#2768 THE INSTITUTE OF PAPER CHEMISTRY
(Hemicelluloses-Iodine Complexes)
Project Reports

PROJECT REPORT FORM

Copies to: Files
Weiner

PROJECT NO. 2768
COOPERATOR
REPORT NO. 1
DATE January 22, 1969
NOTE BOOK 2665
PAGE 1 TO 95
SIGNED N. S. Thompson
B. D. E. Gaillard
A. J. Morak

THE INTERACTION OF POLYSACCHARIDES WITH IODINE

1. Investigation of the General Nature of the Reaction

by

Blanche D. E. Gaillard[1], N. S. Thompson, and A. J. Morak

Abstract

A comparison of the interaction of a commercially available xylan, a galactose-deficient galactoglucomannan from Engelmann spruce holocellulose, a highly branched amyloid from Tamarind seed and a commercial amylose preparation from potato starch in concentrated aqueous calcium chloride solution showed that all four of these polymers reacted with iodine-potassium iodide solution to give a blue colored product which was soluble at low concentrations of reagents. In agreement with data in the literature, other highly branched polysaccharides such as cherry gum and galactose-rich galactoglucomannans did not react with iodine under these conditions. Qualitative tests showed that iodine and calcium ion, as well as polysaccharide were components of the complexes and spectrophotometric measurements showed the dependence of complex formation on the concentration of iodine and polysaccharide as well as on the time and temperature of reaction. Although the polysaccharides reacted to give a dark blue starch-like coloration with iodine, potentiometric titration showed that only the xylan bound iodine in a manner similar to amylose, while galactoglucomannan and amyloid bound

[1]Agricultural College, Animal Husbandry Laboratory, Wageningen, The Netherlands.

iodine in a looser fashion typical of polyvinyl alcohol-iodine complexes. The iodine content of the complexes from xylan, amyloid, and galactoglucomannan, unlike the iodine content of the amylose complex, was found to vary with the concentration of the reactants indicating no chemically unique complexes were formed.

Introduction

Many linear polysaccharides have been shown to give a blue coloration with iodine when they are dissolved in concentrated aqueous calcium chloride solution, although they will not do so when dissolved in salt free aqueous solutions (1). Research has shown that highly branched polysaccharides do not react with iodine under these circumstances, but a limited degree of branching does not prevent the formation of a colored product. It has also been shown that other multivalent ions may be substituted for calcium ion and that bromine may be used instead of iodine in some instances (2). More recent research has shown that a primary requirement for the formation of a blue colored product is the presence of a sequence of at least three (1-4) linked anhydroglucose, anhydroxylose or anhydromannose units in the polysaccharide (3). The role of the various components involved in the formation of the blue color is complicated and explanations based on these qualitative observations have been offered by Gaillard and Bailey (3).

Other polysaccharides such as amylose and various natural occurring amyloids react in aqueous solution with iodine-potassium iodide to give blue to black colored complexes (4,5). Bates and co-workers (6) showed that iodine was bound in the amylose complex within a helical structure of anhydroglucose units and the amount of bound iodine could be determined by potentiometric titration. On the other hand, very little is known about the amyloid com-

plexes except they may be differentiated from the amylose complexes by qualitative tests (7). Colored complexes known to exist between iodine and other organic polymers have been investigated by many researchers. Tebelev and co-workers (8) for example, found by spectrophotometric evidence that the iodine was loosely sorbed or bound in some undefined manner onto the polyvinyl alcohol molecule.

It appears therefore, that iodine may react with a great variety of organic substances to yield colored complexes whose characteristics in many instances have not been precisely defined. The purpose of the present research is to investigate the nature of the interaction of polysaccharides with calcium chloride and iodine in aqueous solution. This report will compare the reaction products of various hemicellulose-like polymers with those of amylose, while subsequent reports will describe in greater quantitative detail the effect of different functional groups and degrees of branching of specific polysaccharides on their ability to form complexes.

Results and Discussion

Introductory Experiments

A commercially available xylan (Pfanstiehl Chemical Co.) was chosen as a representative xylan with a moderate degree of branching. Although the source and the structure of this xylan is not known, it was assumed that it was typical of natural occurring xylans from monocotyledons and that it consisted of a xylan backbone to which were attached single terminal branches of arabinose and uronic acid units. Subsequent research to be described in another publication of this series using xylans of accurately known structures, compositions and sources similar to those speculated for this commercial xylan showed their reactions with iodine to

be similar. A galactose deficient galactoglucomannan isolated from Engelmann spruce holocellulose (9) was chosen as a model for slightly branched hexosans, while an amyloid isolated from Tamarind seed (7) and galactose-rich galactoglucomannans isolated from black spruce and Parana pine were additional examples of highly branched hexosan polysaccharides (10). Examples of linear polysaccharides employed in this investigation were a hydrocellulose and a commercial amylose sample (Superlose) supplied by Stein Hall Inc.

Most of these polysaccharides in concentrated aqueous calcium chloride solution (s.g. 1.3) reacted with iodine to yield a blue reaction product. In agreement with the literature, the highly branched galactose-rich galactoglucomannan from black spruce and Parana pine did not react, whereas the equally highly branched amyloid did react to give a blue color. These results, summarized in Table 1, show that only amylose and amyloid

TABLE I

Qualitative Composition of Certain Polysaccharide-Calcium Chloride-Iodine Complexes

	Reacts in Aqueous Solution	Reacts in Concentrated $CaCl_2$ Solution	Composition of Complex Ca^{++}	I	Forms Methanol Soluble Calcium Complex
Hydrocellulose	—	+	+	+	--
Commercial Amylose (Superlose)	+	+	+	+	—
Amyloid	+	+	n.a.	+	+
Galactose-deficient galactoglucomannan	—	+	+	+	—
Galactose-rich galactoglucomannan	—	—	—	—	—
Commercial xylan	—	+	+	+	—
Cherry Gum	—	—	—	—	+

n.a. = not analysed

react with iodine in aqueous solutions free of calcium chloride and the intensity of the blue color was increased when Na_2SO_4 was added. The addition of calcium chloride to these aqueous solutions brought about no change in the intensity of the amylose-iodine complex but did result in a marked deepening of color when added to the iodine-amyloid complex. Other qualitative observations showed that the addition of a drop of stock iodine solution to amylose solutions caused a blue color to appear immediately, while larger quantities of stock iodine must be added to all other polysaccharides studied here before the blue color appears. When the blue complexes of the latter are diluted with water, the color disappears and a yellowish solution characteristic of aqueous iodine-potassium iodide appears. Unlike the other polymers, the decomposed iodine-amyloid complex can reappear when the diluted solution is cooled to 0° C.

At higher polysaccharide and iodine concentrations, the iodine complexes become insoluble and may be separated from the soluble components by centrifugation. Storage of the isolated complex from xylan, hydrocellulose, and amylose in the air for six weeks brought about no apparent change in the intense dark color. Prolonged storage of the galactoglucomannan complex resulted in a gradual fading of the color of the complex while the amyloid complex decomposed within hours of isolation. After washing the complexes with aqueous calcium chloride to remove excess iodine, they were freed of excess calcium chloride by spreading them on a piece of unglazed porcelain in a dessicator. Analysis of the blue-black greasy products by X-ray diffraction techniques showed no crystalline calcium chloride to be present, although an X-ray powder pattern typical of $CaCl_2 \cdot 2H_2O$ could be obtained from the dry complex which had not been purified on the porcelain plate. Since the blue color could be removed by reducing agents capable of reducing iodine, and

since qualitative tests for iodine could be obtained from the purified complexes (11), iodine is one component of the complex. Since calcium ion (but no crystalline calcium chloride) was found by flame spectrophotometry to be in excess of that required by the carboxyl contents of the polymers, it is likely that calcium ion is also a component of the complex. Quantitative experiments describing the relationships of these components to precisely defined polysaccharides will be the subject of later publications (12).

When the blue precipitates were washed with methanol, the complexes were destroyed, the iodine was liberated and the polysaccharides were isolated as insoluble residues. Quantitative analysis showed no significant differences in composition between the original polysaccharide and these insoluble residues. If on the other hand, methanol saturated with calcium chloride was employed, the polysaccharide-iodine complexes were destroyed and alcohol insoluble fractions were isolated from the amylose, xylan and galactoglucomannan. Amyloid was found to be soluble in the presence of methanol saturated with calcium chloride under these circumstances. Additional tests showed that besides amyloid, cherry gum and black spruce glucurono-arabinogalactan (both highly branched polysaccharides unable to form a blue iodine complex), cannot be precipitated from an aqueous solution containing calcium chloride with methanol saturated with calcium chloride although all three may be precipitated from these solutions with pure methanol.

These results suggest that many polysaccharides can form complexes with iodine in concentrated aqueous calcium chloride solution and that the complexes may be destroyed by adding methanol. Although certain polysaccharides may react with aqueous calcium chloride in such a way that they cannot

be precipitated from solution by the addition of methanol saturated with calcium chloride, this unique characteristic did not ensure the formation of a blue complex when iodine was added to the solution. Conversely, the limited data obtained here suggests that calcium ion is associated with the dark blue complexes when they are formed from concentrated aqueous calcium chloride.

Potentiometric Determinations

The potentiometric titrations employed in this investigation differed in some respects from those described in the literature for the titration of starch (13). Not only were the titrations carried out in the presence of calcium chloride [although Colburn and Schoch claim this makes no difference in starch titrations (14)], but greater iodine concentrations had to be used to ensure reaction of iodine with the polysaccharides. In agreement with the researches of Adkins and Greenwood (15), titrations were conducted at low temperature in an ice bath in order to obtain more satisfactory results. By plotting the amounts of bound iodine against free iodine, as described by Anderson and Greenwood for example (13), it was found that the xylan bound about 9 mg. of iodine per 100 mg. of polymer, which is about half the amount normally found for undegraded amylose under slightly different conditions of titration. The galactoglucomannan and the Tamarind amyloid did not bind iodine in a manner which could be detected by potentiometric titration. These results, which are similar to those of the polyvinyl alcohol-iodine system, suggest that the latter two polysaccharides may react with iodine but that the iodine is not firmly held in these complexes. This observation is supported by the fact that the isolated complexes of these two polymers gradually lose iodine on storage. The firmly bonded starch-iodine, xylan-iodine, or xylan-bromine complexes may be stored for long periods of time without showing signs of decomposition.

Spectrophotometric Determinations

The development of the blue color with time and temperature after the addition of iodine was followed in dilute solutions (0.004% polymer and 0.04% iodine) in order to avoid the complications caused by the formation of an insoluble component. The results shown in Figure 1 demonstrate that the xylan under these conditions gives a maximum extinction within 90 minutes providing the temperature of the solution was cooled in an ice bath before

(FIGURE 1)

the addition of the iodine-potassium iodide solution. The increase of the extinction coefficient for the reaction mixture at 30° C. was more gradual and never reached the same magnitude as that of the cooled solution. The galactoglucomannan (Figure 1) was much less sensitive to differences in initial temperature but it required a higher polymer concentration (0.04% polymer) to develop a measurable, but less intense color. The reaction of the amyloid with iodine under these circumstances is more complex and is shown in Figure 2. The extinction increased very slowly when the reaction was carried out at 30° C. and was still increasing after 7 hours reaction. The addition of Na_2SO_4 to the amyloid resulted in a much faster initial rate of reaction at 30° C., but the maximum value was no higher than that at 30° C. in $CaCl_2$ solution. When the reaction mixture is cooled in an ice bath, a maximum development of color was achieved in 90 minutes which was greater than the maximum after 7 hours at 30° C. The results obtained here may be rational-

(FIGURE 2)

ized by assuming that a complex equilibria exists between the concentrations of the polymer, the calcium complex of the polymer, calcium ion, iodine complex, solubility of the iodine complex, temperature and time of reaction.

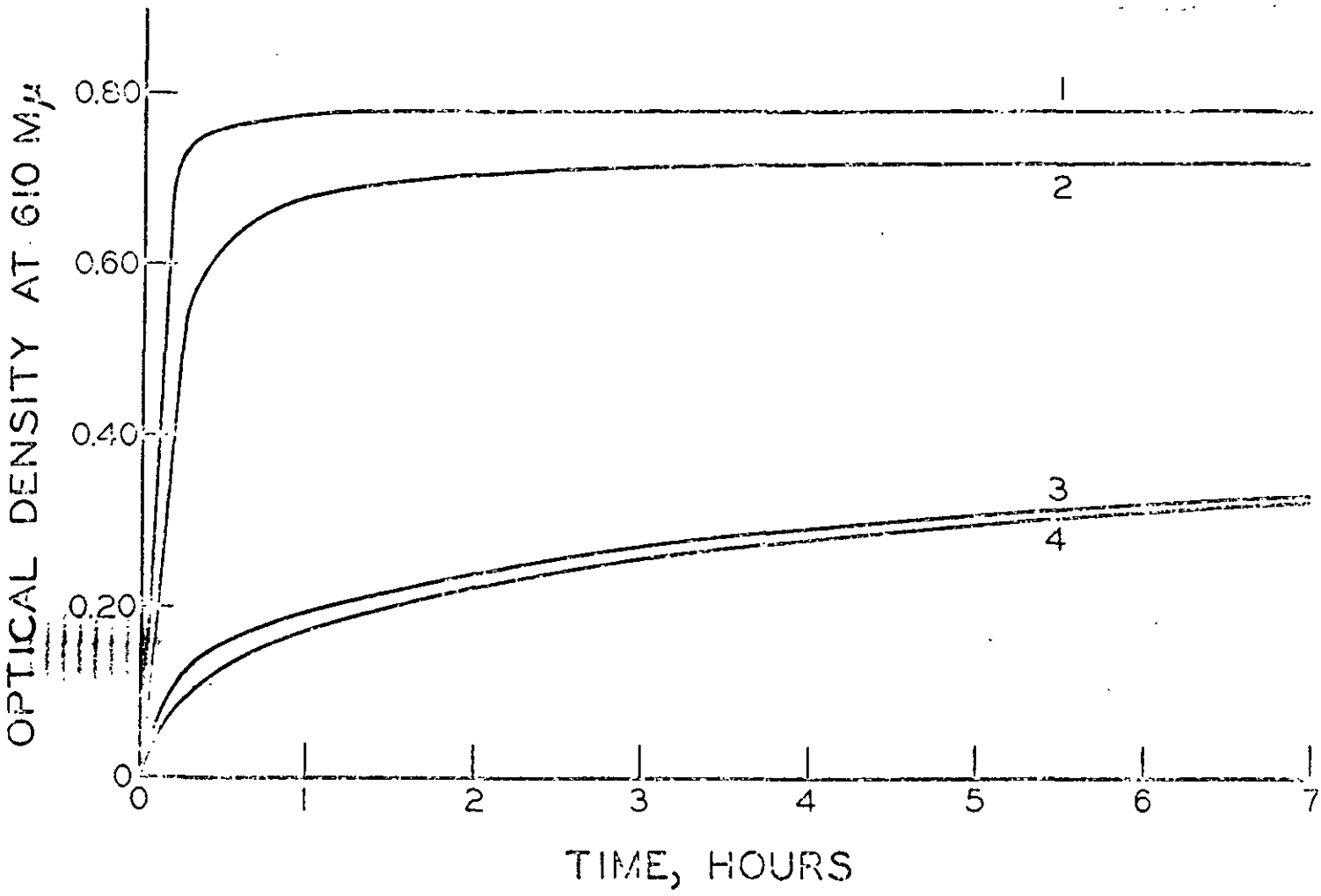


Figure 1. The variation with time of the Optical Density at 610 mμ of the Colored Complex formed by the Reaction of Polysaccharide in Concentrated Aqueous Calcium Chloride Solution (s.g. 1.3) with 0.04% I_2 and 0.16% KI. (1) Commercial xylan (0.004%) at 4° C.; (2) Commercial xylan (0.004%) at 30° C.; (3) Coniferous galactoglucomannan (0.04%) at 4° C.; (4) Coniferous galactoglucomannan (0.04%) at 30° C.

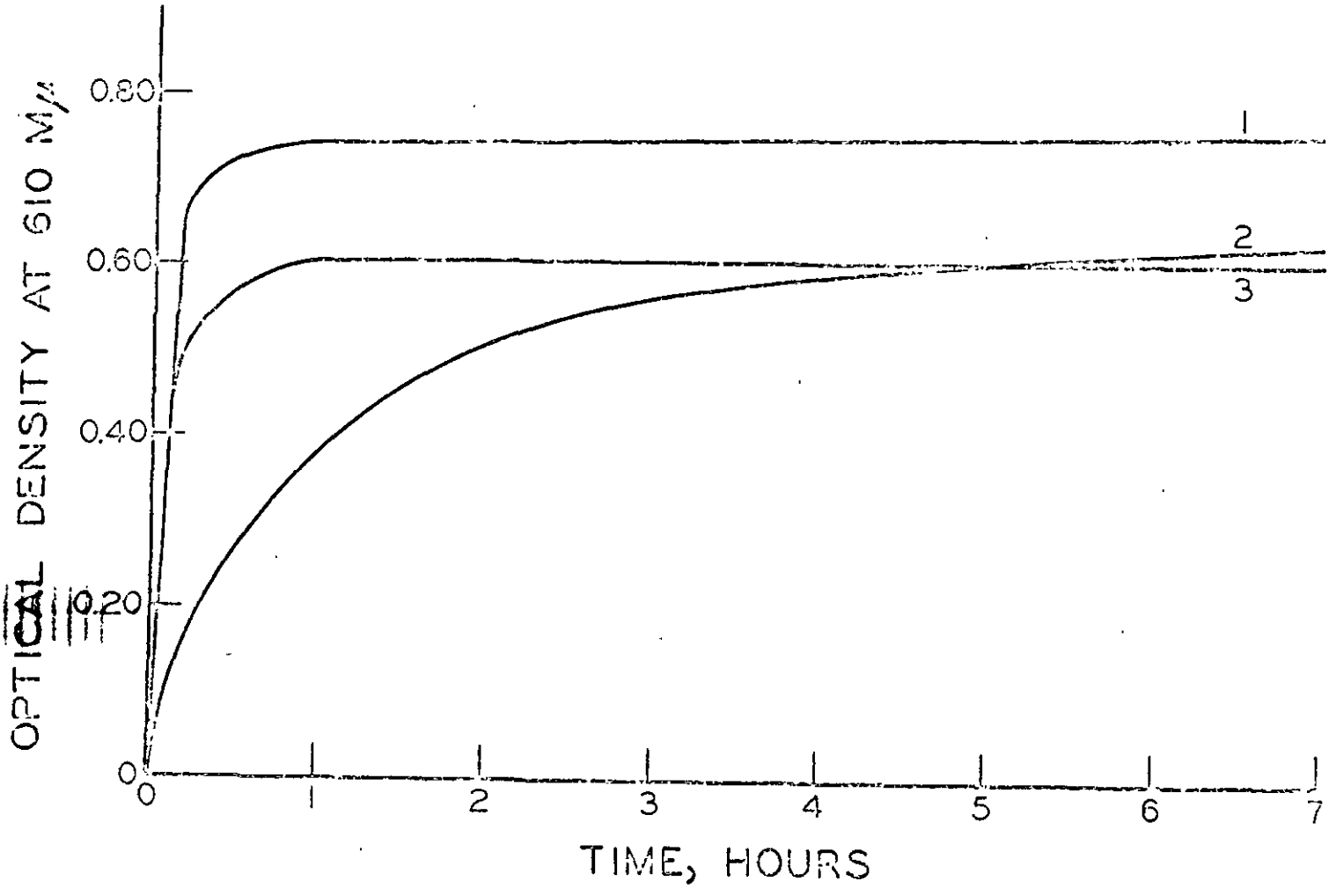


Figure 2. The Variation with time of the Optical Density at 610 mμ of the Colored Complex found by the Reaction of Tamarind "Amyloid" with I_2 under Different Conditions. (1) 0.004% "Amyloid," 0.04% I_2 and 0.16% KI in Aqueous Calcium Chloride (s.g. 1.3) at 4° C.; (2) 0.004% "Amyloid, 0.04% I_2 and 0.16% KI in Aqueous Calcium Chlorate (s.g. 1.3) at 30° C.; (3) 0.004% "Amyloid" with 0.04% I_2 and 0.16% KI in 15% Na_2SO_4.

