PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 149, 294–298 (1975)

Liver Regeneration: An Isolation Perfusion System Employed to Assay Hepatic ^{3}H-Thymidine Incorporation[1] (38792)

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(Introduced by G. D. Hammond)

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Levy and Zeppa (1, 2) have reported a six fold increase in ^{3}H-thymidine incorporation in whole isolated normal rat livers perfused with a blood-like suspension, which had been previously circulated through isolated regenerating rat livers. It is their hypothesis that factor(s) incorporated into the perfusate during circulation through a regenerating liver initiate DNA synthesis in the cells of the normal isolated liver during the period of the second perfusion. Further that these factor(s) may include those which initiate regeneration following liver resection. In this laboratory we have been unable to increase ^{3}H-thymidine uptake in the hepatic cells of whole isolated normal livers by similar perfusions. The method of assessing the ability of hepatic cells to incorporate a labeled DNA precursor employing an isolation-perfusion apparatus has been evaluated.

Materials and Methods. (*a*) *Formation of the perfusate.* Rats (250 g male Sprague–Dawley) were subjected to (a) a 68% hepatic resection or (b) "sham" hepatic mobilization, employing barbiturate anesthesia. (All procedures performed between 9 and 11 AM) At intervals of 24, 26, 27, or 28 hr following the initial procedure the animals were again anesthetized, and the abdominal cavity opened. The livers (post hepatectomy, or "sham"-hepatectomy) were irrigated with cold Lactate Ringers solution through the portal vein *in situ* and then removed and placed in a moist, temperature-controlled chamber for perfusion. The portal vein was canulated for inflow of the perfusate while the outflow from the hepatic veins was collected in a chamber beneath the liver for recycling.

The perfusate employed (Levy and Zeppa, 1, 2) consisted of a Krebs–Ringer bicarbonate solution with the addition of glucose to 180 mg/100 ml, bovine serum albumin Fraction V, 4 g/100 ml, and canine red blood cells to a hematocrit of 20%. The pH of this suspension was 7.4 and the osmolality 303–306 mOsm/kg.

(*b*) *The perfusion system.* The perfusate passed through a "bubble" type oxygenator with a gas mixture of 96% O_2 plus 4% CO_2 propelled by a rotary type sigmamotor pump at a flow rate of 7 ml/min. A millipore filter (pore size 14 mu, dia. 47 mm) was employed. PO_2 electrode monitors were placed in the flow lines entering and leaving the isolated liver. A pH electrode monitor was placed in the in-flow system. The entire apparatus was maintained in a closed incubator at a temperature of 37° ± 1°. Oxygen tension in the hepatic inflow line was maintained between 150–130 mmHg and in the hepatic outflow line was consistently under 50 mmHg. The pH was maintained at 7.4 with bicarbonate solution. The choledochus was canulated and bile output maintained in all preparations. After 60 min of perfusion, the perfusates were collected and assayed for the stimulatory factor.

(*c*) *Assay of the perfusates.* Isolated whole normal, i.e., not regenerating, rat livers (250 g male Sprague–Dawley) were perfused with the two types of suspension: (a) perfusate that had passed through a regenerating liver and (b) perfusate that had passed through a "sham" operated liver, employing the same apparatus and technique described above. In addition, as controls, regenerating livers, (24 hr post 68% hepatec-

[1] This investigation was supported by Public Health Service Research Grant No. AM 08879 from the National Cancer Institute.

tomy) and "sham" operated livers were perfused with fresh perfusate.

In all experiments, after the initial 10 minutes of perfusion, 125 μCi of thymidine-me-^{3}H (26.6 Ci/mmole) was introduced into the perfusate and perfusion continued for 60 min. Seventy minutes after the beginning of perfusion, the livers were removed from the system and flushed with cold Lactate Ringers solution. Small pieces of each liver were fixed for autoradiographic studies. DNA was extracted from the remaining liver by a modification of Marmur's technique (3). Liver tissue was weighed, minced, and homogenized in 5 vol of 0.15 *M* NaCl, 0.1 *M* EDTA, pH 8.0. Sodium dodecyl sulfate was added to give 4% and the homogenates were heated to 60° for 15 min, cooled and NaCl added to give 1.0 *M*.

