congr. Biochem., Vol. 2, p. 211 (1958).

The following statements of NORD and SCHUBERT summarizing 'the process of lignification' are therefore not in clear concordance with the facts of current lignin chemistry: 'This compound (i. e. shikimic acid) is then aromatized into compounds of the type of *p*-hydroxyphenylpyruvic acid. These latter compounds then become the first, or 'primary' lignin building stones'. It ought at least to have been stated that the primary building stones are actually *p*-hydroxycinnamic alcohols.

Furthermore NORD and SCHUBERT give the impression that nothing is known about the oligomeric intermediates of lignin formation. In this respect, attention is called to work done in Heidelberg in recent years in which so far *ten* different intermediates have been isolated. Inexplicably, they have also neglected to mention the well established role of the glucosides, their hydrolysis prior to lignification by a β-glucosidase present between cambium and wood in trees and the dehydrogenative condensation of the free phenolic aglycons brought about by oxidizing enzymes at the site of lignification in the plant.

All in all, NORD and SCHUBERT have merely described a pair of random steps isolated from the complexity of the total process of lignification and hence convey an oversimplified, distorted picture of the overall scheme. For a condensed but more complete survey of the biosynthesis and constitution of lignin, a recent article by FREUDENBERG[13], is recommended.

J. M. HARKIN

Forschungsinstitut für die Chemie des Holzes und der Polysaccharide, Chemisches Institut der Universität Heidelberg, 5. August 1959.

A Rebuttal to the Disputandum of J. M. Harkin

The Disputandum by HARKIN to the paper of NORD and SCHUBERT in this Journal[14] is characteristic of the style of the Heidelberg school, but is unconvincing.

The statement is made that 'The best preparation of wood lignin available at present is A. BJÖRKMAN's milled wood lignin (MWL)'. But until the structure of natural lignin has been proved, one cannot say what is the 'best' preparation of lignin, for until such time as its structure becomes known, one cannot know what the *exact* properties of natural lignin will be, and so one cannot know how closely his own preparation approaches lignin *in situ*. While we do not disparage the value and importance of BJÖRKMAN's preparation, we and indeed others feel that it is questionable to call it, or any other preparation, 'best' at this time.

We do indeed claim that enzymatically liberated lignin is identical with BRAUNS' native lignin, but this certainly carries no implication that it is not identical with the main part of true wood or bagasse lignin. Once again, the author has arbitrarily selected Spruce MWL as a standard of reference, and then he totally confounds the issue by reference to FREUDENBERG's 'biosynthetic lignin', which of course, is not a natural, wood-produced lignin at all, but an artifact[15].

The statement is made 'Brown and Neish were the first to show that radioactive shikimic acid introduced into plants is incorporated into lignin and that radioactive vanillin, etc. is formed on oxidation of such lignin. Independently, EBERHARDT and NORD confirmed this in more elaborate experiments'. To keep the record straight, let it be known that BROWN and NEISH's letter was published in the April 16, 1955 issue of Nature, while the Fordham letter was published in Arch. Biochem. Biophys. in the April, 1955 issue.

Furthermore, no attempt is made to deny that 'The role of shikimic acid in lignin formation is just the same as in all other cases of biosynthesis of C_6–C_3 substances', or that 'Various American authors have shown that shikimic acid is transformed into prephenic acid and thence into phenylpyruvic acid or cinnamic acid or phenyl-alanine'. We simply claim to have called attention (see above), to the intervention of shikimic acid in lignification, as compared with other biosyntheses of aromatic compounds, a possibility which was earlier completely overlooked by FREUDENBERG[16].

The statement is made that coniferin and other glucosides are the 'immediate starting materials for lignification'. But why this arbitrary selection of the glycosides as the starting point of the process? We feel that the origin of the glycosides themselves is just as integral a part of the *total* process of lignification as the eventual conversion of these compounds into lignin.

