Metabolic and Virologic Studies in Primate Liver Organ Cultures[1] (34616)

RAINER LAUFS[2] AND WILLIAM WALKER
(Introduced by F. Deinhardt)

Departments of Microbiology, Presbyterian-St. Luke's Hospital and University of Illinois, College of Medicine, Chicago, Illinois 60612

Organ cultures have been useful for isolating and propagating viruses, including those agents which only multiplied poorly in monolayer cell cultures (1–5). As hepatocytes may be the target cells for human hepatitis viruses, liver organ cultures are the natural choice for isolation attempts of these still unidentified agents. But many attempts to culture dissociated adult, differentiated liver cells have failed and the problems of short parenchymal cell survival and mesenchymal cell overgrowth have not been solved. Kalus *et al.* (6) recently reported a method of organ culture in which they maintained pieces of adult marmoset liver and demonstrated electronmicroscopically the growth of marmoset herpesvirus in hepatocytes of the explants. This paper describes a modification of their organ culture method, the successful cultivation of adult primate liver cells for up to 3 weeks, the histology and the metabolism of the liver explants in organ culture and the use of the method for propagating several DNA and RNA viruses in hepatocytes.

Materials and Methods. 1. Liver organ cultures. Liver tissue was obtained by surgical or needle biopsy from adult marmosets (*Saguinus* (*Oedipomidas*) *oedipus* and *Saguinus nigricollis*) and from adult hospitalized patients who were clinically free of infectious diseases. The tissues were placed in 100 × 15-mm petri dishes containing 5 ml of medium (improved basal medium Eagle in Earles' balanced salt solution supplemented with 10% fetal calf serum, 100 units of penicillin, 50 μg of streptomycin, and 5 μg of fungizone/ml, with the pH adjusted to 7.4 by addition of a 7.5% solution of sodium bicarbonate) (FC-BMEI-E), cut with razor blades into 1–2-mm square fragments, rinsed 3 times with FC-BMEI-E and placed in groups of 8 each on top of two pieces of sterile Gelfoam sponges (No. 7853, The Upjohn Company, Kalamazoo, Michigan), measuring 20 × 7 × 7-mm in each 35 × 10-mm culture dish. The Gelfoam sponges, used in surgery as a hemostatic, were soaked in 1.5 ml of FC-BMEI-E for 2 hr before use. The organ culture dishes were incubated in Torbal anaerobic jars at 35°, the sidearms of the jar were removed and dishes with distilled water were placed in the jars for humidification. The jars were gassed for 5 min with a mixture of 95% room air and 5% CO_2 mixed with various amounts of a mixture of 95% oxygen and 5% CO_2. The gas ratios were monitored by two flowmeters and the final oxygen content of the gas mixtures in the jars was measured by the pyrogallol method (7). For histology, the liver organ pieces together with the supporting sponges were fixed in 10% neutral formalin, embedded in paraffin, sectioned at 6 μ and stained with hematoxylin and eosin. Organ cultures were infected with viruses by either of 2 methods. In the first method, which was used only for infection of fresh liver, 1–2-mm square pieces of liver were incubated in 2 ml of the virus inoculum in a 35 × 10-mm petri dish for 1 hr at room temperature. The pieces were then rinsed 3 times with FC-BMEI-E and placed on sponges as described previously. The second method was used to infect liver pieces which had already been incubated on sponges. The

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[2] Present Address: 34 Göttingen, Hygiene Institute, Western Germany.

original medium was removed from these cultures and 0.3 ml of the virus suspension/culture was dropped onto the liver pieces. These cultures were not rinsed after infection because the liver pieces would have floated off but 1 ml of fresh medium was added to the cultures immediately after infection and the cultures were incubated as previously described. The culture fluids were harvested at various time intervals for determination of interferon levels. For titration of tissue-bound virus the liver pieces were washed 3 times in Hanks' solution, homogenized with a mortar and pestle in 1 ml of FC-BMEI-E, and centrifuged at 600*g* for 10 min. The supernatant was removed and stored at −60°. Serial 10-fold dilutions of the supernatant were inoculated into 4 tissue culture tubes/dilution. End points were determined by appearance of typical cytopathic effects and the $TCID_{50}$ was calculated by the method of Reed and Muench (8). Sendai and mumps virus titrations in human kidney cells were evaluated 7 days after inoculation by hemadsorption with 1% chick red blood cells.

2. *Virus strains.* Marmoset herpesvirus (MHV) was isolated in this laboratory (9) and vaccinia virus was kindly supplied by Dr. J. Gallagher of the University of Illinois. Vesicular stomatitis virus (Indiana) (VSV), Newcastle disease virus (California) (NDV), coxsackie virus type A9, echovirus type 6 (D'Amori), and poliomyelitis virus type 2 (Lansing) were obtained from the American Type Culture Collection, the Sendai strain of parainfluenza type 1 virus has been described previously (10) and the fifth amniotic passage of mumps virus (Jones) was kindly supplied by Dr. G. Shramek of this laboratory.

3. *Cell cultures.* The following cell types were used for virus production and/or virus and interferon assays: MCN subline of L cells (11) for VSV; a marmoset heart fibroblast and a marmoset kidney cell line, established in this laboratory, for vaccinia virus and for interferon assays; human embryonic kidney cells for MHV, NDV, mumps and Sendai viruses; African green monkey kidney cells for poliovirus type 2, Coxsackie virus type A9 and echovirus type 6. Cell culture methods used in this laboratory have been described previously (9).

4. *Interferon assays.* Liver organ culture fluids were assayed for interferon activity after dialysis against pH 2.2 on marmoset kidney cells following essentially the method described by Henle *et al.* (11).

