strains of mycoplasma (*13*). To these we can now add L-glutaminase; our studies indicate that its immunosuppressive properties should be explored in vivo.

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DNA Synthesis and Interaction between Controlled Feeding Schedules and Partial Hepatectomy in Rats

Abstract. *The rate of DNA synthesis has been measured during liver regeneration in rats adapted to a controlled feeding schedule. The results show two different phenomena in the regulation of DNA synthesis. The first is the appearance of a peak of DNA synthesis following the operation itself and independent of the time of the day; the second one is the presence of constant diurnal variations in the rate of DNA synthesis in response to the partial hepatectomy and following the stimulus or stimuli of the controlled feeding schedule.*

The regeneration of rat liver after partial hepatectomy has been extensively studied by many authors since the early experiments performed by Higgins and Anderson in 1931 (*1*). Several reviewers have reported the features of liver regeneration, and the literature is extensive (*2, 3*). As far as DNA biosynthesis is concerned, it is known that it begins after a lag period of about 18 hours and peaks between 20 and 30 hours after partial hepatectomy (*2*). The age of rats (*4*), as well as the percentage of liver excised (*5*), influences the timing of the DNA synthesic period. A second peak of the incorporation rate of thymidine (TdR) into DNA is known to occur between 65 and 72 hours after the operation (*6, 7*). Rhythmic periodicity of mitosis at 24-hour intervals was first observed in regenerating rat liver by Jaffe (*8*), and in unoperated young animals by Jackson (*9*). Halberg and Barnum and their colleagues (*7, 10*) were able to show circadian rhythms in the in vivo labeling of DNA in normal and regenerating rodent liver. In order to conduct a systematic study of the variation in rate of DNA synthesis in rat liver and hepatomas, Potter *et al.* (*11–13*) have developed a "controlled feeding schedule." Under these experimental conditions they found large diurnal variations of the incorporation rate of TdR into DNA in both hepatoma and host liver, using the transplantable Morris hepatoma 7793. Below we report experiments dealing with the diurnal variation of the incorporation rate of TdR into DNA in normal growing liver, and report comparisons with regenerating liver studied in rats adapted to a "controlled feeding schedule."

Male Sprague-Dawley rats obtained from either the Charles River Breeding Laboratories, Inc., the Holtzman Company, or the ARS/Sprague-Dawley Company were used throughout all experiments. Unoperated rats were obtained soon after weaning and were used at about 35 days of age (Fig. 1). Partial hepatectomies were performed on older rats (58 to 62 days old) weighing 230 to 260 g (Fig. 2). The rats were housed on arrival in an air-conditioned windowless room with an inverted and displaced lighting schedule in which lights were on from 8:30 p.m. (20:30) to 8:30 a.m. (8:30) in a 24-hour cycle. The diet contained 30 percent protein (*12*), and food was supplied just before the lights were switched off. Food dishes were removed 8 hours later according to the "8 + 16" feeding schedule developed by Potter *et al.* (*12, 13*). Water was always available. The rats were adapted to this protocol for at least 1 week before being used for the experiments. Partial hepatectomies were performed under ether anesthesia, with removal of the main lobes (66 to 72 percent of the liver was excised) as described by Higgins and Anderson (*1*). The operations were performed at 20:30 ± 30 minutes, at 15:30 ± 30 minutes, or at 11:30 ± 30 minutes, as indicated in Fig. 2, A, B, and C, respectively.

Thymidine-methyl-^{3}H (New England Nuclear Co., specific activity 6.45 c/mmole) was used to evaluate DNA synthesis. At intervals ranging between 15 and 56 hours after the partial hepatectomies, and at different times during the 24-hour day, the rats were injected intraperitoneally with thymidine-methyl-^{3}H (20 μc/100 g of body weight in 0.4 ml of sterile saline) and killed by cervical dislocation exactly 1 hour later. The livers were quickly removed and dropped into cold saline, blotted with absorbent tissue, and homogenized in nine volumes of distilled water with a Polytron homogenizer (*14*). Perchloric acid was added to an aliquot of the homogenate to a final molarity of 0.5. After centrifugation, the supernatant was saved and the residue was washed once with cold 0.5*M* perchloric acid. The supernatants were combined and designated "acid-soluble fraction." DNA in the pellet was separated from RNA as described by Munro and Fleck (*15*) and was assayed by the Ceriotti procedure (*16*) slightly modified (*17*). Radioactivity was measured on a Packard Tri-Carb liquid scintillation spectrometer.

