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Changes in the Concentration of Cytidine 3′,5′ Monophosphate (Cyclic CMP) in Regenerating Rat Liver (40766)

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Liver regeneration after partial hepatectomy PH (6, 7) has been used as a model for studying the molecular events that lead to cell proliferation in a differentiated tissue (1–10). It has been well documented (1–5) that, within 24 hr after hepatectomy in rats, the remaining liver lobe(s) double in size and that within 5–6 days, they regenerate to the original liver weight. The subcellular events responsible for this controlled hyperplasia remain largely unknown; however, cyclic AMP and ornithine decarboxylase have been shown to rise at discrete intervals after PH (6, 7). DNA synthesis takes place between 18 and 24 hr posthepatectomy, and peak mitotic activity of hepatic cells occurs at 28 hr after PH (8, 9).

Bloch (10) has reported that cyclic CMP is increased 50- to 200-fold in regenerating rat liver. A possible role for cyclic CMP in liver regeneration was further suggested by Shoji *et al.* (4), who reported that cyclic CMP phosphodiesterase (C-PDE) activity decreased during liver regeneration. We now report that following PH in rats, the concentration of cyclic CMP in liver increases in a biphasic manner. Cyclic CMP was measured by means of a specific radioimmunoassay (RIA) developed in this laboratory (11). This report verified Bloch's finding (10) that cyclic CMP is increased in regenerating liver and suggests that this cyclic nucleotide may be associated with the regeneration process.

Materials and Methods. Cyclic [^{3}H]CMP (21.0 Ci/mmol) was purchased from Amersham Chemical Corporation. Cyclic [^{3}H]AMP was purchased from New England Nuclear Corporation. Dowex 1-X8 formate resin (200–400 mesh) was purchased from Sigma Chemical Corporation. Thin-layer cellulose plates were purchased from Quantum Industries and polypropylene columns (0.75 × 4.0 cm) were purchased from Bio-Rad.

Thirty-day-old male Sprague–Dawley rats weighing 95–105 g were subjected to partial hepatectomy under light ether anesthesia. Seventy percent of the liver was removed by the procedure of Higgins and Anderson (5). Several animals were sacrificed by decapitation without ether anesthesia to determine the effect of ether anesthesia on cyclic CMP content. All other animals were sacrificed at 4-hr intervals after PH under light ether anesthesia. Control animals were subjected to laparotomy, placement of the liver lobes onto the abdominal surface, and reinsertion of the liver into the abdominal cavity before closing.

Liver samples were extracted as previously described (11). Briefly, the tissue was excised, weighed, placed in tubes, and immediately frozen in dry ice and acetone, lyophilized and stored at −30°C.

The tissues were homogenized in ice-cold water, extracted with $HClO_4$ (final concentration of 1.25 *N*), and centrifuged. After neutralization with KOH and centrifugation to remove $KClO_4$, the supernatant was placed on a Dowex 1-X8 formate column (0.75 × 4.0 cm). The column was washed with 12 ml water and 20 ml of 0.1 *N* formic acid, and cyclic CMP eluted in the 0.1 *N* formic acid fraction. This fraction was lyophilized, reconstituted in a minimum amount of water, and assayed for cyclic CMP content by RIA (11). In addition, aliquots of the solution which had been radioimmunoassayed were chromatographed on cellulose thin-layer plates using ethanol:ammonium acetate 0.5 *M*, 5:2, as the solvent (11). The appropriate section of the plate was extracted with water, and the

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amount of cyclic CMP present was assayed by our previously reported procedure (11). Briefly, 0- to 200-μl aliquots of the eluted cyclic CMP were added to the 100 μl of [^{125}I]-succinyl cyclic CMP tyrosine methyl ester (ScCMPTME, 0.01 pmol or 12,000 cpm) and 100 μl of the antisera (final dilution 1:200,000). The mixture was incubated for 18 hr at 4°C and the antibody bound [^{125}I]-ScCMPTME was separated from free [^{125}I]-ScCMPTME by filtering through a Millipore filter.

Results and discussion. Figure 1 shows the cyclic CMP content of rat liver measured by RIA at different time intervals following surgery. Over the 48 hr of the experiment, the cyclic CMP levels of livers from the sham-operated animals varied from 2.4 to 11.8 pmol/g wet weight. These concentrations do not differ significantly from those found in livers from unoperated animals (7.2 ± 0.87 pmol/g wet wt). In the regenerating livers, cyclic CMP levels were increased, with peak concentrations occurring at 12 and 24 hr. The first significant increase ($P < 0.01$) over prehepatectomy (control) levels was detectable at 8 hr, and a peak concentration of 104.2 ± 19.0 pmol/g wet weight was encountered at 12 hr ($P < 0.01$). This was followed by a fall in cyclic CMP to control levels at 16 hr. A second, less-pronounced peak in the concentration of cyclic CMP occurred at 24 hr, which remained elevated at 28 hr. At 48 hr, the cyclic CMP content of regenerating liver had returned to control levels.

