activated the *p*-nitrophenyl acetate- and *p*-nitrophenyl butyrate-hydrolysing activity of sheep serum at a significantly slower rate than it did the paraoxon-hydrolysing activity. From this and supporting evidence involving the K_m for hydrolysis activity of sheep, rabbit and hog sera and of purified paraoxonase towards *p*-nitrophenyl acetate, it was concluded that a new esterase hydrolysing *p*-nitrophenyl acetate, but not paraoxon, existed in sheep and probably in hog serum. The esterase was called D-esterase.

5. Evidence was presented which suggested that D-esterase was not identical with the phenyl acetate-hydrolysing arylesterase or with the C-esterase of hog kidney.

6. The effect of manganese and the decrease in the diisopropyl phosphorofluoridate-hydrolysing to paraoxon-hydrolysing rate from 1.8 in serum to 0.4 in purified paraoxonase preparations suggested the presence in sheep serum of a diisopropyl phosphorofluoridase similar to that of hog kidney, in addition to paraoxonase.

7. The paraoxonase in sheep serum was responsible for most of the tabun hydrolysis.

8. Sheep-serum and purified paraoxonase preparations hydrolysed *m*-nitrophenyl acetate more rapidly than they hydrolysed *p*-nitrophenyl acetate, and *o*-nitrophenyl acetate was hydrolysed least rapidly.

9. Purified paraoxonase did not hydrolyse tetraethyl pyrophosphate.

The generous help and advice of Dr E. C. Webb, who saw the paper through its revisions and proof reading, is gratefully acknowledged.

REFERENCES

- Aldridge, W. N. (1953*a*). *Biochem. J.* **53**, 111.
 Aldridge, W. N. (1953*b*). *Biochem. J.* **53**, 117.
 Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
 Augustinsson, K. B. & Heimbürger, G. (1954). *Acta chem. scand.* **8**, 1533.
 Bergmann, F., Segal, R. & Rimon, S. (1957). *Biochem. J.* **67**, 481.
 Cohen, J. A. & Warringa, M. G. P. J. (1957). *Biochim. biophys. Acta*, **26**, 29.
 Cohn, E. J., Strong, L. E., Hughes, W. L., Mulford, D. J., Ashworth, J. N., Melin, M. & Taylor, H. L. (1946). *J. Amer. chem. Soc.* **68**, 459.
 Dixon, M. (1953). *Biochem. J.* **55**, 170.
 Huggins, C. & Lapidès, J. (1947). *J. biol. Chem.* **170**, 467.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Main, A. R. (1960). *Biochem. J.* **74**, 10.
 Mounter, L. A. (1954). *J. biol. Chem.* **209**, 813.
 Mounter, L. A. (1955). *J. biol. Chem.* **215**, 705.
 Mounter, L. A. (1956). *J. biol. Chem.* **219**, 677.
 Mounter, L. A., Baxter, R. F. & Chanutin, A. (1955). *J. biol. Chem.* **215**, 699.
 Mounter, L. A. & Chanutin, A. (1953). *J. biol. Chem.* **204**, 837.
 Mounter, L. A. & Chanutin, A. (1954). *J. biol. Chem.* **210**, 219.
 Mounter, L. A. & Dien, L. T. H. (1956). *J. biol. Chem.* **219**, 685.
 Mounter, L. A., Dien, L. T. H. & Chanutin, A. (1955). *J. biol. Chem.* **215**, 691.
 Mounter, L. A., Floyd, C. S. & Chanutin, A. (1953). *J. biol. Chem.* **204**, 221.
 Mounter, L. A. & Whittaker, V. P. (1953). *Biochem. J.* **54**, 551.

Biochem. J. (1960) **75**, 195

Methanol Lignin from *Eucalyptus regnans* F. Muell. and its Purification by Countercurrent Distribution

By D. E. BLAND

Division of Forest Products, C.S.I.R.O., Melbourne, Australia

(Received 5 August 1959)

It is not possible to define lignin satisfactorily from a chemical viewpoint. Brauns (1952) considers it to be a collective term for a group of high-molecular-weight amorphous compounds rather than a constitutionally defined compound. One of the major problems of lignin chemistry is that no simple criterion of purity of a lignin preparation is available.

