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QUANTITATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF ^{14}C-LABELLED PHOTOSYNTHETIC INTERMEDIATES IN ISOLATED INTACT CHLOROPLASTS

CHRISTOPH GIERSCH

Botanisches Institut der Universität Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf (G.F.R.)

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SUMMARY

A rapid quantitative separation of photosynthetic intermediates in spinach chloroplasts is obtained on chemically modified, totally porous, silica gel resins (10 μm). Sugars are separated on a polar resin (LiChrosorb NH_2) by a weakly polar eluent (acetonitrile–water), while phosphorylated compounds are eluted by a phosphate gradient on a strongly basic anion exchanger (LiChrosorb AN). The separation of phosphate esters is complete in *ca.* 1 h. Activity of labelled compounds is monitored on-line by means of a solid scintillator and a multi-channel analyzer.

INTRODUCTION

The chromatographic separation of photosynthetic or glycolytic intermediates on conventional ion exchange resins or by paper chromatography is often employed in studies on metabolism. In conventional chromatography, long elution times are disadvantageous. Furthermore, the quantification of labelled compounds by autoradiography is often not very exact. Recent advances in high-performance liquid chromatography (HPLC) for the separation of multicomponent samples (*e.g.*), containing up to 12 nucleotides, with excellent resolution[1] promise fast separation even of highly complex mixtures of different compounds. The analysis of metabolic regulation in cells or their organelles requires such separations.

This report describes a rapid quantitative separation of soluble compounds labelled during photosynthesis of isolated chloroplasts in the presence of $^{14}CO_2$. The compounds formed are glycolate and phosphate esters[2]. Neutral sugars are minor components, while starch, the storage form of the saccharides, is insoluble in an acidic extract[3]. The phosphorylated intermediates are separated on an anion-exchange resin by a salt gradient, while sugars are eluted on a polar support with a weakly polar eluent. Quantification of the label originating from starch has been described elsewhere[3]. The analysis of collected fractions for activity is accurate but laborious. Hence, activity was quantified on-line by a β-monitor equipped with a glass scintillator cell. Since the detection limit of the on-line measuring system is proportional to the flow-rate, one has to compromise between rapid separation and sensitive detection.

MATERIAL AND METHODS

Ion-exchange resins and columns

A strongly basic anion exchanger (LiChrosorb AN, 10 μm, exchange capacity 0.55 mequiv/g; E. Merck, Darmstadt, G.F.R.) was used for the separation of anions and a polar support (LiChrosorb NH_2, 10 μm) for sugars. Stainless-steel columns (25 cm × 4.6 mm I.D.; Knauer, Berlin, G.F.R.) were packed with the ion-exchange resin as follows: 3.2. g of LiChrosorb AN were suspended in 10 ml of 50m*M* KH_2PO_4, pH 4.5. After sonification for 30 sec, the slurry was pressed into the column filled with the suspension medium at a rate of 20 ml/min for 10 min. The polar support LiChrosorb NH_2 was packed as suggested by the supplier. Before use, columns were flushed with 250 ml KH_2PO_4 (450 m*M*, pH 2.8), 100 ml of 0.1 *N* HCl and 300 ml demineralized water. A prepacked column (Hibar, LiChrosorb AN, 4 mm × 23.5 cm, Merck) was used for comparison. The purity of the packing was routinely checked by inspection of the UV absorption of the column effluent at 195 nm. When chloroplast extracts were analyzed for sugars, the column was flushed with 30 ml of 400 m*M* KH_2PO_4 once or twice a day in order to prevent obstruction due to compounds not eluted by the weakly polar phase.

Chemicals

Enzymes and biochemicals were supplied by Boehringer (Mannheim, G.F.R.) or Sigma (St. Louis, Mo., U.S.A.). Buffer substances were purchased from Merck and used without further purification.

^{14}C-Labelled bicarbonate, glucose, fructose, sodium glycolate, G-6-P*, PGA, F1,6BP, ATP and starch were obtained from Amersham-Buchler (Braunschweig, G.F.R.). Triose phosphates were obtained by enzymatic conversion of F1,6BP with aldolase[5]. Maltose was produced by incubation of starch with α-amylase for 4 h[6].

