Biochemical Studies on the Heartwood Formation

By

Takayoshi HIGUCHI, Kazumi FUKAZAWA and Mikio SHIMADA

Laboratories of Wood Chemistry and Wood Physics,
Faculty of Agriculture, Gifu University,
Kakamigahara, Gifu

心材形成の生化学的研究

樋口隆昌* 深沢和三* 島田幹夫*

Introduction

Heartwood has generally been distinguished from sapwood by the three most distinctive features, the loss of the nucleus in the parenchyma cells, increased extractives content, and a decrease in moisture content[1]. The mechanism of heartwood formation is, however, still obscure, although several workers have endeavored to elucidate the problem.

CHATTAWAY (1952)[2] concluded from her cytological observation of Australian trees that in the formation of heartwood the death of the ray cells is preceded by a period of great physiological activity and then protoplasmic membrane disintegrates to librate the extractives.

On the other hand, FREY-WYSSLING and BOSSHARD (1959)[3] stated that the heartwood formation is an aging process in a living tree, through which the parenchyma cells undergo irreversible changes such as degradation of the protoplasm and the disorganisation of the cell's oxidizing system, without physiological activation during the process.

In any case, the physiological significance of the intermediate wood which is often recognized as a pale color zone between ordinary sap- and heartwood has been noticed recently with special reference to heartwood formation[4),5),6)].

In the present report some of the cytological[7),8),9)] and physiological[10)] characteristics of cambial zone, sap- and intermediate wood are dealt with in relation to heartwood formation. The occurrence of typical heartwood compounds in the "artificial heartwood" obtained by boring through sapwood in standing tree trunks is also described.

The results have indicated that the normal physiological activity concerning cell growth such as protein and nucleic acid metabolism and the respiration con-

* 岐阜大学農学部

cerned rapidly decrease from cambium toward sap- and intermediate wood, and thus the concentration of phenols in heartwood should be the results of change of the metabolic pathway in the ray cells of intermediate wood toward the aromatic biosynthesis.

Materials and Methods

Cytological Observation of Tissues

Several species of conifers (*Cryptomeria japonica* D. Don, *Chamaecyparis obtusa* Sieb. et Zucc., *Abies sachalinensis* Fr. SCHM. *Picea jezoensis* CARR. and *Thujopsis dolabrata* Sieb. et Zucc. etc) and of broad-leaved trees (*Magnolia obovata* Thunb., *Pterocarya rhoifolia* Sieb. et Zucc., *Betula maximowicziana* Regel, *Fraxinus mandshurica* RUPR. and *Quercus crispula* Blume etc) were felled during summer and autumn of 1964 to 1966 and used as samples. The wood discs were taken at certain heights of the trees. The sticks (width, 1 cm) through center of the fresh wood discs were cut off, and sap-, intermediate- and heartwood of the sticks were separated immediately. The respective wood blocks thus obtained were used for preparation of section in fresh and or in fixed, respectively.

Determination of Deoxyribonucleic Acid (DNA)

Radial section (thickness, 15 μ) of the fresh and or fixed wood blocks were prepared by a sliding microtome from outer to inner parts successively and the nuclei of ray cells were stained by Feulgen's reagent. Then, DNA content of the nuclei of the cells were determined according to the plug method of LEUCHTENBERGER[11] by using a microspectrophotometer (MSP–AIV, Olympus Optical Co.).

Detection of DNA and Ribonucleic Acid (RNA)

The ray cells of the wood sections of the fixed material were stained with Azure B. DNA gave green-blue and RNA did purple or dark blue color. In some case, the wood sections were also stained with methyl green and pyronine B. DNA gave blue color and RNA did red color by this procedure. The distribution of RNA and DNA in the ray cells was further confirmed by a selective extraction of DNA and RNA with perchloric acid according to ERICKSON, SAX and OGUR (1949)[12]. In the staining of the tissues thus extracted RNA and DNA were detected more clearly.

Detection of Starch Granules and Oil Droplets

Iodine solution was dropped on the sections of fresh wood and starch granules in the cells were stained purple. Oil droplets in the cells were detected by treating fresh tissues with Sudan III ethanol-glycerol solution, and they gave yellow orange color.

Distribution of Shikimic Acid in Cells

Periodate solution (0.03 M) was dropped on the cross and radial sections of a tree trunk of *Illicium regiosum* Sieb. et Zucc. After several minutes dropping

of ethanol-aniline solution on the section shikimic acid gave pink color[13].

Physiological Investigation of Wood Tissues

Respiration

Sections (0.1 mm thick) of the cambial zone, sap-, intermediate- and heartwood were made in the tangential plane. The sections (300 mg) thus obtained were suspended in 2 mℓ of 0.1 M phosphate buffer (pH 6.8) in a Warburg flask and 0.2 mℓ of KOH (10%) was added in a center well to absorb CO_2. The manometer were shaken at 30°C at the rate of 100 times a minute and oxygen uptake by the tissues was measured for 4 hours.

Measurement of C_6/C_1 Ratio in Glucose Oxidation

Oxidation of glucose-1-^{14}C and -6-^{14}C by tissues was measured at pH 6.8 in 0.1 M phosphate buffer using a Warburg respirometer. Each 0.2 μC of G-1-^{14}C (sp. act. 62.5 μC/mM) and G-6-^{14}C (sp. act. 62.5 μC/mM) was used for 200 mg of tissue sections and 0.2 mℓ of KOH was added in a center well. Oxygen uptake was measured at 30°C for 4 hours. After completion of the measurement the KOH solution in the center well was pipetted into a small test tube quantitatively and potassium carbonate was converted to barium carbonate. The precipitate of barium carbonate was filtered by a glass crucible and the radioactivity of the carbonate was measured by a gas flow counter (Aloka FCI-E). The total activity of the carbonate from respective glucoses and C_6/C_1 ratio were calculated.

Injection of Glucose-1-^{14}C into Cambial Zone of Illicium religiosum

A part of bark (2 cm × 2 cm) of the stem was removed with a knife and 0.1 mℓ of G-1-^{14}C (1 μC, sp. act. 75.6 μC/mM) was injected into cambial zone with a syringe. The treated part was covered with an original bark and a scotch tape. After 24 hours the treated part of the stem was cut off and pulverized. The wood powder (17.5 g) was extracted twice with hot 80% ethanol, solvent was evaporated and the residue was dissolved in a small volume of water. The water soluble part was applied to a column containing Amberlite IRA 410 (carbonic acid form) and the acid fixed on the column was eluted with 1 N ammonium carbonate. The acid solution was condensed *in vacuo* and shikimic acid in the solution was analysed by paper chromatography using phenol-water-formic acid (60 : 20 : 0.8, v/v) as an irrigant. Shikimic acid was detected by a periodate and ethanol-aniline reagent and the corresponding part on the chromatogram was extracted with 80% ethanol. The crude shikimic acid thus obtained was further purified by a second chromatography using butanol-acetic acid-water (4 : 1 : 2.2, v/v) as an irrigant, and then the acid was crystallised from the acetone solution. M.p. 148°C. Radioactivity of shikimic acid was counted by a gas flow counter. Specific activity of the acid. 0.36 μC/mM. From the neutral fraction which was not fixed on the ion exchanger radioactive glucose and xylose were detected by radioautogram.

Phenol Oxidase

The acetone powder of fresh tissues of cambial zone, sap-, intermediate- and heartwood were prepared at −15°C. The acetone powder was extracted for 1 hour with cold 0.1 M phosphate buffer (pH 6.8) in a refrigerator and crude enzyme in the extract was precipitated with ammonium sulfate (70% saturation) at 3°C. The precipitate was dissolved in a small amount of the same buffer and dialyzed against 0.05 M phosphate buffer (pH 6.8).

Assay of Phenol Oxidase

Oxidation of phenolic compounds by the phenol oxidase was measured using a Warburg respirometer at 30°C. Catechol (0.05 M, 0.5 mℓ) solution in the side arm was used as a standard substrate. Enzyme solution (2 mℓ) in main compartment of a flask and 0.2 mℓ of KOH in center well were added, respectively. Oxidation of caffeic acid, ferulic acid, sinapic acid, coniferyl alcohol, sugiresinol and hydroxysugiresinol was measured in the same condition. The effect of CO on the oxidation of catechol and hydroxysugiresinol was also examined using a mixture of 90% CO + 10% O_2 as gas phase of respirometer and air as control.

Phenylalanine Ammonia-Lyase

The acetone powder of tissues was extracted for 1 hour with cold 0.1 M borate buffer (pH 8.8). The enzyme in the extract was precipitated with ammonium sulfate (70% saturation), dissolved in the same buffer and used as enzyme solution after dialysis.

Assay of Phenylalanine Ammonia-Lyase

Enzyme solution was incubated with L-phenylalanine or L-tyrosine in 0.1 M borate buffer (pH 8.8) for 3 hours at 30°C, and the amount of trans-cinnamic or *p*-coumaric acid formed was determined spectrophotometrically[14].

Nitrogen Determination

Wood sections (100 mg) of cambial zone, sap-, intermediate- and heartwood were digested with conc. H_2SO_4 in Kjeldahl flasks. Ammonia formed was steam-distilled in the presence of excess NaOH, titrated with 1/50 N H_2SO_4, and then the amount of crude protein was calculated.

