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PHOTOSYNTHETIC STUDIES ON *ULVA LACTUCA**

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Abstract—The photosynthetic fixation of $^{14}CO_2$ was studied in *Ulva lactuca*. Sucrose was the most highly labelled sugar, although glucose, fructose, xylose and *myo*-inositol also incorporated radioactivity. Starch and the sulphated polysaccharide were extracted and the radioactivity of each was measured, as was that of the constituent sugars of the latter. The residue, after ethanolic and aqueous extraction of the weed, was completely hydrolysed and the activity of the derived sugars was measured. Glucose was the major sugar and had the highest radioactivity. Some conclusions on the biosynthetic pathways of the polysaccharides have been drawn.

INTRODUCTION

FEW PHOTOSYNTHETIC studies with ^{14}C as the carbon source have been carried out on seaweeds. Previously Bidwell[1] and Craigie *et al.*[2] had reported that, after growing *Ulva lactuca*, *Cladophora melagonium*, *Monostroma fuscum* and *Entromorpha intestinales* for 2 hr in $^{14}CO_2$, the low molecular weight carbohydrate most highly labelled was sucrose and that a relatively small amount of radioactivity was also incorporated into glucose and fructose. A considerable amount of radioactivity was also incorporated into the constituents which were insoluble in 80% ethanol. After ethanolic extraction these authors simply hydrolysed the residual weeds and measured the activity of the various amino acids and sugars present in the hydrolysates. Glucose was the most highly labelled sugar in all the solutions, rhamnose came next and then xylose. Since there are at least two glucans and a sulphated polysaccharide in this residue, no conclusions regarding the individual polysaccharides can be drawn from these studies.

In view of our recent structural studies on the water-soluble polysaccharides of *U. lactuca*,[3] it was decided to repeat the culture of this weed in $^{14}CO_2$ medium, to extract and fractionate, where possible, the different polysaccharides and to measure the radioactivity incorporated, not only in the low molecular weight carbohydrates, but also in the different polysaccharides and in their constituent sugars.

While this work was in progress, Patil and Joshi[4] were also studying the photosynthesis of this weed. They cultured it for 6 hr with ^{14}C and removed samples for analysis after 10 and 30 sec, 5 and 30 min and 1 and 6 hr. These authors also found sucrose to be the highest labelled free sugar after short periods of growth, but after 1 hr glucose had incorporated the most radioactivity and this was replaced by fructose after 6 hr.

Patil and Joshi[4] again made no attempt to extract the polysaccharides and examined only the hydrolysates of the residual material from the last four samples. They found that

* Part I in a projected series.

[1] R. G. S. BIDWELL, *Can. J. Bot.* **36**, 337 (1958).

[2] J. S. CRAIGIE, J. MCLACHLAN, W. MAJAK, R. G. ACKMAN and C. S. TOCHER, *Can. J. Bot.* **44**, 1247 (1966).

[3] (a) Q. N. HAQ and E. PERCIVAL, in *Some Contemporary Studies in Marine Science* (edited by H. BARNES), p. 355, Allen & Unwin, London (1966); (b) Q. N. HAQ and E. PERCIVAL, *Proc. 5th Int. Seaweed Symposium, Halifax, Nova Scotia* (edited by E. G. YOUNG and J. L. MCLACHLAN), p. 261, Pergamon Press, Oxford (1965); and references cited therein.

[4] B. S. PATIL and G. V. JOSHI, *Botanica Marina* **XIII**, 111 (1970); *ibid.* **XIV**, 22 (1971).

after 5, 30 and 60 min and 6 hr 33·6, 31·9, 37·8 and 50·0% respectively of the radioactivity in this insoluble residue had been incorporated into the carbohydrates. Again the glucose in the hydrolysate was found to incorporate the most radioactivity and this rose steadily during the period of growth, as did that of glucuronic acid. The quoted figures for rhamnose and xylose are difficult to correlate. Rhamnose had its highest activity after 5 min growth and then dropped 1·5× in 30 min and halved again in 1 hr and rose slightly in 6 hr. Xylose dropped 6-fold from the 5 min to the 30 min growth periods and increased threefold after 1 hr and then dropped again in 6 hr. The authors make no comment about these changes.

RESULTS AND DISCUSSION

In the present study *U. lactuca* fronds were allowed to photosynthesize in the presence of $NaH^{14}CO_3$ for 10 min (A) and for 3 hr (B). After 10 min, nearly half the radioactivity incorporated was in the alcohol soluble fraction, that is in the low molecular weight materials, whereas after 3 hr 64% was present in the water-soluble and insoluble compounds (Table 1) indicating that the polymeric materials are built up from the low molecular weight substances.

