Compartmentation of glucose 6-phosphate in hepatocytes

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Rat hepatocytes were incubated with ^{14}C -labelled hexoses, and the specific radioactivities of glucose 6-phosphate, glucose 1-phosphate and fructose 6-phosphate were determined. (1) When suspensions of freshly isolated hepatocytes were incubated with [^{14}C]glucose, the specific radioactivities of glucose 1-phosphate and fructose 6-phosphate were severalfold higher than that of glucose 6-phosphate. The ratios of the specific radioactivities decreased with time of incubation. These relationships were also found when incubations were carried out with primary cultures of rat hepatocytes or with crude homogenates of hepatocytes, but not with isolated nuclei. (2) When cells were incubated with [^{14}C]fructose, the ratios of the specific radioactivities were higher than with [^{14}C]glucose, and also decreased with time. (3) Paired incubations were carried out with a mixture of galactose and fructose, with one or other sugar being labelled with ^{14}C . The specific radioactivity of glucose released into the medium was greater than that of glucose 6-phosphate when fructose was labelled, but not when galactose was labelled. Furthermore, glucose 6-phosphate and glucose in the medium differed with regard to the distribution of ^{14}C between C-1 and C-6. These results are interpreted as evidence that glucose 6-phosphate in hepatocytes does not exist as a homogeneous pool, but that subcompartments exist which are associated with glucose phosphorylation, gluconeogenesis and glycogenolysis.

INTRODUCTION

Flux rates through metabolic pathways may be governed by regulatable enzymes on the pathway and by metabolic channelling of substrates, leading to metabolism by one pathway in preference to another. Metabolic channelling may be a consequence of, and facilitated by, the presence of multiple pools of a single metabolite, each associated with a particular metabolic pathway. There is evidence that glucose 6-phosphate (Glc6P) is such a metabolite. Sims & Landau [1] showed in rat diaphragm that the ratios of the incorporation of labelled glucose to the incorporation of labelled Glc6P into glycogen, CO_2 and lactate were not identical, as they should have been if glucose entered a homogeneous pool of Glc6P. These and similar results in skeletal muscle [2] could be explained either by postulating the existence of a glycogenic pathway which does not include Glc6P or by compartmentation of the pool of Glc6P. Antony *et al.* [3] arrived at a similar conclusion on the basis of ^{14}C distribution in glycogen glucose and Glc6P. We then demonstrated [4] that, when rat diaphragm was incubated with labelled glucose or pyruvate, the equilibrium specific radioactivities of some of the glycolytic intermediates were not compatible with precursor-product relationships but could be explained by multiple pools of Glc6P and triose phosphates; for each metabolite there was a glycogenic pool and a glycolytic pool. Equilibrium conditions virtually excluded the possibility of pathways which did not require Glc6P or triose phosphates as intermediates. Studies of hepatic metabolism have also demonstrated inhomogeneity of the Glc6P pool. Muntz [5] and Williams & Landau [6] injected rats with fructose labelled in the C-1 or C-6 position and examined the isotope distribution in these carbon atoms of glucose and

Glc6P; the ratio of C-1/C-6 incorporation was consistently higher for glucose than for Glc6P, indicating that the glucose was not derived from the total pool of Glc6P. A kinetic study of [^{14}C]fructose metabolism in liver after intravenous injection provided evidence for compartmentation of Glc6P and triose phosphates into gluconeogenic and glycolytic pools [7]. A similar experiment with [^{14}C]glucose gave results compatible with multiple pools of Glc6P, one of which was on the glycogenic pathway [8].

These studies of intact organs and tissues are complicated by the presence of multiple cell types; apparent inhomogeneity of metabolic pools might be due to differences between cell types with regard to the predominant metabolic pathway and to specific radioactivities of particular substrates. To obtain further information concerning metabolic compartmentation, we have studied hepatocytes in suspension and in primary cell culture.

