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The separation and identification of picomole amounts of intermediates of glucose metabolism by high performance liquid chromatography on pellicular resins

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A column (CarboPac PA1, Dionex) containing an anion-exchange pellicular resin was used for the separation of phosphoryl-hexoses derived from labeled glucose microinjected into individual frog oocytes or from cultures of Escherichia coli. Intermediates were identified by: a) comparison of retention times with those of authentic commercial compounds; b) the use of internal labeled standards; c) incubation of samples with specific enzymes and noting the disappearance of one radioactive peak and appearance of another at a new retention time.

INTRODUCTION

During two independent ongoing investigations on the control of glucose utilization by microinjected frog oocytes (20), and on the effect of mutations of PFK* on the fate of labeled glucose and glycerol in *Escherichia coli* (18), it became necessary to separate and identify metabolic intermediates present in minute amounts. Several procedures using a variety of approaches for the resolution of complex mixtures of glycolytic intermediates have been published (1, 2, 7, 8, 14), but none of them has achieved widespread use either because of low sensitivity or long and cumbersome analytical procedures. The introduction of Hplc for the separation of phosphorylated sugar intermediates has resulted in some cases in efficient analytical methods (15-17). Recently, anion exchange pellicular resins have been introduced for Hplc resolution of sugars (12) and a short abstract (11) has reported its use for the separation of glycolytic phosphate esters. We have employed a Dionex pellicular resin column and would like to report the separations obtained and the strategy used for the enzymatic identification of labeled intermediates (a few of them not readily available as radioactive commercial compounds) produced by individual frog oocytes microinjected with [^{14}C]glucose and by cultures of *E. coli* supplied with labeled glucose or glycerol. Portions of this work were previously presented in abstract form (4).

EXPERIMENTAL PROCEDURE

Materials. Labeled sugars were from The Radiochemical Centre (Amersham). Unlabeled glucose and phosphorylated intermediates, NAD, ATP, glucose-6-P dehydrogenase (*Leuconostoc*), muscle phosphoglucomutase, muscle PFK and yeast hexokinase were from Sigma. [^{3}H]-labeled standards were prepared from [3-^{3}H]glucose incubated with: a) hexokinase and ATP to produce glucose-6-P; b) hexokinase, ATP, glucose-6-P dehydrogenase and NAD^+ to produce 6-phosphogluconate; c) hexokinase, ATP, phosphoglucose isomerase to produce glucose-6-P and fructose-6-P; d) hexokinase, ATP, phosphoglucose isomerase and muscle

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Abreviations: PFK, phosphofructokinase; Hplc, High-performance liquid chromatography; PCA, perchloric acid.

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PFK to produce fructose-1,6-bisP. All incubations were performed at 30° C during 30 min in a medium containing 0.1 M Tris-HCl pH 8, 5 mM $MgCl_2$, and when appropriate 5 mM NAD^+ and/or 1 mM ATP. Enzyme activity was stopped by heating at 95° C for 5 min followed by extraction with PCA as described below. The standard compounds thus prepared were separatelly run on the Dionex colum, to be described below, to assess their clution behavior (Table I). In some experiments (see Fig. 1 for an example) the standards were mixed and added to [^{14}C]-labeled extracts just before the chromatographic run. Other reagents were of analytical grade.

TABLE I

Retention times of glucose and some phosphorylated intermediates in the CarboPac column[a]

	min
Glucose (8)	4.1 ± 0.07
Glucose-1-P (3)	10.5 ± 0.10
Glucose-6-P (6)	28.8 ± 0.24
Fructose-6-P (7)	34.3 ± 0.41
6-phosphogluconate (8)	42.1 ± 0.23
Fructose-1,6-bisP (7)	55.3 ± 0.31

[a] Phosphorylated intermediates were prepared from [^{3}H]glucose using the appropriate commercial enzymes. Hplc analyses were exactly as described under Experimental Procedure. The number of independent chromatographic runs are shown in parentheses. Figures, mean ± S.E.

Cells. Stage VI oocytes were dissected from the ovaries of female frogs (*Caudiverbera caudiverbera*). Strains (DF920 and DF905) of *Escherichia coli* obtained from Dr. Dan Fraenkel have been described elsewhere (3).

