SIMPLIFIED METHOD FOR ISOLATION OF INTACT AVIAN AND RAT LIVER PARENCHYMAL CELLS*

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SUMMARY

A new, simplified procedure is described for the isolation of intact liver cells from cockerel or rat liver. The technique involves a 15 min perfusion of the liver in situ through the portal vein with a solution of collagenase and hyaluronidase, and subsequent incubation of the minced liver in the enzyme solution. The yield of cells was excellent. Most cells excluded a vital stain and were undamaged when viewed with the electron microscope. The cells actively incorporated labeled precursors into lipids and proteins without specific cofactor requirements and responded to insulin in vitro. The biosynthetic capacity of the cells was retained for several days in culture.

INTRODUCTION

Many methods have been described for the isolation of parenchymal cells from rat liver for use in biochemical studies. These procedures have varied in their technical complexity as well as in the total yield, morphological integrity, degree and duration of metabolic activity of the resultant cells. Each method combines some advantages and disadvantages with regard to these parameters; no preparation in use today is ideal in all respects.

The availability of isolated, intact hepatocytes would be extremely useful for studies of metabolic control. The present report details a simple method for obtaining such a preparation. Biochemical data is presented predominately from avian liver cells since one of our objectives was to examine hepatic lipogenesis in birds rather than in the rat where the adipose tissue is the major site of lipid synthesis.

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MATERIALS AND METHODS

All instruments, glass and plastic-ware were autoclaved before use; enzyme solutions and incubation media were sterilized by passage through a millipore filter (0.45 μ). White rock cockerels (3-6 weeks old) and male Sprague-Dawley rats (200 g), all fed ad libitum, were used. Procedures were performed under sterile conditions and at room temperature except where otherwise indicated. A sterile saline minibottle (150 ml) was inverted on a support 40 cm above the operating surface and attached to an intravenous infusion set that consisted of a flexible, volutrole bag (100 ml capacity), plastic tubing with an adjustable clamp, and a butterfly infusion set.

Cockerels were anesthetized by a slow injection of sodium pentobarbital (50 mg/kg) into a wing vein. A transverse incision just below the carina was extended on both sides of the midline to reach the caudal angles of the sternum, with care to avoid damage to the liver. The liver was dissected free of its mesenteric attachments to the thoracic wall but retained in situ, and the gall bladder was carefully removed to expose the underlying portal vein. Two silk ligatures were loosely secured around this vessel using a fine forceps. The butterfly needle (#23 gauge) was directed toward the liver into the portal vein and both ligatures were tied tightly, the proximal one holding the needle in place. After rapid infusion of a few ml of warm saline, the liver blanched, and the hepatic veins were severed. The saline flow was then stopped with a clamp, and 50 ml of collagenase (120 units/ml) - hyaluronidase (0.10%) (both from Sigma Chemical Co.) in Ca^{++} and HCO_3^--free Hanks' solution (1) were added to the volutrole bag through a side arm. The liver was covered with warm, saline-soaked gauze pads while 50 ml of enzyme solution was infused at a rate of 3 to 4 ml per min. The temperature of this solution was controlled by submerging the tubing in a 37° water bath. After 15 min of infusion, the liver was excised, rinsed with serum-free Eagle's medium, and then gently minced in a plastic beaker which contained 20 ml of the collagenase-hyaluronidase solution. This mixture was transferred to a plastic Erlenmeyer flask (125 ml), an

additional 30 ml of enzyme solution was added, and the mixture was stirred magnetically at 40 rpm for 1 hr at 37°. The resultant mixture was filtered through 100-mesh silk cloth and centrifuged in plastic conical tubes at 100 x g for 5 min. The supernatant was discarded and the cells were resuspended in 3 volumes of serum-free Eagle's medium, and recentrifuged under similar conditions. The packed cells were suspended in 40 volumes of Eagle's medium which contained 10% chicken serum (complex medium) and transferred for suspension culture into spinner flasks (200 ml) stoppered with cotton plugs. The medium was not renewed during the culture period. When cells were cultured for more than 24 hr, the cultures were maintained under 5% CO_2-95% air to preserve the pH near 7.4.