Formation of Artificial Heartwood

In spring of 1966 small lateral holes were made by boring through sapwood in standing tree trunks of *Pinus densiflora* and *Cryptomeria japonica* in the Experimental Forest of Gifu University. The holes were washed well with 70% ethanol and sealed aseptically with a wood block and a scotch tape. The trees were felled in autumn of 1966 and the wood discs of the treated part were cut off. Sapwood, the part of heartwood like coloration along the holes in sapwood, and heartwood were separated by a chisel carefully. Each portion was cut into small pieces with a knife and pulverized.

Extraction of Phenolic Compounds

Wood powder (5 g) of each portion of *Pinus densiflora* was extracted with acetone for 8 hours using a Soxhlet extractor. Acetone was evaporated and

the residue was analysed by paper chromatography and thin layer chromatography. As the extract of the artificial heartwood contained a large amount of terpenoid compounds which was formed by a stimulation of boring the extract was treated with cold hexane, hexane soluble part was removed and the insoluble part was dissolved in a small smount of acetone and used for paper- and thin layer chromatography. A mixture of benzene-ligroin-methanol-water (50 : 50 : 1 : 50, v/v) for the former and a mixture of chloroform-acetic acid (9 : 1, v/v) for the latter were used, respectively. Pinosylvin, pinosylvin monomethyl ether, pinobanksin and pinocembrin were used as authentic compounds, and diazotized sulfanilic acid and ferric chloride-ferricyanate solutions were sprayed on the chromatograms as detecting reagents.

Wood powder of *Cryptomeria japonica* was extracted with methanol for 10 hours using a Soxhlet extractor, and the extract was analysed by thin layer chromatography using a mixture of acetone-petroleum ether (2 : 3, v/v) as an irrigant. Sugiresinol and hydroxysugiresinol were used as authentic compounds and they were detected by the same reagents.

Quantitative Determination of Pinosylvin, Pinosylvin Monomethyl Ether, Sugiresinol and Hydroxysugiresinol

Pinosylvin and pinosylvin monomethyl ether on the chromatogram were extracted with hot acetone, respectively and acetone was evaporated. The residue was dissolved in a suitable amount of ethanol and the optical density of the solution at 300 mμ was measured. The amount of pinosylvin and pinosylvin monomethyl ether was calculated consulting the calibration curves of authentic compounds.

Determination of sugiresinol and hydroxysugiresinol[15] was carried out in following way. The methanol extract of *Cryptomeria japonica* was applied to silicic acid column (2 cm × 20 cm) and eluted with a mixture of acetone-petroleum ether (2 : 3, v/v), and the eluent was collected as five mℓ fractions. The fractions of sugiresinol (3–8) and hydroxysugiresinol (8–12) were combined, separately and the solvent was evaporated. Both compounds were purified further by thin layer chromatography and the compounds were dissolved in a suitable amount of ethanol, respectively and the optical density at 225 mμ was measured. The amount of both compounds was calculated consulting the calibration curves of authentic compounds.

Results and Discussion

Cytological Observation

The nuclei of ray parenchyma cells in cambial zone of conifers were globular, and with elongation of the cells to radial direction the nuclei became rod and or ellipsoid shape, and appreciable differences in the shape among species were not observed (Photo 1–3), whereas the nuclei of the ray cells of broad-leaved trees were much smaller than those of conifers and were fusiform and

or globular form, and the variation of the shape was more or less dependent on species (Photo 4–6).

In *Magnolia obovata*, *Acanthopanax sciadophylloides* and *Acer mono* the nuclei of the ray cells in cambial zone were generally globular but in the adjacent xylem the nuclei became fusiform with elongation of the cells, and in 2 or 3 annual rings the nuclei of procumbent, upright and square ray cells were fusiform and or globular and in the more inner part of sapwood the nuclei of all parenchyma cells were globular.

In *Kalopanax ricinifolium* and *Fraxinus mandshurica* the nuclei in the ray cells of the outermost xylem were fusiform but in other parts of sapwood the nuclei became globular form. In *Morus bombycis* the nuclei were small and globular form in whole sapwood and in *Tilia japonica* the nuclei of procumbent ray cells were fusiform and those of short procumbent, and square ray cells towards radial direction at the boundary of annual rings were globular form.

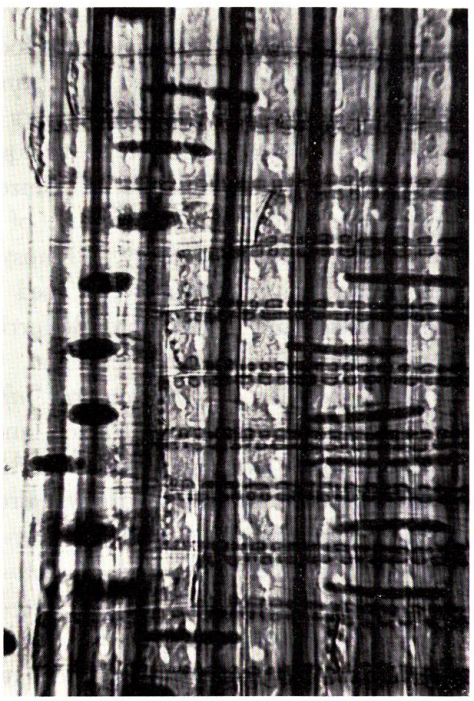

Photo 1. Nuclei of the ray parenchyma cells in cambial zone and in the outermost sapwood of *Abies sachalinensis*.

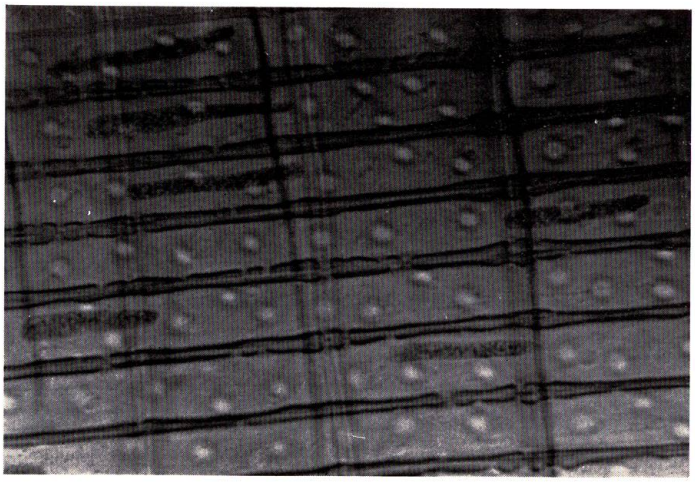

Photo 2. Nuclei of the ray parenchyma cells in springwood of *Picea jezoensis*.

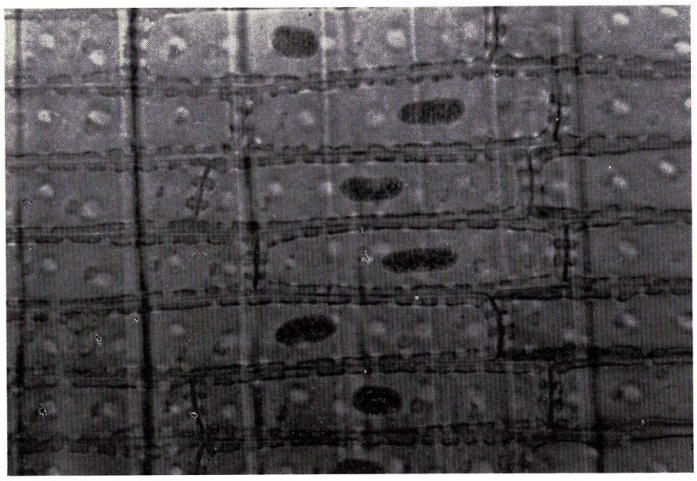

Photo 3. Nuclei of the ray parenchyma cells in summerwood of *Picea jezoensis*.

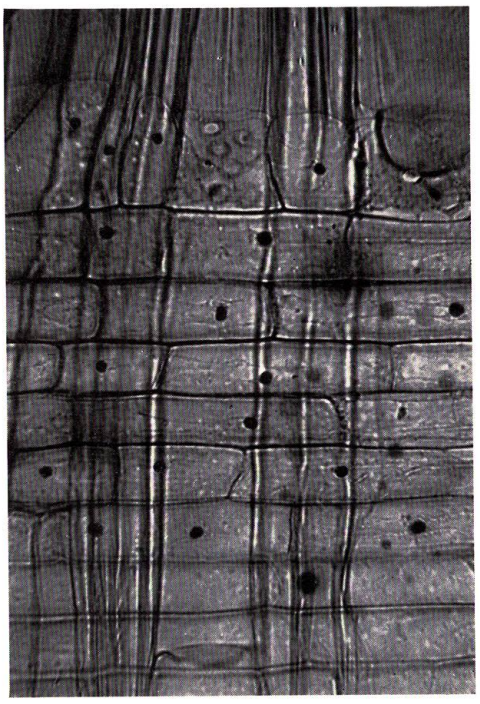

Photo 4–5. Nuclei of the ray parenchyma cells of *Acanthopanax sciadophylloides*.