In view of the above, differences between lignins from different sources and obtained by different

methods of preparation are to be expected. On the other hand, agreement between results on one lignin should be attainable if all substances extraneous to the lignin group were eliminated. The first step towards completing and reconciling apparently conflicting results ought to be the preparation of the substance in question in a satisfactory state of purity. The difficulties besetting the worker in the field of lignin chemistry are such that some compromise usually has to be made. It

was shown by Bland (1958*a*) that paper-chromatographic analysis of lignin extracted from *Eucalyptus regnans* with methanol at 150° disclosed the presence of numerous substances, some of them recognizable as lignin-decomposition products. Chromatography showed that repeated precipitation did not free this lignin from all impurities.

The possible advantages of countercurrent distribution as a method for the purification of lignin make its investigation a matter of considerable importance, although development of a suitable solvent system for the resolution of lignin presents some difficulty. Lignin is insoluble in water and therefore this solvent cannot be employed.

This paper describes solvent systems for the purification by countercurrent distribution of lignin extracted from *Eucalyptus regnans* wood by means of methanol at 150° without the addition of acid catalyst. This lignin is referred to as methanol lignin as it is now believed to be similar to that prepared by treatment of wood with methanol-hydrochloric acid. The composition and some of the properties and reactions of the resultant lignin are described.

EXPERIMENTAL

Materials

The methanol-lignin preparations used in these experiments were extracted from different specimens of *E. regnans* wood. One was from immature wood, the trees being about 12 years old, the other from a composite sample of *E. regnans* mature wood. The wood specimens were ground to pass the 1 mm. screen in a Wiley mill, treated with water at about 55° for 8 hr. on three successive days and then treated in the same way with ethanol.

After thorough drying in air, 1 kg. of the wood meal was heated with methanol (10 l.) at 150° for 4 hr., after which the methanol extract was drained off and a further quantity of methanol added and heating continued for 20 hr. The process was repeated with a third quantity of methanol and a 32 hr. heating period. The combined methanol extracts were concentrated to 2.5 l. and poured with stirring into 25 l. of water at 10°. The precipitated lignin was washed and recovered by decantation and centrifuging. Yield was about 50 g.

Countercurrent purification of methanol lignin

Tests with solvent systems showed that possibly useful ones were mixtures of a hydrocarbon or ether with a hydroxylic solvent which separated into two layers on the addition of small amounts of water. The resolution obtained in preliminary runs was followed with the aid of paper chromatography, specimens from the emergent upper layers and from the tubes of the Craig machine being concentrated in groups. The concentrates were spotted on to Whatman no. 1 paper which had been dipped in 0.05M-sodium tetraborate solution and air-dried. The sheet was irrigated for 6 hr. by the ascending technique with *iso*-butanol-benzene-water (1:9:10), as used by Bland &

Gateley (1954). Efficiency of the countercurrent separation was then roughly assessed by visual inspection of the sheets in ultra-violet light. The two solvents selected as promising were: (1) benzene-methanol-water (200:100:36, by vol.); (2) methanol-*isopropyl* ether-water (2:4:1, by vol.). The lower layer in each system was rich in methanol; in solvent 1, decreasing the proportion of water from 36 to 30 at 20° gave a methanol-rich upper layer.

Progress of the separation was assessed quantitatively by cutting out the visible spots and determining the amounts present by eluting from the paper and measuring the extinction of the eluate. This procedure was similar to that employed by Bland & Stamp (1955) in the determination of oxidation products of lignin by chromatography. Two substances not completely separated by the countercurrent-distribution procedure but separated by paper chromatography could thus be traced and their overlapping distribution curves accurately plotted. When the separation had proceeded to a stage where the chromatogram indicated that the main lignin fraction had been freed from degraded lignin and other materials the emergent upper layers and the contents of the tubes of the Craig machine were grouped and concentrated. The lignin was recovered by precipitation into cold ether from the concentrate of the appropriate tubes. After purification on the Craig machine the main lignin fraction was subjected to further paper-chromatographic examination with benzene containing different percentages of *N*-dimethylformamide. The degraded lignin, which passed into the emergent upper layers, was recovered by extraction of the grouped and concentrated upper layers with aqueous sodium hydroxide followed by precipitation with acid.

