Extracellular metabolites in suspensions of isolated hepatocytes

Emile VAN SCHAFTINGEN,* Louis HUE† and Henry-Géry HERS*

*Laboratoire de Chimie Physiologique and †Hormone and Metabolic Research Unit, Université Catholique de Louvain and International Institute of Cellular and Molecular Pathology, 75 avenue Hippocrate, 1200 Brussels, Belgium

The activity of lactate dehydrogenase and the concentration of several metabolites were measured in a suspension of isolated hepatocytes and in the extracellular medium, obtained after elimination of the cells by centrifugation for 15 s. The initial proportions of ATP, fructose 2,6-bisphosphate and glycogen present in the medium were similar to that of lactate dehydrogenase, and were therefore explained by unavoidable cell breakage occurring during resuspension of the hepatocytes. ATP disappeared from the medium in less than 10 min, being presumably destroyed by membrane nucleotidases. By contrast, the proportions of hexose 6-phosphates and of glycerol 3-phosphate in the medium were several-fold in excess over that of lactate dehydrogenase; under certain conditions, the extracellular value accounted for 80–90 % of the metabolite present in the total suspension, and there was no relationship between the extra- and intracellular concentrations of these metabolites. A potential source of external glycerol 3-phosphate was the hydrolysis of glycerophosphocholine by membranous enzymes. The main conclusion of this work is that the measurement, in isolated hepatocytes, of hexose 6-phosphates, glycerol 3-phosphate and possibly other metabolites that were not investigated, requires the previous separation of the cells from the incubation medium. This conclusion may apply to other cellular suspensions.

INTRODUCTION

Freshly isolated hepatocytes are a convenient model for the study of metabolism. The evaluation of metabolic control in this preparation often requires an accurate measurement of the intracellular concentrations of metabolites. For this purpose, the cells can be separated from the suspending medium by various methods, including centrifugation through a 4 % (w/v) NaCl layer in a simple device as described by Hems *et al.* (1975), or through a layer of silicone (Baur *et al.*, 1975) or of bromododecane (Cornell, 1980). Hems *et al.* (1975) applied their technique for the measurement of intra- and extra-cellular concentrations of various amino acids and of K⁺. Such a separation is, however, not required for metabolites such as adenine nucleotides, which are present exclusively in the cell and the determination of which can therefore be performed on an extract of the whole suspension (Hems *et al.*, 1975). Although it may seem logical to apply a similar rule for the determination of other phosphoric esters, which are generally believed not to cross the cell membrane, we have noticed that the amount of hexose 6-phosphates and glycerol 3-phosphate present in the extracellular medium could, unexpectedly, exceed by several-fold the intracellular amount. This observation is reported and discussed in the present paper, the aim of which is to call the attention of other investigators to a potential source of experimental error.

MATERIALS AND METHODS

Materials

Glycerophosphocholine (Sigma, St. Louis, MO, U.S.A.) was freed from Cd²⁺ ions by chromatography on Chelex 100 (100–200 mesh, Na⁺ form; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Oyster glycogen was

from Sigma, and was gel-filtered on Sephadex G-25 (Pharmacia, Uppsala, Sweden) before use. Phosphatidylcholine and phosphatidic acid (Serdary Research Laboratories, London, Ontario, Canada) were freed from chloroform by evaporation under a stream of nitrogen and dissolved at a final concentration of 4 mg/ml in 50 mM-Tris/HCl buffer, pH 7.4, containing 1 % bovine serum albumin (Sigma). Pyrophosphate:fructose 6-phosphate 1-phosphotransferase (Van Schaftingen *et al.*, 1982b) and fructose 2,6-bisphosphate (Van Schaftingen & Hers, 1981) were prepared as described previously. Collagenase and other biochemicals were from Boehringer (Mannheim, Germany).