4. Fusiform nuclei in 2 nd ring from cambium.
5. Nuclei being globular in inner sapwood.

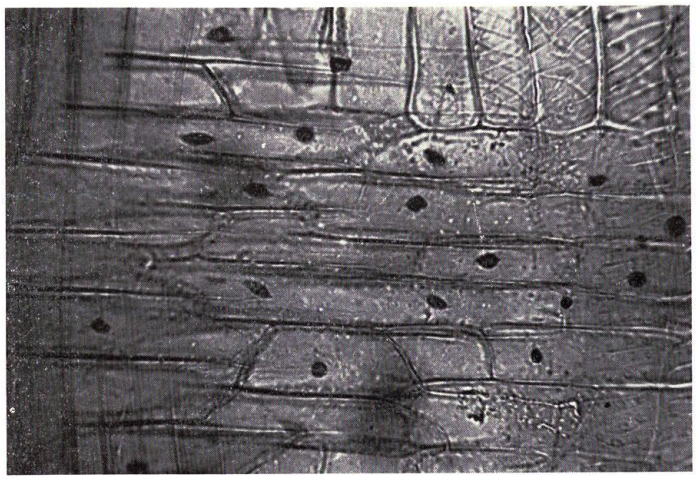

Photo 6. Nuclei of the ray parenchyma cells in outer sapwood of *Fraxinus mandshurica*. Fusiform nuclei are being globular in outer sapwood.

The transition of slenderness ratio (ratio of length to width)[3] of the nucleus from outer sap- to inner sapwood of conifers is shown in Fig. 1. The ratios in spring wood were about two times larger than those in summer wood and this should be attributed to the fact that the ray cells in spring wood were about two times longer comparing with those of summer wood. Both slenderness

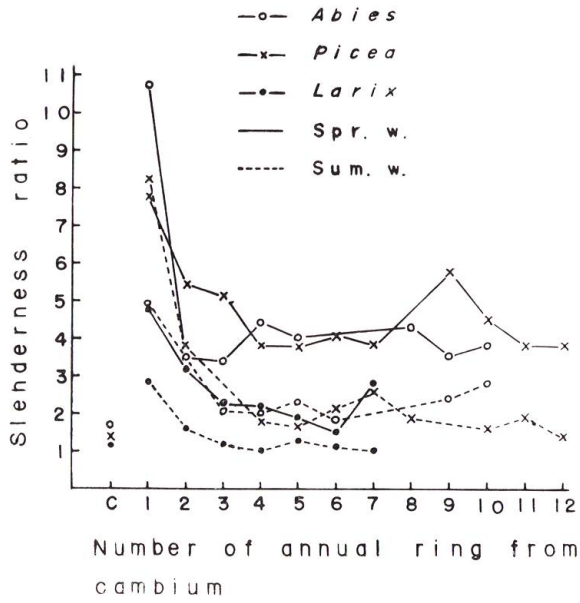

Fig. 1. Slenderness ratio of the nucleus of ray parenchyma cell from outer sap- to inner sapwood of coniferous trees.
Note. C; cambial zone

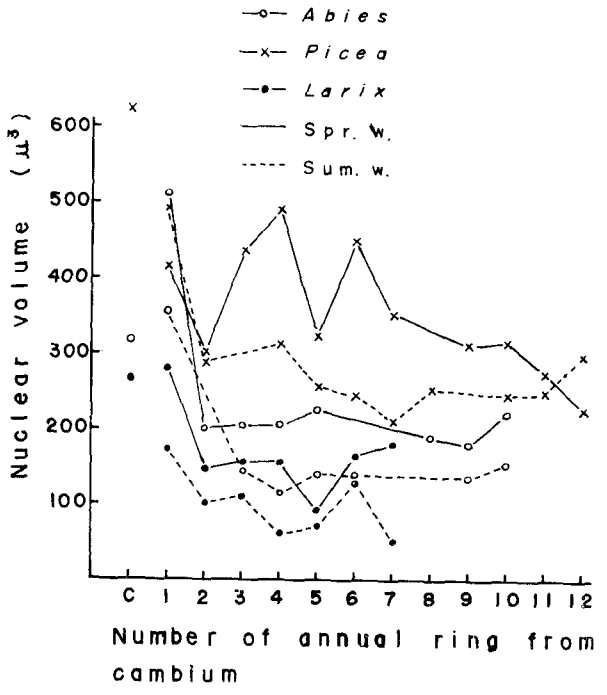

Fig. 2. Nuclear volume of ray parenchyma cell from outer sap- to inner sapwood of coniferous trees.
Note. C; Cambinal zone

ratio and the volume of nuclei in the outermost annual ring decreased abruptly in second annual ring and in the more inner sapwood the values were almost constant (Fig. 2).

In any case the nucleus disappeared in heartwood and resinous heartwood compounds were found in the ray cells of heartwood.

DNA Content in the Nucleus of Ray Parenchyma Cells

In Fig. 3, the absorption spectrum of the nucleus stained with Feulgen's reagent of ray cells of *Cryptomeria japonica* is shown. The figure clearly shows the absorption peak at 560 mμ in the nucleus of sapwood. But, the nucleus in the outermost heartwood did not give any appreciable absorption peak showing the disorganisation of the nucleus. The disorganisation of the nucleus was easily detected by ordinal microscopic observation. The color of the nucleus in the outermost heartwood turned brownish with Feulgen reagent, and in the more inner heartwood the nucleus disappeared.

The DNA content in the nucleus of ray cells is shown in Fig. 4. The DNA content was either nearly constant from cambial zone to intermediate wood or a little higher in intermediate wood and the direct relationship between metabolic activity of ray cells and the DNA content could not be established.

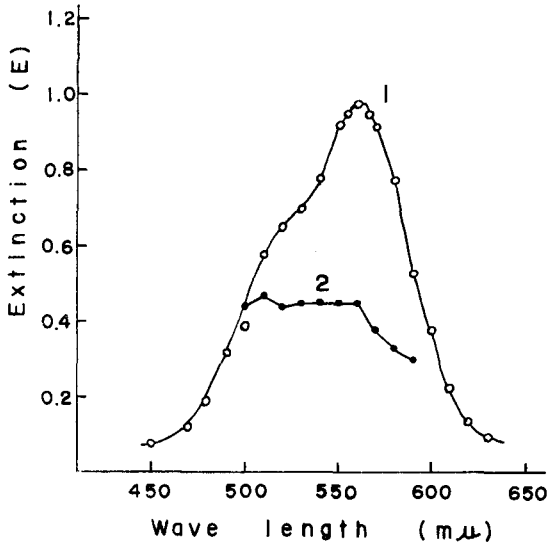

Fig. 3. Absorption spectra of stained nucleus of *Cryptomeria japonica* with Feulgen's reagent.

Note. 1; nucleus in the outermost sapwood
2; disorganized nucleus in the outermost heartwood

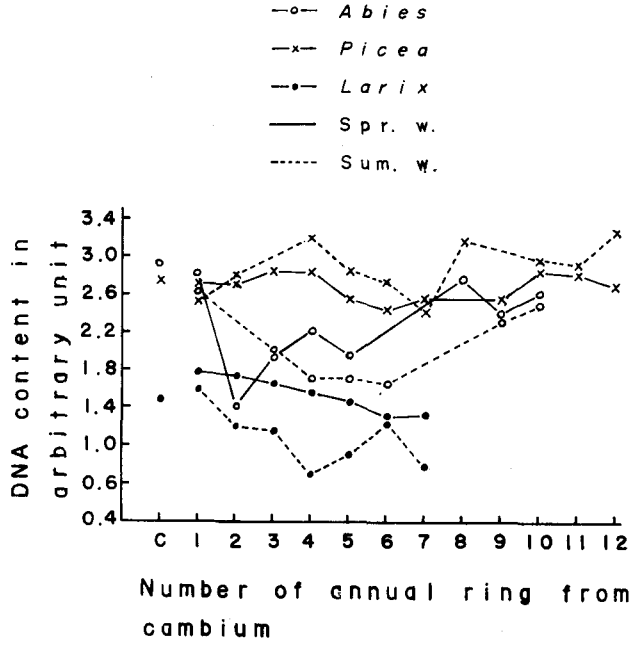

Fig. 4. DNA content in arbitrary unit in the nucleus of ray parenchyma cell of coniferous trees.

Note. C; cambial zone

RNA Content in the Nucleus of Ray Parenchyma Cells

It has been recognized that DNA is in the nucleus and RNA is localized mainly in cytoplasm and in nucleolus. As shown in Photo 7 in the cells of cambial zone and ray parenchyma cells of the outermost sapwood both cytoplasm and nucleus were stained intensely with Azur B, and methyl green and pyronine indicating large amounts of RNA in the cells. However, by extraction of RNA with perchloric acid cytoplasm was stained less intensely comparing with the original section and the nucleus which was not distinguished from the intensive stain of cytoplasm in the original tissues became to be recognized clearly (Photo 8).

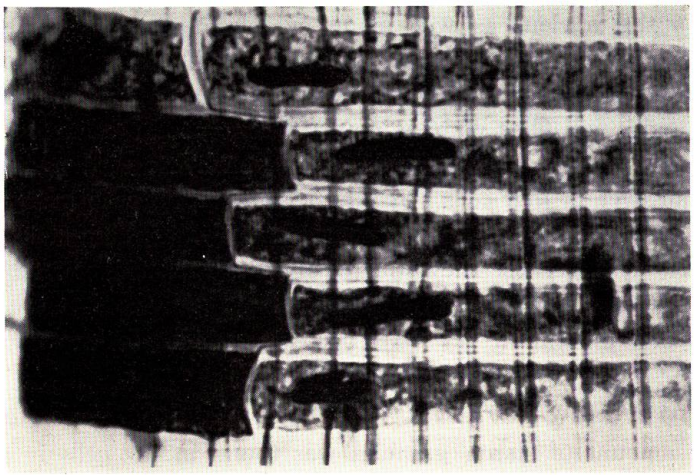

Photo 7. Stained ray parenchyma cells in the outermost sapwood of *Cryptomeria japonica* with Azur B.

