Phytochemistry, 1965, Vol. 4, pp. 383 to 399. Pergamon Press Ltd. Printed in England

# POLYPHENOLS OF PINE POLLENS

## A SURVEY

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(*Received* 31 *July* 1964)

**Abstract**—Pine pollens contain an array of polyphenolic compounds. Those identified include the phenolic acids *p*-hydroxybenzoic, protocatechuic, gallic, vanillic, *cis*- and *trans*-*p*-coumaric, *cis*- and *trans*-ferulic, and 4-glucosyloxybenzoic acid; the flavanonols dihydrokaempferol and dihydroquercetin; the flavanone naringenin; and a series of esters of *p*-coumaric acid which constitute the principal phenolic type present. The phenolic acids and the coumarate esters varied little with the species of pine, but the flavonoid fraction has sufficient variation to warrant further study.

## INTRODUCTION

PRECEDING work[1] in this Laboratory on the constituents of pine pollens (particularly loblolly pine pollen) had concentrated on identification of the lipid materials, but the crude extractives were found to give color tests indicative of phenolic and flavonoid compounds. Since benzene, ether, and methanol extractives were available from eight pine species (*Pinus banksiana* Lamb., *P. echinata* Mill., *P. elliottii* Engelm., *P. palustris* Mill., *P. ponderosa* Laws., *P. resinosa* Ait., *P. strobus* L., and *P. taeda* L.) a paper chromatographic study of the variation of the polyphenols with species was undertaken. Characterization of some new compounds has been made.

Previously the only flavonoid compounds reported to occur in pine pollens have been isorhamnetin and quercetin in *Pinus densiflora* and *P. thunbergiia*;[2] a flavanone with less than five hydroxyl groups in *P. ponderosa*;[3] and a yellow pigment having flavonol properties in *P. montana*.[4] Lubliner-Mianowska[5] found no indication of flavonoid compounds in the yellow pollen of conifers.

A wider variety of flavonoids has been isolated from other plant pollens and identified. Flavonols occur most frequently and are generally in glycosidic form, namely: kaempferol,[6]

* Maintained at Madison, Wis., in cooperation with the University of Wisconsin.

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[2] S. HISAMICHI, *J. Pharm. Soc. Japan* **81**, 446 (1961).
[3] S. L. KRUGMAN, *Forest Sci.* **5**, 169 (1959).
[4] H. VON EULER, L. AHLSTRÖM, B. HÖGBERG and I. PETTERSSON, *Arkiv. Kemi Mineral. Geol.* **19A**, 1 (1945).
[5] K. LUBLINER-MIANOWSKA, *Acta Soc. Botan. Polon.* **24**, 609 (1955). *Bee World* **37**, 118 (1956); *Chem. Abstr.* **50**, 12206 (1956).
[6] H. REZNIK, *Biol. Zentr.* **76**, 352 (1957).

quercetin,[6–14] isorhamnetin,[8, 10, 12, 15–18] robinetin,[19] morin,[19] and myricetin.[9] The flavanone naringenin has been found in *Acacia dealbata* in two glycosidic forms.[9, 20] Four anthocyanins have been identified in the blue pollen of *Anemone coronaria*.[21]

Only two phenolic acids had been formerly reported in pollens, hydrocaffeic in *Lycopodium clavatum* spores[22, 23] and chlorogenic in *Forsythia intermedia*,[6] but recently the glucose esters of *p*-hydroxybenzoic, *p*-coumaric, ferulic and caffeic acids were discovered in petunia pollen.[24] Also quinic and shikimic acids were found in *Ginkgo biloba* pollen.[25]

## RESULTS AND DISCUSSION

Preliminary attempts to prepare well-resolved two-dimensional paper chromatograms of the compounds in the phenolic-rich ether extractives (Fig. 1) of pine pollens failed because of the excessive amounts of one type of material. When nonpolar developing solvents* were used, this material (later identified as *p*-coumarate esters) gave a heavy fast-moving spot trailing over half the sheet; the deep purple color resulting from the spray *p*-nitrobenzenediazonium tetrafluoborate and carbonate*, effectively hid lesser spots. In aqueous solvents it

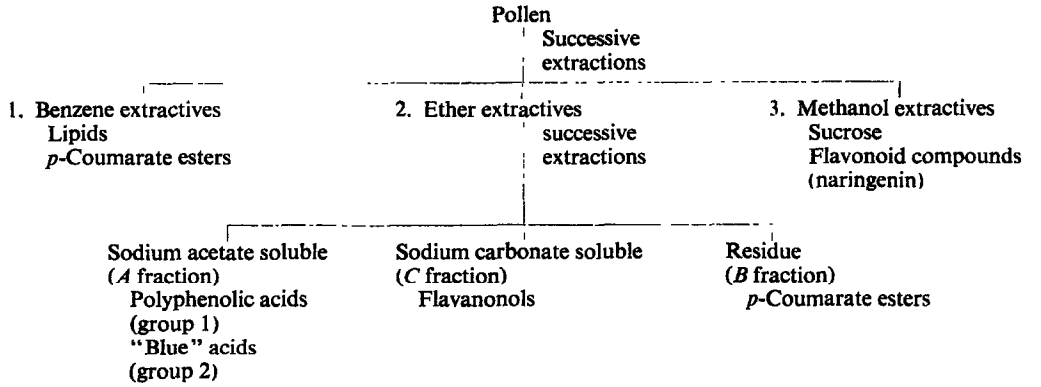


FIG. 1. EXTRACTIVES FOUND IN PINE POLLENS

* See Experimental for description of developing solvents and sprays.

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[15] M. B. MOORE and E. E. MOORE, *J. Am. Chem. Soc.* **53**, 2744 (1931).
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[20] G. TAPPI, A. SPADA and R. CAMERONI, *Gazz. Chim. Ital.* **85**, 703 (1955).
[21] G. TAPPI and A. MONZANI, *Gazz. Chim. Ital.* **85**, 732 (1955).
[22] F. ZETZSCHE and K. HUGGLER, *Helv. Chim. Acta* **10**, 472 (1927).
[23] F. ZETZSCHE and K. HUGGLER, *Ann.* **461**, 89 (1928).
[24] L. BIRKOFER, C. KAISER and H. MEYER-STOLL, *Z. Naturforsch.* **17B**, 352 (1962).
[25] W. TULECKE, L. H. WEINSTEIN, A. RUTNER and H. J. LAURENCOT, *Contrib. Boyce Thompson Inst.* **21**, 291 (1962).

did not move. Fractionation of the extractives by alkaline extractions (Fig. 1) resulted in the separation of two series of minor components which gave good chromatograms and a residue of "purple" compounds which could be handled best by thin-layer chromatography. The benzene and methanolic extractives were briefly investigated, principally for the isolation of specific components.