The plots in Figure 3 demonstrate that the blue colors developed in solutions of increasing polysaccharide content at constant iodine content is a straight line and could be used for quantitative estimation of the hemicellulose content of the solutions. The plots for amylose, xylan, and amyloid are only slightly different whereas the smaller slope of the galactoglucomannan curve is indicative of the less intense nature of the color of the galactoglucomannan-iodine complex.

(FIGURE 3)

If the iodine in a polysaccharide-iodine complex is firmly bound in that complex, the composition may be determined by plotting the relative percent iodine in the reaction (at constant total composition) against the extinction coefficient of the solution for the particular wave length of light which is absorbed by the complex. The composition of iodine which indicates the greatest amount of absorption is a measure of the iodine found in the complex and if for different total compositions of iodine plus polysaccharide the ratio of iodine is always the same, it can be concluded that the complex has a constant ratio of components. This has been found to be the case for the iodine-amylose complex (6) and the results shown in Table 2 show that it is likely to be the case for the iodine complex of amylose formed

TABLE 2

The Composition of Polysaccharide and Iodine at Which Maximum Extinction Coefficient Occurs at Different Total Compositions of Polysaccharide and Iodine

Percent (Polymer and Iodine) of Solution	Relative Percent Iodine at Which Maximum Extinction Occurs				
	4-O-methylglucurone-arabinoxylan	Galactoglucomannan	Amylose	Amyloid (in $CaCl_2$)	Amyloid (in Na_2S
0.01	60	50	20	....	80
0.02	....	40	20	95	50
0.03	50	....	....	70	40

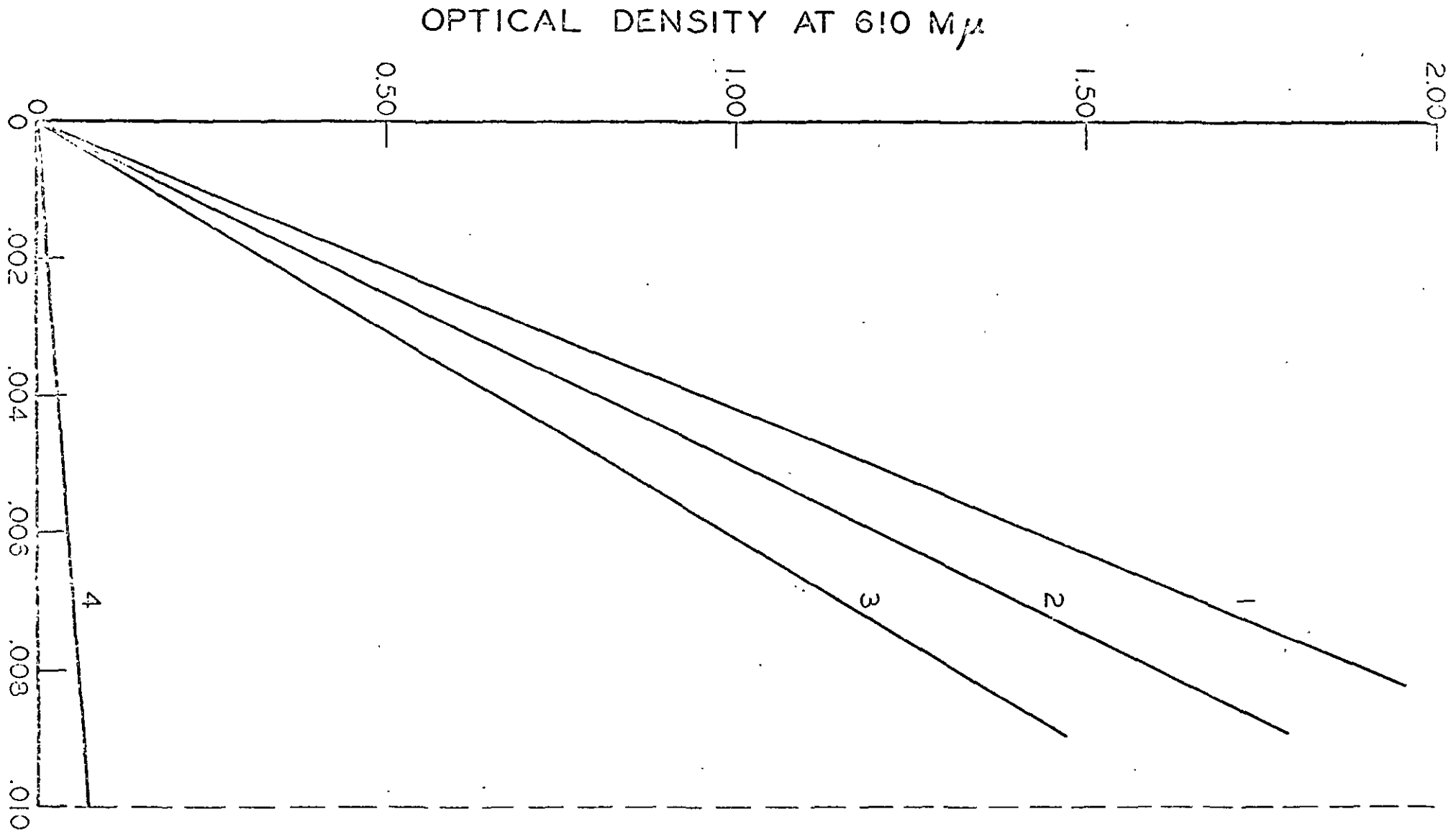


Figure 3. The Variation of Optical Density at 610 mμ of Colored Complexes with Increasing Concentration of Polysaccharide in Concentrated Aqueous $CaCl_2$ Solution (s.g. 1.3) and 0.05% I_2. (1) Commercial amylose, (2) commercial xylan, (3) Tamarind "amyloid", (4) coniferous galactoglucomannan.

in the presence of aqueous calcium chloride. The data given in Table 2 show that the iodine complexes of the other polysaccharides studied here did not react in this manner but reacted in a manner suggesting that the iodine in their complexes was a function of the concentration of the reactants in solution. The plots from which the data for the ratio of xylan to iodine at different total xylan plus iodine concentrations were obtained are shown in Figure 4 and are typical of the plots used to obtain data for the other polysaccharides listed in the table.

(FIGURE 4)

These results confirm the observations in the literature that although certain polysaccharides do not react with iodine in aqueous solution, they will react with it in concentrated aqueous calcium chloride solution. Although previous experience indicated that highly branched polysaccharides would not react under these circumstances, present research demonstrates that the highly branched amyloid from Tamarind seed does do so. Tests suggest the complexes are composed of polysaccharide, iodine and possibly calcium ion, that the stability of the complex varies according to the nature of the polysaccharide and that the iodine content of the complexes is dependent upon the relative concentration of iodine and of polysaccharide in solution. Future research will elucidate the role of calcium ion in the complex and will investigate the effect of polysaccharide structure and functional groups upon complex formation.

Experimental

Preparation of Samples

The amylose employed in this investigation was a commercially available material prepared from potato starch and sold under the trade name

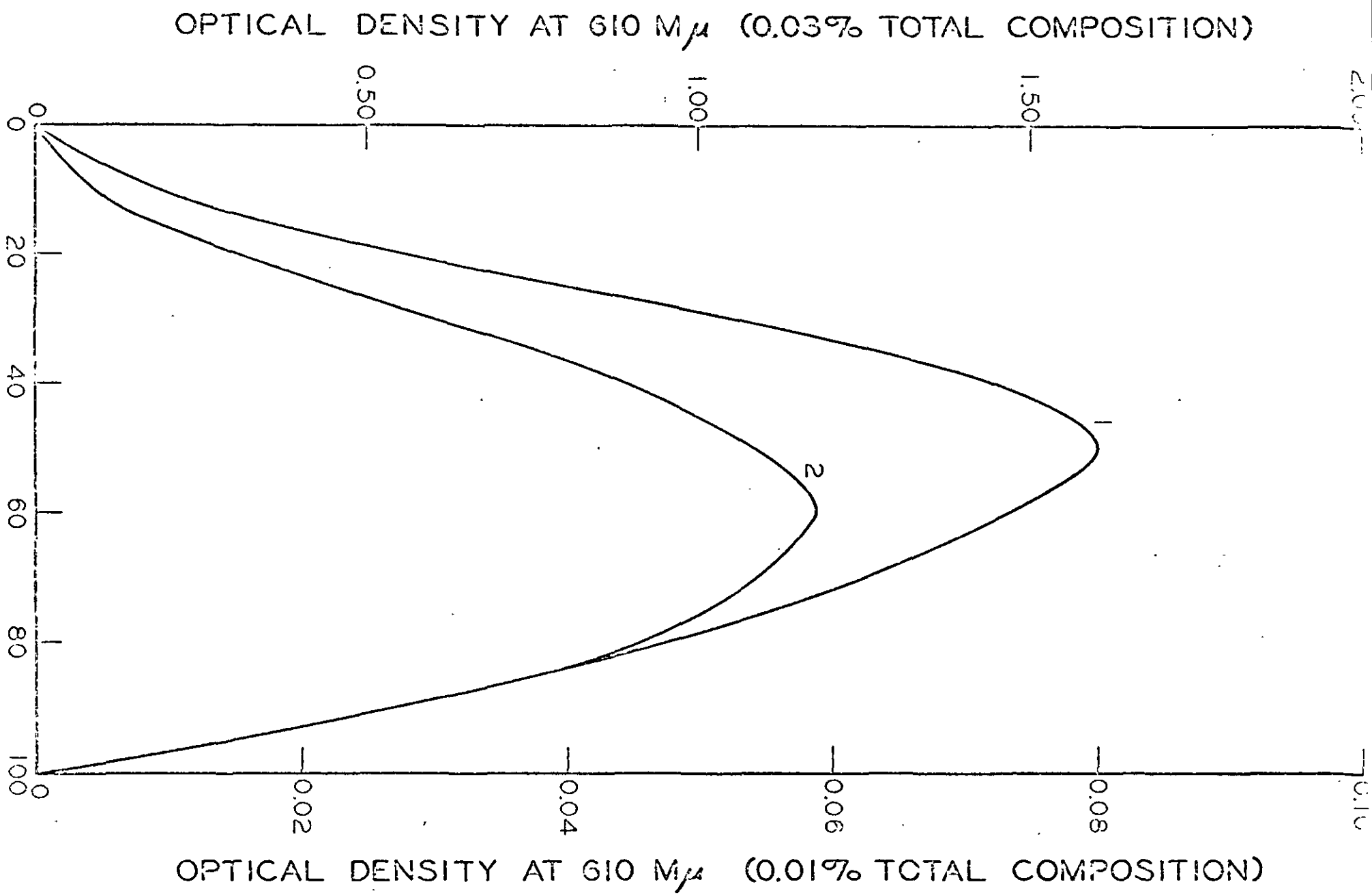


Figure 4. The Variation of Optical Density at 610 mμ of the Colored Complex of Commercial Xylan with Relative Percent I_2 at Constant Total (Xylan + I_2) Composition. (1) Concentration Xylan + I_2, 0.03%; (2) Concentration Xylan + I_2, 0.01%.

of Superlose by Stein Hall Inc. A commercially available xylan sold by Pfanstiehl Chemical Company was also investigated. The source of this xylan was not known and quantitative analysis showed it to contain 73.6% xylose, 12.2% arabinose, 3.9% uronic acid, 0.2% rhamnose, 0.3% mannose, 2.9% galactose, 2.4% glucose, and 4.5% of an acid insoluble lignin like material. Since this polymer had solubility and complexing characteristics similar to those of xylans from vegetable sources of known compositions and structures to be reported in another publication, it was assumed that this xylan was composed of a linear chain of β(1-4) linked anhydroxylopyranosyl residues to which were attached at infrequent intervals single terminal branches of arabinose and uronic acid residues.

The amyloid employed in this investigation was extracted from Tamarind seed with hot water by the technique described by Rao (7). Quantitative analysis indicated 16.9% galactose, 47.6% glucose, 1.3% arabinose, and 34.1% xylose to be present in the hydrolyzates of this polymer. The galactose-deficient galactoglucomannan was isolated from Engelmann spruce holocellulose for use in another study (9) and quantitative analysis showed it to be composed of 7.4% galactose, 21.1% glucose, 64.0% mannose 3.5% xylose, and 4.0% arabinose. The galactose-rich galactoglucomannans were isolated from black spruce and Parana pine holocelluloses by conventional techniques (10) and had compositions approximated by 20% galactose, 20% glucose, and 60% mannose. The hydrocellulose employed in this investigation was prepared by dissolving a suitable cellulose preparation such as Whatman filter paper or Solka Floc (a purified commercial wood cellulose) in 85% phosphoric acid and recovering the hydrolyzed cellulose according to the procedure outlined by Smith and co-workers (16). That fraction of the hydro-

cellulose which dissolved in stock calcium chloride solution was used for this research.

Reagents and Analytical Procedures

The calcium chloride solutions used in this investigation were prepared dissolving 412.1 gm. of analytical grade reagent in 1 l. water to give a specific gravity of 1.3. The stock iodine solutions were prepared by dissolving 2.000 gm. iodine and 8.3 gm. potassium iodide in 100 ml. of water and dilutions were made to required concentrations with stock calcium chloride solution. The stock solutions of polysaccharide were prepared by dissolving 100 to 500 mg. of polymer in 5 ml. of N NaOH, neutralizing with HCl and adding stock calcium chloride solution to 100 ml. The solutions were slightly viscous but remained clear for months without evidence of precipitation or of bacterial decomposition.

The hydrolysis and the quantitative analysis of the polysaccharide was accomplished by the chromatographic method of Saeman et al. (17) while the uronic acid contents of polymers were estimated by the technique of Whistler, Martin, and Harris (18).

The presence or absence of crystalline material in the polysaccharide-iodine complexes was determined by conventional powder techniques using a Debije-Scherrer powder diffraction camera in conjunction with a conventional Norelco X-ray unit.

Samples for calcium analysis were mixed with 50 mg. of $LiCO_3$ and with 180 mg. of graphite that contained tin as an internal standard. A portion of this mixture was arced using an A.C. power source and the spectrum was recorded using a Bausch and Lomb 1.5 meter grating spectrograph. The line

intensities of the elements were measured on a Jarrel Ash microphotometer and the concentrations of elements were derived from the line intensities.

Potentiometric Titration of Bound Iodine

A stock solution containing 40 to 50 mg. of polysaccharide was added to a solution composed of 4.15 gm. of potassium iodide in 5 ml. of water. The solution was made up to 50 ml. with stock calcium chloride solution. A blank solution composed of 4.15 gm. potassium iodide dissolved in water and made up to 50 ml. with stock calcium chloride and the two solutions were stored in the refrigerator until used. Titrations were conducted in an ice bath with undiluted stock iodine solution using a Leeds and Northrup Student type potentiometer in combination with a sensitive galvanometer and bright platinum and normal calomel electrodes.

Spectrophotometric Determinations

All extinctions were measured in a Beckman D U spectrophotometer at 610 m. using conventional 1 cm. cells. The increase in extinction with time was followed using polysaccharide solutions containing 5 mg. of polymer in 10 ml. of standard calcium chloride solution at 4° and at 30° C. After storage overnight, 2.5 ml. of a tenfold dilution of stock iodine with stock calcium chloride solution at the required temperature was added to the polymer solution and the extinctions were measured in the spectrophotometer at 15, 30, 60 minute intervals.

The effect of sodium sulfate on the increase in extinction coefficient of the solution of amyloid and iodine with time was followed by measuring the changes caused by adding 5 ml. of sodium sulfate solution (20 g./100 ml.) to a solution prepared by mixing 0.5 ml. iodine solution

(3 ml. stock iodine solution diluted with water to 10 ml.) with 1 ml. of solution containing 0.26% polymer.

The comparison of the intensities of colors developed at low iodine concentrations was accomplished by preparing a series of polymer solutions in 75 ml. stock calcium chloride solution whose compositions (after the addition of the final iodine solution) ranged from 0.001% to 0.010%. The solutions were cooled to 4° C. and 2.5 ml. of stock iodine solution diluted tenfold with stock calcium chloride solution (also at 4° C.) was added. The extinctions were measured at periodic intervals until they had reached their maximum values.

Stock solutions of polysaccharide and iodine were diluted with stock calcium chloride solution to give concentrations of polymer plus iodine ranging from 0.01% to 0.04% (depending upon the intensities of the colors produced by the polysaccharide). The relative percentage of iodine in each of these constant total composition levels ranged from 10% to 90% iodine. The development of color was measured in the Beckman D U at the point of maximum development with time.

Acknowledgments

The authors would like to acknowledge the assistance provided by Miss M. Hollenback for sugar analysis, A. G. Borchardt for calcium analysis, and Dr. G. Richards for X-ray powder diffraction patterns.

References

1. B. D. E. Gaillard, Nature, 191 (1961) 1295.

2. A. J. Morak, and N. S. Thompson, Nature, 205 (1965) 69.

B. D. E. Gaillard, and R. W. Bailey, Nature, 212 (1966) 202.

4. T. H. Vogel, and M. J. Schleiden, Pogg. Ann. Phys. Chem., 45 (1839) 327.

5. P. Kooiman, Nature, 179 (1957) 107.

6. F. L. Bates, D. French, and R. E. Rundle, J. A. Chem. Soc. 65 (1943) 142.

7. P. S. Rao, "Tamarind", Chapter 19, in "Industrial Gums," R. L. Whistler, Ed., New York, Academic Press, 1959.

8. L. G. Tebelev, G. F. Nikul'skii, E. P. Korchagina, and S. A. Glikman, Vysokomolekul. Soedin. 7 (1965) 123.

9. J. K. Rogers, and N. S. Thompson, Carb. Res. 7 (1968) 66; Svensk Papperstidn. in press (1969).

10. N. S. Thompson, and O. A. Kaustinen, Paperi ja Puu, 47 (1964) 637.

11. C. L. Wilson and D. W. Wilson, "Comprehensive Analytical Chemistry 1A" Elsevier Publishing Company, New York (1959).

12. B. D. E. Gaillard, N. S. Thompson, and A.J. Morak, Research to be Published (1969).

13. D. M. W. Anderson, and C. T. Greenwood, J. Chem. Soc. (1955) 3016.

14. C. R. Colburn, and T. J. Schoch, "Methods in Carbohydrate Chemistry IV" (1964) 161, Ed. R. L. Whistler.

15. G. K. Adkins, and C. T. Greenwood, Carb. Res. 3 (1966) 152.

16. J. D. Geerdes, B. A. Lewis, R. Montgomery, and F. Smith, Anal. Chem. 26 (1954) 264.

17. J. F. Saeman, W. E. Moore, R. L. Mitchell, and M. A. Millet, Tappi 37 (1954) 336.

18. R. L. Whistler, A. R. Martin, and M. Harris, J. Res. Nat'l. Bur. Std. 24 (1940) 13.

PROJECT REPORT FORM

PROJECT NO. 2768
COOPERATOR IPC
REPORT NO. 2
DATE April 16, 1970
NOTE BOOK 2665
PAGE 10
SIGNED N. S. Thompson
N. S. Thompson
B. D. E. Gaillard
B. D. E. Gaillard

Copies to: Central Records
N. S. Thompson (6)
B. D. E. Gaillard
R. H. Atalla
J. P. Brezinski
J. Weiner

THE INTERACTION OF POLYSACCHARIDES WITH IODINE
II. THE BEHAVIOR OF XYLANS IN DIFFERENT SALT SOLUTIONS

Blanche D. E. Gaillard and N. S. Thompson

ABSTRACT

A comparison of the interaction of solutions of 4-O-methylglucuronoxylan from elm, a commercially available xylan, and a highly branched xylan from sapote gum in a variety of Group 2a salt solutions with iodine-potassium iodide solution showed the less highly branched polymers gave a dark blue reaction product similar to that produced by the starch-iodine complex, whereas the highly branched sapote xylan did not react at all. Aqueous calcium chloride solutions were the most effective of the Group 2a salt solutions tested. Solutions of xylans in other uni- and multivalent salt solutions did not give this blue reaction product when iodine was added.