5. *Investigation of liver cell metabolism.* RNA, DNA, and protein synthesis in liver organ cultures was determined by the addition of organ culture media containing either 20 μCi/ml of uridine-5-^{3}H, (24 Ci/mmole) or 1 μCi/ml of thymidine-2-^{14}C, (25–50 mCi/mmole) or 20 μCi/ml of a mixture of L-leucine-4,5-^{3}H (29.1 Ci/mmole), L-proline-3,4-^{3}H (5 Ci/mmole), and L-tyrosine-3,5-^{3}H (>25 mCi/mmole) for 16 hr. Incorporation was terminated by submerging the liver explants in medium containing 10% glycerol precooled to 4° and cultures were frozen in the same medium at −70°.

Density gradient studies were performed on isotope-labeled, cellular components of the liver cells. Ten pieces of liver from a single organ culture dish were combined for each experiment. The frozen tissues were thawed and minced with a razor blade, suspended in 0.8 ml of tris(hydroxymethyl)aminomethane (Tris)–HCl buffer, pH 7.4, containing 1.5 m*M* $MgCl_2$ and 0.05 *M* KCl (TMK) and were held at 4° for 20 min and then homogenized in a Potter-Elvehjem Teflon–glass homogenizer. Sodium desoxycholate and polyoxyethylene lauryl alcohol were added to the homogenate in a final concentration of 0.5% each. The sample was layered onto a previously prepared and chilled linear, 10 to 60% (w/v) sucrose–TMK gradient. The gradient was centrifuged at 40,000 rpm for 2.5 hr at 4° in a SW 41 rotor in a Spinco model L-2 ultracentrifuge. The gradients were fractionated from the bottom of the tube and the optical density for each fraction was recorded at 254 mμ. Purified ribosomes were used as markers for this sucrose gradient. The 30 S ribosomes from *E. coli* were found in fraction 9 and the 50 S ribosomes in fraction 11. Single ribosomes from Chang's conjunctiva cells (12), used as markers, were found in fractions 13 and 14 and ^{14}C-thymidine-

labeled nuclei from the same cells in fraction 20–21. Bovine serum albumin (0.3 ml, 100 μg) and 0.6 ml of 15% trichloroacetic acid (TCA) were added to each 0.5-ml fraction. The precipitates that formed at 4° overnight were collected on Bac-T-Flex membrane filters with interstices of 0.45 μ and were washed with 5% TCA and water. The membranes were placed into glass vials, dried, and then 15 ml of toluene containing 5 g/liter of 2,5-diphenyloxazole and 0.1 g/liter of dimethyl 1,4-bis-2-(5-phenyloxazolyl)-benzene were added. Radioactivity was determined in a Tri-Carb liquid scintillation spectrometer.

Radioautographic studies were performed by dipping the unstained slides, obtained after regular histological preparation of the labeled liver explants, in NTB-3 (Kodak) emulsion and exposing them for 14 days at refrigerator temperature. After development and fixation the slides were stained through the emulsion with hematoxylin and eosin.

Results. 1. Conditions necessary for successful liver organ culture. Two critical factors for successful culture of the liver explants were controlled oxygen supply and proper support for the liver pieces.

Marmoset liver explants were cultured in gas mixtures of room air with 40, 50, 60, and 75% oxygen and in room air without additional oxygen, each gas containing 5% CO_2. The liver pieces were examined histologically after 3, 8, 11, and 21 days of culture. In the central portions of the liver explants, cultured in the different gas mixtures, degeneration with pyknosis of nuclei and granular eosinophilia of the cytoplasm was observed with zones of living hepatic parenchymal cells in the periphery. The nuclei of these hepatocytes were often enlarged and the cytoplasm was slightly basophilic. The degeneration in the center of the liver explants and the changes in the peripheral hepatocytes varied, increased with the length of the culture period, but were found less and later in 60 liver pieces cultivated in a gas mixture containing 60% oxygen. The tissue architecture (with the exception of the small central necrotic areas) remained intact in all of these liver explants. The central necrotic area, which was always present when air without additional oxygen was used was completely absent in 5% of the cultures maintained under 60% oxygen and was much smaller and developed later in the remaining 95% of these cultures as compared to cultures maintained in room air. The hepatocytes showed fewer changes and remnants of necrotic cells were less frequent. Pure oxygen with 5% CO_2 or hyperbaric conditions with various gas mixtures at 3 atm were toxic for the hepatocytes. After liver cells were cultured in pure oxygen, they showed enlarged nuclei with prominent nucleoli and dispersed chromatin, while tissues cultured under hyperbaric conditions became necrotic near the gas–organ interphase although cells near the center of the liver pieces still appeared viable.

A Gelfoam sponge, without special pretreatment, was used as the support material for the liver explants instead of the usual organ culture grids and the recently described human fibrin sponge (6). The liver pieces adhered quite securely to the surface of the Gelfoam sponges and no or only little pressure necrosis was observed.

The best results obtained by using a gas mixture with 60% oxygen and Gelfoam sponge as matrix for the long-term cultivation of marmoset liver explants are presented in Fig. 1 and 2. After 11 days (Fig. 1) the liver explant showed no central necrosis, bile ductules and central veins survived, and the nuclei and cytoplasm of the hepatocytes appeared almost histologically unchanged. A few hepatocytes showed eosinophilic degeneration. After 21 days of culture (Fig. 2), some of the hepatocytes and the epithelium of the bile ductules were still viable and morphologically unaltered, but many hepatic parenchymal cells were enlarged and vacuolated with increase in nuclear size. In the periphery and the center of the liver explant some hepatocytes had degenerated, the cytoplasm of these cells had become eosinophilic and their nuclei were pyknotic. Small necrotic areas were scattered between the surviving cells. Only occasionally were hepatocytes in mitosis found during the 3 weeks of culture.