*Acid Fraction of the Ether Extractives*

The acid fractions (designated as the *A* fractions), containing compounds soluble in sodium acetate, were most thoroughly studied. A total of 26 *A* spots were finally differentiated on the two-dimensional chromatograms, different pollens exhibiting from half to almost the entire array. These spots could be divided into two groups; (1) *A* spots which gave colors with the diazonium sprays (I and II); and (2) *A* spots which appeared fluorescent white, blue, or greenish in u.v. light in the presence of ammonia fumes.

The six prominent *A* spots of group 1 and two weaker ones (which were, however, important in *Pinus strobus* pollen) were identified by their color reactions and by cochromatographic work as the six common phenolic acids listed in Tables 1 and 2. Among the five collections of *P. taeda* pollen these acids varied principally in relative amount. Gallic acid, however, was lacking from three; two of these were samples which had been subjected to more or less elevated temperatures and humidity because of improper aeration of the male flowers after collection. A study of variation of the acids among the other seven pines studied (Table 2) revealed few clear-cut distinctions since slight differences in concentration were ignored. The lack of gallic acid in the pollens of *P. strobus* and *P. elliottii* may have no taxonomic meaning as shown by the more extensive work on *P. taeda* pollen. The lack of vanillic acid in the pollens of *P. ponderosa* and *P. echinata* may however be taxonomically significant since this acid was still present in the poorly handled samples of *P. taeda* pollen. *P. strobus* pollen shows probably the most fundamental differences from the other pollen in respect to the phenolic acids present. It has relatively larger amounts of the cinnamic acid derivatives, *p*-coumaric and ferulic acids, and less of the corresponding benzoic acid derivatives, *p*-hydroxybenzoic acid (the single most important phenolic acid in all but one of the other pollens) and vanillic acid. It is perhaps significant that *P. strobus* is the only haploxylon pine[26] in the list. The discovery of these phenolic acids in pine pollens seems to be in no way unusual, as recent work shows that they occur and co-occur widely in various plants,[27–29] and several have been reported in other pine tissues.[30, 31]

The group 2 *A* spots included eight prominent but still unidentified spots which fluoresced a vivid turquoise blue in u.v. light in the presence of ammonia. These did not couple with diazonium salts; pale lavender and pale rose colors shown by two of the spots seem to be caused by overlying spots (see later discussion of spot *A*-10*b*). Considerable variation between the eight species may not be taxonomically significant, since instability of the two most prominent spots was evidenced by their absence from or very low concentration in the extractives of the older and poorly handled samples of *P. taeda* pollen and by their loss in isolation and hydrolysis work. This group from *P. echinata* pollen was the most outstanding, as it showed

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[27] R. K. IBRAHIM and G. H. N. TOWERS, *Arch. Biochem. Biophys.* **87**, 125 (1960).
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[30] W. E. HILLIS (Editor) *Wood Extractives*, Academic Press, N.Y. (1962).
[31] H. OKSANEN, *Suomen Kemistilehti* **34**, B-91 (1961).

six spots in high concentration. The spots occurred in pairs, separable by aqueous developing solvents. Attempts to isolate one of these compounds by preparative paper chromatography and to hydrolyze it failed to produce fluorescent compounds. It is postulated that they represent hydroxycinnamic acid derivatives, probably *cis-trans* pairs. They may be phenolic

TABLE 1. TWO-DIMENSIONAL PAPER CHROMATOGRAPHIC DATA ON IDENTIFIED PHENOLIC ACIDS (4 SPOTS)

| Spot No. | Identity* of acid | ($R_f$ × 100) BzAW/aNaCl† | Colors observed‡ in UVA | with sprays Ia | Ib | II |
|---|---|---|---|---|---|---|
| *A*-2 | vanillic | 81/52 | C | O | V | OR |
| *A*-3 | *p*-hydroxybenzoic | 71/65 | C | lY | Ro | dOY |
| *A*-4 | *trans p*-coumaric | 74/38 | bBl | O | BlGy | lRo |
| *A*-11 | *cis p*-coumaric | 75/74 | bBl | O | BlGy | lRo |
| *A*-13 | gallic§ | 37/27 | C | pY | pOY | C |
| *A*-16*b* | protocatechuic§ | 46/48 | C | pY | pYG | pPkW |
| *A*-29 | *trans* ferulic | 72/26 | lTuBl | lPk | pGyPk | |
| 4-32 | *cis* ferulic | 78/70 | pTuBl | pPk | pPk | |

* Authentic samples mixed with *A* fractions showed no separation of spots or differences in color.

† See Table 9 for solvents. $R_f$ values approximate only in BzAW.

‡ UVA = u.v. light in the presence of ammonia fumes. For sprays see Table 10. Abbreviations of colors: B = brown, Bl = blue, C = colorless, G = green, Gy = gray, O = orange, P = purple (i.e., absorbing), Pk = pink, R = red, Ro = rose, Tu = turquoise, V = violet, W = white, Y = yellow, b = bright, d = deep l = light, p = pale.

§ Gives characteristic gray-green with 1% ferric chloride spray.

TABLE 2. DISTRIBUTION OF IDENTIFIED PHENOLIC ACIDS IN *Pinus* POLLENS*

| Species | *p*-Hydroxy-benzoic acid | *p*-Coumaric acid *cis* | *p*-Coumaric acid *trans* | Vanillic acid | Proto-catechuic acid | Gallic acid | Ferulic acid |
|---|---|---|---|---|---|---|---|
| *P. taeda*† (loblolly) | + + + | + + | + + | + + | + | + ‡ | + |
| *P. strobus* (white) | + + | + + + | + + + | ± | + | | + + |
| *P. resinosa* (red) | + + + | + + | + + | + + | + + | + | + |
| *P. elliottii* (slash) | + + + | + + | + + | + + | + + | | + |
| *P. banksiana* (jack) | + + + | + + | + ± | + + | + | + | + |
| *P. ponderosa* (ponderosa) | + + + | + + + | + + + | + | + | + | ± |
| *P. echinata* (shortleaf) | + + | + + | + + | | + | + | |
| *P. palustris* (longleaf) | + + + | + + | + ± | + + | + + | + + | ± |

* Key: + + + most prominent spot on sheet, + + = strong spots, + = weak spots, ± = seen only if heavier application used. Amounts relative only for each sample, not necessarily between samples

† Most representative results from several samples.