Examination of purified lignins

Sulphuric acid treatment. A specimen (0.2 g.) of the lignin was stirred at 20° for 2 hr. with 72% sulphuric acid (15 ml.), which was then diluted to 3% and boiled for 2 hr. The lignin was recovered by filtration on an alundum crucible, washing with hot water and drying at 105°. The resultant lignin was analysed for C, H, O and O·CH₃.

Infrared spectra. The infrared-absorption spectra were determined in potassium chloride disks as described by Higgins (1957).

Determination of phenolic groups. Phenolic groups were determined by a modification of the method of Butler & Czepl (1956), which involves potentiometric titration of lignin with sodium hydroxide in methanol with *N*-dimethylformamide as solvent. The solvent was titrated to the point where the observed e.m.f. ceased to rise rapidly; a quantity of the lignin sample (about 0.1 g.) was then introduced and the titration continued until the e.m.f. again ceased to rise. A weighed quantity of the lignin (about 0.2 g.) was then introduced and the titration continued until the e.m.f. reached a steady value. A plot of observed e.m.f. against volume (ml.) of alkali added gave a discontinuous curve with two rising parts after each addition of lignin. On each one of these the point of inflexion could be distinguished and the titre of the second quantity of lignin was taken as the difference between the titres at the two points of inflexion (see Fig. 3). This method eliminated uncertainty of the starting point of the titration. Phenolic groups were also determined by the barium chloride method of Enkvist, Alm & Holm (1956) and by the spectrophotometric method of Aulin-Erdtman (1954).

Total hydroxyl content. This was determined by the method of Hillis (1954).

Detection of hydroxybenzyl alcohol group. These were tested for by the quinonemonochloroimide method of Gierer (1954).

Nitrobenzene oxidation. The lignin (0.05 g.) was placed in a stainless-steel bomb (10 ml. capacity) with 2N-sodium hydroxide (5 ml.) and 0.2 ml. of nitrobenzene. The sealed bomb was heated for 3 hr. at 160°, cooled, opened and the contents were acidified to about pH 3.0 with hydrochloric acid and allowed to stand overnight. The mixture was then centrifuged and the residue washed with water (3 × 30 ml.). The supernatant solution and washings were combined and extracted with chloroform (4 × 10 ml.) and the chloroform extracts were evaporated to dryness at room temperature. The dry residue was dissolved in ethanol (2 ml.) and the aldehydes were separated chromatographically on borate paper with methanol-*isopropyl* ether (peroxide-free)-water (1:1:1, by vol.) and determined according to Bland & Stamp (1955).

Spectrophotometric determination of syringyl and guaiacyl units. The molecular extinction coefficient, ϵ , based on the mol.wt. of the C_9 unit of the purified lignins, was determined and an estimate made of the ratio of syringyl to guaiacyl units by comparison with the molecular extinctions of syringyl alcohol and vanillyl alcohol. The $\Delta\epsilon$ ($\epsilon_{\text{alkaline}} - \epsilon_{\text{neutral}}$) curves for the lignins were determined and an estimate of the number of phenolic units was made by comparison of $\Delta\epsilon_{\text{max}}$ for the lignins with $\Delta\epsilon_{\text{max}}$ for the two alcohols. The same $\Delta\epsilon$ curves were used to obtain an estimate of the proportion of syringyl units in the total phenolic units. The determinations were made in neutral and alkaline (0.1N-potassium hydroxide) methanol with an Optica recording spectrophotometer.