Preparation and handling of hepatocytes

Hepatocytes were isolated from the liver of fed or overnight-fasted male Wistar rats by a modification (Bartrons *et al.*, 1983) of the method described by Seglen (1973). Some experiments were performed with hepatocytes prepared as described by Hue *et al.* (1978). Both methods gave similar results. After disruption of the liver, the cells were washed in 3 × 100 ml of a Krebs–Henseleit (1932) bicarbonate buffer equilibrated with O₂/CO₂ (19:1) as gas phase. In our hands, resuspending a cell pellet takes 1–2 min. Incubation of the cells was started as soon as resuspension was complete, and was performed at 37 °C in a shaking water bath.

At the indicated times, samples of the cell suspension were mixed with 1 vol. of 10 % (v/v) HClO₄ (for the determination of phosphate esters other than fructose 2,6-bisphosphate), or frozen in an acetone/solid-CO₂ cooling mixture (for the determination of glycogen and of fructose 2,6-bisphosphate), or kept at 0 °C (for the determination of LDH). Similar samples were also taken from the supernatant resulting from centrifugation for 15 s at 1000 g. For the determination of Trypan Blue

Table 1. Concentration of metabolites in a suspension of hepatocytes

Hepatocytes were incubated at a concentration between 54 and 145 mg wet wt./ml and with or without 20 mM-glucose. For each time, the first column shows the concentration of metabolite normalized for a cell concentration of 100 mg/ml; the second column shows the concentration of the metabolite in the extracellular medium, expressed as a percentage of that in the complete cellular suspension; the third column shows the ratio of this value to the percentage of external LDH; values shown are means $\pm$ S.E.M. or individual values; n.d., not detectable. Although only one value is given for ATP and for fructose 2,6-bisphosphate, these values are in agreement with others obtained in similar experiments.

Metabolite	Dietary condition	Glucose added (mM)	Time (min) ...			0			10			60		
			n	Total concn. (μ M or mg/ml*)	Extra total (%)	Extra LDH	Total concn. (μ M or mg/ml*)	Extra total (%)	Extra LDH	Total concn. (μ M or mg/ml*)	Extra total (%)	Extra LDH	Total concn. (μ M or mg/ml*)	Extra total (%)
ATP	Fed	0	1	269	3.1	0.7	303	n.d.	n.d.	273	n.d.	n.d.	273	n.d.
Fructose 2,6-bisphosphate	Fed	0	1	0.80	6.6	1.4	1.85	2.2	0.3	1.06	1.1	0.1	1.06	0.1
Hexose 6-phosphates	Fed	0	3-5	82 $\pm$ 13	24 $\pm$ 3	3.6 $\pm$ 0.4	54 $\pm$ 11	88 $\pm$ 2	3.9 $\pm$ 1.0	26 $\pm$ 5	80 $\pm$ 5	4.5 $\pm$ 0.3	26 $\pm$ 5	4.5 $\pm$ 0.3
	Fed	20	1 or 2	102, 81	21, 71	2.9, 5.7	59, 61	67	4.5	24, 32	47, 53	4.7, 3.9	24, 32	4.7, 3.9
Glycerol 3-phosphate	Fasted	20	8	-	-	-	19 $\pm$ 3	44 $\pm$ 5	-	25 $\pm$ 4	47 $\pm$ 3	-	25 $\pm$ 4	-
	Fed	0	4 or 5	43 $\pm$ 14	25 $\pm$ 3	3.6 $\pm$ 0.1	44 $\pm$ 3	49 $\pm$ 12	2.6 $\pm$ 0.3	53 $\pm$ 10	81 $\pm$ 4	5.3 $\pm$ 0.8	53 $\pm$ 10	5.3 $\pm$ 0.8
Glycogen	Fed	20	2	79, 40	21, 32	2.6, 2.9	72, 62	33, 56	2.2, 3.6	58, 53	44, 53	4.4, 3.9	58, 53	4.4, 3.9
	Fed	0	4 or 5	2.5 $\pm$ 0.3*	9.5 $\pm$ 2.3	1.4 $\pm$ 0.2	2.0 $\pm$ 0.4*	13.0 $\pm$ 3.6	0.7 $\pm$ 0.1	1.4 $\pm$ 0.3*	6.5 $\pm$ 1.6	0.4 $\pm$ 0.1	1.4 $\pm$ 0.3*	0.4 $\pm$ 0.1
	Fed	20	2	3, 3.2*	6.9, 12	1.0, 1.0	2.7, 2.8*	8.9, 12.7	0.6, 0.8	3.1, 3.2*	8.5, 3.2	0.8, 0.2	3.1, 3.2*	0.8, 0.2