2. Metabolism of the cultured liver cells.

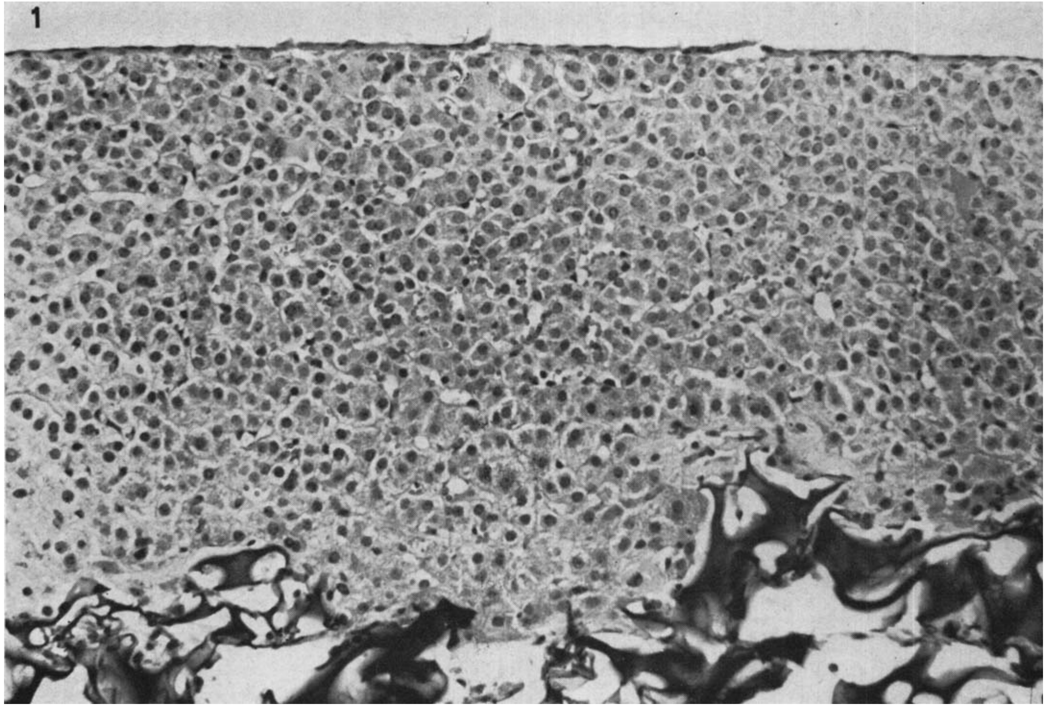


FIG. 1. Adult marmoset liver explant after 11 days of culture (×200, hematoxylin and eosin).

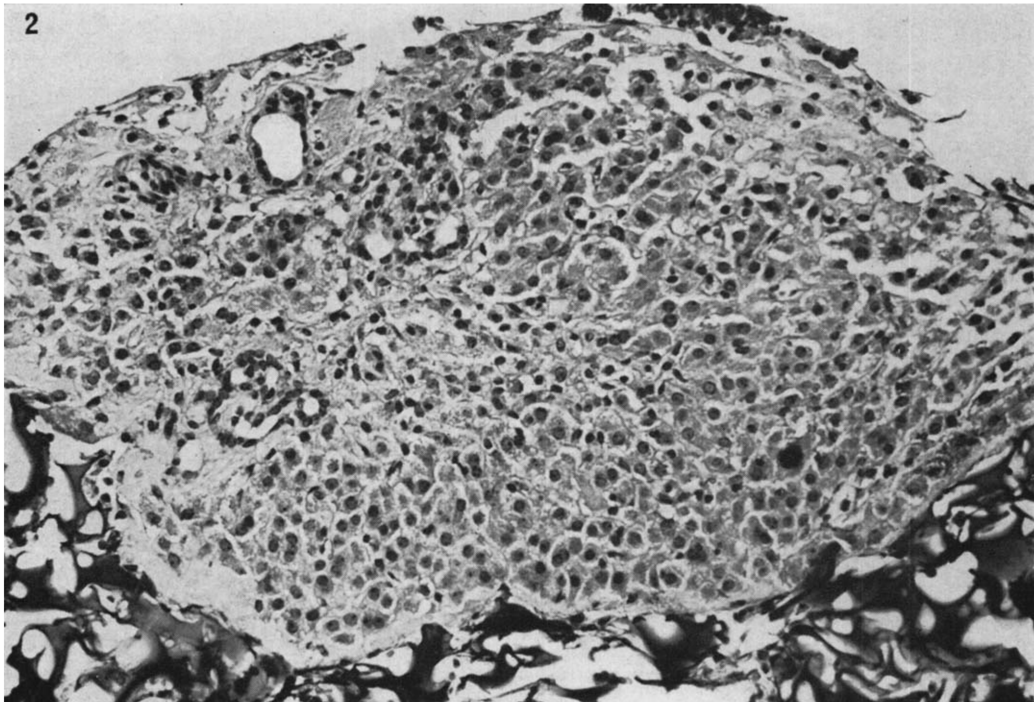


FIG. 2. Adult marmoset liver explant after 3 weeks of culture (×180, hematoxylin and eosin).

