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RADIOACTIVITY DETECTION SYSTEM WITH A CaF_2 (Eu) SCINTILLATOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative ^{14}C detection system with solid CaF_2 (Eu) scintillator was applied to the analysis of aqueous metabolites by high-performance liquid chromatography. CaF_2 (Eu) is an excellent scintillator compared with lithium glass, with a high efficiency for ^{14}C counting and a low background rate. Counting efficiencies of the detector with CaF_2 (Eu) for ^{14}C are approximately 57% in the absence of orthophosphate and 54% in the presence of 50 m*M* phosphate in the effluent, while those with lithium glass are 20% and 16%, respectively. The CaF_2 scintillator is chemically stable without any adsorption of salts or metabolites tested. Calibration on the CaF_2 system established a rapid, reliable and quantitative system for the determination of free sugars in the study of photosynthate partitioning and carbohydrate metabolism in green plants.

INTRODUCTION

Two-dimensional paper chromatography[1], paper electrophoresis[2] and ion-exchange chromatography[3] have been widely used for the separation of a variety of intermediates, especially for metabolic studies on photosynthesis in green plants. Recent advances in high-performance liquid chromatography (HPLC) have provided great advantages in rapid separations of these compounds with excellent resolution.

Simultaneous measurements with solid scintillators of liquid chromatographic separations of ^{14}C-labelled compounds have been performed in several laboratories. The system with a lithium glass scintillator has been well characterized[4] and applied to various metabolites in aqueous solution, *e.g.*, phosphate esters[5], amino acids[6] and organic acids[7]. Recently, Albro *et al.*[8] reported a simultaneous monitoring system equipped with a flow cell packed with CaF_2. However, the results were preliminary and the radiolabelled compounds used were in non-aqueous systems.

This paper describes a quantitative radioactivity detection system equipped with europium-impregnated CaF_2 scintillation material for the separation of compounds in aqueous solution by HPLC. The inert scintillator is shown to be superior to the lithium glass scintillator for the efficient and reproducible monitoring of ^{14}C

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β-radiation, the scintillation efficiency of solid CaF_2 (Eu) being approximately four times greater than that of the glass scintillator[9–11]. The basic characteristics of the CaF_2 (Eu) cell determined by using ^{14}C-labelled glucose and nucleotides as standard samples are reported.

The paper also deals with the rapid determination of free sugars produced during photosynthetic $^{14}CO_2$ fixation in green plants. The system would be useful for studies of the regulation of ^{14}C-photosynthate partitioning and carbohydrate metabolism in plants.

EXPERIMENTAL

Procedures for HPLC

Free sugars were determined with a Toyo Soda (Tokyo, Japan) HLC-803 HPLC system fitted with a TSKgel NH_2-60 column (30 cm × 4.0 mm I.D.) using 65% aqueous acetonitrile as the eluent at a flow-rate of 0.7 ml/min, unless indicated otherwise. Carbohydrate peaks were detected with a Model RI-8 differential refractometer. For the separation of nucleotides, anion-exchange chromatography was performed with a TSKgel DEAE-2SW column (30 cm × 4.0 mm I.D.) using 50 m*M* potassium phosphate (pH 6.0) in 25% (v/v) acetonitrile as the eluent at a flow-rate of 0.7 ml/min, unless indicated otherwise. The peaks were monitored with a Model UV-8II UV detector at 254 nm. In all experiments the column temperature was kept constant at 30°C and the eluents were degassed by continuous bubbling with helium gas.