‡ In two out of five; but its absence may have been due to poor handling (see Text)

glycosides of the common acids, since previous workers[32,33] reported handling such compounds with considerable care and hydroxycinnamic acids are unstable to hot 5 N acid.[34] On the other hand, the glucoside of *p*-coumaric acid does not fluoresce under the conditions used.[33] Note that a similar fluorescence was shown by the group *B* spots, identified as *p*-coumarate esters. They do not seem to be depsides of the chlorogenic acid type, as no quinic or shikimic acid was found among the hydrolysis products.[35]

One additional acid was identified in this work, 4-glucosyloxybenzoic acid (*p*-hydroxybenzoic acid glucoside) (spot *A*-41), not previously characterized as a natural product. It was discovered in solution in attempts to isolate a sample of an acid belonging to group 2, and since it is invisible on paper, it has been impossible to decide from the data available whether *A*-41 underlay *A*-10 or was its decomposition product. In order to visualize *A*-41, it must be studied on fluorescent silica thin-layer plates or on silica plates sprayed with periodate–silver (Table 3). Note that the glucoside of *p*-coumaric acid is similarly reported to be invisible on paper.[33] The new glycoside absorbs at an extremely short wavelength in u.v. light, and the maximum shows a hypsochromic shift on the addition of base (Table 4). Such phenomena are characteristic of *p*-substituted aromatic carboxylic acids; compare *p*-anisic acid[36] and the hypsochromic base shifts of the glucosides of ferulic and *p*-coumaric acids.[33] Its glycosidic nature was proven by its hydrolysis in acid but not in base to yield *p*-hydroxybenzoic acid and glucose, identified by spectral and chromatographic methods (Tables 4 and 5). Although the occurrence of the glucoside of *p*-hydroxybenzoic acid in plants has been assumed previously in several cases[37–39] from hydrolysis results, the compound itself has not been described as a natural product, probably because of the difficulty of detecting it on paper chromatograms.

A trace of a second acid (*A*-10b), detected in solution during the above work, was not hydrolyzed by dilute hydrochloric acid, resembled *p*-hydroxybenzoic acid in color reactions, in $R_f$ values on thin-layer plates (Table 3), and in spectral properties (Table 4), but was clearly separated from this acid on paper (Table 5) since its ($R_f \times 100$) values in A-2 and BzAW* were respectively 80 and 54. It could possibly be a compound such as 4-(4′-hydroxyphenoxy)-benzoic acid which has been reported to absorb in 10% dioxane in hexane at 256 m$\mu$ with a shoulder at 290 m$\mu$.[40] Although this derivative of diphenyl ether has not been reported to occur naturally, others have been found in plants, for example: 4,4′-dihydroxydiphenyl ether in *Taxodium distichum*, Rah.[41]

### *Carbonate Soluble Fraction of the Ether Extractives*

*Flavonoids.* The carbonate soluble materials (designated as the *C* fractions) were less easily studied than the acid fractions because: (1) the amounts of material available were quite

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[34] S. L. TOMPSETT, *J. Pharm. Pharmacol.* **13**, 747 (1961).
[35] E. SONDHEIMER, in *Plant Phenolics and Their Industrial Significance* (Edited by V. C. RUNECKLES), p. 15, Proc. Symposium of The Plant Phenolics Groups of North America (held 1962, Corvallis, Oreg.) (1963).
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[37] C. F. VAN SUMERE, C. VAN SUMERE-DE PRETER, L. C. VINING and G. A. LEDINGHAM, *Can. J. Microbiol.* **3**, 847 (1957).
[38] I. A. PEARL, S. F. DARLING, H. DEHAAS, B. A. LOVING, D. A. SCOTT, R. H. TURLEY and R. E. WERTH, *Tappi* **44**, 475 (1961).
[39] E. C. BATE-SMITH, in *Wood Extractives* (Edited by W. E. HILLIS), p. 147, Academic Press, N. Y. (1962).
[40] H. E. UNGNADE, E. E. PICKETT, L. RUBIN and E. YOUSE, *J. Org. Chem.* **16**, 1318 (1951).
[41] H. L. HERGERT, *Abstr. Am. Chem. Soc. Meet.*, Div. Cellulose, Wood, Fiber Chem., p. 9D, Los Angeles, April (1963).

TABLE 3. THIN-LAYER CHROMATOGRAPHIC DATA

| Compound | ($R_f$ × 100) with* | | | | | | | | | Distinctive color reactions with sprays† |
|---|---|---|---|---|---|---|---|---|---|---|
| | CA95 | CA90 | CAM75 | CAM55 | CAM45 | BzAM | CM9 | CD | CMF | |
| Spot *A*-41 | 0 | 0 | 38 | | | | | | | Ia and Ib, colorless<br>VII, reduced in 6 min<br>VIII, relatively large amount required |
| Acid from *A*-41 | 33 | 56 | | | | | | | | Ib, rose<br>II, light orange-yellow |
| *p*-Hydroxybenzoic acid, authentic | 33 | 58 | 91 | | | | | | | Ib, rose<br>II, light orange-yellow |
| Spot *A*-10*b* | 36 | | 91 | | | | | | | Ia, pale yellow<br>Ib, violet-pink |
| Sugar from *A*-41 | | | | 38 | 46 | 66‡ | | | | IX, pale yellow (in u.v., pale brown-yellow)<br>VIII, positive |
| Glucose | | | | 41 | 51 | 68 | | | | IX, yellow (in u.v., brown-yellow) |
| Unknown alcohol§ | | | | | | | | | | |
| from *B*-1 | | | | | | | 41 | | | VII, reduced in 10–20 min |
| from *B*-4 | | | | | | | 40 | 64 | 35 | VII, reduced in 8–20 min |
| Cyclohexanediols§ | | | | | | | | | | |
| 1,2-, *cis* | | | | | | | 44 | 49 | | VII, reduced in 4–11 min |
| 1,3-, *cis* | | | | | | | 32 | 46 | 23 | VII, reduced in 9–23 min |

* See Table 9 for solvents. † See Table 10 for sprays. ‡ Galactose simultaneously 64. § Average values.

TABLE 4. ULTRAVIOLET ABSORPTION SPECTRAL DATA*

| Solutions | 4-Glucosyloxy-benzoic acid (A-41) | *p*-Hydroxybenzoic acid (from *A*-41) | *A*-10b ($HOC_6H_4OC_6H_4COOH$) (?) | *p*-Coumaric acid (from *B*-4) | *p*-Coumaric acid Authentic |
|---|---|---|---|---|---|
| Water (pH) | 245 (5) | 251 (7) | 258; 258, sh. 290† (5) (1) | 285, sh. 295, sh. 305 | 288, sh. 297, sh. 305 |
| +NaOH (pH 12·5–13) | 240 | 278 | 281 | 307, 330 | 332 |

| Solutions | Naringenin (from pollen) | *p*-Coumarate esters B-1 | B-2 | B-3 | B-4 | Methyl |
|---|---|---|---|---|---|---|
| 95% alcohol | 290 | 311, sh. 303 | 311, 300 | 311, sh. 302 | 311, sh. 303 | 312, sh. 300 |
| $+NaOC_2H_5$ | 326 | 364, 310, sh. 303 | 363, sh. 312, sh. 304 | 362, 312 sh. 304 | 364, 311, sh. 306 | 365, 312, sh. 302 |
| $+AlCl_3$ | 308‡ | | | | | |
| $+NaOOCCH_3$ | 327 | | | | | |
| *Fluorescence* (in alcohol+$NaOC_2H_5$) | | 448 | 448 | 447 | 447 | 444 |

* $\lambda_{max}$ in m$\mu$; sh. = shoulder.
† After attempted acidic hydrolysis.
‡ After standing one half-hour.