The lower portion of Fig. 1 shows the 1-hour incorporation rate of TdR into DNA in the normal, growing, young rat liver during the course of the day. Rats 35 days old were used for these experiments in order to detect some DNA synthesis which is almost completely abolished in adult rats. The data are expressed as percentage of total uptake (defined as the sum of the radioactivities in the DNA and the acid-soluble fraction at 1 hour after injection). This way of express-

ing the data is felt to be the most suitable, as it takes into consideration the total amount of labeled thymidine which entered the cell. This permits us to minimize the changes in the uptake due to changed permeability of the cellular membrane or to a changed transport of this molecule which would alter the specific activity of the thymidine triphosphate precursor pool for DNA. The changes in the acid-soluble pool and in blood after injection of [^{3}H]TdR have been reported by Chang and Looney (*18*). They found a rapid disappearance of the labeled TdR from the blood in the first 10 minutes, followed by a rapid incorporation of the radioactivity into the acid-soluble fraction and DNA. A constant ratio of the radioactivities in thymidine monophosphate, thymidine diphosphate, and thymidine triphosphate was found in the acid-soluble fraction during the first hour after the intravenous administration of the labeled precursor.

From the data (Fig. 1) it appears that the normal growing liver does follow a diurnal rhythm for DNA synthesis. In fact, the percentage of TdR incorporated into DNA rises to about 40 percent of total uptake starting from a rather constant level of about 20 percent. This peak of incorporation occurs between 19:30 and 21:30 under our conditions, and it seems interesting to point out that the change from the dark period to the light period is at 20:30 (see *11*). The total uptake of the labeled precursor does not show any significant variation (see upper portion of Fig. 1).

In Fig. 2 we report the pattern of the incorporation rate of TdR into DNA after partial hepatectomy for which the operations have been performed at different times of day. The lower graph (solid line) of each portion of Fig. 2 (A, B, and C) represents the radioactivity in DNA; these data are expressed as the percentage of total uptake, as in Fig. 1. The upper graph (dashed line) represents the total uptake of the labeled precursor into the liver cells.

When the operation is carried out at 20:30 (Fig. 2A), the incorporation rate of TdR into DNA shows a lag period of about 18 hours, peaking thereafter at 23 hours after the operation and apparently coinciding with the diurnal peak in unoperated animals shown in Fig. 1. This first sharp rise of the incorporation rate reaches a maximum of 75 percent of total uptake and is followed by a rapid decrease at 31 hours to the lowest level of 25 percent. After that the rate of DNA synthesis increases again to reach a second peak between 45 and 48 hours. At 51 hours the incorporation rate is down again to a value of 45 percent of the total uptake.

Figure 2B shows the pattern of DNA synthesis following partial hepatectomy when the operation is performed at 15:30. Also in this case we have found a lag period of about 18 hours and a first sharp rise peaking at 23 hours but now *independent of the time of day*. This first postoperative peak was followed by a decrease at 25 hours and by a second peak between 27 and 29 hours after the operation. The second peak reflects environmentally entrained DNA synthesis that coincides with the peak seen in Fig. 1. Another large increase in the incorporation rate of TdR into DNA has been observed between 51 and 54 hours after the operation. This last peak in Fig. 2 coincides (in terms of time of the day) with the one found between 45 and 48 hours when the operation was performed at 20:30 (see Fig. 2A) and precisely between 18:30 and 21:30, again indicating entrainment by the schedule of controlled feeding and lighting.

In order to confirm the pattern reported in Fig. 2B, one lot of rats was operated 4 hours earlier (at 11:30) and the results are reported in Fig. 2C. Again, as in the two previous experiments, we have found a first sharp rise of incorporation rate of TdR into DNA peaking at 23 hours after a lag period of about 18 hours (the first postoperative peak) followed, this time, by a second peak between 31 and 33 hours. This second peak coincides (in terms of time of the day) with the peaks observed in the two previous experiments (see Fig. 2, A and B) and occurs between 18:30 and 20:30, and is evidently environmentally entrained.