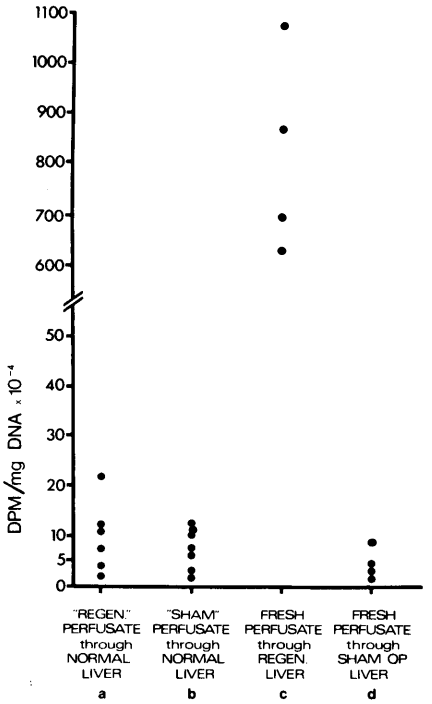

FIG. 1. Analysis of specific activity of DNA from four types of isolated livers: (a) normal liver perfused with perfusate previously circulated through regenerating livers, (b) normal livers perfused with perfusate previously circulated through "sham" hepatectomy livers, (c) regenerating (24 hr post-hepatectomy) livers perfused with fresh, i.e., not previously circulated, perfusate and (d) "sham" hepatectomy livers (24 hr post "sham" procedure) perfused with fresh perfusate. Methods (see text).

TABLE I[a]

SPECIFIC ACTIVITY (DPM × 10^{-4}/MG DNA) IN TISSUE FROM 18 NORMAL, ISOLATED RAT LIVERS PERFUSED WITH THE TWO DIFFERENT PERFUSATES PLUS ^{3}H-THYMIDINE

Pair No.	Nine livers (a) perfused with a perfusate previously circulated through a regenerating liver.	Nine livers (b) perfused witha perfusate previously circulated through a liver post "sham" procedure.
1	2.0	2.3
2	1.2	1.7
3	3.9	12.8
4	11.4	5.8
5	7.0	6.1
6	21.7	9.6
7	3.3	3.0
8	1.5	0.6
9	1.4	2.3

[a] ^{3}H-Thymidine incorporation in isolated normal livers perfused with a suspension previously circulated through (a) an isolated regenerating liver or (b) an isolated "sham" procedure liver. In pairs No. 1–6 inclusive, both partial hepatectomy and a "sham" procedure were performed 24 hr prior to the assay perfusion. In pair No. 7, this interval was 26 hr; in pair No. 8, 27 hr; and in pair No. 9, 28 hr. All of these results are very low when compared with the uptake in liver tissue known to be regenerating (650 to 1085 dpm × 10^4 dpm/mg DNA). See Fig. 1.

The solution was extracted with an equal volume of chloroform:isoamyl alcohol (24:1), and DNA precipitated from the aqueous layer with one volume of ethanol. The precipitate was redissolved in $\frac{1}{10}$ SSC (SSC = NaCl 0.15 *M*, NaCitrate 0.015 *M*) containing 2 m*M* EDTA and extracted twice with chloroform:isoamyl alcohol as above. DNA was precipitated from the aqueous layer with 2 vol of ethanol, washed successively in 70% and 100% ethanol, and finally dissolved in a small volume of $\frac{1}{10}$ SSC, 2 m*M* EDTA, at a concentration of approximately 300–400 μg/ml. Aliquots of the DNA solution were assayed for DNA content (Burton's Method) (4) and TCA-precipitable radioactivity and specific activities calculated.

