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SPECIFIC TOTAL STARCH DETERMINATIONS IN CONIFER TISSUES WITH GLUCOSE OXIDASE

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Abstract—Techniques are described for accurate total starch analysis in low starch, physically and chemically difficult conifer tissues, based on starch glucose measurement by glucose oxidase–peroxidase–chromogen, or glucose oxidase–iodide reagent systems. Sample preparation involves acid hydrolysis of the ethanol-insoluble fraction, neuturalization, and enzyme inhibitor removal. Enzyme reagent inhibitors in foliage and buds were tentatively classed as lower molecular weight phenolics derived from polyphenols during preparation, but removable by adsorption on charcoal or alumina. Non-specific carbohydrate reactions with anthrone methods resulted in 250 per cent overestimation of total starch, while seasonal variations in amylose content of twig bark starch of from 45 to 85 per cent resulted in smaller but highly variable errors with iodine staining methods.

INTRODUCTION

IN STUDIES of carbohydrate food reserves in plants, accurate starch determination is of equal importance to that of soluble sugars. Though soluble sugars can be considered as transient in nature, available for immediate metabolic demands, stored starch represents a surplus of photosynthates over current growth and energy requirements. As such, starch levels represent a valuable diagnostic tool in plant physiology. Regrettably, starch analysis of many plant tissues has not markedly improved since Sullivan[1] stated in 1935 that the reason for the numerous starch methods is that few were reliable, even in limited cases.

Whelan[2] recommended only two methods of starch analysis as specific: Steiner and Guthrie's[3] optical rotation method, and Pucher's *et al.*[4] precipitation and acid hydrolysis method. Both required a lengthy series of purification steps, involving the risk of sample loss, which rendered them unsuitable for rapid analysis of small samples of low-starch tissue. The iodine staining method of Neilson[5] and the anthrone method of McCready *et al.*[6] appeared more convenient for rapid analysis but extremely unspecific. McCready and Hassid[7] had adapted the iodine staining reaction for what could be considered its more specific role; the analysis of the amylose component of starch. However, the use of anthrone for total starch appeared suitable only for high-starch content tissues and seriously overestimated it in low-starch, woody tissues with high levels of other interfering polysaccharides and cell-wall contaminants.

[1] J. T. SULLIVAN, *Ind. Engng Chem. Anal. Ed.* **7**, 311 (1935).
[2] W. J. WHELAN, in *Modern Methods of Plant Analysis* (edited by K. PAECH and M. V. TRACEY), Vol. 2, p. 145. Springer-Verlag, Berlin (1955).
[3] E. T. STEINER and J. D. GUTHRIE, *Ind. Engng Chem. Anal. Edit.* **16**, 736 (1944).
[4] G. W. PUCHER, C. W. LEAVENWORTH and H. B. VICKERY, *Anal. Chem.* **20**, 850 (1948).
[5] J. P. NIELSEN, *Ind. Engng Chem. Anal. Edit.* **15**, 176 (1943).
[6] R. M. MCCREADY, J. GUGGOLZ, V. SILVIERA and H. S. OWENS, *Anal. Chem.* **22**, 1156 (1950).
[7] R. M. MCCREADY and W. Z. HASSID, *J. Am. Chem. Soc.* **65**, 1154 (1963).

These problems were overcome, and extraction and purification steps simplified by Keston's[8] glucose oxidase–peroxidase–chromogen system for selective measurement of glucose after acid hydrolysis of ethanol-extracted Soxhlet residues.[9] As no other Douglas-fir polysaccharide appeared to liberate free glucose on mild hydrolysis, this method could also be considered starch-specific.

Glucose oxidase reagents have been extensively used in clinical chemistry[10,11] and to a lesser extent for glucose analysis of hydrolyzates of concentrated plant products.[12–17] Their apparent lack of use in plant biochemistry may be a result of problems similar to those we have encountered in formulating a balanced reagent, and from variable inhibition of the reagent enzymes by plant phenols. Simple inhibitor removal steps have been incorporated in sample preparation procedures, and the glucose oxidase reagent improved by deletion of peroxidase and chromogen in favor of a conventional colorimetric adaptation of the Malmstadt *et al.*[18,19] glucose oxidase–iodide reaction reagent.

These latest procedures have permitted confident analysis of total starch content of small samples of difficult conifer tissues and should be widely applicable.

METHODS

Sample Preparation

Duplicate 100 mg freeze-dried, 40-mesh samples are weighed into thimbles carefully folded from 9 cm Whatman GF/A glass-fibre filter paper. Soluble sugars and the bulk of phenolic substances interfering with starch analysis are removed by extraction in micro-Soxhlet assemblies with 20 ml of 80% ethanol for 4 hr. The extracts may be utilized for analysis of total soluble sugars, as separately described.[20] The thimbles containing the ethanol insoluble residue and polysaccharides are air-dried for immediate use, or filed in 90-compartment polyethylene ice-cube trays in desiccated boxes.