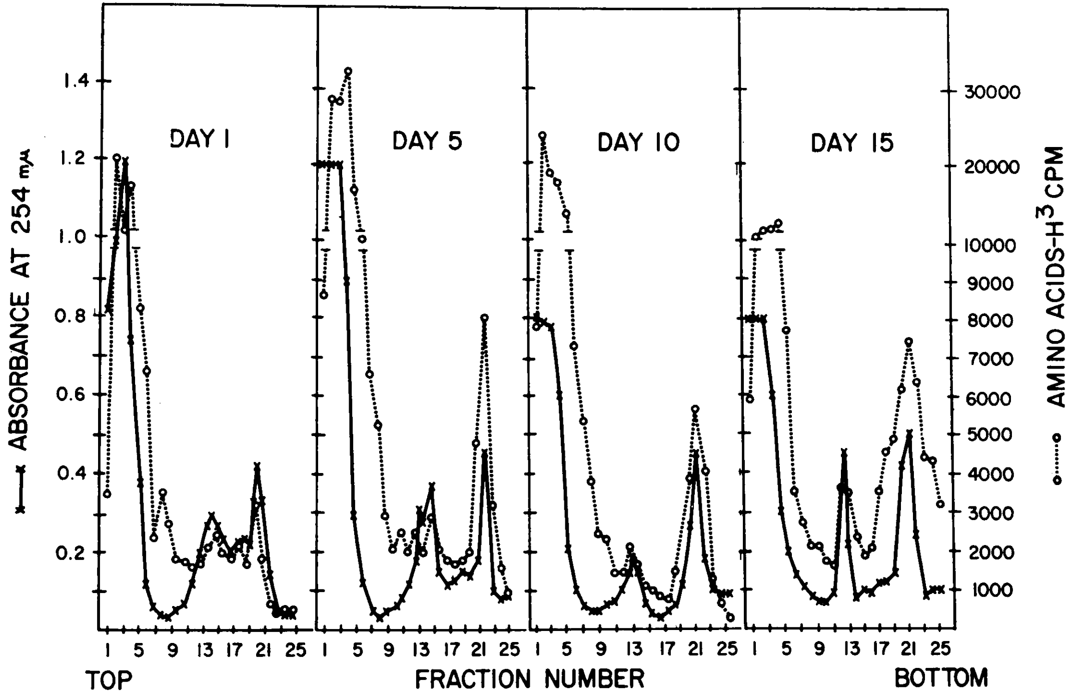


FIG. 3. Sucrose gradients of liver cell homogenates labeled with ^{3}H-amino acids (O=radioactivity; × = optical density.)

The metabolism of the liver explants and its alteration during prolonged cultivation was investigated by measuring the incorporation of radioactive precursors into cellular macromolecules of the liver explants at day 1, (labeled precursors added within 1–4 hr after explantation), 5, 10, and 15 of cultivation. The optical density and radioactivity of the fractions are presented in Figs. 3–5.

a. Protein synthesis (*Fig. 3.*). Proteins labeled with tritiated amino acids were distributed throughout all cellular components. A high peak of radioactivity was found in the soluble portion of the gradient (fractions 1–5). Radioactivity was associated with the ribosomal portion (fractions 13–15) and with fractions 20–22 which contained the nuclei and mitochondria. Neither the distribution nor the amount of radioactive-labeled proteins in cytoplasmic or nuclear cell compartments differed remarkably at day 1, 5, 10, and 15 of culture.

b. RNA synthesis. (*Fig. 4*). Newly synthesized RNA was found in cytoplasmic and nuclear cell components after labeling with tritiated uridine. One peak of radioactivity was associated with fractions 20–22 which contained the cell nuclei and considerable amounts of radioactivity were also found in the ribosomal portion (fractions 13–15). Some radioactivity was detected in ribosomal subunits (fractions 7–12) and a high activity in the soluble portion of the gradient. The amount and the distribution of the synthesized RNA in the liver cells were similar in 1-, 5-, 10-, and 15-day-old liver organ cultures and the RNA synthesis was completely inhibited by treating the liver explants with actinomycin D (2 μg/ml), 1 hr prior to the labeling with ^{3}H-uridine.

c. DNA synthesis (*Fig. 5*). The uptake of ^{14}C-thymidine was much lower than the uptake of ^{3}H-uridine and ^{3}H-amino acids. The liver organ explants synthesized only small amounts of DNA, present in fractions 20–22, at day 1, 5, 10, and 15 of culture.

d. Radioautography. Radioautographic studies were performed to identfy the locality of protein, RNA, and DNA synthesis within the different cells of liver explants and

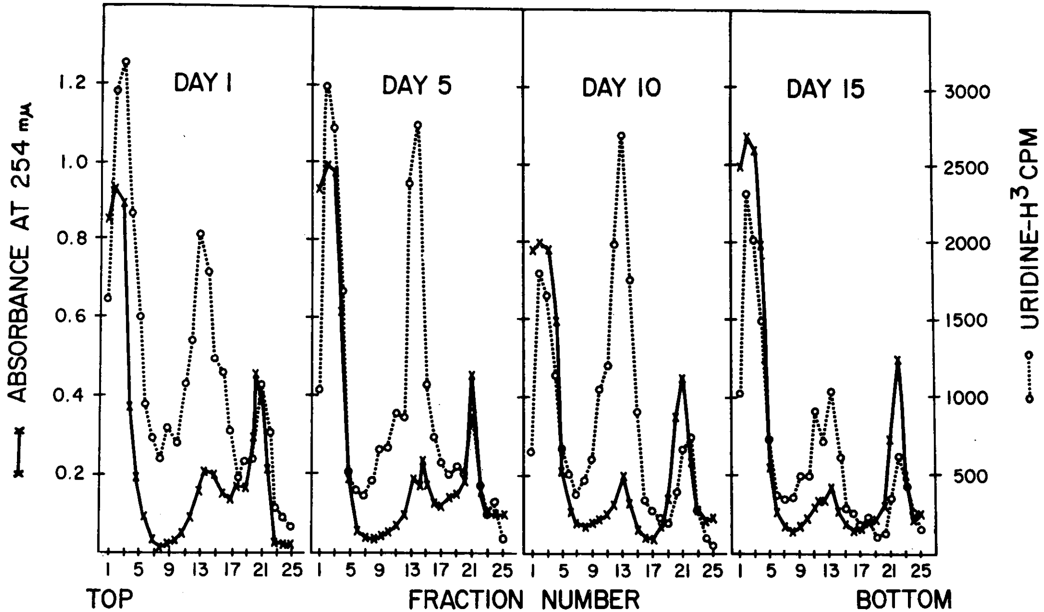


FIG. 4. Sucrose gradients of liver cell homogenates labeled with ^{3}H-uridine (O = radioactivity; X = optical density.)

