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Hormonal stimulation of DNA synthesis in primary cultures of adult rat hepatocytes

(epidermal growth factor/insulin/glucagon/hepatocyte cultures)

ROBERT A. RICHMAN*, THOMAS H. CLAUS*, SIMON J. PILKIS*, AND DANIEL L. FRIEDMAN†‡

* Department of Physiology and † Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37232

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ABSTRACT **Adult rat hepatocytes have been previously isolated and maintained in monolayer culture, but attempts to stimulate DNA synthesis have been unsuccessful. Hormonal conditions are now described which induce DNA synthesis in cultured hepatocytes from partially hepatectomized rats. DNA synthesis was determined autoradiographically by the incorporation of [^{3}H]thymidine into nuclei of morphologically distinct hepatocytes. Insulin (4–4000 nM) or epidermal growth factor (10 ng/ml) alone caused significant increases in the labeling index. The two hormones together acted synergistically to produce labeling indices of 35–50% on the third day of culture, compared with 2–7% in control cultures. The addition of glucagon (400 nM) further increased the labeling index. Dexamethasone (80 ng/ml) inhibited DNA synthesis but, under certain conditions, enhanced cell attachment. Growth hormone and triiodothyronine had no significant effect on DNA synthesis. The mixture of epidermal growth factor, insulin, and glucagon also stimulated incorporation of [^{3}H]thymidine into phenol-extracted DNA. Although DNA synthesis was stimulated, cell division occurred infrequently. These data suggest a prominent role for epidermal growth factor in promoting hepatic DNA synthesis by acting in concert with insulin and glucagon.**

Liver regeneration after partial hepatectomy has been employed widely as an experimental model of mammalian cell division (1, 2). However, the regulatory mechanisms that stimulate quiescent hepatocytes to proliferate are poorly understood. Cross circulation studies between partially hepatectomized rats and intact animals suggest the involvement of humoral stimuli (3). Although several hormones have been implicated (4–9), definitive studies have been difficult to carry out because of complex hormonal interactions *in vivo*. The development of cell cultures of normal adult hepatocytes would provide a system to characterize those factors necessary for DNA synthesis and cell division. However, while fetal liver (10) and hepatoma cells (11) can be grown in culture, there have been, as yet, no successful attempts to induce DNA synthesis or cell division in adult liver cells in culture. Studies with hepatocytes from partially hepatectomized rats have shown that DNA synthesis declines progressively with the time in culture (12), and that only 50–60% of the isolated hepatocytes adhere to the substratum.

We report conditions that increase to 90% the number of hepatocytes that adhere to flasks as well as conditions which stimulate DNA synthesis. Epidermal growth factor (EGF), in combination with insulin and glucagon, has been found to greatly enhance DNA synthesis.

MATERIALS AND METHODS

Isolation and Plating of Liver Cells. Male rats (150–300 g, Sprague–Dawley, Madison, Wisc.) were subjected to partial hepatectomy (13) and then starved for 18–24 hr. The remaining liver fragments were perfused with collagenase and hyaluronidase as previously described (14), except that after catheterization of the portal and superior vena cava veins the fragments were excised from the abdomen and placed on a stainless steel mesh. This procedure helped to ensure sterility. Isolated hepatocytes were washed twice with minimal essential medium (MEM) and suspended in MEM supplemented with amino acids, 5 mM sodium lactate, 0.5 mM sodium pyruvate, 10% calf serum, and 80 ng/ml of dexamethasone. The amino acid supplement consisted of a single strength mixture of basal Eagle's medium amino acids, 0.5 mM glycine, 1 mM alanine, and 0.5 mM serine. Preparations contained more than 95% hepatocytes (14).

Aliquots (2.5 ml) of the cell suspension (0.8×10^6 cells per ml) were added to falcon flasks (25 cm^2 surface area) with the appropriate hormone additions. The flasks were gassed briefly with 95% air–5% CO_2 and incubated at 37°. The medium was changed daily.

