Thymidine Kinase and Deoxyribonucleic Acid Metabolism in Growing and Regenerating Livers from Rats on Controlled Feeding Schedules

By HAROLD A. HOPKINS,* HAROLD A. CAMPBELL, BRUNO BARBIROLI† and VAN R. POTTER‡

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wis. 53706, U.S.A.

(Received 23 July 1973)

Rats accustomed to eating during the first 8 h of a daily 12 h dark period re-established about 80% of intact liver weight, protein and DNA within 4 days following partial hepatectomy; further increases were not observed. Liver thymidine kinase activity and thymidine incorporation into liver DNA exhibited marked daily oscillations during liver regeneration. Maximum values were observed near the end of the dark period both in intact growing rats and in rats partially hepatectomized 2 h before the end of the dark period. The time of day of surgery affected thymidine kinase activity and thymidine incorporation into DNA at specific times following partial hepatectomy. This seriously affects the interpretation of reports of experiments where the time of day of killing has been held constant and time of surgery varied. Highly significant correlation coefficients were observed for thymidine incorporation before killing versus thymidine kinase activity at time of killing and for thymidine versus orotic acid incorporation into DNA of livers from rats partially hepatectomized 2 h before the end of the dark period and killed at 12 h intervals. Thymidylate phosphatase activity returned to the normal amount at a rate similar to that for liver protein. Thymidylate phosphatase did not affect the validity of the thymidine kinase assay. The relationship of [^{14}C]orotic acid to [^3H]thymidine incorporation into liver DNA varied with the time of day, with the age of the rat and during the regeneration of the liver.

The process whereby the liver mass is restored following surgical removal of a portion of the liver or administration of toxic agents continues to attract the attention of investigators from many branches of biology. The chemical stimulus responsible for initiating the events leading to hyperplasia after partial hepatectomy has not been established. However, several agents, such as tri-iodothyronine (Lee *et al.*, 1968; Short *et al.*, 1972), glucagon (Short *et al.*, 1972), insulin (Younger *et al.*, 1966) and papain (Kambara *et al.*, 1964), when administered to intact rats have given responses similar to those following partial hepatectomy, except diminished in amount. The literature pertaining to liver regeneration is extensive and has often been reviewed in recent years (Harkness, 1957; Bucher, 1963; Leduc, 1964; Bucher, 1967*a,b*; Bresnick, 1971; Bucher & Malt, 1971).

Even though the operative procedure itself is simple and reproducible (Higgins & Anderson, 1931) many environmental factors appear to modify the

response one observes after partial hepatectomy (Bucher & Malt, 1971). Among these are hormonal and dietary factors that are themselves influenced by environmental lighting and modified by stress. As a result of these and perhaps other factors, large variation is frequently seen among animals seemingly treated alike and sometimes conflicting results arise from different (or even single) laboratories.

The uniformity of magnitude of many biochemical parameters among rats adapted to controlled feeding and lighting schedules when examined at specific times of day (Potter *et al.*, 1968; Watanabe *et al.*, 1968; Baril & Potter, 1968; Potter, 1970) suggested that much of the individual variation in regenerating liver experiments might result from just those environmental factors that are held more nearly constant with the controlled feeding and lighting regimen. The results presented here establish the usefulness of the controlled feeding schedule in understanding the relationship between normal diurnal rhythms in metabolic processes and the hyperplasia following partial hepatectomy. Environmental entrainment of animals and varying the times of onset of DNA synthesis by varying the time of operation offer refinement of the regenerating liver system, already considered 'nearly ideal for the study of gene control' (Mayfield & Bonner, 1972).

* Present address: Department of Pediatrics, Division of Radiobiology and Biophysics, University of Virginia School of Medicine, Charlottesville, Va. 22901, U.S.A.

† Present address: Istituto di Chimica Biologica, Università di Bologna, Bologna, Italy.

‡ To whom requests for reprints should be sent.

Materials and Methods

Environmental entrainment of animals

White male rats were obtained from the Sprague-Dawley Company (Madison, Wis., U.S.A.) at 21 days of age and were housed usually two per cage in an air-conditioned windowless room lighted from 20:00h to 08:00h. A pelleted diet containing 30% protein and 61% carbohydrate (Watanabe *et al.*, 1968) was supplied daily from 08:00h to 16:00h; this corresponded to the first 8h of the dark period. This regimen has previously been designated '8+16' (Potter *et al.*, 1968; Potter, 1970). The inverted lighting and feeding schedule allows these nocturnally active creatures to sleep with minimum disturbance and permits feeding during usual working hours.