Apparatus for analysis system

Fig. 1 shows the experimental arrangement for HPLC and a schematic diagram of the electronics for measuring radioactivity. The eluate from the HPLC column and the refractometer (10-μl flow cell) or UV detector (10-μl flow cell) passed through a solid scintillator cell. The flow cell (8 cm × 2 mm I.D.) was packed with CaF_2 (Eu) particles (150–250 mesh). In some experiments, the CaF_2 was replaced with lithium glass particles (about 150 mesh). The flow cell–photomultiplier tube assembly was suitably fixed and shielded to decrease the radiation background. The photons from the solid scintillator were divided equally between two Philips PM2232B photomultiplier tubes (2 in. diameter). The bias supplies for the photomultipliers with CaF_2 and glass scintillators were 880 and 1000 V, respectively. The pulses from the detectors were first amplified by charge-sensitive low-noise preamplifiers and then by linear amplifiers. The amplified pulses were led to single-channel analysers to eliminate the many small noise pulses by lower discrimination. The shaped outputs then passed to the coincidence circuit with a resolving time of 500 nsec. In this way, background pulses were mostly removed from the circuit, as the pulses arising within the tubes are independently random and do not produce coincident signals in the two phototubes (discussed below, see Fig. 2). The outputs of the coincidence circuit were counted by the scaler. The counting time was usually 10 sec, unless indicated otherwise. For the analysis of spectra of ^{14}C sample and background pulses with CaF_2 (Eu) and lithium glass scintillators, a multi-channel analyser was used in combination with a linear gate connected to the delay circuit.

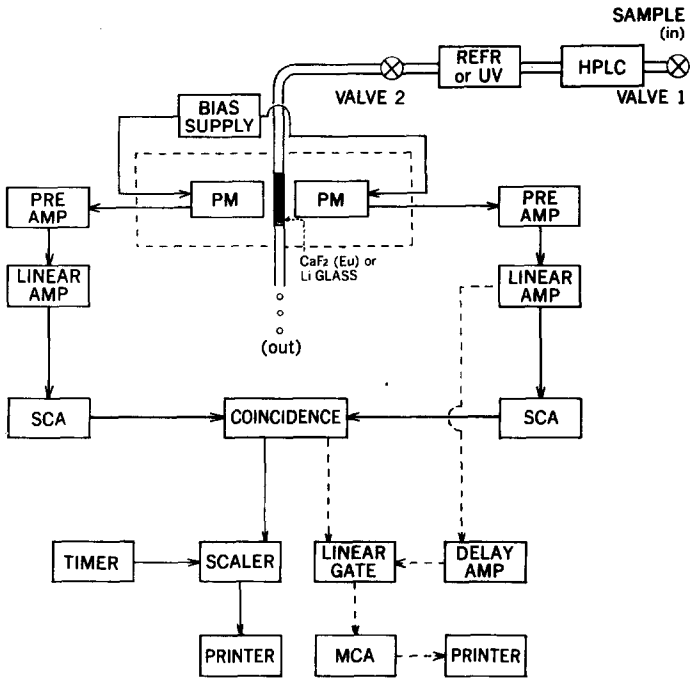


Fig. 1. Schematic diagram of HPLC and ^{14}C counting apparatus. Samples for analysis by HPLC were injected through valve 1. For measurement of the counting efficiency of solid scintillators, a small volume (2 μl) of [^{14}C]glucose solution was injected through valve 2. REFR, differential refractometer; PM, photomultiplier; PRE AMP, preamplifier; LINEAR AMP, linear amplifier; SCA, single-channel analyser; MCA, multi-channel analyser.

Plant materials and ^{14}C labelling experiments

Chlorella vulgaris 11 h cells were grown photoautotrophically in an inorganic culture medium with a photoperiod of 16 h at about 23°C with bubbling of air enriched with 3% (v/v) CO_2 as described previously[12]. Three hours after the start of the last light period, cells were harvested and suspended in one tenth the volume of the culture solution at a cell density of 2 μl packed cell volume per millilitre. A 10-ml volume of the cell suspension was placed in a flat-sided glass vessel ("lollipop") (20 ml) immersed in a flat plastic tank filled with circulating water with a controlled temperature of 20°C. The lollipop was illuminated with a metal halide lamp at 24 000 lux. After photosynthesis for 20 min with continuous bubbling with air containing 3000 μl/l of $^{14}CO_2$ at a rate of 100 ml/min, the light was switched off and then the temperature was quickly increased to 38°C. Incubation in the dark was continued for 5 min. At selected intervals (20 and 25 min), 4-ml portions of the suspension were rapidly transferred into 16 ml of methanol that had been cooled with dry-ice.