Determination of Cell Attachment. The cells that did not adhere to the flask were obtained by combining the culture medium that covered the monolayer and a subsequent 2.5 ml rinse with warm (37°) 0.9% NaCl. After the saline rinse, the monolayer was incubated at 37° for 10 min in 5 ml of an isotonic mixture (0.14 M NaCl, 5.4 mM KCl, 6.9 mM $NaHCO_3$, and 5.6 mM glucose) containing 0.6 mM EDTA and 10% calf serum. The presence of serum eliminated cell lysis. Cells were detached by vigorous shaking and counted with a Coulter Counter.

[^{3}H]Thymidine Incorporation into DNA. Tritiated thymidine ([^{3}H]dT; 0.5 μCi/μmol, 8 μCi per flask) was added to the monolayer cultures. After 24 hr, the flasks were rinsed with 5 ml of cold isotonic NaCl and incubated with 7% trichloroacetic acid for 10 min at 4°. After two rinses with 7% trichloroacetic acid, the flasks were incubated with 70%, and then with 95% ethanol, each for 10 min at 4°. After drying overnight, flasks were cut into 2.5 × 6 cm pieces, dipped into photographic emulsion (15), and exposed for 4 days prior to development. At least 400 hepatocytes with normal morphology were counted for each sample. Rounded cells (Fig. 1), epitheloid cells (Fig. 2B), and fibroblasts were excluded. The labeling index is defined as the percentage of morphologically distinct hepatocytes with grains over their nuclei. All labeled nuclei had at least 20 grains, and most had more than 100 grains (Fig. 2A), whereas the background was less than 3 grains.

[^{3}H]dT incorporation into DNA was estimated by the phenol extraction method of Morley and Kingdon (16). Prior to extraction, cells were removed with EDTA solutions as described above.

Materials. Pure EGF from mouse was generously supplied by Stanley Cohen. Insulin, triiodothyronine, and dexametha-

Abbreviations: EGF, epidermal growth factor; MEM, minimal essential media.

‡ To whom requests for reprints should be sent.

Table 1. Factors influencing cell attachment

	Cells in culture			
	Intact liver		Partial hepatectomy	
Cell wash	− Serum	+ Serum	− Serum	+ Serum
Buffer	12	39	34	85
Buffer + Dex	8	36	40	94
Medium	12	21	47	86
Medium + Dex	18	56	78	94

Cells were prepared as described in *Materials and Methods* from intact liver or from a liver remnant 24 hr after partial hepatectomy. Cells from each liver were divided into four groups. Each group was washed with one of the solutions as shown. The medium was complete with 10% serum. The buffer was Krebs-Henseleit at pH 7.4 containing 1% bovine serum albumin. Cells from each wash condition were subdivided. Half of the cells were cultured in medium and half in medium + 10% calf serum. Cells washed with dexamethasone (Dex, 80 ng/ml) were also cultured in its presence. Cell attachment was determined after 24 hr in culture as described in *Materials and Methods*. The results are the mean from triplicate flasks. Values are percent of cell attachment.

sone were from Sigma Chemical Co. Glucagon was a gift of Eli Lilly Co. [^{3}H]dT was from ICN Pharmaceuticals, Inc.

RESULTS

Factors Influencing Cell Attachment. To find conditions that would increase the number of isolated hepatocytes that adhere to the flask, we investigated the effect of several washing procedures and plating conditions with liver cells from both intact and partially hepatectomized rats (Table 1). The results indicate that several factors contribute to cell adherence, the two most important being partial hepatectomy and the presence of serum in the culture medium. These factors each gave 2- to 5-fold greater attachment than with most other conditions examined. Dexamethasone also improved attachment in all cases except with cells from intact liver washed with buffer. Washing cells in medium rather than buffer may also have had a small favorable effect. Under optimal conditions, over 90% of the cells from partially hepatectomized rats adhered to the flask, compared with 56% from cells from intact liver.