Metabolic labeling. Oocytes were microinjected with about 50 nl of buffered amphibian saline (5) containing 0.5 nmol of [U-^{14}C]glucose (about 40,000 cpm) and incubated in groups of four cells in 75 µl of saline, as previously described (13). At the end of the incubation period oocytes were plunged into liquid nitrogen and then homogenized in cold 3% PCA. Precipitated material was removed and the supernatant neutralized

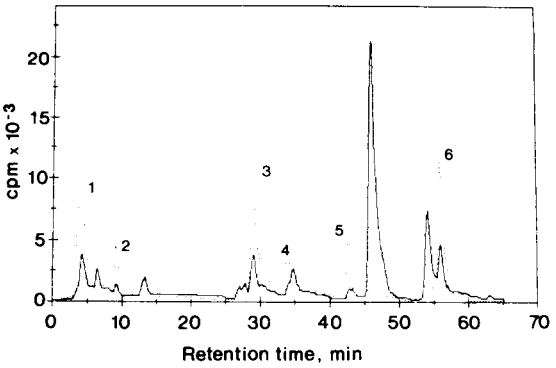

Fig. 1: Hplc (CarboPac Pa1) separation of labeled metabolites from PCA extracts of *E. coli* DF905 incubated with [U-^{14}C]glucose. Cultures of *E. coli* were incubated during 60 sec with a medium containing [U-^{14}C]glucose as described under Experimental Procedures. A mixture of [^{3}H]-labeled standards was added to the [^{14}C]-labeled PCA extract before analysis. The solid line represents radioactivity from ^{14}C; the dotted line records radioactivity from ^{3}H. The numbers identify the elution positions for the following compounds: 1. glucose; 2. unidentified; 3. glucose-6-P; 4. fructose-6-P; 5. 6-phosphogluconate; 6. fructose-1,6-bisP.

with KOH. Perchlorate was discarded by centrifugation and ethanol added to precipitate glycogen. Aliquots of glycogen-free PCA extracts were concentrated to dryness under a stream of nitrogen and dissolved in 0.1 M NaOH just before processing the sample by Hplc. In the case of metabolic labeling in *E. coli*, strain stocks were kept on agar plates at 4° C. Overnight cultures were grown in Luria-Bertani medium, diluted 1:100 in fresh medium and grown until a density corresponding to an absorbance at 580 nm of 0.5 was reached. Then, cultures were centrifuged, resuspended in minimal medium M9 (9), washed once more in the same and finally resuspended to an absorbance of 2.0. At t=0 the cells were provided with either [U-^{14}C]glucose (6 x 10^6 cpm/ml) or [U-^{14}C]glycerol (5 x 10^6 cpm/ml), vortexed and 0.3 ml samples (medium and cells) were taken at 30 and 60 sec. Afterwards, sample manipulation was the same as described above for oocyte extracts except that $KHCO_3$ was used to neutralize PCA extracts.

Hplc analysis. A Dionex CarboPac PA1 column with a AG-6 guard column was used. The Hplc resin has been succinctly described (12,19). The column was operated at room temperature and controlled through a LKB Hplc system. A Rheodyne injection valve equipped with a Tefzel rotor seal was used. The outlet was connected to a Beckman L171

scintillation liquid detector for on-line measurement of radioactivity in the effluent. The column was equilibrated with 90% solution A (100 mM NaOH) and 10% solution B (1 M sodium acetate-100 mM NaOH) and eluted at a flow rate of 0.5 ml/min with a gradient program as follows: T_0=10%; T_{20}=15%; T_{21}=20%; T_{51}=50%; T_{65}=50% (subscript numbers are time in minutes; percent figures refer to amount of solution B). Radioactivity measurements were taken every 30 sec and readings were stored in a Compaq PC to be processed later using the ChromatoGraphics program supplied by Beckman. Under the conditions used ^{14}C and ^{3}H counting efficiencies are about 85% and 30%, respectively.

RESULTS AND DISCUSSION

Separation of labeled intermediates from E. coli cultures

At the low concentrations of precursors used (about 2 μM), uptake and metabolism of glucose or glycerol are very fast in the strains employed. Already after 30 sec, clear peaks representing products can be resolved accounting for as much as 30% of the added label (data not shown) the remainder being lost as $^{14}CO_2$ or as PCA-insoluble material. The time course of appearance and disappearance of each peak and its usefulness for the understanding of glucose utilization either in *E. coli* or in oocytes will be reported elsewhere. In this report we will focus on the chromatographic separation and on the strategy used for the identification of the fractions resolved.