Plants of wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) were grown in vermiculite in a growth chamber under 16 h of light at 25°C and 8 h of dark at 20°C. Light was provided by a combination of incandescent and fluorescent lamps at 20 000 lux. Three individuals of the 7-day-old plants taken late in the morning were sealed in a glass chamber in an open-circuit system. The temperature was main-

tained at 25°C by immersing the chamber in a plastic water-bath and light was provided by a metal halide lamp at 20 000 lux. After injection of ambient air for 30-min at a flow-rate of 400 ml/min, it was replaced with $^{14}CO_2$ gas (0.04% by volume) at the same flow-rate. After photosynthetic $^{14}CO_2$ fixation for 10 min, the plants were quickly immersed in liquid nitrogen. The three primary leaves (about 10 cm long) were removed 2 cm from the base and stored overnight in 80% (v/v) methanol that had been cooled with dry-ice. The plant leaves were homogenized with a mortar and pestle.

Analysis of labelled compounds

The 80% methanol-soluble and -insoluble fractions were prepared as described previously[12]. The neutral fraction containing free sugars was obtained as the eluate after passing the soluble fraction through a cation-exchange resin (Bio-Rad AG 50W-X8, 200–400 mesh, hydrogen form) and then through an anion-exchange resin (Bio-Rad AG 1-X8, 200–400 mesh, formate form), by the method of Atkins and Canvin[13]. When necessary, the neutral fraction was treated with β-fructosidase to hydrolyse [^{14}C]sucrose, as described previously[14].

The portion of the insoluble fraction was sealed and heated at 120°C with 2 *N* trifluoroacetic acid for 2 h. The resulting free sugars were concentrated *in vacuo* and subjected to HPLC analysis.

Chemicals

Standard samples of D-[U-^{14}C]glucose, [U-^{14}C]AMP, [U-^{14}C]ADP and [U-^{14}C]ATP were purchased from Amersham, U.K. All other chemicals used were of analytical-reagent grade.

RESULTS AND DISCUSSION

Comparison of ^{14}C spectra between CaF_2 and glass scintillators

When CaF_2 (Eu) was used as the solid scintillator for ^{14}C counting, the distribution curve for the signals passing through the coincidence circuit was apparently distinguished from that for the noise pulses arising in the anti-coincidence circuit (Fig. 2A). In contrast, the peak of the coincident beta pulses with the lithium glass scintillator was overlapped by the large number of the random noise pulses (Fig. 2B). Therefore, a subtle adjustment of the circuit is necessary in order to decrease the background rate without serious influencing the counting efficiency for ^{14}C. Nevertheless, the background rate was significantly higher in the glass detector than in the CaF_2 detector. The dramatic difference in spectra between the CaF_2 and glass scintillators is due to the fact that the scintillation efficiency of the former scintillator is approximately four times higher than that of the latter[9–11]. Hence CaF_2 (Eu) scintillator is an excellent ^{14}C detector, with a high counting efficiency, low background counts and stable pulse analysis.

Efficiency

In an attempt to measure the efficiency of the analysis system, a small volume (2 μl) of [^{14}C]glucose was injected as a standard sample through injection valve 2 (Fig. 1). The sample was led into the flow cell with the aid of the HPLC system