Spectrophotometric determination of the intensity of the color formation indicated that the reaction depended upon the concentration of the salt solution as well as on the nature of the salt and the concentration and type of xylan in solution. Since the intensity of the color reaction was enhanced by increasing the potassium iodide concentration and since xylans dissolved in concentrated aqueous potassium iodide solution did not yield a blue coloration when iodine was added, it was concluded that potassium iodide enhanced the formation of a necessary iodine-iodide intermediate, but did not react with the xylan in such a manner as to permit it to react with iodine.

the methods used to isolate them. These polysaccharides offer the opportunity to study the effect of chemical and structural parameters on the ability of the polymer to react with iodine in the presence of various salt solutions. The present investigation was initiated to take advantage of this opportunity.

The polysaccharides chosen for this comparative study of xylan behavior are described in Table I and include an acid-hydrolyzed commercial xylan (5), bamboo (6), elm (7), and sapote gum (8), as well as a commercial xylan of unstated origin (but thought to be oat hulls) provided by Pfanstiehl which was not the source of the acid-hydrolyzed xylan mentioned earlier. Of these polymers, only the xylan from elm has been extensively characterized by conventional chemical and physical techniques. Studies on this polymer by Timell and coworkers (9, 10) show it to consist of a chain of approximately 200 anhydro-D-xylopyranosyl residues linked to one another by β(1-4) glycosidic bonds. Apparently randomly attached to this filiform structure by α(1-2) glycosidic bonds are anhydro-4-O-methyl-D-glucopyranosyl uronic acid residues, and it is likely this polymer is very similar to other hardwood xylans (11). It is likely that the acetyl groups associated with xylan are missing in the polymer employed here since it was extracted from elmwood with 10% potassium hydroxide.

Two new polysaccharides not differing appreciably in chain length from the original were prepared from the elm xylan by altering the nature and the number of the branches attached to the anhydro-D-xylopyranosyl residues of the main chain. In one case, the anhydro-4-O-methyl-D-glucopyranosyl uronic acid residues were reduced by a modification (7) of a diborane reduction procedure (12) to anhydro-4-O-methyl-D-glucopyranosyl residues in over 90% conversion. A comparison of the behavior of this polymer with that of the unreduced control demonstrates the effect of the functional group of a branch on complex formation. In the other preparation,

TABLE I

THE COMPOSITION OF VARIOUS XYLOSE-RICH POLYSACCHARIDES

	Sapote Gum Xylan	Commercial Xylan	Acid-Hydrolyzed Xylan	Bamboo Xylan	Reduced Elm Xylan	Elm Xylan	Degraded Elm Xylan
Anhydro-D-xylose, %	45.3	73.6	99	78.8	88.7	86.7	98.1
Anhydro-L-arabinose, %	22.9	12.2	trace	6.9	0	0	0
Anhydro-4-O-methyl-D-glucuronic acid, %	16.1	3.9	0	13.4	1.1	13.3	1.8
Anhydro-4-O-methyl-D-glucose, %	0	0	0	0	10.1	0	0
Others, %	11.3[a]	5.8[b]	0	0	0	0	0
Viscosity, dl./g.	--	1.13	--	0.69	1.20	0.96	0.85

[a] Anhydro-D-glucuronic acid.

[b] A mixture of anhydro-D-glucose, anhydro-D-galactose, and anhydro-D-mannose.

advantage was taken (13, 14) of the known alkali lability of the glycosidic bond linking uronic acid residues to aglucones at temperatures greater than 100°C. to convert the 4-O-methylglucuronoxylan of elm into a linear xylan substantially devoid of uronic acid branches (7). A slight decrease in the viscosity of the polysaccharide occurred as a result of this treatment, but the loss would not invalidate the use of the polymer for determining the effect of the absence of branches on complex formation.

A highly branched xylan was isolated from sapote gum according to procedures described in the literature (8, 15). This polymer is thought to be composed of a linear sequence of anhydro-D-xylopyranosyl residues linked by β(1-4) glycosidic bonds to which are attached anhydro-L-arabinopyranosyl residues by (1-2) linkages and anhydro-D-glucopyranosyl uronic acid residues (and its 4-O-methyl ether) by α(1-2) glycosidic bonds. This polysaccharide is a xylan in which there are no nonterminal D-xylopyranosyl residues that are not branch points; all the components have a pyranose ring and it is therefore analogous in many respects to the hexosan-guaran.

The structure of the bamboo xylan has been investigated by Matsuzaki and coworkers (16) who showed the polysaccharide to be composed of a linear sequence of β(1-4)-linked anhydro-D-xylopyranosyl residues to which are attached branches of L-arabinofuranosyl residues and uronic acid residues. As can be seen in Table I, the bamboo xylan employed in this investigation has the same quantity of uronic acid as elm xylan, has a lower viscosity, but has appreciable L-arabinofuranosyl residues, making it more branched than the elm xylan. The amount of information available about the bamboo xylan is much less than that available for the elm xylan. Very little is known of the origin and structure of the commercial xylan obtained from Pfanstiehl except that it may have been isolated from oat hulls. If it is

assumed the L-arabinose and uronic acid are terminal branches attached to a chain of D-xylopyranosyl residues, the commercial xylan should be equivalent to the bamboo xylan with the difference in viscosity being the major variable.

Other polysaccharides were employed as controls in this investigation and include a xylan prepared from commercial xylan by acid hydrolysis (5), a commercial amylose preparation (Stractan), and a commercial amylopectin (Ramalin) obtained from Stein, Hall & Co., Inc.

RESULTS AND DISCUSSION

INTRODUCTORY EXPERIMENTS

Previous experiments indicate that solution of polysaccharides in many salt solutions besides aqueous calcium chloride will react with iodine to yield a blue-colored product (1-4). The initial experiments therefore involved a qualitative survey of solutions of commercial xylan in various salts to find those where the most intense reactions with iodine might occur. The results showed that xylan dissolved in solutions of calcium chloride, calcium nitrate, calcium iodide, magnesium chloride, barium chloride, strontium chloride, aluminum nitrate, and zinc chloride would react with iodine to form blue complexes. Xylan did not react with iodine in solutions of aluminum chloride, mercuric chloride, lead chloride, cupric chloride, cadmium chloride, cobalt chloride, ferric chloride, potassium iodide, potassium chloride, or sodium iodide. These results indicate the nature of the cation is a predominating (but not the only) factor in complex formation.

A quantitative measurement of the intensity of the color produced by the interaction of commercial xylan with iodine in various salt solutions such as calcium chloride, calcium nitrate, and magnesium chloride is given in Fig. 1. The results demonstrate that at a given molar concentration the extent of complex

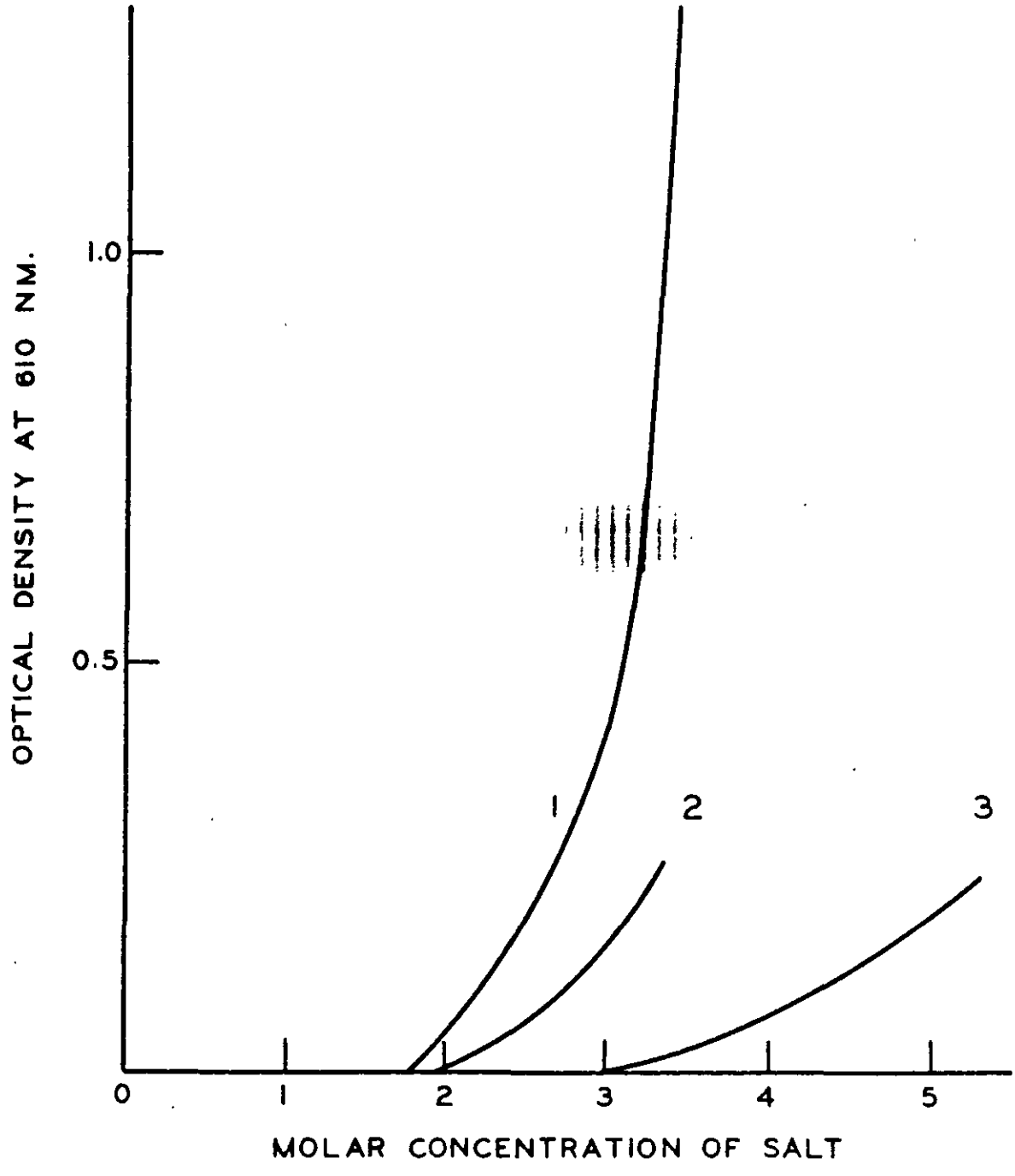


Figure 1. Variation of Optical Density at 610 nm. of Colored Iodine Complexes of Commercial Xylan (0.004%) in Different Concentrations of Different Aqueous Divalent Salt Solutions with 0.05% I_2 and 0.21 KI. (1) Calcium Chloride, (2) Calcium Nitrate, (3) Magnesium Chloride

formation will depend upon both anion and cation components of the salt, and help rationalize the qualitative behavior of the aluminum sulfate and aluminum chloride solutions of xylan reported above.

Since additional tests have shown that elm and other hardwood xylans yield a different order of solvent effectiveness in promoting complex formation, the possibility exists that even greater significant differences of salt effectiveness may exist between structurally different polymers such as hexosans and pentosans: These possibilities are being investigated in the case of glucomannans and softwood xylans. The maximum intensity of the reaction of xylan with iodine in Fig. 1 is limited by the solubility of the solvating salt in water, and it is not known if a constant intensity would ever be achieved at very high salt concentrations.

The data in Fig. 2 demonstrate that a linear relationship exists between the intensity of the color produced and the concentration of polysaccharide in solution in either calcium chloride or strontium chloride solutions. In agreement with the previous data, the intensity of the reaction at a given polymer concentration varies with the salt involved. In the case of the commercial xylan, bamboo, and elm xylans, the intensity of the color reaction was always greater in calcium chloride than in other salt solutions, and this system was used in all further studies of xylan behavior. Differences of behavior in other salt solutions were observed, with the extreme being the slight reaction of elm xylan in aqueous magnesium chloride solution with iodine in contrast to the more significant behavior of commercial xylan.

Since the intensity of color formation is dependent upon the nature and the concentration of the salt as well as on the nature and the concentration of the polysaccharide, it is likely that the complex equilibria existing between the

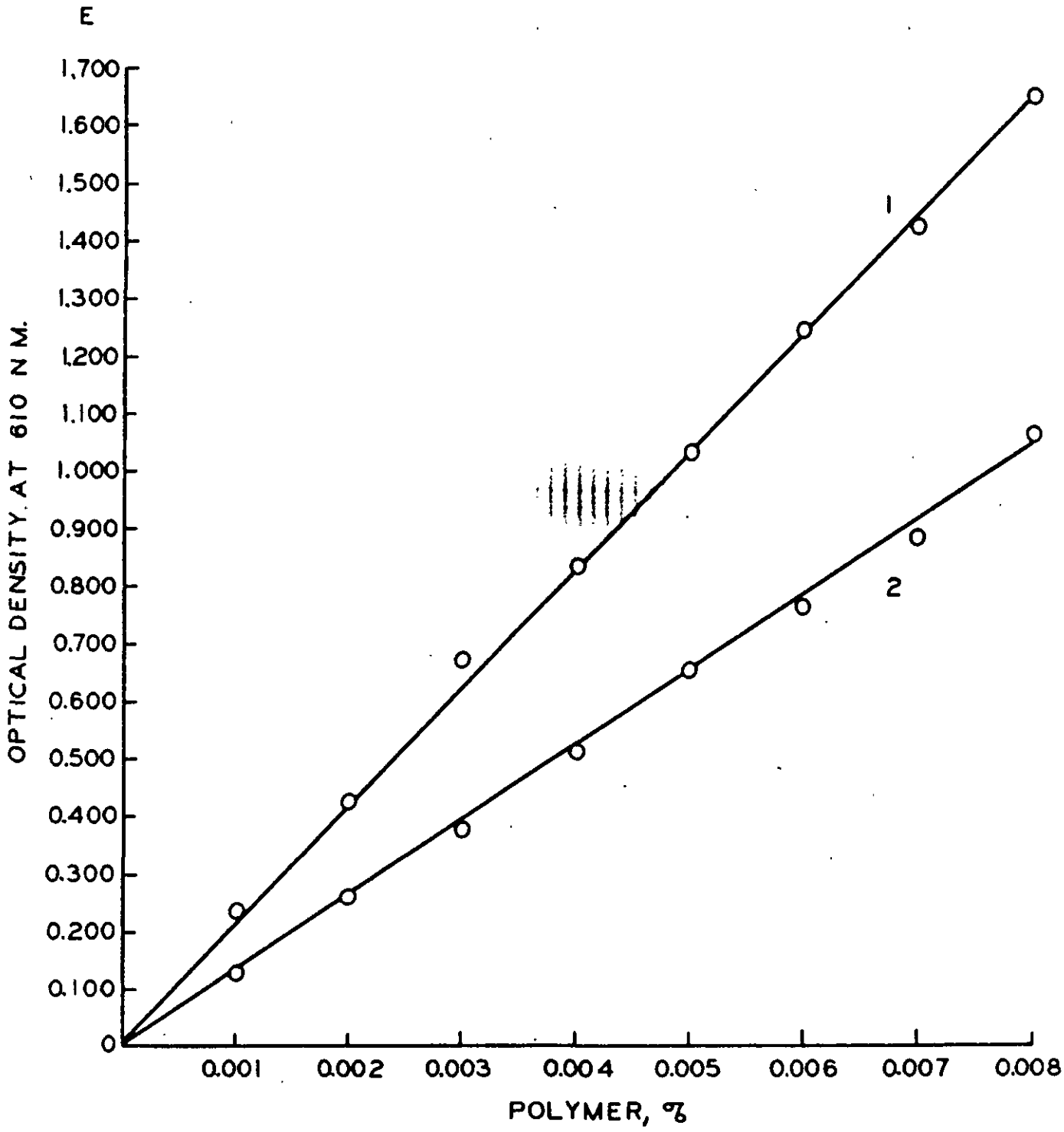


Figure 2. Variation of Optical Density at 610 nm. of Colored Iodine Complexes of Elm Xylan with Increasing Xylan Concentration with 0.05% I_2 and 0.21% KI. (1) Aqueous Calcium Chloride Solution (3.7M), (2) Aqueous Strontium Chloride Solution (3.7M)

complex and the various entities in solution are affected by the physical parameters of these entities. In the previous communication, the suggestion was made on the basis of qualitative solubility observations for the existence of an intermediate complex formed by the interaction of calcium ion and polysaccharide. The observations obtained so far in this investigation can be rationalized by assuming the ionized salts facilitate the formation of a polysaccharide conformation which permits the attachment of a linear array of iodine atoms in a manner analogous to that of starch. The extent of formation of this intermediate complex may depend upon the nature and concentration of the salt involved and thus may control the extent of formation of the iodine complex.

The intensity of the color produced by a given polysaccharide-salt system under controlled conditions is also dependent upon the concentration of the potassium iodide in solution. This dependence is similar to the dependence of starch-iodine complex formation and is illustrated in Fig. 3 in the case of the reaction with commercial xylan.

The role of the iodide ion in the amylose-iodine reaction has been the subject of much debate (17) and theories have been postulated showing that it may enhance the formation of a necessary iodide-iodine species (18, 19) or that it may be necessary for the formation of a starch helix (20). Since xylans in potassium iodide solution do not react with iodine to form a blue color, it is obvious that potassium iodide does not influence xylan conformation, but may enhance the formation of iodide-potassium iodide species necessary for sorption or reaction with the polysaccharide as has been suggested by others (17-19).