Regarding *p*-hydroxyphenylpyruvic acid, it is claimed that its role is 'rather uncertain as it has been shown that its incorporation into conifer lignin proceeds rather unsatisfactorily'. But NEISH[17] states that '*p*-hydroxyphenylpyruvic acid-3-C^{14} can be converted to guaiacyl or syringyl lignin in wheat but not in buckwheat (*Fagopyrum tataricum*) or sage (*Salvia splendens*)'. This difference in biosynthetic abilities among different species was later interpreted[11] as follows: 'Neither *p*-hydroxyphenylpyruvic acid nor *p*-hydroxyphenyl lactic acid is a general intermediate in lignification, and differences noted here and in previous papers between grasses and non-grasses probably result from the unique ability of grasses to convert *p*-hydroxyphenyl lactic acid to *p*-hydroxy cinnamic acid'. Since the sugar cane plants employed in our experiments are considered to be 'grasses', they are thus able to utilize *p*-hydroxyphenylpyruvic acid for lignin biosynthesis, as has been demonstrated in this laboratory[15].

The statement is made that 'It ought at least to have been stated that the primary building stones are actually *p*-hydroxycinnamic alcohols'. But, if this was not explicitly stated, it was certainly clearly implied. Thus, on p. 252 of the paper in this Journal, it was stated that 'The building stone of lignin possesses a phenylpropane carbon structure (Figure 4b). Lignin building stones, although having the same basic phenylpropane carbon structure, may be of at least three types, the vanillyl (Figure 12a), syringyl (Figure 12b), or *p*-hydroxyphenylmethyl (Figure 12c) structure'. Hence, obviously the reader who superimposed the phenylpropane carbon structure of Figure 4b upon the vanillyl structure of Figure 12a would have deduced the structure of coniferyl alcohol, which in fact was presented in Figure 2 on page 246.

[13] K. FREUDENBERG, Nature *183*, 1152 (1959).

[14] F. F. NORD and W. J. SCHUBERT, Exper. *15*, 245 (1959).

[15] F. F. NORD, W. J. SCHUBERT, and S. N. ACERBO, Naturwissenschaften *44*, 35 (1957).

[16] K. FREUDENBERG *et al.*, Liebigs Ann. *575*, 145 (1952); *584*, 54 (1953).

[17] A. C. NEISH, in the Discussion of the paper of G. BILLEK in *Biochemistry of Wood*, Vol. 2 of the Proceedings of the Fourth International Congress of Biochemistry, p. 213 (London 1959).

Relative to the oligomeric intermediates in lignin formation, comments on them were made on p. 253 of the original paper. No reference was made to the 'intermediates' isolated at the Heidelberg school since the authors wished to restrict themselves to investigations of the processes occurring in woody plants, whereas the 'intermediates' referred to are the result of the *in vitro* action of an ill-defined enzyme system from mushrooms, which results may in fact have no relation at all to the process of lignification.

Accordingly, the concluding statement of the Heidelberg authors' regarding the Fordham picture of the overall scheme of lignification (as also reviewed by EVANS[18]) may be dismissed as a purely subjective analysis.

CARMINE J. COSCIA

Department of Organic Chemistry and Enzymology, Fordham University, New York, September 24, 1959.

In reply to the rebuttal by COSCIA of my objections to the paper by NORD and SCHUBERT[14], I should like to clarify the following points.

I repeat that BJÖRKMAN's milled wood lignin (MWL) is the best lignin preparation *available at present*, since it can be obtained repeatedly in yields of over 50%[1] and is likely to be more representative of natural lignin *in situ* than BRAUNS soluble lignin, the yield of which represents only 2–3% of the total lignin. If enzymically liberated lignin is identical with BRAUNS lignin, which is not identical with MWL, then it cannot be identical with the main part of wood lignin. The conformity of BRAUNS lignin and enzymically liberated lignin is also open to question because of the low methoxyl content of the latter[19]. FREUDENBERG's biosynthetic lignin is chemically identical with BJÖRKMAN lignin but not with BRAUNS lignin.

It is for the sake of simplicity in experimentation that spruce lignin, which contains practically only guaiacylpropane residues, is preferred by us as a reference material for lignin studies.

Relative to the discovery that shikimic acid is a precursor of lignin, my comments are in accordance with statements by KRATZL[20]. Furthermore, the letter by EBERHARDT and NORD 'on the phase sequence of methyl *p*-methoxycinnamate and its possible relation to lignification'[6a] contains no report of shikimic acid's being an intermediate in the formation of lignin. It states that 'the formation of shikimic acid is presumed' in the formation of methyl *p*-methoxycinnamate – which in turn *may* be related to lignification – and in a reaction flow sheet, shikimic acid appears in brackets with a question mark, but is otherwise unmentioned. NORD's *practical* work relating shikimic acid to lignin only appeared in 1956[6b], whereas NEISH's 1955 publication was complete with experimental data. The papers by FREUDENBERG[16] cited by COSCIA, although dealing with shikimic acid, describe studies relative to its stereochemical configuration and do not have the remotest connection with lignin chemistry.