to determine the time needed for the medium to diffuse from the periphery to the central portion of a liver explant.

Five-day-old liver organ cultures were sampled after 1, 2, 3, 4, and 6 hr of labeling. In all experiments, more than 95% of the radioactivity was found in the hepatocytes and the radioactivity had reached all hepatocytes throughout the entire liver piece after 1 hr. After 4 hr of labeling with ^{3}H-amino acids all hepatocytes were loaded with silver grains and the greatest amount of the radioactivity was localized in the cytoplasm (Fig. 6). The ^{3}H-uridine-labeled liver explants after the

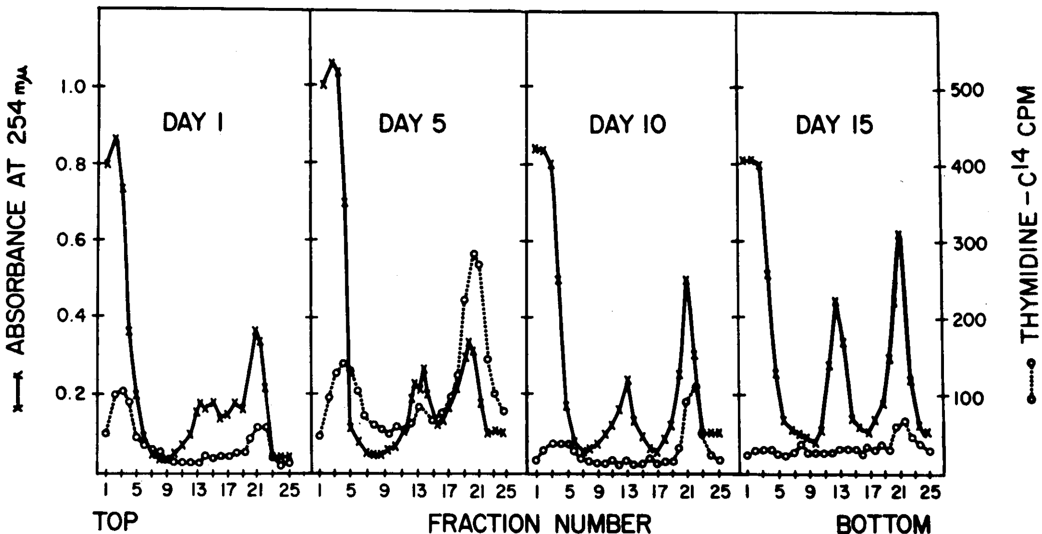
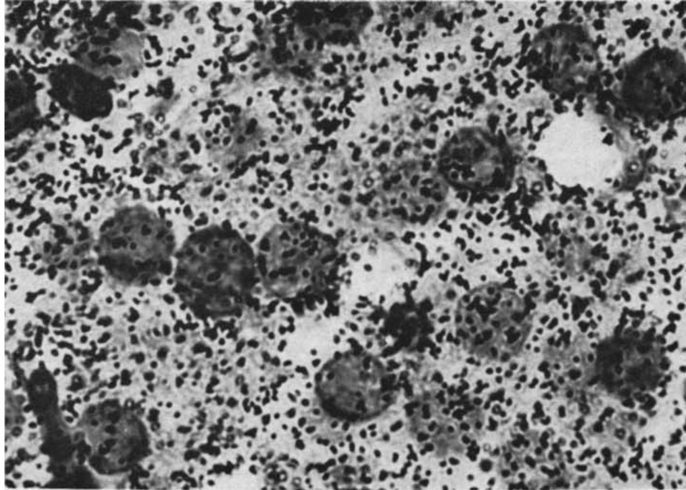
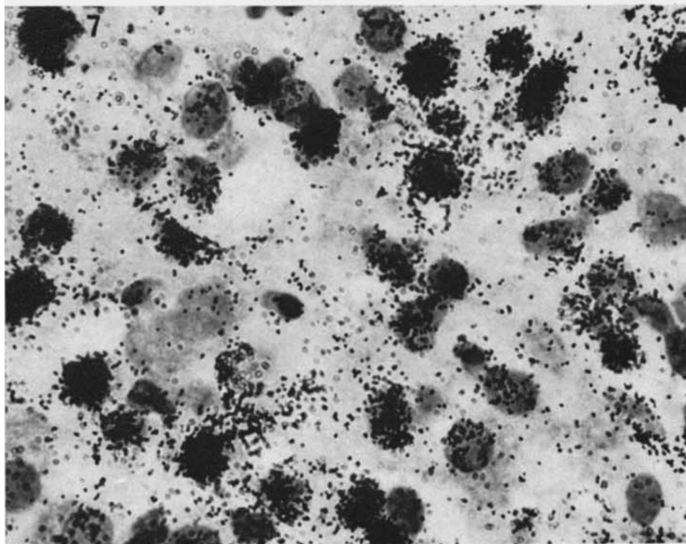


FIG. 5. Sucrose gradients of liver cell homogenates labeled with ^{14}C-thymidine (O = radioactivity; X = optical density.)