Results. The normal whole isolated livers perfused with either (a) perfusate previously circulated through "sham" operated livers or (b) perfusate previously circulated

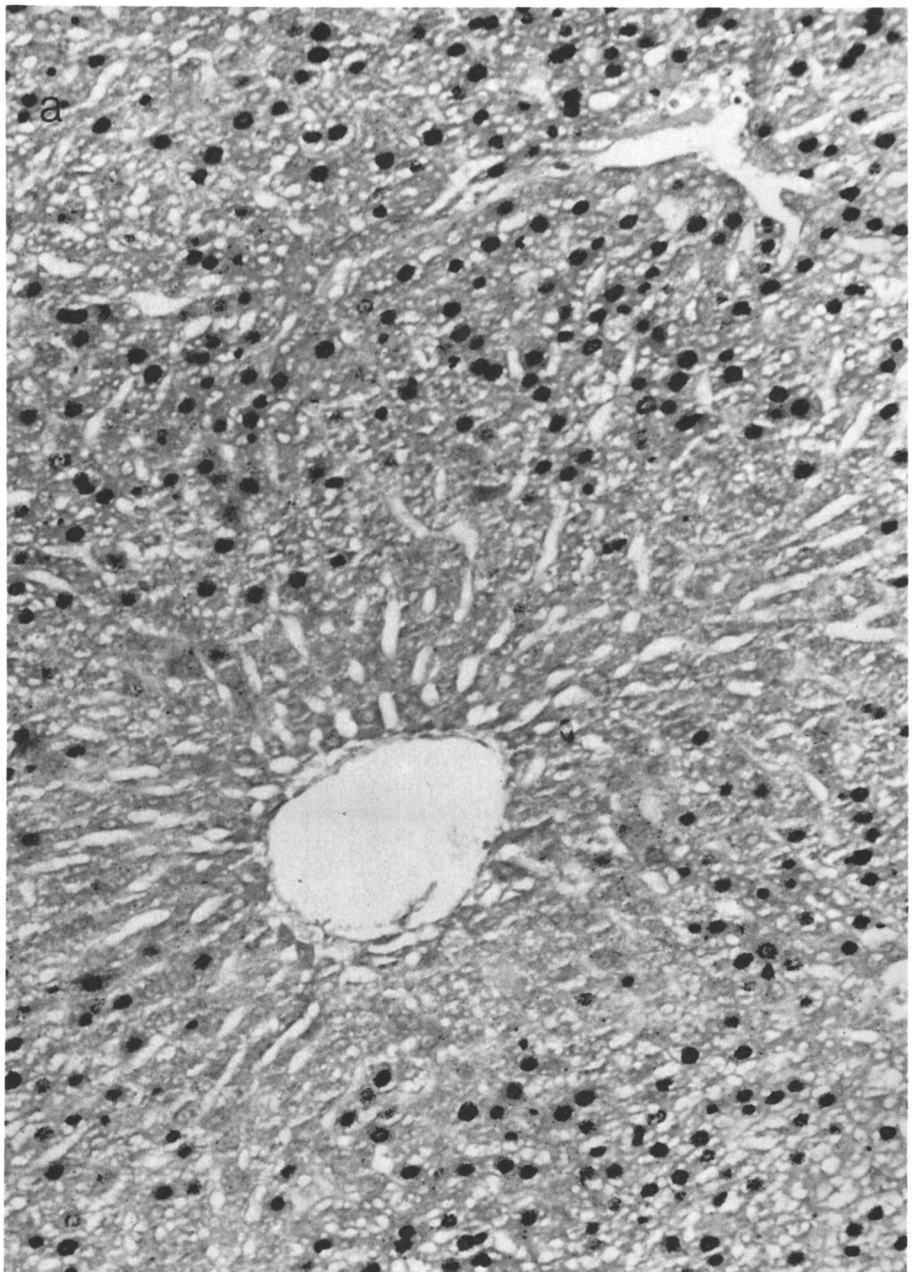

FIG. 2a.