RESULTS

Some difficulty was experienced with the benzene-methanol-water mixture as it tended to produce a coarse emulsion of the upper and lower layers, the separation of which was imperfect even after a settling period of 15 min. Nevertheless, as preliminary experiments showed that it brought about a definite resolution of the crude methanol lignin its use was continued. With this solvent system the partition coefficient of the main lignin fraction was such that it always passed mainly into the methanol-rich phase. When this was the upper (mobile) phase the lignin therefore passed rapidly along the machine into the emergent upper layers, when no further separation was possible. When the methanol-rich phase was the lower (stationary) phase the main lignin fraction remained in the first few tubes of the train and a large number of transfers could be employed. The number of transfers is, however, limited by other factors such as losses by spillage resulting from imperfect separation of the two layers. The results of such a resolution with 200 transfers are shown graphically in Fig. 1, from which it may be seen that the substances of low R_F remained in the first 30 tubes, whereas those of

higher R_F were carried forward. Only the substances of low R_F had u.v.-absorption spectra typical of lignin.

The fraction which passed forward in the top layer of the solvent could not be recovered by ether precipitation, since it was soluble in ether. This material was spread over a large range of the emergent upper layers, from which it was recovered by extraction with sodium hydroxide and reprecipitation with acid. This precipitate was obtained almost ash-free after exhaustive washing with water. Paper-chromatographic analysis of this product showed it to consist of materials of a wide range of R_F and not to correspond to any one of the lignins distinguished chromatographically in the original crude lignin. Its ultraviolet-absorption spectrum lacked the typical maximum of lignin in the 275 m μ region. The infrared-absorption spectrum (potassium chloride disk, Fig. 2) may be seen by comparison with the curve of the main lignin

Fig. 1. Distribution curves of crude methanol lignin with benzene-methanol-water solvent system. R_F values were determined with *isobutanol*-benzene-water (1:9:10, by vol.).

Fig. 2. Infrared-absorption curve of the lignins I-IV showing removal of band at 6 μ by borohydride reduction and absorption curve of degraded lignin.

Table 1. *Composition of lignins from mature and immature Eucalyptus regnans wood after purification by countercurrent distribution with different solvents*

Nature of wood	Solvent	Lignin	Absorption maximum (m μ)	Composition (%)				C ₉ formula
				C	H	O	O·CH ₃	
Immature	Benzene-methanol-water	I	275	58.78	6.21	32.9	26.01	C ₉ H _{8.08} O _{2.70} (O·CH ₃) _{1.86}
Immature	isoPropyl ether-methanol-water	II	275	59.02	6.26	33.6	26.21	C ₉ H _{8.13} O _{2.77} (O·CH ₃) _{1.87}
Mature	Benzene-methanol-water	III	272	58.68	6.25	34.2	25.40	C ₉ H _{8.30} O _{2.82} (O·CH ₃) _{1.81}
Mature	isoPropyl ether-methanol-water	IV	272	59.11	6.29	33.5	27.11	C ₉ H _{8.05} O _{2.71} (O·CH ₃) _{1.84}

Table 2. *Chromatographic resolution of main lignin fraction*

Paper chromatography with dimethylformamide-benzene, of the lignin fractions (Table 1) gave two spots. The relative amount of material in each spot depended upon the composition of the solvent, as shown.

Percentage of dimethylformamide in solvent mixture	Percentage of lignin	
	R _F 0	R _F 0.95
5	83	17
7	69	31
10	23	77
15	16	84

fraction to have the same general shape, but the bands are less well defined. An additional band is present at 5.9 μ and this may correspond to the generation of a carbonyl group, as found by Sarkanen & Schuerch (1957) to take place during alcoholysis of lignin. Unlike the main lignin fraction, this material, which appears to be correctly termed degraded lignin, could not be prepared to a constant composition.