Apparatus

A schematic diagram of the apparatus is given in Fig. 1. The gradient is formed by switching a valve between the diluted and concentrated sovents by means of a low-pressure mixer (Mixograd, Gilson). The switching cycle time is 6 sec. The mobile phase is delivered by a dual reciprocating pump (Knauer). When a reciprocating pump is used in combination with this type of gradient mixer irreproducibilities in retention times are observed and have been reported[7]. This is due to beats between the frequencies of the pump and that of the valve, causing the actual mobile phase concentration to oscillate around the programmed one. A simple, effective method of avoiding this

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* Abbreviations: DAP = dihydroxyacetone phosphate; F-6-P = fructose 6-phosphate; F1,6BP = fructose 1,6-bisphosphate; GAP = glyceraldehyde phosphate; G-1-P = glucose 1-phosphate; G-6-P = glucose 6-phosphate; PGA = 3-phosphoglycerate; P_i = inorganic phosphate; PP_i = pyrophosphate; R-5-P = ribose 5-phosphate; Rul,5BP = ribulose 1,5-bisphosphate; S-7-P = sedoheptulose 7-phosphate; S1,7BP = sedoheptulose 1,7-bisphosphate; ATP = adenosine 5′-triphosphate; ADP = adenosine 5′-diphosphate; AMP = adenosine 5′-monophosphate; HMP = hexose monophosphates; PMP = pentose monophosphates; TP = triose monophosphates.

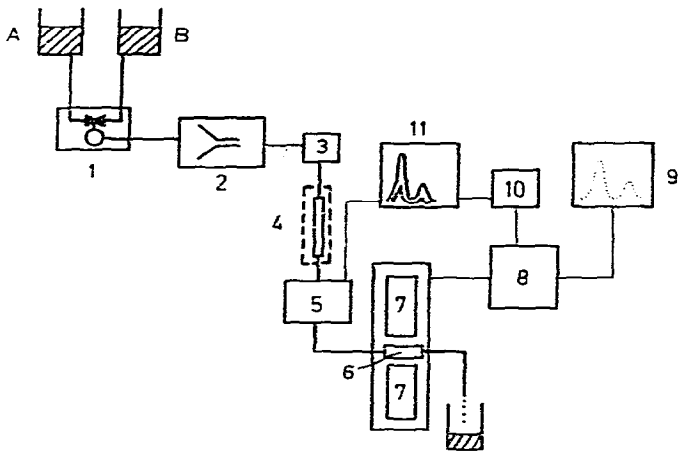

Fig. 1. Schematic diagram of experimental set-up. A, B = Solvent reservoirs, containing degassed mobile phase at ambient temperature; 1 = low pressure gradient mixer with switching valve and mixing chamber; 2 = pump; 3 = sample injection valve; 4 = column with water jacket; 5 = variable-wavelength spectrophotometer; 6 = solid scintillator flow cell; 7 = photomultipliers; 8 = high-voltage supply, impulse-forming and amplification unit; 9 = multi-channel analyzer; 10 = linear ratemeter; 11 = two-pen recorder.

problem when using a mixing device with fixed switching time is to choose a mixing cell with a volume V_m such that

$$V_m \approx 2\, T_b v_p$$

where $T_b = 1/\nu_b$ (min) is the beat time, ν_b (min^{-1}) is the beat frequency and v_p (ml/min) is the flow-rate. The mixing chamber has to be large compared to the volume delivered within the time interval T_b.

