A SIMPLE METHOD FOR THE PERIFUSION OF ISOLATED LIVER CELLS

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1. Introduction

The study of regulatory mechanisms in liver cell metabolism has been greatly facilitated recently by the availability of physiological competent preparations of isolated liver cells [1], with which it is possible to study several parameters simultaneously using uniform material. Furthermore, the determination of intracellular concentrations of metabolites at frequent intervals is facilitated in comparison with the liver perfusion technique. However, the procedure normally used, in which cells are incubated in a vessel, has at least one intrinsic drawback: high, non-physiological substrate loading in a closed system occurs, and no true steady-state condition can be reached. This procedure also lacks some of the more specific experimental possibilities of the flow-through liver perfusion technique [2,3] e.g. (1) Reversible alteration of the extracellular environment. (2) Monitoring parameters like extracellular pH and PO_2. (3) Use of physiological and constant levels of substrates.

Since these three points are highly relevant to the study of hepatic metabolism under near-physiological conditions, we have attempted to combine the advantages of the use of isolated liver cells with those of the flow-through perfusion by developing a perifusion technique for isolated rat-liver cells. It was realized that the most important criterion in assessing the usefulness of such a technique is whether the viability of the isolated cells is preserved during the perifusion.

Hitherto, no satisfactory perifusion technique for isolated liver cells has been described. The perifusion methods for isolated fat cells reported recently [4,5] suffer from the disadvantage that it is not possible to take reproducible samples of the cell suspension. This also applies to the method described by Gronov [6] for perifusing isolated kidney tubules. The procedure described by Walter et al. [7] for liver cells leads to loss of viability.

In this paper a simple method for perifusion of isolated liver cells is described, in which the viability and physiological competence of the cells are preserved. A preliminary account of this study has been given [8].

2. Materials and methods

Rat liver cells were prepared from livers of 24-h fasted rats (Sprague-Dawley) essentially according to the procedure of Berry and Friend [1]. A sample of these cells (70–150 mg dry weight) was transferred to the perifusion system schematically depicted in fig.1. The perifusion chamber is a modification of an Amicon Stirred Cell (model 12). The water-jacketed chamber (thermostatted at 37°C), with a volume of 10 ml, contains a Teflon stirring bar close to the separating membrane in the bottom of the chamber, so that the cell suspension is kept homogeneous, and clogging of the filter is prevented. For the separation of the cells and the perifusate we have selected a nylon membrane filter (NS Duralon from Millipore) with a pore size of 7 μm and a high porosity. This filter is placed on a polypropylene support. In the top of the chamber there is an orifice for sampling of the cell suspension in order to determine intracellular metabolite concentrations.

A Krebs bicarbonate buffer (pH 7.4), thermostatted

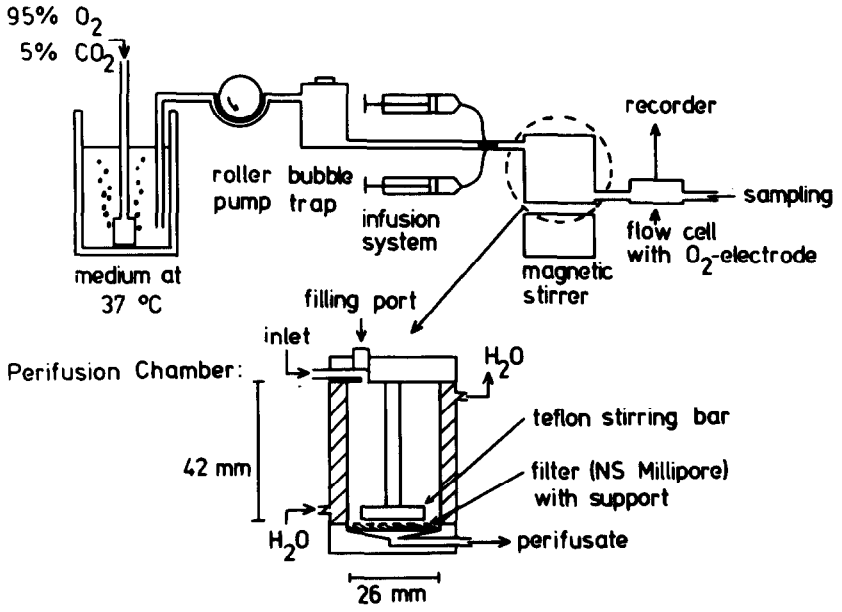

Fig.1. Diagram of perifusion system for isolated liver cells.

at 37°C, was pumped through the perifusion chamber at the desired flow rate and with several substrate additions (see fig.4). The oxygen activity in the perifusate was measured with a Clark electrode positioned in a thermostatted flow cell close to the exit port of the perifusion chamber.