The main lignin fractions, recovered from immature wood with solvents 1 and 2, and from mature wood with solvents 1 and 2, have been numbered I to IV respectively, as shown in Table 1. When recovered by precipitation into ether they were light buff or almost white. They were recovered in a yield of 40–50 % of the original precipitated lignin and chromatographic analysis showed them to be free from the low-molecular-weight material associated with the original lignin. However, resolution on plain paper with benzene-dimethylformamide for 6 hr. gave two fractions as shown in Table 2. It can be seen by reference to this table that when the two lignins were estimated by elution and determination of $E_{275\text{ m}\mu}$ the relative amounts depended on the percentage of dimethylformamide in the solvent mixture. It thus appears that countercurrent distribution gave a lignin of

narrow partition-coefficient limits but containing substances of differing dissociation constants. They did not show a definite melting point but shrank at about 130° and softened over a range of roughly 20°. It is clear that the lignins isolated were not pure substances in the chemical sense but were purified lignins in Brauns's sense of lignin as a group of substances. The examination of these lignins is of interest to establish the basic structure of eucalypt lignin and to compare with other lignins.

The analyses presented in Table 1 show no significant difference between lignins recovered with either of the two solvent systems or between lignins from immature or mature wood. There is, however, a difference in the position of the ultra-violet-absorption maximum of lignin from mature and immature wood and this is the same as observed earlier by Bland (1958b) for lignin separated by paper chromatography, i.e. the maximum of the immature-wood lignin lies at 275 m μ and that of the mature-wood lignin at a shorter wavelength, here 272 m μ . The infrared-absorption spectra of the four lignins were identical, the curve being given in Fig. 2.

The molecular weights of lignins I and IV determined by the Rast method were 2235 and 2180 respectively. The sulphuric acid treatment of lignin I yielded 90 % of acid lignin [Found C, 60.68; H, 5.80; O, 31.30; ash, 1.57; O·CH₃, 22.37 %; equivalent to C₉H_{7.48}O_{2.57} (O·CH₃)_{1.48}]. This corresponds to the loss of 0.41 methoxyl group per C₉ unit during the acid treatment and probably represents the methoxyl group introduced during the methanol extraction.

The results of the determination of hydroxyl groups in lignins I and IV are shown in Table 3. The phenolic hydroxyl-group content of lignin I, determined by the potentiometric method as illustrated in Fig. 3, corresponds to 0.35 hydroxyl group per C₉ unit, whereas the total hydroxyl-group content corresponds to 1.40, giving the aliphatic hydroxyl-group content by difference as

1.05 per C₉ unit. It appears that the C₉ formula of lignin I may be expressed as

but this requires further examination.

Treatment of the lignins I-IV with *p*-quinone-monochloroimide according to Gierer (1954) gave no colour, thus showing *p*-hydroxybenzyl alcohol groups to be absent. The original wood gave a positive reaction corresponding to less than 0.01 *p*-hydroxybenzyl alcohol group per phenylpropane unit; this agrees with the conclusion of Adler (1958) about spruce lignin. This indicates that the *p*-hydroxybenzyl alcohol groups were methylated during the extraction but as the proportion of them in the original lignin was very low this reaction

accounts only for a very small part of the introduced methoxyl group.

Adler & Gierer (1955) found that in Brauns's native lignin from spruce, treatment with methanol-hydrochloric acid, either at room temperature or under reflux, did not cause the disappearance from the infrared spectrum of the carbonyl-group band at about 1660 cm.⁻¹ (6 μ), but reduction with sodium borohydride caused its disappearance. From the spectra of lignin I in Fig. 2 it is clear that this band is intact and shows that the carbonyl groups did not react with methanol at 150° but were removed by reduction with borohydride.

The phenolic hydroxyl-group content of the eucalypt methanol lignin per C₉ unit, 0.35, is similar to that found by Bland (unpublished work) in eucalypt milled-wood lignin (about 0.37) and to that found in spruce milled-wood lignin (0.35) by Enkvist *et al.* (1956). There is no indication therefore of any change in the number of phenolic groups during the methanol extraction. Adler (1958) concluded that in conifer lignin the introduction of 0.42 methoxyl group per original methoxyl group took place at arylcarbinol and arylcarbinol-alkyl ether groups. It would appear, therefore, that the reaction of eucalypt lignin with methanol at 150° was similar to the reaction with methanol-hydrochloric acid at room temperature, and it may be concluded that the methylation took place most probably at arylcarbinol or arylcarbinol-alkyl ether groups. However, the 'total hydroxyl' determined by acetylation probably includes the introduced methoxyl groups, as these were removed by the sulphuric acid treatment and similar ethyl groups were unstable even in dilute acid (Bland, Billek, Gruber & Kratzl, 1959). These results suggest that the composition of the original lignin in the wood may be expressed as:

This C₉ formula may be compared with those derived for spruce lignin by various workers. Erdtman, Lindgren & Petterson (1950), on the assumption that sulphonation occurred by substitution of hydroxyl groups by sulphonic acid groups, deduced the formula

for protolignin. Arlt, Sarkanen & Schuerch (1956) deduced $\text{C}_9\text{H}_{7.6}\text{O}_{2.6}(\text{O}\cdot\text{CH}_3)_{0.95}$ from the composition of their ethanol lignin. Björkman & Person (1957) found

for milled-wood lignin from *Picea abies* and

for milled-wood lignin from *Betula verrucosa*. Merewether (1954) reached a formula for eucalypt

Table 3. *Hydroxyl-group contents of lignins*

	Phenolic hydroxyl group (%)		Total hydroxyl group (%) (Acetylation)
	Potentiometric	BaCl ₂	
Lignin I	2.85	2.03	11.27
	2.86	2.26	10.74
	2.58	2.13	—
Lignin IV	2.69	2.51	—
	2.72	2.30	—
	2.84	2.38	—

Fig. 3. Specimen curves of potentiometric determination of phenolic groups in lignin (*t*, titre).

Table 4. Spectrophotometric data on methanol lignins and related compounds

Substance	Neutral		Difference spectrum ($\Delta\epsilon$)				$\Delta\epsilon_{\max.}$ 9245
	$\lambda_{\max.}$ (m μ)	$\epsilon_{\max.}$	$\lambda_{\max.}$ (m μ)	$\Delta\epsilon_{\max.}$	$\lambda_{\min.}$ (m μ)	$\Delta\epsilon_{\min.}$	
Eucalypt lignin I	275	2060	258	1 940	282	587	0.21
Eucalypt lignin IV	272	2140	258	1 820	282	482	0.20
<i>P. radiata</i>							
Methanol lignin	280	3100	251	1 985	280	78	—
Vanillyl alcohol	280	2900	253	10 090	278	0	—
Syringyl alcohol	272	1100	260	8 400	283	3858	—

protolignin which, expressed on the same basis, becomes $C_9H_{7.17}O_{2.90}(O \cdot CH_3)_{1.37}$ or

Nitrobenzene oxidation of lignin I gave 26.9 % of syringaldehyde and 5.9 % of vanillin; lignin IV gave 24.3 % of syringaldehyde and 5.8 % of vanillin. These results demonstrate the presence of syringyl and guaiacyl residues but do not disclose their relative numbers in the lignin. Freudenberg & Schlüter (1955) have pointed out that the low yield of vanillin is connected with the linkage of guaiacyl residues by C-C bonds at the position *ortho* to the hydroxyl group whereas this is not possible for the syringyl residues.

An independent estimate of the relative numbers of syringyl and guaiacyl units can be obtained from the spectrophotometric data on lignins. Aulin-Erdtman (1953*a*, 1957) reported that for vanillyl (guaiacyl) alcohol and syringyl alcohol $\log \epsilon_{280} = 3.47$ and $\log \epsilon_{271} = 3.05$ respectively, and that for spruce methanol lignin $\log \epsilon_{280} = 3.5$. The ϵ values for lignins I and IV together with those for *Pinus radiata* methanol lignin and vanillyl alcohol and syringyl alcohol determined under the same conditions are shown in Table 4. It may be seen by reference to this Table that $\epsilon_{\max.}$ of lignins I and IV corresponds to the presence of approximately equal numbers of guaiacyl and syringyl units.