Samples were injected by means of a six-way valve equipped with a 70-μl sample loop (Rheodyne). The eluate was monitored by a spectrophotometer at 195 nm (LC 55, Perkin-Elmer) equipped with a 7-μl flow cell. At this wavelength all compounds investigated show some, or even appreciable, absorption. The eluate passed a solid scintillator cell (cerium-activated lithium glass; Berthold, Wildbad, G.F.R.) with an effective volume of 150 μl. This type of cell has the advantage that the eluate can be used for further studies; care must be taken to keep the background low (see *Quantification*). Whenever the spectrophotometer was used in combination with the β-monitor, the maximum pressure limit of the solvent delivery pump was set to a value well below the maximum tolerable pressure of the spectrophotometer, in order to avoid damage of the latter due to possible obstruction of the solid scintillator cell. The column temperature was generally maintained at 20° by a water jacket. Activity was recorded on a multi-channel analyzer equipped with a parallel printer (Silena, Milan, Italy). The counting time for one channel was 10 or 20 sec. Dead time between adjacent channels is *ca.* 35 μsec. The content of the memory of the multi-channel analyzer could be recorded on a strip chart recorder at a rate of about 1 channel per sec. A linear ratemeter (Berthold) was used to obtain on-line recordings of activity.

Quantification

The efficiency of the system is given by

$$E(\%) = \frac{c_n u}{\mathrm{dpm} \cdot v_c} \cdot 100 \qquad (1)$$

where c_n = net counts resulting from a known activity dpm, u = flow-rate and v_c = effective volume of the flow cell. The efficiency of the solid scintillator cell is 22.5 ± 1% during elution with the salt gradient; there is probably a slight quenching by increasing salt concentrations which does not exceed 1%. In the less polar eluent acetonitrile–water the efficiency is 39 ± 2%. The higher efficiency in the less polar phase may be due to the altered partition coefficient of the label between the mobile phase and the solid scintillator which also causes an increase of background (see below).

Fig. 2 shows the correlation between the activity and the counts of the corresponding peaks as measured with the solid scintillator cell. The detection limit is *ca.* 1 nCi; the signal-to-noise ration is low at these low activities and the error in quantification may be appreciable. Linearity of the response is good and may even be established in the 10-μCi region. Higher activities caused a drastic increase of the background by adsorption of the label at the scintillator. Activities expected to exceed several hundred nanocuries in one peak were therefore diluted before analysis. The background is *ca.* 170 cpm in the salt gradient; elution with the less polar eluent causes an increase of background due to stronger adsorption of label at the solid

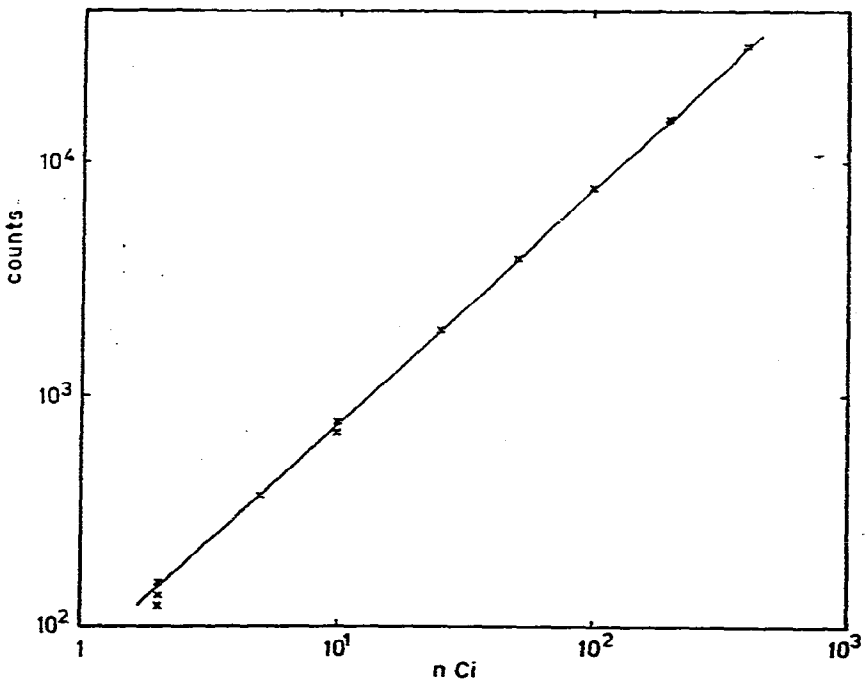

Fig. 2. Calibration curve for ^{14}C-labelled glucose. Known amounts of activity were eluted with 5 m*M* KH_2PO_4.

scintillator. A reduction in the background can be achieved by flushing the cell with *ca.* 100 ml of hot (90°) commercial detergent (*e.g.*, RBS35, 5 vol.% aqueous solution; C. Roth, Karlsruhe, G.F.R.) followed by 100 ml of 0.1 *N* HCl. This procedure does not affect the efficiency of the system. Chromatograms were evaluated on the basis of the data from the parallel printer which records counts in individual channels. The activity corresponding to a peak was calculated from the net counts (counts minus background) according to eqn. 1.