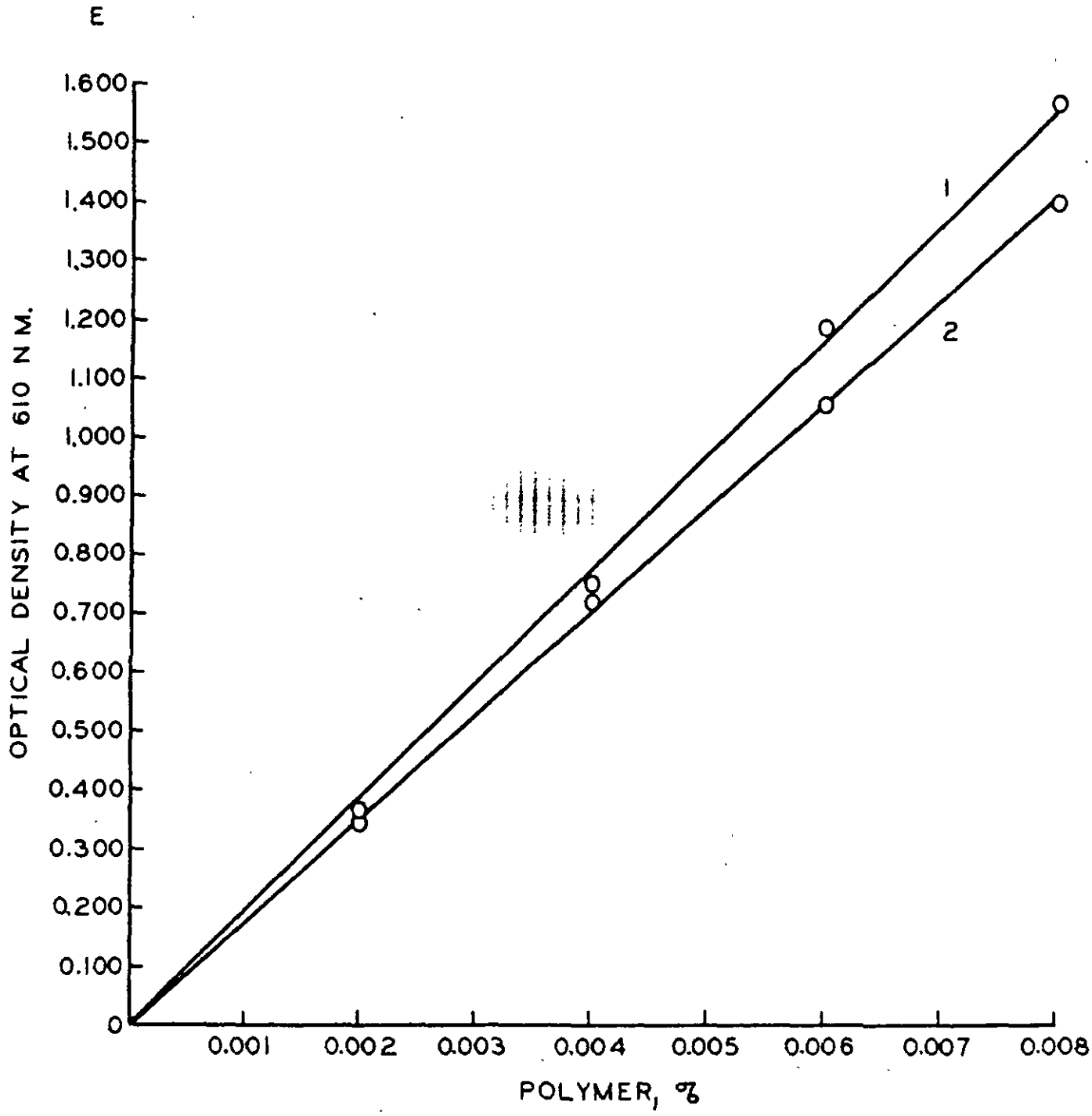


Figure 3. Variation of Optical Density at 610 mm. of Colored Iodine Complexes of Commercial Xylan with Increasing Xylan Concentration in 3.7M Calcium Chloride. (1) 0.05% I_2 and 0.21% KI, (2) 0.05% I_2 and 0.07% KI

A COMPARISON OF THE BEHAVIOR OF DIFFERENT XYLANS

Xylans which differed in the nature and extent of the branches associated with them were dissolved in concentrated aqueous calcium chloride solutions and reacted with iodine-potassium iodide under similar controlled conditions. The change in absorption at 610 nm. with changing polymer concentration for each of these different xylans is plotted in Fig. 4. The highly branched xylan from sapote gum did not react at all with iodine, and this behavior was similar to that of most other highly branched hexosans such as guaran under these circumstances. The other less highly branched xylans reacted with iodine to give the expected blue coloration. The elm xylan and the alkali-degraded elm xylan reacted in an almost identical manner to give the most intense absorptions at 610 nm. Since it had been expected the unbranched degraded xylan would have given the more intense reaction than observed in Fig. 4, it must be assumed that the uronic acid groups of the undegraded elm xylan do not hinder complex formation. On the other hand, reduction of the uronic acid groups of this xylan to primary alcohol groups (producing a 4-O-methyl gluco-xylan) reduced the extent to which the polysaccharide would react with iodine. The nature of the branch in this polysaccharide system has an important influence on the extent of reaction, and it can only be assumed at this point that the formation of the calcium salt of the uronic acid in some manner counteracts the inhibitory action of the branch. Although these speculations can be successfully applied to rationalize the behavior of the commercial xylan in Fig. 4, it cannot be used to explain the behavior of the bamboo xylan if it indeed has the simple structure assigned to it by Matasuzaki and coworkers (16).

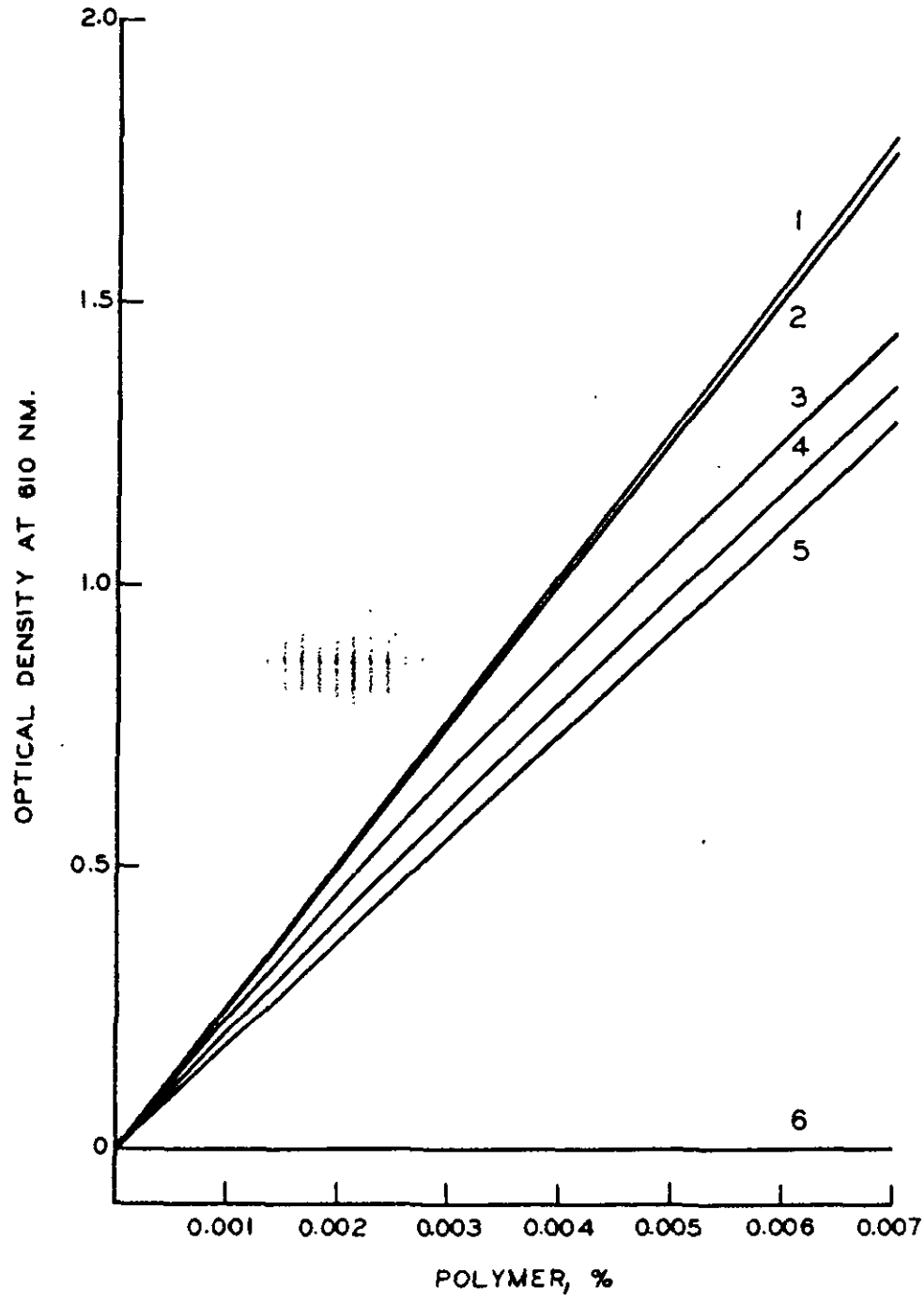


Figure 4. Variation of Optical Density at 610 nm. of Colored Iodine Complexes of Different Xylans with Increasing Concentration of Xylan in 3.7M Aqueous Calcium Chloride Solution and 0.05% I_2 and 0.21% KI. (1) Elm Xylan, (2) Alkali-Degraded Elm Xylan, (3) Reduced Elm Xylan, (4) Commercial Xylan, (5) Bamboo Xylan, and (6) Xylan Isolated from Sapcte Gum

Potentiometric titration has been used as a sensitive tool for the determination of the degree of iodine reaction in the case of amylose-iodine and amylopectin-iodine interactions (21). The difference in oxidation potential of the sample and the control can be used to calculate an "iodine number" which is characteristic of a given sample. Potentiometric titration of the interaction between other non-starch polysaccharides and iodine in calcium chloride solution has also been carried out, and it was observed that only commercial xylan reacted in a manner similar to starch and that the more highly branched amyloid and galactoglucomannan polymers did not cause any change in iodine potential (4). The supposition arose that this behavior of the hexosans was due to the bulky nature of their branches preventing the formation of extended sequences of iodine atoms in association with the polymer. The results of the potentiometric titration of the various xylans employed in this investigation are recorded in Table II, and they show that the reduced elm xylan, even though it reacts with iodine under these circumstances to form a blue complex, does not react in such a manner as to change the oxidation potential of the system from that of the control. Thus, the behavior of this polymer is similar to the behavior of glucomannan, amyloid, and poly(vinyl alcohol) and it does not yield an "iodine number" as a result of the titration. The alkali-degraded xylan, which is composed primarily of a linear, unbranched sequence of anhydro-D-xylopyranosyl residues, does react with iodine, however, to produce a greater change in oxidation potential (and hence in "iodine number) than the unmodified elm xylan control. The comparison of the behavior of these three closely related xylans demonstrates that the "iodine numbers" are dependent upon the number and type of short terminal branches which are associated with the polymer.

TABLE II

A COMPARISON OF THE IODINE CONTENT OF VARIOUS "XYLAN"-IODINE COMPLEXES

	Iodine, %, Found by:		Calcium Content of Precipitated Complex
	Potentiometric Titration[a]	Analysis of Precipitated Complex	
Acid hydrolyzed xylan	6	21.9	--
Commercial xylan	10	16.6	6.8
Bamboo xylan	4	14.5	--
Elm xylan	7	19.3	--
B_2H_6-reduced elm xylan	0	17.9	--
Alkali-degraded elm xylan	12	18.5	18.2
Amylose	15.5[b]	23.5	11.5
Amylopectin	7.6[c]	--	--

[a] These values were determined in aqueous calcium chloride solution.

[b] 17.8% in water.

[c] 9.6% in water.

It is impossible to support this hypothesis of elm xylan behavior by the behavior of the other xylans studied in this investigation since the "iodine numbers" obtained by potentiometric titration cannot be related to their conjectured degrees and types of branching. Until more is known of these parameters of these xylans and the effect of the parameters upon the reaction of a polysaccharide with iodine, little can be done with the data.

Amylose and amylopectin were both employed as controls in these experiments. The "iodine numbers" of the two starch polymers were determined in aqueous

solution, and the values obtained here differ little from those in the literature. The "iodine numbers" determined in aqueous calcium chloride solution were somewhat less than those determined in water, indicating no enhancement of the complexing of starch and iodine was produced by the salt.

When the concentration of iodine in the solutions from the potentiometric determinations is increased, the iodine complex will precipitate and it can be isolated by normal carbohydrate techniques. After storage in vacuum in a desiccator on a porous plate until no water or free iodine is detected, the complexes from the xylans become dark-blue films which can be stored in the open in the laboratory for prolonged periods of time without obvious signs of decomposition. The analysis of these complexes shows them to contain more iodine than can be accounted for by potentiometric titration. Some of the difference might be due to the greater amount of iodine present in solution at the time of precipitation (increasing the iodine content of the complex), and some is probably due to the presence of iodine in the complexes able to cause coloration but not able to affect the potentiometric titration. Although appreciable calcium ion was present in the isolated iodine complexes as determined by flame spectrometry, x-ray diffraction techniques showed no crystalline component to be present. It is possible that the calcium ion is a component of the complex, but it also might be present as uncrystallized calcium chloride. The lack of suitable manipulating and purifying techniques at this time makes it impossible to examine these speculations in any detail.

An attempt was made to determine whether the iodine in the complexes was firmly bound to the polymer in a definable ratio as is the case of starch, or if no definite chemical compound was formed. This determination may be obtained by plotting the variation of optical density of the colored complex of the polymer at 610 nm. against the relative percentage of iodine at different total (xylan + iodine)

compositions. In the case of starch, maximum optical density always occurs at a constant iodine composition, indicating a preferred stoichiometry. Although the alkali-degraded elm xylan is a linear polymer like starch, the data in Table III show that these polymers do not have a preferred stoichiometry but behave instead in a manner typical of the branched xylans.

TABLE III

THE COMPOSITION OF "XYLAN" AND IODINE AT WHICH MAXIMUM EXTINCTION COEFFICIENT OCCURS AT DIFFERENT TOTAL COMPOSITIONS OF "XYLAN" AND IODINE

Percentage (Polymer and Iodine) of Solution	Relative Percentage of Iodine at Which Max. Extinction Occurs				
	Commercial Xylan	Bamboo Xylan	Acid-Hydrolyzed Commercial Xylan	Elm Xylan	Reduced Xylan
0.01	60	--	--	70	--
0.02	--	70	60	60	65
0.03	50	60	50	--	55
0.04	--	50	--	--	--

On the basis of the results obtained here, it may be postulated that the stability of the iodine-xylan complexes is not due to the bonding of the iodine to the polymer since the existence of the color does not coincide with changes in oxidation potential ("iodine number") of the polysaccharide-salt-iodine system. The important influence of the structural configuration of the components attached to the chain of anhydro-D-xylopyranosyl residues suggests the stability of the complex which is able to react with iodine is a controlling factor in complex formation. The absence of a preferred stoichiometry of these complexes in contrast to those of starch is therefore due to the less stable nature of the configuration of the xylan forced on it by the solvating salt solution compared to the corresponding helical starch configuration.

EXPERIMENTAL

PREPARATION OF SAMPLES

The amylose and amylopectin used in this investigation were commercially available materials sold by Stein, Hall & Co., Inc. under the trade names of Superlose and Ramalin, respectively. A commercially available xylan sold by Pfanstiehl Chemical Company was employed as a control, but as discussed in the previous investigation, nothing is known of the structure of this material.

The highly branched sapote xylan used here was isolated by extracting sapote gum (150 g.) with 10 liters of water. After filtering through glass wool to remove particulate matter, the viscous solution was bleached for two days with 10 g. of sodium chlorite at pH 4. After bleaching, 100 ml. of 37% hydrochloric acid was added; the clear solution was stirred for 30 min., poured into 30 liters of 95% ethanol, and the precipitated gum was freed of supernatant liquor by decantation, washed with 95% ethanol, and redissolved in 3 liters of distilled water. To this solution was added 30 ml. of hydrochloric acid, and after standing for 45 min., it was poured with mixing into 9 liters of 95% ethanol. The resulting precipitate was thoroughly washed with anhydrous ethanol, was redissolved in water, freed of ethanol by evaporation, and isolated by freeze drying.

The elmwood xylan used in this study was isolated by Ross and Thompson (7) for a study of the effect of alkali at elevated temperatures on polysaccharides. After extraction from the wood with 10% potassium hydroxide and suitable purification, the polysaccharide was isolated by freeze drying. A portion of the polymer was acetylated and then reduced to a 4-O-methylglucoxylan by a modification of the diborane technique described by Smith and Stephen (12). The modification of the earlier Smith reduction process was necessary in order to prevent the reduction of

a few of the C-acetyl groups of the polymer to O-ethyl groups. A quantity of this polysaccharide remaining from the earlier studies was used in this investigation to study the effect of functional groups on complex formation. A xylan devoid of most of the uronic acid branches was also prepared from the unmodified elm xylan by heating it in 1.55N sodium hydroxide at 140°C. for two hours. The analyses of these polysaccharides is given in Table I.

REAGENTS AND ANALYTICAL SOLUTIONS

The various salt solutions employed in this investigation were prepared from analytical-grade reagents using distilled water. The stock calcium chloride solutions used for spectrophotometric and potentiometric experiments were prepared by making up 112.1 g. of analytical-grade reagent in 1 liter of water to give a specific gravity of 1.3. Strontium chloride solutions of similar molality were also prepared. The stock iodine solutions were prepared by dissolving 2.000 g. of iodine and 8.3 g. of potassium iodide in 100 ml. of water, and dilutions were made to required concentrations with stock calcium chloride or other salt solutions. The stock solutions of polysaccharides were prepared by dissolving 100 to 300 mg. of polymer in 5 ml. of N sodium hydroxide, neutralizing with hydrochloric acid and adding stock salt solution to 100 ml. These solutions, like those previously described (4), were slightly viscous and did not precipitate or decompose on standing.

The hydrolysis and the quantitative analysis of the polysaccharides were accomplished by the chromatographic method of Saeman et al. (22), while the uronic acid contents of the polymers were estimated by the technique of Whistler, Martin, and Harris (23). Methoxyl analyses were accomplished by conventional procedures (24).

The presence or absence of crystalline material in the polysaccharide-iodine complexes were determined by conventional powder techniques using a Debije-Scherrer powder diffraction camera in conjunction with a conventional Norelco x-ray unit, as described in the previous communication (4).

Samples for calcium analysis were mixed with 50 mg. of lithium carbonate and with 180 mg. of graphite that contained tin as an internal standard. A portion of this mixture was arced using an a.c. power source, and the spectrum was recorded using a Bausch and Lomb 1.5-meter grating spectrograph. The line intensities of the elements were measured on a Jarrel Ash microphotometer, and the concentrations of elements were derived from the line intensities.

POTENTIOMETRIC AND SPECTROPHOTOMETRIC DETERMINATIONS

The procedures used for the potentiometric and spectrophotometric examination of the iodine complexes for the various complexes were identical to those used in the previous report (4). The iodine complexes from the potentiometric titrations of xylans could be isolated by adding 1 ml. of stock iodine solution to the titration mixture, and the precipitate was isolated by centrifugation. Excess salt, water, and iodine were removed by placing the sample on a porous plate over a desiccant under vacuum for several weeks until the odor of iodine had disappeared.

The iodine content of the blue precipitates was accomplished by collecting by centrifugation, washing twice with standard $CaCl_2$ solution. Thereafter it was dissolved in water and the iodine was titrated with 0.1N sodium thiosulfate.

ACKNOWLEDGMENTS

The authors acknowledge the assistance provided by Mrs. B. Turley, A. G. Borchardt for sugar and calcium analyses, and Dr. G. Richards for x-ray powder diffraction patterns.

LITERATURE CITED

1. Gaillard, B. D. E., Nature 191:1295 (1961).

2. Morak, A. J., and Thompson, N. S., Nature 205:69 (1965).

3. Gaillard, B. D. E., and Bailey, R. W., Nature 212:202 (1966).

4. Gaillard, B. D. E., Thompson, N. S., and Morak, A. J., Carb. Res. 11:509 (1969).

5. Gaillard, B. D. E., unpublished research. The Netherlands, Agricultural University, 1969.

6. Morak, A. J., and Ward, K., Tappi 43:413 (1960).

7. Ross, J. R., and Thompson, N. S., Tappi 48:376 (1965).

8. Lambert, R. D., Dickey, E. E., and Thompson, N. S., Carb. Res. 6:43 (1968).

9. Göring, D. A. I., and Timell, T. E., J. Phys. Chem. 64:1426 (1960).

10. Gillham, J. K., and Timell, T. E., Can. J. Chem. 36:410, 1467 (1958).

11. Timell, T. E., Adv. Carbohydrate Chem. 19:247 (1964).

12. Smith, F., and Stephen, A. M., Tetrahedron Letters 7:17 (1960).

13. Semke, L. E., Thompson, N. S., and Williams, D. G., J. Org. Chem. 29:1041 (1964).

14. Hamilton, J. K., Partlow, E. V., and Thompson, N. S., Tappi 41:805, 811 (1958).

15. Smith, F., and Montgomery, R. The chemistry of plant gums and mucillages. A. C. S. Monograph Series. p. 301. New York, Reinhold, 1959.

16. Matsuzaki, K., Moriya, M., Ishida, A., and Sobue, H., J. Chem. Soc. Japan, Ind. Chem. Sect. 63, no. 6:987 (1962).

17. Foster, J. F. Physical properties of amylose and amylopectin in solution. In Whistler and Pashall's Starch. Vol. 1, chap. 15. New York, Academic Press, 1965.