Fig. 1 shows the pattern obtained after chromatography of a [^{14}C]-labeled *E. coli* extract containing added [^{3}H] internal standards (prepared enzymatically from [^{3}H]glucose as described under Experimental Procedure). Based on the co-elution pattern we have identified glucose as peak 1, glucose-6-P as peak 3, fructose-6-P as peak 4, 6-phosphogluconate as peak 5 and fructose-1,6-bisP as peak 6. The presence of glucose is expected, since the PCA extract was prepared with the complete culture (medium and cells). Peak 2 in the mixture of standards has not been identified but it may be the result of some contaminating enzyme activity present in the commercial enzymes used. Four additional peaks show retention times that do not coincide with any standard. Efforts are being made to identify them.

Another approach for peak identification followed the strategy described by Hammerstedt (6) using appropriate enzymes. Fig. 2A shows the elution pattern obtained with strain DF920 30 sec after supplying it with [U-^{14}C]glycerol. Fig. 2B is the same extract after treatment with fructose-1,6-bisphosphatase. This result confirms the identification of the peak eluting at about 55 min (see Fig. 1) as fructose-1,6-bisP, since the label moves to peaks at 29 min and 34 min, which were tentatively identified as glucose-6-P and fructose-6-P, respectively. Glucose-6-P must result from contamination of the bisphosphatase with phosphoglucose isomerase. Other peaks were not modified.

Experiments (not shown) indicate that overall recovery of radioactivity is generally better than 98%. The standard compounds used did not show differential stability under the alkaline conditions employed. Other experiments (not shown) revealed that, with the elution program used, glucose-1-P and glyc-

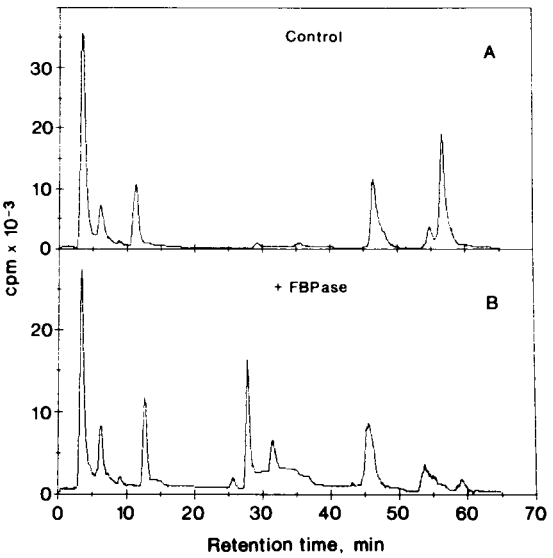

Fig. 2: Identification of fructose-1,6-bisP in Hplc chromatograms of *E. coli* PCA extracts. A. Elution pattern of a PCA extract of *E. coli* strain DF920 obtained after 30 sec incubation in a mixture containing [U-^{14}C]glycerol, as described in Experimental Procedure. B. Elution pattern of a sample identical to that in A) but incubated for 30 min at 30° C with fructose-1,6-bisphosphatase.

erol-3-P have equal retention times (10.5 min). Also, non-phosphorylated hexoses or derivatives (glucose, fructose, sorbitol) coelute at about 4.1 min. They can be separated under isocratic conditions (ref. 12 and our own unpublished data).

Separation of labeled intermediates from micro-injected frog oocytes

After [^{14}C]glucose injection into oocytes and incubation for different time periods, a few peaks were discerned by Hplc (Fig. 3). Based on the retention times of standard compounds and the spiking of PCA extracts with authentic compounds, we have identified glucose, glucose-1-P, glucose-6-P and fructose-6P. Also, after incubation of a PCA extract with hexokinase and ATP, the glucose peak disappeared and the radioactivity eluted after 29 min (Fig. 4). In this experiment, the sample was spiked with [3-^{3}H]glucose in order to check if the enzymatic conversion of glucose to glucose-6-P was complete. This procedure identifies glucose and confirms that 29 min is the retention time for glucose-6-P. Glucose-1-P was identified as the peak eluting at 10.5 min since it decreased by incubation of the PCA extract with phosphoglucomutase; concomitantly, a new small peak appeared at 44 min marking the retention time for glucose-1,6-bisP (Fig. 5). Treatment of the extracts with glucose-6-P dehydrogenase and NAD^{+} decreased markedly the radioactivity of the peak eluting at 29 min with the concomitant