TABLE 5. PAPER CHROMATOGRAPHIC DATA ON DERIVED PHENOLIC ACIDS*

| Acid | Source | ($R_f$ × 100) with† A2 | aNaCl | AtW | BzAW |
|---|---|---|---|---|---|
| *p*-Hydroxybenzoic | From *A*-41 | 69 | | 88 | 75 |
| | Authentic | 68 | | 88 | 76 |
| *p*-Coumaric, *trans* and *cis* | From *B*-1 | | 38, 69 | | |
| | From *B*-4 | 45, 73 | 39, 71<br>35, 66 | 74, 85 | 77 |
| | Authentic | 45, 76 | 38, 69<br>36, 66 | 74, 86 | 77 |

* Spots of knowns and unknowns matched in color properties; see Table 1 for descriptions.
† See Table 9 for solvents.

limited in several cases; (2) color distinctions were poor so that key spots were difficult to locate in comparing chromatograms from different samples (the benzene–acetic acid–water solvent gives excellent separation but poor reproducibility[42] of $R_f$ values); and (3) the useful spray, Benedict's reagent (IV),[43] was not used until some samples were exhausted. Differentiation between species, however, was most marked with this fraction, and it should be studied in greater detail.

The two-dimensional chromatograms of the eight species showed the following distinguishing characteristics: *P. elliottii* and *P. palustris*, very few spots; *P. echinata*, a large number of minor spots, plus an extensive lightly colored area (¼ of sheet) when viewed in u.v. light or sprayed; *P. ponderosa*, similar lightly colored area; *P. strobus*, three apparently

TABLE 6. TWO-DIMENSIONAL PAPER CHROMATOGRAPHIC DATA ON CERTAIN CARBONATE SOLUBLE MATERIALS (*C* SPOTS)

| Spot No. | Identity | ($R_f$ × 100)* BzAW/A20 | Colors observed† UV | UVA | Sprays III(UV) | Ia | Ib |
|---|---|---|---|---|---|---|---|
| *C*-1 | Aurone (?) | 34/9 | pY | bOPk | pGY | lPkO | lOY |
| *C*-6 | Dihydrokaempferol | 60/54 | C | C | lbYG | lOY | pOY |
| *C*-12 | Dihydrokaempferol | 48/68 | P | P | lbY | lO | YO |
| *C*-7 | Dihydroquercetin | 40/60 | P | P | lY‡ | YO | OY |
| *C*-10 | | 44/48 | P | P | lY | C | C |
| *C*-11 | | 39/54 | C | C | lYG | C | C |
| *C*-14 | Ellagic acid (?) | 32/8 | blBl | lYG | lYG | C | C |
| *C*-17 | | 53/8 | pY | OY | lBlG | C | pOY |

* See Table 9 for solvents.
† See Table 1 for explanations and abbreviations; see Table 10 for sprays.
‡ This color changes characteristically with age, becoming more yellow-orange in 2–3 weeks. A re-observation aids differentiation from other spots.

[42] I. SMITH, *Chromatographic and Electrophoretic Techniques*, Vol. I, p. 322, Interscience Publishers, Inc., N.Y. (1960); E. WONG and A. O. TAYLOR, *J. Chromatog.* **9**, 449 (1962).
[43] H. REZNIK and K. EGGER, *Z. Anal. Chem.* **183**, 196 (1961)

different spots; *P. resinosa*, very prominent *C*-7 and *C*-12 spots (Table 6); and *P. taeda*, the characteristic and different *C*-1 spot.

Of the 18 *C* spots occurring in more than traces, 7 respond to aluminum chloride spray (Table 6) and probably represent strongly acidic flavonoid compounds with the 5,7-dihydroxyl grouping. Of these, *C*-7 was shown to be dihydroquercetin and *C*-12 and *C*-6 two isomers of dihydrokaempferol; spray (IV) clearly differentiates these rather closely running spots. Dihydrokaempferol was present in all of the pollens although in low concentration in *P. taeda* and *P. palustris*. In *P. resinosa* a large amount of the major isomer (*C*-12) and none of the minor (*C*-6) showed. Dihydroquercetin, similarly, produced an outstanding spot on the *P. resinosa* chromatogram, was definitely present in *P. taeda* and *P. echinata*, but its concentration was low or its identity doubtful in the other pollens. No other definite identifications were made, although it was postulated from their typical color reactions that *C*-14 (present in all but *P. elliottii*) was ellagic acid[44] and that *C*-1 (characteristic of *P. taeda*) was an aurone.[45] With this fraction, also, *P. strobus* showed some unique spots, such as *C*-17 and "off-color" *C*-7 and *C*-11 spots.

TABLE 7. PAPER CHROMATOGRAPHIC IDENTIFICATION OF NARINGENIN

| | | | | | Colors observed† | | | |
|---|---|---|---|---|---|---|---|---|
| | ($R_f \times 100$)* | | | | | With sprays | | |
| Compound | A30 | CAW | BAm | BAW | UV and UVA | Ia | Ib | III(UV) |
| Flavanone from *P. taeda* pollen | 69 | 69 | 47 | 95 | C | OY | OB | lG |
| Naringenin | 70 | 69 | 47 | 95 | C | OY | OB | lG |
| Eriodictyol | 68 | 48 | 14 | 89 | C | O | OY | lG |
| Homoeriodictyol | 75 | 73 | | | C | OY | YB | lG |
| Liquiritigenin | 75 | 49 | | | C | Y | YPk | pdBl |
| Pinocembrin | 75 | | 62 | | P | O | OY | lG |

* See Table 9 for solvents.
† Abbreviations as in Table 1; see Table 10 for sprays.

The finding of dihydrokaempferol and dihydroquercetin in pine pollens is not surprising since both have been reported in the wood and bark.[46] Other less important spots may represent related compounds such as dihydromyricetin or the simpler flavanones and flavanonols, also reported in pine wood and bark.[46] None of the spots showed the color reactions characteristic of quercetin or isorhamnetin, the flavonols previously reported in pine pollens.[2] An attempt to repeat this work, as described on 200 mg of pollen,[2] gave a concentrated methanolic solution still too dilute to reveal any flavonoid spots even though a time of extraction used was considerably longer than recommended.

To continue the search for the reported flavonols[2] mentioned in the preceding paragraph, the low concentration of flavonoid material contaminating sucrose, extracted by methanol

[44] L. JURD, in *Wood Extractives* (Edited by W. E. Hillis), p. 243, Academic Press, N.Y. (1962); L. HÖRHAMMER, H. WAGNER and H. SCHILCHER, *Arzneimittel-Forsch.* **12**, 1 (1962).
[45] T. A. GEISSMAN, J. B. HARBORNE and M. K. SEIKEL, *J. Am. Chem. Soc.* **78**, 825 (1956).
[46] H. L. HERGERT, in *The Chemistry of Flavonoid Compounds* (Edited by T. A. Geissman), p. 553, Pergamon Press, Oxford (1962).

from *P. taeda* pollen, was concentrated by lead precipitation and studied chromatographically. The outstanding flavonoid spot was identified as naringenin by cochromatography and spectral methods (Tables 4 and 7). Note that two glycosides of this flavanone have been previously isolated from *Acacia* pollen.[20] If flavonols are present in the loblolly pollen, their concentration is thus much less than that of flavanoid materials.