Reproducibility of the method is primarily determined by the degree of recovery of the starch-containing residue, reduction of the residue to a cell-free state to render starch grains rapidly accessible to reagents, and by the minimizing of transfer steps. To commence, carefully unfold the glass paper thimble with forceps. Transfer the residue to a heavy wall, conical, glass to glass tissue grinder (Kontes Glass Co., Vineland, N.J., Duall size C, 15 ml capacity) with the aid of a small, wide stem, seamless aluminium funnel. Sample loss can be

[8] A. S. KESTON, Proceedings, 129th Meeting Am. Chem. Soc., Dallas, Texas, Abst. p. 31C (1956).
[9] L. F. EBELL and B. O. MYHRE, Proceedings, 3rd Meeting Can. Soc. Plant Physiologists, Winnipeg, Manitoba, Abst. p. 19 (1963).
[10] H. U. BERGMEYER and E. BERNT, in *Methods of Enzymatic Analysis* (edited by H. U. BERGMEYER), p. 123, Academic Press, New York (1963).
[11] F. W. FALES and D. SELIGSON, in *Standard Methods of Clinical Chemistry* (edited by D. SELIGSON), Vol. 4 p. 101, Academic Press, New York (1963).
[12] J. P. COMER and H. F. BRICKLEY, *Anal. Chem.* **31**, 109 (1959).
[13] K. TAEUFEL, H. RUTTLOF and R. FRIESE, *Staerke* **14**, 309 (1962); from *Chem. Abst.* **58**, 7016h (1963).
[14] K. TAEUFEL, U. BEHKE and H. WERSUHN, *Nahrung* **9**, 287 (1965); from *Chem. Abst.* **63**, 15082h (1965).
[15] K. R. L. MANSFORD and R. K. OPIE, *Analyst* **88**, 646 (1963); from *Chem. Abst.* **59**, 13002e (1963).
[16] A. G. ROHWER, E. R. HENSCHEL and C. E. ENGEL, *J. Assoc. Off. Agr. Chemists* **48**, 844 (1965); from *Chem. Abst.* **64**, 1270c (1966).
[17] P. THIVEND, CH. MERCIER and A. GUILBOT, *Staerke* **17**, 276 (1965); from *Chem. Abst.* **63**, 15083e (1965).
[18] H. V. MALMSTADT and H. L. PARDUE, *Anal. Chem.* **33**, 1040 (1961).
[19] H. V. MALMSTADT and S. I. HADJIIOANNOU, *Anal. Chem.* **34**, 452 (1962).
[20] L. F. EBELL, Variation in total soluble sugar of conifer tissues with method of analysis. *Phytochem.* **8**, 227 (1968).

restricted to a few particles by systematic tapping and scraping of the thimble. Add 2 ml of 1 N HCl with a bulb-type automatic pipet, and grind the sample for 3–5 min for complete disintegration. To power the pestle, a low-speed, timer-controlled variable-speed stirrer may be used. To rinse the tube walls add 2 ml of acid and continue homogenizing for an additional 2 min; the last 30 sec use a third portion of acid to rinse the pestle as it is withdrawn from the tube.

A 5 ml beaker, set into the grinder tube, acts as a condensing surface. Starch is converted to glucose by a 2-hr hydrolysis in a covered water-bath at 98°.

One ml of a 2 mg/ml glucose standard may be similarly treated with 5 ml of 1·2 N HCl as a check on the possibility of glucose destruction or loss during hydrolysis and subsequent handling. Such losses have ranged from nil to a high of 4·7 per cent. Pirt and Whelan[21] have shown that glucose losses are less with H_2SO_4 than with HCl, but the former acid has not been tested for use with the present procedure.

The hydrolyzed samples are neutralized to pH 7·0 with a pH meter or titrimeter and combination glass-calomel electrode. Thirty per cent NaOH is delivered, either manually or automatically,[22] from a 2 ml micrometer syringe through an 8-in., 24-gauge teflon needle. Natural plant indicators in the residue turn from red to black at about pH 6, and are an aid in neutralization. Effective magnetic stirring may be obtained by cutting a flexible magnetic refrigerator door seal strip to form stirring bars to fit the bottom of the conical grinding tube. It is desirable to avoid overshooting the end point as varying degrees of sugar isomerization may result in alkaline solution.[23]

The stirring bar remains in place with plant tissues having interfering levels of enzyme inhibiting substances, a 0·2 g scoop of powdered bone charcoal is added, and the samples stirred magnetically or vortexed for 20 min. The last 5 min of stirring, the sample is diluted with water to about 25 ml. Stirring is followed by brief centrifuging, with the grinding tubes held by adapters in 100 ml shields in a horizontal rotor. The supernatant is passed through 1-in., 0·45 μ pore size cellulose triacetate membrane filters directly into 50 or 100 ml volumetric flasks held in vacuum filtrators. The solids are washed by resuspending in a further 15–30 ml of water, re-centrifuging, and combining the filtered supernatants. Filtration is aided, without affecting results, by substituting 0·2 N NaCl for water in the washing step. The filter holder is rinsed and the flask contents brought to volume, ready for analysis. Filters of greater than 0·45 μ pore size were found to pass enough charcoal to reduce absorbance of the reaction products, affecting repeatability of results.

Glucose Measurement with Glucose Oxidase–Peroxidase–Chromogen System

For each 100 ml of glucose oxidase reagent, dissolve (1) approximately 150 units of a purified grade of glucose oxidase (i.e. 1·5 mg Nutritional Biochemicals Co. "pure" grade, or equivalent), (2) 4 mg peroxidase (Sigma type II, or equivalent), and (3) 1 ml of *o*-dianisidine-HCl (reagent grade 3,3′-dimethoxybenzidine dihydrochloride, 100 mg dissolved in 10 ml H_2O, kept in dark and refrigerated), in 0·1 M, pH 7·0, phosphate buffer (8·70 g Na_2HPO_4 + 5·30 g KH_2PO_4 made to 1 l. with water).

