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Rapid Separation of Isolated Hepatocytes or Similar Tissue Fragments for Analysis of Cell Constituents

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A simple device is described for the rapid centrifugal separation of isolated hepatocytes or similar tissue fragments from the suspending medium. Rapid separation is essential if meaningful information on the concentrations of cell constituents and of their distribution between cell and suspension medium is to be obtained. The usefulness of the technique is illustrated by measurements of hepatocyte constituents (glutamate, aspartate, alanine, K^+) which show large concentration gradients against the extracellular medium.

Isolated hepatocytes have proved a valuable tool in the study of liver metabolism, their metabolic characteristics being almost identical with those of the isolated perfused liver. In experiments on the perfused liver it is often necessary to determine tissue metabolite concentrations, which can be done on the freeze-clamped tissue. Hepatocytes must be separated from the suspension medium before measurements of the intracellular metabolite concentrations can be made. A method is described in this paper that achieves a rapid separation of the cells from the medium. This has proved useful in studying the distribution of metabolites and inorganic ions between cells and medium and of the permeability characteristics of the plasma membrane. The technique is applicable to suspensions of isolated tissue fragments generally, e.g. kidney tubules.

Experimental

Design of separation tubes

A special centrifuge tube (Fig. 1) consists of a bulb containing $HClO_4$, separated by a capillary from an upper chamber. The capillary contains NaCl solution and the suspension is placed in the upper part of the tube. On centrifugation the cells migrate rapidly through the saline into the bulb and the cell-free suspension medium remains in the upper part of the tube. The concentrations of $HClO_4$ and of NaCl in the capillary are so adjusted that the specific gravity increases in the direction of the bottom of the tube. In consequence the three layers remain separated on centrifugation.

Handling of the tubes

The following description applies to 4ml suspension containing 70–80mg wet wt. of cells. The dimensions of the tube must be closely adhered to. A calibration mark is made at a point 1–2mm above the junction of the lower bulb with the capillary, and the volume of the bulb up to the mark is determined with mercury. Before use, and at intervals, the inner surface of the tube is treated with a solution of dimethyldichlorosilane in carbon tetrachloride (2%, v/v). The lower bulb is filled with a solution containing 4.0% (v/v) $HClO_4$ and 3.0% (w/v) NaCl by means of a hypodermic syringe and needle. Any droplets of the solution which remain in the capillary are removed with a pipe cleaner. The capillary is then filled, again by means of a hypodermic syringe, with 4.0% NaCl, leaving a small air bubble near the top and bottom of the column. The lower bubble prevents mixing of the acid in the bulb and the upper bubble allows the incubation medium to be placed rapidly on to the upper chamber without danger of mixing with the contents of the capillary.

When a series of samples is incubated they are conveniently handled in pairs, with an interval of 2min between pairs. At the end of the incubation period the suspension is poured into the separating tubes which are standing ready in the centrifuge. The time required to complete the spin is rather less than 2min.

The tubes are centrifuged at 3000rev./min at 1500*g* for 1min. Acceleration should be as rapid as possible. An M.S.E. Super Minor Centrifuge with a fly-out head and an electric brake is suitable. After centrifugation the cells form a packed sediment in the bulb and the clear cell-free supernatant remains in the upper chamber. The transfer of the cells to the bulb forces some of the NaCl solution into the upper chamber, and when collecting supernatant for analysis care must be taken that the NaCl solution in the bottom layer of the upper chamber is excluded from the sample. When 4ml-cell suspensions are centrifuged the yield of the cell-free supernatant, collected by a Pasteur pipette, is up to 3.5ml.