The rats were entrained to the feeding and lighting schedules for a period of 3 weeks and then, at approx. 6 weeks of age (150–170g body wt.) they were subjected to 70% partial hepatectomy (Higgins & Anderson, 1931) under ether anaesthesia.

Preparation of homogenates and supernatant fractions

Rats were killed by decapitation at various intervals after partial hepatectomy. The livers were quick-frozen in liquid N₂ and stored at -70°C for periods of 1–30 days. Weighed portions of frozen pulverized liver were homogenized in 9 vol. of buffer (0.25M-sucrose in 0.05M-Tris-HCl, pH8.0) in a Polytron homogenizer (Kinematic G.m.b.H., Lucerne, Switzerland). This procedure disrupts all cell nuclei and probably all other subcellular organelles as well.

Supernatant fractions were prepared from the homogenates by centrifugation at 150000g_{av.} for 1h at 4°C with a Beckman type 50 Ti rotor in a Beckman L2 ultracentrifuge. The supernatant fractions used for enzyme determinations were taken from near the centre of the centrifuge tube, avoiding both the top of the liquid where protein and thymidine kinase are being cleared from solution (unpublished work) and the top of the pellet where protein and thymidine kinase are being concentrated. These supernatant fractions were kept frozen at -20°C until enzymic activities were determined. Under these conditions thymidine kinase activity was not appreciably affected by repeated freezing and thawing or by prolonged storage.

Analytical methods

For determination of thymidine or orotic acid incorporation into DNA, [*Me*-³H]thymidine (Schwarz-Mann, Orangeburg, N.Y., U.S.A.) or [6-¹⁴C]orotic acid hydrate (New England Nuclear Corporation, Boston, Mass., U.S.A.) were injected intraperitoneally. Non-radioactive thymidine (PL Biochemicals, Milwaukee, Wis., U.S.A.) in 0.9% NaCl was used to dilute the [³H]thymidine for injection.

Protein was determined by the procedure of Lowry *et al.* (1951) after removal of cold-trichloroacetic acid-soluble compounds; bovine serum albumin, fraction V (Sigma Chemical Co., St. Louis, Mo., U.S.A.) was used as the protein standard.

DNA was extracted by an adaptation (Hopkins *et al.*, 1972) of the procedures of Schmidt & Thannhauser (1945) and of Schneider (1945). A standard reference homogenate, prepared by pooling a number of individual liver homogenates, was stored at -20°C. Thawed portions from this reference homogenate were subjected to extraction and analysis with each group of samples to ensure that determinations made at different times were comparable. DNA was measured in the trichloroacetic acid extracts by the method of Burton (1956), but H₂SO₄ was omitted, diphenylamine was increased to 2g per 100ml and HClO₄ was present at a final concentration of 0.4M. Highly polymerized DNA (Calbiochem, Los Angeles, Calif., U.S.A.) was the standard. Radioactivity in the DNA extracts was measured in Scintisol (Isolab Inc., Akron, Ohio, U.S.A.) on a Packard Tri-Carb liquid-scintillation spectrometer by using external standardization. Neither of two additional alkali treatments followed by precipitations with cold acid before hydrolysis of the DNA decreased the ¹⁴C d.p.m. from [6-¹⁴C]orotic acid relative to the ³H d.p.m. from [*Me*-³H]thymidine in DNA in experiments utilizing both precursors.

Thymidine kinase [EC 2.7.1.21] in the high-speed supernatants was assayed by the procedure of Ives *et al.* (1969) at 37°C. The composition of the thymidine kinase assay reaction mixture was: high-speed supernatant, 10–50μl; 100mM-Tris-HCl, pH8.0; 10mM-ATP; 7mM-MgCl₂; and 0.1mM-thymidine, in a total volume of 0.2ml containing 2μCi of radioactivity from [*Me*-³H]thymidine. One munit is equivalent to the enzymic activity resulting in a reaction rate of 1nmol per min, under the conditions of the assay.

Thymidylate phosphatase* activity of the high-speed supernatants was measured by an adaptation of the method of Ives *et al.* (1969). The composition of the thymidylate phosphatase assay reaction mixture was: high-speed supernatant, 25μl, 100mM-Tris-HCl, pH8.0; 7mM-MgCl₂; and 0.02mM-dTMP in a total volume of 0.2ml containing 0.4μCi of radioactivity from [*Me*-³H]dTMP (Schwarz-Mann)

* Thymidylate phosphatase is used here in an operational sense, representing the enzymic hydrolysis of dTMP at concentrations relevant to conditions in the thymidine kinase assay. It is possible and even probable that more than one phosphatase is responsible for dTMP hydrolysis by these high-speed supernatants. To guide our thinking the kinetic constant relating the rate of hydrolysis with the dTMP concentration was determined: apparent $K_m = 0.25$ mM-dTMP.