The difference values, $\Delta\epsilon_{\max.}$, for lignins I and IV shown in Table 4 indicate that 0.2, i.e. one in five, of the C_9 units of these two lignins are phenolic and ionized in methanolic 0.1N-potassium hydroxide. This is considerably lower than the total phenolic hydroxyl-group content determined by the potentiometric method, which indicated one in three of the C_9 units to be phenolic. The low results given by the $\Delta\epsilon$ method for phenolic content have previously been discussed by Freudenberg & Dall (1955).

The $\Delta\epsilon_{\min.}$ at about 280 m μ gives an estimate of the fraction of syringyl units in the total phenolic units ionized in methanolic 0.1N-potassium hydroxide, as Aulin-Erdtman (1953*b*, 1957) has shown that the $\Delta\epsilon_{\min.}$ for vanillyl alcohol is close to zero but for syringyl alcohol $\Delta\epsilon_{\min.}$ has a positive value.

However, it may be seen from Table 4 that *Pinus radiata* methanol lignin gave a small positive value for $\Delta\epsilon_{\min.}$, although Bland, Ho & Cohen (1950) showed that *P. radiata* contained no syringyl groups. This may be due to the influence of conjugated chromophores in the lignin, and as the long-wavelength (200–300 m μ) portion of the $\Delta\epsilon$ curve for *P. radiata* lignin was similar to that of the eucalypt lignins, the $\Delta\epsilon_{\min.}$ value of the *P. radiata* lignin was used as a first approximation to correct the $\Delta\epsilon_{\min.}$ values of the eucalypt lignins. This led to the conclusion that about 60 % of the ionized phenolic groups were syringyl groups.

DISCUSSION

There was no reason for assuming that counter-current resolution of a lignin preparation would give one or more clearcut lignin fractions. A continuous distribution of partition coefficients in the lignin would have led to a continuous distribution of lignin along the train, but in fact the main lignin fraction appeared as the usual type of distribution curve. However, it cannot be claimed that counter-current distribution provides a method whereby lignin can be prepared as a pure substance in the strictly chemical sense. Nor would the methods described necessarily apply to other lignin preparations, especially those of higher molecular weight, such as milled-wood lignin. On the other hand, where applicable as a method of purification it is probably more satisfactory than repeated precipitation as it eliminates adsorption of impurities by the lignin. The 'pure' lignin obtained had properties in accordance with Brauns's conception of lignin as a group of amorphous compounds.

The results of chemical examination of this lignin can be accepted as referring to the lignin itself and not partly to adsorbed degradation products or other impurities. The results of Bland *et al.* (1959) suggest that lignin extracted with methanol at high temperatures is an altered lignin, as when labelled methanol was used not all of the activity could be recovered by Zeisel distillation. However, comparison of results on this eucalypt

lignin with those of other workers on spruce lignin show the eucalypt lignin to have striking resemblance in chemical composition and properties to spruce lignin and suggests that, despite the presence of syringyl groups, the basic structure of the two lignins is fundamentally the same.

SUMMARY

1. Crude methanol lignin from immature and mature *Eucalyptus regnans* has been separated by countercurrent distribution into lignin and degraded lignin.

2. Chromatographic analysis of the separated lignin indicates the presence of a number of substances of differing dissociation constants. This is in accord with Brauns's conception of lignin as a group of amorphous substances.

3. The lignin of immature *E. regnans* is shown to possess the empirical formula based on a C_9 unit of $C_9H_{6.68}O_{1.30}(OH)_{1.05}(OH)_{0.35}(O \cdot CH_3)_{1.45}$, where the aliphatic hydroxyl-group content is represented by 1.05 and the phenolic hydroxyl-group content by 0.35. The lignin of mature *E. regnans* does not differ significantly from this.

4. An approximately equal number of syringyl and guaiacyl units are present in the lignin of *E. regnans*.

5. Potentiometric determination indicates that one in three of the C_9 units is phenolic and ionized in dimethylformamide, whereas spectrophotometric determination shows one in five to be ionized in methanolic 0.1N-potassium hydroxide.

