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THE MAINTENANCE OF LIVER CELLS *IN VITRO* FOR VIROLOGICAL AND OTHER STUDIES

D. RUBENSTEIN*, M. R. BAKER*, E. J. STOTT† AND A. S. TAVILL

From the Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ

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Summary.—Cells from the livers of rats and monkeys were dispersed by enzyme treatment and maintained for weeks in roller tube cultures. Rat liver cells were shown to synthesize urea and monkey cells apparently synthesized RNA and protein—rat cells supported the growth of Sendai virus and monkey cells supported yellow fever virus. Organ cultures of human embryo liver supported the growth of adenovirus type 5 and herpes simplex virus. The Australia antigen serum hepatitis agent failed to grow in human or monkey liver cultures.

ALTHOUGH isolated liver perfusion, slices and cell suspensions have proved to be valuable tools for biochemical investigation of hepatic metabolism, these techniques in their present form have had limited application in virological studies because of their short life span. Tissue cultures of very thin liver slices or dissociated cells in high concentrations of oxygen have retained viability by enzymatic and morphological criteria for some days (Campbell and Hales, 1971; Dickinson, 1971) while virus growth may be detectable in squashed fragments of foetal liver over a similar period of time (Zuckerman *et al.*, 1972).

Our objectives in this present study were to maintain hepatocytes on a glass surface in the roller tube cultures which are so valuable in virological studies, and to evaluate the growth of viruses in these cells and in organ cultures of liver.

MATERIALS AND METHODS

Organ cultures.—As a result of preliminary experiments, we used 5 mm cubes of human liver from foetuses aged 15–20 weeks. These were placed on stainless steel grids in 50 mm plastic petri dishes containing 2·5 ml of medium. The dishes were incubated in humidified plastic boxes in 95% O_2 in air at 37°. A number of synthetic media were tested and the largest number of well preserved cells were seen in fragments maintained in Liebowitz's (L15) medium containing 0·2% bovine plasma albumin. No serum was used since this stimulated the appearance of fibroblasts.

Monolayer cultures

(1) *Rhesus monkey.*—Immediately after death the liver of an adult monkey was perfused *via* the portal vein with 15–20 ml of a solution of 0·05% collagenase (B.D.H. or Sigma Type I) and 0·1% hyaluronidase (Sigma Type I) in calcium-free Kreb's ringer phosphate (KRP) solution (modified method of Howard and Pesch, 1968). The liver was removed, chopped with scissors, and incubated in 80 ml of the collagenase–hyaluronidase solution at 37° with

* Present address: Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QQ.

† Present address: Institute for Research on Animal Diseases, Compton, near Newbury, Berkshire RG16 0NN.

stirring, and in an atmosphere of 95% O_2 in air, for 1½–2 hours. The remaining lumps were then squashed with a spatula to release the cells and the mixture filtered through sterile surgical gauze and then through a 60 μm nylon filter. The cell suspension was centrifuged, washed twice in KRP and the cells resuspended in culture medium at a concentration of 10^6 viable cells per ml. One ml of suspension was inoculated into roller tubes, which were rolled immediately. Refractile rounded cells attached to the glass and remained there for many days. The cells survived best in Eagle's basal essential medium (BEM) containing 10% calf serum, pH 7, at 37°. For virus studies, this was replaced with 1% foetal calf serum after the cells had attached. Cell viability was assessed by exclusion of Trypan blue.

(2) *Rat liver cells.*—Isolated parenchymal cells were prepared by *in situ* perfusion with 0·05% collagenase + 0·1% hyaluronidase in calcium-free KRP, according to the method of East, Louis and Hoffenberg (1973).

Urea biosynthesis.—Sodium [^{14}C] carbonate (Radiochemical Centre, Amersham, >50 mCi/mmol/l) was diluted with 0·9% saline to give a final concentration of 200 μCi in 3 ml and stored in sealed ampoules under nitrogen. To the explant or monolayer cultures 1·0 ml of this solution was added and the incubation continued under culture conditions for periods up to 3 hours. At the end of the incubation period, incorporation was inhibited by chilling and 1·0 ml of a standard urea solution (1·424 mg/ml) was added. The cells and debris were centrifuged at 1000 *g* and the supernatant solution was acidified and dried down under vacuum in a rotary evaporator. The urea solution was then reconstituted with pyrophosphate buffer (pH 7·0), reacted with urease at room temperature for one hour and the liberated CO_2 measured and counted as described previously (Tavill, Craigie and Rosenoer, 1968).