The dTMP concentration (0.02 mM) is below a saturating one of substrate and was selected in the upper range of dTMP formed in the thymidine kinase activity assay. The reaction times were 15 and 30 min; the reaction was stopped by placing the tube in boiling water for 5 min. At this low concentration of dTMP the measurement of the dTMP remaining, by using the procedures employed in the thymidine kinase activity assay, gave more reliable results than the measurement of the thymidine formed in the reaction. The initial reaction rate at 0.02 mM-dTMP concentration was calculated by using the formula $v_0 = s_0/t \cdot \ln s_0/s_t$ where v_0 = initial reaction rate, s_0 = initial dTMP concentration, t = reaction time and s_t = final dTMP concentration. Both reaction times gave the same value for the initial reaction rate. Thus under the conditions of the assay the reaction rate is consistent with first-order kinetics. One munit is equivalent to the enzymic activity resulting in an initial reaction rate of 1 nmol/min, at a substrate concentration of 0.02 mM-dTMP.

Radioautography

Radioautography was performed employing NTB-3 emulsion (Eastman Kodak Co., Rochester, N.Y., U.S.A.) according to the basic procedures outlined for this emulsion by Rogers (1967).

Results

DNA metabolism in intact liver

Livers from intact rats that had been entrained for 3 weeks to the controlled feeding schedule and killed at about 6 weeks of age exhibited maximum thymidine incorporation and thymidine kinase activity at the end of the dark period (Fig. 1). The magnitudes of the oscillations of these parameters were small but at two different times of day in growing rats of three different ages (Table 1) these parameters also exhibited maximum activities at the end of the dark period. The correlation coefficient of 0.91 was found for thymidine incorporation into DNA versus thymidine kinase for growing rats at the times examined. Incorporation of orotic acid into liver DNA also was higher at 18:00h than at 06:00h for these intact rats. The liver DNA contents of these intact growing rats are given in Table 2.

Restoration of liver mass following partial hepatectomy

The rapidity with which the liver mass is restored in rats adapted to controlled feeding schedules and subjected to 70% partial hepatectomy is shown in Fig. 2. Liver weight, protein and DNA are restored to about 85, 77 and 80% respectively of initial amounts within 4 days following surgery and remain

Fig. 1. DNA content, $[^3\text{H}]$ thymidine incorporation into DNA and thymidine kinase activities in intact control livers of rats adapted to the '8+16' feeding schedule

Each rat received a single 0.7 ml intraperitoneal injection containing $88 \mu\text{Ci}$ of $[\text{Me-}^3\text{H}]$ thymidine in 140 nmol of thymidine 1 h before killing. Each point is the mean for five rats killed at that time of day. The vertical bars represent the S.E.M. associated with each mean. Liver DNA (▲) has been normalized to 08:00h starved body weights. ●, $[^3\text{H}]$ Thymidine incorporation; ■, thymidine kinase activity. In this and subsequent figures cross-hatched bars represent the dark periods in 24h cycles and the open bars below them represent the periods when food was available.

Table 1. *Thymidine and orotic acid incorporation into DNA and thymidine kinase activity of livers of intact rats*

At 4h and again at 2h before being killed each rat received 0.3 ml of radioisotopically labelled DNA precursors (13 nmol of thymidine containing 7.5 μ Ci from [Me - 3 H]thymidine and 133 nmol of orotic acid containing 7.5 μ Ci from [6 - 14 C]orotic acid in 0.3 ml of 0.9% NaCl). Entrainment to the '8+16' controlled schedule began at 21 days of age. Thymidine kinase activity is expressed as munits/liver per 100g body wt. Each value is the mean and s.e.m. for three rats.

Age (days)	$10^{-5} \times$ Thymidine incorporation (d.p.m./liver)		$10^{-4} \times$ Orotic acid incorporation (d.p.m./liver)		Thymidine kinase activity	
	06:00h	18:00h	06:00h	18:00h	06:00h	18:00h
28	1.92 $\pm$ 0.20		3.69 $\pm$ 0.25		18.1 $\pm$ 2.84	
28		2.55 $\pm$ 0.15		10.1 $\pm$ 0.90		24.5 $\pm$ 2.11
29	1.30 $\pm$ 0.14		2.90 $\pm$ 0.45		17.1 $\pm$ 3.53	
35		1.65 $\pm$ 0.48		8.75 $\pm$ 2.35		22.5 $\pm$ 7.57
36	0.54 $\pm$ 0.08		1.42 $\pm$ 0.20		8.48 $\pm$ 0.97	
36		1.57 $\pm$ 0.41		8.28 $\pm$ 2.69		16.5 $\pm$ 2.83
47		0.99 $\pm$ 0.10		4.37 $\pm$ 0.95		10.0 $\pm$ 1.05
48	0.52 $\pm$ 0.09		1.07 $\pm$ 0.15		6.93 $\pm$ 0.83	
48		0.80 $\pm$ 0.17		3.54 $\pm$ 1.07		8.98 $\pm$ 1.74

Table 2. *DNA content of livers of growing rats*

Entrainment to the '8+16' controlled feeding schedule began at 21 days of age. Body weights were taken near the end of the 16h 'fast' on the day of killing. Each value is the mean and s.e.m. for three rats.