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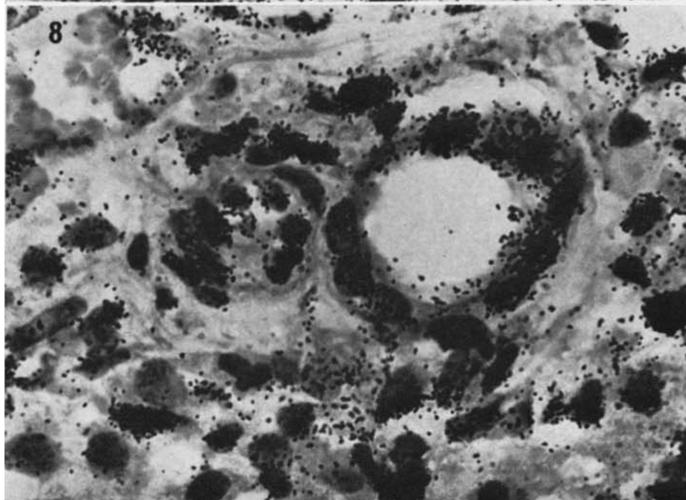
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FIG. 6. Radioautograph of a 5-day-old marmoset liver organ culture labeled for 4 hr with ^{3}H-amino acids (×1200).

FIG. 7. Radioautograph of a 5-day-old marmoset liver organ culture labeled for 4 hr with ^{3}H-uridine (×800).

FIG. 8. Radioautograph of a periportal field of a 5-day-old marmoset liver organ culture labeled for 4 hr with ^{3}H-uridine (×800).

same time period showed the greatest amount of radioactivity in the nuclei (Figs. 7 and 8). In the ^{14}C-thymidine labeled liver explants however, only a few nuclei of the hepatocytes showed incorporation of radioactivity even after labeling for 6 hr.

3. Replication of viruses. a. In marmoset liver. The liver organ cultures were infected with several DNA and RNA viruses to investigate their ability to support the growth of viruses. Each day after virus infection one culture was taken for histology and another for titration of the tissue bound virus or virus released into the culture fluids (NDV and mumps). The growth curves of the nine different virus strains used for the infection of marmoset liver organ cultures are shown in Table I. The DNA viruses (MHV and vaccinia) and RNA viruses (VSV, coxsackie type A9, NDV, Sendai, mumps, echo type 6, and poliomyelitis type 2) replicated in the liver cells to high titers and the size of the inoculum had little influence on the multiplication of MHV and VSV. Virus titers usually reached their maxima by the fifth day after infection and slowly decreased thereafter. The infected liver pieces showed characteristic cytopathic effects which progressed from the periphery towards the center of the liver explants and the virus titers increased with the progression of the cytopathic effects. MHV produced typical inclusion bodies in the nuclei of the hepatocytes (Fig. 9) followed by complete cell necrosis. Six days after infection with VSV the cytoplasm of the hepatocytes was eosinophilic, the nuclei had become pyknotic (Fig. 10) and the whole liver explant underwent necrosis during the following days.

In order to determine whether liver tissue could continue to support virus replication when infected after various lengths of time in *in vitro* culture the liver pieces were infected at 1, 5, and 10 days after explantation. On each of the 6 succeeding days following infection one culture was harvested for virus titration. The length of the cultivation period before infection did not influence the ability of the liver cells to replicate MHV and VSV (Fig. 11) and the tissue-bound virus reached similar titers after infection of either fresh liver explants or 5- and 10-day-old cultures.

TABLE I. Multiplication of DNA and RNA Viruses in Marmoset Liver Organ Cultures.

Inoculum		Virus titer at time after infection							
Virus	Titer	1 hr	days: 1	2	3	4	5	6	7
MHV	7.8[a]	4.3	4.0	6.0	7.0	7.5	7.8	7.0	6.8
MHV	3.8						7.5		
Vaccinia	6.8	4.0	4.3	4.8	6.0	6.5	7.0		6.3
VSV	4.8	2.8	3.8	6.0	6.5	6.5	7.3		6.3
VSV	2.8	<2.5			5.3		5.3		4.3
Coxsackie A9	6.8	5.0	5.8	6.8	7.5	7.8	8.3		6.5
NDV[b]	7.3	3.5		5.3			6.1		
Sendai	8.5	4.3	5.3	5.5	6.3	5.8	6.5	5.5	5.3
Mumps[b]	5.8	2.8		4.8			7.5		
ECHO 6	8.8	6.3	6.5	7.8	8.0	7.8	7.3		7.0
Polio 2	6.5		5.5	6.5			5.8	6.3	

[a] $TCID_{50}$/ml (log 10).

[b] Virus assays were performed only on culture fluids.