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN VARIOUS FRACTIONS

Time in $NaH^{14}CO_3$	dpm × 10^{-4} 80% Ethanol-soluble	Hot water-soluble	Residue
After 10 min	7830 (48·1%)	2420 (14·9%)	6020 (37%)
After 3 hr	31 350 (35·5%)	13 670 (15·5%)	43 070 (49%)

The alcohol soluble fraction in each experiment was separated on resin into charged and neutral substances. The proportion of radioactivity in the charged fraction was higher after 10 min than it was after 3 hr indicating that many of the charged materials are probably synthesised before the neutral compounds. The neutral substances were separated by paper

TABLE 2. TOTAL RADIOACTIVITY IN THE NEUTRAL COMPONENTS IN THE 80% ETHANOL-SOLUBLE FRACTION

Time	dpm × 10^{-4} Neutral extract	Oligo-saccharide	*Myo*-inositol	Sucrose	Glucose	Fructose	Xylose	Ribose	Unknown
10 min	3159	366	25	1437	720	429	47	73	53
3 hr	21 690	9979	499	10 540	3666	3146	521	933	132

chromatography and identified (Table 2). In agreement with the results of earlier workers,[1,2] sucrose was the most highly labelled free sugar, and contrary to the findings of Patil and Joshi[4] this was the case after 3 hr. Glucose was the next highly labelled sugar, closely followed by fructose. Unidentified oligosaccharides also incorporated a high proportion of radioactivity. Less highly labelled was xylose and a tentatively identified ribose. *Myo-*

inositol had incorporated very little activity after 10 min, but this increased 20-fold in the 3 hr compared with a 7-fold increase in sucrose (Table 2).

The hot water-soluble extract, which contained 15% in (A) and 15·5% in (B) of the total radioactivity incorporated, comprised starch, sulphated glucuronoxylorhamnan and a certain amount of protein together with some low molecular weight substances which were lost during dialysis (Table 3). Also during the dialysis a small precipitate formed inside the sac and this was separated and its activity measured (Table 3); it contained little carbohydrate and was not examined further.

TABLE 3. TOTAL RADIOACTIVITY PRESENT IN THE VARIOUS CONSTITUENTS OF THE HOT-WATER EXTRACTS

Time	Total extract	Dialysate	Precipitate in sac	Polysaccharide	Starch	Glucurono-xylorhamnans
			dpm × 10^{-4}			
10 min	2400	1270	326	779	354	249
3 hr	13 670	2575	2307	8948	1745	5593
	Weight (as carbohydrate mg)			478	*ca.* 12	466
			Specific activity/mg carbohydrate			
10 min					29	0·5
3 hr					145	12·0

After measurement of the radioactivtiy in the solution recovered from the dialysis sac, the starch was destroyed by α-amylase. It was found in the 10 min experiment that the proportion of radioactivity remaining in the glucuronoxylorhamnan was lower than that of the starch. After 3 hr the acitvity in the starch had increased 5-fold and that in the glucuronoxylorhamnan 22-fold (Table 3). Considering that the proportion of starch is only about 1·5% of that of the glucuronoxylorhamnan,[3b] it can be deduced from these facts that the starch is synthesized very rapidly and also probably transformed into other products whereas the glucuronoxylorhamnan is synthesised more slowly and laid down as a permanent storage product under the conditions of these experiments.

TABLE 4. PROPORTIONS AND RADIOACTIVITY IN THE SUGARS IN THE POLYSACCHARIDE

Time	Glucose	Xylose	Sugar Rhamnose	Glucuronic acid	Oligosaccharide
			Relative proportions		
	1	4·55	5·7	2·28	—
			dpm × 10^{-4}		
10 min	49·4	38·6	123·7	28·9	8·98
3 hr	313·2	1376	2417	1102	391·4

Before we consider the radioactivity in the individual sugars in the sulphated polysaccharide, the molar proportions of the sugars (Table 4) should be considered and the fact that glucuronosyl-rhamnose forms a major structural feature of this polysaccharide and is probably the major constituent of the oligosaccharide fraction (Table 4). The proportion of rhamnose to glucuronic acid is roughly 2:1. It is surprising therefore that the activity in the rhamnose is nearly 5 times that of the glucuronic acid after 10 min photosynthesis and after 3 hr this has increased about 20-fold in the rhamnose and 40-fold in the glucuronic acid

bringing the specific activity of each to about the same level (Table 5). It is tempting to conclude from this that the rhamnose is laid down first and the glucuronic acid transferred to the polysaccharide later.