FIG. 2. (× 185). Autoradiographic demonstration of ^{3}H-thymidine uptake in isolated regenerating (a) and normal (b) liver tissue. Twenty-four hours following a 68% hepatic resection, liver (a) was perfused with a blood-simulating solution containing 125 μCi of thymidine-me-^{3}H for 60 min. Liver (b) was from an animal in which a "sham" hepatectomy had been performed 24 hrs. previously, followed by perfusion with the same label in the same solution for 60 minutes. Following removal segments of both livers were fixed with formalin, paraffin embedded, sectioned with a microtome and mounted on glass slides. These were then dipped in photographic emulsion (Kodak Nuclear Tract Emulsion, NTB^{2}), and stored in darkness at 5° for 24 days before development.

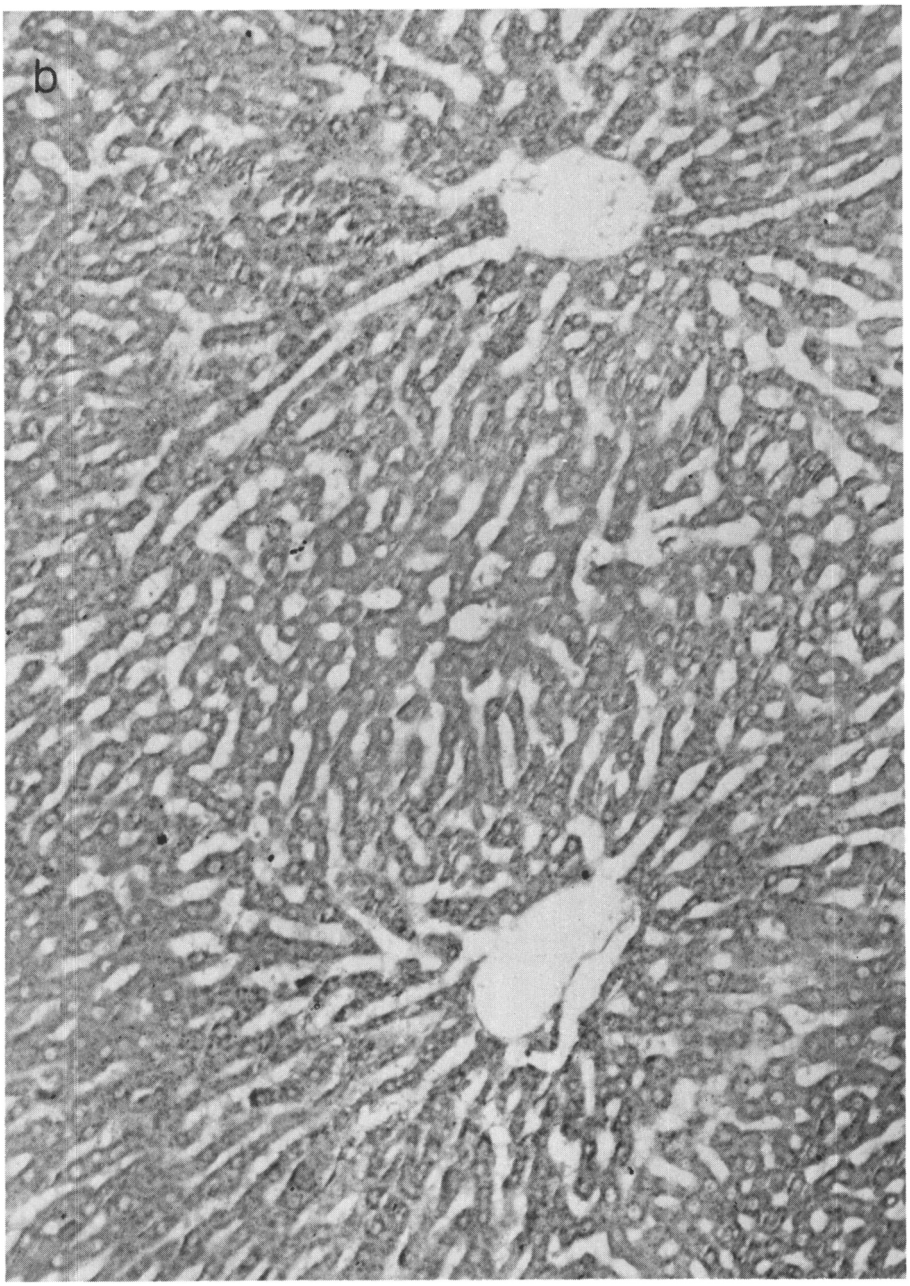

FIG. 2b.

