THE METABOLIC INTEGRITY OF HEPATOCYTES IN SUSTAINED INCUBATIONS

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1. Introduction

Over the past few years, suspensions of isolated hepatocytes have become accepted as important systems for studying hepatic metabolism and its control [1]. Several methods that yield hepatocyte suspensions physiologically similar to intact liver have been published [2–4]. Although this similarity is apparent immediately after isolation, incubation of hepatocytes at 37°C in simple salt solutions is accompanied by substantial deterioration after 2–3 h [2,5]. Thus, although the preparation is suitable for investigation of short-term events [6,7], study of long-term processes under these conditions will be of limited validity.

Various workers have attempted to increase hepatocyte viability by incubating cells under culture conditions [8,9]. During colony formation, however, many parenchymal cells are lost completely and, although cells in culture maintain some of the properties of hepatocytes in intact liver, many functions are altered [10,11]. Many of these studies do not apply sufficiently stringent biochemical tests to assess cellular viability throughout culturing. A system for the preparation of biochemically viable non-dividing parenchymal cells and their maintenance for incubations of 8–12 h. would prove a valuable tool in the study of hepatic anabolism.

Complex physiological mixtures of amino acids, vitamins and serum have advantages over Krebs-Henseleit buffers for polysome aggregation [12], albumin synthesis [13] and inhibition of ribonuclease activity following hepatocyte isolation [14]. This paper details the biochemical stability of isolated parenchymal hepatocytes incubated in minimum essential medium (Eagle) plus foetal calf serum and the advantages of that medium over Krebs-Henseleit bicarbonate supplemented with bovine serum albumin.

2. Materials and methods

Hepatocytes were isolated from fed or 48 h -starved rats by the procedure described [4]. Following dispersion of hepatocytes in Krebs-Henseleit bicarbonate buffer containing 2% fatty acid-free dialysed bovine serum albumin (medium A), the suspension was centrifuged for 2 min at 40 × *g*. The cell pellets were washed twice and resuspended in either medium A or minimum essential medium (Eagle) containing 2 g $NaHCO_3$/litre supplemented with 10% foetal calf serum (medium B). Both media contained streptomycin (100 μg/ml) and penicillin (120 μg/ml). Fractions (0.3 ml) of the stock cell suspension were added to 1.7 ml medium in siliconized glass scintillation vials; all were equilibrated for 2 min with O_2:CO_2 (95%:5%) and incubated at 37°C in an orbital shaking water bath (Infors, type WTR1; diameter of rotation 12.5 mm) at 130 rev/min. Triplicate samples were analysed at zero time and at the time points specified.

Dry weights of cells were measured as described [15].

For estimation of glucose production and ATP and total adenylate content of hepatocytes, incubation was terminated by addition of perchloric acid (final 2%). Glucose was measured with glucose oxidase [16]. After neutralization of the acid extracts, adenylates were measured by chemiluminescence [17]. Total adenylates were measured as ATP following the conversion of ADP and AMP to ATP in the presence of phosphoenolpyruvate, pyruvate kinase and adenylate

kinase. The activities of lactate [18] and glutamate [19] dehydrogenases were measured in the cell medium after centrifugation and in the cell pellet following glass-on-glass homogenization in 100 mM triethanolamine–HCl (pH 7.4) containing 0.5% (v/v) Triton X-100.

Intracellular K^+ contents of hepatocytes were determined by flame emission spectrometry following cell–medium partition in separation tubes [20]. The contamination of cells by medium K^+ was estimated by the carryover of [^{3}H]inulin from the medium into the separation bulb. The incorporation of L-[4,5-^{3}H] leucine into TCA-precipitable material was obtained by the filter-disc method [21]. The conversion of [1,5-^{14}C]citrate to $^{14}CO_2$ was measured by entrapment of $^{14}CO_2$ in centre wells containing 0.25 ml 2-phenylethylamine:methanol (1:1, v/v).