Whereas partial hepatectomy and serum had the greatest influence on cell attachment, the addition of several hormones increased the attachment of hepatocytes from intact liver (Table 2). When cultured in serum, the combination of EGF, insulin, and dexamethasone almost doubled the percentage of attached cells, whereas the three hormones alone had only small effects. When the cells were cultured without serum, the hormone combination was ineffective.

Effect of Hormones on DNA Synthesis. During the first 24 hr of culture, the hepatocytes obtained from partially hepatectomized rats begin to flatten out and migrate together to form clusters or islands (Fig. 1) which appear morphologically similar to hepatocytes *in situ*. The same process occurs with cells from intact liver at a slower rate. The addition of hormones (insulin, EGF, and glucagon) accelerates the flattening process and the cells almost completely cover the flask surface within 3 days. In the first few days of culture, there are still many adherent cells that have not flattened and appear rounded and refractile. In cultures from partially hepatectomized rats, the number of these cells is as high as 50% after the first day and decreases to less than 10% by the third day in culture. Also observed in cultures after the second day are clusters of apparently rapidly dividing cells of either epitheloid or fibroblastic morphology. These cells are found in spaces between the islands of liver cells. Autoradiography revealed that a high proportion of these cells are labeled (Fig. 2B). Both these cells and the rounded cells were disregarded in all of the autoradiographic studies to be reported.

Table 2. Effect of hormones on cell attachment

Hormone addition	− Serum	+ Serum
None	12	36
Insulin (40 nM)		39
Dex (80 ng/ml)		45
EGF (10 ng/ml)		52
EGF + insulin		47
EGF + insulin + Dex	16	64

Cells were prepared from an intact liver and washed in buffer as in Table 1. Half of the cells were cultured in MEM and the other half in MEM + 10% calf serum. Hormones were added as shown. Cell attachment was determined after 24 hr in culture as described in *Materials and Methods*. The results are the means from triplicate flasks. Values are percent of cell attachment.