Protein and nucleic acid synthesis.—Tube cultures containing either 2×10^6 monkey liver cells or 2×10^5 L132 cells were washed twice in Hank's balanced salt solution. A proportion of the tubes were then immersed in a boiling water bath for 5 min. The cells were then overlaid with 1 ml of cold Hank's balanced salt solution containing 0·1% sodium bicarbonate, 2% dialysed calf serum and either 1 μCi/ml of [^{3}H] uridine (2000 mCi/mmol; Radiochemical Centre, Amersham) or 0·1 μCi/ml [^{14}C] protein hydrolysate (>45 mCi/milliatom carbon; Radiochemical Centre, Amersham). The cultures were then incubated at 37° and 1, 2, 3 and 5 hours later 4 replicate cultures of each cell type were chilled to 4°, washed twice in cold saline and frozen at −70° in 4 ml of distilled water. After thawing, the contents of each 4 replicate tubes were pooled and the nucleic acid and protein precipitated with an equal volume of cold 0·5 mol/l perchloric acid. The precipitate was washed twice in 10 ml of 10% trichloroacetic acid and dissolved in 1 ml distilled water, 1 ml 1N NaOH and 5 ml 0·125 mol/l sodium pyrophosphate. The nucleic acids and proteins were then reprecipitated with 10% trichloroacetic acid and washed on a fibreglass filter with 5% trichloroacetic acid. The filters were then dried, placed in scintillation vials, covered with Triton–toluene scintillation fluid and the acid precipitable radioactivity counted in a Packard Tricarb Spectrometer.

Virus growth.—The viruses used were: (1) poliovirus type 2 attenuated Sabin vaccine strain, supplied by Dr D. I. Magrath; (2) adenovirus type 5 prototype strain grown in HeLa cells and obtained from Dr H. G. Pereira; (3) yellow fever virus 17D vaccine strain; (4) herpes simplex virus passaged in WI-38 cells, a type 1 strain obtained from Mrs B. Zisman.

In a few experiments strains of Semliki Forest and West Nile virus grown in suckling mouse brain and supplied by Dr J. S. Porterfield were used, and a strain of Sendai parainfluenza 1 virus was used which had been obtained from rats and propagated in chick embryos (Tyrrell and Coid, 1970).

In the standard experiment, virus was diluted to contain about 1000 infectious doses and this was inoculated in a volume of 0·1 ml into a culture containing medium. After 1–3 hours of incubation the culture was drained, washed 3 times and incubated with fresh medium. Medium was harvested at intervals of one or 2 days, mixed with equal volumes of nutrient broth and stored at −70° for subsequent titration.

RESULTS

First of all, we assessed the success of cultures in maintaining the viability of liver cells. The inner zone of the organ cultures of foetal human liver de-

generated within 24 hours but the peripheral tissue was normal histologically for up to 6 days (Fig. 1). Electron microscopy showed that these cells were largely intact. In the monolayer cultures a substantial proportion of the added cells remained stuck to the glass. They remained rounded and refractile and did not flatten out; about 10% excluded dye for at least 14 days but most cells appeared abnormal on electron microscopy. Fibroblasts were not seen in any culture showing virus growth.

Urea biosynthesis

Tube cultures maintained for 2 days were individually pulsed with 1·0 ml of the [^{14}C] carbonate solution and rolled for a further one hour and 3 hours in the presence of labelled medium. Similar cultures maintained for 14 days were incubated for one and 6 hours in the presence of labelled precursor.

On both occasions there was incorporation of ^{14}C from sodium [^{14}C] carbonate into urea and on both occasions there appeared to be increasing specific activity with the length of incubation (Table I). No inferences can be drawn from a

TABLE I.—*Synthesis of Urea by Rat Liver Cell Monolayers*

Experiment number	Maintenance period (days)	Period of incubation with [^{14}C] carbonate (hours)	[^{14}C] urea specific activity (d/min/mg C)
1	2	1	$4{\cdot}07 \times 10^2$
	2	3	$11{\cdot}4 \times 10^2$
2	14	1	$25{\cdot}3 \times 10^3$
	14	6	$31{\cdot}7 \times 10^3$

comparison of specific activities in the 2-day and 14-day cultures since these may be influenced by variables such as relative cell survival, the size of the pre-existing unlabelled urea pool and the amount of metabolic and atmospheric CO_2 in individual cultures.