In rats insertion of the butterfly needle (#21 gauge) was much simpler because of their wider and more accessible portal vein. The yield of rat liver cells was substantially increased, without adversely affecting their integrity, by an initial slow infusion of 50 ml of Ca^{++}-free Locke's solution (2) containing 0.027 M sodium citrate (37°) followed by 30 ml of the enzyme solution. The minced rat liver was stirred in 25 ml of the enzyme solution for 1 hr. Subsequent steps were the same as those described for cockerel liver cells.

Incubation Conditions: Aliquots of suspended cells (4 ml) were incubated under air in complex medium or were gently centrifuged and resuspended for incubation in a similar volume of HCO_3^--free Hanks' solution (simple medium). 1-^{14}C-sodium acetate (2.0 mc/mM) or U-^{14}C-L-serine (125 mc/mM), both from New England Nuclear Corp., were used for studies of lipid or protein synthesis respectively. Incubations were carried out in 25 ml plastic Erlenmeyer flasks in a rotary shaker at 130 rotations per min in a 37° room.

RESULTS

Under optimal conditions the recovery of liver cells (ml of packed cells/g of liver) was 40 to 60%. When diluted 1:40 with incubation medium, the cell concentration ranged from 2.4 to 3.5 x 10^6 cells per ml (cockerel liver) and 0.75 to 1.25 x 10^6 cells per ml (rat liver). About 90% of the cells excluded nigrosin dye (0.05%) for the first 3 days in culture. Samples prepared (3) for

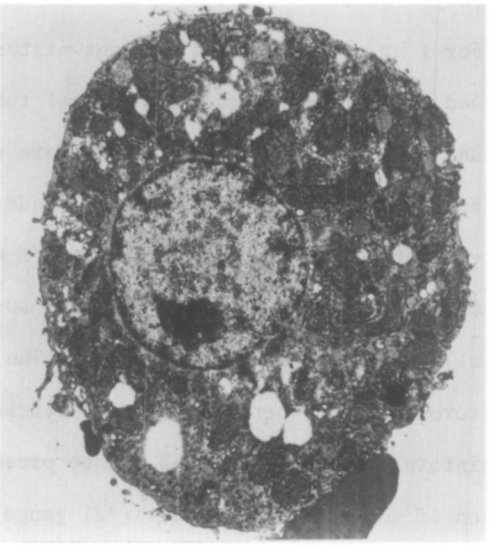

Figure 1. Isolated cockerel liver cell. X 4000

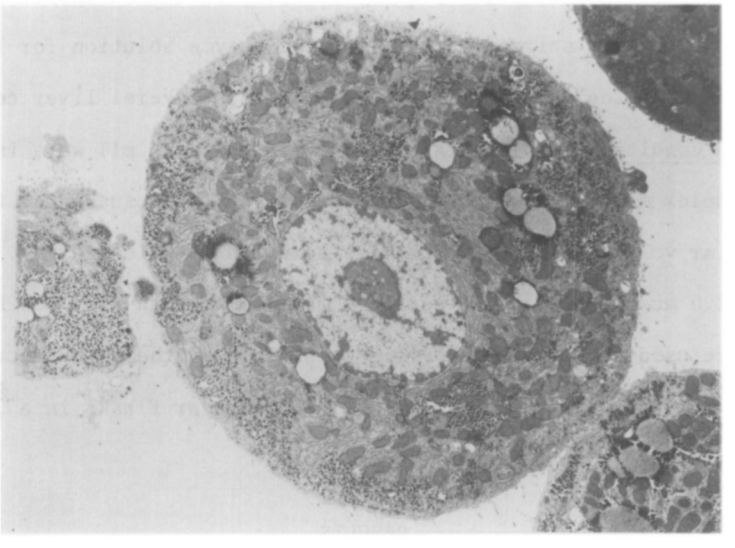

Figure 2. Isolated rat liver cell. X 2000

electron microscopic examination demonstrated that at least 50% of the cells were structurally intact, with uninterrupted plasma membranes and normal sub-

cellular organelles (Figs. 1 and 2). The cells doubled in number over the first 24 hrs in culture, but changes in cell number were minor after this period.