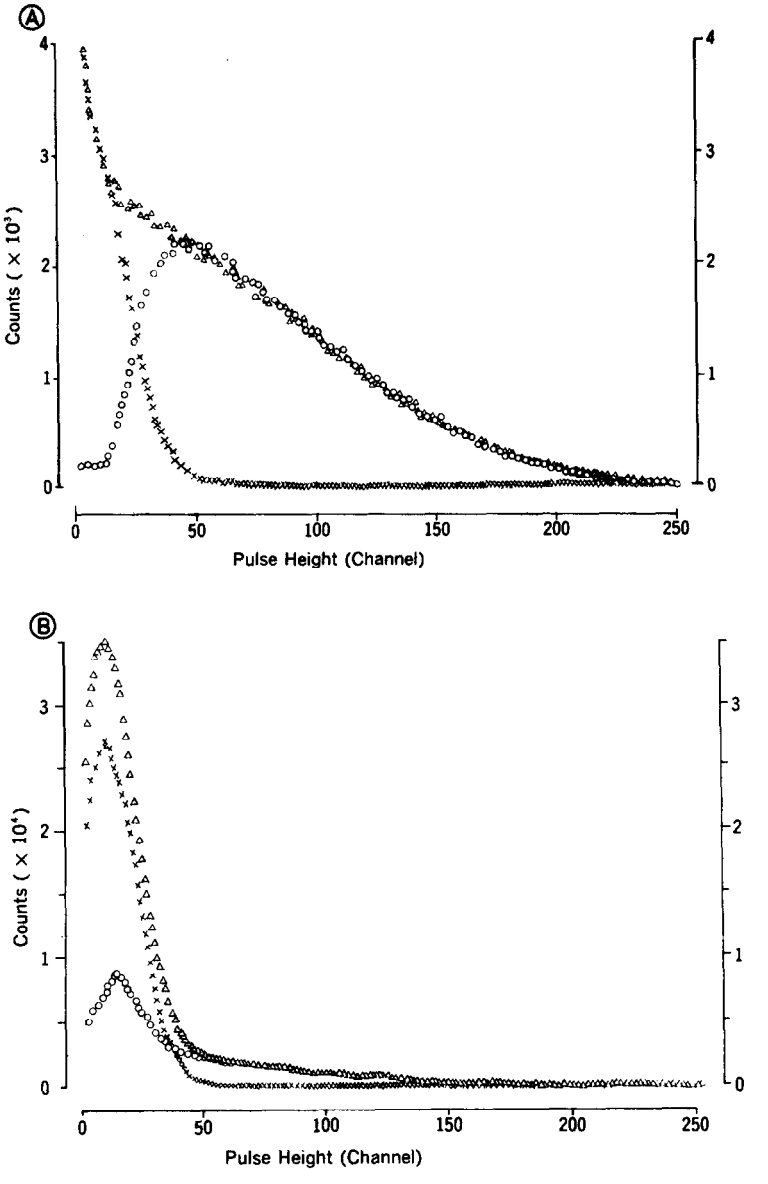


Fig. 2. Fluorescence spectra for ^{14}C detected by the solid scintillation counting system. Standard [^{14}C]glucose in 65% (v/v) acetonitrile was injected through valve 2, slowly eluted with the same eluent until maximum counting was obtained and then stopped for radiation measurement. The output pulses generated by the pulse-selection system were counted for 5 min. Spectra were expressed as distribution of pulse height. (A) CaF_2 (Eu) scintillator. Bias supply, 880 V. Symbols show spectra of pulses which output without coincidence (△), through coincidence (○) and anti-coincidence (×) circuits, respectively. (B) Lithium glass scintillator. Bias supply, 1000 V. Symbols as in A.

equipped with a solvent delivery pump at a minimum flow-rate of 0.01 ml/min. As the standard sample moves with a restricted volume, continuous measurement of the radioactivity gave a broad or flat peak, the sample remaining within the central region of the photomultiplier tubes (Fig. 3). By comparing the average net counts per minute of the peak with the known disintegrations per minute of the given sample (15 900 dpm as measured by liquid scintillation counting), the efficiency was calculated to be 56.6 ± 3.0% from three separate injections. In the same way, the efficiency of the lithium glass scintillator for ^{14}C counting was 20.4 ± 1.7% from the three separate injections.