METHODS

Hepatocytes were obtained from male Sprague-Dawley rats weighing 175–250 g, previously maintained on Purina rat chow and water *ad lib*. Cells were prepared by non-recycling perfusion with collagenase as previously described [9]. Hepatocytes were used either as suspensions of freshly isolated cells or in primary cultures. Cells for culture were plated in collagen-coated plastic culture dishes at a density of about 1.5×10^6 cells/dish in medium L-15 without galactose, containing 0.5 nM-insulin, 14 nM-dexamethasone and 10% (v/v) fetal-bovine serum. The following morning the medium was changed to L-15 with insulin and 140 nM-dexamethasone but no serum. Incubations were carried out with

Abbreviations used: Glc6P, glucose 6-phosphate; Glc1P, glucose 1-phosphate; Fru6P, fructose 6-phosphate.

Table 1. Specific-radioactivity ratios after incubation with [¹⁴C]hexose

Values shown in this and subsequent Tables are means $\pm$ S.E.M.; ND = not done (insufficient Fru6P for accurate measurement). * $P < 0.05$; ** $P < 0.025$; *** $P < 0.001$. Isolated hepatocytes in suspension (5×10^7 cells/ml) and hepatocytes maintained in primary culture (3.4×10^7 cells per 150 mm dish) were incubated in L-15 medium modified to omit galactose and to contain 1% bovine serum albumin and either 8 mM-[U-¹⁴C]glucose (2–3 μ Ci/ μ mol) or 8 mM-[U-¹⁴C]fructose (0.5 μ Ci/ μ mol). Unless otherwise indicated, incubations with labelled substrates were for 60 min.

Hexose	Preparation	<i>n</i>	Glc1P/Glc6P	Fru6P/Glc6P
Glucose	Hepatocytes	5	$3.67 \pm 0.29^{***}$	$3.42 \pm 0.21^{***}$
	Cultured hepatocytes (fed rats)	5	$2.21 \pm 0.14^{***}$	1.45 ± 0.19
	Cultured hepatocytes (starved rats)	10	$3.43 \pm 0.38^{***}$	$1.88 \pm 0.14^{***}$
	Hepatocyte homogenate	3		
	Whole homogenate		$4.03 \pm 0.44^{**}$	$6.3 \pm 1.2^{***}$
Fructose	Nuclei		$0.39 \pm 0.01^{***}$	ND
	Hepatocytes			
	1 h incubation	3	$4.48 \pm 0.59^*$	$11.07 \pm 2.7^*$
	2 h incubation	3	1.9 ± 0.55	3.8 ± 0.3

suspensions of freshly isolated hepatocytes and with 48 h cultures, in L-15 without galactose, containing [¹⁴C]hexose as indicated for each experiment; for cultured cells, the culture medium was removed and the cells were rinsed in Dulbecco's phosphate-buffered saline (Gibco) before addition of incubation medium. In experiments with subcellular fractions, liver was homogenized and fractionated as described by Widnell & Tata [10] to obtain crude homogenate and purified nuclei. Incubations were carried out in 0.1 M-Tris buffer, pH 7.4, containing 0.01 M-MgCl₂, 0.005 M-ATP, 8 mM-glucose and [¹⁴C]glucose.

Isolation of phosphorylated intermediates

These were isolated by a modification of the procedure previously described in detail [11]. Incubation of cell suspensions were terminated by chilling in an ice bath; the cells were then separated by centrifugation (25 *g* for 5 min) at 4 °C, washed with cold phosphate-buffered saline to remove residual medium, and then suspended in cold 0.5 M-HClO₄. For cells in culture, the incubation medium was removed by aspiration and cells were rapidly rinsed three times with cold phosphate-buffered saline; they were then scraped from the dish after the addition of cold 0.5 M-HClO₄. The cells in HClO₄ were homogenized with a Polytron homogenizer; after standing for at least 1 h, the precipitate was removed by centrifugation (2500 *g* for 10 min) at 4 °C, and the supernatant was decolorized with 20 mg of activated charcoal. The pH was adjusted to 8.0 by titration with 5 M-KOH, and the precipitated KClO₄ was removed by centrifugation (2500 *g* for 10 min). The clear supernatant was evaporated to dryness, then redissolved in 2 ml of cold water and clarified by centrifugation. A portion was used to measure Glc6P; the remainder was applied to a column of Dowex-1 (X8; formate form), 1 ml of packed resin being used per 125 nmol of Glc6P. The column was eluted with 10 vol. of water to remove free glucose, with 5 vol. of 0.5 M-formic acid to remove lactate and then with 20 vol. of 1 M-formic acid to obtain the phosphorylated metabolites. The 1 M-formic acid fraction was dried, then redissolved in 2 ml of water. Carrier hexose (2 μ mol) of the type used during the incubation (see below) was added, the extract was applied to a second column of Dowex-1 (formate form) and eluted as

