Loss of Cell Constituents from Hepatocytes on Centrifugation

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In studies of the metabolism of isolated hepatocytes, it is often necessary to measure the concentrations of cell constituents both in cells and medium. When hepatocytes are separated in the special tubes of Hems, Lund & Krebs (1975) (*Biochem. J.* **150**, 47–50), they lose much glucose, urea and Na^+ , whereas there is no loss of K^+ , glutamate, aspartate and adenine nucleotides. Cell water is also lost, as measured by the distribution of $^3\text{H}_2\text{O}$. This loss is mainly due to an exchange of cell water with the aqueous solution in the stems of the tubes through which the cells pass on centrifugation. In general, substances are lost only when the intracellular concentration is equal to, or lower than, the extracellular concentration. Probably solutes are lost because they travel with the water unidirectionally out of the cell. A loss of solute does not occur when the cells are centrifuged in conical tubes with a layer of silicone oil between the cell suspension and the deproteinizing layer. The reasons for the loss occurring in the special separation tubes are discussed.

In the study of hepatic metabolism it is often important to measure concentrations of cell constituents within cells and in extracellular fluid. The special centrifuge tube designed by Hems *et al.* (1975) has proved satisfactory for the measurement of many hepatocyte constituents, particularly for those that do not readily permeate plasma membranes, such as ATP, ADP, AMP, glutamate, α -oxoglutarate, aspartate and malate. However, when testing the distribution of urea, glucose and water, we found the procedure unsatisfactory. For example, the concentration of urea in cells was only one-quarter of that in the medium, although it is known that *in vivo* the concentration of urea is equal in cells and medium. This paper is concerned with the causes of this loss of urea and other substances, and with ways and means of preventing the loss.

Experimental

Preparation and incubation of cells

Hepatocytes were prepared as previously described (Cornell *et al.*, 1973; Krebs *et al.*, 1974).

Hepatocytes (about 80 mg wet wt.) from 48 h-starved rats were incubated at 37°C in 4 ml of saline medium containing 2.5% dialysed bovine serum albumin for 15–20 min before they were separated either in the special tubes described by Hems *et al.* (1975) or in 15 ml conical centrifuge tubes as described below.

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Estimation of intracellular water content and correction for adhering fluid

Intracellular water was estimated by two methods.

Method (a). Distribution of $^3\text{H}_2\text{O}$ with corrections for adhering water. Before separation of the cells from the medium, $^3\text{H}_2\text{O}$ (1.0 μCi) and inulin-[^{14}C]-carboxylic acid (0.5 μCi) were added to the suspension. After separation, samples of cell and medium extracts (0.1 ml) were counted for radioactivity in a scintillation counter and corrections were made for quenching and for ^{14}C present in the ^3H channel. Intracellular water space was calculated from total cell water (total $^3\text{H}_2\text{O}$) minus adhering water (i.e. inulin space):

$$\text{Intracellular H}_2\text{O} = \text{total H}_2\text{O} - \text{extracellular H}_2\text{O}$$

$$\text{i.e. } \left[\frac{^3\text{H}_2\text{O (d.p.m.)/pellet}}{^3\text{H}_2\text{O (d.p.m.)/ml of supernatant}} \right] - \left[\frac{\text{inulin (d.p.m.)/pellet}}{\text{inulin (d.p.m.)/ml of supernatant}} \right]$$

Method (b). Measurement of wet weight/dry weight ratio with corrections for adhering fluid. After separation of the cells from the medium in the conical tubes, the cell pellet was weighed to give the total wet weight of cells. This was dried for 4 h at 100–110°C. Adhering fluid (obtained from inulin space) and dry weight were subtracted from the total wet weight to give intracellular water space. The simplifying assumption was made that 1 mg of cells occupies a volume of 1 μl . Thus:

Intracellular H_2O = total wet wt. –
extracellular H_2O – dry wt.

Inulin-space measurements were also used for corrections of cell (pellet) values of solutes when the concentrations of the solutes were equal or higher in the medium than in the cells, e.g. for Na^+ or when the substance to be measured had been added to the medium.

Separation of cells from the medium

Method I. Separation in conical centrifuge tubes. After incubation, cell suspensions were poured into 15 ml conical Pyrex centrifuge tubes and spun in an MSE 'Minor' centrifuge for 1 min at 1700g. The model of the centrifuge proved important as discussed below. Immediately after centrifugation, 2.0 ml of the supernatant was withdrawn and mixed with 0.2 ml of 20% (v/v) $HClO_4$, and the tube was inverted to drain off adhering fluid. The tube and pellet surface were dried carefully with absorbent paper held in Spencer-Wells forceps. The pellet was resuspended in 1.5 ml of 4% $HClO_4$. In some experiments, where stated, cells were separated in conical tubes that contained 1.5 ml of 4% $HClO_4$ plus aq. 8.5% glycerol beneath a layer of silicone fluid (2.0 ml) of specific gravity 1.03. The cell suspension was poured on top of this immediately after incubation and centrifuged.