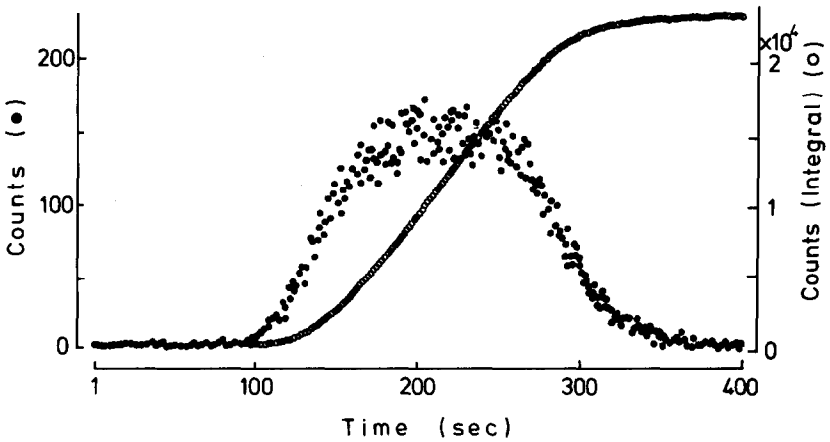


Fig. 3. Time course of counting rate when a ^{14}C-labelled sample passes through a CaF_2 (Eu) scintillator cell. Standard [^{14}C]glucose (15 900 ± 743 dpm as measured by liquid scintillation counting) in 2 μl of 25% (v/v) acetonitrile was injected through valve 2 and eluted with the same eluent at a minimum flow-rate of 0.01 ml/min. The counting time was 1 sec. The figure represents one set of experimental results. The average net counts of the peak shown in this figure as calculted from the net linear slope of the integral counts are 149 ± 12 cps. Therefore, the counting efficiency is 56.2 ± 0.5%.

Giersch[5] reported that the efficiency of a glass scintillator for counting ^{14}C decreased from 39 to 22.5% when the acetonitrile–water was replaced with a salt eluent containing 5–400 m*M* orthophosphate, although quenching on increasing the salt concentration did not exceed 1%.

To test the effect of salt on the counting efficiency of the CaF_2 cell, a standard sample of [^{14}C]glucose was injected through valve 2 and eluted with 50 m*M* potassium phosphate in 25% acetonitrile solution. The efficiency of the CaF_2 scintillator for ^{14}C counting decreased slightly to 54.4 ± 2.3% from three separate injections (data not shown). In contrast, the counting efficiency of the lithium glass scintillator measured in the same way decreased considerably to 16.0 ± 0.9% from three separate injections in the presence of 50 m*M* phosphate (data not shown). The slight decrease in the counting efficiency of the CaF_2 scintillator in the presence of the salt is very advantageous because a salt gradient in the elution buffer is frequently required for maximum resolution of compounds by HPLC.

Peak characteristics

Fig. 4A shows the correlation between the peaks of [^{14}C]glucose as measured with the refractometer and the CaF_2 scintillation detector. It was found that presence of the solid scintillator cell slightly increased the half-maximum width of the peak, but neither distorted the symmetrical shape of the peak nor increased the background counts owing to adsorption, if any, of the radioactivity on CaF_2 particles. On the other hand, the absorbance at 254 nm and the radioactivity derived from ^{14}C-labelled standard AMP, ADP and ATP with known concentrations and the same radioactivity were monitored. Fig. 4B indicates a good correlation between the peaks obtained from the two detectors. Similarly, it is evident that the nucleotides did not adhere to the solid scintillator when they were eluted with the salt buffer solution. In addition, the integral counts of the peak were identical, irrespective of the different peak shapes among these nucleotides.