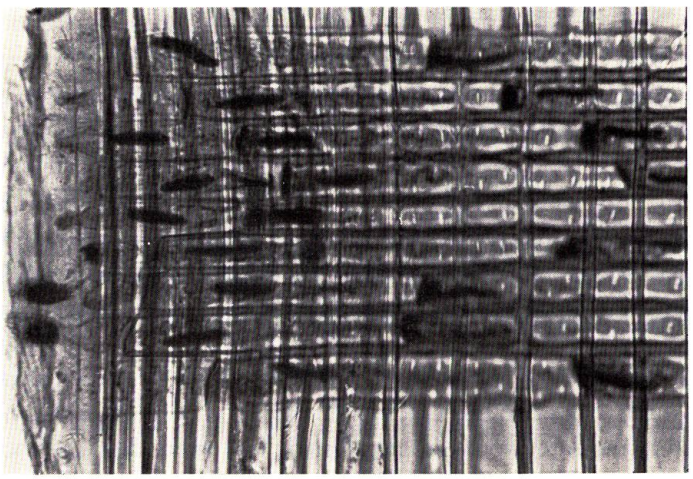

Photo 8. Stained ray parenchyma cells in the outermost sapwood of *Chamaecyparis obtusa* with Azur B after the extraction of RNA by perchloric acid.

Nucleus and RNA in cytoplasm were located to one side of the outer tangential wall of ray parenchyma cells in the outermost sapwood of conifers. The nucleus in this stage seemed to contain a considerable amount of RNA, as nucleus giving a dark red color with methyl green and pyronine, and RNA in cytoplasm was recognized in small granules like ribosome. RNA in cytoplasm of ray parenchyma cells decreased rapidly from cambial zone toward the inner sapwood within about 1–2 ring or 3–5 mm in width.

At this stage the localization of nucleus shifted from the outer tangential wall to the central part of ray cells, and with methyl green and pyronine the nucleus was stained blue, indicating the decrease of RNA. These results suggest that RNA in the nucleus as well as in cytoplasm disappeared rapidly during aging of wood tissues. In the inner sapwood, RNA in the cytoplasm of ray cells was found slightly. During this stage, the nucleus in parenchyma strand disappeared, and it was filled with resinous substances. In the innermost sapwood and intermediate wood, some cells in ray parenchyma died and were stained partially or all over in that cells. In the heartwood, all cells in ray parenchyma tissues died and were stained rather homogeneously. These stained substances are not nucleic acid, because they still remained in the sections after the selective extraction of RNA. The results obtained should indicate that the synthesis of nucleic acid and protein in ray cells decrease rapidly in the region of outer sapwood.

Localization of Starch Granules, Oil Droplets and Shikimic Acid

A large amount of starch granules was found in ray cells in younger sapwood and it decreased towards older sapwood. Conversely the content of oil and fat in cells increased from younger sapwood towards older sapwood and even in heartwood the compounds stained with Sudan III were found. However, as Sudan III is not specific for fat at least a part of the compound in the older sapwood and heartwood should be a terpenoid compound or a disintegrated cell constituent. The results, in any case, suggest that oils and fats in the ray cells might be synthesised from starch as well as sugars translocated from cambium.

Shikimic acid was located in the cytoplasm of ray parenchyma cells suggesting the conversion of sugars to shikimic acid by the enzyme system in cytoplasm.

FREY-WYSSLING and BOSSHARD[3] found that the volume and slenderness ratio of nuclei of ray parenchyma cells of conifers diminish gradually in the direction from the sapwood to the heartwood, and in the intermediate wood the volume of the nuclei is at a minimum. They also found that active mitochondria are located only in a few annual rings inwards from the cambium. From these observation FREY-WYSSLING and BOSSHARD concluded that the ray cells of the intermediate wood do not display an intensified metabolism against CHATTAWAY's hypothesis[2] but in the intermediate wood the nuclei gradually lose control, granules are hydrolysed, the enzymes of the sapwood parenchyma disorganize, starch and an oxido-polymerization of the phenols becomes possible.

The present experiment has confirmed most of their observation. However, the DNA content in the nuclei of the ray cells does not vary significantly in cambial zone and in sapwood, and then the volume of nuclei may not be a good index of metabolic activity of the ray cells as well.

RNA content in cytoplasm and in nucleus, on the other hand, is much related to the metabolic activity of the ray cells, and abundant RNA were found only in the cells of cambial zone and the outermost xylem tissues. In the innermost sapwood and intermediate wood RNA could not be found any more. These results and the occurrence of shikimic acid in the cytoplasm of ray cells should indicate that in the heartwood formation the metabolic activity of the ray cells in the intermediate wood is not activated but the pattern of metabolism of the ray cells changes from cambial zone to intermediate wood toward aromatic biosynthesis.

Physiological Investigation

Respiration of Tissues

The results of experiments on oxygen uptake by the tissues of cambial zone, sap-, intermediate- and heartwood are shown in Fig. 5–8. The rate of oxygen uptake by cambial tissue which is richly protoplasmic is most rapid when expressed on a wet weight basis, and the rate (about 150 $\mu\ell$/g/hr) is almost the same in *Chamaecyparis obtusa* and *Thujopsis dolabrata*, but in *Cryptomeria japonica* the oxygen uptake amounted to 300 $\mu\ell$/g/hr. The adjacent outermost xylem tissue has a lower rate and inner sapwood and intermediate wood have very

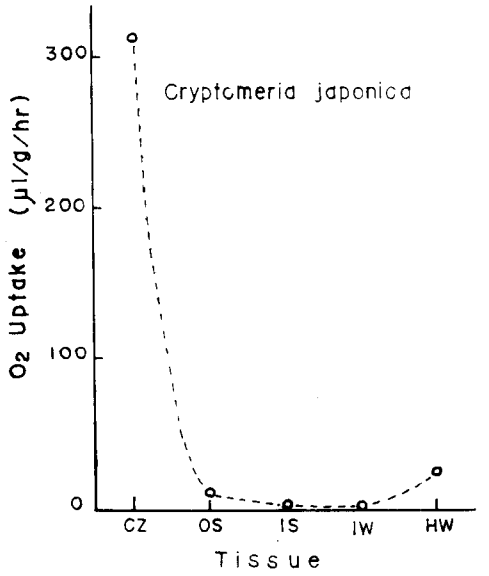

Fig. 5. Respiration of tissues.

Note. CZ; cambial zone, OS; outer sapwood, IS; inner sapwood, IW; intermediate wood, HW; heartwood.

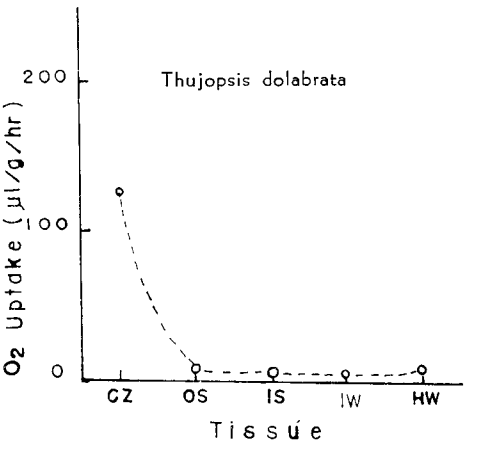

Fig. 6. Respiration of tissues.

Note. CZ; cambial zone, OS; outer sapwood, IS; inner sapwood, IW; intermediate wood, HW; heartwood.

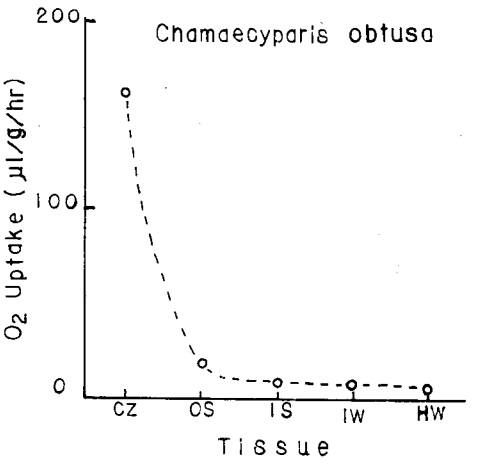

Fig. 7. Respiration of tissues.

Note. CZ; cambial zone, OS; outer sapwood, IS; inner sapwood, IW; intermediate wood, HW; heartwood.

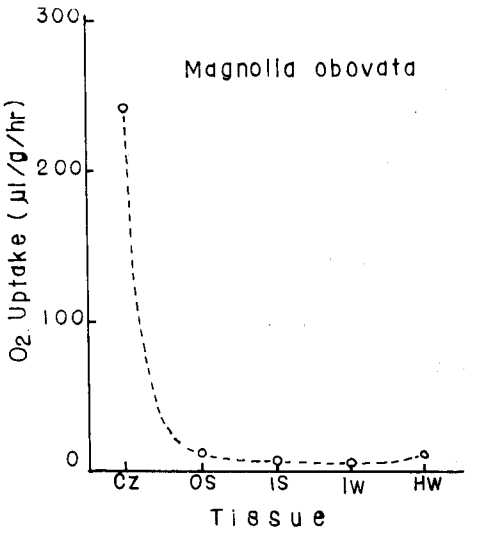

Fig. 8. Respiration of tissues.