Protein and nucleic acid synthesis

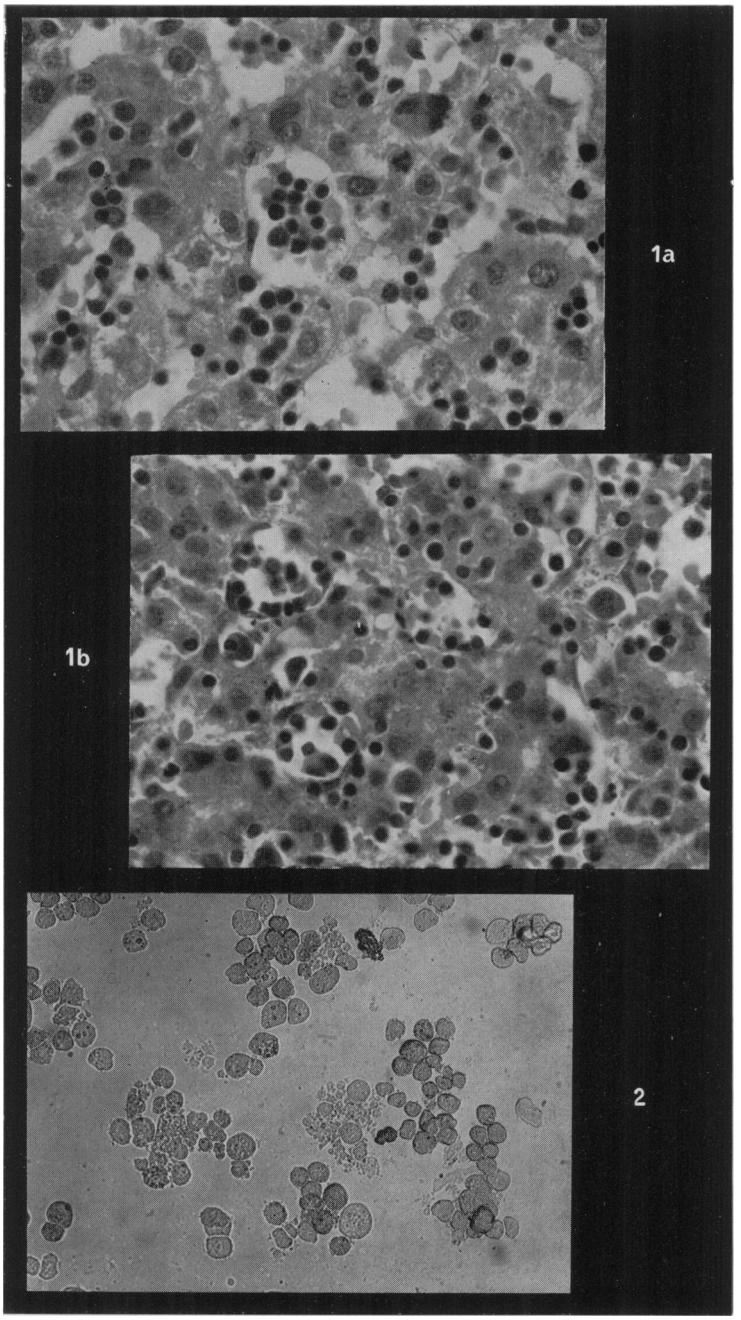
The synthesis of protein and nucleic acid was studied after adding radioactive precursors to the culture medium. Total acid precipitable counts recovered from live liver cells labelled with [^{3}H] uridine are significantly higher than those found in boiled cells, and increase with time, indicating that RNA synthesis occurs in these liver cells (Table II). However, synthesis is only 2–3% of that of L132 cells (Table II). Since both types of cultures contained approximately the same number of viable cells (2×10^5) by Trypan blue exclusion, this result suggests that either the liver cells have a much lower turnover of RNA or that many of the cells were metabolically inactive.

Protein synthesis, as indicated by incorporation of [^{14}C] amino acids into

EXPLANATION OF PLATE

FIG. 1.—Section of human embryo liver organ culture (a) on the day of preparation and (b) after incubation for 4 days. H. & E. ×470.

FIG. 2.—A tube culture of monkey liver cells incubated for 3 weeks; some cells are degenerating. Unstained. ×105.


1a
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2

acid precipitable material, also occurs in the monkey liver cells, but again is only about 6% of that found in L132 cells (Table III).

TABLE II.—*RNA Synthesis by Monkey Liver and L132 Cell Monolayers*

Period of incubation with [^{3}H] uridine (hours)	Total acid-precipitable ct/min			
	Monkey liver cells		L132 cells	
	Normal	Boiled	Normal	Boiled
1	208	ND*	6608	ND
2	372	69	15800	323
3	431	ND	20163	ND
5	528	72	27130	112

* ND = not done.

TABLE III.—*Protein Synthesis by Monkey Liver and L132 Cell Monolayers*

Period of incubation with [^{14}C] amino acids (hours)	Total acid-precipitable ct/min			
	Monkey liver cells		L132 cells	
	Normal	Boiled	Normal	Boiled
1	2429	ND*	46485	ND
2	4246	41	70012	177
3	4967	ND	111773	ND
5	8320	10	111131	81

* ND = not done.