Method II. Separation in the special tubes. The stem of the special tube was filled with one of the following fluids, all having a specific gravity of 1.03: aq. glycerol (11%, v/v); silicone fluid, obtained by mixing 42.8 g of MS 550 with 8.7 g of MS 200/2CS silicone fluids; 4% NaCl as used by Hems *et al.* (1975). The bulb of the special tube contained an aqueous solution with 8.5% (v/v) glycerol and 4% $HClO_4$. The cell suspension was poured into the upper chamber and centrifuged at 1700g for 1 min. During centrifugation, the cells migrate through the stem into the bulb where they are immediately deproteinized. A 2.0 ml sample of the medium was removed from the upper chamber and the remaining medium and the stem contents were carefully removed with a Pasteur pipette. A pipe cleaner was then introduced into the stem to remove any remaining fluid and the upper chamber was dried with absorbent paper to minimize contamination of the pellet fraction with the medium. Careful removal of adhering fluid is of special importance when large corrections of pellet values for adhering fluid have to be made, as for Na^+ . The bulb contents were resuspended and removed from the bulb with a Pasteur pipette.

Centrifugation

The positioning of the special tubes in the centrifuge was found to have a significant effect on both

intracellular water content and inulin space of the pellet fraction. The MSE 'Minor' centrifuge (model BA 100A) has a swing-out head with four double trunnions, each carrying two buckets. The 'leading' bucket of the two is that which, relative to the direction of spin, travels ahead of the 'trailing' bucket. Cells separated in tubes in the leading buckets carried down significantly ($P < 0.005$) smaller amounts of adhering fluid [$6 \pm 0.048 \mu\text{l}/78 \text{ mg wet wt. of cells (mean} \pm \text{S.E.M., } n = 4)$] than those in the trailing buckets ($21 \pm 0.09 \mu\text{l}/78 \text{ mg wet wt. of cells}$). The MSE 'Super Minor' centrifuge exhibited similar behaviour. When conical tubes instead of special tubes were used the minor differences seen were significant only by paired *t* tests. In all experiments, the leading bucket was used for separations with the special as well as conical tubes.

Determination of metabolites and inorganic ions

Urea, glucose and alanine were determined on neutralized $HClO_4$ extracts by the methods of Bernt & Bergmeyer (1970), Slein (1963) and Williamson (1974) respectively. Na^+ and K^+ were measured on acid extracts with a Perkin-Elmer atomic absorption spectrophotometer, model 103.

^3H and ^{14}C radioactivity was counted in a Beckman scintillation counter in vials containing 10 ml of scintillation fluid, containing 5.5 g of Permablend III (Packard Instrument Co., Caversham, Berks., U.K.), 60 g of naphthalene, 600 ml of toluene and 400 ml of methoxyethanol. Corrections were made for quenching and for ^{14}C present in the ^3H channel.

Animals and materials

Rats. Female Wistar rats starved for 48 h with water *ad libitum*, and weighing approx. 200 g, were used.

Reagents. Standard analytical-grade reagents were obtained commercially. Enzymes were the products of Boehringer Corp. (London) Ltd., London W.5, U.K., except urease (type VI), which was from Sigma (London) Chemical Co., Poole, Dorset, U.K. ^3H_2O and inulin- ^{14}C carboxylic acid were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Silicone fluids were obtained from Hopkin and Williams, Chadwell Heath, Essex, U.K.

Results and Discussion

Effect of centrifugation on cell solutes

When hepatocytes were centrifuged in the special tubes through an aqueous medium (aq. 11% glycerol), much urea, some glucose but very little alanine were lost from the cells before they reached the bulb (Table 1). When the aqueous solution was replaced by silicone fluid, the loss was greatly decreased but not fully abolished. After centrifugation in the

Table 1. *Effects of various techniques of separation on the distribution of some metabolites and ions between hepatocytes and medium*

Before separation, cells were incubated for 10–20 min in the presence or absence of added substrate as indicated. Values are means $\pm$ S.E.M. for the numbers of determinations in parentheses of the ratio concentration of metabolite or ion in intracellular water/concentration of metabolite or ion in medium. Values for intracellular water were derived from measurements of wet weight, dry weight and inulin space on cells separated by Method I on samples of the same suspensions.