The pressure in the perifusion chamber is registered by means of a pressure transducer (Elema Schönander EMT 35) inserted between the bubble trap and the infusion system. The transducer was connected to an electromanometer (Elema Schönander EM 31) and a recorder.

Samples of the perifusate were taken for the determination of lactate dehydrogenase (LDH) activity. Metabolite assays were carried out in samples of the cell suspension and the perifusate, which were acidified with cold $HClO_4$ (final concentration 3–4%) and subsequently neutralized with 2 M KOH–0.3 M morpholinopropane sulphonic acid (MOPS). LDH activity and metabolities were determined according to standard procedures [9,10].

3. Results and discussion

One of the time-consuming bottlenecks in the development of this technique was the selection of a suitable filter for the separation of the cells and the perifusate.

Such a filter has to fulfill two criteria: firstly, during the course of the perifusion (up to 2.5 h) the filter should not clog. Secondly, intact cells should not pass through the filter.

The first criterion could be tested by measurement of the pressure in the perifusion chamber. In fig.2 a pressure registration is depicted for a typical experiment. At the start of the perifusion the NS filter was placed in such a way, that the dull side of the filter was in contact with the suspension. After the addition of cells there was a slight increase in pressure to a constant level which was reached after about 5–10 min. At this stage, the perifusion was interrupted, the suspension removed from the chamber and the filter inverted, so that the shiny side of the filter was in contact with the suspension. After the addition of the same cell suspension a rapid pressure increase was registered indicating that clogging of the filter was occurring.

This procedure was repeated three more times. After each inversion, there was a further slight increase in the steady-state pressure when the dull side of the filter was uppermost. Apparently this is due to an

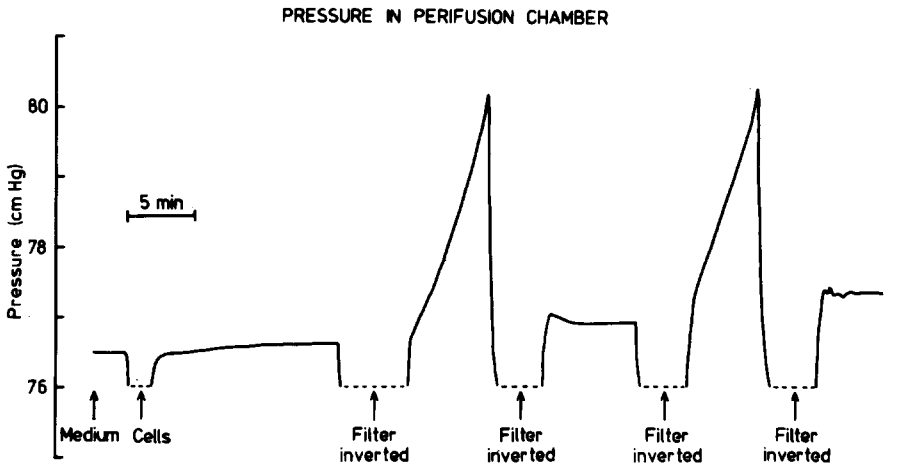

Fig.2. Registration of pressure in perifusion chamber. Rat-liver cells (70 mg dry wt) were in a Krebs-Ringer bicarbonate medium (volume 8 ml). The gas phase was 95% O_2 –5% CO_2 and the flow rate, 5.0 ml/min. For other details, see text.

irreversible penetration into the pores of the filter by cells when the shiny side is uppermost.

This experiment demonstrates that the NS filters are anisotropic with respect to separation of cells and perifusate, but that they do not clog provided the dull side of the filter is in contact with the suspension.

That the second criterion was fulfilled was concluded from microscopic examination of the perifusate; no cells could be detected.

A useful criterion in assessing cell viability is determination of extracellular LDH activity [11]. The graph in fig.3 shows the time course of the appearance of LDH activity in the perifusate. Due to dilution in the 'dead volume' behind the perifusion chamber, there is at first an increase in LDH activity in the perifusate. However, this increase is followed by a rapid decay, levelling off to a barely detectable activity. The $t_{1/2}$ of the rapid decay of the LDH activity was 65 sec and was in close agreement with a theoretical $t_{1/2}$ of 61 sec for the volume/flow ratio in this experiment. The inset table in fig.3 shows a comparison of the extracellular LDH in an Erlenmeyer incubation and in the perifusion system, using samples of the same preparation of cells. During the incubation in the Erlenmeyer flask, extracellular LDH increased from 10% to the fairly high level of 35% of total activity. In contrast, the total LDH activity in the accumulated perifusate was approximately equal to the extracellular LDH already present at zero time. It should be pointed out that the medium contained no albumin.