18. Gilbert, G. A., and Mariott, J. V. R., Trans. Faraday Soc. 44:84 (1948).

19. West, C. D., J. Chem. Phys. 15:689 (1947).

20. Kuge, T., and Ono, S., Bull. Chem. Soc. Japan 33:1269, 1273 (1960).

21. Bates, F. L., French, D., and Rundle, R., J. Am. Chem. Soc. 65:142 (1943).

22. Saeman, J. F., Moore, W. E., Mitchell, R. L., and Millett, M. A., Tappi 37:336 (1954).

23. Whistler, R. L., Martin, A. R., and Harris, M., J. Res. Nat. Bur. Std. 24:13 (1940).

24. Penniston, Q. P., and Hibbert, H., Paper Trade J. 109:46 (1939).

PROJECT REPORT FORM

PROJECT NO. 2768
COOPERATOR IPC
REPORT NO. 3
DATE June 16, 1971
NOTE BOOK 2170
PAGE 62 TO 114
SIGNED N. S. Thompson

N. S. Thompson for A. J. Morak

Copies to: Files
Green
Thompson
Weiner

LABELING OF TREMBLING ASPEN, RED PINE, AND BALSAM FIR TREES WITH CARBON-14

The main objective of this project is the continued enrichment of earlier already (1967 and/or 1966) labeled young red pine (Pinus resinosa) and aspen (Populus tremuloides) trees as also the labeling to a high specific activity of an unlabeled thus far red pine and of a balsam fir (Abies balsamea) tree with carbon-14 by employment of radioactive carbon dioxide with a view of making labeled wood components of coniferous and deciduous trees for eventual studies available.

A further objective is the comparison of the effect of one and/or of two season labeling of red pine and of balsam fir; similarly, a comparison of the effect of labeling of aspen labeled in two and three seasons respectively.

Another objective is to establish a material balance so to find a utilization factor for carbon-14 spent.

As a final objective shall an evaluation of experience made with this growth chamber be used for improvements for any possible later labeling studies.

Experimental

The same growth chamber, size 60" x 70" high with a 12" peek having 10 mil thick PVC-sheeting built and used in 1967 was employed after a thorough inspection for leaks and replacement of the tray lining forming

the support for the tree pots and after completion of the following additions. An eight-inch diameter fan was suspended from inside of the roof center and kept at medium speed throughout the growing season, this in order to continuously equalize temperature, moisture and $C^{14}O_2$-concentration, the latter especially with a view of proper sampling in the continuous analysis. A further addition was a (suspended beneath this fan) copper cooling coil of about 8 sq. ft. area provided with a solenoid feed valve controlled thermostatically allowing to maintain a temperature of below 95° F. in the chamber. A further provision was a small 3" fan located in a recirculating line that was mounted on the chamber side wall. In this line a Geiger counter was mounted. Other stoppered openings in the chamber housing served the addition of water, the withdrawal of water and gas-samples, the mounting of a Geiger counter at base level, the withdrawal of chamber air from the top and return to the base and for a thermometer.

Watering with city water was done in the same crude way as in 1967, that is, several times during the growth period the tray was charged to a level of about 3 cm. height. The 30 tree pots were thus almost constantly submerged in this approximately 3 cm. depth of water providing that way certainly a higher soil moisture than used in photosynthesis experiments (15 - 18%) otherwise.

Apart from spot checks directly on the chamber, C^{14} of the growth chamber air was almost continuously measured by means of an assembly consisting of a Masterflex tubing pump (Cole-Parmer Instrument Co.) which

charged, at a rate of 130 ml. per min., a home made flow assembly of 8 cm^2 area and 12.6 ml. capacity, made of Plexiglas, provided with an O-ring to form a snug fit for a Geiger thin-window counter Model 108, Serial 1466, Nuclear-Chicago, of 1.4-2 mg/cm^2 window wall. This assembly was housed in a RCL counting chamber, Mark 3; tubing from the chamber to the pump and back again was Tygon S 50 HL 1/16"-wall, for the pump itself were all the offered three types, that is Tygon, Silicone and Viton tubing used, but even the latter, most expensive one, required frequent replacement. The Geiger counter counts were registered on a Nuclear-Chicago Model 182 scaler and each 256 counts were recorded on an Esterline-Angus 9-1 ma. recorder. Several times air was withdrawn from the chamber by means of a gas pump with reservoir and analyzed either for total CO_2 by GLC or for C^{14} by trapping in a diluted NaOH-solution and determining same by liquid scintillation counting using a PPO-dioxane cocktail.

For the GLC determination of CO_2, 10 ml. of the air sample were injected in the Aerograph 1520B with thermal conductivity detector using a Poropak Q 80/100 mesh, 8' x 1/4" s.s. column and 50 ml./min. helium flow as carrier gas (22); injector, column and detector were kept at room temperature. The height of the CO_2-peak was measured and expressed as a ratio to the height of the CO_2-peak obtained by injecting 10 ml. of atmospheric air under the same conditions. The attained accuracy of the ratio was $\pm$ .1%. The same column and conditions with filament current of 275 ma., using hydrogen as carrier gas, was used for several helium gas determinations where again the concentration was determined by assigning to a peak produced in the growth chamber with a known helium concentration a value

of unity and subsequently drawn samples were expressed as a ratio. GLC was finally also used for O_2 determinations employing the Aerograph 1520-B with thermal conductivity detector, 220 ma. filament current, 55 ml./min. He, Molecular sieve 5A column, 2 meters x 1/4" s.s (23). Here, the O_2 peak height of atmospheric air served for establishing of O_2 concentration ratios with air samples drawn from the growth chamber. The attained ratio accuracy was ±.05%.

Arrangement of Trees in Growth Chamber,

N

P_1 , P_2 , P_3 , P_4 , P_5

P_6 , P_7 , P_8 , P_9 , P_{10}

P_{11}, P_{12}, P_{13}, P_{14}, P_{15}

W 1S , 2S , 3S , 4S , 5S E

6S , 7S , 8S , 9L , 17L,

18L, 19L, 20L, B_1 , B_2

S

Designation	Description
P_1 , P_2 , P_3 , P_4 , P_5, P_6 , P_7 , P_8 , P_9 , P_{10}, P_{11}, P_{12}, P_{13}, P_{14}	Potted 3 years old red pine seedlings, treated in 1967 (Thesis John Walkush, 115 mc C^{14} for 15 red pine and 10 aspen seedlings treated in 1966 but not harvested and 5 aspen seedlings also previously treated and harvested). Condition: poor, did not develop shoots from brownish colored buds; needles partly of brownish color. At that time (June, 1968) it was assumed that winter damage might be responsible for this unhealthy condition.
P_{15}	Potted 3 years old red pine seedling (1967 control) condition: had developed new shoots, showing few brownish needles only.

1S, 2S, 3S, 4S, 5S, 6S, 7S, 8S, 9L	Potted 4 years old triploid trembling aspen seedlings, harvested after 2 years (1966, 1967) $C^{14}O_2$ treatment. Stem stumps of about 7 inches above soil developed one, sometimes 2 shoots having fairly well developed leaves.
17L, 18L, 19L, 20L	Potted 4 years old triploid trembling aspen seedlings, harvested after 1 year treatment (1966) with $C^{14}O_2$ (8 mc C^{14} for 20 seedlings). The stem stumps about 7 inches long showed a single shoot developed to above 20 inch length having adequate yet somewhat smaller size leaves.
B_1, B_2	Potted 5 years old balsam fir seedlings, purchased via Genetics Group, healthy, stocky trees (no control was set aside).

A rough check of pre-treated trees for radioactivity was made as follows:

Placing the Geiger counter directly onto the top of the needles of the red pine trees gave counts from 6,400-14,000 cpm., while the base of the needle (near twig) showed counts from 1,200-2,900 cpm.

Placing the Geiger counter close to the top of the sawed-off stem (stump) of aspen trees 1S-8S and 9L, counts of 4,500 to 4,700 cpm. were obtained while trees 17L-20L showed from 1,270-1,440 cpm.

The growth chamber was then sealed on July 12, 1968, the trees watered with a layer of about 3 cm. city water in the PE-lined tray and the first $C^{14}O_2$ labeling was begun. In all eleven treatments with a total of 120 mc. C^{14} according to the schedule shown in Table I were made. [There had been 130 mc. $BaC^{14}O_3$ in 10 ml. vials of 10 mc. each ordered from Mallinckrodt Nuclear Corp. at a price of $5 per mc. The specific activity

was 30.6 mc./m M, each vial containing 65 mg. $BaC^{14}O_3$. Used were 120 mc. and the unused 10 mc. $BaC^{14}O_3$ are stored in the Radiochemical Laboratory.] For generating the $C^{14}O_2$ an assembly was prepared; decomposition was done with diluted sulfuric acid (once with glacial acetic acid), the developed $C^{14}O_2$ was pushed in the growth chamber by air pressure (twice done with unlabeled CO_2); by 30-min. heating of the assembly on a water bath the complete decomposition of the carbonate was checked.

TABLE I: Schedule of $C^{14}O_2$ - Application

Trt. no.	Date '68	Time	Quantity mc. C^{14}	Hours Between Application
1	7/12	2 pm	10	235
2	7/22	9 am	10	167
3	7/29	9 am	10	73
4	8/1	8 am	10	96
5	8/5	9 am	10	72
6	8/8	8 am	10	96
7	8/2	9 am	10	47
8	8/14	9 am	10	120
9	8/19	8 am	10	55
10	8/21	3 pm	10	116
11 & 12	8/26	11 am	20 until Oct. 3 = about 38 days.	
		Total	120 mc. C^{14}	

Note: The chamber was thus left sealed for a total of 83 days.

The recorded course of radioactivity in the eleven treatments and thus the $C^{14}O_2$ - assimilation is shown in the two attached graphs (Fig. 1 and 2). The recorded count found in the flow assembly is shown in cpm. units. Except for the first $C^{14}O_2$-treatment graph, there appears a fairly regular pattern of assimilation, showing the typical degressing though rise in the dark cycle and even after four days of treatment, still the presence of radioactivity in the growth chamber air.

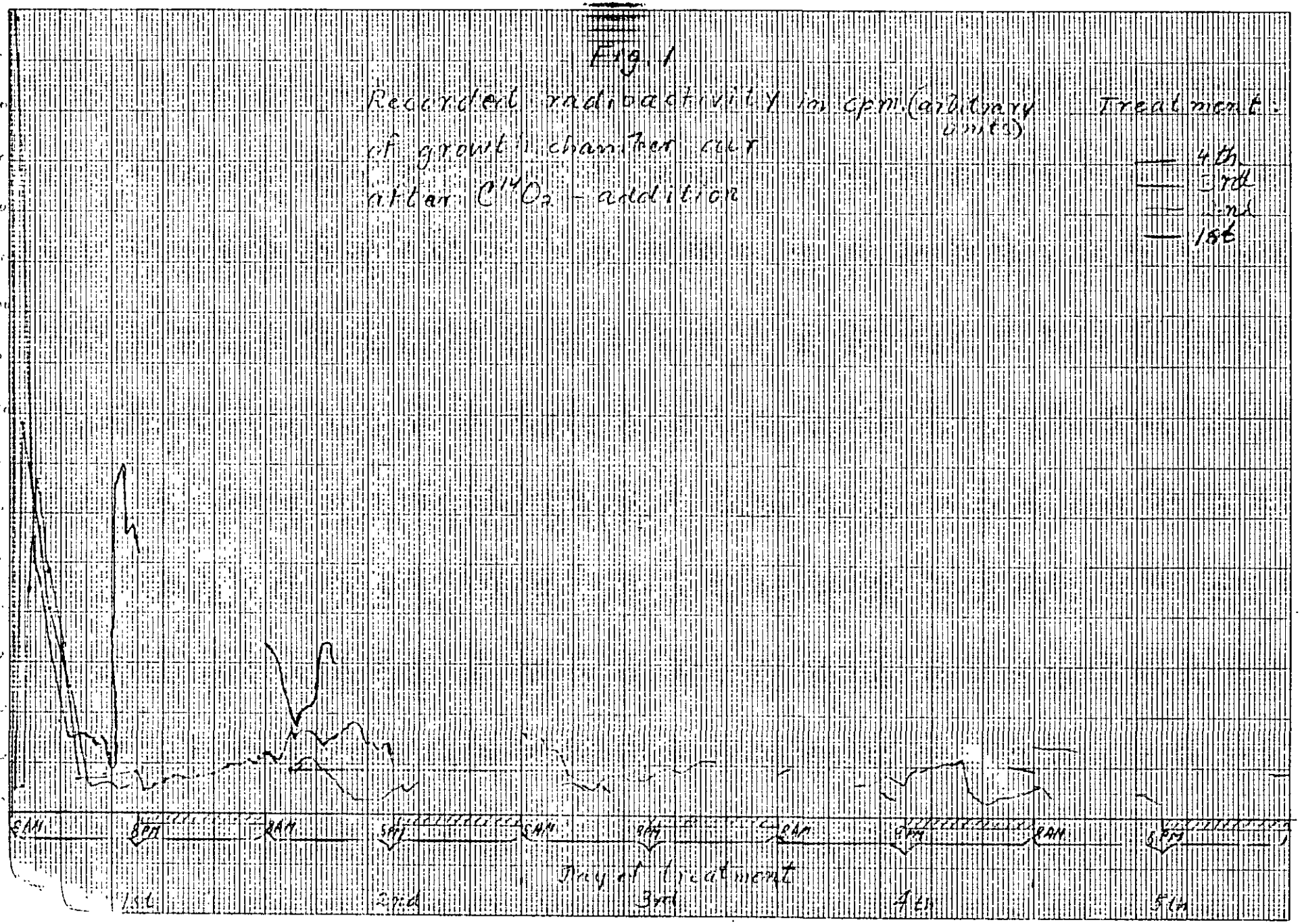
On August 27 a total of about nine liters water was withdrawn from the growth chamber tray and absorbed in a concentrated aqueous Ba(OH)2-solution. Also one month later a dish with a saturated Ba(OH)2-solution was placed inside the growth chamber. The precipitates from these two sources were filtered off and washed. A fairly white powder of 33.34g $BaC^{14}O_3$ resulted. By liquid scintillation counting of the air-dry, through a 100-mesh screen passing, powder a specific activity of 8.16 μc/g or 1.61 μc/m $\underline{M}$, $BaC^{14}O_3$ equal to a total of 272.05 μc C^{14} was found which is 0.23% of the totally applied activity. This material (together with earlier by Thesis student John Walkush recovered 291.11 μc C^{14} and the unused this year ordered 10mc. C^{14}, together thus 10.56316 mc. C^{14} are available for any further assimilation study, the two smaller quantities admittedly at low specific activity.)

Test results of the growth chamber air are shown in Table II.

TABLE II: Testing of Growth Chamber Air

Date '68	CO_2 ratio	A.M.	CO_2 ratio	P.M.	O_2 ratio	Helium ratio
7/18	0.8					
7/24	5.9					
7/24			1.2			
7/25	6.2		0.9			
7/30	3.8		1.2	(5:30)		
7/31	1.2	(8:30)				
7/29	1.8	(9:30)			1.0	
7/30	5.4	(10:30)	1.2		1.0	
8/1	5.3	(9:30)				
8/5	1.4	(8:00)			1.0	
8/15	1.4	(10:30)			1.0	
8/6			7.7	(1:00)		
8/7			1.6	(1:00)	1.0	
8/7			1.5	(8:00)	1.0	
8/8			2.4			
8/12	1.5	(8:00)				
8/12			3.4	(8:00)		
8/13	3.7	(9:00)				
8/15	2.1	(11:00)				
8/15			2.0	(1:00)		
8/15			2.4	(3:00)		
8/16			1.4	(3:00)		
8/18			3.2	(4:00)		0.3
8/19	3.6	(8:15)				0.3
8/19	2.9	(8:20)				0.4
8/19	4.6	(11:30)				0.4
8/20			1.4	(1:00)	1.0	0.1
8/20			1.3	(4:00)	1.0	0.1
8/21	1.0	(8:30)			1.0	0.1
8/21			2.1	(12:45)		(see
8/21			3.6	(3:45)		permeability
8/23	0.9	(9:30)			1.0	of PVC)
8/23			1.0	(1:45)	1.0	
8/26			4.2	(8:00)		
8/27	1.4	(9:30)				
8/28	3.7	(9:45)				
8/28			3.6	(1:00)	1.0	
9/24			1.5	(1:00)	1.0	
9/24			1.0	(4:00)	1.0	

The CO_2-ratios found differ in several cases from the expected values with samples drawn early morning and late evening showing due to excessively high temperatures high dark cycle respiration ratios;



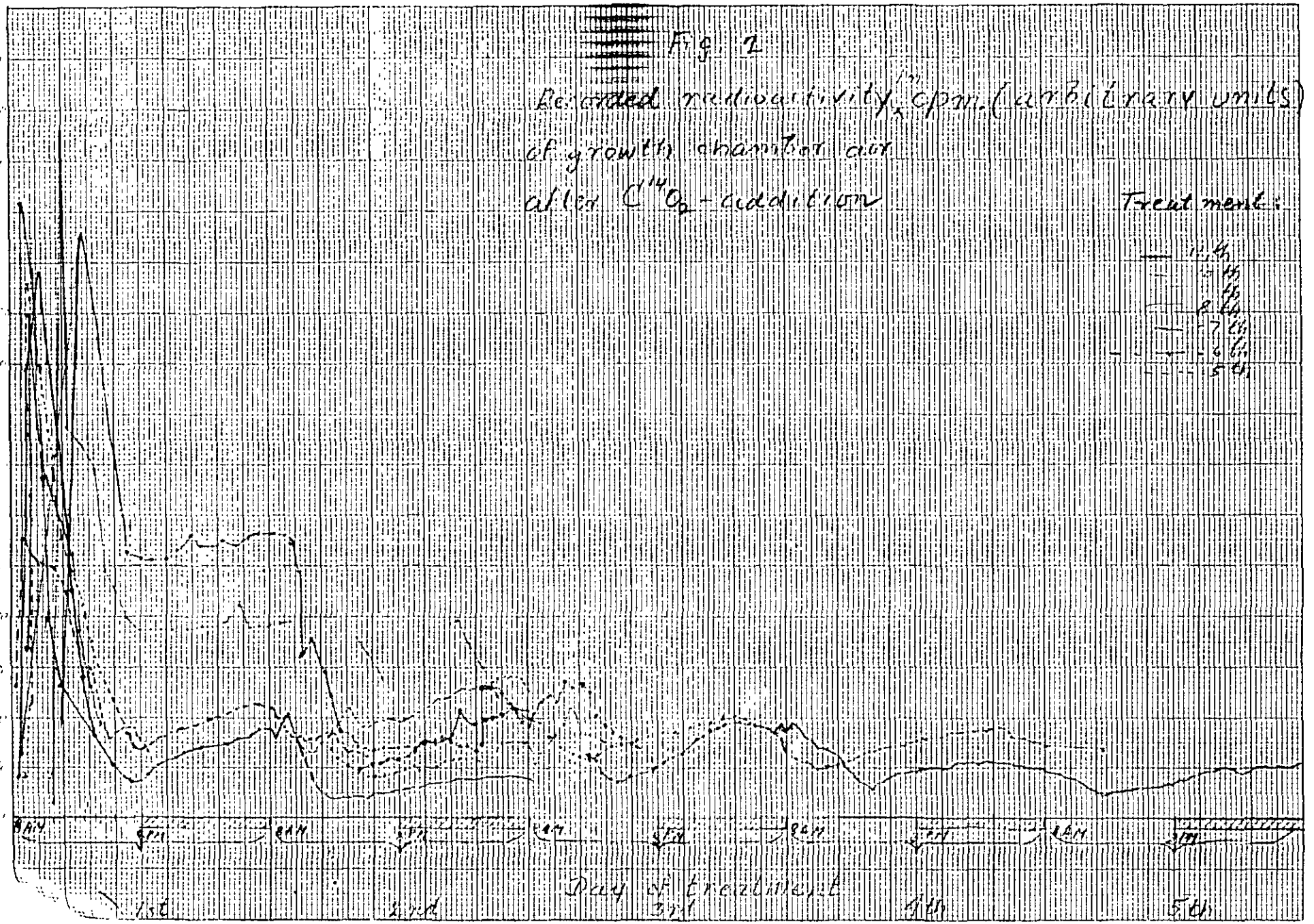
Project 2768

Fig 1

Recorded radioactivity, cpm. (arbitrary units) of growth chamber air after $C^{14}O_2$ - addition

Treatment:

1 day
2 "
3 "
4 "
5 "
6 "
7 "
8 "
9 "
10 "



with samples drawn on fore- and afternoons a low assimilation ratio would then have to be expected.