above. The 1 M-formate fraction was again dried, dissolved in 1.0 M-HCl and heated for 6 min at 100 °C to hydrolyse Glc1P; it was then dried, redissolved in water, applied to a column of Dowex-1 (X8; formate form), eluted with 20 vol. of water to remove the glucose released from Glc1P, and then with 20 vol. of 2 M-formate to obtain the unhydrolysed hexose phosphates. The latter were dried, redissolved in 1.5 ml of water and applied to a column (13 cm $\times$ 0.5 cm) of Dowex-1 (X8; Cl⁻ form). This was washed with 3 vol. of 1 mM-NH₃, then eluted with a concave gradient of NH₄Cl in K₂B₄O₇ [12] at a rate of 0.3 ml/min to isolate Glc6P and Fru6P. Recoveries of standard Glc1P, Glc6P and Fru6P were > 85%. Glucose was isolated from the incubation medium by two procedures. (a) A portion of medium was treated with HClO₄, then neutralized as described above for cells. The neutralized extract was desalted by passage through sequential columns of Dowex-50 (H⁺ form) and Dowex-1 (formate form), then concentrated on a rotary evaporator. Sugars were separated by t.l.c. on cellulose, in formic acid/butanone/2-methylpropan-2-ol/water (3:6:8:3, by vol.) [13]; three successive chromatographic runs were used to increase the separation of the hexoses. Standards run simultaneously were located with aniline/diphenylamine (Sigma). The glucose band was removed from the plate, eluted with water, measured enzymically and assayed for radioactivity. (b) A sample of medium was incubated with hexokinase, then applied to a column of Dowex-1 (formate form); after washing the column with water, Glc6P was eluted with formic acid, dried, assayed enzymically and counted for radioactivity. The procedures gave virtually identical results.

Quantitative measurements

Enzymic procedures were used for the measurement of glucose [14], Glc6P and Fru6P [15]. The ¹⁴C in C-6 glucose was determined as formaldehyde [16]. Radioactivity was measured in a liquid-scintillation counter.

RESULTS

Incubation with [¹⁴C]glucose

When suspensions of isolated hepatocytes were incubated with 8 mM-[¹⁴C]glucose, the specific-radioactivity

Fig. 1. Time course of the ratios of specific radioactivities of Glc1P and Fru6P to Glc6P in isolated hepatocytes incubated with [^{14}C]glucose

Hepatocytes were incubated at 37 °C in L-15 medium modified to omit galactose and containing 8 mM-[U- ^{14}C]-glucose (1.5 $\mu\text{Ci}/\mu\text{mol}$) and 1% bovine serum albumin. The cell density was 3×10^7 cells/ml. At various times of incubation, portions of the cell suspension were removed and assayed for phosphorylated intermediates as described in the Methods section.

ratios of Glc1P/Glc6P and Fru6P/Glu6P were consistently greater than 1 (Table 1). The ratios fell with time of incubation, but even after 1 h were still about 4 (Fig. 1). Cultured hepatocytes had somewhat lower ratios, particularly when the cells used for culture were obtained from fed rats. Elevated ratios were observed when cell homogenates were incubated with [^{14}C]glucose; however, similar incubation with isolated nuclei failed to yield increased ratios.

Incubation with [^{14}C]fructose

The incorporation of fructose into Glc6P involves prior conversion of the hexose into fructose 1-phosphate, triose phosphates as well as fructose 1,6-bisphosphate and Fru6P. After incubation of hepatocytes with 8 mM-[^{14}C]fructose, the specific radioactivity of Fru6P at 1 h was much greater than that of Glc6P, but the difference decreased with time (Table 1). The specific radioactivity of Glc1P was also consistently greater than that of Glc6P, though not by as much as that of Fru6P.