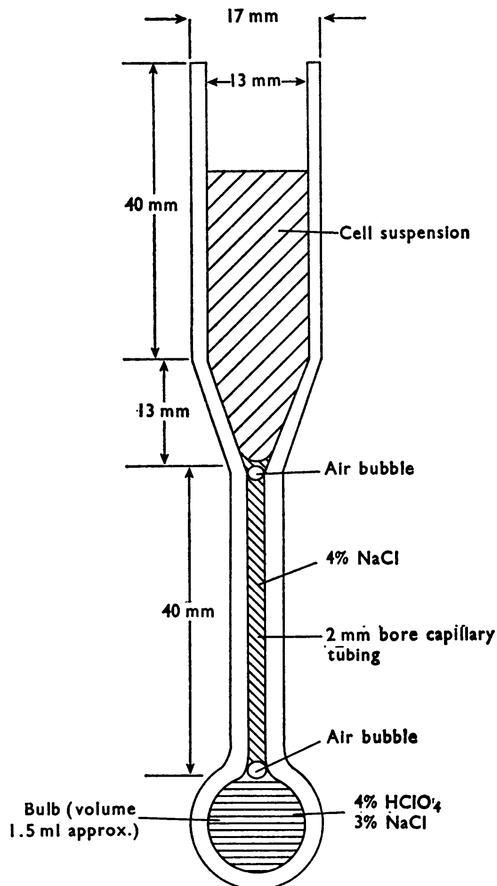

Fig. 1. *Tube for rapid separation of hepatocytes from incubation medium*

The dimensions given are suitable for 4ml of suspension containing up to 80mg wet wt. of hepatocytes. Full details are given in the text.

The remaining contents of the upper chamber are removed by a Pasteur pipette attached to a suction pump and by a swab of absorbent paper. Then the capillary is emptied with a new Pasteur pipette to the calibration mark and dried with a pipe cleaner.

The pellet of cells is resuspended in the $HClO_4$ solution with the help of a thin stirrer consisting of a length of 20-gauge stainless-steel wire inside polythene tubing attached to an overhead battery-driven motor. The end of the polythene tubing is about 5mm longer than the steel wire and is closed by heating and crimping. The short length of unsupported tubing is flexible and aids mixing.

After resuspending the pellet and standing for about 10min on ice (to allow for even distribution of cell contents and suspension medium) the tubes are re-centrifuged and the clear supernatant is collected with a Pasteur pipette.

When tubes of the dimensions given in Fig. 1 are used for the centrifugation of 4 ml containing about 80mg of cells (wet wt.) about 80μl of $HClO_4$ solution enters the capillary and is removed by the above procedure. However, this loss of the $HClO_4$ solution can usually be neglected because its content of cell constituents is negligible. Only on stirring and standing are the cell constituents released. Evidence that enzyme activities cease immediately is provided by determinations of the adenine nucleotides. A loss of ATP and gains of ADP and AMP are early indicators of 'postmortal' changes. Values of 1.75, 0.76 and 0.12μmol per g for ATP, ADP and AMP respectively were obtained in the cell extract after incubation without substrate. In a parallel flask, in which the whole-cell suspension was deproteinized by the addition of $HClO_4$, the corresponding values were 1.55, 0.86 and 0.15μmol per g.

A small amount of the original suspension medium adheres to the sedimented cells and is carried into the bulb. The amount of this contamination is measured by the addition of ^{14}C-labelled inulin to the suspension just before the initial centrifugation of the cells. When the mean weight of cells was 77.7mg the volume of medium carried into the bulb was 11.35μl $\pm$2.3 S.E.M. for 10 samples. The analytical values obtained for the cell contents have to be corrected on the basis of this contamination with the medium. However, unless the concentration of the metabolite in the medium is relatively high, the correction may be neglected.

The tube dimensions have to be changed when larger volumes of cells are to be handled. In this case the diameter of the capillary must be increased to accommodate the displaced acid. Beyond a certain diameter it becomes difficult to retain the air bubbles; therefore a short length of narrow tubing at top and bottom of the capillary is essential. The measurements of a tube based on a 50ml centrifuge bucket capable of handling 16ml of incubation medium and 320mg of cells is shown in Fig. 2.