### p-*Coumarate Esters*

The relatively large amount of phenolic material remaining in the ether extractives after removal of the two acidic fractions already discussed and the phenolic material in the benzene extractives was separated into clearly defined spots by thin-layer chromatography on silica. Neutral and preferably non-alcoholic developing solvents were used since the compounds are very sensitive to transesterification; chloroform–acetone mixtures were most satisfactory. By two-dimensional thin-layer chromatography, five spots giving the typical purple-red color with the diazonium spray (Ib) and a fluorescent blue or turquoise color under u.v. light in the presence of ammonia were discovered and designated as *B*-1 to *B*-5 (Table 8).

TABLE 8. TWO-DIMENSIONAL THIN-LAYER CHROMATOGRAPHY OF *p*-COUMARATE ESTERS*

| Spot No. | ($R_f \times 100$) CA2/EAF† | Light or spray‡ | Colors observed for all *B* spots§ |
|---|---|---|---|
| *B*-1 | 86/90 | UVS* | Dark |
| *B*-2 | 69/86 | UVA | Fluorescent turquoise blue |
| *B*-3 | 63/77 | Ia | Yellow-orange |
| *B*-4 | 35/58 | Ib | Pink-purple |
| *B*-5 | 11/25 | V | Orange-yellow |

* On fluorescent silica plates; UVS = seen in short-wave u.v. light.
† See Table 9 for solvents.
‡ See Table 10 for sprays.
§ Concentrations varied greatly.

A study of the variation with species of the four most important of these *p*-coumarate esters in the pollen, easily carried out on one-dimensional chromatograms, showed that the variation was principally one of different relative concentrations. *P. strobus* pollen contained so little of these esters that they were completely extracted by the benzene, the ether extractive containing none. Since this same pollen was exceptional in containing *p*-coumaric acid as the principal free phenolic acid, the enzyme(s) responsible for esterification may be lacking. The slow running spot, *B*-4, was the most important spot observed in the ether extractives of the other pollens with the exception of *P. taeda* which had an equally prominent fast spot, *B*-1. *P. taeda* also showed the intermediate spots, *B*-2 and *B*-3, clearly. The other pollens possessed much lower concentrations, if any, of the last three compounds. A preliminary test on three pollens suggested that the benzene extractives were richer in all *B* spots, particularly in *B*-1.

An attempt to isolate crystalline samples of one or more of the four esters for analysis from a rich benzene extractive of *P. taeda* failed, apparently because adhering lipids are not easily removed. In addition, the esters are unstable to laboratory conditions (as well as to acid, base,

and alcohols), and particularly to heat, for samples which have been preserved or which have been extracted with boiling hydrocarbon solvents show increasing amounts of material which streaks and which does not move from the origin on thin-layer plates; the latter was always present when the crude extractives were chromatographed. Apparently the compounds are very sensitive to auto-oxidation.

For structural studies, micro samples of the compounds responsible for the four principal *B* spots were isolated from the *P. taeda* pollen by preparative thin-layer chromatography. Spectral studies (Table 4) gave indistinguishable results for the four, and values almost identical with those of methyl *p*-coumarate determined simultaneously. These values also check those reported for the glucose ester of *p*-coumaric acid.[32] The *B* spots, however, show one distinct difference from methyl *p*-coumarate; they give a yellow color when sprayed with acidic 3,5-dinitrophenylhydrazine while the spot of the simple ester remains colorless. The *p*-coumaric acid moiety of the unknown esters was substantiated by basic hydrolysis of *B*-1 and *B*-4 and by spectral (Table 4) and chromatographic proof (Table 5) of the identity of the acidic product as *p*-coumaric acid. No water soluble alcoholic moiety could be discovered, so the esters are not the glycosyl type reported with increasing frequency,[32,47,48] even recently in a pollen.[24] From a second hydrolysis of a considerably larger sample of *B*-4, a few milligrams of a solid, water insoluble alcohol were isolated. Small amounts of this same alcohol were visible on chromatographic plates from the hydrolysis of a crude sample of *B*-1.

This alcohol has not been completely identified, but the following data have been determined. The pure colorless crystals melt at 89·5°; too little was obtained for an analysis. The compound showed only end absorption in the u.v., but it gave an i.r. spectrum with prominent sharp bands, in $cm^{-1}$, at: 3280, 2880, 1480, 1458, 1348, 1118, 1085, 1055, 1035, 1023, 995, 982, 972, 909, 852, 818, 729, but no sign of a methyl band at 1370–1380 $cm^{-1}$ or a carbonyl band. It was invisible on thin-layer plates prepared with silica containing a fluorescent additive, but it moved with chloroform–methanol solutions like unsubstituted diols such as a hexanediol and 1,2-, 1,3- and 1,4-cyclohexanediols and reduced the permanganate spray (VII) in approximately the same length of time. With chloroform–dioxane (CD), which because of its greater basicity seemed to sort compounds subject to intramolecular hydrogen bonding differently than chloroform–methanol did and which was shown to separate the *cis* and *trans* isomers of the cyclohexanediols, the unknown moved considerably faster than the cyclohexanediols. Spots of the unknown on plates reacted very slowly (an hour or more) with the acidic dinitrophenylhydrazine spray (V) at room temperature; the cyclohexanediols do not react. With the sulfuric acid spray (VI), the u.v. fluorescent orange color which finally develops resembles the color shown by 1,3- and 1,4-cyclohexanediol and differs from the duller green shades exhibited by *cis* and *trans* 1,2-cyclohexanediol.

The data above, plus their distinctive (though similar) i.r. spectra, definitely eliminate *cis* 1,2-cyclohexanediol, m.p. 98°,[49] 95°[50] (found as a constituent of grandidentatin from the bark of *Populus grandidentata*)[50] and *cis* 1,3-cyclohexanediol, m.p. 86°.[49] Saturated terpenediols appear to be eliminated from consideration by the lack of a methyl band in the i.r. spectrum. In conclusion, a saturated alicyclic diol still seems to be the structure which accounts best for the properties, but other groups or other arrangements than those mentioned above must be

[47] S. Asen and S. L. Emsweller, *Phytochem.* **1**, 169 (1962); L. Birkofer, C. Kaiser, W. Nouvertné and U. Thomas, *Z. Naturforsch.* **16B**, 249 (1961).
[48] J. J. Corner, J. B. Harborne, S. G. Humphries and W. D. Ollis, *Phytochem.* **1**, 73 (1962).
[49] I. Heilbron and H. M. Bunbury (Editors), *Dictionary of Organic Compounds*, Vol. II, O.U.P., N.Y. (1953).
[50] I. A. Pearl and S. F. Darling, *J. Org. Chem.* **27**, 1806 (1962).

present. For example, a methoxy group[51] would absorb only near 1450 $cm^{-1}$ and could account for the mobility in the dioxane solvent.