We reported in the preliminary account of this

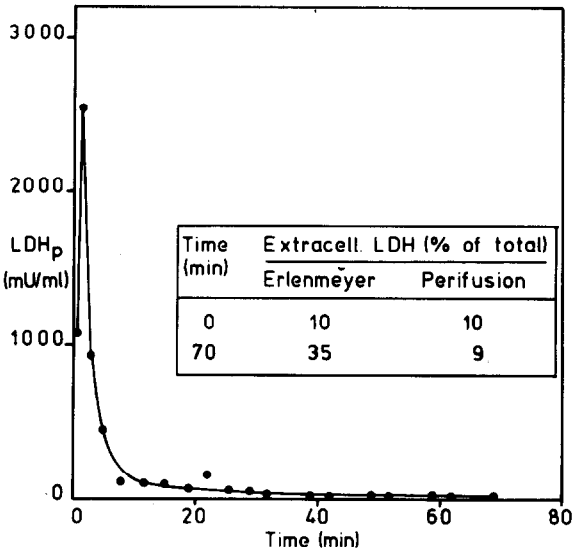

Fig.3. Measurement of extracellular lactate dehydrogenase activity in perifusate during perifusion of rat-liver cells, and in medium during incubation of cells in an Erlenmeyer flask. Flow rate in perifusion system, 4.8 ml/min. The cells (116 mg dry weight) were incubated in Krebs-Ringer bicarbonate containing 10 mM alanine. Gas phase, 95% O_2 –5% CO_2. No albumin was present. Abbreviation: LDH_p, lactate dehydrogenase in perifusate.

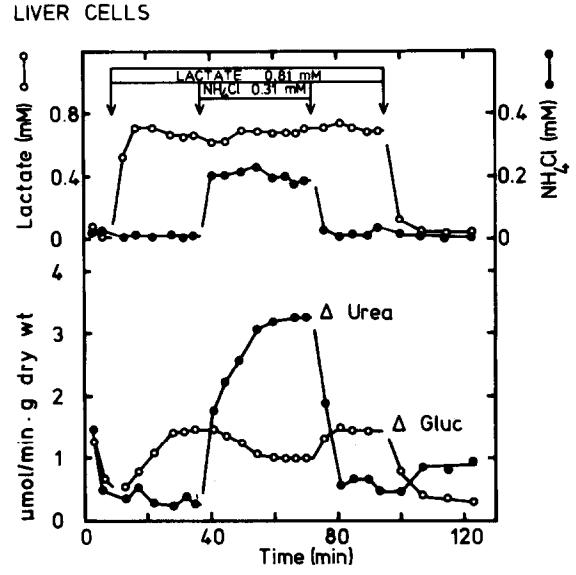

Fig.4. Measurement of glucose production during perifusion of rat liver cells. Flow rate, 4.8 ml/min. The cells (108 mg dry weight) were incubated in an initial volume of 10 ml in Krebs-Ringer bicarbonate. No albumin was present. Gas phase, 95% O_2 –5% CO_2.

study [8], that the metabolic capacity of the cells is high and remains constant during the course of the perifusion. This is a second criterion, the fulfilment of which is a further indication that the viability of the cells is certainly not diminished in the perifusion system as compared with incubations in Erlenmeyer flasks. Recently, Jeejebhoy et al. [12], too, have reported that stirring a suspension of rat liver cells does not influence their performance in the negative sense.

In order to measure the oxygen consumption of isolated liver cells, Krebs et al. [13] employed the Warburg manometer technique, using specially designed flasks. With the perifusion system we observed [8] rates of O_2 uptake and glucose production with lactate and oleate as substrates that were in good agreement with the values reported by Krebs et al. [13]. Thus, the perifusion system offers an additional method for determination of O_2 consumption is isolated liver cells. This makes it possible to relate O_2 consumption not only to metabolic fluxes but also to changes in intracellular metabolite concentrations.