Age (days)	DNA (mg/liver per 100g body wt.)		DNA (mg/liver)	
	06:00h	18:00h	06:00h	18:00h
28	13.9 $\pm$ 0.12		8.87 $\pm$ 0.14	
28		16.4 $\pm$ 0.32		11.3 $\pm$ 0.18
29	15.3 $\pm$ 1.07		10.5 $\pm$ 0.64	
35		16.4 $\pm$ 0.53		16.3 $\pm$ 1.39
36	15.9 $\pm$ 0.06		16.0 $\pm$ 1.38	
36		14.7 $\pm$ 0.32		15.0 $\pm$ 0.08
47		14.5 $\pm$ 0.35		23.7 $\pm$ 0.73
48	13.7 $\pm$ 0.44		21.8 $\pm$ 1.86	
48		14.9 $\pm$ 0.73		24.6 $\pm$ 1.21

essentially unchanged over the subsequent 10-day period.

DNA metabolism in regenerating liver

Thymidine incorporation into DNA and thymidine kinase activity in regenerating livers of rats on controlled feeding schedules and partially hepatectomized at 18:00h are shown in Figs. 3(a) and 3(c). At 18:00h (24h following the operation) mean thymidine incorporation into DNA and thymidine kinase activities were elevated some 23- and 22-fold respectively compared with that found in normal liver. The most striking feature of Figs. 3(a) and 3(c) is the marked diurnal nature of both thymidine

incorporation into DNA and thymidine kinase activity, with both parameters being high in the evening and low in the morning on our inverted feeding and lighting schedule. Rhythmic periodicity of mitosis at 24h intervals was first observed in regenerating rat liver by Jaffee (1954) and rhythms in the labelling of DNA *in vivo* have been observed by Barnum *et al.* (1957), Halberg *et al.* (1958) and others. The diurnal periodicity in thymidine kinase activity has previously been reported by Gebert (1967) for intact rat livers, host livers and certain Morris hepatomas. This periodicity has not been reported by others even though the short half-life of the enzyme (which makes it a likely candidate for marked diurnal oscillations) was reported by Bresnick *et al.*

Fig. 2. Restoration of liver weight (a), protein (b) and DNA (c) in rats adapted to the '8+16' feeding schedule and subjected to 70% partial hepatectomy at 18:00h

The alternate shaded and open areas at the top represent the 12h dark and light periods respectively. The open areas under the shaded areas represent the 8h feeding periods that coincided with the first 8h of darkness. Each point represents the average for three rats, except two rats at zero time in Expt. 1. Average initial amount for Expt. 1 (●) were 5.29g of liver/100g body wt., 872mg of protein/liver per 100g body wt. and 14.1mg of DNA/liver per 100g body wt.; initial amounts for Expt. 2 (○) were 5.83g of liver/100g body wt., 900 mg of protein/liver per 100g body wt. and 14.5mg of DNA/liver per 100g body wt. A longer storage at -70°C resulted in somewhat greater dehydration of the livers in Expt. 1. Liver weights were determined at the end of storage, therefore total liver protein and DNA were not affected. The points at 120, 168 and 336h after partial hepatectomy, all at 18:00h, have been connected by straight lines, but marked daily oscillations in liver weight and smaller oscillations in liver protein occur.

(1967). Half-lives of 2.6, 3.7 and 3.8h have been reported for thymidine kinase from intact, regenerating and neonatal rat liver respectively (Bresnick *et al.*, 1967; Bresnick & Burleson, 1970). The decrease of enzyme activity in Fig. 3(c) is consistent with these values.