Collagenase (grade II), collagen and glucose oxidase were obtained from the Boehringer (London) Lewes, Sussex. Firefly lantern extract, streptomycin, penicillin and soybean trypsin inhibitor (type II-S) were from the Sigma (London), Norbiton, Surrey. Sera were purchased from Gibco:Bio-Cult, Paisley England and minimum essential medium from Flow Laboratories, Irvine, England. Bovine serum albumin was obtained from Armour Pharmaceutical, Eastbourne, Sussex, and was freed of fatty acids and other contaminating materials [22]. All radiochemicals were from the Radiochemical Centre, Amersham, Bucks. England.

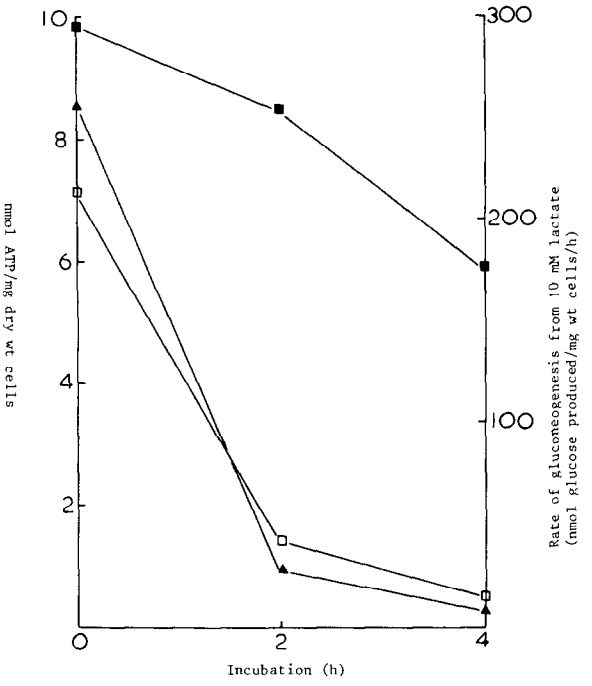

Fig.1. Effect of incubation at 37°C in medium A on ATP content of hepatocytes from fed (■) and 48 h-starved (□) rats and the rate of gluconeogenesis from starved rat cells (▲). Glucose output was measured between 30 min and 90 min after the addition of L-lactate (10 mM final) at the times indicated.

3. Results and discussion

Gluconeogenesis from lactate is acknowledged to be a valuable indicator of the metabolic integrity of isolated liver preparations [23]. The gluconeogenic capacity in hepatocytes from 48 h-starved rats decreased rapidly during a 4 h incubation (fig.1) and this correlated with decreased hepatocyte ATP content. Incubation of fed-rat hepatocytes also resulted in lowered ATP contents (fig.1). The rate of ATP decrease varied with individual preparations but could generally be assigned a half-life of 2–5 h. Storage of cell suspensions on ice slowed the rate of metabolic deterioration by approx. 300%. The close correlation between ATP content and the rate of gluconeogenesis indicates that ATP content may be applied as an estimate of the functional metabolic capacity of hepatocytes. All suspensions used in this set of experiments had initial ATP contents in the physiological range (8–13 nmol ATP/mg dry wt of cells) [1]. Earlier observations have suggested that trypan blue exclusion may not be an accurate reflection of hepatocyte viability [4,5]; in any case, the clumping of hepatocytes noted during long-term incubations make it difficult to obtain homogeneous samples of cells without physical damage during pipetting.

The lowering of hepatocyte ATP content could theoretically be due to a lack of substrates or hormones during incubations, and resultant deterioration of metabolic function. Nevertheless, addition of all the factors detailed in table 1 singly or in combination failed to prevent ATP loss. Filtration of all media to remove bacteria, constant gassing with O_2:CO_2 (95%:5%) and use of extremely dilute cell suspensions were also ineffective. Over the time periods studied, we were unable to detect any appreciable contamination by microorganisms.