Note. CZ; cambial zone, OS; outer sapwood, IS; inner sapwood, IW; intermediate wood, HW; heartwood.

low rate.

The rate of oxygen uptake by tissues of broad-leaved trees is also similar in *Magnolia* and in *Populus* and the highest rate is found in cambial zone (about 250 $\mu\ell$/g/hr).

The oxygen uptake by the intermediate wood of conifers and broad-leaved trees was much lower than that of cambial tissue, and no intensified respiration was observed in the all species tested. As shown in the figures a very low oxygen uptake takes place even in the heartwood. The oxygen uptake by heartwood must be due to non-enzymatic oxidation of heartwood compounds because the rate of oxygen was not affected by boiling.

Table 1 indicates the oxygen uptake per hour per mg of nitrogen in the

Table 1. Respiration of wood tissues ($\mu\ell$ O_2/hr/mg N).

Samples	CZ	OS	IS	IW	HW
*C. *japonica*	70.4	15.5	17.2	16.3	29.7
**C. *obtusa*	52.1	25.7	16.4	14.6	9.3
*T. *dolabrata*	51.4	17.4	13.5	10.6	19.7
**M. *obovata*	43.9	11.9	15.1	14.1	25.0

Note. CZ; cambial zone, OS; outer sapwood, IS; inner sapwood, IW; intermediate, wood, HW; heartwood.
*; Sample collected from the campus of Gifu University,
**; Sample collected from the Experimental Forest of Gifu University.

tissues is a more satisfactory measure of actual living protoplasm, although some of the nitrogen in the sapwood and all of the nitrogen in the heartwood must be derived from dead cells. As shown in the table oxygen uptake on this basis by cambial zone is about 60 $\mu\ell$/mg N/hr, and the rate is much lower in the outer- and inner sapwood suggesting the occurrence of a large amount of physiologically inactive nitrogen compounds in the tissue.

GOODWIN and GODDARD[16] reported that the oxygen uptake by the cambial zone of *Acer rubrum* and *Fraxinus nigra* is most rapid, the values for the adjacent xylem are somewhat lower, and inner sapwood towards heartwood the values become progressively lower. They also found that the rate of oxygen uptake in heartwood is very low and is not affected by boiling.

The experimental results have shown conclusively that the metabolic activity of cambial zone, which may be correlated with growth and secondary wall formation, is considerably high but the activity of the sapwood and intermediate wood is very low and no acceleration of respiration is found contrary to CHATTAWAY's supposition.

Oxidation of Glucose-1-^{14}C and -6-^{14}C

It can be recognized in Fig. 9 that total amount of oxygen uptake in the respiratory breakdown of glucose by tissues of *Cryptomeria japonica* decreased from cambial zone towards intermediate wood.

The C_6/C_1 ratio in cambial zone is 0.8–0.9 and the ratio abruptly decreased in sapwood and the values decreased gradually toward intermediate wood (Fig. 10).

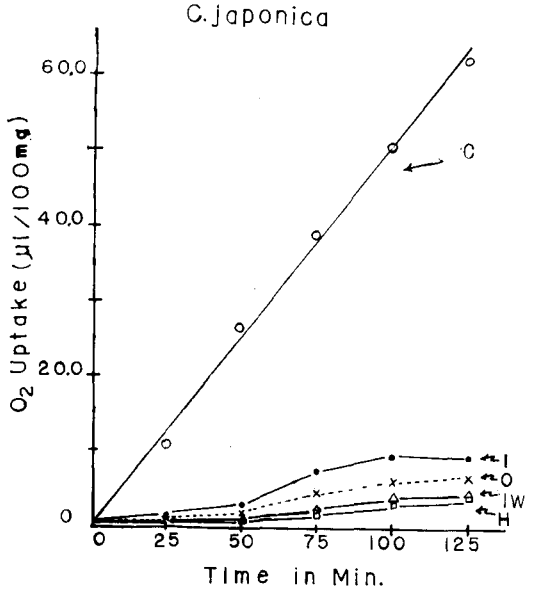

Fig. 9. Respiration of tissues in the presence of glucose.
Note C; cambial zone, O; outer sapwood, I; inner sapwood, IW; intermediate wood, H; heartwood.

This phenomenon is very similar to that observed in bamboo shoot[17] in which upper parts the greater majority of respiration is performed through the glycolysis-TCA cycle system, whereas in the tissues of lower parts the pentose phosphate pathway largely contributes to the respiration. C_6/C_1 ratio in cambial zone obtained from the wood block (50 cm length) with its cut ends covered with paraffin and kept for about 1 month at about 15°C, was 0.45, and also the value of C_6/C_1 in cambial zone of fresh wood felled in November gave about 0.4–0.5. These results indicate that the pattern of metabolism changes towards pentose phosphate pathway during storage or the dormant season.

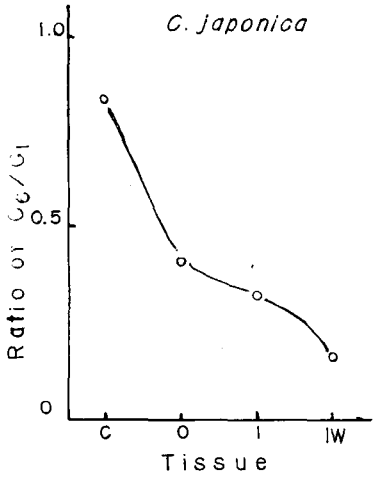

Fig. 10. Pattern of respiratory breakdown of labeled glucose.

Note. C; cambial zone, O; outer sapwood, I; inner sapwood, IW; intermediate wood.

Anyway the results in the present experiment show that respiratory breakdown of glucose through the glycolysis-TCA cycle system in cambial zone gradually changes to that through the pentose phosphate pathway which may be mainly related to the lignification of cell wall and partly to the biosynthesis of phenolic compounds other than lignin.

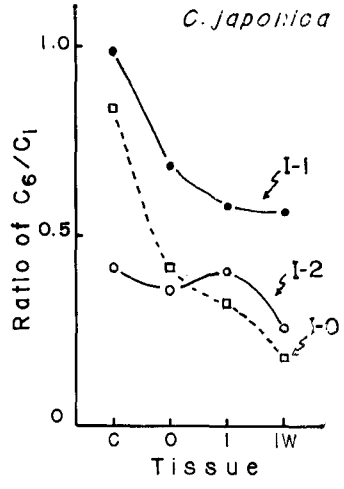

Fig. 11. Effect of 2, 4-dinitrophenol and arsenite on glucose breakdown.

Note. I-O; control, I-1; 2, 4-dinitrophenol as an inhibitor, I-2; sodium arsenite as an inhibitor, C; cambial zone, O; outer sapwood, I; inner sapwood, IW; intermediate wood.

The effect of dinitrophenol and arsenite on the respiration[18] of the tissues is also given in Fig. 11. Dinitrophenol, which indirectly induces an increased rate of glycolysis in the tissues, increases the contribution of C_6 and the ratio comes closer to 1. As seen in the figure the effect of dinitrophenol was much stronger in the tissue of sapwood than in cambial tissues. On the other hand, arsenite which blocks pyruvate utilization, increases the relative contribution of C_1 to the CO_2. The effect of arsenite on the respiration of the tissues especially of cambial zone was quite clear, and the C_6/C_1 ratio of the tissues with the inhibitor, is considerably lower than those of the tissues without inhibitor. Thus the results further support the contribution of pentose phosphate pathway,

which favours shikimic acid synthesis, in the glucose metabolism of ray cells.

HILLIS and INOUE[19] also used arsenite as an inhibitor of acetate utilization by wood tissue of *Rhus succedanea* and found a marked increase in the formation of flavonoids by this inhibitor. They explained this phenomenon as blockage of TCA cycle which releases acetate to enable the formation of the A ring of flavonoid. Present experiment, however, has indicated that pentose phosphate pathway is rather predominant in the carbohydrate metabolism in the ray cells of sapwood and that also arsenite mostly contributes to change the rate of glycolysis towards pentose phosphate pathway which is favor to synthesis of the B ring of flavonoids.

Conversion of Glucose to Shikimic Acid in Wood Tissues

Conversion of glucose to shikimic acid in the wood tissues was clearly established by injection of glucose into the cambial zone of *Illicium religiosum*. The occurrence of radioactive shikimic acid in the injected stem indicates that the conversion is effected in the wood tissue without the contribution of leaf and is accordant with the fact that glucose-1-^{14}C was converted with tissue culture of strob pine[20]. Localization of shikimic acid in the cytoplasm of ray cells detected by cytological observation further supports that the synthesis of shikimic acid is mediated with the enzyme system in cytoplasm of ray cells.