The results reported in Fig. 2 clearly show two phenomena in the regulation of liver DNA synthesis following partial hepatectomy. The first is the constant appearance of the first postoperative peak of DNA synthesis 23 hours after the operation independent of the timing of the operation in the "controlled feeding schedule." This result is

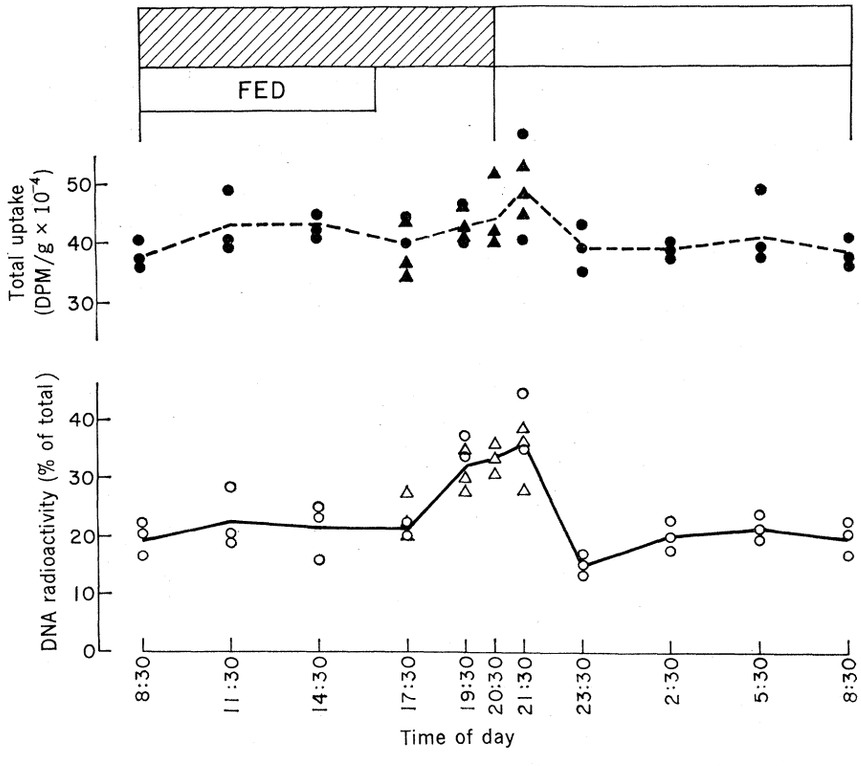

Fig. 1. Incorporation of labeled thymidine into liver DNA as a function of time of day. Rats were injected intraperitoneally with thymidine-methyl-^{3}H and pulsed for 60 minutes. Rats were killed at the times indicated in the figures. The upper curve (dashed line) with closed symbols (▲, ●) represents the total uptake expressed as disintegrations per minute per gram of liver × 10^{-4}; the lower curve (solid line) with open symbols (△, ○) reports the percentage of label in DNA. ● or ○, Charles River rats; ▲ or △, Sprague-Dawley rats. Each point represents one animal and the curves are drawn through the means.

in agreement with previous work from this (*6*) and other (*2*) laboratories using uncontrolled feeding schedules. It seems valid to conclude that the duplication of the first lot of liver cells has a high priority and is not sensitive to external stimuli and environmental changes. The second peak reveals the presence of constant diurnal variations of the rate of DNA synthesis in response to the stimulus or stimuli of the controlled feeding schedule to which the rats have been subjected before the operation. This peak is insensitive to the length of time after the operation, but nevertheless represents a response to the operation itself. This diurnal variation is represented by a peak of DNA synthesis constantly present in the 4-hour period between the removal of food and the beginning of the light, and namely, in the present feeding schedule, around 19:30. When the operation is performed at 20:30, the first peak of incorporation rate of TdR into DNA occurs in the time of the day in which there is the peak of activity due to the diurnal variation (Fig. 2A). But it is possible to obtain two separate peaks by operating at earlier times of the day, that is, 11:30 or 15:30 (Fig. 2, B and C).