In both complex and simple media, incorporation of ^{14}C-acetate by fresh cockerel hepatocytes was very rapid for 60 min, but reached a plateau by about 120 min (Fig. 3). The fall in acetate incorporation after 1 hr did not result from diminished cellular viability since a similar initial rate of incorporation occurred when ^{14}C-acetate was added to cells at any time during the initial 24 hr in culture. Since the linear period of acetate incorporation was not prolonged by a 10-fold increase in acetate concentration in the incubation medium, the decline in incorporation did not result from lack of labeled substrate. Cockerel hepatocytes maintained in complex medium synthesized lipids from ^{14}C-acetate for 3 to 4 days, but the initial rate of incorporation gradually declined after the first 24 hr in culture.

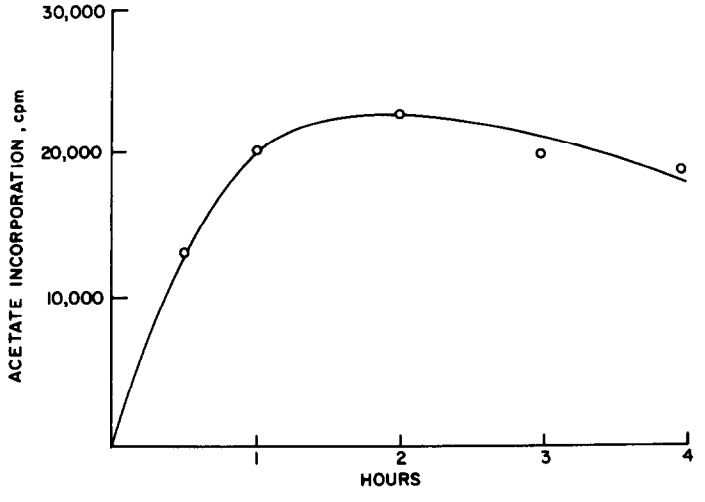

Figure 3. Time course of ^{14}C-acetate incorporation into cellular lipids in complex medium. Each point represents an average of values from two experiments and is expressed in lipid cpm per 10^7 cells. 2.0 μc of ^{14}C-acetate were used. At the end of incubation the flasks were placed on ice and 0.1 ml of unlabeled 10% sodium acetate solution was added. Cells were separated from medium by centrifugation at 2000 x g for 10 min. Labeled cellular lipids were extracted (4), dried, and counted in 10 ml of POPOP-PPO toluene scintillation fluid. Efficiency of the scintillation spectrometer for ^{14}C was 68%.

Incorporation of ^{14}C-serine into cockerel cellular protein was consistently linear for at least 3 hr (Fig. 4). ^{14}C-serine incorporation was strikingly