Recently DIETRICHS[21], and HASEGAWA and SHIROYA[4] showed the transformation of sucrose -^{14}C into shikimic acid and quinic acid in the wood tissues

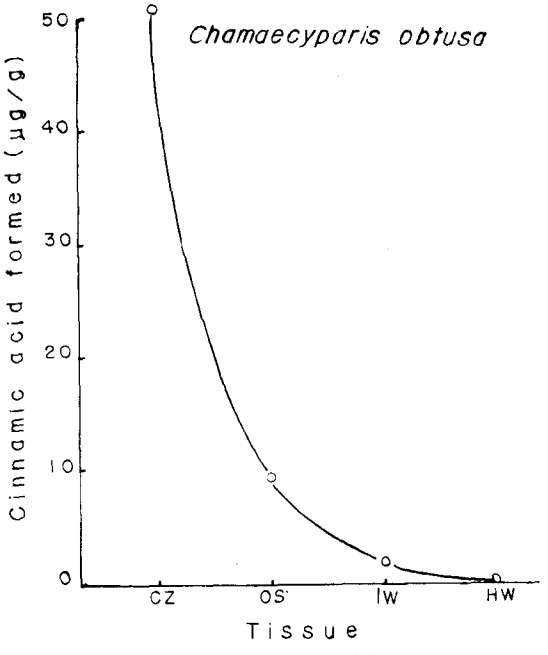

Fig. 12. Localization of phenylalanine ammonia lyase in wood tissues.

Note. CZ; cambial zone, OS; outer sapwood, IW; intermediate wood, HW; heartwood.

of a beech and a cherry tree, respectively. These results suggest a possible participation of the "shikimic acid pathway" in the biosynthesis of heartwood phenols.

Localization of Phenylalanine Ammonia-Lyase in Wood Tissues

A typical example of the distribution of phenylalanine ammonia-lyase in cambial zone, sap-, intermediate- and heartwood of *Chamaecyparis obtusa* is shown in Fig. 12. A considerable amount of enzyme was found in the cambial zone and the activity markedly decreased towards sapwood. In the intermediate wood the enzyme activity further decreased and no activity was found in the heartwood. A similar result was obtained for the tissues of *Cryptomeria japonica.*

The heartwood phenols including stilbene, flavonoids and condensed tannins are known to be formed via both shikimic acid and acetate pathway. HASEGAWA and SHIROYA[4] found the incorporation of sucrose into phenylalanine and heartwood phenols in a cherry wood. Acetyl-CoA required to synthesize phloroglucinol ring of the flavonoids and stilbenes may be produced not only from sugars but also from fatty acid by the β-oxidation, which is widely distributed in the parenchyma cells of sapwood to intermediate wood.

The results of the present experiment have shown that the activity of phenylalanine ammonia-lyase is highest in the cambial zone and decreases rapidly towards the sap- and intermediate wood. The high activity in the cambial zone should be mainly related to the production of precursors of lignin. However, in the sap- and intermediate wood the cells have been completely differentiated and lignified, and thus the phenylalanine ammonia-lyase in these tissues should be mostly responsible for the synthesis of heartwood phenols.

Phenol Oxidase in Wood Tissues

The oxidation of catechol by the tissues of cambial zone, sap-, intermediate- and heartwood of *Cryptomeria japonica* is shown in Fig. 13. The rate of oxidation of catechol by the tissues of cambial zone is quite high but the oxidation by the other tissues is quite feeble. The oxidation of

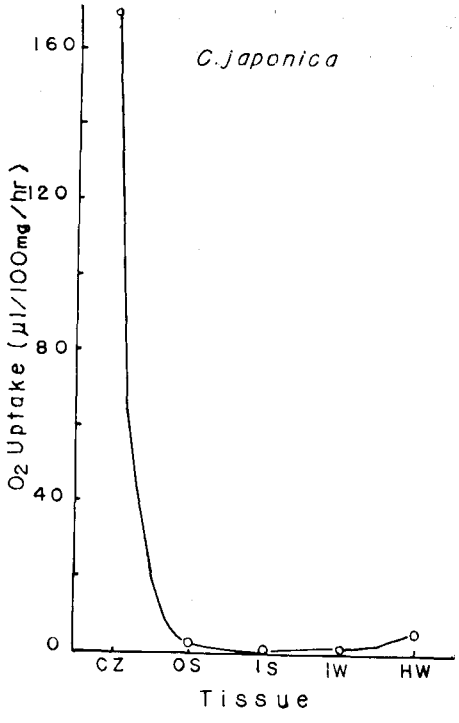

Fig. 13. The oxidation of catechol by the wood tissues.

Note. CZ; cambial zone, OS; outer sapwood, IS; inner sapwood, IW; intermediate wood, HW; heartwood.

catechol by the tissues of heartwood is due to the non-enzymatic one, because the rate of oxidation is not affected by boiling.

KONDO et al.[6] found a somewhat higher activity in the intermediate wood than in the sapwood of *Cryptomeria japonica* in the oxidation of catechol. However, quite a long period oxidation (40 hours) was carried out in their experiment. The oxidation of catechol by phenol oxidase is quite rapid and within 30 minutes the oxidation is generally completed under ordinary condition, and therefore their experiment results should be re-considered.

Thus the present experiment could not find any activation of phenol oxidase in the intermediate wood. However, as LAIRAND[23] found a marked peroxidase activity in the zone adjacent to the heartwood of some conifers it will be still possible to postulate some activation of phenol oxidase in the intermediate wood in some physiological condition.

Substrate specificity of the enzyme in cambial zone of *Cryptomeria japonica* is given in Fig. 14. Caffeic acid and catechol are oxidized very rapidly, but the rate of oxidation of hydroquinone, guaiacyl- and syringyl compounds are very low. Fig. 15 shows the CO inhibition of the enzyme activity in the oxidation of catechol. As expected from the substrate specificity the oxidation of catechol

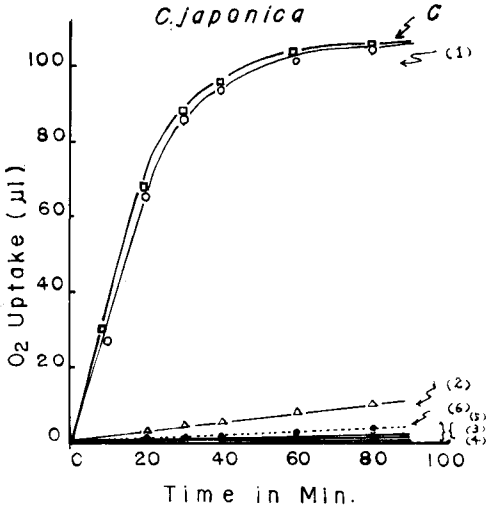

Fig. 14. Substrate specificity of phenol oxidase.

Note. C; catechol, (1); caffeic acid, (2); hydroquinone, (3); coniferyl alcohol, (4); ferulic acid, (5); synapic acid, (6); syringaldehyde.

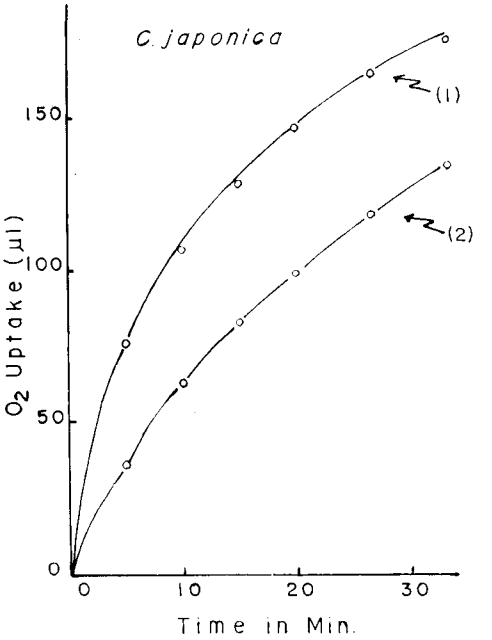

Fig. 15. Carbon monoxide inhibition of catechol oxidation by phenol oxidase.

Note. (1); air phase, (2); mixture of CO gas (95%) and 0_2 gas (5%).

was inhibited by CO and the results indicate that the phenol oxidase in the cambial zone of *Cryptomeria japonica* is a *o*-diphenol oxidase against the finding of *p*-dihydroxyphenol oxidase in the leaves of *Cryptomeria japonica* by CAMBIE

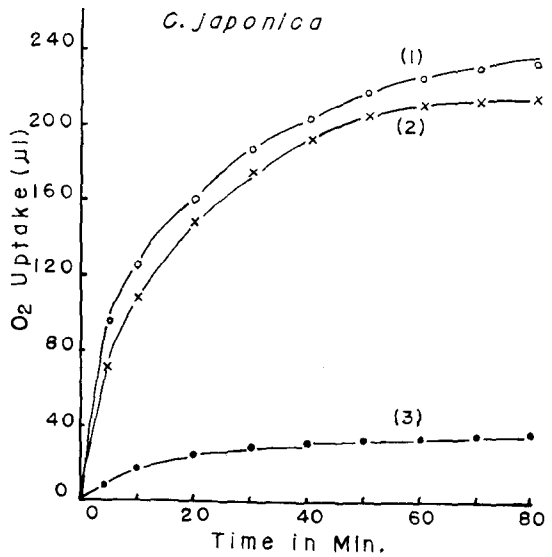

Fig. 16. Oxidation of sugiresinol and hydroxysugiresinol by phenol oxidase;

Note. (1); hydroxysugiresinol, (2); mixture of hydroxysugiresinol and sugiresinol (5 : 1), (3); sugiresinol.