Peak identification

The metabolic pattern of photoreducing chloroplasts is known[2,8], as is the order of elution of the compounds from conventional chromatographic analysis[8–10]. Identical retention times are taken to indicate that the substance in question forms part of the peak that has to be identified. The homogeneity of peaks was checked by enzymatic conversion when an identified compound was believed to be eluted together with other substances: the identified compound was enzymatically converted[11] and the resulting sample rechromatographed.

RESULTS AND DISCUSSION

Mobile phase conditions

Phosphorylated compounds were eluted by a mobile phase gradient. The influence of ionic strength and pH (2.5–4.0) was checked for the following counter ions: $H_2PO_4^-$, Cl^-, NO_3^-, CH_3COO^- (cation, K^+ throughout; pH adjusted with H_3PO_4); and ammonium formate (pH adjusted with formic acid). Selectivity is good with ammonium formate and acetate, reasonable with $H_2PO_4^-$ and poor with the other compounds tested. The elution with ammonium formate–formic acid requires very concentrated mobile phases. $H_2PO_4^-$ was chosen as counter ion since strongly retained compounds were eluted with the best resolution (smallest peak width as compared to peak distance). Moreover, this mobile phase causes the least baseline drift at 195 nm. The somewhat poor resolution of sugar monophosphates can be overcome by careful gradient design. By trial and error (increasing pH has no drastic effect but slightly reduces resolution), following conditions were found to be most suitable for the separation of soluble (mainly phosphorylated) compounds: linear gradient 5–400 m*M* KH_2PO_4, 2.88%/min, pH 2.8; flow-rate, 0.94 ml/min. To increase resolution of peaks and sensitivity of detection, the flow-rate and gradient steepness were decreased in some cases to 0.47 ml/min and 1.44%, respectively. The separation of phosphorylated compounds is complete within *ca.* 40 min (0.94 ml/min). The regeneration time for LiChrosorb AN is *ca.* 2 h. Three columns were used in parallel, and regeneration was performed by a separate pump with the low concentration of the eluent. The separation of sugars is less critical; fructose, glucose and maltose are separated by isocratic elution with acetonitrile–water = (80:20), pH 3.0 (adjusted with H_3PO_4; flow-rate, 2.0 ml/min).

Chromatography of known intermediates

Table I gives the relative retention times of some sugars as eluted by acetonitrile–water and of more polar compounds as eluted by the salt gradient. Phosphorylated ketose generally show higher absorption at 195 nm compared to the corre-

TABLE I

RELATIVE RETENTION TIMES OF KNOWN COMPOUNDS

Depending on the specific extinction coefficient, 0.025–1 mg of the known compound were chromatographed on LiChrosorb NH_2 (sugars) or LiChrosorb AN (glycolate and phosphorylated compounds). Labelled substances were adjusted to contain 25 nCi in the sample volume of 50 μl. Flow-rate for separation of phosphorylated compounds, 0.94 ml/min. Reproducibility of retention time: *ca.* 3% between individual runs; <0.5% for peak distances within a chromatogram.

Compound	*Relative retention*
*(a) Sugars**	
Fructose	54.2
Glucose	59.7
Maltose	100.0
*(b) Other compounds (mainly phosphorylated)***	
Unretained compounds (sugars)	9.6
Glycolate****	11.6, 15.4, 22.6, 21.9
AMP	14.6
S-7-P	41.0
G-6-P, G-1-P, F-6-P	42.4
Erythrose 4-phosphate	43.8
R-5-P	45.5
DAP	50.6
ADP	65.2
PGA	77.6
S1,7BP	85.2
F1,6BP	90.0
Ru1,5BP	100.0
ATP	141.8

* Eluted with acetonitrile–water on LiChrosorb NH_2 (25 cm × 4.6 mm).