The reagent may be prepared in quantity for use over a week or more if kept refrigerated and dispensed cold, and reagent blank and standards are included with each set of determinations. Martinek[24] found that a few drops of $CHCl_3$ would stabilize a commercially prepared,

[21] S. Pirt and W. J. Whelan, *J. Sci. Food Agr.* **2**, 224 (1951).
[22] L. F. Ebell, *Anal. Chem.* **37**, 446 (1965).
[23] J. A. Johnson and R. M. Fusaro, *Anal. Biochem.* **13** 412 (1965).
[24] R. G. Martinek, *J. Am. Med. Technol.* **29**, 257 (1967).

clinical glucose oxidase reagent for at least 25 weeks. Instability of the *o*-dianisidine chromogen, as well as microbiological contamination, is a factor limiting reagent stability.

Pipet 1 ml aliquots of water blank, standards and samples into 20 × 150 mm tubes, add 5 ml glucose oxidase reagent from an accurate repetitive dispenser to each tube of the test series; incubate at 37° for 30 min, transfer to a cold water bath and quench reaction by rapid addition of 2 ml of 50% H_2SO_4 from an automatic dispenser; cool tubes and read absorbance at 530 nm against the distilled water-reagent blank. Calculate glucose from a standard curve prepared with 10, 20, 40, 60, 80, and 100 μg/ml glucose standards made up in 0·25% benzoic acid.

Glucose Measurement with Glucose Oxidase–Iodide System

Due to varying stability on dilution or standing, the reagent is made up from three components, easily mixed for each day's analysis.

Component A. Buffer-molybdate catalyst. 41 g KH_2PO_4, 21 g K_2HPO_4, 6·5 g $(NH_4)_6 Mo_7 O_{24} \cdot 4H_2O \cdot$, made to 1 l. with water. Stable for long periods.

Component B. 2 g KI in 100 ml water. Fresh daily or a stock solution is useable for a week or more if protected from photo-oxidation.

Component C. 1000 units purified grade glucose oxidase (i.e. 10 mg Nutritional Biochemicals Co. "Pure" grade, or equivalent). Weigh fresh daily or a stock solution, stable if refrigerated, can be prepared by dissolving in 1 ml of 0·002% thimerosal preservative per multiple weight of enzyme.

Composite reagent. Dissolve component C in a mixture of 100 ml each of component A and component B, and dilute to 250 ml with water. Final buffer strength is 0·15 M, and pH 6·0. Reagent is useable for several days if refrigerated, protected from light, and standards are included with each set of determinations.

Pipet 1 ml of water blank, standards and samples into 20 × 150 mm tubes; add 8 ml of composite reagent from an accurate repetitive dispenser to each tube of the test series, and incubate in the dark at room temperature (25°) for 40 min or until maximum color is developed. Immediately read absorbance at 353 nm against the distilled water-reagent blank. Errors due to slow fading of the reaction products can be held to less than 0·01 absorbance if samples are read within 10–15 min of maximum color development. Calculate glucose from a standard curve prepared with 10, 30, 60, and 90 μg/ml glucose standards made up in 0·25% benzoic acid.

Reagent Notes

Kingsley and Getchell[25] have studied the optimum conditions of pH, incubation temperature and time, and final acid concentration for maximum sensitivity of the glucose oxidase–peroxidase system. Their findings, and the outline by Bergmeyer and Bernt,[10] were adapted for use with plant materials. However, trials of a range of quantities and grades of components and of reaction conditions, in an effort to formulate a balanced glucose oxidase–peroxidase reagent, did not result in a combination giving standard curves that were completely linear for low and high glucose concentrations. Martinek[24] attributed part of the non-linearity problem to interference from catalase, a common contaminant of glucose oxidase. Our observations appeared to implicate the peroxidase and/or the chromogen, and elimination of these is the chief virtue of the glucose oxidase-iodide system.

[25] G. R. KINGSLEY and G. GETCHELL, *Clin. Chem.* **6**, 446 (1960).

With the glucose oxidase–iodide reagent, the hydrogen peroxide formed in the enzymatic oxidation of glucose reacts immediately with excess iodide, in the presence of molybdate catalyst, to produce an equivalent amount of iodine. This iodine further reacts with excess iodide to form iodate which is easily measured at its 353 nm absorption peak to provide quantitative equivalent values of initial glucose.[18,19]