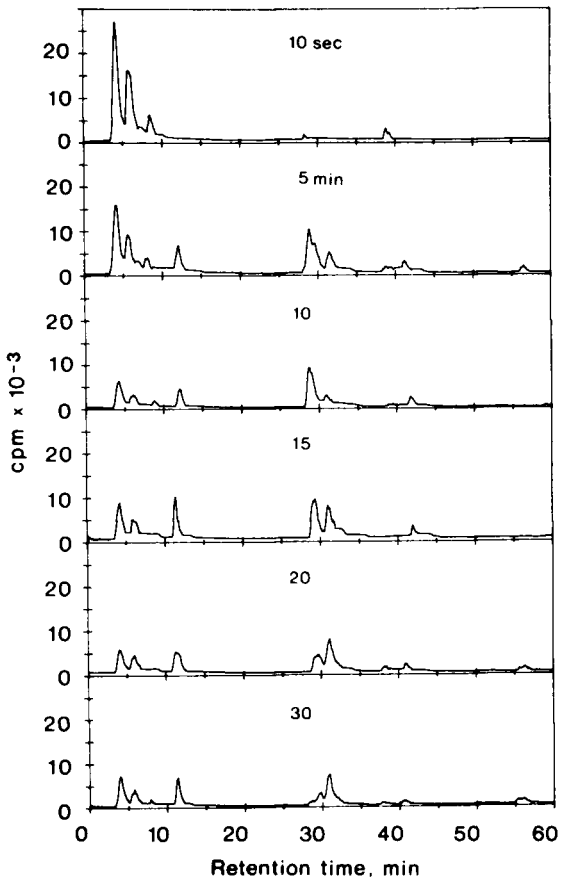

Fig. 3: Hplc separation of labeled metabolites from PCA extracts from oocytes injected with [U-^{14}C]glucose. Oocytes were injected with 50 nl of saline containing 0.5 nmol of [U-^{14}C]glucose (75000 cpm/nmol) and incubated for the indicated times. After plunging in liquid nitrogen, PCA extracts were prepared, and freed from glycogen by ethanol precipitation. Then, extracts were evaporated and resuspended in 0.1 ml of solution A just before Hplc separation. Other details as in the legend of Fig. 1. Note the very fast conversion of glucose to other compounds readily visible at 10 sec after injection, the shortest time interval which could be studied. Note also the sluggish appearance of glucose-6-P, fructose-6-P or glucose-1-P. The identification of some of the peaks appearing early after glucose injection will be reported elsewhere.

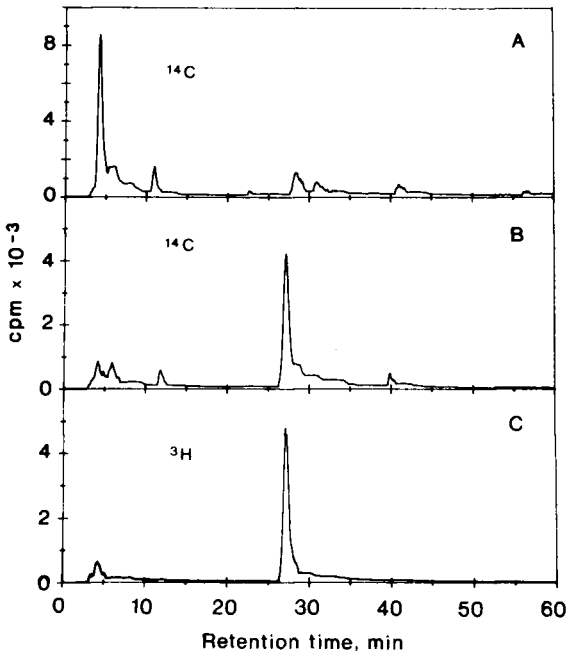

Fig. 4: Identification of glucose in PCA extracts from oocytes injected with [U-^{14}C]glucose. Oocytes were incubated for 20 min after injection and processed as described in the legend of Fig. 3. An aliquot (0.3 ml) of the PCA extract was evaporated, resuspended in 0.1 M Hepes pH 8 and applied to a CarboPac column (A). Another aliquot (0.1 ml) was similarly treated except that [6-^{3}H]glucose was added as an internal standard. Then, 0.2 nM ATM $MgCl_2$ and 2 mUnits of hexokinase were added. After incubation for 60 min, the sample was applied to the column and eluted as described in the legend of Fig. 3. Dual-label on-line radioactivity counting is shown in panel B (^{14}C) and panel C (^{3}H).