Table 1
Additions to hepatocyte incubations in albumin-supplemented Krebs-Henseleit buffer (Medium A)

	Final concentration
Glucose	20 mM
Physiological amino acid mixtures [13]	
L-Lactate	5 mM
Pyruvate	0.5 mM
Palmitate	2 mM
Dialysed, heat-inactivated horse serum	8% and 15%
Newborn heat-inactivated calf serum	10%
Hypoxanthine	10 mM
Allopurinol	1 mM
Insulin	100 nM
Glucagon	50 nM

Contaminants in batches of collagenase and their possible attachment to hepatocytes have been advanced as being responsible for hepatocyte degeneration during incubation [24,25]. The presence of soybean trypsin inhibitor during collagenase perfusions, as recommended in [9], in our hands greatly retarded hepatocyte liberation without lessening subsequent deterioration during incubation. Addition of antibody directed against commercial collagenase was also ineffective at any stage. Resuspension of hepatocytes in fresh medium and addition of Hepes (*N*-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid) buffer to the medium during incubations equally did not increase ATP retention. During hepatocyte 'culturing', authors [26] have commented on the advantages of cell adherence to collagen plates or films. In our experiments, we found that the presence of colloidal collagen (0.8 mg/ml) during incubation produced dramatic association of hepatocytes with a concomitant fall in ATP content. Thus we believe that the aggregation of cells observed under normal incubation conditions with medium A may indicate decreased metabolic capacity.

Reports of long-term maintenance of isolated hepatocytes have stressed the requirement for culture media supplemented with serum [8,9]. We found the isolation procedures of [9] to be relatively slow, whereas that described in [8] seemed to complex to permit the study of many variables with one preparation. We decided to use our own method of hepatocyte preparation [4] because of its simplicity and rapidity, and to investigate the advantages of incubation of cells in culture media.

Waymouth's medium [27] supplemented with 15% dialysed heat-inactivated horse serum offered no advantage over incubation in medium A. In contrast with others [8], we found that medium B markedly prolonged the viability of hepatocytes (fig.2). The ATP content of hepatocytes in medium B was maintained for 8 h incubation and, even after 16 h, the ATP content was 2.5-times that in cells isolated from the same livers and incubated in medium A.

Metabolic characteristics of hepatocytes incubated in medium B were analysed to assess the integrity of the preparation for metabolic studies (fig.3). Over 8 h, ATP and total adenylates were maintained in the physiological range in medium B; both parameters fell

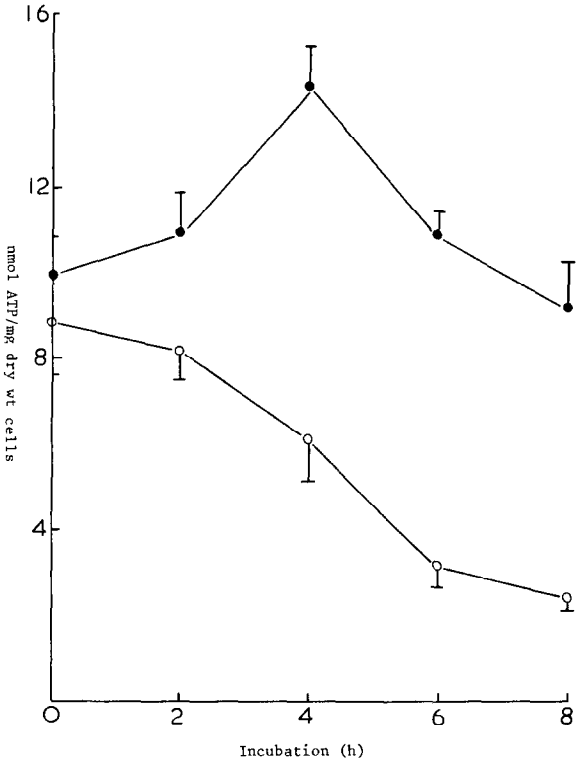

Fig.2. Comparison of the effectiveness of media A and B on the maintenance of hepatocyte ATP. Results are from a single experiment (representative of 6), and are expressed as means ± SD. Cells were prepared from fed animals. (○) Medium A. (●) Medium B.