To probe the possibility that hormones might stimulate DNA

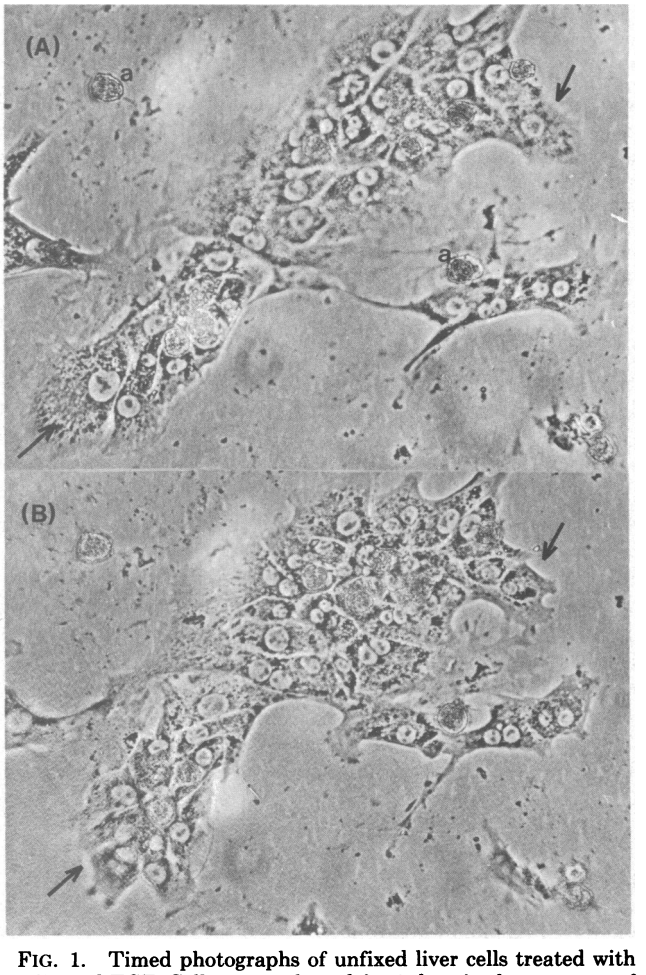

FIG. 1. Timed photographs of unfixed liver cells treated with insulin and EGF. Cells were cultured for 3 days in the presence of insulin (40 nM) and EGF (10 ng/ml). Flasks were kept at 37° while cells were photographed at 100× under an inverted microscope. The two panels represent identical fields except that (B) was photographed 4 hr after (A). The arrows indicate cells that apparently divided during this 4-hr period. It may also be noted that many cells have two nuclei as is the case with adult liver cells. Also denoted (a) are "rounded" cells.

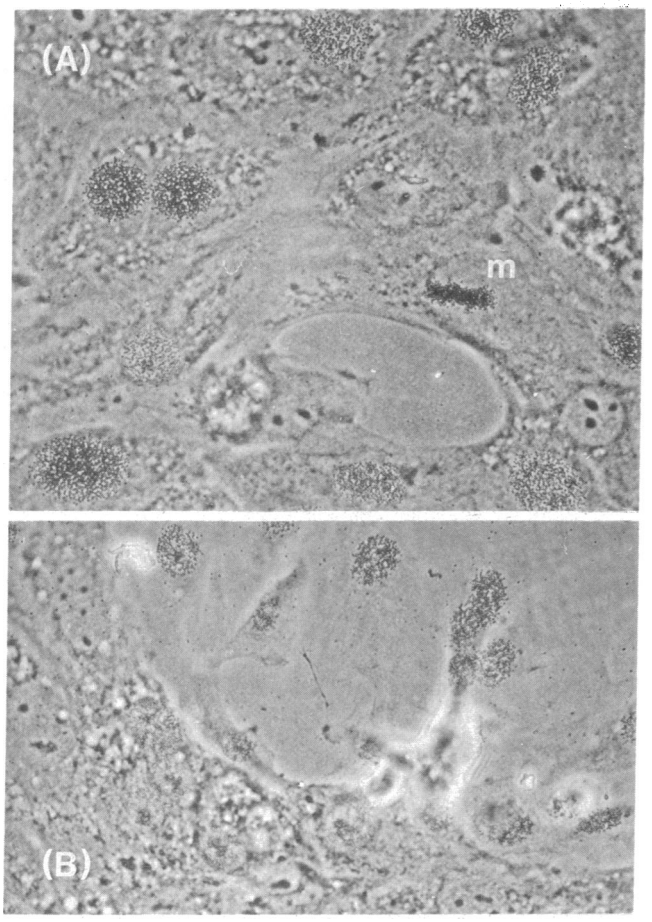

FIG. 2. Autoradiograms of cultured cells from partial hepatectomized rats. Cells were cultured with insulin, EGF, and glucagon and labeled as in Table 3. Cells were fixed and prepared for autoradiography as described in *Materials and Methods*. In (A) labeled and unlabeled liver cell nuclei may be observed. Cell boundaries are unclear due to fixation. A labeled metaphase cell is observed (m). In (B) the upper right-hand area is clear (unoccupied by liver colonies) except for the presence of labeled cells with clear cytoplasm. These cell types were not included in labeling index determinations. They are easily distinguished from liver cells, which have granular cytoplasm and which migrate together to form colonies of tightly packed cells.