exclusion, a final concentration of 0.25% of the dye was used and approx. 200 cells were counted in an haemocytometer. The weight of cells was calculated from the concentration of protein (Lowry *et al.*, 1951) by assuming that 1 g of cells contained 200 mg of protein.

Assay of enzymes and of metabolites

LDH was measured as described by Vassault (1983). Hexose 6-phosphates (Lang & Michal, 1974), ATP (Lamprecht & Trautschold, 1974), glycerol 3-phosphate (Michal & Lang, 1974) and UDP-glucose (Keppler & Decker, 1974) were determined spectrophotometrically on HClO_4 extracts as described in the cited references. For these measurements, an Aminco-Chance DW2 spectrophotometer was used in the dual-wavelength mode (340–400 nm); taking into account the dilution of the extracts in the assay and the value of the blanks, the limit of detection of these metabolites is in our hands about 1 nmol/ml of cell suspension. Glycogen was measured as described by Lust *et al.* (1975). Fructose 2,6-bisphosphate was determined in alkaline extracts by the method of Van Schaftingen *et al.* (1982b) as modified by Van Schaftingen & Hers (1983).

RESULTS

Distribution of metabolites between cells and the suspension medium

In freshly isolated hepatocytes, 5–10% of the total LDH was present in the extracellular fluid, and this proportion approximately doubled after incubation for 1 h. Since LDH was found to be stable in the extracellular medium under our experimental conditions, we can assume that its presence reflects the proportion of cells broken during their initial resuspension and during their subsequent incubation. The proportion observed is in good agreement with the viability value determined by the Trypan Blue exclusion method (results not shown). We have also measured the concentrations of several metabolites in the total suspension and in the external medium, and expressed the latter value as a percentage of that found in the total cell suspension (second column for each experimental time in Table 1). This percentage was then related to the same parameter calculated for LDH (third columns in Table 1), bearing in mind that a ratio close to 1 indicates that cell rupture entirely accounts for the presence of the metabolites in the medium.

We found that the proportions of glycogen and of fructose 2,6-bisphosphate present in the medium at the beginning of the experiment were not greatly different from that of LDH and tended to decrease in the course of the 1 h incubation period, reflecting a more rapid disappearance of these compounds from the medium than from the cells. About 3–5% of ATP was outside the cells at zero time, and then this nucleotide became undetectable, being probably rapidly destroyed by membrane nucleotidases. Extracellular UDP-glucose remained below the limit of detection throughout the incubation (results not shown).

Unexpected values were observed for hexose 6-phosphates and glycerol 3-phosphate. Indeed, already in the fresh preparation of hepatocytes obtained from fed rats, their extracellular concentration exceeded by more than 3-fold the value that could be explained by the

Fig. 1. Effect of glucose on the extra- and intra-cellular concentrations of hexose 6-phosphates in a suspension of hepatocytes isolated from a fasted rat

The cells were incubated at a concentration of 10 mg of protein/ml in the absence (a) or in the presence (b) of 20 mM-glucose. Total and extracellular (Extra) hexose 6-phosphates were measured; the 'intracellular' value (Intra), given as nmol/ml of suspension, is the difference between the two first values. The concentration of hexose phosphates inside the hepatocytes is 20 times this value. 90 and 80 % of the cells excluded Trypan Blue respectively at the beginning and at the end of the incubation.

rupture of cells. This abnormality may be explained by the fact that, because of anoxia, the intracellular concentration of these esters was much higher in the freshly prepared suspension (zero time in Table 1) than after some time of reoxygenation. It was presumably still higher in the pellet, precisely at the time when the cells were damaged by resuspension. During the subsequent incubation, the extracellular proportion of both metabolites became greater, sometimes exceeding 80 %. It can be calculated from Table 1 that this resulted from both an increase in the extracellular concentration, particularly evident for glycerol 3-phosphate, and a decrease in the intracellular value. Table 1 also shows that the proportion of extracellular metabolites was slightly lower when glucose was included in the medium, mainly as a result of an increase in the intracellular pools. These results were not affected by the inclusion of albumin in the suspension medium (results not shown).