If a more concentrated extract of the cells is required (because of low metabolite concentrations) the size of the bulb shown in Fig. 2 can be decreased to about 1.5ml. In this case it is advisable not to discard the $HClO_4$ which has entered the capillary and to remove from the capillary only the NaCl solution. This is easy because the interface between the NaCl and the $HClO_4$ is clearly visible. In consequence the volume of the $HClO_4$ extract is larger than the calibrated volume by approximately the wet wt. of the cells. This increase must be allowed for in the calculation of the intracellular metabolite concentrations. The extra volume may be taken, by first approximation, to be equal to the wet wt. of the cells.

The concentration of $HClO_4$ in the bulb must be adjusted according to the amounts of cells used to ensure effective deproteinization. The final concentration of $HClO_4$, after dilution by the centrifuged cells, should be 4% (v/v). If the initial concentration of $HClO_4$ is increased, it is necessary to modify the amount of NaCl accordingly, to maintain the same specific gravity by using, for example, 6% (v/v) $HClO_4$ with 2.5% (w/v) NaCl.

Cleaning of the separating tubes

A convenient device for washing the tubes can be made from a rubber bung to fit the tube. Through the top of the bung are placed two hypodermic needles: in the centre a 19-gauge needle to which is attached a piece of polythene tubing long enough to reach into the bulb, and next to it a larger-gauge needle cut off almost flush with the bung. To the connector of the long needle is attached a piece of polythene tubing about 20cm long, and to the connector of the short needle a piece of pressure tubing leading to a water suction pump. The separating tube is held upside down and cleaned by the jet of hot washing fluid sucked into the tube by the pump. This is followed by thorough rinsing and drying with the help of acetone.

General comments

Cells (or mitochondria) have been separated before on the basis of similar principles, but non-aqueous solvents or silicone oils of suitable specific gravity were used to separate the deproteinizing solution from the suspension in ordinary centrifuge tubes (Werkheiser & Bartley, 1957). The new procedure is an elaboration of a principle, i.e. the use of a capillary for separating the suspension from the deproteinizing agent as described by Crompton & Chappell (1973). It is a decisive advantage of the new technique that the suspension can be poured rapidly into the tube instead of having to be carefully layered on top of the non-aqueous phase. Speed in separation is very essential because the suspension can rapidly become anaerobic; this disturbs the distribution of substances between cells and medium, as well as intracellular redox systems.

Examples illustrating the use of the technique

Table 1 shows the use of the tubes for the determination of the distribution of metabolites when there are large gradients between tissue and medium, as in the case of glutamate, alanine and aspartate. In the freshly prepared cells the amino acid content is low compared with that *in vivo* (see Krebs *et al.*, 1974), amino acids having been lost from the cells during the isolation procedure. The values *in vivo* (from freeze-clamped liver) after 48h starvation are, for glutamate, alanine and aspartate, 2.38, 0.46 and 0.76μmol per g wet wt. respectively (Williamson *et al.*, 1967; 1969). On incubation without nitrogenous substances the concentrations of the amino acids rise somewhat on account of proteolysis. Incubation with NH_4Cl and lactate causes marked increases in, and a release of, alanine into the medium. Because of the large volume ratio of medium/cells, the tissue/medium ratios for amino acids are higher than the tissue/plasma ratio *in vivo*. Incubation with 1mM-alanine shows that a more