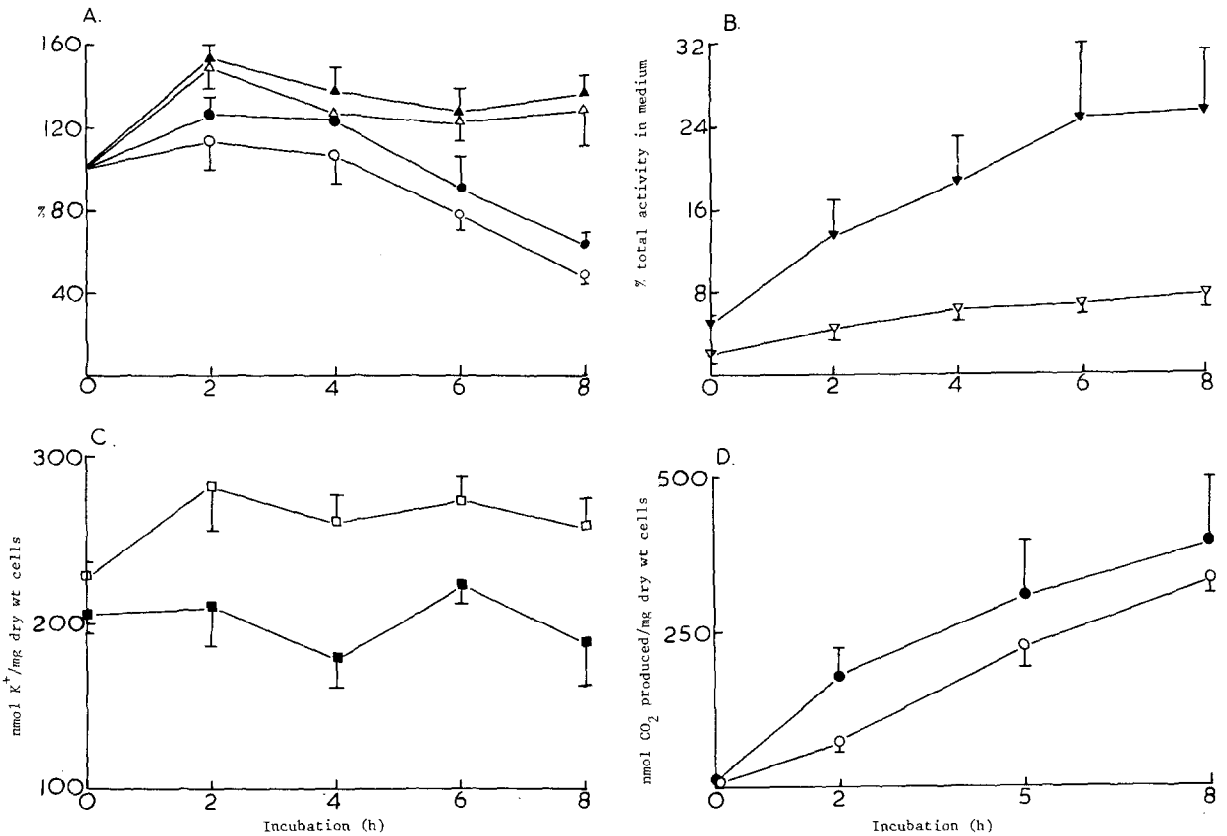

Fig.3. Alteration of metabolic characteristics of hepatocytes incubated in media A and B. Each point represents the mean of 3 experiments ± SEM. (A) ATP: (•) Medium A; (▲) Medium B and total adenylate; (○) Medium A; (△) Medium B contents. (B) Lactate dehydrogenase leakage: (▼) Medium A; (▽) Medium B. (C) K^+ content: (■) Medium A; (□) Medium B. (D) Conversion of [1,5-^{14}C] citrate to $^{14}CO_2$. Final spec. act. citrate 1.53 μCi/μmol. (●) Medium A; (○) Medium B.

in medium A. The initial cellular activities of lactate dehydrogenase and the ratio of lactate to glutamate dehydrogenases were similar in cells incubated in both media, but the subsequent leakage of lactate dehydrogenase into the medium was greatly retarded in medium B (fig.3B). Although cellular K^+ content was maintained in both media A and B (fig.3C), that of cells in medium B was higher throughout. (Using dry: wet weight ratio 1:3.7, and with our somewhat conservative measurement of cell dry weights, we observed a mean of 73 μmol K^+/g wet wt of cells, well within the published range [1,28].)