Independent formation of intra- and extra-cellular phosphoric esters

Hepatocytes from fasted rats were used to gain more information on the formation and origin of hexose 6-phosphates and of glycerol 3-phosphate. Fig. 1 shows that, when the cells were incubated in the absence of

glucose, the concentration of hexose 6-phosphates was initially at the lower limit of detection and then increased linearly at a rate of approx. 0.8 nmol/min per g of hepatocytes, all of these hexose 6-phosphates being extracellular. In the presence of glucose, the amount of hexose 6-phosphates formed was several-fold higher, but this was mostly due to an increase in the intracellular concentration, with little effect on the extracellular value.

We show in Fig. 2 that, in the absence of glucose, glycerol 3-phosphate was also formed essentially extracellularly, but at a rate approx. 4-fold greater than that of hexose 6-phosphates. Intracellular glycerol 3-phosphate was formed in large amounts when glucose, and even more when ethanol, was added to the incubation mixture, but this caused only a slight increase in the extracellular concentration of the phosphate ester.

Fate of added metabolites

Remarkably, the incubation of cells of fasted rats in the presence of 0.8 mM-glycero-phosphocholine led to the formation of glycerol 3-phosphate at a rate of 100 nmol/min per g of protein (Fig. 3), which is about 5-fold the rate of spontaneous formation of glycerol 3-phosphate; incubation with phosphatidic acid or phosphatidyl-glycerol (40 μg/ml) did not increase the formation of glycerol 3-phosphate above its basal value (results not shown). Other results (not shown) were as follows: when added at an initial concentration of 0.5 mM to a suspension of hepatocytes obtained from fed rats, ATP and UDP-glucose were completely destroyed within 10 min, the latter being converted (at least 50 %) into hexose 6-phosphates; hexose 6-phosphates disappeared at an initial rate of 0.7 μmol/min per g of protein, a rate which is only partially accounted for by the activity of glucose-6-phosphatase, expected to be present in the extracellular fluid, knowing that there is normally about 10 units of this enzyme/g of liver and that its K_m is close to 2.3 mM (Arion *et al.*, 1972). At lower concentrations of hexose 6-phosphates, as found in the extracellular fluid, the rate of hydrolysis would be proportionally decreased. In contrast, glycerol 3-phosphate and fructose 2,6-bisphosphate (initial concn. 10 μM) were stable. When added to a suspension of hepatocytes isolated from a fasted rat, glycogen (3.5 μmol of glucose units/ml) was destroyed at a rate of 6 μmol/min per g of protein, only a small part of this (0.5 %) being accounted for by the hexose 6-phosphates formed.

DISCUSSION

From the data presented in this paper and also from the literature, it seems reasonable to assume that approx. 5–10 % of hepatocytes are broken during their initial resuspension and another 5–10 % during the subsequent 1 h of incubation at 37 °C, resulting in a parallel release of their content of enzymes and metabolites. It must be noted that, because of the transient anoxia, the concentration of some metabolites in cells may reach a maximum when the pellet is resuspended, precisely at the time when the cells are ruptured. This would cause an excess of these metabolites relative to LDH in the extracellular fluid, as was actually observed for hexose 6-phosphates and glycerol 3-phosphate. Subsequently, the released enzymes, as well as those retained inside the cell ghost or present in the outer surface of the plasma

Fig. 2. Effect of glucose and of ethanol on the extra- and intra-cellular concentration of glycerol 3-phosphate in a suspension of hepatocytes isolated from a fasted rat

Same experiment as in Fig. 1, except that the cells were also incubated in the presence of 20 mM-glucose + 20 mM-ethanol (c).