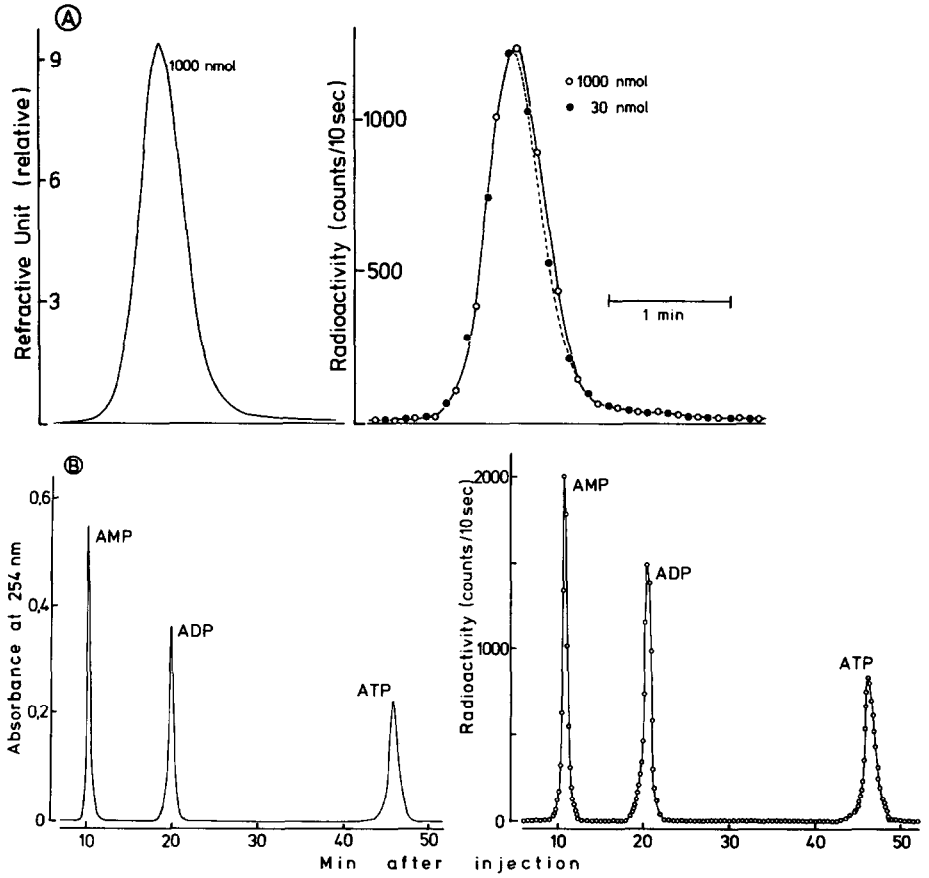


Fig. 4. (A) Comparison of peak shapes of chromatogram of [^{14}C]glucose monitored by a refractometer and CaF_2 (Eu) scintillation detector. The radioactivity of [^{14}C]glucose was 100 000 dpm. The amount of the standard glucose was 30 or 1000 nmol as indicated. (B) Comparison of peak shapes of chromatograms of [^{14}C]nucleotides monitored by a UV detector and CaF_2 (Eu) scintillation detector. The radioactivity (17 700 dpm) was the same for AMP, ADP and ATP. The amounts of AMP, ADP and ATP were 30, 40 and 40 nmol, respectively.

These results show that CaF_2 cell is an excellent detector for radioactivity without any serious loss in resolution of the peaks obtained by HPLC.

Calibration

A lower flow-rate caused a marked increase in the net counts (the gross counts minus background counts) resulting from a fixed radioactivity, owing to the longer retention time of the sample in the solid scintillator cell, as shown in Fig. 5.

Fig. 6 shows that with a fixed concentration of glucose the net counts were proportional to the radioactivity injected, at least up to 150 nCi. When standard glucose with a constant specific radioactivity (about 1 nCi per 4 nmol) was added, the same linear relationship was obtained (data not shown). The lower limit for quantification was about 0.5 nCi when [^{14}C]glucose was chromatographed at a flow-rate of 0.7 ml/min. A similar value was also obtained with [^{14}C]AMP at the same flow-rate of the phosphate solution.

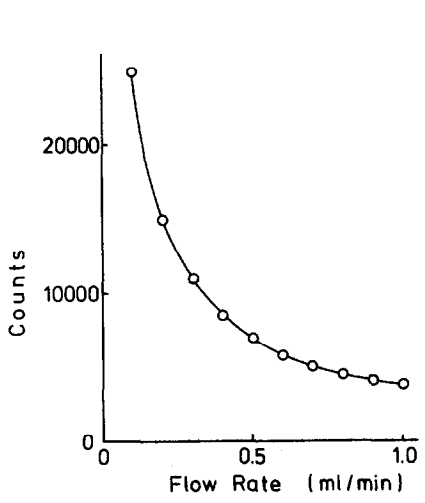
The same net counts were detected when [^{14}C]glucose with constant radioactivity was added to the system in the range from 0.17 to 1000 nmol (data not shown). As shown in Fig. 7, the same value of the net counts was obtained even when the counting time was as short as 0.5 sec, which would be effective in detecting a sharp peak with a small width.