Only tentative suggestions on the structure of this series of unstable *p*-coumarate esters can be made from the data available. They may be simply esters of a series of closely related alcohols, an idea supported by the lack of any intermediate during the hydrolysis. On the other hand, a study of the $R_M$ values[52] gave a straight line for the series, with the values for *B*-2 and *B*-3 falling close together as if they were isomeric. (Later work suggested that *B*-4 can be separated into two isomers in line with the reported separation of stereoisomers by thin-layer chromatography.)[53] This may indicate a stepwise increase in one component of the compound. Complex derivatives of the hydroxycinnamic acids have recently been discovered in *Polygala senega* root,[48] the structure of which is still unsettled.

The occurrence of *p*-coumaric acid derivatives in pollens is not surprising considering the prevalence of the derivatives which have already been identified and their probable importance.[28] A recent study on oat kernels[54] has uncovered a group of still incompletely identified esters of at least caffeic and ferulic acids which seem to resemble the pollen *p*-coumarate esters, while the occurrence of a pinoresinol ester of *p*-coumaric acid in resin of *Pinus laricio* has been reported.[55]

## EXPERIMENTAL

### *Material*

Pollen from eight pines was employed: loblolly (*Pinus taeda*), slash (*P. elliottii*), longleaf (*P. palustris*), shortleaf (*P. echinata*), jack (*P. banksiana*), red (*P. resinosa*), ponderosa (*P. ponderosa*), and white (*P. strobus*). Large quantities (100–1600 g) from collections (0·8–5 kg) of carefully dried and preserved pollen, each derived from many trees in a single location, had been previously extracted with benzene, diethyl ether, and methanol in succession.[1] The extractives were available in the form of gummy or oily residues which had been stored in a freezer for 2·75–4·0 years. In addition, extractives from several samples of *P. taeda* pollen, which had been collected in three different years and some of which had been dried without proper ventilation, were tested for comparison.

### *Fractionation of Ether Extractives*

The small ether residues were dissolved in mixtures of ether and butanol and extracted exhaustively, first with 2% sodium acetate and then with 20% sodium carbonate. The remaining butanol–ether layers were each evaporated to dryness *in vacuo* yielding fractions *B*. The aqueous layers were acidified and extracted with butanol. Evaporation of these butanol layers left respectively fractions *A* and *C*. The extractions were followed by spot testing with spray Ib (see Table 10). The dried gummy residues available for study weighed as follows: fraction *A*, 11 to 119 mg; fraction *C*, 4 to 75 mg; and fraction *B*, 68 to 454 mg for the different samples of pollen.

[51] L. H. BRIGGS, L. D. COLEBROOK, H. M. FALES and W. C. WILDMAN, *Anal. Chem.* **29**, 904 (1957).
[52] E. C. BATE-SMITH and R. G. WESTALL, *Biochim. Biophys. Acta* **4**, 427 (1950).
[53] G. PASTUSKA and H. J. PETROWITZ, *J. Chromatog.* **10**, 518 (1963).
[54] D. G. H. DANIELS, H. G. C. KING and H. F. MARTIN, *J. Sci. Food Agr.* **14**, 385 (1963).
[55] M. BAMBERGER and A. LANDSIEDL, *Monatsh. Chem.* **18**, 481 (1897).

*Paper Chromatography (PC)*

Whatman No. 1 paper was used, and papers were viewed in u.v. light with and without ammonia fumes. Developing solvents and chromogenic sprays are listed in Tables 9 and 10.

Two-dimensional chromatograms of the *A* spots were developed first with BzAW, then with aNaCl. BzAW must be carefully prepared and at least six sheets run simultaneously in a large tank in order to obtain a good spread of acid spots with ($R_f \times 100$) values from 33 to 84.[42] $R_f$ values obtained with it were almost impossible to reproduce, and the acids were better characterized by their relative positions and their distinctive color reactions.

TABLE 9. DEVELOPING SOLVENTS FOR PAPER CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

| Abbreviation | Composition | Type of compound | Technique* | Ref. No. |
|---|---|---|---|---|
| A2 | 2% Acetic acid | Phenolic acids | PC | |
| A20 | 20% Acetic acid | Flavanonols | PC | |
| A30 | 30% Acetic acid | Flavanones | PC | |
| aNaCl | 3% Sodium chloride in 0·1 N hydrochloric acid | Phenolic acids | PC | |
| AtW | Acetone:water (1:1) | | PC | |
| BAm | Butanol:2 N ammonia (1:1) | Flavanones | PC | |
| BAW | Butanol:27% acetic acid (1:1) | Flavanones | PC | |
| BzAM | Benzene:acetic acid:methanol (2:2:6) | Sugars† | TLC | 56 |
| BzAW | Benzene:acetic acid:water (125:72:sat.) | Phenolic acids<br>Flavanonols | PC | 42 |
| CA90 | Chloroform:acetic acid (90:10) | Phenolic acids (monohydroxy-monocarboxy) | TLC | |
| CA95 | Chloroform:acetic acid (95:5) | | TLC | |
| CAM45 | Chloroform:acetic acid:methanol (45:20:35) | Sugars and related compounds | TLC | |
| CAM55 | Chloroform:acetic acid:methanol (55:10:35) | | TLC | |
| CAM75 | Chloroform:acetic acid:methanol (75:5:20) | Phenolic acid glycosides | TLC | |
| CAt2 | Chloroform:acetone (2:1) | *p*-Coumarate esters | TLC | |
| CAt4 | Chloroform:acetone (4:1) | | TLC | |
| CAW | Chloroform:acetic acid:water (2:1:1) (lower layer) | Flavanones | PC | 57 |
| CD | Chloroform:dioxane (1:1)‡ | Diols | TLC | |
| CM2 | Chloroform:methanol (2:1) | Sugars and related compounds | TLC | |
| CM9 | Chloroform:methanol (9:1) | Diols, *p*-coumarate esters | TLC | 58 |
| CMF | Chloroform:methyltetrahydrofuran (1:1) | Diols | TLC | |
| EAF | 2% Dimethylformamide in ethyl acetate | *p*-Coumarate esters | TLC | |

* PC = paper chromatography; TLC = thin-layer chromatography; † On borate impregnated plates; ‡ Dioxane peroxide-free.

The flavanonols in fraction *C* were developed with BzAW in the first direction and A–20 in the second.

*Thin-Layer Chromatography (TLC)*

Silica plates, made with silica containing an inorganic fluorescent additive (silica gel $HF_{254}$, Brinkmann Instruments, Inc., N.Y.), were used for most of the work, and they were viewed

[56] E. STAHL (Editor), *Dünnschicht-Chromatographie*, p. 476, Springer-Verlag, Berlin (1962).
[57] K. EGGER, *J. Chromatog.* **5**, 74 (1961).
[58] L. D. BERGELSON, E. V. DYATLOVITSKAYA and V. V. VORONKOVA, *Doklady Akad. Nauk S.S.S.R.* **141**, 84 (1961); *J. Chromatog.* **10**, D17 (1963).

under both short-wave and long-wave u.v. light, 254 mμ and 366 mμ respectively, and for the *p*-coumarate esters in the presence of ammonia fumes. Sugars were also tested on silica plates impregnated with 0·1 N boric acid.[56] Developing solvents and sprays are listed in Tables 9 and 10.