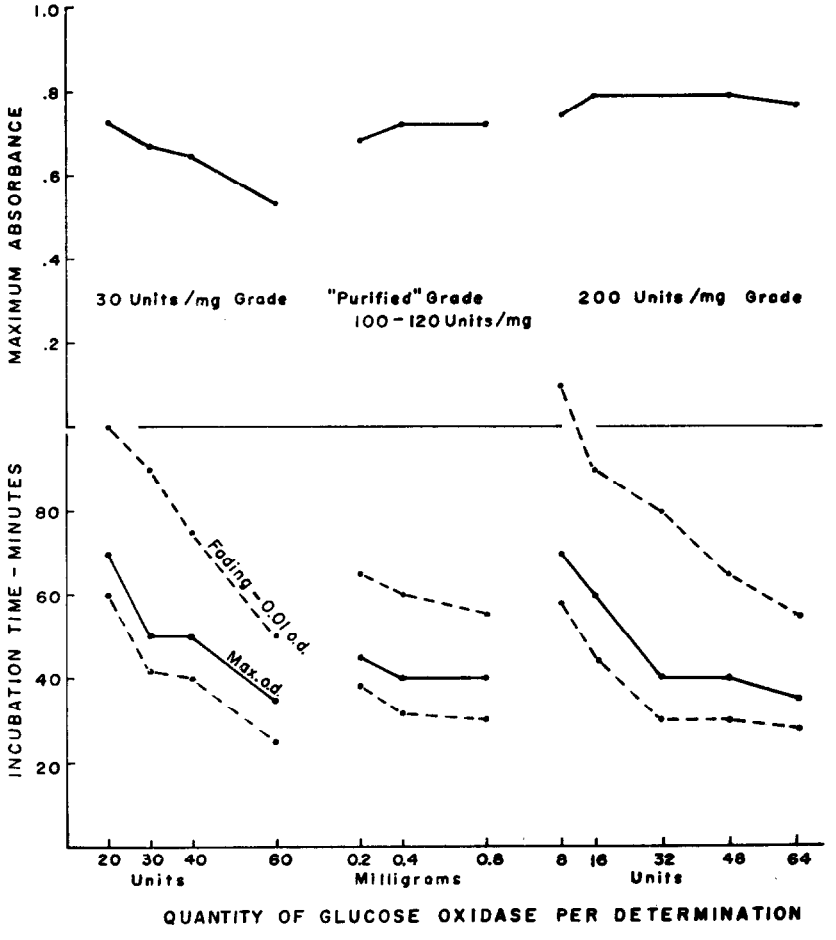

FIG. 1. INFLUENCE OF GLUCOSE OXIDASE QUANTITY AND PURITY ON ABSORBANCE, TIMES FOR MAXIMUM ABSORBANCE, AND TIMES PRIOR TO AND AFTER MAXIMUM FOR Δ0·01 ABSORBANCE.
1 ml (60μg) glucose: 8 ml iodide system reagent reacted at 25°.

Malmstadt and Hadjiouannou[19] optimized their original reagent and conditions to provide fast reaction rates for rapid analysis with their colorimetric reaction rate measurement apparatus. Fading was not a factor influencing results. For adaptation to conventional colorimetry, we found it necessary to reduce reaction rates by halving reagent strength, and by incubating at room temperature. Absorbance of the reagent read against distilled water was reduced from 0·26 to 0·06, and standard curves were obtained which cut the origin at zero and were linear over the entire absorbance scale. Time for maximum color development

was halved by incubation at 40°, compared to 25°, but absorbance was decreased by 20 per cent, and some difficulty in retaining linearity at higher temperatures was experienced.

Reaction times at 25°, with a range of concentrations of three grades of glucose oxidase, are shown in Fig. 1. Optimum amounts (approximately 30 glucose oxidase units per determination) were those giving maximum color in about 40 min. Absorbance values were decreased with increasing amounts of the less pure 30 unit/mg grade. Time for maximum color development was also increased, and standard curves were not linear below 10 μg/ml concentrations of glucose. Though the purer grades are thus to be preferred, the less expensive ones still

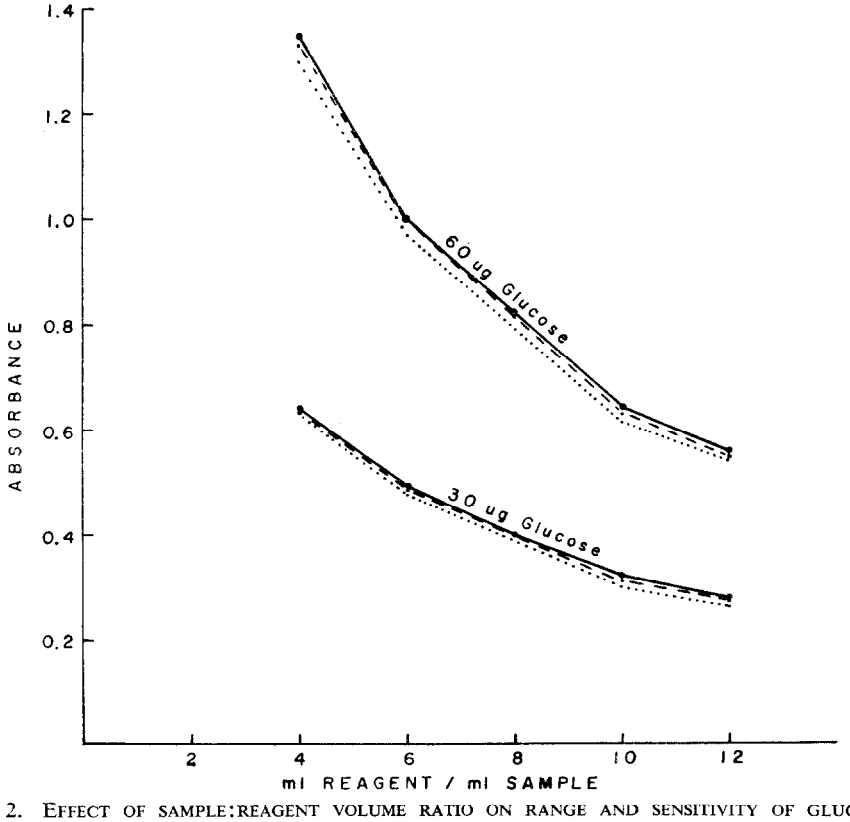

FIG. 2. EFFECT OF SAMPLE:REAGENT VOLUME RATIO ON RANGE AND SENSITIVITY OF GLUCOSE OXIDASE–IODIDE SYSTEM.
Read at: ——— 40 min; - - - - - 50 min; ..... 60 min.