Metabolite or ion	Substrate added	Separation in the conical tubes (Method I)		Separation in the special tubes (Method II)	
		No layering	Suspension layered over silicone fluid	Aq. 11% glycerol in stem	Silicone fluid in stem
Urea	Urea (2mM)	0.94 $\pm$ 0.063 (3)	—	0.28 $\pm$ 0.025 (3)	0.64 $\pm$ 0.014 (3)
Glucose	Glucose (2mM)	1.15 $\pm$ 0.11 (4)	—	0.75 $\pm$ 0.13 (4)	0.82 $\pm$ 0.09 (4)
Alanine	Alanine (2mM)	3.96 $\pm$ 0.50 (4)	—	3.35 $\pm$ 0.22 (4)	3.58 $\pm$ 0.34 (4)
K ⁺	None	25.8 $\pm$ 0.56 (12)	—	25.7 $\pm$ 0.67 (12)	—
Na ⁺	None	0.17 $\pm$ 0.011 (16)	0.19 $\pm$ 0.05 (3)	0.056 $\pm$ 0.008 (11)	0.097 $\pm$ 0.022 (7)

Table 2. *Effect of the method of separation of cells from medium on the intracellular water content of hepatocytes*
All tubes were placed in leading buckets of the centrifuge for separation (see the text). Intracellular water [by Method (b)] was 158 $\pm$ 9.4% of dry weight ($n = 6$). Values are means $\pm$ S.E.M. for the numbers of determinations in parentheses.

Method of separation	Intracellular water of dry wt. (%)	Extracellular water of dry wt. (%)
1. Conical tube [Methods I and (a)]	168 $\pm$ 8.0	95.6 $\pm$ 8.5 (7)
2. Conical tube + 4% HClO ₄ and silicone fluid	180 $\pm$ 12.4 (4)	168 $\pm$ 13.1 (4)
3. Special tubes (Method II)		
(a) 11% Glycerol in stem 4% HClO ₄ + 8.5% glycerol in bulb	7.8 $\pm$ 1.2 (7)	32.0 $\pm$ 3.5 (7)
(b) 4% NaCl in stem 4% HClO ₄ + 8.5% glycerol in bulb	5.1 $\pm$ 0.25 (3)	48.7 $\pm$ 2.3 (3)
(c) Silicone oil in stem 4% HClO ₄ + 8.5% glycerol in bulb	109 $\pm$ 4.2 (6)	78.0 $\pm$ 5.7 (6)

conical tubes, the concentrations of glucose and urea in cells and in the medium were about equal, whereas the concentration of alanine was about four times higher in the cells. This is to be expected on the basis of measurements *in vivo*. Thus separation in conical tubes (Method I) gives the correct intracellular/extracellular ratios (Table 1). Hence the conical tubes were used as a reference standard.

Although there was no loss of K⁺ from cells separated in the special tubes, intracellular Na⁺ was lost with glycerol in the stem decreasing in concentration from 25 to 8.8mM, as reflected by a greatly decreased intra-/extra-cellular ratio (see Table 1). Even when glycerol was replaced by silicone fluid, 50% of the intracellular Na⁺ was still lost. However, this was not the case when the suspension was

layered over silicone fluid, when the ratio was similar to the reference standard. The different behaviour of Na⁺ and K⁺ on centrifugation suggests that the ions are not evenly distributed within the intracellular space. A non-uniform distribution of Na⁺ in rat hepatocytes has been postulated by Hooper & Dick (1976), who produced evidence that the Na⁺ concentration in the nucleus is much higher than in the cytosol.

Effect of centrifugation on cell water

Since solutes are lost during centrifugation, a loss of intracellular water may also be expected (Table 2). The amount of water in the pellet is expressed as a percentage of the dry matter of cells. The wet weight is unsuitable as a reference because the

water content of cells changes with metabolic activity (Sainsbury, 1978).

When measured (in conical tubes) by Methods (a) and (b), the intracellular water content was 168 and 156% of the dry wt. respectively, i.e. there was no difference within the limits of error. Nor was there a loss of cell water when cells were separated in conical tubes through a silicone layer into an acid layer. In contrast, after separation through aqueous solutions (glycerol or NaCl) in the stem of the special tubes (Method II), the cell water, as measured by Method (a), was only 5–8% of the dry wt. Therefore a rapid exchange between labelled intracellular water and unlabelled water in the stem solution must have occurred. To confirm this, a non-aqueous medium, silicone fluid, was substituted for the aqueous glycerol in the stem. Under these conditions the loss of intracellular water was much less (35%) than with separation through a glycerol solution (95%) (Table 2). Since there can be no exchange between intracellular $^3\text{H}_2\text{O}$ and unlabelled water in the stem in these conditions, this 35% loss is difficult to explain. However, this may represent net loss of water from the cells.

The practical difficulty was overcome by abandoning the special tubes for the separation of substances whose intracellular concentrations were equal to or lower than the extracellular concentration and using standard conical tubes with silicone fluid between cell suspensions and the HClO_4 solution. Under

these conditions there is no loss or degradation of adenine nucleotides (see Hems *et al.*, 1975).

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