The *p*-coumarate esters (*B* spots) were developed with CAt4 or CAt2 depending on whether a good assortment of the fast or slow spots was desired. For two-dimensional work CAt2 was combined with EAF. CM9 was used to subdivide spot *B*-4 into two close-lying spots.

TABLE 10. SPRAYS USED FOR PAPER CHROMATOGRAPHY AND THIN-LAYER CHROMATOGRAPHY

| No. | Composition and method | Type of compound | Technique* | Ref. No. |
|---|---|---|---|---|
| Ia | 0·05% *p*-Nitrobenzenediazonium tetrafluoborate† (stable 6 weeks if refrigerated)‡ | Phenolic acids<br>Flavonoids | PC<br>TLC | 59 |
| Ib | Ia oversprayed with 2–20% sodium carbonate (after complete drying) | *p*-Coumarate esters | | |
| II | Diazotized sulfanilic acid oversprayed with 20% sodium carbonate | Phenolic acids | PC | 60 |
| III | 1% Alcoholic aluminium chloride | Flavonoids | PC | |
| IV | Benedict's reagent (basic cupric citrate complex) | Flavonoids | PC | 43 |
| V | 0·05% Dinitrophenylhydrazine in 2·4 N hydrochloric acid | *p*-Coumarate esters | TLC | 60 |
| VI | Approx. 30% conc. sulfuric acid in ether (heat slowly from 100–150° and observe§) | *p*-Coumarate esters<br>Hydroxy compounds | TLC | 61 |
| VII | 0·3% Potassium permanganate in 0·7% sodium carbonate (not heated) | All hydroxy compounds (alcohols, diols, polyols, sugars)<br>Unsat. compounds | TLC | 60 |
| VIII | 0·02 M Sodium metaperiodate ($NaIO_4$) oversprayed after ½ hr or more with 0·1 N silver nitrate | Sugars, glycosides<br>Unsat. compounds | TLC | 62 |
| IX | *p*-Anisidine phosphate in 2% phosphoric acid in aqueous alcohol (heat at 110–130°) | Sugars | TLC | 60 |

* PC = paper chromatography; TLC = thin-layer chromatography.
† From Eastman Kodak Co.
‡ This spray could not be used when the sheets had been previously exposed to ammonia.
§ Characteristic color changes appear.

Hydroxy compounds (alcohols, diols, polyols and sugars) were developed with various mixtures of chloroform and methanol of which CM9 separated diols cleanly from the faster running monohydroxy compounds and the slower running triols and polyols. The routine spray used for these compounds was (VII), used without heating; the speed of development of the yellow spots clearly differentiated the immediate reducing spots of unsaturated compounds from the rapidly reducing spots of the polyols (~ 3–5 min), from the slowly reducing spots of the diols (~ 10–20 min) and the very slowly reducing spots of monohydroxy compounds (~ 30–40 min).

[59] I. A. PEARL and P. F. MCCOY, *Anal. Chem.* **32**, 1407 (1960).
[60] R. J. BLOCK, E. L. DURRUM and G. ZWEIG, *A Manual of Paper Chromatography and Paper Electrophoresis*, Academic Press, N.Y. (1958).
[61] D. F. ZINKEL and J. W. ROWE, *J. Chromatog.* **13**, 74 (1964).
[62] R. L. WHISTLER and M. L. WOLFROM, *Methods in Carbohydrate Chemistry*, Vol. I p. 28, Academic Press, N.Y. (1962).

*Absorption Spectra*

Ultraviolet absorption spectra were determined on a Beckman DK Recording Spectrophotometer. Spectral shifts were measured by adding the following reagents:[63] (1) dilute sodium hydroxide to aqueous solutions to pH 12·5; (2) 1 N sodium ethoxide to alcoholic solutions; (3) solid sodium acetate; and (4) 5% alcoholic aluminum chloride. Fluorescence spectra were observed by the use of the Beckman 73500 Fluorescence Attachment.

*Infrared Spectra*

Infrared spectra were determined on a Baird Infrared Recording Spectrophotometer.

*Two-Dimensional Paper Chromatography of Acid Fractions (Fraction* A*) and Flavonoid Fractions (Fraction* C*)*

The *A* and *C* fractions were dissolved in methanol (0·05 g/ml), and 10–50 $\mu$l of the solutions were spotted on paper in triplicate, the amount applied being adjusted to produce at least one strong spot. For cochromatographic comparison the samples were overspotted with similar solutions of known phenolic acids and flavanonols, respectively. The papers were developed and sprayed as described above.

*Thin-Layer Chromatography of* p*-Coumarate Esters* (B *Spots*)

The *B* fractions of the ether extractives were carefully freed of traces of butanol *in vacuo*, 10 mg samples of the brown gums were dissolved in 0·2 ml acetone, and 2–12 $\mu$l of these solutions were applied to the fluorescent silica plates for one- and two-dimensional chromatography by the methods described above. Samples of the benzene extractives of several pollens were similarly dissolved and studied.

*Isolation of* 4*-Glucosyloxybenzoic Acid and Its Characterization*

The remaining fraction *A* from longleaf pollen was streaked on large sheets of Whatman No. 1 paper and developed with 2% acetic acid. Two light "blue", fast-running bands were cut from the paper, eluted with water, and the concentrated eluates purified by rebanding with BzAW. From the still fainter "blue" bands resulting, two aqueous eluates were obtained which were studied spectrophotometrically in neutral and basic solution (pH 5 and 12·5, respectively) and after heating on the steam bath at pH 1 and 13 for 7–15 hr. After this work showed that the absorbing compounds present in the eluates, called *A*-41 and *A*-10b, were not responsible for the "blue" color used in their isolation, a thin-layer chromatographic method using fluorescent silica plates was developed for studying them, particularly *A*-41, since it could not be visualized satisfactorily on paper (see section on TLC). The combined residues containing *A*-41 were dissolved in 0·5 ml water, treated with 0·5 ml 2·0 N hydrochloric acid and the mixture heated 8·5 hr on the steam bath, a paper blank being carried through the hydrolysis procedure. The hydrolysis solutions were extracted with peroxide-free ether, and the residue of the ether solution studied by spectral and chromatographic methods. The aqueous layer from the hydrolysis was freed from acid by repeated evaporation and studied by TLC. The trace of spot *A*-10b was similarly studied.

[63] L. JURD, in *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 107, Pergamon Press, Oxford (1962).