Another interesting point appears from the patterns of total uptake of TdR into the liver cells, which is reported in the upper portion of Fig. 1 and Fig. 2, A–C, by a dashed line connecting the mean values at each time point. In the normal growing liver (Fig. 1) the total uptake, as we have already pointed out, does not show significant changes during the day, whereas regenerating liver shows a pattern similar to that of DNA synthesis. The data suggest that the uptake of the labeled precursor does not change when the percentage incorporation into DNA changes within a certain range. This range seems to have

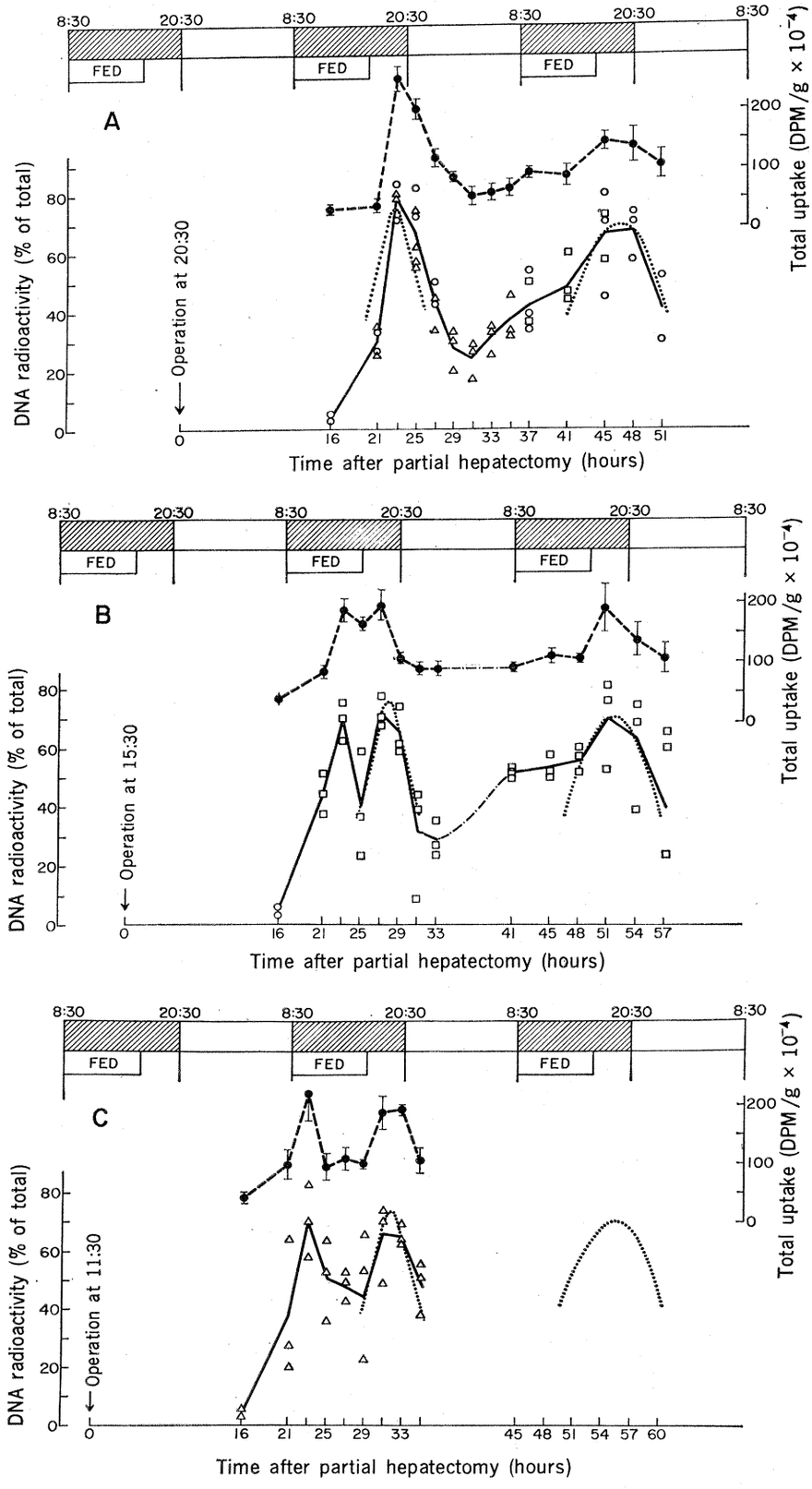

Fig. 2. Incorporation of labeled thymidine into liver DNA after partial hepatectomy as function of time of day. The operation was carried out (A) at 20:30, (B) at 15:30, and (C) at 11:30. Rats were injected intraperitoneally with thymidine-methyl-^{3}H and pulsed for 60 minutes. Rats (58 to 62 days old) were killed at the time reported in the figure. The total uptake (upper curves) expressed as disintegrations per minute per gram (*DPM/g*) of liver $\times 10^{-4}$ is shown by solid symbols with the standard error of the mean reported by vertical bars, with the ordinate scale on the right. Solid lines represent the average percentage of radioactivity found in DNA (ordinate scale on the left) in the corresponding animals. Each open symbol represents a single animal, and standard errors are not shown. ○, Charles River rats; △, Sprague-Dawley rats; □, Holtzman rats. The first peaks in dotted lines at 20:30 local time in A, B, and C was drawn by averaging the data from the corresponding peaks in B and C to show the agreement among the experiments. The second peak in dotted lines in A, B, and C was drawn from the average of corresponding data in A and B. The abscissa showing time after hepatectomy (lower scale) comes at different clock time (upper scale) in each experiment.

an upper limit around 40 percent. Above this presumed limit it seems that the TdR transport system is more active and more TdR is transported by the cell in response to the large increase in DNA synthesis. This suggestion is supported by the facts that the total uptake in the normal growing liver does not change during the day and the requirement of TdR due to the diurnal increase of DNA synthesis hardly goes over 40 percent of total uptake, and in the regenerating liver when the incorporation into DNA does not exceed 40 percent, the uptake is constant and about at the same level as normal liver, that is, about 40×10^4 disintegrations per minute per gram of fresh tissue (see data at 16 and 21 hours in Fig. 2A and at 16 hours in 2B and 2C).

Since our findings present clear evidence for a constant diurnal rhythm of DNA synthesis both in normal and in regenerating rat liver, we believe that this emphasizes the need for a precise knowledge and control of the feeding schedule on which the animals are maintained in order to obtain more useful information from the experiments.