The growth chamber of about 5000 liters, would, at 25°C. and at a CO_2 ratio of 1.0, hold (18, according to Table 8.II p. 174 and p. 189) about 2.80g CO_2. The approximate 10 liter tray, soil and sap water, would hold 0.0005g CO_2 and about 0.0012g CO_2 would be held by the leaves. Variations of CO_2 in the air and thus changes of dissolved CO_2 in water and sap, effected by a changing temperature which enriches air with CO_2 during the day and depletes it during the dark cycle, would have meant a masking effect on the expected data; however, this could have been just insignificant and we must assume that high CO_2 ratios in mid-morning were probably caused by cloudy and cool, and such at noon and afternoon by excessive sunshine and high temperature (photorespiration) which conditions lead to inhibition of assimilation.

The oxygen-ratios found in 15 tests did not indicate any change of concentration at all. Night samples, which would have shown the lowest oxygen ratios, were not drawn and no ready explanation is available regarding the constant remaining ratios.

There remained the suspicion that coarse leaks or permeability might have influenced the composition of the atmosphere. The thorough overhaul of the framework, lining and air proofing of the chamber, makes the former unlikely and as regards permeability some considerations and tests were made. According to Sacharow (20) "oxygen generally permeates four times as fast as nitrogen, carbon dioxide, however, about twenty-five times

as fast at any constant temperature." The permeability of polyvinyl chloride in cc. (S.T.P.)/cm^2/sec/cmHg at 25°C. x 10^{-8} (0% R.H.) is for carbon dioxide 0.010, for oxygen 0.012 and for nitrogen 0.004. Gas transmission in cc. (S.T.P.)/cm^2/sec/cmHg at 25°C. x 10^{-8} for 0.003 inch thick polyvinyl chloride is 0.13 for carbon dioxide, 0.16 for oxygen and 0.05 for nitrogen. Loudenslagel et al. (21) quote the following transfer rates for helium in cc./sq. in/year/atmosphere:

Caliper of PVC film, mil	Determined by Mass Spectrometry	Determined Volumetrically
6.3	261	244
13.4	123	137
16.8	98	100
24.7	64	67

The oxygen transmission for PVC is lower than that for medium weight PE. For the latter, helium diffuses about 2.5 times faster and carbon dioxide about 4 times faster than oxygen.

The recheck of a sample of our PVC film showed 9.6 mil thickness. This sample was tested by the Polymer Chemistry Group on the Gas drive equipment using a shimed D porosity disk; the test area had a diameter of 3/4 in., dry carbon dioxide was used as gas at minimum measurable flow of 200 cc./min. No measurable amounts of gas were transmitted at pressures up to 70 p.s.i.

If applying the above helium data for our growth chamber, which was filled in a permeability test with about 12 liters (at ambient air conditions) or to a concentration of about 3.4% b.v. with helium which concentration was found to have decreased after 48 hours by 70% and by 90% after 96

hours we find, when selecting a transfer rate of 172 cc. helium for a 10 mil PVC-film for our growth chamber of 23,280 sq. in. surface area, that some higher than the prevailing pressure would have been needed to cause the helium to permeate; at 15 lb. pressure the total helium quantity of 12 liters would have permeated within 10.9 hours. Of course, some of the helium might have gotten trapped by intercellular plant material and even by soil. Still, the possibility of some leaking cannot be excluded.

Results and Discussion

The results of harvested trees are shown in Table III and IV.

TABLE III: Harvest and Weights of Labeled Plant Material

Trees Designation	Number	Removed from pot on	Airdry weight per tree, grams				
			Roots	Stem	Shoots (Twigs)	Leaves Needles	Total
Aspen							
9L	1	Oct. 16, 1968	36.9	22.4	9.8	19.6	88.7
1S - 3S	8	Nov. 19, 1968	32.1	16.4	13.8	14.6	77.9
17L - 20L	4	Nov. 19, 1968	41.2	19.8	25.1	9.7	95.8
Red Pine							
P_{10}	1	Oct. 16, 1968	9.7	3.6	4.7	25.3	44.3
P_{15}	1	Oct. 16, 1968	24.0	13.0	5.4	45.8	87.7
P_1-P_9, P_{11}-P_{14}	13	Oct. 17, 1968	9.8	8.0	5.5	24.4	47.7
Balsam fir							
B_1	1	Oct. 16, 1968	37.5	22.2	44.0	71.2	174.9

Leaves of the 13 aspen trees had changed color almost entirely by October 4; they dried up and had fallen off. Only a few green leaves remained on the shoots.

One of such a green leaf, weighing airdry 0.74 g of 38 cm^2 area and one dried up brown leaf, weighing airdry 0.35 g of 25 cm^2 were withdrawn and exposed to Kodak M-54 emulsion for autoradiography. Both leaves showed a uniform distribution of radioactivity over the entire leaf surface, veins, showing also strong radioactivity, were barely distinguishable.

TABLE IV: Individual Weight and Stem Dimensions of 13 Red Pine Trees After Harvest

Designation	roots, g:	stem(unbarked) g:	diameter, m/m base:	top:	length, cm:	twigs (unbarked) g:	needles g:	total g:
P_1	12.8	7.9	11.5	7.5	18	8.3	34.8	63.8
P_2	17.1	12.5	10.5	9.5,7.0	16+13	8.3	39.4	77.3
P_3	8.1	9.5	8.3	7.5,6.5	19+9	3.4	17.5	38.5
P_4	15.1	13.4	10.5	9.6,8.0	13+10	6.7	28.5	63.7
P_5	10.3	6.5	8.0	7.4,5.5	13+10	2.8	21.8	41.4
P_6	15.2	11.5	10.5	8.8,6.5	16+14	5.5	36.4	68.6
P_7	10.5	7.1	8.5	8.5,6.0	10+16	3.5	22.3	43.6
P_8	7.5	8.3	8.5	8.0,7.5	13+13	3.5	17.3	36.6
P_9	14.1	9.1	9.8	8.9,7.5	12+13	5.1	29.4	57.7
P_{11}	9.2	6.9	9.0	7.0,5.5	11+14	4.2	21.7	42.0
P_{12}	14.4	7.7	9.9	6.5,5.5	9+13	6.2	24.3	52.6
P_{13}	17.0	6.7	9.2	7.6,6.2	13+12	4.5	25.0	53.2
P_{14}	9.8	4.8	8.5	7.5,5.5	11+9	4.8	23.2	42.6
Average:	12.4	8.6	8.44	7.96,6.43	13.4+12.1	5.14	26.3	52.44
%:	23.65	16.40				9.80	50.15	

The weight of leaves from these 13 aspen trees at collection was for:

9L: 1S-8S: 17L-20L:
22.8g 17.8g (142.4g total) 9.8g (37.8g total), thus
14, 13 and 1% water was lost on

drying in a strong current of warm air in the hood These leaves were then ground to a powder and stored.

Red Pine "o P_{14}"

Earlier already a pine tree, treated just in 1967 with $C^{14}O_2$ and designated as "old P_{14}", of poor, dried-up condition, was removed from the pot and analyzed. Its earlier needle top count was just 3,100 cpm., less than one half of the remainder of the pine trees at that time Weights prior to and after drying for 18 hours at 105° C in an atmospheric oven (stems at 68° C in vacuo) are as follows:

	Roots:	Stem:	5 Twigs:	Needles.	Total:
before drying, g	21 0(=31%)	13 4 (unbarked/=20%)	2 9 (unbarked/=5%) 0 92(unbarked)	29 9(/=44%)	67.2
after drying, g	7.5	4 3 (barked)	0.4(barked)	8.3	20 5
LOSS %	64			50.0	70

The stem, 27.5 cm long, holding 16 5g needles and 5 twigs of 0 12, 0 27, 1.09, 0 19, and 0.70 g with 2 72, 2 51, 4 32, 1 66, and 2 14 g needles had a 12 cm long next shoot, carrying at its head 5 dark brown colored buds which had remained encapsuled and did not indicate any sprouting.

Barking resulted in 4 9 g bark (of about 32% moisture) and 7 9 g barked stem. The bark was extracted for 54 days at room temperature with a 2:1 benzene-alcohol mixture resulting in 2 40 g residue (82%) and 0.5138 g extractives (18%) Bark of the 5 twigs of 1 6 g yielded similarly 0 925 g residue (78%) and 0.2456 g extractives (22%)

The stem of the old P_{14} red pine tree, had a specific activity of 4.28 μc/g. at the base, 8 96 μc/g at the center while the stem top had just 6.53 μc/g

Its five twigs had an average specific activity of 8 22 μc/g with 7 09 μc/g. as minimum and 10.21 μc/g as maximum, the needles an average specific activity of 6 60 μc/g with 2.67 μc/g as minimum and 10 11 μc/g as the maximum The extracted twigs showed 9 83 μc/g , the twig extract 28 07 μc/g The roots had an average specific activity of 1 02 μc/g and the soil in the pot 0.022 μc/g. All these data were determined by liquid scintillation counting, using 15 ml of a 0 3% PPO-toluene cocktail and 0 6 g Cabosil per vial. Counting was begun after two minutes shaking of the vials and thirty minutes of de-airing in the desiccator. This technique was developed by Cluley for $BaCO_3$ (24) and it appears to give satisfactory results with the various wood components. The effect of the fineness of the sample was not specifically investigated, however, satisfactory data were obtained with powders passing a 100-mesh screen.

Some 4.0 g. of stem, 2 2 g of extracted bark and 0.4 g of extracted twigs, apart from 11 needle samples (caps designated from 20 through 30), having the above specific activity, are stored together with the other project plant material

Aspen "10L"

Similarly was a triploid aspen tree, treated with $C^{14}O_2$ in 1966 and 1967 designated "10L", showing also a very poor condition and no new shoots, removed from the pot and analyzed. Weights prior to and after similar drying as above were:

	roots	stem	total
before drying, g	52 7	31.5 (unbarked) 16.6 (barked)	84.2
after drying, g	16 0	8 14 (barked)	24.14
water lost, %	70	51 (barked)	

The stem of just 18 5 cm length, of 14 3 mm bottom and 12.9 mm. top diameter gave some 47% bark which resulted, after a similar extraction as described above, in 92% residue and 8% extractives.

The stem of the 10L aspen tree treated in 1966 and 1967 with $C^{14}O_2$ had a specific activity of 5.61 μc/g at the base, 9 04 μc/g at center and just 7 51 μc/g at the stem top. Its extracted bark showed 2.71 μc/g and the extract 8 13 μc/g specific activity, the roots 4 19 μc/g and the surprising high value of 0 88 μc/g. for soil

An unbarked and barked shoot of the above tree was separated into bottom and top parts, the respective specific activities were 14 08, 17.02 and 15 07, 16 24 μc/g

A prepared Klason lignin of the above tree had a specific activity of 9.8 μc/g.

Some 7.5 g of stem, 4 5 g. extracted and 9.2 g. unextracted bark, also some ground unbarked and barked shoot (1966) parts of the above specific activity, are stored together with other project plant material

Autoradiography

Short blocks cut from the barked base, center, and top of the stems of old P_{14} and needles of same were placed in a killing and fixing solution

(consisting of 35% water, 50% ethanol, 5% glacial acetic acid and 10% formaldehyde) for a day and then washed with distilled water and stored in approximately 50% ethanol (5). Microtome sections of 50μ for stem and twigs and of 30μ for the needles were cut by first removing the ethanol and heating slightly the materials in distilled water. Needles were taken from almost the top and they required embedding in groups of 5 or more in butyl methacrylate. Longitudinal and cross sections of stems and just cross sections of twigs and needles were cut. The sections were then mounted for autoradiography onto 1/4" thick polished plate glass dried covered with Mylar Scotch tape No. 853 to hold them in position (later just Saran wrap was employed). With the polyester 87%, with Saran wrap 35%, and with a light-weight Mylar cover, 26% of radioactivity were shielded off. The Kodak x-ray film emulsion M-54 was sandwiched between the sample and another 1/4" polished glass plate and the plates secured around all 4 sides with Scotch tape for firm hold and to avoid any film shifting. Three sets of such sandwiches were prepared and the required autoradiography exposure time determined.

Some of these sections are shown in Fig. 1 and 2.

Comparative counts of unshielded microtome sections of the old P_{14} red pine and the 10L aspen tree were as follows.

		net
Cross section of old P_{14}, stem P_3,		120 cpm.
Cross section of old P_{14}, twig B_1		60 cpm.
Cross section of old P_{14}, twig B_5		69 cpm.
Cross section of old P_{14}, needles in butyl methacrylate embedded:		
	N_1	31 cpm.
	N_4	28 cpm.
	N_8	22 cpm.
Radial section of 10L, stem	A_2	194 cpm.
stem	A_3	134 cpm.

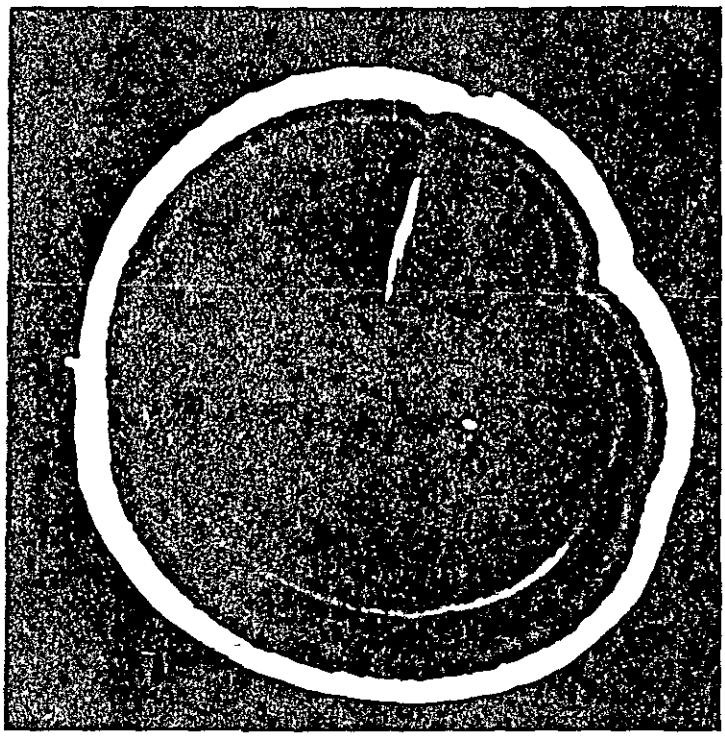


Figure 3 Cross-section of Center of Barked Aspen (10L) Stem Treated in 1966 and 1967.

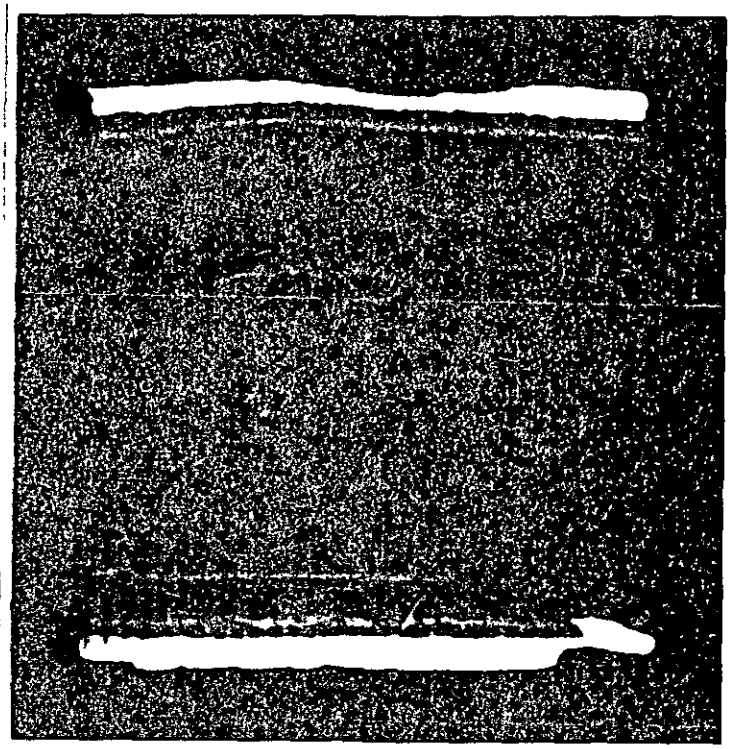


Figure 4 Transverse Section of Center of Barked Aspen (10L) Stem Treated in 1966 and 1967.

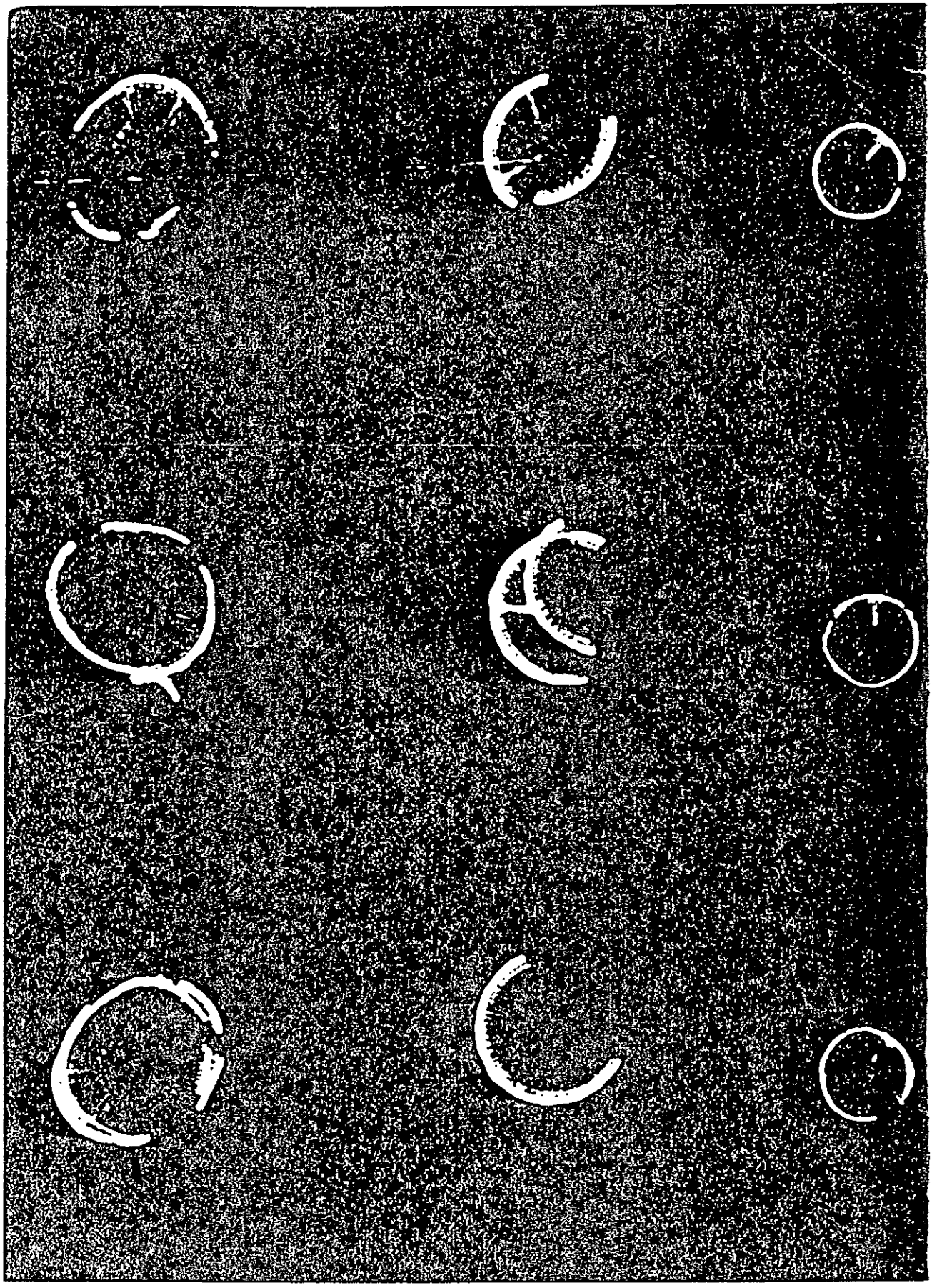


Figure 1 Cross-sections of Barked Base, Center, and Top of Stem of Red Pine ($o.P_{14}$) Treated 1x(1967)