** Eluted with a salt gradient on LiChrosorb AN (25 cm × 4.6 mm).

*** Solutions in 11% formic acid, 1 *N* $HClO_4$, 66% methanol and water, respectively. See text for explanation.

sponding aldoses because the specific extinction coefficient for the $\pi \rightarrow \pi^*$ transition is one to two orders of magnitude higher for ketoses[12]. Sugars are practically not retained in the salt gradient. The retention time of glycolic acid is strongly dependent on the medium in which the sample was prepared: for a solution of glycolic acid in water, the retention time is 12.6 min; in 66% methanol it is about the same, in 11% formic acid and in 1 *N* $HClO_4$ which are frequently used for chloroplast extraction there is only slight retention (*cf.*, Table I). This shift in retention times apparently reflects the competition between glycolic acid and the other anions present in the sample ($HCOO^-$, ClO_4^-) for the binding sites of the stationary phase. For sugars and for phosphorylated compounds no comparable shift in retention times is observed: the affinity of sugars is too low and that of phosphate esters too high to cause competition with other sample anions.

When samples extracted in $HClO_4$ are assayed for glycolate, neutralization is obligatory and potassium perchlorate has to be removed quantitatively in order to obtain the proper retention time and an acceptable peak form (competition with ClO_4^- caused strong tailing). G-6-P, G-1-P and F-6-P are eluted in one peak which is designated as hexose monophosphates. Together with R-5-P, some other pentose

monophosphates may be eluted. The triosephosphates DAP and GAP are not separated. In chloroplasts the ratio DAP:GAP is *ca.* 20:1 as a consequence of the equilibrium constant of triosephosphate isomerase (EC 5.3.1.1). The retention times of the adenine nucleotides are given for comparison with the separations of ^{32}P-labelled compounds[8,9]; the resolution of triose, tetrose, pentose, hesoxe and heptose monophosphates will be incomplete in practice. If desired, the separation can be improved at the expense of time by decreasing the flow-rate (*cf.*, *Mobile phase conditions*).

The separation of sugars is described in the application literature of several suppliers[13–15]. Fructose levels in chloroplats are low, and the separation of glucose from maltose yields sufficient information for studies on starch metabolism. It should be mentioned that glycolate is also eluted in this system. For chloroplasts extracted in 1 *N* HCl (ref. 3), the retention time of glycolate is very close to that of glucose. In 11% formic acid it decreases to a relative retention of 35.4 (*cf.*, Table Ia), and so quantification of glucose and maltose is possible. In principle, it is possible to separate sugars from each other and from phosphorylated compounds in one single run by starting the salt gradient after elution of the sugars. In practice, this method requires a strict timetable for regeneration times in the respective mobile phases, otherwise reproducibility will be poor. The performance of the stationary phase may deteriorate faster due to the drastic changes in mobile phase polarity.

Chromatographic separation of ^{14}C-labelled intermediates in pea chloroplasts

Fig. 3 shows the metabolic spectrum of intact pea chloroplasts which were allowed to reduce $^{14}CO_2$ for 20 min. Beside starch, the main body of labelled intermediates consists of sugar, glycolate, triose phosphates and PGA. At the chosen specific activity the label of these compounds ranged from *ca.* 1 to *ca.* 3 μatoms ^{14}C per mg chlorophyll; hexose monophosphates, pentose monophosphates and the sugar bisphosphates contained 0.2–0.6 μatoms ^{14}C per mg chlorophyll. The separation of sugar monophosphates depends on the relative heights of these peaks. Levels of S-7-P, which is eluted just in front of HMP, are low. Separation from HMP was complete in cases where S-7-P could be detected, whereas erythrose 4-phosphate vanishes in the HMP–PMP–TP group. The detection limit of the system is *ca.* 50 natoms per mg chlorophyll (S1,7BP and unidentified compound X) at the chosen specific activity of 5 mCi/mmol and a flow-rate of 0.47 ml/min (*cf.*, legend to Fig. 3). This is clearly sufficient for a study on the interconversion of metabolites in the course of starch synthesis and degradation. It may be poor for a study of compartmentalization problems where activities of a few nanocuries have to be quantified. To quantify very low activities, conventional techniques such as collection of fractions, autoradiography or thin layer chromatography (TLC) may be used. Although the efficiency of the β-monitor used in TLC and autoradiography (30%, ref. 17) is comparable to that of the flow cell used in this work, the detection limit of the former methods can be increased by decreasing the scanning time without affecting the separation procedure. Alternatively, the detection limit of HPLC analysis may be increased by a factor of *ca.* 10 (detection limit, 5 nCi per mg chlorophyll) by simultaneously decreasing the flow-rate by a factor of two, increasing the efficiency to *ca.* 90% by mixing the eluate with liquid scintillator in another type of flow cell and by increasing the specific activity. Details will be given in a subsequent paper.