6. The spectrophotometric data suggest that of the phenolic groups ionized in the 0.1N-potassium hydroxide 60% are syringyl groups.

The author is grateful to Mr M. John and Mr P. Carson

for assistance and to Mr K. Harrington for determination of infrared spectra. The microanalyses were done by the Australian Microanalytical Service and the hydroxyl determinations by Mr M. Menshun.

REFERENCES

- Adler, E. (1958). *Proc. 4th int. Congr. Biochem.*, Vienna, vol. 2, p. 137.
 Adler, E. & Gierer, J. (1955). *Acta chem. scand.* **9**, 84.
 Arlt, H. G., Sarkanen, K. & Schuerch, C. (1956). *J. Amer. chem. Soc.* **78**, 1904.
 Aulin-Erdtman, G. (1953a). *Svensk Papp-Tidn.* **56**, 91.
 Aulin-Erdtman, G. (1953b). *Svensk Papp-Tidn.* **56**, 287.
 Aulin-Erdtman, G. (1954). *Svensk Papp-Tidn.* **57**, 745.
 Aulin-Erdtman, G. (1957). *Svensk Papp-Tidn.* **60**, 671.
 Björkman, A. & Person, B. (1957). *Svensk Papp-Tidn.* **60**, 158.
 Bland, D. E. (1958a). *Holzforschung*, **12**, 19.
 Bland, D. E. (1958b). *Holzforschung*, **12**, 36.
 Bland, D. E., Billek, G., Gruber, K. & Kratzl, K. (1959). *Holzforschung*, **13**, 6.
 Bland, D. E. & Gatley, F. M. (1954). *Nature, Lond.*, **173**, 32.
 Bland, D. E., Ho, G. & Cohen, W. E. (1950). *Aust. J. sci. Res.* **3A**, 642.
 Bland, D. E. & Stamp, C. (1955). *Aust. J. appl. Sci.* **6**, 353.
 Brauns, F. E. (1952). *The Chemistry of Lignin*, p. 14. New York: Academic Press Inc.
 Butler, J. P. & Czepiel, T. P. (1956). *Analyt. Chem.* **28**, 1468.
 Enkvist, T., Alm, B. & Holm, B. (1956). *Paperi ja Puu*, **38**, 1.
 Erdtman, H., Lindgren, B. O. & Petterson, T. (1950). *Acta chem. scand.* **4**, 228.
 Freudenberg, K. & Dall, K. (1955). *Naturwissenschaften*, **22**, 606.
 Freudenberg, K. & Schlüter, H. (1955). *Chem. Ber.* **88**, 617.
 Gierer, J. (1954). *Acta chem. scand.* **8**, 1319.
 Higgins, H. G. (1957). *Aust. J. Chem.* **10**, 496.
 Hillis, W. E. (1954). *J. Soc. Leath. Tr. Chem.* **38**, 177.
 Merewether, J. W. T. (1954). *T.A.P.P.I.*, **37**, 483.
 Sarkanen, K. & Schuerch, C. (1957). *J. Amer. chem. Soc.* **79**, 4203.

Biochem. J. (1960) **75**, 201

The Spontaneous and Induced Recovery of Fly-Brain Cholinesterase after Inhibition by Organophosphates

BY D. C. MENGLE AND R. D. O'BRIEN*

Department of Entomology, University of Wisconsin, Madison, Wisconsin, U.S.A.

(Received 7 September 1959)

The widely held belief is that organophosphates exert their insecticidal action through the inhibition of cholinesterase (Spencer & O'Brien, 1957; Fukuto, 1957). Mengle & Casida (1958), with 17 organophosphates in amounts corresponding to the

* Present address: Pesticide Research Institute, London, Ontario, Canada.

LD₅₀, showed that cholinesterase *in vivo* was usually largely inhibited within a few hours, but always recovered in the survivors to 90% or more of normal within 1 day. These recoveries are greater than those found in the mammal *in vivo* (Davison, 1955). There appeared to be no connexion between the nature of the alkoxy substituents and