Cellular thymidine kinase can be separated into three fractions by polyacrylamide-gel electrophoresis (Taylor *et al.*, 1972). Thymidine kinase of the cyto-

Fig. 3. $[^3\text{H}]$ Thymidine (a) and $[^{14}\text{C}]$ orotic acid (b) incorporation into DNA and thymidine kinase (c) activities in livers of rats adapted to the '8+16' feeding schedule and subjected to 70% partial hepatectomy at 18:00h

Each point represents the mean for three rats, except two rats at zero time for Expt. 1; S.E.M. values are indicated. Two experiments are shown (●, Expt. 1; ○, Expt. 2). At 4h and again at 2h before killing, each rat received intraperitoneal injections of 0.3ml of radioisotopically labelled DNA precursors in 0.9% NaCl. In Expt. 1 13nmol of thymidine containing 7.5 μCi from $[\text{Me-}^3\text{H}]$ thymidine and 133 nmol of orotic acid containing 7.5 μCi from $[\text{6-}^{14}\text{C}]$ orotic acid were administered in each 0.3ml injection. In Expt. 2 13 nmol of thymidine containing 7.5 μCi from $[\text{Me-}^3\text{H}]$ thymidine but no orotic acid was administered in each 0.3ml injection. Other results for these animals are summarized in Fig. 2.

plasmic fraction is predominantly of the slowly migrating forms, but that of the mitochondrial fraction is almost exclusively the fast-migrating form

(Taylor *et al.*, 1972). Adelstein *et al.* (1971) from studies with 10-day-old mice have suggested that thymidine kinase from the soluble fraction turns over much more rapidly than the enzyme from the mitochondrial fraction. The increase in 06:00h thymidine kinase activity up to 60h after partial hepatectomy (Fig. 3c) and the decrease after 60h is a pattern similar to that of pyruvate kinase type III in regenerating liver (Bonney *et al.*, 1973). Of the several possibilities, this pattern of thymidine kinase activities could result from appearance and disappearance of a form of the enzyme that has a longer half-life than the one responsible for the daily maximums at 18:00h or could also result from proliferation of non-parenchymal cells (see below under 'Cell populations in regenerating liver').

Correlation coefficients were calculated for thymidine incorporation over the 4h period before death (as abscissa) versus thymidine kinase activity at the time of killing by using the method of least squares. Correlation coefficients of 0.84 and 0.94 and slopes of 4.74 and 4.05 were obtained respectively for Expts. 1 and 2 in Fig. 3. Since thymidine must be phosphorylated before it can be incorporated into DNA, does this highly significant correlation indicate that the oscillations in DNA labelling from thymidine result from oscillations in the amount of thymidine kinase instead of oscillations in DNA synthesis? We have four independent lines of evidence that suggest that this is not the case. (1) Orotic acid incorporation into DNA also oscillates in phase with thymidine incorporation (Fig. 3b and Table 1). A correlation coefficient of 0.94 was found between thymidine incorporation (Fig. 3a, Expt. 1) and orotic acid incorporation (Fig. 3b) into DNA of regenerating liver. (2) Chemical determinations of DNA suggest that liver DNA accumulates faster during the dark periods of maximum thymidine incorporation (Table 3). (3) Thymidine kinase activity assays indicate the presence of the enzyme in both regenerating and intact livers of all rats so far examined. (4) Thymidine incorporation into DNA during the

1h period before death is well under way, although thymidine kinase at the time of killing is still relatively low under some conditions [compare the first peak in Fig. 4(a) with 4(b), and Fig. 4(c) with 4(d)].

Interaction between time of operation and labelling of DNA

The time of onset of thymidine or orotic acid incorporation into DNA after partial hepatectomy has been variously reported as 13, 18 or 22h (Bucher *et al.*, 1964; Lieberman, 1970; Hecht & Potter, 1956; Barbason & Lelievre, 1972). It can be seen from Figs. 4(a), 4(c) and 4(e) that the time of day at which rats are partially hepatectomized influences the response one sees in thymidine incorporation at various times after the operation. As the time of operation is shifted from 05:00h to 11:00h to 21:00h the amount of incorporation one sees at 15–16h after the operation varies and for these operating times it was at a minimum for the 11:00h operation. This observation apparently explains the various times of onset of incorporation found by the various laboratories. Barbason & Lelievre (1972) also observed differences in time of onset of incorporation when the time of operation was varied with respect to lighting schedules. Both Barbason & Lelievre (1972) and Burki *et al.* (1971) observed differences in the extent of precursor incorporation into DNA following partial hepatectomy when the time of operation was varied. It should be noted that, whereas the time of onset differed according to the time of operation in the present experiments, the time of the first maximum did not, but occurred at 20–23h after surgery, regardless of the time of operation. This is compatible with the assertion by Bucher & Malt (1971) that the first mitotic wave always appears at a constant time after surgery.

For the 05:00h operations (Fig. 4a) the second maximum for thymidine incorporation is near the end of the dark period, only about 18h after the first maximum. This is the same time of day that the

Table 3. *DNA content of regenerating livers*

Partial hepatectomies (70%) were done at 18:00h on rats entrained to the '8+16' controlled feeding schedule for about 3 weeks. Each value is the mean and S.E.M. for these rats.