provide highly reproducible results and may be utilized for routine analyses on large numbers of samples.

Both glucose oxidase reagent systems require similar amounts of glucose oxidase for similar reaction rates and color production. Since the glucose oxidase–peroxidase–chromogen reaction with glucose can be stopped before completion by addition of acid, less glucose oxidase can be used and the slope of standard curves varied widely by this means. However, addition of acid to the glucose oxidase–iodide system promotes rapid oxidation of iodide to iodate, resulting in falsely elevated readings.

The simplest means of regulating the slope of the glucose oxidase–iodide system curves is by changing the sample volume: reagent volume ratio. The results of reacting 1 ml of 30

and 60 μg/ml glucose standards in 4, 6, 8, 10, and 12 ml of reagent is shown in Fig. 2. The 1:8 ratio was selected in terms of volume convenience and range of glucose concentrations of our samples. Time for maximum color development was approximately 40 min for all ratios and rate of fading was also similar. All combinations gave linear curves after 15 min reaction time.

Removal of Enzyme Inhibitors

Certain reducing substances, such as ascorbic acid and glutathione, cause a reduction in glucose values by competing with the chromogen as hydrogen donors for peroxidase.[11] Before utilizing the glucose oxidase–peroxidase system for starch analysis of conifer tissues, recovery tests were applied to determine whether inhibitors of the enzyme reagent were present. These inhibitor checks consisted of adding 20–40 μg glucose (as a 1 μg/μl solution) to the 1 ml sample aliquot prior to analysis, by means of a 0·2 ml micrometer pipet.

Complete recoveries of glucose added to twig bark and twig wood hydrolyzates were obtained, and these tissues were confidently analysed without additional treatment. A 10–15 per cent loss of glucose was encountered with 1-yr-old foliage, and loss of glucose added to bud hydrolyzates was found to vary from less than 10 per cent to a more usual range of 20–35 per cent. Since bud starch levels varied seasonally, apparent glucose values before inhibitor removal treatment were depressed by a wider range of from 5 to 70 per cent. Inhibition varied between duplicate extractions of the same sample, indicating varying degrees of inhibitor formation or release due to subtle differences in handling during preparation or time of standing before analysis.

Some indication of the nature of inhibiting substances was gained during trials of various methods of removal. The inhibitors co-eluted with glucose when bud soxhlet residue hydrolyzates were passed through an ion-exchange column consisting of Amberite IRA-400-Na over Amberite IR-120-H. This treatment has been recommended for removal of ascorbic acid and glutathione from urine.[10] Therefore, it is unlikely that inhibitors were similar reducing substances, since those not removed by ethanol extraction should have been removed by such ion-exchange treatment. Inhibitors were soluble in both ethanol and hot water. Only 6 per cent recovery was obtained from a 30 μg glucose addition to aliquots of ethanol soxhlet extracts of the first 2 hr. Recovery rose to 47 per cent in the second 2-hr extraction period but remained at this low level in extract fractions collected between 4–8, and 8–16 hr. Only 5 per cent recovery of added glucose was obtained from 2-hr water soxhlet extraction, which followed 4 hr of ethanol extraction. Recoveries rose to 45, 77, 89, 88, 91 and 94 per cent in succeeding 2-hr water extract fractions. These prolonged extractions indicated that additional inhibiting substances were being continually formed or released. This, together with small decreases in apparent bud starch glucose, ruled out prolonged extraction as a means of inhibitor removal from the soxhlet residue. A deep red color of the hot water extracts, suggestive of oxidized phenolic substances, could be removed by animal charcoal treatment with improvement in glucose recoveries.

Phenolics in ethanol extract fractions and hot water extract fractions chromatographed similarly, except that additional ferric chloride positive substances appeared after prolonged water extraction. Bands eluted from silica gel chromatographs were inhibitory in positions which corresponded to some of these phenolics. Inhibition from bud extract chromatographs were strongest at the solvent (80% ethanol) front, whereas inhibitors from bud soxhlet residues were also present in bands from R_f 0·2–0·5. Substances remaining at the origin were not inhibitory. These observations suggest that glucose oxidase reagent inhibitors of

the present procedure are lower molecular weight phenolics derived from polyphenols during hydrolysis of the soxhlet residue. Interference from phenolics is further supported in that there is an almost total inhibition of glucose oxidase reagent by tannin-rich tea extract, and by the problems caused by phenolic substances in the isolation of plant enzymes from disrupted tissues.[26–28]