TABLE 5. SPECIFIC RADIOACTIVITY IN THE SUGARS IN THE POLYSACCHARIDE

Time	Glucose	Xylose	Rhamnose	Glucuronic acid
10 min	49·38	48·5	21·7	12·7
3 hr	313·2	302	422	483

Very little activity is incorporated into the xylose in the first 10 min but this increases at about the same rate as the glucuronic acid in the 3 hr, supporting the evidence from structural studies of the mutual linkage of glucuronic acid and xylose. Glucose only comprises 1 part in about 14 in this polysaccharide. That it is not derived from starch which has resisted enzymic attack has been proved from the structural studies[5] and evidence that it is 1,3-linked has also been obtained. Although all attempts at fractionation have failed, no conclusive evidence has been obtained that glucose is part of the macromolecule of the sulphated polysaccharide. It is more highly labelled than the other sugars after 10 min growth and the activity only increased 6-fold after 3 hr indicating a slower rate of incorporation of radioactive glucose into the polysaccharide than for the other sugars. From this it might be tentatively deduced that a separate polysaccharide is being synthesized, and, degraded during the time of the experiment.

The residual material after aqueous extraction contained 37% of the total radioactivity after 10 min and 49% after 3 hr. In order to ensure complete hydrolysis of the polysaccharides in this residue, it was treated with 72% sulphuric acid before hydrolysis of the derived fragments with N sulphuric acid at 100°. A proportion of the constituents are inevitably lost during this drastic treatment so that the results on this material cannot be regarded as absolutely quantitative. Somewhat less than half of the radioactivity was present in charged materials (amino acids, perhaps a little undegraded glucuronic acid, etc.) after 10 min growth and this was less after 3 hr. The sugars contained about 23 and 33% of the total activity in the alga after 10 min and 3 hr growth respectively showing that the polysaccharides in the residue are being fairly rapidly synthesized.

TABLE 6. RADIOACTIVITY IN THE HYDROLYSATE AND DERIVED SUGARS FROM THE RESIDUE

			dpm × 10^{-4}			
Time	Total radioactivity	Neutral fraction	Glucose	Mannose	Xylose	Rhamnose
10 min	6024	3780	2510	877	220	174
3 hr	43 070	29 500	22 350	3894	1357	1917

Glucose was the major sugar and had the highest radioactivity both after 10 min and 3 hr. This activity had increased almost 10-fold in the 3 hr sample (Table 6). Mannose, xylose and rhamnose were also detected in the hydrolysates (Table 6). The extent of their labelling was

[5] E. PERCIVAL and R. H. MCDOWELL, *Chemistry and Enzymology of Marine Algal Polysaccharides*, p. 179, Academic Press, New York (1967).

in that order and this increased roughly 4-, 6- and 10-fold respectively in the 3 hr. The rhamnose and xylose probably originated from residual sulphated polysaccharide, not extracted by the hot water. Since the extent of their labelling after 3 hr appeared to be lower than that of the water-soluble material, it is probable that a high proportion of this insoluble fraction was laid down earlier in the cell-wall.

This is the first time that mannose has been reported in this alga.

EXPERIMENTAL

Material. The alga, *Ulva lactuca*, was collected at Point Pleasant, Halifax, Nova Scotia, Canada, in August 1969. It was freed from epiphytes and stored at 15° in filtered seawater overnight.

Experimental conditions. (A) *Ulva lactuca* fronds (50 g, blotted weight) was placed in 1500 ml filtered, sterile seawater at 18° and illuminated for 15 min. $NaH^{14}CO_3$ (1·0 mCi) was added and the sample illuminated for a further 10 min. It was then taken out of the medium, rinsed thoroughly in fresh seawater and pulverized in liquid nitrogen. (B) Fronds (50 g as for A) were treated similarly except that the volume of seawater was 6·0 l., containing 2·0 mCi $NaH^{14}CO_3$ and the illumination period was 3 hr.

General methods. Evaporations were carried out at 40° under reduced pressure. Paper chromatography was performed on Whatman No. 1 paper in (a) EtOAc–HOAc–HCO_2H–H_2O (18:3:1:4), (b) *n*-BuOH–pyridine–H_2O (6:4:3). The sugars were detected with aniline oxalate spray, and reducing as well as non-reducing compounds with $AgNO_3$–NaOH.[6] The sugars in the ethanolic extracts and in the hydrolysates of the polysaccharides were identified by PC and GLC.