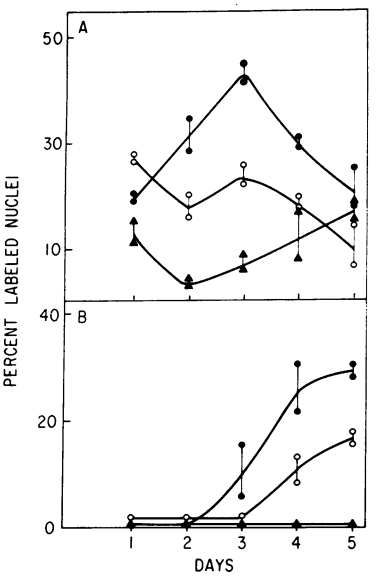

FIG. 3. Effect of hormone mixtures on the [^{3}H]thymidine labeling index. Hepatocytes were prepared from a liver remnant 22 hr after partial hepatectomy (A) and from an intact liver (B). Cells cultured with insulin (400 nM), EGF (10 ng/ml), human growth hormone (1 μg/ml) and dexamethasone (80 ng/ml), —○—; cells cultured with the same mixture but dexamethasone was removed after the first 24 hr, —●—; cells cultured with triiodothyronine (44 ng/ml), glucagon (400 nM), and heparin (44 milliunits/ml), —▲—. Media with hormones were replaced daily. Each day [^{3}H]dT was added to two flasks from each group. After 24 hr the cells were harvested and the percentage of labeled nuclei was determined as described in *Materials and Methods*. Each data point represents the results from one flask.

synthesis, we tested the effect of several combinations of hormones on the [^{3}H]dT labeling index of morphologically distinct hepatocytes from partially hepatectomized as well as intact rats (Fig. 3). With hepatocytes from partially hepatectomized rats (Fig. 3A), 19–27% of the cells were labeled during the first day in culture when 40 μM insulin, 10 ng/ml EGF, 1 μg/ml of human growth hormone, and 10 μg/ml of dexamethasone were present. If, after 24 hr, the dexamethasone was removed, the labeling index increased further. With these conditions, at the peak of DNA synthesis on the third day, over 40% of the cells were labeled. The labeling index then declined over the next 2 days. In contrast to the enhancement of DNA synthesis by this hormone mixture, the addition of glucagon (40 nM), triiodothyronine (44 ng/ml), and heparin (44 milliunits/ml), a combination similar to that previously employed to stimulate DNA synthesis *in vivo* (5), resulted in a lower labeling index on day one than with the other hormone combinations. The labeling index fell on the second day to 3% but then gradually rose over the next 3 days to reach values of about 18%.

The same hormone mixtures were tested on hepatocytes from intact rats (Fig. 3B). Only about 1% of the cells were labeled on the first day and the addition of glucagon, triiodothyronine, and heparin did not significantly increase the labeling index over the course of the experiment. Addition of the other hormone mixture resulted in the appearance of increasing numbers of labeled cells from days 3–5. The continual presence of dexamethasone appeared to delay the onset of DNA synthesis for 1 day.

To determine which hormones were responsible for the increase in labeling index, we studied the individual hormones alone and in various combinations (Table 3). Hepatocytes from partially hepatectomized rats were cultured for 3 days with hormones, and the labeling index was determined after incubation of cells with [^{3}H]dT from the second to the third day. When added alone, only EGF, insulin, and possibly glucagon, stimulated DNA synthesis. The effect of insulin plus glucagon was slightly greater than their individual effects. However, the combination of either glucagon or insulin, but particularly insulin, with EGF gave dramatic synergistic effects. With the combination of all three hormones, as many as 70% of the cells were labeled. Growth hormone and triiodothyronine were without effect either by themselves or in combination with insulin and EGF.