Fig.4 illustrates the use of this technique for the study of the interaction between gluconeogenesis and ureogenesis at physiological concentrations of lacetate and ammonium chloride. The substrates were added by means of the infusion system. After a lag phase typical for this substrate [14], the rate of glucose production from lactate reached a steady-state value of 1.5 μmol/min · g dry weight, in good agreement with the values reported for the flow-through liver perfusion [15]. A subsequent infusion of a low concentration of ammonium chloride resulted in a urea production of 3.3 μmol/min · g dry weight. This rate is very close to the values reported for the recirculating liver perfusion and for isolated cells incubated in flasks, in both cases at much higher substrate concentration [16,17]. Concomitantly with the increase in urea production

Table 1
Intracellular concentration of metabolites during perifusion of isolated rat-liver cells

Time	Infusion with	ATP	Glutamate	Malate
(min)		(μmoles/g dry weight)		
8	No additions	9.4	1.4	< 0.1
36	Lactate	11.2	7.1	0.8
71	Lactate + NH_3	7.5	8.3	0.3
94	Lactate	10.1	8.1	0.8
124	No additions	9.2	5.8	< 0.1

At the times indicated in the table, samples of the cell suspension in the experiment of fig.3 were taken for the determination of the intracellular concentration of metabolites.

there was an inhibition of glucose production of more than 30% (see [18]). This inhibition was completely reversible; after cessation of the ammonium chloride infusion the rate of glucose production returned to the original value.

During this experiment, samples of the cell suspension in the chamber were taken in order to determine the intracellular concentration of metabolites. The results are depicted in table 1. The ATP concentration increased after the lactate infusion and decreased in a reversible manner on infusion with ammonium chloride. After the lactate infusion, there was an increase in the glutamate concentration, which did not change very much subsequently. As expected, the malate concentration increased during the lactate infusion. During the ammonium chloride infusion, the malate concentration was lowered; this effect was reversible. The nature of this interaction between glucose production and urea synthesis (cf. ref. [18]) at more physiological substrate levels is under study.

4. Conclusions

The perifusion technique described in this paper offers a new and simple method for studying liver cell metabolism in a more dynamic way. It has potential use in hormonal response studies, pulse-chase experiments and many other types of investigations. Finally, the perifusion technique may be applicable to other isolated cell preparations as well. The method combines some advantages of the flow-through technique with the great advantages of using isolated liver cells, apparently without disturbing the viability of the cells.

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References

[1] Berry, M. N. and Friend, D. S. (1969) J. Cell. Biol. 43, 506–520.
[2] Ross, B. D. (1972) Perfusion Technique in Biochemistry, Oxford University Press, Great Britain.
[3] Lindros, K. O. (1974) in: Regulation of Hepatic Metabolism (Lundquist, F. and Tygstrup, N., eds.) pp. 778–786, Munksgaard, Copenhagen.
[4] Allen, D. O., Largis, E. E., Miller, E. A. and Ashmore, J. (1973) J. Appl. Physiol. 34, 125–127.
[5] Lowry, P. J. and McMartin, C. (1974) Biochem. J. 142, 287–294.
[6] Gronow, G. and Weiss, Ch. (1976) in: Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (J. M. Tager, H. D. Söling and J. R. Williamson, eds.) pp. 391–394, NorthHolland, Amsterdam.
[7] Walter, P., Nyfelder, F., Fasel, P., Von Glutz, G., Moser, U. K. and De Sagarra, M. R. (1976) in: Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (J. M. Tager, H. D. Söling and J. R. Williamson, eds.) pp. 167–174. NorthHolland, Amsterdam.
[8] Van der Meer, R., De Haan, E. J. and Tager, J. M. (1976) in: Use of Isolated Liver Cells and Kidney Tubules in Metabolic Studies (J. M. Tager, H. D. Söling and J. R. Williamson, eds.) pp. 159–164, NorthHolland, Amsterdam.
[9] Bergmeyer, H. U. (1970) Methoden der Enzymatischen Analyse, Verlag Chemie, Weinheim.
[10] Williamson, J. R. and Corkey, B. E. (1969) Methods Enzymol. 13, 434–513.
[11] Seglen, P. O. (1976) in: Methods in Cell Biology (Prescott, D. M., ed.) in press.
[12] Jeejeebhoy, K. N., Ho, J., Greenberg, G. R., Philips, M. J., Bruce-Robertson, A. and Sodtke, U. (1975) Biochem. J. 146, 141–155.
[13] Krebs, H. A., Cornell, N. W., Lund, P. and Hems, R. (1974) in: Regulation of Hepatic Metabolism (Lundquist, F. and Tygstrup, N., eds.) pp. 726–750, Munksgaard, Copenhagen.
[14] Cornell, N. W., Lund, P., Hems, R. and Krebs, H. A. (1973) Biochem. J. 134, 671–672.
[15] Exton, J. H. and Park, C. R. (1967) J. Biol. Chem. 242, 2622–2636.
[16] Krebs, H. A., Hems, R. and Lund, P. (1973) Biochem. J. 134, 697–705.
[17] Stubbs, M. and Krebs, H. A. (1975) Biochem. J. 150, 41–45.
[18] Meijer, A. J., Gimpel, J. A., DeLeeuw, G., Tager, J. M. and Williamson, J. R. (1976) J. Biol. Chem., submitted for publication.