The nature and the timing of the stimulus or stimuli responsible for the diurnal rhythm of DNA synthesis both in normal growing and regenerating rat liver are not known. Nevertheless, the present study makes it reasonable to believe that the stimuli may vary in relation to food intake and changes from the dark period to the light period of the day or vice versa. It appears that the first postoperative peak may represent a population of cells that has passed an environmentally controlled point in the cell cycle, while the following peak appears to reveal another population of cells that depend on environmental controls in order to move into the cell replication cycle (*19*).

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22. This study was supported in part by departmental grant CA-07175 and training grant T01-CA-5002 from the National Cancer Institute, PHS. Address correspondence to V.R.P. at the McArdle Laboratory.
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Cigarette Smoking: Objective Evidence for Lung Damage in Teen-Agers

Abstract. *High school students with 1 to 5 years' smoking experience have excessive cough, sputum production, and shortness of breath. When maximum expiratory flow is plotted against maximum expired volume, the curves of nonsmokers and smokers differ in shape. The smokers have lower flow rates at mid-vital capacity and at lower lung volumes. This probably reflects small airway obstruction in the smokers.*

We present objective evidence of functional changes in the lungs of teen-agers who smoke only a few years. At least part of this damage to the lungs might be reversible on cessation of smoking. However, permanent effects, including premature arrest of lung development, cannot be excluded at this time.

Although the effects of smoking as a cause of lung cancer and other diseases appear to be well known among young people, antismoking propaganda does little to reduce smoking (*1*), and it is even suspected that smoking among teen-agers may increase rather than decrease (*2*). College seniors and others in their twenties who smoke have reduced lung function (*3*), but at that age smoking habits may already be fixed (*4*). Since objective evidence of damage to their own lung function might be more convincing to teen-agers than the faraway danger of lung cancer, we therefore looked for such evidence in 365 students of four high schools in the New Haven area. There were 195 boys and 170 girls, aged 15 to 19 years; of these 50 percent of the boys and 37 percent of the girls were regular smokers. Most of the girls (23 percent) were light smokers, with only 1 percent smoking more than 20 cigarettes daily. Most of the boys (26 percent) were moderate smokers (11 to 20 cigarettes per day), with 8 percent smoking more than 20 cigarettes daily.

In selecting a sensitive test for lung function we used the fact that expiratory flow rates are nearly independent of the subject's effort during the last portion of a maximally forced expiration after a full inspiration. During that maneuver, the large intrathoracic airways are compressed because of the high pressures generated in the chest. Under these conditions, more effort does not result in higher flow rates. Rather, the supply of air from the al-