Radioactivity was measured by Liquid Scintillation counting in a Tracerlab Coru-matic machine. The radioactivity of solutions were measured by spotting a measured aliquot on a filter paper (2 × 4 cm) and this was placed in a vial containing the scintillant (PPO–POPOP-toluene). Sugars and alcohols were located on paper chromatograms by means of guide strips and the corresponding areas cut out and placed in a vial as for solutions and counted as previously described.

GLC. GLC of the sugars and their respective alcohols was carried out after conversion into their respective trimethylsilyl ethers (TMS derivatives). The following columns were used (1) 7·5% Apiezon K coated on acid and alkali washed, HMDS treated, Gas Chrom W. at 175° (2) 3% SE 30 on HMDS treated Celite at 162°. Quantitative GLC was performed on column (1) of the TMS-derivatives of the sugar-alcohols. The peak areas were measured, and the amount present found from appropriate standard graphs.[7]

Reduction of the sugars was carried out with 2% KBH_4. The reaction was left overnight, excess borohydride was then destroyed with Amberlite IR 120 (H^+) resin and boric acid removed by evaporation with methanol.

Extraction of the Alga. The finely ground powder was extracted with 80% EtOH, by refluxing for 6 hr (3 times), followed by exhaustive extractions with hot water on a boiling water-bath in an atmosphere of CO_2. The residue after the aqueous extractions was completely hydrolysed with 72% H_2SO_4.[8] The radioactivities in the different fractions were measured (Table 1).

80% *Ethanol-soluble fraction.* The combined extracts were deionized with Biodeminrolite resin in carbonate form, to remove amino acids, sugar phosphates and other charged compounds. The constituents of the derived neutral fraction were analysed by PC and GLC.

Hot-water extract. The combined extracts were dialysed against a closed system and the activity of the dialysate measured. During the dialysis a precipitate (3·4 mg carbohydrate in A and 16·7 mg carbohydrate in B) was deposited in the dialysis sac. This was removed before freeze-drying the polysaccharide (805 mg, carbohydrate content 50%). Previous studies had shown that the latter was a mixture of starch and sulphated glucuronoxylorhamnans.[3] After measurement of the radioactivity the starch was destroyed by treatment of a solution of the mixture with α-amylase. The resulting solution was then dialysed against a closed system. The activity in the dialysate (derived from the degraded starch) and in the solution inside the dialysis sac were measured (Table 3). The glucuronoxylorhamnan in the latter was recovered by freeze-drying.

Because the glucuronic acid is highly degraded during normal acid hydrolysis, the polysaccharide was first given a mild hydrolysis with 2·25% oxalic acid at 100° for 4 hr. After neutralization, the derived neutral and acidic oligosaccharides were separated on Amberlite IR-45($HCOO^-$) resin and eluted respectively with water and M-formic acid. The acidic fraction was converted into its methyl ester methyl glycosides and then reduced to the corresponding sugars. After further separate hydrolysis of the neutral and of the reduced acidic fractions with 90% formic acid, aliquots of the derived syrups were subjected to PC in solvent (a) and the radioactivity present in each sugar was measured. Separate aliquots were reduced and the ratio of the derived alditols present was determined by GLC. The results obtained from the two fractions correlated with

[6] W. E. Trevelyan, D. P. Procter and J. S. Harrison, *Nature, Lond.* **166,** 444 (1950).
[7] P. G. Johnson and E. Percival, *J. Chem. Soc.* **906** (1969).
[8] J. D. Blake and G. N. Richards, *Carbohy. Res.* **14,** 375 (1970).

the carbohydrate content of each fraction were combined to find the ratio of the sugars present in the polysaccharide and the radioactivity present in each sugar (Table 4). The relative specific radioactivity per sugar unit was calculated (Table 5).

Residual material. After hydrolysis the radioactivity of the hydrolysate was measured. The solution was then deionized with Biodeminrolite resin in carbonate form and an aliquot of the derived neutral syrup was examined as for the hydrolysate of the water-soluble polysaccharide (Table 6). Glucose was the major sugar in this hydrolysate.

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Key Word Index—Ulva lactuca; Ulvaceae; biosynthesis; polysaccharides; photosynthesis; incorporation of $^{14}CO_2$.