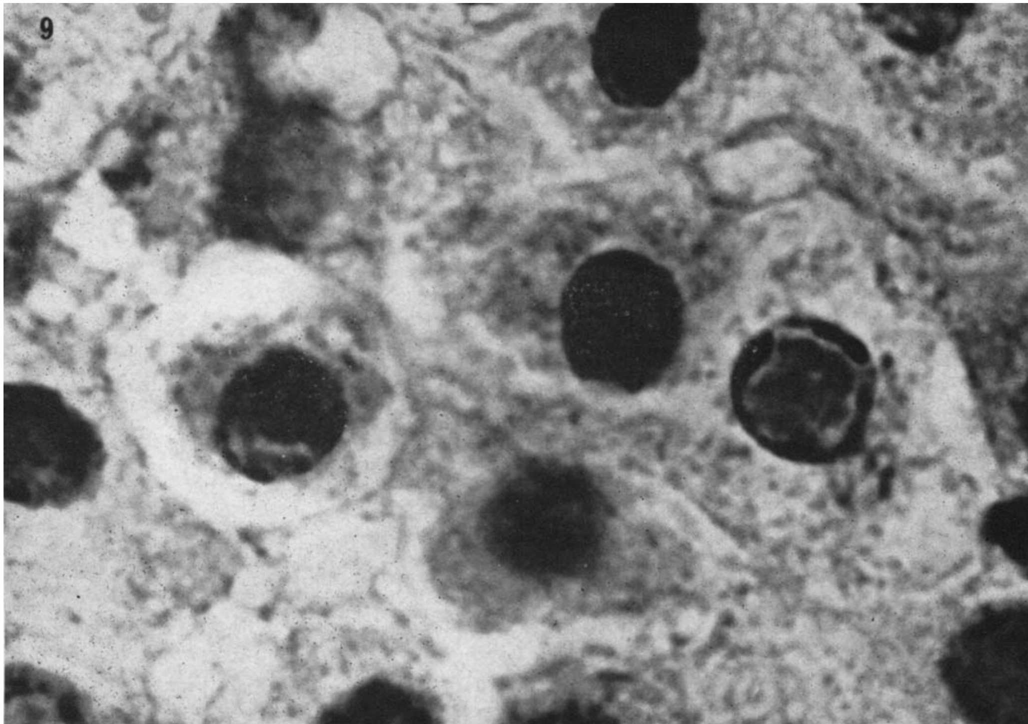

FIG. 9. Marmoset liver organ culture 2 days after infection with MHV (×2800, hematoxylin and eosin).

b. In human liver. Human liver was cultured and infected with MHV and VSV in the same way as marmoset liver. The human liver explants supported the growth of these viruses (Table II).

4. Interferon production. Three different myxoviruses were used for the induction of interferon in marmoset liver explants. One, 48, and 120 hr after infection the liver organ culture fluids were harvested for interferon assay and titration of infectivity. Table III shows that NDV, Sendai, and mumps viruses induced interferon production and replicated in the lower organ cultures. The highest interferon titer, with up to 1000 interferon units/ml, was found in individual experiments in liver organ culture fluids 5 days after infection with Sendai virus.

TABLE II. Multiplication of MHV and VSV in Human Liver Organ Cultures.

Inoculum			Virus titer at time after infection		
Virus	Titer	days:	1	3	5
MHV	6.8[a]		3.5	5.0	5.5
VSV	5.5		3.0	4.3	5.8

[a] $TCID_{50}$/ml (log 10).

The interfering activity in liver organ culture fluids from Sendai virus infected cultures was completely lost after incubation with 0.05 mg/ml of crystalline trypsin for 1 hr at 37° and heating at 80° for 1 hr. Lowering the pH to 2.2 for 24 hr or ultracentrifugation at 100,000*g* for 2 hr had no effect on the interfering activity. Cultures of mouse L cells (MCN subline) and chick embryo fibroblasts were not protected against infection with VSV by the same culture fluids.

Discussion. The maintenance of the tissue and cellular identity and, presumably, the maintenance of metabolism of the liver explants in culture were influenced by correct oxygen tension and the use of a synthetic sponge as the supporting matrix. Cells of small marmoset liver explants showed the

least histological change when maintained in an atmosphere of room air with 60% oxygen and 5% carbon dioxide. The optimal oxygen content for maintenance of human liver explants was not investigated but as the optimal concentration of oxygen for maintenance of tissue slices *in vitro* in some mammalian species has been shown to be inversely proportional to body weight (13), it is probable that results with human liver organ cultures could be further improved by lowering the oxygen content of the gas phase. Replacement of the organ culture grids usually used as support material by synthetic sponge reduced pressure necrosis, regulated the supply of medium and made the system considerably easier to handle. Some central necrosis still developed in explants although this was reduced by decreasing the size of liver explants.

Study of the protein, RNA, and DNA synthesis of the liver cells in this culture system showed that their metabolism also was maintained for about the same period of time. The ^{3}H-uridine, ^{3}H-amino acids and ^{14}C-thymidine incorporation into different cellular components was very similar at the 1st, 5th, and 10th day of culture. The synthesis