Fig. 3. Extracellular formation of glycerol 3-phosphate from glycerophosphocholine in hepatocytes isolated from a fasted rat

The cells were incubated at a final concentration of 9.3 mg of protein/ml with or without 0.8 mM-glycerophosphocholine (GroPCho).

membrane of intact cells, act on the extracellular metabolites.

The fact that added UDP-glucose and ATP were rapidly undetectable in the extracellular medium is in agreement with previous observations by Hems *et al.* (1975). It is easily accounted for by the high capacity of the cell suspension to split these nucleotides, presumably due to membrane ATPases and nucleotidases (Stanley *et al.*, 1982). External glycogen, of endogenous or exogenous origin, is degraded more slowly; the phosphorolytic pathway seems quantitatively minor in this process, since the hexose 6-phosphates accounted for less than 1% of the degraded glycogen. More than 80% of the extracellular fructose 2,6-bisphosphate initially present (50 nM) in the experiment reported in Table 1 was degraded in 60 min, whereas no decrease was apparent within 60 min when 10 μM-fructose 2,6-bisphosphate was added: this indicates that a low-capacity low- K_m enzyme, i.e. fructose-2,6-bisphosphatase, is involved in the degradation of extracellular fructose 2,6-bisphosphate (Van Schaftingen *et al.*, 1982a).

To account for the relatively high concentration of extracellular hexose 6-phosphates at zero time of incubation of cells obtained from fed rats compared with that of LDH, one can evoke the rapid hydrolysis of UDP-glucose (which could account for 1.5–3 μM) and, as

stated above, the effect of anoxia. During incubation, extracellular hexose 6-phosphates were destroyed, possibly by glucose-6-phosphatase, since glycerol 3-phosphate and fructose 2,6-bisphosphate were more stable. They were also formed by an unknown mechanism, as illustrated by the experiment performed with hepatocytes from fasted rats (Fig. 1). The intracellular value decreased markedly in the fed condition or remained very low in the fasted condition in the absence of glucose. As a result of these modifications, external hexose 6-phosphates eventually made up as much as 80–90 % of the total amount. Glycogenolysis presumably contributes to the formation of extracellular hexose 6-phosphates in cells from fed rats, but not in cells from fasted rats, since in the latter glycogen was not detectable. We have therefore no simple explanation for the appearance of a small but detectable amount of extracellular hexose 6-phosphates under the latter condition.

The high relative concentration of glycerol 3-phosphate at zero time is presumably, like that of hexose 6-phosphates, explained by anoxia. Subsequently, glycerol 3-phosphate was formed in the extracellular medium at similar rates in hepatocytes from fed (Table 1) and from fasted (Fig. 2) rats. This formation was, as for hexose 6-phosphates, independent of the intracellular concentration and was presumably the result of the hydrolysis of extracellular glycerophosphocholine and glycerophosphoethanolamine. These two compounds are indeed found at relatively high (0.5–1 $\mu\text{mol/g}$) concentrations in the liver of both fed and fasted rats (Dawson, 1955, 1956b). Furthermore, the liver contains a phosphodiesterase able to split these compounds into glycerol 3-phosphate and choline or ethanolamine (Dawson, 1956a; Fig. 3); part of this enzyme is bound to the plasma membrane (Fowler & de Duve, 1969; Lloyd-Davies *et al.*, 1972).

Whatever the mechanisms, the important conclusion is that there is no simple relationship between the intra- and the extra-cellular concentrations of several phosphate esters in suspensions of isolated hepatocytes. This confirms the general belief that the cell membrane is not permeable to phosphate esters. For some of them, mainly hexose 6-phosphates and glycerol 3-phosphate, but possibly also for others which were not investigated in the present work, the determination of the intracellular concentration requires the separation of the cells from the medium. Various techniques are available for this purpose (see the Introduction). This requirement is potentially applicable to other cell types.

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