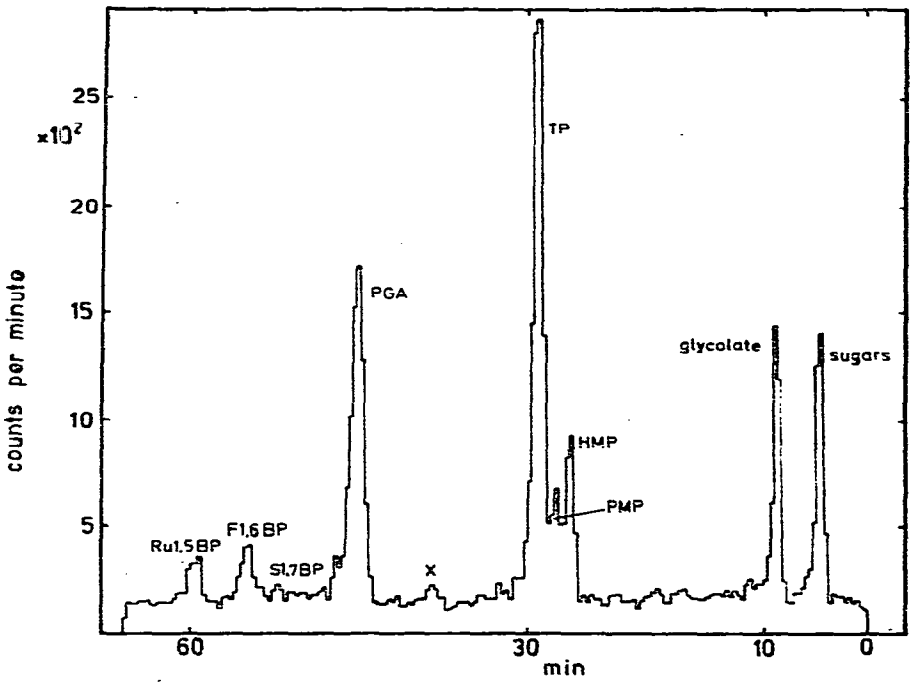

Fig. 3. Chromatogram of ^{14}C-labelled intermediates in pea chloroplasts. Chloroplasts were isolated according to Robinson and Wiskich[16] and suspended in a medium containing 0.33 *M* sorbitol, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 2 m*M* ethylenediaminetetraacetate, 0.1 m*M* P_i and 50 m*M* N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), pH 7.6. Catalase (1500 I.U./ml) was added; substrate concentration ($NaH^{14}CO_3$, 5 mCi/mmol), was 2 m*M*; chlorophyll concentration, 100 μg/ml. Furthermore, 0.1 m*M* PGA, 0.4 m*M* ATP and 5 m*M* PP_i, which are known to stimulate starch synthesis in pea chloroplasts [16], were added. Photosynthesis was stopped after illumination for 20 min with 200 W/m² white light by injecting an aliquot into 0.5 *N* HCl (final concentration). After extraction for 10 min, the sample was spun down and chromatographed; flow-rate, 0.47 ml/min; gradient, 1.44%/min; prepacked column (Hibar); multi-channel analyzer, 20 sec per channel.

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