Various amounts of potential inhibitor adsorbants were shaken with 10 ml of filtered, made to volume, soxhlet residue hydrolyzates of a May bud sample. Recoveries of 20 μg glucose additions were then determined on the filtrates. The results of three test series are shown in Table 1. Our initial interest in the glucose oxidase–iodide system was as a means

TABLE 1. PERCENT APPARENT STARCH GLUCOSE AND PERCENT RECOVERY OF 20 μg ADDED GLUCOSE FROM A MAY DOUGLAS-FIR BUD SAMPLE; ON ANALYSIS WITH GLUCOSE OXIDASE–IODIDE OR –PEROXIDASE SYSTEMS; AFTER VARIOUS INHIBITOR REMOVAL TREATMENTS APPLIED TO 10 ml OF MADE TO VOLUME HYDROLYZATES. MEANS OF DUPLICATE TREATMENTS FROM SEPARATE HYDROLYZATES FOR EACH SERIES

Inhibitor removal treatment	% Starch glucose		% Glucose recovery	
	Iodide	Peroxidase	Iodide	Peroxidase
Series A				
Untreated	2·05	2·01	80	78
400 mg charcoal	2·47	2·43	103	96
400 mg alumina	2·51	2·52	106	96
Series B				
Untreated	1·71		86	
50 mg charcoal	2·50		99	
100 mg charcoal	2·54		100	
50 mg alumina	2·44		98	
100 mg alumina	2·60		96	
50 mg insoluble PVP	2·17		95	
100 mg insoluble PVP	2·19		93	
Series C Mean of				
10 hydrolyzates untreated	1·77 ± 0·24*		—	
100 mg charcoal	2·50 ± 0·05*		99·7 ± 2·4*	

* Standard deviation.

of assessing the relative inhibition of the two enzymes of the glucose oxidase–peroxidase system. Inhibition of the glucose oxidase–iodide reagent was only slightly, but consistently, less than with the glucose oxidase–peroxidase reagent. The slight peroxidase inhibition that was indicated provided further justification for its substitution by iodide, for measurement of H_2O_2 formed in enzymatic oxidation of glucose.

Both charcoal and basic alumina were efficient adsorbants of inhibiting substances. However, glucose recovery from alumina treated hydrolyzates was more variable at a range of concentrations, and in other tests showed a tendency to elevate apparent glucose. In addition, spectra of treated and untreated bud hydrolyzates (Fig. 3) showed that charcoal removed more of the u.v. absorbing substances which appeared to be associated with poor glucose recoveries. For these reasons charcoal was selected for use in the final procedure.

[26] J. L. GOLDSTEIN and T. SWAIN, *Phytochem.* **4**, 185 (1965).
[27] J. D. JONES, A. C. HULME, and L. S. C. WOOLTORTON, *Phytochem.* **4**, 659 (1965).
[28] W. D. LOOMIS and J. BATTAILE, *Phytochem.* **5**, 423 (1966).

Although insoluble polyvinylpyrollidone (PVP) (Polyclar AT) has been shown by Loomis and Battaile[28] to strongly adsorb phenolic substances, thereby permitting isolation of plant enzymes, it was not applicable in the present procedure. Loomis and Battaile mention that some phenolic compounds do not form strong H-bonded complexes with PVP, and may remain in extracts as latent inhibitors. Of the three treatments, PVP was the least efficient for removal of u.v. absorbing substances. The lower apparent glucose values after PVP treatment appeared to be related to effect on the glucose oxidase reagent itself rather than to adsorption of sugars, as confirmed by trials with glucose standard solutions and anthrone reagent.

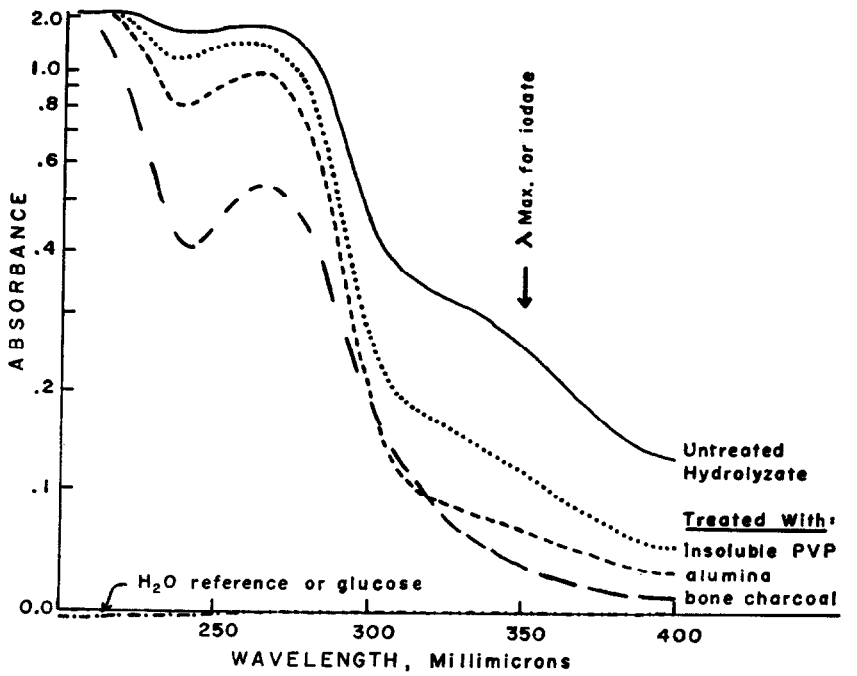

FIG. 3. CHANGE IN U.V. SPECTRA OF MADE TO VOLUME BUD SOXHLET RESIDUE HYDROLYZATE AFTER TREATMENT WITH 100 mg INHIBITOR REMOVAL ADSORBANTS PER 10 ml.