Time (h) after partial hepatectomy	DNA (mg/liver per 100g body wt.)		DNA (mg/liver)	
	06:00h	18:00h	06:00h	18:00h
48		9.1 ± 0.06		13.3 ± 0.62
60	9.3 ± 0.24		13.0 ± 0.99	
72		11.4 ± 0.38		16.6 ± 1.4
84	11.5 ± 0.47		16.9 ± 5.5	
96		12.9 ± 0.24		17.6 ± 2.3
108	13.5 ± 0.65		18.7 ± 2.6

Fig. 4. [^3H]Thymidine incorporation into DNA and thymidine kinase activity in livers of rats adapted to the '8+16' feeding schedule and subjected to 70% partial hepatectomy at different times of day

Rats were subjected to 70% partial hepatectomy at about 05:00h (a and b), 11:00h (c and d), 21:00h (e and f) or to no operation (g and h). At various times of day each rat received a single 0.6–0.7 ml intraperitoneal injection of [$\text{Me-}^3\text{H}$]thymidine in 0.9% NaCl. After 1 h the rat was killed and [^3H]thymidine incorporation into DNA ($\circ$, $\bullet$) and thymidine kinase activity ($\square$, $\blacksquare$) was determined. Two experiments are shown ($\bullet$, $\blacksquare$, Expt. 1; $\circ$, $\square$, Expt. 2) for both the 05:00h and the 11:00h operations. For Expt. 2 in (a) and (b), each point represents the mean for two to three rats, with s.e.m. values indicated for three rats; in (g) and (h) each point represents the mean for four to five rats; in all other cases each point represents the mean for five to six rats. For (a) 130 nmol of thymidine containing 75 μCi of ^3H was injected per rat in Expt. 1 ($\bullet$) and 12 nmol of thymidine containing 75 μCi of ^3H was injected in Expt. 2 ($\circ$). For (c) 120 nmol of thymidine containing 30 μCi of ^3H was injected per rat in Expt. 1 ($\bullet$) but each mean and its s.e.m. has been multiplied by 2.5; in Expt. 2 ($\circ$) 12 nmol of thymidine containing 75 μCi of ^3H was injected. [Note: the remaining difference in extents of incorporation of [^3H]thymidine for the two experiments shown in (c) is considered to result from loss of DNA in Expt. 1 during storage at one point in the DNA extraction procedure. This procedural difficulty was avoided in all other experiments with '8+16' entrained rats.] For (e) 120 nmol and (g) 140 nmol of thymidine containing 88 μCi of ^3H was injected per rat.

intact rats showed a maximum incorporation (Fig. 1 and Fig. 4g). For the 18:00h operations (Fig. 3a) the incorporation values at 12h intervals would not permit recognition of shifts in the maxima of several hours, but within this limitation the results are consistent with a 24h rhythm of thymidine incorporation with maxima near the end of the dark periods. For rats accustomed to feeding for only one 8h period in each 48h [an '8+40' lighting and feeding schedule (Watanabe *et al.*, 1968)] the peak of thymidine

Fig. 5. DNA content (Δ), [^3H]thymidine incorporation ($\bullet$) into DNA and thymidine kinase activity ($\blacksquare$) in livers of rats adapted to the '8+40' feeding schedule and subjected to 70% partial hepatectomy at 21:00h

Partial hepatectomies were performed at about 21:00h either on the day the rats had access to food (a) or on the day the rats were starved (b). Each point represents the mean for five rats, except four rats in (b) at 32h. Each rat received a 0.5ml intraperitoneal injection containing 110nmol of thymidine ($62\mu\text{Ci}$ of ^3H) 1h before being killed. The DNA contents for these '8+40' entrained rats are lower than those reported herein for '8+16' entrained rats, but this may be attributable to the procedural difficulty noted in the legend for Fig. 4, since similar storage procedures were used in the two experiments. The open bars below the cross-hatched bars represent periods when food was available and the stippled bars indicate days on which food was withheld.

incorporation occurred near the end of the dark period about 23h after the operation whether the rat received food or not on the day following the operation (Fig. 5). Rats on the '8+16' schedule but starved on the day of operation and fed normally the next day show a pattern for thymidine incorporation similar to that in Fig. 5(b) (results not shown).

For the 11:00h operations the first maximum occurs near the start of the dark period, about 23h after the operation. For the two experiments reported here no maxima occurred near the end of the dark period about 32h after the operation; the incorporation of thymidine remained elevated in comparison with the 32h point for the 21:00h operation (compare Figs. 4c and 4e). Barbiroli & Potter (1971), by using the same protocol, observed a second maximum of incorporation near the end of the dark period. The present experiments were with rats 47 days old at the time of partial hepatectomy, but the rats in the experiments reported by Barbiroli & Potter (1971) were 58–62 days old.