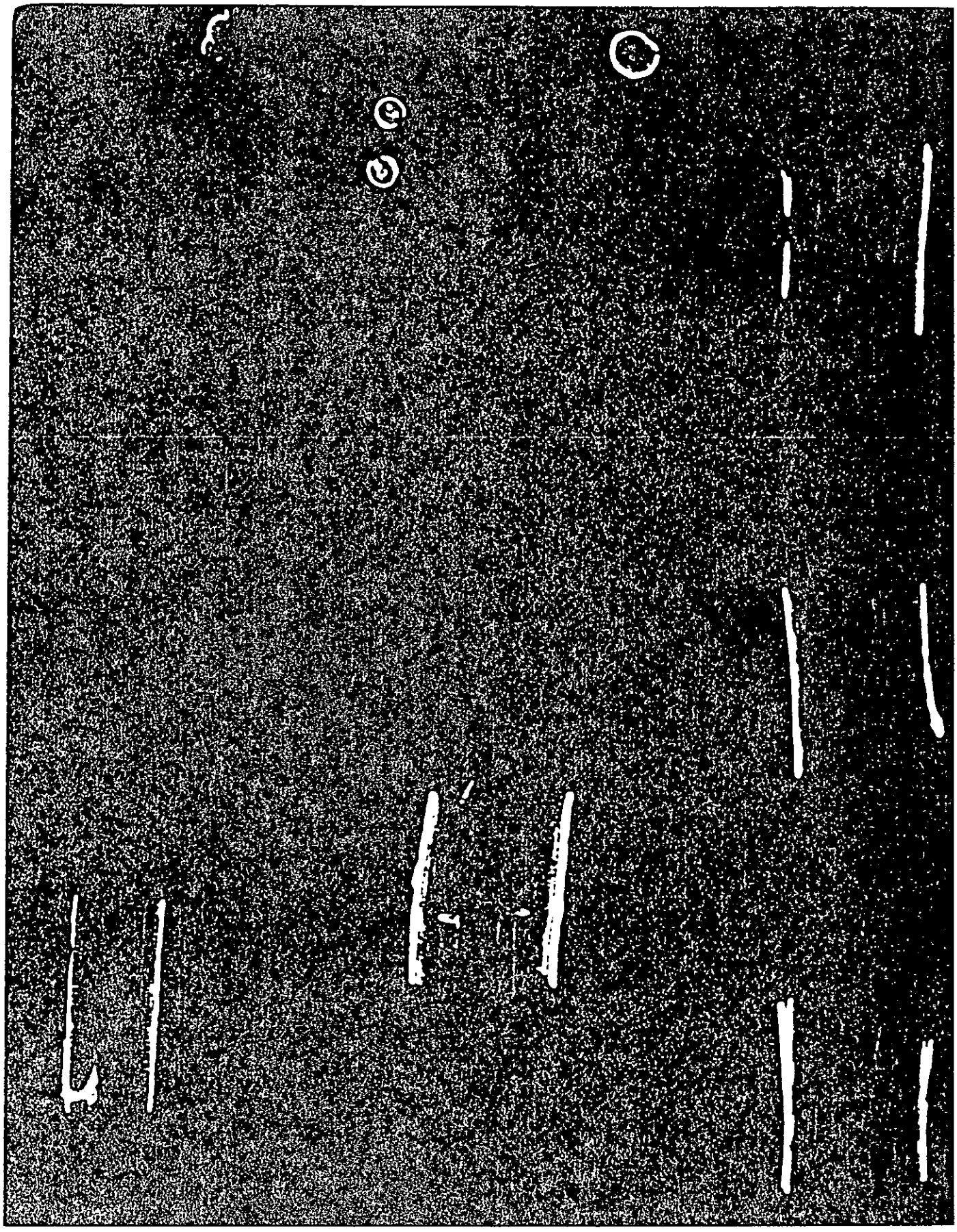


Figure 2 Transverse Sections of Barked Base, Center, and Top of Stem and of 4 Twigs of Red Pine (o.P_{14}) Treated 1x(1967)

The radioactivity of the above stem sections is thus of the same order of magnitude, that of the twigs just less than one-half and of the needles just one-sixth of that of the stems. Such information is helpful in autoradiography technique.

Based on measurements taken from a magnified autoradiograph (Fig. 3 and 4) of a 10L stem cross section of 40μ, layers .075 (= 8.2%), 0.25 (= 16.4%), 0.14 (= 9.5%) 0.14 (= 9%) mm thick were cut off on a lathe and ground, together with the remaining core (57.1%) on a 20-mesh Wiley mill screen.

The stem part had 5.30 μc/g. specific activity and the layers, in the above order, 14.63, 5.22, 2.54, 1.81 μc/g., while the core showed 0.47 μc/g.

The aim of this work was to 1) locate the radioactivity and 2) to design the peeling-off of the respective radioactive layers by mechanical means, and 3) obtain a semiquantitative information of the labeling effect. Also, since no such work had been done at the Institute before, it was considered worthwhile to study this technique for possible further applications (19). Developing of the exposed films was done for 8 minutes with an x-ray developer (and replenisher), treated with x-ray fixer with hardener. Certain, well representative sections were magnified about 5 times, and it became possible to measure clearly labeled zones.

On September 24, twenty-eight days after the final $C^{14}O_2$-addition, when practically all aspen leaves had fallen off, chamber air activity was measured with a Geiger tube inserted at the base of the front panel showing at 1 p.m. a residual net count of 450 cpm. This air had a CO_2-ratio of 1.5 and at 4 p.m. the ratio was 1.0.

On September 25 (at a morning chamber temperature of 12°C.) the net count was:

at 9:10	9:30	11:30 a.m. :	at 12:55	2:15 p.m. :
430	470	380	350	495

on September 26 at 4:00 p.m.
430, 420, 430, and

on September 27 at 8:35 a.m.
512, 500, 500 cpm.

The air of 450 cpm., corresponding to a count of 70 cpm. in the flow assembly showed an absorbing with 0.05N NaOH (4 ml.) when counted with a dioxane-PPO-cocktail at 80% efficiency, an activity of 2666 cpm./10 ml. equal to 0.1712 μc/liter at a count of 100 cpm. in the assembly. (The arbitrary unit of 100 cpm. in the two graphs showing the course of radioactivity in the growth chamber during treatments with $C^{14}O_2$ corresponds thus to a total of 0.856 mc. and plotted there maxima of 1000 cpm. to 8.56 mc. which is in agreement with the 10 mc. of C^{14} actually applied in each of the treatments.)

Similarly, a sample drawn from the chamber on October 1, giving a net count of 350 cpm., showed a flow assembly reading of 50 cpm. which is about the same ratio as found above.

During the period from August 16 till September 24, fifteen water samples were tested for radioactivity. The results are shown in Table V.

TABLE V: Radioactivity Growth of Chamber Water

Designation	Date		Found uc/liter
W5	8-16	3:00 PM	2.9
W6	8-19	8:00 AM	3.1
W7	8-19	8.20 AM	3.3

(Table V continued)

W8	8-19	2·00 PM	8.5
W9	8-20	1:00 PM	2.6
W10	8-20	4 30 PM	5.6
W11	8-21	8·20 AM	2.0
W12	8-21	1 00 PM	6.9
W13	8-23	9·30 AM	12.1
W14	8-23	1 45 PM	11.0
W15	8-26	10.00 AM	5.1
W16	8-26	3:00 PM	9.3
W17	8-27	9:00 AM	16.1
W18	8-28	10·00 AM	16.5
W19	9-24	1:00 PM	0.4

In the approximately 9 liter of water slightly with organic material contaminated withdrawn on August 27, a total of 272 μc or a specific activity of about 30 μc/liter was found. There is no ready explanation for the existing discrepancy with the above data available.

Red Pine

Trees P10 and P15, the latter labeled just in 1968, were removed on October 16 from the pots, the remaining three-year-old thirteen trees P1-9 and P11-14 on October 17.

The following data and specific activities were found and are recorded in Table VI, they were simultaneously compared with tree o. P14, labeled in 1967.

TABLE VI: Results with Red Pine Trees

	In 1967 labeled o P14	In 1967 and 1968 labeled P1-10, P11-14 (14 trees)	P10 (separate)	In 1968 labeled P15
Stem, length, cm.	15.5 + 12	13.4 +12.1	(26 + 10)	24 + 15

(Table VI continued)

Stem, diameter, base, mm.	9	8.5	(9.5)	10.5
Stem, diameter, center, mm.	8	8.0	(8.5)	8.2
Stem, diameter, top, mm.	6	6.4	(6.0)	6.0
Soil, μc/g.	0.02	0.32	(0.95)	0.28
Roots, average, μc/g.	1.02	16.8	(15.3)	28.0
Stem, base, μc/g.	6.7	12.9	(6.3)	29.2
Stem, center, μc/g.		9.0	(13.4)	--
Stem, top, μc/g.		7.8	(20.2)	59.4
Bark, μc/g.			(13.8)	26.6
Twigs, large, μc/g.	8.2	12.5	(10.0)	42.5
Twigs, small, μc/g.			(26.8)	55.0
Needles, μc/g.	6.6	14.4	(31.0)	15.2
Per/gram of total plant material, μc/g.	4.70	12.9	(2414)	22.9
Weight of one tree, g.	21.0	45.9	(44.3)	88.2

In 1968, by using the closed growth chamber, at least a doubling of the 1967 labeling effect was expected. Except for the needles, this effect was not attained. If disregarding roots, the factor for the activity per/gram of total plant material becomes just barely 2.0. Tree P10, the healthiest of the fourteen trees shows very high activity of the needles which is indicative of the availability of chlorophyll throughout the season and of an improved plant vigor. Still, it must now be admitted

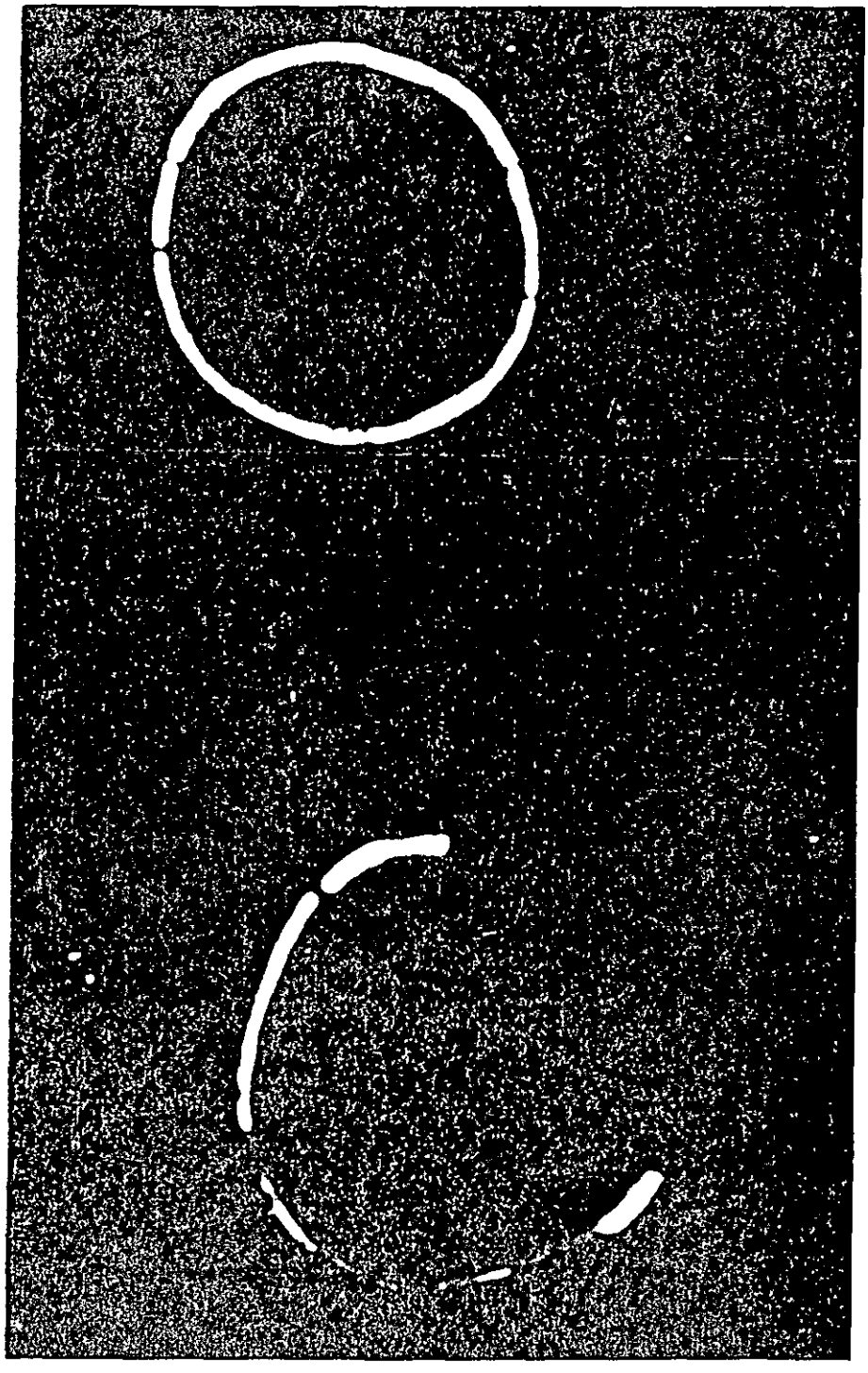


Figure 5 Cross-sections of Stems of Red Pine

Upper: P_{10}, treated 2x(1967,68)

Lower: P_{15}, treated 1x(1968)

that all these fourteen pines, incapable of forming new shoots this year, had rather been damaged in 1967 already by an overdose of $C^{14}O_2$ radiation and had not suffered any winter damage as it was earlier supposed to be the case.

Tree P15, exceedingly healthy (last year's control), gave (except for needles) very good labeling results. The needles were still of a healthy appearance after the final labeling. This tree did better than the twice labeled P10 as can be seen from the almost equal specific activity per/gram total plant material. The high assimilation is a result of an almost double as great tree weight and of an excellent plant vigor.

No treated pine is left for further labeling. For the future, it is advisable not to exceed with pines a tolerable radiation dose in labeling with C^{14} (1,15,16,17).

Also, it should be pointed out that from the entire harvested plant material unbarked stems amount to about 14% (104 g.) and that the activity is to be found just at the periphery and in the bark (4).

Stems of trees P10 and P15, covered with Mylar of 2.5 µc. thickness, were exposed, just as o. P14 earlier, for 7 days to Kodak x-ray film M-54 emulsion. From such magnified autoradiographs the thickness of the concentric layers may easily be determined as such layers will have to be pealed off in order to obtain high specific activity holocellulose, hemicelluloses and lignin. Also, the intensity of the autoradiographs of these stem sections reflects very well their specific activity of 13.0 vs. 30.0 µc/g. found by counting. Some activity, but of a much lower degree, is to be found in the inner stem parts (wood rays).

Stored are:

128.2 g. (21.4%), roots of P1-9, 11-14 of about 11.4 μc/g. spec. act.

9.7 g. (21.9%), roots of P10 of about 15.3 μc/g. spec. act.

24.0 g. (27.2%), roots of P15 of about 28.0 μc/g. spec. act.

84.0 g. (14.0%), stems of P1-9, 11-14 of about 8.8 μc/g. spec. act.

3.6 g. (8.1%), stems of P10 of about 13.0 μc/g. spec. act.

13.0 g. (14.7%), stems of P15 of about 30.0 μc/g. spec. act.

41.8 g. (11.5%), twigs of P1-9, 11-14 of about 12.0 μc/g. spec. act.

5.7 g. (12.9%), twigs of P10 of about 18.0 μc/g. spec. act.

5.4 g. (6.2%), twigs of P15 of about 48.0 μc/g. spec. act.

317.9 g. (53.1%), needles of P1-9, 11-14 of about 13.1 μc/g. spec. act.

25.3 g. (57.1%), needles of P19 of about 31.0 μc/g. spec. act.

45.8 g. (51.9%), needles of P15 of about 15.2 μc/g. spec. act.

Triploid Trembling Aspen

In this investigation a comparison of four-year-old aspen labeled twice and three times respectively was foreseen and a tree labeled in 1966 and 1967 (harvested before the start of this year's labeling) is also shown in this comparison.

The following activities were found and listed in Table VII:

TABLE VII: Results with Trembling Aspen

	In 1966 and 1967 labeled 10L	In 1966 and 1968 labeled 17L-20L (4 trees)	In 1966, 1967 and in 1968 labeled 1S-8S (8 trees)	9L
Soil, μc/g.	0.88	0.75	2.28	0.25

(Table VII continued)

Roots, coarse, μc/g.			17.5	
Roots, medium, μc/g.	4.2	32.0	35.3	35.3
Roots, fine, μc/g.			68.6	39.2
Stems, average, μc/g.	5.3	4.9	11.2	20.4
Stems, base, μc/g.				14.0
Bark, outer, μc/g.				23.0
Shoots, μc/g.	15.0	19.0	69.3	45.2
Leaves, μc/g.		52.0	46.0	30.0
Per/gram of total plant material, μc/g.		24.96	35.85	
Average weight of total tree		95.58	78.26	

Three-times labeled trees show a 1.44-fold greater specific activity (the rough comparison of the activity of the top of stems prior to labeling showed a factor of 3.6). Leaves of the four trees 17L-20L were of small size, weighing, in spite of strong and heavy shoots, just one-half as much as the others. Still, the rootwork of these four trees is 1.11-fold and the total weight 1.22-fold greater and better labeling would have resulted with a greater leave area.

No thrice-labeled aspen is left for further labeling. The tolerance toward radiation damage appears to be much greater than that of red pine.

For delignification some 148 g. unbarked thrice- and 79 g. twice-labeled stems are available, the former having thus a 2.3 fold higher specific activity than the latter. Shoots of about 5 mm. diameter and above should be also a suitable pulping material, especially since their specific activity is 4 to 6 times greater than that of the stems.

Microtome sections of the 9L stem show in an enlarged autoradiograph with Kodak M-54 emulsion the activity distributed just on the periphery (for comparison part of bark with its more concentrated effect of radioactivity is also shown) and for pulping of high activity holocellulose and other plant materials the dimensions of the layer to be peeled off may be taken from this graph.

Stored are:

257.0 g. (38.6%), roots of 1S-8S of about 30.3 μc/g. spec. act.

36.9 g. roots of 9L of about 35.3 μc/g. spec. act.

176.0 g. (43.1%), roots of 17L-20L of about 32.0 μc/g. spec. act.

148.0 g. (22.1%), stems of 1S-8S of about 11.5 μc/g. spec. act.

stems of 9L of about 20.4 μc/g. spec. act.

79.0 g. (20.7%), stems of 17L-20L of about 4.9 μc/g. spec. act.

120 g. (18.0%), shoots of 1S-8S of about 69.3 μc/g. spec. act.

shoots of 9L of about 45.2 μc/g. spec. act.

100.5 g. (26.2%), shoots of 17L-20L of about 19.0 μc/g. spec. act.