Conversion of [^{14}C]fructose and [^{14}C]galactose into glucose

The preceding experiments were concerned with interconversions between Glc6P and its proximal intermediary metabolites, each of which can be formed from other sources. We also studied the conversion of Glc6P into glucose, an end product for which Glc6P is the only source. Duplicate cell suspensions were incubated in glucose-free medium (5×10^7 cells in 10 ml) containing 5 mM-fructose and 5 mM-galactose; [U- ^{14}C]fructose was added to one portion of cells and [1- ^{14}C]galactose to the other. Specific radioactivities of the Glc6P in the cells and of the glucose released into the medium were determined. Accumulation of glucose in the medium was rapid (Fig. 2), and after 1 h of incubation was

Fig. 2. Release of glucose into the medium during incubation of isolated hepatocytes with a mixture of galactose and fructose

Hepatocytes were incubated at 37 °C in Hanks buffered salt solution containing 5 mM-galactose, 5 mM-fructose and 1% bovine serum albumin. The cell density was 5×10^6 cells/ml. At various times of incubation, portions of the cell suspension were removed and centrifuged at 50 g for 5 min to remove cells, and the supernatants were treated with HClO_4 (0.5 M final concn.) and then neutralized with KOH. Glucose was determined in the neutralized supernatants as described in the Methods section. Values shown are means $\pm$ S.E.M.

Table 2. Specific-radioactivity ratios after incubation of hepatocytes with fructose and galactose

Isolated hepatocytes in suspension (5×10^6 cells/ml) were incubated in Hanks buffered salt solution containing 1% bovine serum albumin, 5 mM-galactose and 5 mM-fructose, and either [U- ^{14}C]fructose (0.4 $\mu\text{Ci}/\mu\text{mol}$) or [1- ^{14}C]galactose (0.4 $\mu\text{Ci}/\mu\text{mol}$). Cells were isolated by centrifugation and analysed for the specific radioactivities of the phosphorylated intermediates as described in the Methods section. Results are means $\pm$ S.E.M. (except †, mean of two experiments); *** $P < 0.005$.

	Glc1P/ Glc6P	Fru6P/ Glc6P	Glucose/ Glc6P
[^{14}C]Fructose	1.5†	4.5 $\pm$ 1.2	2.8 $\pm$ 0.1***
[^{14}C]Galactose	2.1 $\pm$ 0.2	2.9 $\pm$ 0.5	0.8 $\pm$ 0.1

23 ± 1 μmol , equivalent to 23% of the original hexose content (fructose + galactose) of the medium. Radioactivity from fructose also appeared rapidly; after 1 h the glucose contained $28 \pm 2\%$ of the starting glucose radioactivity. The specific radioactivity of the glucose was severalfold higher than that of Glc6P. After incubation for 1 h with labelled galactose only $11 \pm 1\%$ of the label had appeared in the glucose, and the specific radioactivity was the same as that of Glc6P (Table 2). It can be calculated that, of the 23 μmol of glucose produced, 14 μmol was derived from fructose, 5 from galactose and 4 from endogenous sources.

The ^{14}C radioactivity in C-6 of glucose in the medium and in the glucose moiety of Glc6P was determined

Table 3. Radioactivity in C-6 of glucose derived from [1-¹⁴C]-galactose

Incubations were performed with [1-¹⁴C]galactose as described for Table 2.

Time of incubation (min)	Radioactivity (% of total)	
	Glucose of Glc6P	Glucose in medium
15	2.3	1.4
30	1.9	0.9
60	2.3	0.4

during the course of the incubation of hepatocytes with [1-¹⁴C]galactose. The proportion of radioactivity in C-6 (C-6/total) in the glucose released into the medium fell during the period of incubation, whereas that of the glucose moiety of Glc6P was constant, and higher than that of glucose in the medium. The proportion was low in all samples (Table 3).

DISCUSSION

As observed previously with hemidiaphragm, when isolated hepatocytes were incubated with labelled glucose the specific radioactivity of the Glc6P was lower than that of both Glc1P and Fru6P, metabolites that are derived from Glc6P.