TABLE IV.—*Results of Attempts to Grow Viruses in Liver Cell Cultures*

Type of culture	Species	Virus	Number of times virus grew*	Maximum titres† in medium	
				Geometric mean	Range
Organ	Human	Adenovirus 5	4/4	4·1	2·5–5·5
		Herpes hominis	1/1	4·0	—
Monolayer	Monkey	Yellow fever	3/6	4·1	2·5–6·3
		West Nile	0/2	—	—
		Semliki Forest	0/2	—	—
		Herpes hominis	0/3	—	—
		Adenovirus 5	0/1	—	—
		Poliovirus 1	0/1	—	—
	Rat	Parainfluenza 1 (Sendai)	4/6	4·3	2·2–5·2

* Numerator = number of experiments in which growth was detected; denominator = number of experiments done.
† Titre = $\log_{10}$ TCD_{50}/ml.

Virus growth

It was shown that organ cultures would support the growth of adenovirus type 5 in 4/4 attempts and herpes virus on the only attempt (Table IV). Monkey liver monolayers supported the growth of yellow fever virus on 3/6 attempts, but the other viruses which had grown in other cultures failed to multiply, probably reflecting the limited susceptibility of the intact monkey liver. Sendai virus grew in rat liver cell monolayers.

Experiments with Australia antigen

Attempts were made to support the multiplication of Australia antigen in explants of human embryo liver and monolayers of rhesus monkey cells. The antigen (kindly supplied by Dr June Almeida) contained a high concentration of Dane particles and was inoculated onto explant cultures immediately after preparation, and onto monolayers 24 hours after preparation. The cultures were incubated for 5 hours or 24 hours to allow adsorption, and then washed 3 times to remove unabsorbed antigen.

Medium was harvested after 4 and 7 days' incubation at 37° and stored at −70°. Explants and cells were fixed for electron microscopy at the same time.

The presence of antigen in the supernatant was tested by complement fixation and no evidence of an increase in titre was observed. Electron microscopy of thin sections of the explants and pellets of the monolayer cells showed no evidence of Australia antigen, Dane particles or internal components (Almeida, Rubenstein and Stott, 1971).

DISCUSSION

Many cultures of hepatic tissue contain cells derived from fibroblasts or reticuloendothelial cells, but few contain true hepatocytes. We think that there is morphological evidence that the liver explants contained healthy liver parenchymal cells, and there is biochemical evidence for such cells in the monolayer cultures although the activity is not very high.

The lowest common denominator of the carbon of urea is the carbon of CO_2 and bicarbonate which becomes fixed *via* the ornithine–arginine cycle (Krebs and Henseleit, 1932). Arginine is the intermediate in the cyclic series of reactions whereby urea is formed by condensation of CO_2 and NH_3 with ornithine (Krebs and Henseleit, 1932; Krebs, 1942). Whilst guanidine labelled arginine may give rise to labelled urea outside the liver, it appears that the complete ornithine–arginine cycle exists only within the liver since no incorporation of ^{14}C into urea from sodium [^{14}C] carbonate could be detected in hepatectomized dogs (Craigie *et al.*, 1965; Tavill, Craigie and Rosenoer, 1968; Tavill *et al.*, 1973). Since the ornithine–arginine cycle is the only known pathway of CO_2 fixation into urea in mammals, and is present in its complete form only in the liver, the present data strongly support both the hepatic origin and the maintenance of hepatic function in these cultured cells. However, the enzymes of the urea cycle in foetal rat liver are only slightly active and, over 20–30 days of neonatal life, urea cycle activity increases to a plateau as a switch occurs from pyrimidine to urea synthesis (Räihä and Kretchmer, 1965; Räihä and Suihkonen, 1968; Visek, 1972). It is therefore possible that the increased incorporation of $^{14}CO_2$ into urea as cells are maintained in culture may be partly explained by analogous maturation *in vitro*. Confirmation of this hypothesis will require sequential analysis of urea cycle enzyme activity in the mitochondria and cytosol of the hepatic cells maintained in tissue culture.

Viruses grew consistently in the human embryo liver organ cultures but only occasionally in the monkey or rat liver monolayer cultures. This latter finding may be related to the low level of protein and RNA synthesis found in the monolayer cultures, or to the fact that only yellow fever was adapted to growth in hepatocytes. The infectious agent of hepatitis B did not appear to replicate in

either the organ cultures or monolayer cultures, as judged by complement fixation tests or electron microscopy. While this work was in progress, Zuckermann and his colleagues (1972) reported the apparent replication of Australia antigen in organ cultures of human embryo liver. The reasons for our failure to confirm their work are not obvious since the methods were similar. However, there were small differences in the medium, the inoculum and the source of the liver material. More work is clearly needed in this field, but we think that the techniques described are worthy of further development and that they may assist in the further evaluation of infection and other pathological processes in the liver.

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