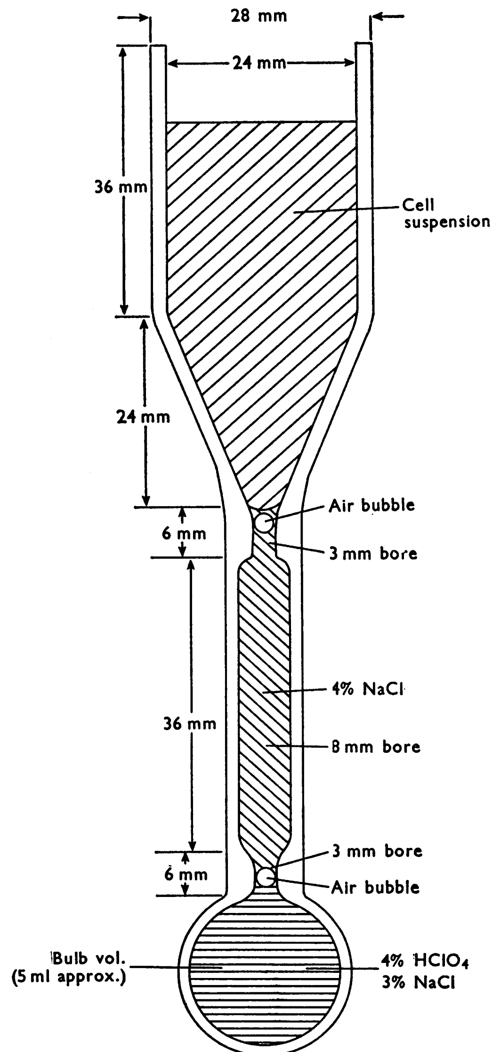

Fig. 2. *Dimensions of separating tube handling 16ml of cell suspension containing up to 400mg wet wt. of cells*

Full details are given in the text.

Table 1. *Intracellular/extracellular distribution of amino acids after incubation with various substrates*

Initial concentrations were 10mM-lactate, 5mM-NH_4Cl and 1mM-L-alanine. Each flask contained 16ml of suspension (335mg wet wt. of cells from a 48h-starved rat). Cells were separated from incubation medium as described in the text, in tubes of 16ml capacity and bulb size 1.4ml. Assay of amino acids was carried out as described by Cornell *et al.* (1974).

			Amino acid found (μmol/g of cells or μmol/ml)			Cell/medium gradient	
Substrates added	Incubation time (min)	Compartment	Glutamate	Alanine	Aspartate	Glutamate	Alanine
None	0	Cells	0.37	0.05	0.48		
Lactate	20	Cells	1.20	0.70	0.53	60	37
		Medium	0.02	0.02	<0.002		
Lactate+NH_4Cl	20	Cells	2.46	3.08	1.38	123	18
		Medium	0.02	0.17	<0.002		
Alanine	20	Cells	3.74	0.94	0.88	125	3
		Medium	0.03	0.28	<0.002		

Table 2. *K^+ gradients between hepatocytes and suspension medium*

Cells (80mg in 4ml of medium) were incubated at 37°C for 10min anaerobically and then for 40min aerobically. After separation K^+ was measured by flame photometry (experiment by Mrs. Gillian Sainsbury of this laboratory).

	K^+ content	
Experimental conditions	Medium (mequiv./litre)	Cells (mequiv./kg wet wt.)
Freshly prepared cells	5.50	78.9
After 10min anaerobiosis	5.72	69.0
After 20min re-oxygenation	5.23	100
After 40min re-oxygenation	5.34	99.5

physiological concentration gradient of 3 for alanine is reached after 20min.

Unless there is information on the distribution of metabolites between cell and medium, determinations of cell constituents in the whole suspension given no information on the metabolite content of the cells. When it is established that a metabolite is present, under given test conditions only within cells (as is true for ATP, ADP and AMP) no separation of cells from the medium is necessary.

The second example (Table 2) shows measurements of the K^+ gradient between liver cells and the suspension medium. K^+ is lost during anaerobiosis and rapidly regained by the cells on admission of O_2. The gradients observed are of the order known to occur *in vivo*. The use of isolated cells has the advantage that extra- and intra-cellular spaces are clearly defined, whereas in the intact liver the separation of hepatocyte spaces, 'extracellular' spaces and the spaces occupied by other types of cells and vascular systems is not clear-cut.

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