This situation might arise if the cell population were heterogeneous with regard to the pool sizes of the metabolites and to the equilibrium specific radioactivity of the Glc6P, even though the specific radioactivities of the metabolites were identical within individual cells. Such heterogeneity might be due to the presence within the liver of several cell types, or to zonation of metabolic activity within the hepatocyte population [17]. We believe that both of these possibilities can be excluded, for several reasons. (a) The proportion of endothelial cells and Kupffer cells to hepatocytes is greatly decreased during the isolation of hepatocytes [18], and most of the remaining non-epithelial cells die out when hepatocytes are maintained in primary culture. However, the high specific-radioactivity ratios persisted in these primary cultures. (b) The high ratios were noted with fructose as labelled substrate. This sugar is not known to be taken up by cells other than hepatocytes. (c) Glycolytic and gluconeogenic states of hepatocytes are interconvertible, depending on culture conditions [19]; the oxygen partial pressure and the presence of insulin in the culture medium employed in the present experiments would have converted all cells into the glycolytic type [19]. (d) Homogenization would obliterate any distinctions among cell types and create a single metabolic pool, yet high specific-radioactivity ratios were observed when cell homogenates were used in these experiments. Thus the phenomenon appears to be characteristic of all hepatocytes and not the result of mixing several different cell types.

The occurrence of a higher specific radioactivity in Glc1P and Fru6P than in Glc6P might also be explained by the conversion of glucose directly into Glc1P and Fru6P without passing through Glc6P; apart from the

fact that there is no known evidence for such pathways, it has been shown that after very short-term incubations virtually all metabolized glucose is present as Glc6P, indicating that this compound is an essential first product [20]. Thus it may be concluded that Glc6P formed by phosphorylation of glucose does not mix rapidly with that formed from other sources. With increasing duration of incubation the specific-radioactivity ratios fell, presumably as the slow interchange of metabolites progresses.

Isolated nuclei had a specific-radioactivity ratio (Glc1P/Glc6P) of 0.39; since there was no precursor of Glc1P other than glucose, this low value was not due to dilution of the label arising from Glc6P, and was therefore due to a low phosphoglucosyltransferase activity. This result does not permit any conclusion about compartmentation within nuclei.

When cells were incubated with fructose and galactose, each hexose was converted into glucose; this necessitated prior conversion into Glc6P, but by different pathways. The marked difference in the glucose/Glc6P specific-radioactivity ratios noted on incubation with [¹⁴C]-fructose and [¹⁴C]galactose demonstrates that the Glc6P derived from each hexose was distinct from that derived from the other. Further information is obtained from the distribution of radioactivity in the glucose molecule. Since the galactose was labelled only in C-1, introduction of ¹⁴C into the C-6 position of glucose is an indication of the activity of the futile cycle Glc6P → triose phosphates → Glc6P. This was evidently a very minor pathway, since glucose in the medium had only 1.4% of its total radioactivity in C-6, and this amount fell with time. Although the corresponding value for glucose of Glc6P was also low, it was higher than that of glucose in the medium, and was constant throughout the incubation. These differences in extent and time course of C-6 labelling indicate that the small amount of galactose that traversed the triose phosphate futile cycle was not distributed into a single pool of Glc6P from which glucose was derived.

These results demonstrate that intra-hepatocyte Glc6P does not constitute a homogeneous pool. It is suggested that in hepatocytes, as in diaphragm, there are multiple pools of Glc6P. This is in line with previous evidence for compartmentation of Glc6P and triose phosphates in liver and muscle [1–8], and with more recent evidence of compartmentation of Glc6P in vascular-smooth-muscle cells [21] and of UDP-glucose in liver [22]. The results with fructose and galactose suggest that there is one pool of Glc6P associated with the pathway from galactose 1-phosphate to Glc6P, and another with the pathway from fructose 1-phosphate to Glc6P. Since each of the other metabolites for which there is evidence of compartmentation is also common to two or more metabolic pathways, it is possible that compartmentation of the metabolites is a consequence of compartmentation of integrated pathways. It has been considered that in the intact cell all enzymes are associated with membranous structures and/or cytoskeletal elements [23]. Such association might provide a basis for the organization or grouping of enzymes associated with a specific pathway, and thus to a multiplicity of pools of a metabolite common to several pathways.

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