through regenerating livers, had relatively low and similar uptakes of ^{3}H-thymidine (Fig. 1a and b and Table I). Radioautographic studies revealed little uptake in liver cell nuclei, with labeling of only a few cells situated adjacent to inflow vessels. This result was not significantly influenced by whether the regenerating livers employed in the first perfusion were 24, 26, 27, or 28 hr post hepatectomy (Table I).

When regenerating livers were perfused with fresh (not previously circulated) perfusate, the resulting ^{3}H-thymidine uptake in the regenerating livers (four animals) was

over 6 × 10^6 DPM/mg DNA, i.e., >100 times that found in similarly perfused "sham" operated livers (Fig. 1c and d). Labeling of the nuclei in the radioautographs of regenerating livers perfused with fresh perfusate was impressive and widespread throughout the liver segments, excluding only areas immediately adjacent to the central vein (Fig. 2a and b).

Discussion. No evidence of a factor which can rapidly increase the ^{3}H-thymidine uptake in a perfused normal liver was detected in these studies. The hepatic perfusion system described is capable of delivering a large amount of ^{3}H-thymidine to an isolated liver, permitting significant incorporation of label and a high tissue specific activity, as demonstrated by the studies which employed regenerating livers for assay of the perfusate. Thus, this represents an alternative method of demonstrating the capacity for active DNA synthesis in liver cells, employing perfusion as a substitute for intravenous or intraperitoneal injection of labeled compounds in intact animals. Differences between the technique employed in these studies and those of Levy and Zeppa were as follows: (1) The livers were not left in the animal carcass during the perfusion. The *in situ* perfusion technique (1, 2), resulted in a highly variable amount of perfusate loss and was replaced by the one described. The initial volume of the perfusate could then be reduced because it was not being lost into the carcass. If this factor explains the differences in the results obtained in these two reports, it suggests that a stimulatory factor may be extracted by the perfusate from the carcass rather than from the liver. (2) Perfusates in this laboratory were assayed in the fresh state, whereas those employed by Levy and Zeppa were frozen and thawed. (3) Rats weighing approximately 250 g were employed rather than 160–200 g animals, to facilitate the perfusion connections. Small increments in weight make slight differences in the magnitude of the hepatic regenerative response, but not in the range of those found in these studies. (4) There were methodological differences in the extraction and assay of DNA.

Campagno and Grisham (5) have also perfused isolated normal (rat) livers with a perfusate previously circulated through 70% hepatectomized or "sham" hepatectomized isolated livers without demonstrating a difference in ^{3}H-thymidine uptake. These investigators also employed isolated 12–18 hr. regenerating remnants as the assay livers without demonstrating a stimulatory effect from these perfusates.

A theoretical argument against acceptance of the hypothesis advanced by Levy and Zeppa is based on the observation that in all other forms of experimental stimulation of DNA synthesis in liver cells (including cross-circulation studies) a minimum of 6–8 hr is required from the time of the stimulus to the beginning of significant amounts of DNA precursor incorporation (6). In accepting the hypothesis of Levy and Zeppa, one would have to assume that the sequence of events prior to the "S" phase of the cell cycle can be compressed into a single hour.

Summary. Isolated normal livers perfused with ^{3}H-thymidine containing suspensions which had been previously circulated through isolated livers either (a) regenerating or (b) "sham" operated, showed equal and relatively low levels of both tissue specific activity and nuclear labeling by autoradiography.

When such blood-simulating perfusates, containing ^{3}H-thymidine, are circulated through whole isolated regenerating livers, nuclear uptake is apparent and specific activity is increased >100 × over levels obtained when the same perfusate is circulated through nonregenerating livers.

The able technical assistance of Mrs. Marie Krempin is acknowledged.

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Received October 7, 1974. P.S.E.B.M. 1975, Vol. 149.