*Isolation of Naringenin From Methanolic Extractives*

The small amount of flavonoid material in a 20 g sample of the methanolic extractive from loblolly pollen was separated from the large amounts of sucrose by precipitation with neutral and then basic lead acetate.[64] The two samples of moist, mixed salts were suspended in methanol and treated overnight with Dowex-50 (H form) to remove the lead. Paper chromatography of the residues of the two methanolic filtrates showed principally one flavonoid compound derived from the neutral lead fraction with less in a more complex mixture from the basic lead fraction. This compound was isolated from the 0·2 g of the first residue by twice banding on paper, first with CAW, then with A30. The bands were detected by spray (III) and were eluted first with methanol and second (for spectral work) with 95% ethanol, a paper blank being prepared in the latter case.

The u.v. absorption spectrum and spectral shifts were determined (Table 4), and then the sample was tested by paper cochromatography with samples of all the common flavanones which show the same absorption spectrum (Table 7).

*Isolation of* p-*Coumarate Esters and Their Characterization*

(*a*) *Study in solution.* The remaining fraction *B* from the *P. taeda* 1960 pollen was streaked on two fluorescent silica plates (thickness of layer, 0·25 mm) and the bands developed with CAt2. The bands were located most clearly by observation in long-wave u.v. light in the presence of ammonia fumes; a definite concentration boundary divided *B*-2 and *B*-3 on the more heavily loaded plate. Isolation of the separated constituents *B*-1, *B*-2, *B*-3, and *B*-4 was made by sucking the adsorbents carrying the materials from the plate and eluting the esters with acetone (0·15–0·2 ml) in a micro eluting apparatus.[65]. The traces of pale yellow oils obtained on evaporation were studied chromatographically and spectrophotometrically. A 2·5 mg sample of *B*-4, similarly isolated on two plates from *P. taeda* 1960 pollen, was subjected to alkaline hydrolysis; see section (*c*) for preferred method. This reaction mixture, however, was acidified with a cation ion-exchange resin before ether extraction. The acid component was isolated from the filtrate by ether extraction, and the partly crystalline residue was identified as *p*-coumaric acid by a spectral study (Table 4) and by paper chromatography (Table 5). No water soluble hydroxylated compound could be found in the aqueous layer except a trace of glycerol.

(*b*) *Isolation from benzene extractives.* A 25 g sample of the waxy benzene extractive of *P. taeda* pollen was partly freed from lipids by dissolving it in acetone, cooling the solution in the freezer, centrifuging, and reworking both the supernatant and the precipitate until, after 17 separations, the esters were concentrated in the 10·5 g of residue obtained by evaporating the last supernatant. The residue was dissolved in 25 ml water-washed chloroform, added to 44 × 3 cm silicic acid column (prepared with the same solvent), and another 4·0 g of waxy materials washed out by chloroform. The column was then developed for the *B* compounds and eluted in 15 ml fractions with CAt4. Analysis by TLC showed that *B*-1 was concentrated in tubes 50–52, *B*-22 in tubes 55–57, *B*-3 in tubes 68–70, and *B*-4 in tubes 92–108. When all the eluates were evaporated, the weights of residues obtained in the intermediate tubes were not significantly less than those in the ester-rich tubes, proof that the latter were undoubtedly still badly contaminated. Attempts to obtain crystalline esters from the waxy

[64] T. R. SESHADRI, in *The Chemistry of Flavonoid Compounds* (Edited by T. A. GEISSMAN), p. 17, Pergamon Press, Oxford (1962).

[65] M. A. MILLETT, W. E. MOORE and J. F. SAEMAN, *Anal. Chem.* **36**, 491 (1964). M. K. SEIKEL, M. A. MILLETT and J. F. SAEMAN, *J. Chromatog.*, **15**, 115 (1964).

residue containing *B*-1 or the oily residue with *B*-4 by recrystallization or extraction with petroleum ether failed. TLC studies showed deterioration of the individual spots originally obtained when the fresh eluates of the column were tested. The *B*-4 sample was rechromatographed on a silicic acid column; 37 mg of colorless oil resulted. (Found: C, 70·99; H, 9·8. Calc. for $C_{15}H_{18}O_4$: C, 68·9; H, 6·9%). Part of this material was further purified by banding on silica plates developed with CM9. Two close-lying "blue" bands were obtained, $(R_f \times 100) = 58$ and 54. The oily residues, of a milligram or less, obtained from these by elution with acetone and evaporation were shown by basic hydrolysis to produce *p*-coumaric acid and the alcohol described below.

(*c*) *Hydrolysis and isolation of unknown alcohol.* The recommended hydrolysis procedure for the *p*-coumarate esters follows. The sample was dissolved in a 1:1 mixture of methanol and 2 N sodium hydroxide, and the solution was refluxed until paper and thin-layer tests conducted on acidified test samples showed that the reaction was complete; *B*-4 required about 1 hr, *B*-1 seemed to require 5 hr. The methanol was boiled from the reaction mixture which was then cooled and extracted with peroxide-free ether. Evaporation of the ether left a solid alcohol. The pHof the aqueous layer was reduced to 4–5 with hydrochloric acid, it was in turn extracted with ether, and evaporation of the ether left an oily residue of *p*-coumaric acid.

The oily acid residue from the saponification of a relatively large sample of *B*-4 was purified by extracting it from an ether solution with bicarbonate, acidifying, and reextracting with ether. The partly oily, tan residue from evaporation of the ether was purified by decolorization with Norite and several recrystallizations from water. The white fronds, freed of the insoluble oil, decomposed at 213° and did not lower the decomposition point of authentic *p*-coumaric acid.

The few milligrams of solid alcohol from *B*-4 were purified by extraction with ether, which removed unchanged ester and then by recrystallization from either ether or acetone–water, m.p. 88·5°–89·5°. This material was studied by i.r. spectra and by thin-layer chromatography with various solvents and sprays (Table 3) in comparison with many similar straight-chain, branched-chain, and cyclic aliphatic alcohols and diols.

## CONCLUSIONS

(1) Trees offer an abundant supply of pollen for study of the phenolic constituents.

(2) Pine pollens have been shown to have a much wider variety of these compounds than was previously suspected, but the phenolic acids, flavonoids, and esters which have been identified (*a*) have already been discovered in pine wood or bark, (*b*) have been identified in other pollens, (*c*) are widely present in other plants, or (*d*) are of type present in other plants.

(3) Complete identification of the *p*-coumarate ester series may lead to interesting new results, and their role as the principal phenolic material in the pollen should be investigated.

(4) Chemotaxonomically, only the carbonate soluble material from the ether extractives and perhaps the flavonoid fraction from the methanolic extractives, both small fractions, show promise of considerable variation between the species.

(5) Pollen must be handled carefully to prevent changes in the phenolic constituents. High temperatures and especially high humidity caused by the bulking of male flowers during collection must be avoided. After pollen is air dried, it should be stored in a cool, dry place. Differences reported in the literature may be caused by improper handling.

*Acknowledgements*—We wish to thank Dr. R. W. Scott for his continued interest in the pollen problem and for his advice on the handling of lipids, Dr. I. A. Pearl for furnishing the sample of *cis* 1,2-cyclohexanediol, and Mr. Nicholas Mirov for the opportunity to see his bibliography on pine pollen.