All these results support the conclusion that the radioactivity of a sample can be calculated from the net counts corresponding to the peak.

Analysis of ^{14}C-labelled sugars in plants

The radioactivity detection system equipped with the CaF_2 (Eu) cell was applied to the rapid quantitative separation of the neutral fractions prepared from plant tissues fed with $^{14}CO_2$ during photosynthesis.

Fig. 8A shows distribution of ^{14}C among sugars in the neutral fraction ob-



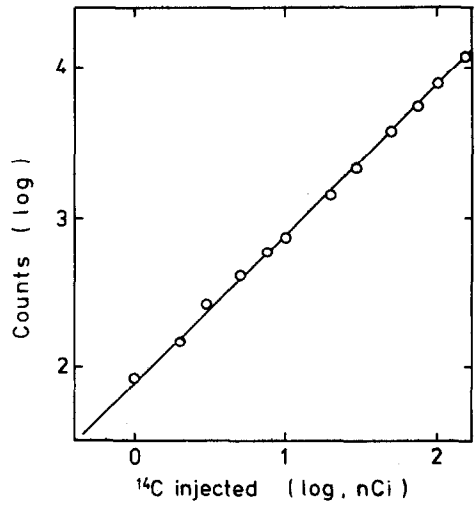


Fig. 5. Effect of flow-rate on the net ^{14}C counts with the CaF_2 (Eu) scintillation detector.

Fig. 6. Calibration graph for the net ^{14}C counts with the CaF_2 (Eu) scintillation detector.

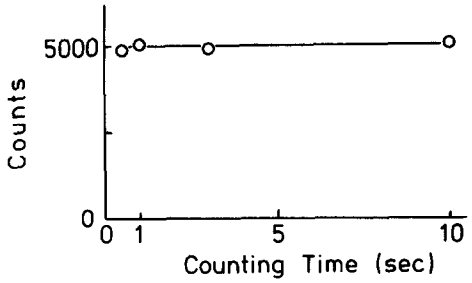


Fig. 7. Effect of counting time on the net counts with the CaF_2 (Eu) scintillation detector.

tained from a *Chlorella* cell suspension, which had been incubated for 5 min at 38°C after photosynthesis for 20 min at 20°C. Our previous work[14] indicated that conversion of starch to sucrose was greatly enhanced under these conditions. Fig. 8A shows that virtually all of the radioactivity was found in sucrose. The fact that little radioactivity was detected in glucose and maltose suggests that α-glucan phosphorylase, but not amylase(s), is involved in the temperature-dependent conversion of starch to sucrose. Fig. 8B also shows that when the neutral fraction was incubated with β-fructosidase, ^{14}C was split evenly into fructose and glucose, indicating *de novo* synthesis of both the moieties of sucrose from starch.

Table I summarizes the distribution of ^{14}C in the neutral and methanol-insoluble fractions in *Chlorella* cells and higher plant leaves. In *Chlorella*, partitioning of ^{14}C-photosynthates into starch was much higher (about 7.8-fold) than that into sucrose. On the other hand, about 60–70% of the total ^{14}C fixed during photosynthesis