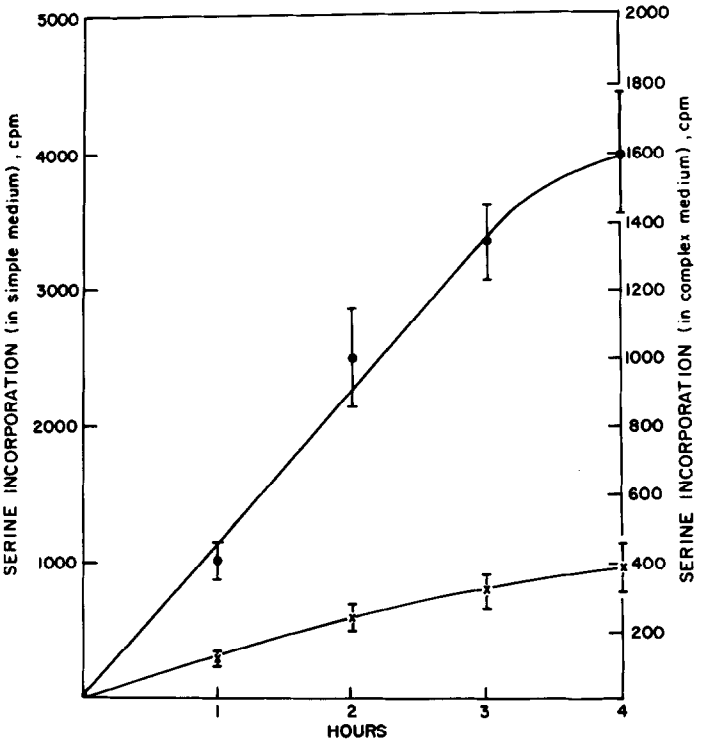

Figure 4. Time course of ^{14}C-serine incorporation into cellular protein using cells incubated immediately after preparation. Mean values from four experiments and standard deviations of the mean are depicted. Values are expressed in protein cpm per 10^7 cells. At the end of incubation, 0.1 ml of 0.3 M unlabeled serine solution was added to each sample which was placed on ice. After centrifugation of cells, the packed cells were lysed in 2% Triton; cell lysates were applied to filter paper disks, washed, and counted (5).

x--x--x, in complex medium.
o--o--o, in simple medium.

higher when freshly isolated cells were incubated in simple medium rather than in complex medium, which contained no unlabeled serine. ^{14}C-serine incorporation in complex medium steadily improved as cells were maintained in this medium so that by 72 hr the rate of incorporation was comparable in the two media. Cells incorporated labeled serine into protein for at least 7 days.

Insulin stimulated cellular lipid synthesis by about 15% (Table 1). The addition of 3', 5' cyclic AMP inhibited acetate incorporation by 87%. In the presence of insulin the inhibitory effect of 3', 5' cyclic AMP was diminished by 57%. These effects of insulin and 3', 5' cyclic AMP on hepatocyte lipid

TABLE 1

Effect of Insulin and Cyclic 3', 5' AMP on ^{14}C-acetate Incorporation into Cellular Lipids

Addition	Relative Incorporation %
None (6)	100
Insulin (6), 0.05 u/ml	115 ± 13*
Cyclic 3', 5' AMP (2), 10^{-4}M	13
Insulin plus Cyclic 3', 5' AMP (2)	30

*Mean ± standard deviation

( ) indicates number of livers studied

Insulin or cyclic 3', 5' AMP were incubated with freshly prepared cells in complex medium for 30 min. ^{14}C-acetate (2.0 µc) was added and incorporation carried out for a 30 min period. Incubation conditions are described in the text, and methods of labeled lipid isolation and counting are indicated under Fig. 3.

synthesis are compatible with data obtained in liver perfusion (6) and slice experiments (7).

DISCUSSION

Electron microscopic examination of freshly isolated cells has indicated that structurally intact cells are obtained only by enzymatic methods, but a major drawback of these techniques has been the recovery of 5% or less of the total hepatocytes (8,9). Berry and Friend (10) introduced the use of a closed, recirculating perfusion of rat liver by collagenase-hyaluronidase, followed by EDTA solution. This procedure increased the recovery of hepatocytes to about 50%. However, their method is technically difficult and requires the use of highly specialized and costly equipment.

The present report describes a procedure which is far simpler and requires

no specialized equipment, yet produces similar results. The recovery of hepatocytes was 50% and at least half of these cells were intact by electron microscopic examination. Moreover, this technique has been successfully applied to the cockerel as well as the rat. Finally, cells isolated in this manner maintain metabolic activity for several days in suspension culture.

Although earlier reports indicate maintenance of rat liver cells in culture after preparation with tetraphenylboron chelation (11) or citrate-mechanical disruption (12), we have found that cells isolated by these methods show severe structural damage. Furthermore, their rate of lipid and protein synthesis, which was very low initially (3), did not improve after 24 hr in complex medium.

The present technique has many advantages for studies of hepatocyte metabolism since it combines simplicity of preparation with an excellent recovery of metabolically active, structurally intact liver cells. In addition, their responsiveness to insulin and maintenance of lipid and protein synthesis in suspension culture renders them potentially useful for *in vitro* study of physiological regulatory mechanisms.

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