142.4 g. (21.3%), leaves of 1S-8S of about 48 μc/g. spec. act.

leaves of 9L of about 30 μc/g. spec. act.

37.8 g. (10%), leaves of 17L-20L of about 52 μc/g. spec. act.

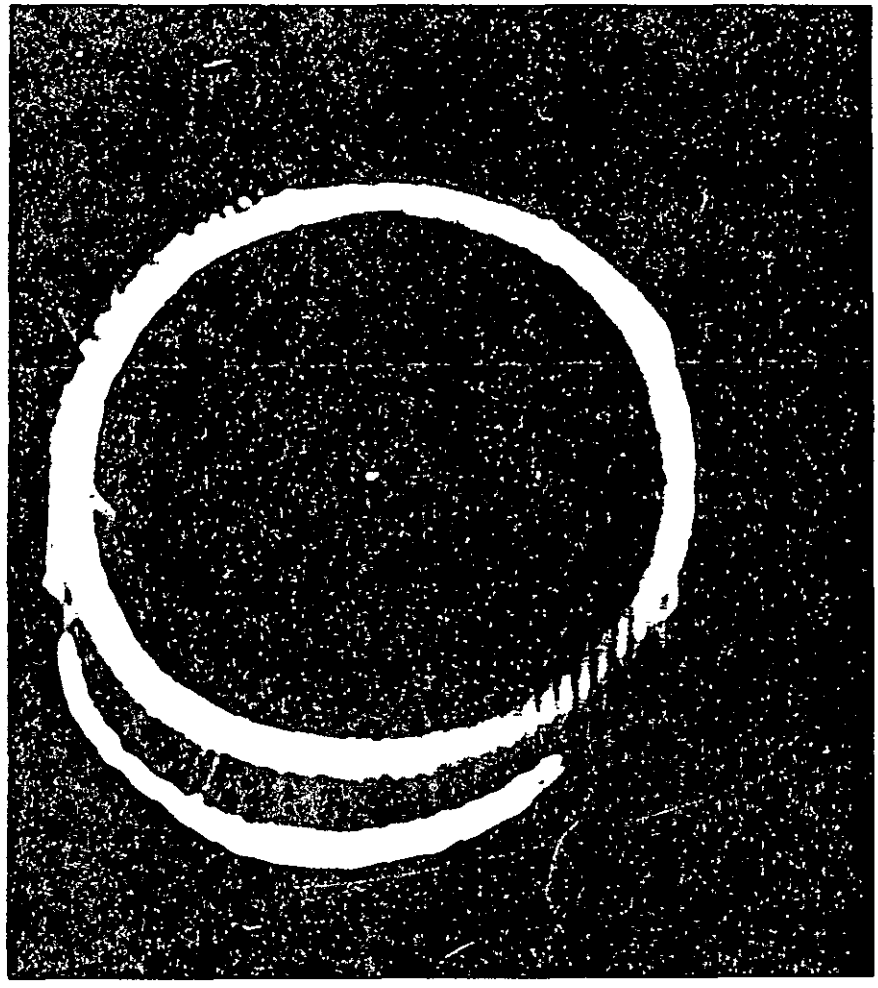


Figure 6 Cross-section of Barked Stem of Aspen (9L) (Treated in 1966, 1967 and 1968) (Showing part of Bark)

Balsam Fir

Two five-year-old trees were labeled in 1968 of which B1 was removed from the pot for analysis, the other, B2, handed over to winter storage by the Genetics Group.

Both trees showed their healthy appearance also after labeling, except for several unexplained spots of about 2 x 2 inches of dried up, brown twigs. In spring, the condition of the winter-stored tree shall become a further and crucial measure of tolerance toward C^{14}.

The results are listed in Table VIII:

TABLE VIII: Results with Balsam Fir

Stem length, overall, cm.		34.0		
Stem diameter base, mm.		14.2; 1/3 = 12.0; 1/2 = 10.0;		
Weight at harvesting, g.	: roots :	76.0, stem 33.5, twigs, needles : 201.5		
Weight airdried, g.:	roots :	42.4, stem 22.2	44.0	7.10
Weight loss	: %	45	34	53
Soil, µc/g.		0.38		
Roots, µc/g.	:	coarse : 15.0, coarse barked 13.7, bark 9.7		
Roots, µc/g.	:	medium 19.0		, bark 39.8
Roots, µc/g.	:	fine 21.0		
Stem diameter, mm:	11.5 :	8 :	5 :	
Stem, µc/g. unbarked:	24.0	23.0	17.0	
Stem, µc/g. bark inner:	20.0	25.0	31.0	
Stem, µc/g. : bark (20%):	27.0	18.0	23.0	
Stem, µc/g. : barked	19.0	19.0	22.0	

Cross sections of root and stem parts are shown in Figure 7.

(Table VIII continued)

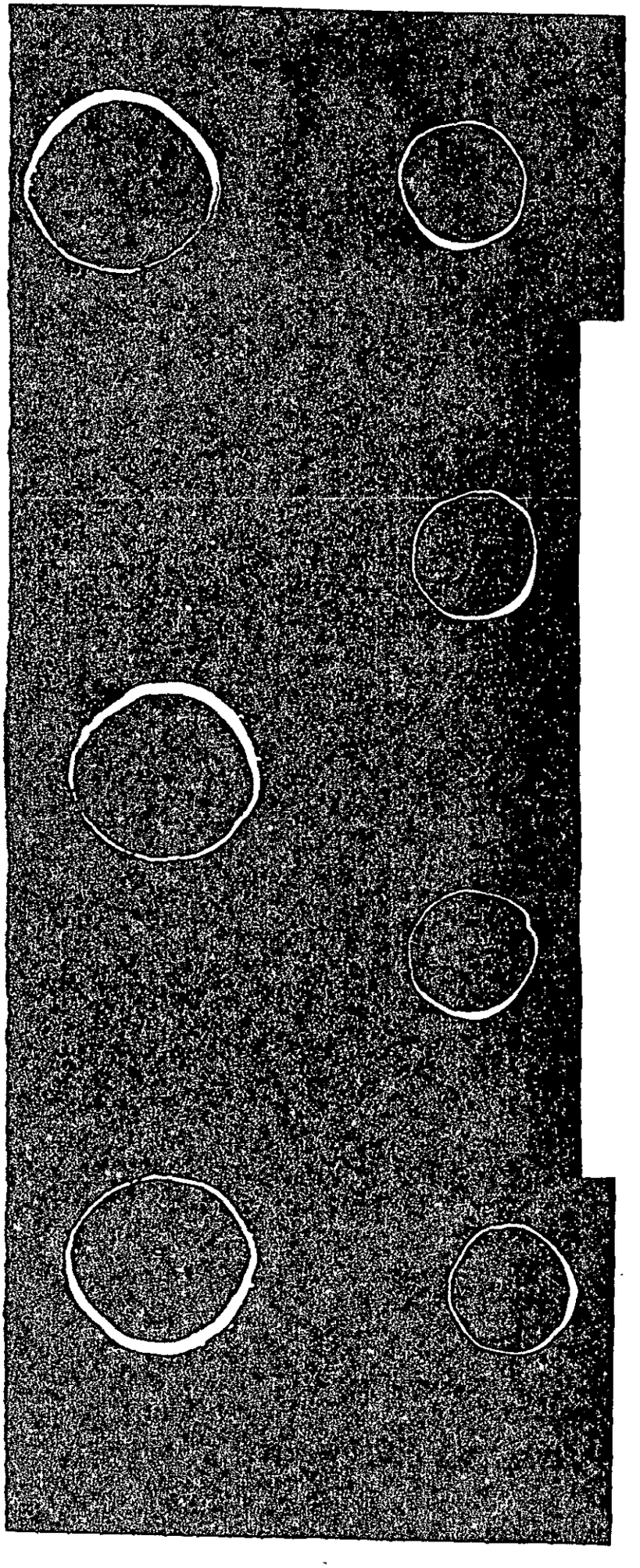
Twigs, coarse, μc/g. :	18
Twigs, fine, μc/g. :	34
Needles, fallen off during season green μc/g. :	3.2
Needles, fallen off during season, brown μc/g. :	13.0
Needles, green, removed from twig top μc/g. :	24.0, 29.0, 28.0, 51.0
Needles, green, removed from twig base μc/g. :	23.0, 5.0, 7.0
Needles, green, removed from branch base μc/g. :	6.0
Needles from damaged twigs μc/g. :	17.0, 19.0, 14.0, 13.0
Needles randomly collected, green μc/g. :	2.7, 2.1, 9.5, 9.8, 1.1

It should be noted that it is difficult to obtain consistent data without grinding of the entire respective plant material. Also, with such truly representative samples the more accurate combustion method should then be applied.

In setting an average specific activity of 20.3 μc/g. for roots, of 25.4 for the stem, of 26.0 for the twigs and 28.5 μc/g. for the needles, a specific activity of 25.72 μc/g. per 1 gram of the total plant material results. This compares well with the figure of 22.9 μc/g. obtained for red pine tree P15 that was labeled just in 1968 also. The total dry weight of B1 was 174.9 g., P15 weighted 88.2 g.

Microtome sections, 40μ thick, of the coarse root part and of three stem sections were autoradiographed as above. Again, no radioactivity neither within the root nor the stem parts was detectable and all the activity was found peripherally concentrated in the youngest growth.

This is shown in Fig. 7.



For pulping, 22 g. unbarked stem, besides partly usable twigs (44 g) and roots (37.5 g) are available. For obtaining high radioactive material are the removal of the outer layer and its separate delignification again imperative.

Stored are:

37.5 g. (21.4%) roots of approximately 20 μc/g. spec. act.

70.0 g. (20.7%) stems of approximately 25 μc/g. spec. act.

44.0 g. (25.1%) twigs of approximately 26 μc/g. spec. act.

71.2 g. (20.8%) needles of approximately 28 μc/g. spec. act.

and 12.0 g. needles separately.

Conclusions and Recommendations for any Future Work

The specific activities obtained vary at any given position of the plant component which should be kept in mind when using the reported data. It must be stressed again that complete destruction (grinding) and intimate mixing of the plant material would provide a more uniform sampling and more accurate results would be obtained by combusting and gas proportional counting.

Except for the fourteen pine trees, the main objective of this project was fulfilled.

It is clear now that these trees have almost succumbed to radiation damage. Pine trees appear to be less tolerable to labeling than aspen. Pertinent information comes forth from a recent publication (1) and in any further labeling work more close attention to the possible biological effects of radiation should be devoted and less than a tolerable dose be

applied (15,16,17).

The further objective of this project does indeed clearly allow the comparison of the labeling effect between one or two and two or three labeling seasons.

The objective of establishing a material account of the applied C^{14} was achieved to a satisfactory degree. However, the high radioactivity of the roots (4) and especially that of the soil was unexpected.

If assuming that 1/3 of the found activity of the fourteen pine trees P1-14, 1/3 of that of the four aspen trees 17L-18L, and 2/5 of the nine aspen trees 1S-8S and 9L was present at the start of this year's labeling already, and that the available to the trees C^{14} would have been distributed according to plant weight (which definitely hardly was the case with the fourteen pine trees P1-P14), we find that of the available C^{14} some 16.5% (5.74 mc.) were utilized by pine trees P1-14, some 40.4% (2.02 mc) by pine trees P15, some 38.3% (15.15 mc.) by aspen trees 1S-8S, 9L, some 30.0% (6.30 mc.) by aspen trees 17L-20L, and some 45.6% (9.00 mc) utilized by balsam firs B1 and B2.

Of the totally applied 120 mc. some 38.20 mc. or an average of 31.8% were utilized by the trees in the photosynthesis.

Since we did not mix the entire soil of each pot to prepare truly representative samples and had withdrawn just from about the center of the pot some soil, a proper determination of the total activity of the 30 pots, each holding at least 7 kg. soil, is difficult to establish. If we assume that just 3 kg. soil of each pot contained the radioactivity as

was found in the reported concentrations, we arrive at a total of some 77 mc. which would give a total of 115 mc. accounted for. (The insignificant quantity of recovered 0.272 mc. C^{14} in $BaC^{14}O_3$ need not be considered here.)

It appears, therefore, that just small losses might have resulted with the permanently closed growth chamber during the entire growth season, that is, that the permeability and other leakage losses must have been rather small, below some 4% or so, maybe even less.

The phototransmission of the 10-mil PVC-sheet used with the growth chamber in the past 3 years was determined and compared with that of sheets of 3.5 and 4.5 mil prepared for this comparison:

Region, mμ	Transmission, % 3.5-mil.	4.5-mil.	10-mil.
290	2.0	0.0	0.0
295	28.0	7.0	0.0
300	75.0	55.0	0.0
310	93.0	87.0	0.0
320b	94.0	90.0	0.0
350			0.0
360			3.0
370			20.0
380			50.0
390			73.0
400			82.0
425			88.0
from 680 on			90.0
down at 1720			to 74%
up again at 1900 mμ			to 89%
down at 2300 mμ			to 42%
up again at 2700 mμ			to 75%

Note: The prepared sheets were cast from 100 p. Marvinol VR 56 (U.S. Rubber Co.)

75 p. dioctyl phthalate

2 p. stabilizer 1212A (Ferro-Chem Co.), curing 3 minutes at 380° F.

An inquiry regarding UV-transmitting window glass was answered negatively by Libby-Owens and the Corning Glass Works recommended to us VYCOR (R) brand 7910 UV transmitting glass which would transmit at 2 mm thickness 70% at 254 mμ, and less than 1% at 185 mμ. The price of such glass would probably be prohibitive.

Watering of the plants would require more controlled conditions since our method employing the tray filled with some quantity of water, causing the pots to be partly submersed in it, has disadvantages. This water will contain radioactive $C^{14}O_2$ which finds its way into the soil and the roots. The high specific activity of both of our softwoods and of aspen and in some cases very high activity of the soil are indicative that reactions between dissolved CO_2 (HCO_3^-) and inorganic soil compenents and labeling of roots has taken place by simply enriching the sapwater of the roots with C^{14}. Addition of water should not exceed the normally in such experiments maintained soil moisture of 15 to 18%. Another disadvantage with the above watering is the small though masking effect temperature changes might bring about by lower CO_2 - solubility in the tray water during warm and a greater one at colder temperatures coinciding with periods of maximum CO_2 - assimilation in the light cycle and maximum CO_2 - respiration in the dark cycle as mentioned already.

A good example of a well controlled photosynthesis chamber may be found in a journal article by Lister et al. (2).

The presence of considerable quantities of organic soil material presents another difficulty, inasmuch, as organic CO_2 becomes gradually available from the soil by enzymatic action, providing thus an uncontrolled

source of unlabeled CO_2, replacement of soil with an inert material and feeding with isotonic solutions would eliminate the above drawback

Autoradiography using Kodak x-ray film M-54 emulsion has proved well in these investigations. Kodak nuclear emulsion NTB-3, used recently by Saleh et al. (3), was not tried. It should be noted that the latter emulsion possesses a radiation radius small enough to allow the detection of the exact locus of radioactivity inside the dimension of a single cell, here, labeling with tritium is preferred.

The listed literature contains useful texts and articles pertinent to this project.

Highly specific information has been published in the journal Radiation Botany (started in 1962, a quarterly publication, Pergamon Press, London) and in the Annales of Botany (also a quarterly, started in 1887, Oxford University Press, London). Neither of these two journals is available at our library.

LITERATURE

1. D. J. Ursino, V. Slankis, and G. Krotkov. Effects of radiation from $C^{14}O_2$ absorbed by Pinus strobus L. seedlings during photosynthesis on their subsequent growth and metabolism. Can. J. Biochem. 46:919-923 (1968).

2 G. R. Lister, G. Krotkov, and C. D. Nelson. A closed-circuit apparatus with an infrared CO_2 analyzer and a geiger tube for continuous measurement of CO_2 exchange in photosynthesis and respiration. Can. J. Botany, 39:581-591 (1961).

3. T. M. Saleh, L. Leney, and K. V. Sarkanen. Radioautographic studies of cottonwood, douglas fir and wheat plants. Holzforsch 21:116-120 (1967) Aug.

4. T. Shiroya, G R. Lister, V. Slankis, G. Krotkov, and C. D. Nelson. Translocation of the products of photosynthesis to roots of pine seedlings. Can. J. Botany 40:1125-1135 (1962).

5. A. A. Selders, B. A. Staskievicz, Jean P. Picard. Carbon-14 labeled wood cellulose. Tappi 42:409-412 (1959) May.

6. Zhuravlev, Yu. N. Products of photosynthesis in pine needles of various ages. Tr. Inst. Biol. Akad. Nauk SSSR, Ural' skii Filial no. 35:85-92 (1963). A.B.I.P.C. 36:(2) Aug. 1965, no. 851.

7. Yushkov, P. I. Post-photosynthetic distribution of carbon-14 in scotch pine. Tr. Inst. Biol. Akad. Nauk SSSR, Ural' skii Filial no. 35:77-84 (1963) A.B.I.P.C. 36:(2) Aug. 1965, no. 850.

8. Tinus, R. W. The role of oleoresin in the metabolism of Pinus ponderosa laws. Ph.D. Thesis Univ. of California, Berkeley, 1966, 114 p. A.B.I.P.C. 37:(10) April 1967, no. 7545.

9. Tissut, M., Rozier, C. Quantitative study of flavonols in potato and in beech in the greenhouse and under different illumination. Compt. Rend. Ser. D264, no. 25:2895-8 (June 19, 1967) A.B.I.P.C. 38:(9) March 1968.

10. San Pietro, A., Greer, F. A., Army, T. J. (ed.) Harvesting the sun-photosynthesis in plant life (Symposium sponsored by International Minerals & Chem. Corp., Chicago, Oct. 5-7, 1966) N.Y., Acad. Press, 1967, 342 p.

11. Dursan, D. J. Nitrogen metabolism of Picea glauca. Can. J. Botany 46:no. 7:909-19; 921-8; 929-37 (July 1968).

12. Bykhovskii, E. G., Sokolov, A. V., Fomina, E. V., Kabanov, V. V. Use of radioactive isotopes for measuring the rate of translocation of oleoresin and assimilates along the pine trunk. Girdroliz. Lesokhim. Prom. 21, no. 2:16-18(1968) A.B.I.P.C. 39 (1968) no. 806.

13. Chistyakov, A. R., Levkhina, T. A., Uspenskii, E I. Physiological condition of spruce regrowth of different vitality. Lesnoi Zh. 11, no. 1:10-12 (1968) A.B.I.P.C. 39 no. 1709

14. Calvin, M., and Bassham, J. A. The photosynthesis of carbon compounds. Benjamin, N. Y., 1962.

15. P. F. Bourdeau, and G. M. Woodwell. Field measurements of CO_2 exchange of Pinus rigida trees exposed to chronic gamma radiation. Ecology 45:403-406 (2) 1964).

16. G. W. Woodwell. Effects of ionizing radiation on terrestrial ecosystems. Science 138:572-577.

17. L. P. Zill, and N. E. Tolbert. The effect of ionizing and ultraviolet radiations in photosynthesis. Arch. Biochem. Biophys. 76:196-203.

18. E. I. Rabinowitch in Photosynthesis and related processes, vol. I, Interscience Publishers, N. Y., 1945.

19. A. W. Rogers. Techniques of autoradiography. Elsevier Publishing Co., N. Y., 1967.

20. S. Sacharow. Permeability of flexible plastic films. Plastics Design and Processing, 5:20-23 (1965) Sept.

21. K. P. Loudenslagel, and S. F. Roe. Gas transmission in thick plate sections. Modern Packaging, 40:139-146 (1967) Feb.

22. W. H. Wilhite, and O. L. Hollis. The use of porous-polymer beads for analysis of Martian atmosphere. J. Gas Chromatogr. 6:84-88 (1968) Feb.

23. T. L. Chang. Chromatographic analysis of mixtures containing oxygen and argon by elimination of oxygen. J. Gas Chromatogr. 4:371-375 (1966) Oct.

24. H. J. Cluley. Suspension scintillation counting of carbon-14 barium carbonate. Analyst 87:170-176 (1962) March.