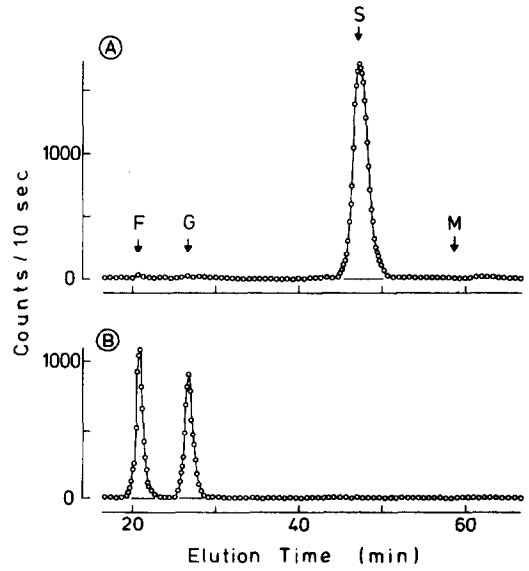


Fig. 8. Chromatogram of ^{14}C-labelled products of *Chlorella vulgaris* 11 h. The *Chlorella* cell suspension was incubated for 5 min at 38°C in the dark after photosynthetic $^{14}CO_2$ fixation for 20 min at 20°C. The neutral fraction was prepared (A) and then treated with β-fructosidase (B), as described under Experimental. Conditions with CaF_2 (Eu) scintillator as in Experimental, except that the eluent was 80% (v/v) acetonitrile and the flow-rate 0.55 ml/min. Arrows indicate the elution time of fructose (F), glucose (G), sucrose (S) and maltose (M).

TABLE I

DISTRIBUTION OF MAJOR ^{14}C-LABELLED PRODUCTS IN A VARIETY OF GREEN PLANTS

Product	*Chlorella*		*Wheat, 10 min, light, 25°C*	*Barley, 10 min, light, 25°C*
	20 min, light, 20°C	*20 min, light, 20°C; 5 min, dark, 38°C*		
80% Methanol-insoluble fraction (starch)*,**	63.2	45.8	14.2	17.2
80% Methanol-soluble fraction**	36.8	54.2	85.8	82.8
Neutral fraction**	8.1	24.6	69.2	62.6
Fructose***	n.d.§	0.2	0.7	1.0
Glucose***	n.d.	0.2	0.9	1.1
Sucrose***	99.7	98.9	98.4	97.6
Maltose***	0.3	n.d.	n.d.	0.2

* Almost all of ^{14}C in the insoluble fractions was found in starch.
** Results are % of total ^{14}C.
*** Results are % of ^{14}C in neutral fraction.
§ Not detected.

was incorporated into sucrose in wheat and barley leaves. Higher percentage incorporations into sucrose and lower percentage incorporations into starch during photosynthesis in higher plant leaves have been reported by many investigators. Mechanisms to explain the great difference in photosynthate partitioning between algae and higher plant leaves should be resolved in further experiments.

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REFERENCES

1 J. A. Bassham and M. Calvin, *The Path of Carbon in Photosynthesis*, Prentice-Hall, Englewood Cliffs, NJ, 1957.
2 P. Schürmann, *J. Chromatogr.*, 39 (1969) 507.
3 H. W. Heldt, A. R. Portis, R. M. Lilley, A. Mosbach and C. J. Chon, *Anal. Biochem.*, 101 (1980) 278.
4 L. N. Mackey, P. A. Rodriguez and F. B. Schroeder, *J. Chromatogr.*, 208 (1981) 1.
5 C. Giersch, *J. Chromatogr.*, 172 (1979) 153.
6 S. Mori, *Agr. Biol. Chem.*, 45 (1981) 1881.
7 S. Mori, *Plant Cell Physiol.*, 23 (1982) 703.
8 P. W. Albro, J. Lorenzo and J. Schroeder, *J. Liq. Chromatogr.*, 2 (1984) 310.
9 J. B. Birks, *The Theory and Practice of Scintillation Counting*, Pergamon Press, Oxford, London, Edinburgh, New York, Paris, Frankfurt, 1964, Ch. 13, p. 541.
10 O. Gilar, Z. Pavlicek and L. Jursova, *TESLA Electron.*, (1981) 53.
11 *Harshaw Data Sheet*, Harshaw Chemical Co., Cleveland, OH, 1966.
12 Y. Nakamura and S. Miyachi, *Plant Cell Physiol.*, 21 (1980) 765.
13 C. A. Atkins and D. T. Canvin, *Can. J. Bot.*, 49 (1971) 1225.
14 Y. Nakamura and S. Miyachi, *Plant Cell Physiol.*, 23 (1982) 333.