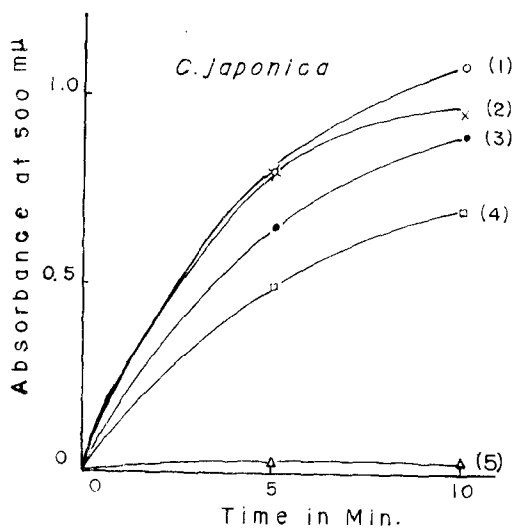

Fig. 17. Coloring of the mixture of hydroxysugiresinol and sugiresinol by phenol oxidase.

Note. (1); hydroxysugiresinol, (2), (3) and (4); mixture of hydroxysugiresinol and sugiresinol in the ratio of 3 : 1, 2 : 1 and 1 : 1, respectively, (5); sugiresinol.

and BOCKS[23].

Sugiresinol and hydroxysugiresinol were isolated from heartwood of *Cryptomeria japonica* and their structure was determined by KONDO et al.[24] and KAI[15] and have been presumed to be precursors of coloring substances of heartwood of this species.

The oxidation of sugiresinol and hydroxysugiresinol with phenol oxidase is shown in Fig. 16, respectively. The rate of oxidation of sugiresinol is quite low and the color of the oxidation product is pale yellowish brown, whereas the rate of oxidation of hydroxysugiresinol is considerably high and the color of the oxidation product is dark brown. As can be seen in Fig. 17 the rate of oxygen uptake and the darkness of the oxidation products are almost proportional to the amount of hydroxysugiresinol in the mixture of both compounds.

Thus, the present experiment supports the view that both compounds especially hydroxysugiresinol should be precursors of coloring substances in the heartwood of *Cryptomeria japonica*. However, as stated previously the enzyme activity could not be found in the intermediate- and heartwood so the coloration by the oxidation of both compounds might be due to non-enzymatic oxidation lasting for a long time.

Nitrogen Content in Wood Tissues

As shown in Table 2, through conifers and a broad-leaved tree the amount of nitrogen in cambial zone is highest and abruptly drops in sapwood and decreases towards heartwood. Against expectation from CHATTAWAY's hypothesis, the result could not show any increased synthesis of protein in the intermediate wood. BECKER[25] also found that the protein content at sapwood-heartwood boundary of pines does not increase. Recently LAIDLAW and SMITH[26] examined the distribution of protein content in the wood tissues of Scots pine and obtained quite similar result to the present experiment.

Table 2. Nitrogen content in wood tissues (mg N/g of wet weight).

Samples	CZ	OS	IS	IW	HW
**C. japonica*	4.13	1.31	0.32	0.35	0.63
***C. obtusa*	2.93	0.68	0.70	0.71	0.81
***T. dolabrata*	2.43	0.86	0.74	0.57	0.76
***M. obovata*	5.57	1.05	0.76	0.39	0.52

Note. CZ; cambial zone, OS; outer sapwood, IS; inner sapwood, IW; intermediate wood, HW; heartwood.
*; Sample collected from the campus of Gifu University,
**; Sample collected from the Experimental Forest of Gifu University.

Formation of Artificial Heartwood

The first experiment of the formation of heartwood by boring through sapwood into heartwood of beech tree trunk had carried out by YAZAWA and

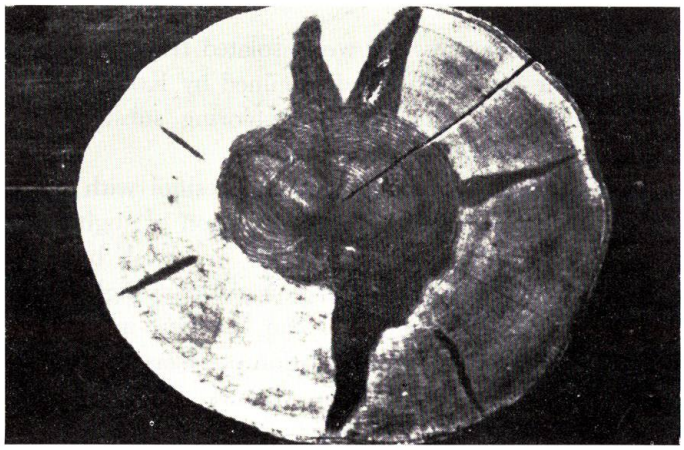

Photo 9. Artificial false heartwood formed by lateral boring (*Fagus crenata*).

HIGUCHI[27]. By this treatment coloration like heartwood along the hole is initiated and finally heartwood with irregular shape containing initiation part by boring is formed. The authors called this "artificial heartwood". The experiment has been continued by YAZAWA and ISHIDA[5] for many conifers and broad-leaved trees. By these experiments the morphorogical and cytological similarity between natural heartwood and artificial heartwood has been established but the identification of the compounds in artificial heartwood has not yet been carried out. (Photo 9).

In the heartwood of *Pinus densiflora*, pinosylvin, pinosylvin monomethyl ether, pinobanksin and pinocembrin are generally found[28]. In the sapwood, however, very small amount of pinosylvin and pinosylvin monomethyl ether have been found.

Acetone extract of heartwood and artificial heartwood of *Pinus densflora* contains a substantial amount of pinosylvin and pinosylvin monomethyl ether. Pinocembrin and pinobanksin are contained in fairly amount in the heartwood extract but in the artificial heartwood the amount of the compounds are very small. Table 3 shows the amount of pinosylvin and pinosylvin monomethyl ether in the heartwood, in the artificial heartwood and in the sapwood, respectively. As can be seen in the table the amount of both compounds in the artificial heartwood were still less than those in the heartwood but is considerably high, compared with those in the sapwood.

The amount of sugiresinol and hydroxysugiresinol in the methanol extract of heartwood and artificial heartwood is also shown in Table 3. Again in *Cryptomeria japonica*, the amount of both compounds in the artificial heartwood is rather comparable with those in the heartwood and a clear difference in the amount of both compounds is recognized between artificial heartwood and sapwood. Thus the results indicate that at least in *Cryptomeria japonica* and

Table 3. The amount of phenolic compounds in natural and artificial heartwood.

C. japonica	N. HW	A. HW	Sapwood
sugiresinol	0.27	0.15	trace
hydroxysugiresinol	0.69	0.31	trace
P. densiflora			
pinoslyvin	0.12	0.02	trace
pinosylvin monomethyl ether	0.14	0.01	trace

Note. N. HW; natural hearwood, A. HW; artificial heartwood.
The amount of the compounds was expressed in mg/g dry matter.

Pinus densiflora a quite similar pattern of metabolic pathway which functions in the formation of natural heartwood might be induced in the artificial heartwood by boring, and then artificial heartwood should be a good model in research of metabolism as well as morphorogical characteristics in heartwood formation.

Recently JORGENSEN[29] found that formation of heartwood substances is induced in the affected parts of the sapwood by mechanical damage of bark and cambium of *Pinus resinosa*. He detected pinosylvin and pinosylvin monomethyl ether in affected sapwood and concluded that these compounds are formed by living cells in the sapwood under the influence of desiccation and or aeration.

However, in some woods especially broad-leaved trees the formation of some artefacts other than natural heartwood compounds might be induced by boring and then analytical results of the compounds should be carefully considered in such cases.

Summary

The mechanism of heartwood formation has been studied by means of cytological observation and biochemical investigation of the metabolic pattern of the ray cells from cambial zone towards intermediate wood.

The nuclei of ray parenchyma cells in cambial zone of conifers were globular, and with elongation of the cells to radial direction the nuclei became rod and or ellipsoid. The volume and slenderness ratio (ratio of length to width) of nuclei of conifers decreased abruptly in second annual ring and in the more inner sapwood the values were almost constant.

The nuclei of ray cells of broad-leaved trees, however, were much smaller than those of conifers and were fusiform and or globular form, and the variation of the shape was more or less dependent on species.

The DNA content in the nucleus was either nearly constant from cambial zone to intermediate wood, and these facts may not necessarily relate to the metabolic activity. However, in cambial zone a large amount of RNA was

found and in the innermost sapwood and intermediate wood RNA scarcely found, and thus the amount of RNA seems closely related to the metabolic activity of the ray cells.

Through conifers and broad-leaved trees the rate of oxygen uptake by respiration of cambial tissue was most rapid, the adjacent outermost xylem tissue had a lower rate and inner sap- and intermediate wood had very low rate. The oxygen uptake by intermediate wood was much lower than that of cambial tissue and no intensified respiration was observed in the all species tested.

The C_1/C_6 ratio of the radioactivity of CO_2 formed by respiratory breakdown of glucose-1-^{14}C and -6-C^{14} in cambial zone was 0.8-0.9 and the ratio abruptly decreased in sapwood and the values further decreased gradually towards intermediate wood. The results support the more contribution of pentose phosphate pathway which is favor to shikimic acid synthesis in the older ray cells.

Occurrence of shikimic acid in the cytoplasm of ray cells and conversion of glucose-1-^{14}C to shikimic acid-^{14}C were established, and the results suggest a possible participation of shikimic acid pathway in the biosynthesis of heartwood phenols.