Because EGF and insulin were the most effective stimuli of DNA synthesis, the effect of various combinations of the two agents were investigated (Fig. 4). In the presence of either EGF (10 ng/ml) alone or insulin (40–4000 nM) alone the labeling index increased from a control value of 2% to about 12%. The combination of 0.1 or 1 ng/ml of EGF with 4–4000 nM insulin was no better than insulin by itself. However, the addition of 10 ng/ml of EGF produced synergistic effects at all insulin concentrations. EGF at 100 ng/ml was not any more effective than was the 10 ng/ml dose. The optimal insulin concentration

Table 3. Effect of various hormones on [^{3}H]thymidine labeling index in hepatocytes from partially hepatectomized rats

Exp. no.	Additions	% Labeled nuclei
1	None	2.8
	Ins (400 nM)	4.7
	EGF (10 ng/ml)	14.7
	Human GH (1 μg/ml)	3.2
	T_3 (44 ng/ml)	1.5
	Dex (80 ng/ml)	0.8
	EGF + Ins	37.5
	EGF + Ins + GH	32.4
	EGF + Ins + GH + T_3	25.6
	EGF + Ins + GH + T_3 + Dex	24.8
2	Bovine GH (4 μg/ml)	1.2
	Glucagon (400 nM)	4.5
	Ins (40 nM)	11.4
	EGF (10 ng/ml)	12.5
	EGF + Ins	52.5
	EGF + Glucagon	25.4
	Ins + Glucagon	18.2
	EGF + Ins + GH	49.2
	EGF + Ins + Glucagon	70.9

The results of two separate experiments are shown. Culture conditions were as described in Fig. 3. Labeling index was performed on days 2–3 as described in Fig. 4. Ins, insulin; GH, growth hormone; T_3, triiodothyronine; Dex, dexamethasone.

in the presence of EGF was about 40 nM, and observable effects were seen with concentrations as low as 0.4 nM.

The effect of the combination of EGF, insulin, and glucagon on both the labeling index and on incorporation of [^{3}H]dT into DNA isolated by the phenol extraction method of Morley and Kingdon (16) were compared. In the absence of hormones, about 8000 cpm per flask were incorporated. The presence of EGF (10 ng/ml), insulin (40 nM), and glucagon (0.4 μM) increased the incorporation to about 50,000 cpm. These results paralleled the changes seen in the labeling index, which increased from 7% in controls to 47% in the presence of the hormone mixture.

Cell Numbers. Although DNA synthesis was stimulated by combinations of insulin, EGF, and glucagon, an increase in the cell number could not be demonstrated with the Coulter Counter. With the hormone mixture, the cell number remained relatively constant over the 3-day culture period, while in the absence of hormones, the cell number declined about 20%. While there was no net cell number increase, repeated photographs of specific microscopic fields at different time intervals demonstrated that on the third day a small fraction of cells did divide in the presence of EGF and insulin (Fig. 1A and B). Furthermore, labeled mitotic figures (Fig. 2A) comprising 1–2% of the labeled cells were observed on the autoradiograms.

DISCUSSION

Autoradiography was used in these studies to estimate DNA synthesis. This method tends to minimize effects of hormones that are related to precursor uptake or to changes in precursor pools and, further, cells other than morphologically distinct hepatocytes can be disregarded. Though we have shown a rough correlation between labeling index and thymidine incorporation into isolated DNA, autoradiography is the method of choice in cultures that may contain different cell types with different rates of DNA synthesis.

Previous studies with primary adult hepatocytes have shown

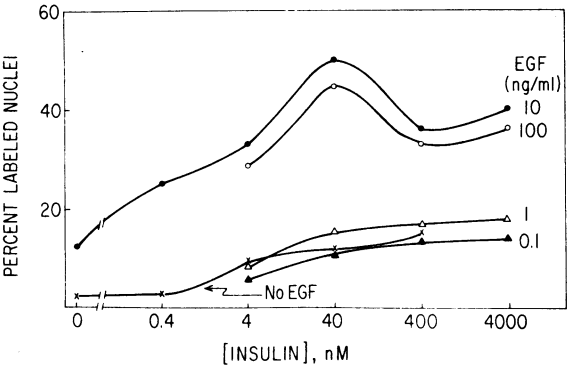

FIG. 4. Effect of various concentrations of EGF and insulin on [^{3}H]thymidine labeling index in cells from partially hepatectomized rats. Culture conditions were as described in Fig. 3. EGF and insulin were added in the concentrations shown. [^{3}H]dT was added to flasks on the second day in culture and the cells were harvested for autoradiography 24 hr later. The data points are the averages of duplicate determinations.