Conversion of citrate to CO_2 estimates the contamination of preparations with hepatocytes with damaged membranes [23]; this method resembles that involving measurement of malate production from exogenous succinate [29]. The rates of CO_2 production were very low and did not differ significantly between the two media studied (fig.3D).

Conversion of radiolabelled leucine into trichloroacetic acid (TCA)-insoluble material is commonly used to assess general protein synthesis of preparations in vitro. Hepatocytes isolated from fed rats incorporated L-[4,5-^{3}H]leucine into TCA-insoluble material at a constant rate for 8 h; the rate then slowed for the subsequent 4 h (fig.4). During the latter stages of the incubation, ATP decreased, but only marginally. A decrease of incorporation rate of labelled valine by cells after a similar period has been reported [8]. Such incorporation includes both synthesis of

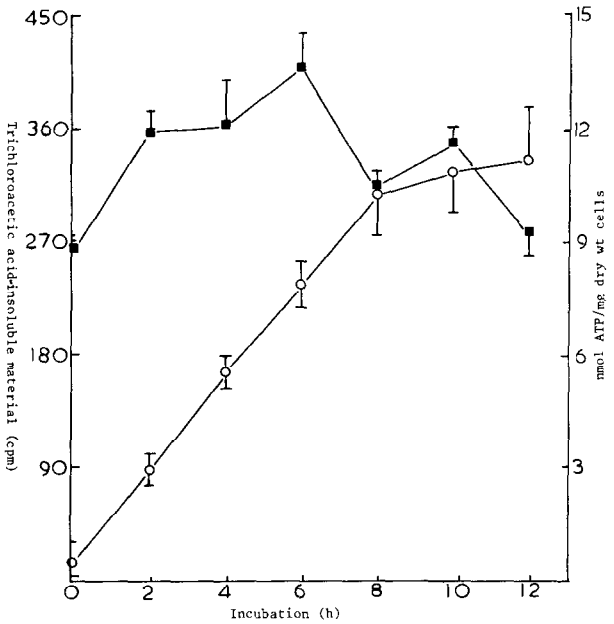

Fig.4. Incorporation of L-[4,5-^{3}H]leucine into TCA-precipitable material (○) and correlation with cell ATP content (■) during incubation in Medium B. Values are means ± SD. Leucine specific activity was 0.249 μCi/μmol.

secreted protein, which is linear for up to 20 h, and incorporation of label into intracellular protein, which may be linear for a much shorter period [9]. As we have measured total (cells plus medium) TCA-insoluble radioactivity, the pattern we observed is probably a composite of the two synthetic processes. That intracellular protein synthesis remains patent for an appreciable period is shown by the maintained steroid-induced increase in assayable tyrosine aminotransferase for at least 6 h (Marston, F. A. O., unpublished).

Foetal calf serum is a complex medium containing glucose and large and variable amounts of lactate and fructose [30]. Experiments on glucose production and several other metabolic processes, however, clearly require more defined conditions. We have investigated the behaviour of fed-rat hepatocytes in medium B containing dialysed serum, and have found results similar to those obtained with untreated serum (not shown). This suggests that, firstly, the factors involved in cell 'stabilization' may be of high molecular weight or tightly bound to serum protein and, secondly, medium B with dialysed serum may be a suitable medium for extensive metabolic studies.

Our findings indicate that isolated hepatocytes can be maintained in physiological states for periods up to 8 h with little alteration in viability, and for a subsequent 8 h with greatly reduced deterioration. Medium B appears to be the medium of choice. Serum addition increases the glucagon sensitivity of gluconeogenesis [32] and insulin-stimulated, and basal, lipogenesis [33] as well as inhibiting total nitrogen loss [34]. Serum may possibly prolong cell viability by stabilizing plasma membranes such that hormonal responsiveness is enhanced and cell autolysis inhibited.

Acknowledgements

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