Interaction between time of operation and thymidine kinase activity

The patterns obtained for thymidine kinase activity when rats on controlled feeding schedules were partially hepatectomized at 05:00, 11:00 and 21:00h (Figs. 4b, 4d and 4f respectively) have much in common with those obtained for thymidine incorporation into DNA. In each case there is a maximum of thymidine kinase activity near the end of the dark period. In addition, when there is a maximum in thymidine incorporation into DNA at a time other than near the end of the dark period, then the thymidine kinase activity is also elevated but is delayed about 3h behind the thymidine incorporation. For the 05:00h operations the maximum occurs at about 23h after the operation and the second peak near the end of the dark period is at 37h after the operation. For the 11:00h operation the thymidine kinase activity elevations occur about 3h after the thymidine incorporation maximum; this results in a leading shoulder or broad peak with a maximum of activity near the end of the dark period.

Phosphatases in regenerating liver

Reports that phosphatase amounts are decreased in regenerating liver compared with intact liver (Maley & Maley, 1961a,b; Beltz, 1962) prompted us to explore the possibility that the apparent thymidine kinase activities at various times after partial hepatectomy were influenced by the amount of phosphatase activity in the high-speed supernatants. Assays employing dTMP as the substrate in amounts similar to those generated in the thymidine kinase assay and under the conditions of the thymidine

kinase assay, except ATP and thymidine omitted, were done on supernatant fractions previously assayed for thymidine kinase. In relationship to the amount of remaining or regenerating liver we find (Fig. 6) no evidence for a significant decrease in thymidylate phosphatase activity following partial hepatectomy, although the possibility that this occurs at 24 or 36h cannot be excluded. The restoration of thymidylate phosphatase activity corresponds to the rate of restoration of liver weight, protein and DNA (compare Fig. 6 and Fig. 2). The phosphatase activity appears to be completely restored at 108h after operation, the liver thymidylate phosphatase activities for three intact rats at the same time of day and age (18:00h and 48 days old) were 439, 508 and 642munits/liver per 100g body wt.

Even though thymidylate phosphatase activity in regenerating liver is not decreased the thymidine kinase assay is not noticeably affected. ATP at the concentration used in the thymidine kinase assay decreases the phosphatase activity by 98%, being slightly more effective than 15mm-NaF in this respect (Table 4). This resolves the dilemma of Beltz (1962), who found the thymidine kinase assay to be unaffected by 16mm-NaF even though phosphatases that hydrolysed dTMP were known to be present in the assay mixtures. The decreased phosphatase activity in the presence of ATP or AMP (Table 4) is not a result of rephosphorylation of the thymidine produced, since AMP is almost as effective an inhibitor as ATP but does not act as a phosphate donor for thymidine kinase. Also these results are not consistent with the inhibition being a result of the depletion of the dTMP by further phosphoryla-

tion, since the thymidylate kinase and thymidine disphosphate kinase are reported to require ATP rather than AMP as the phosphate donor. The inhibition of phosphatases by ATP is not a result of chelation of Mg^{2+} , since ATP is an effective inhibitor even in the presence of large excesses of Mg^{2+} (results not shown). It is not known whether the decrease in thymidylate phosphatase activity is a result of the ATP or AMP serving as a competitive substrate or is a result of some other mechanism.

These results establish that the differences in amount of dTMP observed in the thymidine kinase assays that are reported here result from differences in the activity of thymidine kinase in the high-speed supernatants, not from various combinations of synthesis and degradation of dTMP. These results also establish that the activity is not the non-specific nucleoside phosphotransferase recently shown (Deng & Ives, 1972) to have confused the thymidine kinase literature, especially that relating to higher plants.

Cell populations in regenerating liver

Radioautographs prepared by the methods described by Rogers (1967) from the rats subjected to partial hepatectomy at 21:00h indicated that the initial peak of thymidine incorporation (Fig. 4e) for this time of operation represents primarily incorporation into parenchymal cells. At 16, 23, 27, 32 and 37h

Fig. 6. Thymidylate phosphatase activities in livers of rats adapted to the '8+16' feeding schedule and subjected to 70% partial hepatectomy at 18:00h

Each point represents the mean for three rats; S.E.M. values are indicated. ○, Thymidylate phosphatase activity; ---, liver protein.