A considerable amount of phenylalanine ammonia-lyase was found in the cambial zone, the activity markedly decreased towards sapwood, the enzyme activity in the intermediate wood further decreased and no activity was found in the heartwood. The enzyme in the ray cells of sap-and intermediate wood should be mostly responsible for the biosynthesis of heartwood phenols.

The *o*-diphenol oxidase was abundant in the cambial tissue, but the enzyme activity in other tissues was quite low. No activity of the enzyme in the intermediate wood was found.

Sugiresinol and hydroxysugiresinol which are assumed to be precursors of coloring substances of heartwood of *Cryptomeria japonica* were oxidized with the *o*-diphenol oxidase. Sugiresinol was slowly oxidized to a pale yellowish brown compound and the hydroxysugiresinol was rapidly oxidized to a dark brown compound. However, as the enzyme activity could not be found in the intermediate wood both compounds should be oxidized to coloring substances non-enzymatically.

Nitrogen content in the cambial zone was highest and the amount was abruptly dropped in sapwood and decreased towards heartwood.

In artificial heartwood of *Pinus densiflora* formed by boring through sapwood into heartwood substantial amounts of pinosylvin and pinosylvin monomethyl ether were detected. Similarly in the artificial heartwood of *Cryptomeria japonica* considerable amounts of sugiresinol and hydroxysugiresinol were found and the amount was comparable with those in heartwood. Thus artificial heartwood should be a good model in research of metabolism as well as morphorogical characteristics in heartwood formation.

The results of the present experiment conclusively indicated that the physio-

logical activity of the ray parenchyma cells of intermediate wood of conifers and broad-leaved trees was quite low, and in the formation of heartwood no increase of normal physiological activity as in cambium occurred in the intermediate wood. Therefore, the formation of large amounts of heartwood extractives must be the results of the change of metablic pathway towards the synthesis of secondary products and the activation of some enzymes involved.

Acknowledgement

The authors wish to express their thanks to Dr. A. SATO of Wood Research Laboratory, Kyoto University and Dr. Y. KAI of Shizuoka University for the authentic samples of pinosylvin, pinosylvin monomethyl ether, sugiresinol and hydroxysugiresinol.

Literature cited

1) HILLIS, W. E.: Wood Extractives Ed. W. E. HILLIS. *Academic Press.* P 60 (1962).
2) CHATTAWAY, M. M.: *Australian Forestry*, **16**, 25 (1952).
3) FREY-WYSSLING, A. and BOSSHARD, H. H.: *Holzforsch.*, **13**, 129 (1959).
4) HASEGAWA, M. and SHIROYA, M.: Preprint of Meeting of IUFRO Section **41**, Oct. (1965).
5) YAZAWA, K. and ISHIDA, S.: Journal of the Faculty of Agriculture Hokkaido University, 54, Pt. 2 (1965).
6) MATSUKUMA, N., KAWANO, H. SHIBATA, Y. and KONDO, T.: *J. Japan. Wood Res. Soc.*, **11**, 227 (1965).
7) HIGUCHI, T., FUKAZAWA, K. and NAKASHIMA, S.: *J. Japan Wood Res. Soc.*, **10**, 235 (1964).
8) FUKAZAWA, K. and HIGUCHI, T.: *J. Japan Wood Res. Soc.*, **11**, 196 (1965).
9) FUKAZAWA, K. and HIGUCHI, T.: *J. Japan Wood Res. Soc.*, **12**, 221 (1966).
10) HIGUCHI, T. and FUKAZAWA, K.: *J. Japan Wood Res. Soc.*, **12**, 135 (1966).
11) LEUCHTENBERGER C.: *Chromosoma.* **3**, 449 (1950).
12) JENSEN, W. A.: *Boatanical Histochemistry*, W. H. FREEMAN & Co., P 251 (1962).
13) YOSHIDA, S. and HASEGAWA, M.: *Arch. Biochem. Biophys.* **70**, 377 (1957).
14) HIGUCHI, T.: *Agr. Biol. Chem.*, **30**, 667 (1966).
15) KAI., Y.: *J. Japan. Wood Res. Soc.*, **11**, 23 (1965).
16) GOODWIN, R. H. and GODDARD, D. R.: *Amer. J. Bot.*, **27**, 234 (1940).
17) HIGUCHI, T. and SHIMADA, M.: *Plant & Cell Physiol.*, **8**, 71 (1967).
18) AP REES, T. and BEEVERS, H.: *Plant Physiol.*, **35**, 830 (1960).
19) HILLIS, W. E. and INOUE, T.: *Phytochem.*, **5**, 483 (1966).
20) HASEGAWA, M., HIGUCHI, T. and ISHIKAWA, H.: *Plant & Cell Physiol.*, **1**, 173 (1960).
21) DIETRICHS, H. H.: *Holzforsch.*, **18**, 14 (1964).
22) LAIRAND, D. B.: *Drevarsky Vyskum* No. 1, 1 (1963).
23) CAMBIE, R. C. and BOCKS, S. M.: *Phytochem.*, **5**, 391 (1966).
24) FUNAOKA, K., KURODA, Y., KAI, Y. and KONDO, T.: *J. Japan Wood Res. Soc.*, **9**, 139 (1963).
25) BECKER, G.: *Holz als Roh- u. Werkstoff*, **70**, 368 (1962).
26) LAIDLAW, R. A. and SMITH, G. A.: *Holzforsch.*, **19**, 129 (1965).
27) YAZAWA, K., HIGUCHI, T. and MACHII., S.: *Studies on the properties of beech wood from*

the natural forest under the control of Nagoya Regional Forestry Office, Nagoya Rigional Forestry Office. (1958).
28) HATA, K. and SOGO, M.: *J. Japan Forest. Soc.*, **36**, 8 (1954).
29) JORGENSEN, E.: *Can J. Botany*, **39**, 1765 (1961).

心材形成の生化学的研究

樋口隆昌・深沢和三・島田幹夫

要　約

心材形成機構に関して，形成層から移行材部に至る放射柔細胞の細胞学的研究及び代謝パターンの生化学的研究を行なった。

針葉樹形成層部の放射柔細胞の核は球状であり，細胞が放射方向に伸長するに従い核は楕形或るいは楕円形となることが判った。針葉樹における細胞の核の体積及び細長比は第2年輪では急激に低下し，更に内部に入ると，その値はほぼ一定値を示した。しかしながら広葉樹放射組織細胞の核は，針葉樹に比べてはるかに小さく，紡錘形或るいは球形を示していた。またその形の変動は，多かれ少なかれ樹種によって異なっていることが判った。

核内にある DNA 含量は，形成層部から移行材部に至るまでほぼ一定であった。しかし RNA 含量については，形成層部で最も高く，内部辺材及び移行材部では殆んど認められなかった。従って RNA 含量が，放射組織細胞の代謝活性とより密接な関係にあるように思われる。

針葉樹，広葉樹共に，形成層組織片の呼吸による酸素吸収速度は極めて早いが，これに比べて隣接外部辺材組織片ではやや劣り，内部辺材，移行材部では極めて遅かった。特に移行材部では，どの樹種についても顕著な呼吸現象を見るに至らなかった。

形成層組織片の呼吸によってグルコース-1-^{14}C，-6-^{14}C から生じた CO_2 の放射能の C_6/C_1 比は 0.8～0.9 であった。この比は辺材部では急激に落下し移行材部に向って徐々に減少した。この結果から，移行材に近い内部辺材の放射組織では，ペントースフォスフェイト経路がシキミ酸の合成と関連して，かなりの役割を演じているようである。

放射組織細胞質中にシキミ酸が存在すること及び，グルコース-1-^{14}C がシキミ酸に転換されること等から，心材フェノール成分生合成に“シキミ酸経路”の関与することが判った。

フェルアラニンアンモニアリアーゼが樹木形成層部にかなり多量に存在しており，その活性は，辺材部に向って顕著に減少し，移行部では極めて低く，心材部では全く認めら

れなかった。

o-ディフェノールオキシダーゼは形成層部で多量に存在するが，他の部位では極めて低く移行材部では認められなかった。

スギ心材の着色物質の前駆体と考えられているスギレジノール，ヒドロキシスギレジノールは *o*-ディフェノールオキシダーゼにより酸化された。この結果前者は薄い黄褐色物質に，後者は速やかに酸化され，黒褐色物質に変った。移行材部において本酵素の活性を認めることができなかったことから，両スギレジノール化合物が心材着色物質に変化するとすれば，非酵素的な酸化重合によるものと考えられる。

窒素含量については，形成層において最も高く，辺材，心材に向って著しく減少した。

アカマツの辺材部から心材部にたっする穴をあけることによって生じた，人工心材からかなりのピノシルビン及びそのモノメチルエーテルが抽出された。同様にスギの人工心材にかなりの量のスギレジノール及びヒドロキシスギレジノールの存在することが判った。従って人工心材は，心材形成に伴う形態学的研究のみならず代謝変動の研究に対しても良いモデルと考えられる。

本実験結果から，針葉樹，広葉樹のいずれにおいても，移行材組織の生理活性は極めて低く，形成層で営まれる正常な生理活性は，移行部では増大しないことが明らかになった。従って心材成分生成に際し，細胞の生理活性増大というよりはむしろ，第二次代謝産物合成の方向に代謝が変移するものと考えられる。