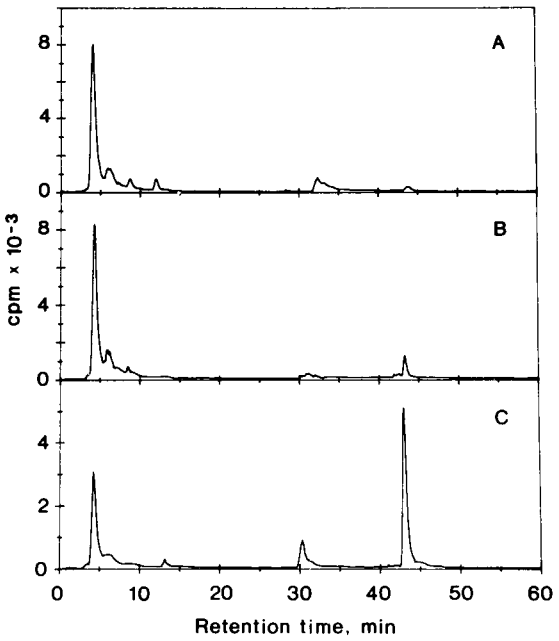

Fig. 5: Identification of glucose-1-P in extracts from oocytes injected with [U-^{14}C]glucose. Oocytes were injected, incubated for 20 min and processed as described in the legend of Fig. 3. An aliquot (0.3 ml) of the PCA extract was evaporated, suspended in 0.1 M Hepes pH 8 and applied to a CarboPac column (A). Another aliquot (0.3 ml) was incubated for 60 min with 0.5 mM $MgCl_2$, 2 mUnits of phosphoglucomutase (B) and 24 µM glucose-1,6-bisP. A third aliquot (0.1 ml) was treated as in B, except for the addition of 36 nmol of [U-^{14}C]glucose-1-P (C), which was quantitatively converted into [^{14}C]glucose-1,6-bisP and a trace of [^{14}C]fructose-6-P (eluting at 32 min) a result which will be further studied because of its relevance to the understanding of the mechanism of phosphoglucomutase.

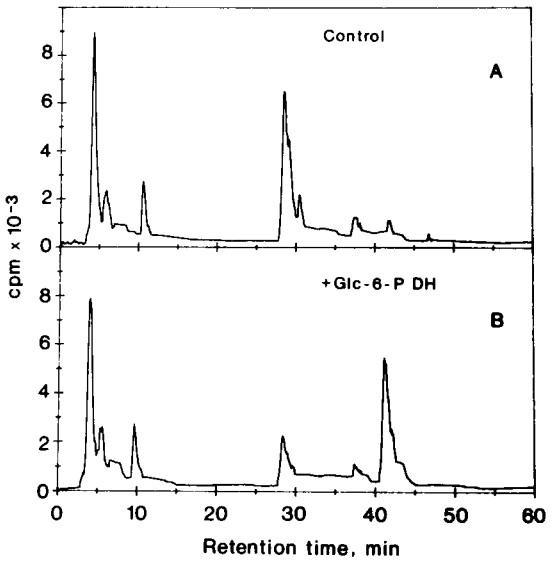

Fig. 6: Identification of glucose-6-P in peak 5 of PCA extracts from oocytes injected with [U-^{14}C] glucose. Oocytes were injected, incubated and processed as described in the legend of Fig. 3. An aliquot (0.3 ml) of the PCA extract was evaporated, resuspended in 0.1 M Hepes pH 8 and applied to a CarboPac column (A). Another aliquot (0.3 ml) was incubated overnight at 22°C with 0.8 mM $MgCl_2$, 0.5 mM NAD^+ and 2.4 mUnits of glucose-6-P dehydroge-nase (B).

appearance of a peak at 42 min, marking the retention time for 6-phosphogluconate (Fig. 6).

The sharpness of separation depends of course on the elution program. We have not performed a detailed systematic study to optimize the resolutions obtained. A summary of the retention times for glucose and several phosphorylated intermediates is shown in Table 1.

It should be noted that, because of the very low levels of glucose– or glycerol–derived intermediates in the samples used, it is not possible to measure the absolute amount of each metabolite in order to calculate specific radioactivities to obtain estimates of pool size. On-line pulsed amperometric detection (12) of intermediates in conjunction with the techniques described in this paper, could in principle be used for that purpose. We are presently trying to develop such an approach.

While this paper was being prepared, we learned that Ogushi et al. (10) have employed a similar column for the separation of phosphorylated intermediates of glucose metabolism in brain. No comparison with the present work is possible because only a few experimental details were presented. Retention times are very different to those reported in this paper.

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