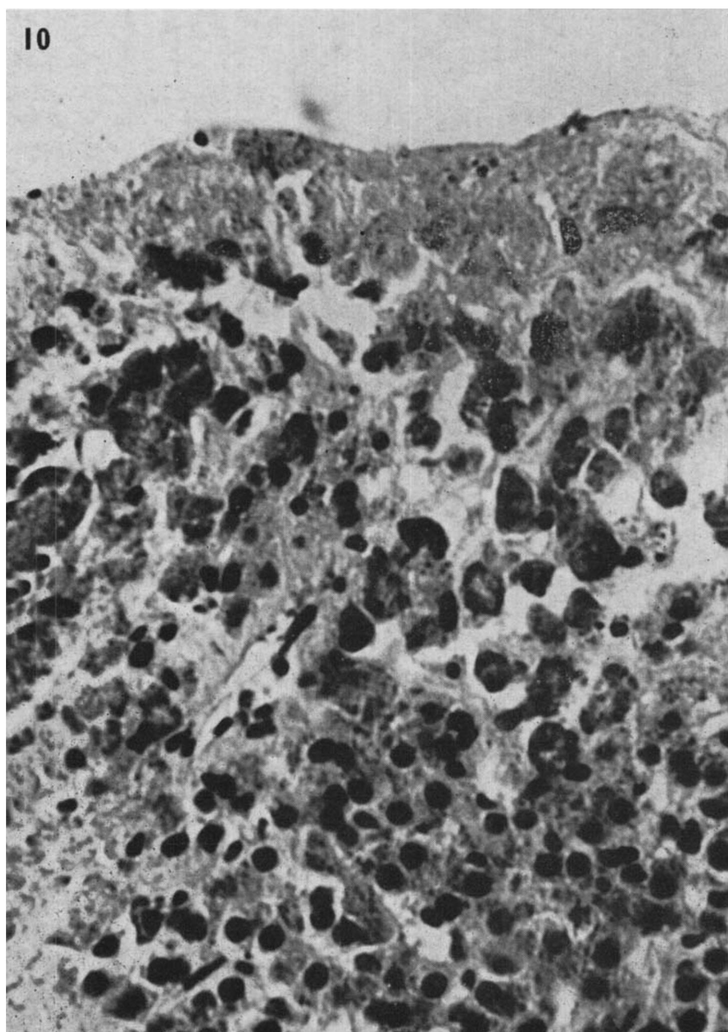

FIG. 10. Marmoset liver organ culture 6 days after infection with VSV (×620, hematoxylin and eosin).

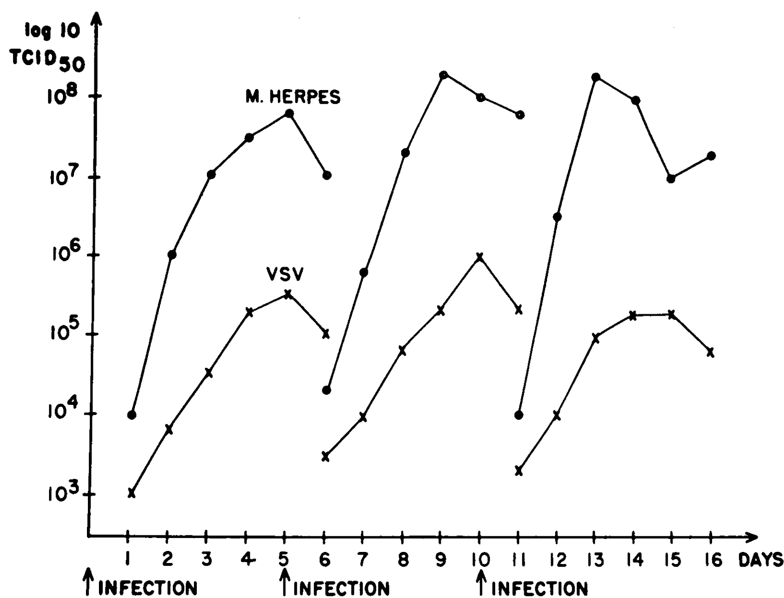

FIG. 11. Growth curves of MHV (⊙), and VSV (×), after inoculation of 1-, 5-, and 10-day-old marmoset liver organ cultures.

of RNA (which was inhibited by actinomycin D), protein, and DNA was slightly decreased at the 15th day. Incorporation of ^{14}C-thymidine over the entire period of cultivation was low, indicating a low-DNA synthesis corresponding with the histological finding of only occasional hepatocytes in mitosis. As the metabolism of whole liver explants was studied, and as these explants contain mesenchymal cells as well as hepatocytes, radioautography was used to recognize the cells actually incorporating labeled RNA, DNA, and protein precursors. More than 95% of the activity was found in the hepatocytes, indicating that the biochemical studies reflected the hepatocyte metabolism. By radioautography, it was also found that labeled compounds perfused the entire liver pieces in less than 1 hr and labeling times shorter than a 16 hr pulse could obviously be used in further, more detailed studies of liver cell metabolism itself and of the influence of viruses on liver cell metabolism.

Two DNA and 7 RNA viruses multiplied in the liver cells to high titers, and produced characteristic cytopathic effects which ultimately destroyed the whole liver explant. These results clearly demonstrated that hepatocytes are as susceptible to viral infection as other marmoset epithelial cells (14) and

TABLE III. Interferon Production in Marmoset Liver Organ Cultures.

Inoculum		Titers at hours after infection					
		2		48		120	
Virus	Titer	Virus	Interferon	Virus	Interferon	Virus	Interferon
NDV	7.3[a]	3.5	4[b]	5.3	26	NT	
Sendai	8.5	2.8	2	5.3	29	5.5	400
Mumps	5.8	2.8	4	4.8	31	7.5	54

[a] $TCID_{50}$/ml (log 10).

[b] Interferon units/ml, i.e., reciprocal of the highest dilution of the interferon preparation giving a 50% reduction of VSV plaques. All values are averages from 2 to 6 individual experiments.

that liver explants can support the growth of viruses for the same length of time that they maintain their histological structure and their metabolism. In addition, 3 myxoviruses induced interferon production in marmoset liver organ cultures.

The purpose of this study was the maintenance of mature primate liver explants in as near normal a state as possible for virological studies. This was achieved as the explanted liver pieces maintained a normal structure and there was no overgrowth by mesenchymal cells or de-differentiation of the liver cells in the peripheral portions of the organ cultures and the metabolism continued almost unchanged. As hepatocytes may be the target cells for the agents of viral hepatitis, this method of liver organ culture is likely to be useful for studies of human viral hepatitis, and especially of the hepatitis in marmosets described by this laboratory (15, 16) and this possibility is being explored.

Summary. Adult primate liver explants were maintained in culture for up to 3 weeks. The liver explants maintained their histological identity, the cellular metabolism remained active, and the explants supported the multiplication of a number of DNA and RNA viruses during the period of *in vitro* cultivation.

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