Without inhibitor removal there was an unaccountably poor correspondence between apparent starch glucose and recovery of added glucose. An example of this is shown in Table 1, by comparison of Series A and Series B, untreated hydrolyzates of the same bud sample. In Series C, charcoal treatment, compared to no inhibitor removal treatment, resulted in complete recoveries, elevation of apparent glucose from 1·8 to 2·5 per cent of dry bud weight, and a five-fold improvement in reproducibility of starch glucose values. The 95 per cent confidence limit for reproducibility after charcoal treatment of this bud sample was 4·5 per cent of the mean value.

Whole conifer buds were found to be the most difficult tissue to analyse for starch glucose. Reproducibility of the final procedure in routine analysis of twig tissue of another conifer, *Abies grandis*, is summarized in Table 2. Standard deviation and the F-test for comparison

TABLE 2. REPRODUCIBILITY OF STARCH GLUCOSE IN *Abies grandis* FOLIAGE AND TWIG AXIS SAMPLES ANALYSED BY GLUCOSE OXIDASE–IODIDE SYSTEM

Statistic	Foliage	Twig axis (wood, bark, buds)
No. of samples, duplicate analysis	34	34
Range of starch glucose (% of dry weight)	1·18–6·98	1·61–6·80
Overall mean	3·88	3·33
Standard Deviation $S=\frac{\Sigma\text{ (differences between duplicates}^2)}{\text{Total No. of determinations}}$	0·076	0·051
Coefficient of variation (C.V.)	1·95%	1·52%
95% limit ($t=2{\cdot}03+$C.V.)%	$\bar{x}\pm3{\cdot}96\%$	$\bar{x}\pm3{\cdot}09\%$
$F=\frac{\text{Variance of foliage duplicates}}{\text{Variance of twig duplicates}}=$	2·24	(Significant difference at 95% level)

of results from foliage and twig axis samples analysed in duplicate were calculated according to methods presented by Henry and Dryer.[29] These samples included 1–4-yr-old tissue, and were from nine sets of determinations conducted over a period of several weeks. Thus, the data indicate maximum variability rather than a more limited within-run variability that can be expected from the method. The 95 per cent limits predict a better than 4 per cent agreement between duplicate determinations of either tissue. Reproducibility was significantly better for twig axis (wood, bark, buds) samples than for foliage samples, which in turn appeared better than that found for Douglas-fir buds. In practice, starch glucose values were accepted when duplicate determinations were in agreement by 5 per cent or better. Only two repeat determinations were found necessary in analysis of 98 *A. grandis* foliage and twig axis samples.

Between Method Variation in Conifer Starch Levels

As previously discussed, enzyme inhibitors encountered in Soxhlet residue hydrolyzates of conifer tissue appeared to be easily removed by adsorption on charcoal. Some reservations, however, might be properly expressed over the use of an enzymatic method for glucose measurement in tissues in which variable inhibitor levels may be a potential threat to accuracy and precision. Results from preliminary trials comparing use of glucose oxidase and practical alternative methods, given in Table 3, showed that such alternatives were not applicable with conifer tissue. The same extract from each of the three preparation methods was used for each measurement method, with the addition of a HCl hydrolysis step on a portion of the perchloric acid extracts for glucose measurement with glucose oxidase.

Values from anthrone reagent, used similarily to McCready *et al.*,[6] and the chromogenic acid reagent for hexose sugars of Klein and Weissman,[30] were variable and unrealistically high, due to non-specific reaction with cell-wall polysaccharides in addition to starch glucose. Bennet[31] observed that perchloric acid in the concentrations used for starch extraction also acted as a non-selective solvent for cornstalk hemicellulose, removing variable amounts.

[29] R. J. HENRY and R. L. DRYER, in *Standard Methods of Clinical Chemistry* (edited by D. SELIGSON), Vol. 4, p. 205, Academic Press, New York (1963).
[30] B. KLEIN and M. WEISSMAN, *Anal. Chem.* **25**, 771 (1953).
[31] E. BENNET, *Plant Physiol.* **30**, 562 (1955).

The cold perchloric acid extraction and iodine staining method of Neilson,[5] using potato starch standards, corresponded most closely to starch values by the proposed glucose oxidase method. With both methods, fine grinding with the extracting solvent, to assure intimate contact and mixing with the substrate, was found essential for good results and reduction of variability. Results also varied with closeness of fit of less efficient glass to glass grinders used in this early work. Under the microscope, Douglas-fir starch grains dissolved within 2 min of exposure to 4·7 N perchloric acid. However, quantitative extraction from glass-ground woody tissues proved difficult, in contrast to the ease of extraction reported for soft, high starch content tissues.[5,6] Four 5-min grindings with 2-ml portions of 4·7 N perchloric, with water rinses of the glass pestle, centrifuging, and each supernatant made to volume and analysed separately, extracted 71, 15, 8 and 6 per cent respectively, of the total starch value of 8·1. Four extractions with 7·2 N perchloric yielded 80, 13, 7 and 0 per cent respectively, of a larger total starch value of 9·0. Similarly, Humphries and Kelly[32] found optimum perchloric acid concentration to be 7·2 N for starch extraction from 200-mesh eucalyptus wood.