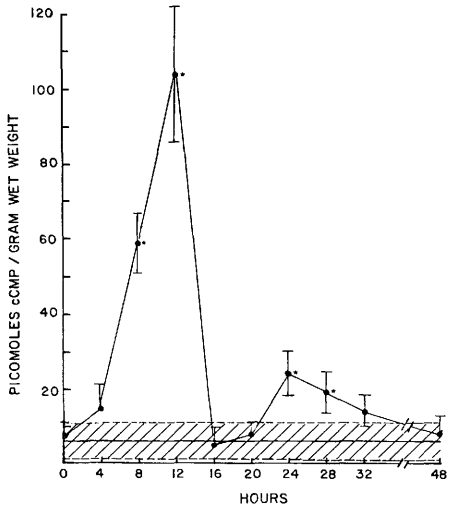

FIG. 1. Changes in the cyclic CMP content of rat liver following 70% hepatectomy. Male Sprague–Dawley rats (30 days old) were subject to partial hepatectomy and the cyclic CMP content of regenerating liver assayed by RIA. Each point represents the mean ± SD for 3–4 animals. The mean value for 20 sham-operated animals (horizontal line) ± SD (diagonal lines) are also shown, for sham, $n = 2$ at each time point. *$P < 0.01$ as related to prehepatectomy control.

Cyclic AMP has also been reported to increase at 12 hr (12), but the peak concentration of the immunoreactive material which we measured at 12 hr was not cyclic AMP since Dowex 1-X8 chromatography performed on all samples prior to assay separates cyclic AMP from cyclic CMP (11) and since the identity of the immunoreactive material (0.1 *N* formic acid fraction) from 12-hr PH livers is further established by thin-layer chromatography in which the radioimmunoreactive material cochromatographed with cyclic CMP (R_f 0.53) and not with cyclic AMP (R_f 0.43). In addition, the amount of cyclic CMP measured by RIA was approximately the same before and after tlc, within the variation of the assay. Using this procedure, we found no cyclic CMP radioimmunoassayable material at the R_f of cyclic AMP.

Freezing of tissues in dry ice–acetone mixtures provided adequate protection against enzymatic degradation of cyclic CMP because it has been shown that cyclic CMP phosphodiesterase activity is of a much lower order than that of cyclic AMP phosphodiesterase in the same tissue (13). Cyclic CMP phosphodiesterase was not a significant problem in our experiments with liver homogenates (11).

The effect of ether anesthesia on cyclic CMP levels in rat liver was found to be negligible. The liver cyclic CMP content was 6.2 ± 4.1 pmol/g wet weight ($n = 3$) in rats killed by decapitation and 7.2 ± 0.87 pmol/g wet weight after light ether anesthesia.

The 14-fold increase found by us at 12 hr after PH was not as large as that reported by Bloch (10). However, he did not report the age of the animals he used, and we decided to examine the cyclic CMP concentrations in the liver of older (6 months) ani-

mals as well. Two days after PH (14), the concentration of cyclic CMP in the regenerating livers of these rats was 180 times greater than that found in the controls, amounting to 413.4 ± 244 vs 2.27 ± 1.1 pmol/g wet weight (n = 4), respectively. The time at which this increase occurs coincides with the time at which Shoji *et al.* (4) observed the maximum depression of C-PDE. A similar delayed increase in ornithine decarboxylase activity has been shown to occur in older rats after PH (14).

Analogous to cyclic CMP, the concentration of cyclic AMP has also been shown to increase after PH in a biphasic manner. Peak concentrations of cyclic AMP occur at 2–6 and 12–14 hr posthepatectomy (12). In contrast, cyclic GMP has been reported to increase during the first 10–20 min after PH (15). The increase in the concentrations of the cyclic purine nucleotides after PH are considerably smaller than those we found with cyclic CMP.

This is the first report demonstrating the changes in the concentration of cyclic CMP that occur in regenerating liver as a function of time. Cyclic CMP increases in a biphasic manner. The first peak at 12 hr is coincident with the decrease in C-PDE described by Shoji *et al* (4), and also with previously reported increases in the concentration of cyclic AMP and the activity of ornithine decarboxylase (6, 7, 14). The second cyclic CMP peak occurs at 24–28 hr. Other investigators (8, 9) have reported that the peak in mitotic activity in the liver of PH rats occurs at 28 hr. Although the relationship between these biochemical events and cyclic CMP is unknown, these findings suggest that this cyclic nucleotide may play a role in liver regeneration.

Summary. The entire CMP content of regenerating rat liver was measured by radioimmunoassay. The concentration of cyclic CMP in normal liver was 7.2 ± 0.87 pmol/g wet weight. Eight hours after hepatectomy, this level had increased to 58.9 ± 10.0 pmol/g wet weight, and at 12 hr had reached a peak of 104.2 ± 19.0 pmol/g wet weight. By 16 hr, the cyclic CMP concentration had fallen to prehepatectomy levels. A second, smaller increase in the level of cyclic CMP occurred at 24–28 hr after operation, and by 48 hr the cyclic CMP had returned to prehepatectomy levels.

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