that cells maintain morphology and function in monolayers (17–19). However, attempts to stimulate DNA synthesis and division have been unsuccessful (20). When cells were prepared from liver remnants 12–24 hr after partial hepatectomy, apparently only cells in S-phase at the time of isolation continued to synthesize DNA and, by 48 hr in culture, the labeling index declined to low levels (12, 20). The present studies define hormonal conditions which result in continued DNA synthesis. The fact that in cultures from partially hepatectomized rats the labeling index increased 2-fold by the third day (Fig. 3A) suggests that many cells have been stimulated to initiate S-phase. However, this result is inconclusive because of the presence of numerous rounded cells (Fig. 1A), which cannot be included in the autoradiographic analysis because of poor visualization of grains. The rounded cells may be hepatocytes that have not yet flattened since their number diminishes to low levels by the third day in culture. Clearly, if the rounded cells have a different labeling index than the flattened cells this might affect the temporal pattern of the labeling index. Perhaps better evidence for initiation was obtained with cells prepared from intact rats in which the hormone mixture containing insulin and EGF stimulated the labeling index from nearly zero after 2 days to about 17% on the fifth day in culture (Fig. 3B).

Previous studies have implicated pancreatic hormones in liver growth *in vivo*. Starzl *et al.* (21) demonstrated, by employing vascular transpositions, that only blood from the pancreas stimulates DNA synthesis in regenerating liver. Bucher and Swaffield (7) demonstrated that insulin and glucagon act synergistically in promoting DNA synthesis in eviscerated, partially hepatectomized rats, but that other undefined factors also play a role. Short *et al.* (5) showed that infusion of a mixture of amino acids, glucagon, triiodothyronine, and heparin stimulate DNA synthesis in the liver of intact rats. Although pancreatic hormones apparently play a role in liver proliferation, total pancreatectomy or alloxan diabetes does not prvent regeneration (7, 22). The role of extrapancreatic glucagon is not known. Hypophysectomy (23) and parathyroidectomy (9), though delaying or attenuating the response, do not prevent regeneration. As suggested by many investigators, the various data imply a complex interplay between many hormonal factors in regulation of liver cell proliferation. In agreement with Bucher and Swaffield (7), the present studies support a role for both insulin and glucagon in stimulating liver DNA synthesis.

EGF involvement in liver regeneration has as yet not been

studied, but the present report suggests that it may play a prominent role in stimulating DNA synthesis. The sites of EGF production are not known and a radioimmunoassay for rat plasma EGF has not been developed. Thus, it is not yet possible to study the effect of EGF on liver regeneration by the classical techniques of removing its source or by studying plasma levels of hormone. The dramatic effects of EGF reported here suggest that it may be one of the heretofore unidentified factors affecting liver cell proliferation, possibly by acting in concert with hormones such as glucagon and insulin. EGF has previously been shown to stimulate growth and DNA synthesis in cultured fibroblasts (24), and it acted synergistically with insulin in that system (25).

Despite the stimulation of DNA synthesis, cell division occurred infrequently and a net increase in cell number was not achieved. This deficiency may be due to an extremely long S-phase and/or to an absence of culture conditions or factors required for initiation of mitosis. Leffert *et al.* (26) have reported that it is possible to dissociate effects of growth promoting agents on initiation of DNA synthesis from stimulation of mitosis in cultured fetal liver cells. Stimulation of mitosis was obtained by addition of a factor that could be purified from the culture medium. Studies are currently underway to find such factors and to define the conditions necessary to maintain dividing cultures of adult rat hepatocytes.

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