If iodine color, read against amylose standards at 700 nm to minimize amylopectin interference, is taken as a measure of amylose content, and results by glucose oxidase accepted as total starch, the proportion of amylose in Douglas-fir bark starch would be 60–70 per cent. Since potato starch contains only approximately 20% amylose,[7] its use as a standard would result in the overestimation by the iodine method shown in Table 3. Cashen and

TABLE 3. PERCENT STARCH IN ETHANOL INSOLUBLE PORTION OF DOUGLAS-FIR TWIG BARK AND COEFFICIENTS OF VARIATION (% OF MEAN OF SIX DETERMINATIONS) BY VARIOUS PREPARATION AND MEASUREMENT METHODS

Preparation method	Measurement method				
	Iodine staining				
	Amylose standards (700 mu)	Potato starch standards (590 mu)	Glucose* oxidase-peroxidase	Anthrone*	Chromotropic* acid
Hydrolysis—I N HCl,					
40-mesh sample	0·0	0·0	6·6±14	15·7± 6	11·3±14
Glass-ground sample	0·0	0·0	6·8± 1	—	—
Two extractions 4·7 N $HClO_4$ 40-mesh sample	3·3±6	6·3±5	4·8±14	12·4±12	12·4±11
Four extractions 7·2 N $HClO_4$ Glass-ground sample	4·4±3	9·4±3	7·6± 3	—	—

* Glucose found multiplied by 0·90 for conversion to starch.

Friloux[33] have listed reasons why percent deviation between amylose present in a standard starch and sample starch may not coincide with differences in starch values between the methods employed.

The amylose:amylopectin ratio is not necessarily fixed within a species, as assumed in methods employing correction factors for relative iodine staining capacity.[32–34] McCready *et al.*[6] found the proportion of amylose to increase in peas as they mature, and Cashen and

[32] F. R. HUMPHREYS and J. KELLY, *Anal. Chim. Acta* **24**, 66 (1961).
[33] N. A. CASHEN and J. J. FRILOUX, *Agri. Food Chem.* **14**, 434 (1966).
[34] J. P. NIELSEN and P. C. GLEASON, *Ind. Engng Chem. Anal. Edit.* **17**, 131 (1945).

Friloux reported a range of 22–52 per cent amylose in starch of sugar cane juice, depending upon variety, season and stage of development. Likewise, amylose content of Douglas-fir twig bark has been found to vary seasonally as shown in Fig. 4, from a low of 45 per cent to

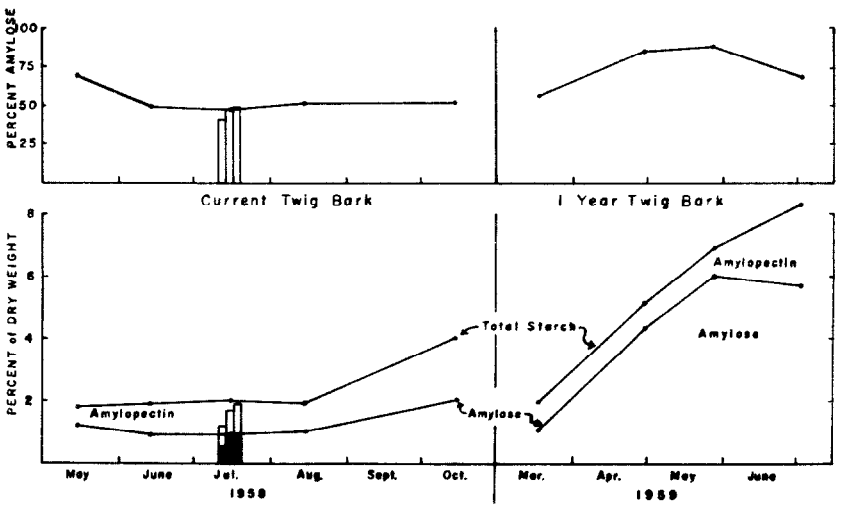

FIG. 4. SEASONAL CHANGES IN AMYLOSE AND AMYLOPECTIN IN DOUGLAS-FIR TWIG BARK STARCH. Histograms compare mid-July values for 1-yr, 2-yr, and 3-yr twigs.

a high of 85 per cent of the total starch. Amylose content of starch of current 1-, 2-, and 3-year twig bark, sampled in mid-July, was found to be very similar. This suggests that less variation may be expected between various tissues at various phases of starch accumulation or utilization. Such changes in composition of starch can be predicted to be universal in plants, invalidating iodine colorimetric techniques for immature tissues.

More specific starch methods are all based on use of the component glucose as an indirect standard, since starch glucose does not change with starch composition. Utilization of glucose oxidase for glucose measurement in the acid hydrolysed, ethanol insoluble fraction, provides further specificity and reasonable convenience for analysis of total starch. The range of applicability and accuracy of the proposed method is limited only by a need for detection and adequate removal of any enzyme inhibitors, such as encountered in conifer foliage and buds.