The glucosides were not designated as the ultimate starting point of the *total* process of lignification – which is in fact atmospheric CO_2 – but as the 'immediate starting materials for lignification'; they are the culminating substances of a large number of phenylpropane derivatives which are all potential precursors of lignin. They are, however, the only precursors to have been so far isolated from plants and are present in much higher stationary concentration than any of the others. The transformation of phenylalanine into coniferin by spruce saplings[7] illustrates the important central position of coniferin in lignification. The eventual conversion of the *p*-hydroxycinnamic alcohols into lignin is lignification in the strict sense; the conversion of, for example, shikimic acid into *p*-hydroxyphenylpyruvic acid is not; this latter process occurs in many organisms (e.g. *Lentinus lepideus*) which are by no means lignified.

The inability of plants other than Gramineae to convert *p*-hydroxyphenylpyruvic acid into lignin is the uncertainty implied in my original statement; it can only be metabolized if converted into *p*-hydroxycinnamic acid in the plant[11]. We have restricted ourselves for reasons mentioned above to conifer lignin, in which this transformation does not occur. It is indeed some progress that NORD *et al.* now consider that the *p*-hydroxycinnamic alcohols are intermediates of lignin formation.

The references to 'the preliminary stage of lignification' at which 'dimerization or trimerization has already occurred' convey no picture of what the oligomeric intermediates of lignin are like or how they have been formed. The dehydrogenative polymerization of coniferyl alcohol reported by FREUDENBERG *et al.* is an attempt at an *in vitro* reproduction of the *in vivo* process occurring in woody plants and uses the same enzymes as occur there. We call the first (dimeric) products of this dehydrogenation intermediates of lignin formation or secondary building stones. Careful investigations suggest that these intermediates are conclusive for conifer lignin in nature.

The enzyme – laccase – used to produce biosynthetic lignin can no longer be termed 'ill-defined'[21], and is of widespread occurrence in plants. More recent work uses H_2O_2 and crystalline peroxidase, a system exactly equivalent to that of laccase with oxygen.

The article by EVANS[18] is a review of the metabolism of aromatic compounds by lower plants, not of the overall scheme of lignification.

The *experimental* work done by NORD *et al.* is not at variance with our conception of the biogenesis and constitution of lignin, but it only partially covers the problems at issue. It is mainly the *interpretation* which NORD *et al.* give to their results that we cannot accept.

Professor FREUDENBERG stresses that he agrees with my first and this second Disputandum. I wish to call attention to his recent survey[22].

J. M. HARKIN

Forschungsinstitut für die Chemie des Holzes und der Polysaccharide, Chemisches Institut der Universität Heidelberg, 19. Oktober 1959.

[18] C. EVANS in *Encyclopedia of Plant Physiology*, vol. 10 (Springer Verlag, Heidelberg 1958), p. 498.

[19] A. APENITIS, H. ERDTMAN, and B. LEOPOLD, Svensk kem. Tidskr. *63*, 195 (1951). – W. MÜLLER-STOLL, Proc. 4th Intern. Congress Biochem. *2*, 206 (1959).

[20] K. KRATZL, Proc. 4th Intern. Congress Biochem. *2*, 262 (1959).

[21] B. G. MALMSTRÖM, R. MOSBACH, and T. VANNGÅRD, Nature *183*, 321 (1959). – B. G. MALMSTROM, G. FAHREUS, and R. MOSBACH, Biochim. biophys. Acta *28*, 652 (1958). – T. HIGUCHI, J. Biochem. *45*, 515 (1958); Physiol. Plantarum *10*, 356 (1957). – K. FREUDENBERG, J. HARKIN, M. REICHERT, and T. FUKUZUMI, Chem. Ber. *91*, 581 (1958).

[22] K. FREUDENBERG, Chem. Ber. *92*, LXXXIX (1959).