Table 4. Inhibition of thymidylate phosphatase activity

The composition of the reaction mixture was: high-speed supernatant (25μl), 100mm-Tris-HCl (pH 8.0), 7mm-MgCl₂, 0.02mm-dTMP, in a total volume of 0.2ml containing 0.4μCi of radioactivity from [Me-³H]dTMP. Inhibitors were added at the following concentrations: 0.1mm-thymidine; 15mm-NaF; 10mm-ATP; 10mm-AMP; 10mm-EDTA.

Additions to reaction mixture	Decrease in activity (%)
None	0
Thymidine	8
Thymidine + ATP*	98
Thymidine + AMP*	93-95
Thymidine + EDTA	95
NaF	92
Thymidine + NaF	87-91
Thymidine + ATP + NaF	100

* In control experiments performed concurrently with these experiments, the AMP preparation did not serve as a phosphate donor in the thymidine kinase assay, whereas the ATP preparation resulted in a reaction rate of 11.3munits/g of liver.

after partial hepatectomy and 1 h after injection of [^3H]thymidine the labelled cells of each cell type as percentages of total cells counted (means and s.e.m. for six rats) were for parenchymal cells 14 ± 3.6 , 15 ± 4.1 , 3.9 ± 0.37 , 3.4 ± 0.42 and 4.3 ± 0.34 and for non-parenchymal (ductal and littoral) cells 0.97 ± 0.24 , 1.5 ± 0.38 , 2.2 ± 0.35 , 2.5 ± 0.48 and 3.2 ± 0.36 respectively. These patterns are entirely consistent with those reported by Grisham (1962), where 2 h after injection of [^3H]thymidine maximum labelling of parenchymal cells occurred at about 20 h and maximum labelling of both ductal and littoral cells occurred between 36 and 42 h after partial hepatectomy.

In view of the slow gradual increase in labelling of non-parenchymal cells until at least 37 h after partial hepatectomy, it is possible that the 24 h rhythm in thymidine incorporation is occurring only within the parenchymal cell population.

Salvage versus 'de novo' pathways

We have been unable to influence the radioactivity found in DNA of regenerating liver by dilution of the [^3H]thymidine precursor with non-labelled thymidine. This is apparent from Fig. 4(a) where similar amounts of radioactivity but different amounts of total thymidine were injected in the two experiments. The identity and concentration of non-radioactive thymidine preparations from two different sources were verified spectrophotometrically and both of these preparations effectively competed with [$\text{Me-}^3\text{H}$]thymidine in a tissue-culture system.

Direct comparisons of three thymidine concentrations were made in conjunction with Expt. 2, Fig. 4(c). Injections of 12, 130 and 250 nmol of thymidine containing 75 μCi of [$\text{Me-}^3\text{H}$]thymidine gave incorporations of $7.7 \times 10^6 \pm 0.99 \times 10^6$, $6.4 \times 10^6 \pm 0.62 \times 10^6$ and $7.1 \times 10^6 \pm 0.51 \times 10^6$ d.p.m./liver at 23 h after partial hepatectomy and $3.8 \times 10^6 \pm 0.63 \times 10^6$, $3.6 \times 10^6 \pm 0.91 \times 10^6$ and $2.6 \times 10^6 \pm 0.71 \times 10^6$ d.p.m./liver at 32 h respectively. It is apparent that these changes in incorporation are minor in relation to the changes in the specific radioactivity of the thymidine injected (a comparison of means using Student's *t* test gave values of $P = 0.3$ to $P = 0.9$). In another experiment with the same operating schedule as for Fig. 4(c), injections of 8 nmol of thymidine or 1000 nmol of thymidine containing 50 μCi of radioactivity from [$\text{Me-}^3\text{H}$]thymidine gave the same d.p.m. in the liver DNA. Gebert (1967, p. 111) reported that 'within the range (4–400 nmol) the specific radioactivity of the liver DNA appears to be independent of the total amount of thymidine injected and a function only of the number of μcuries injected. Beyond this limit a further dilution of the radioisotope with unlabelled thymidine produced a decrease in the specific radioactivity of the liver DNA'. In Gebert's experiments

the intact rats were killed 30 min after the intraperitoneal injection of the radioisotope. In the experiments reported here the partially hepatectomized rats were killed 60 min after the radioisotope injection.

The fact that the amount of radioactivity incorporated into liver DNA is quite independent of the specific radioactivity of the injected thymidine over a considerable range of nmol seems paradoxical but is the resultant of several factors. First, it appears that at the cellular location of thymidine kinase the concentration of thymidine is sufficiently low when low amounts of thymidine are injected that an increase of substrate results in a proportional increase in the rate of its conversion into dTMP, a situation that has its counterpart in enzyme kinetics *in vitro*. Secondly, at the high end of the range used the time required for catabolic degradation of thymidine is prolonged, permitting the thymidine to be available for phosphorylation for a longer period (Morse, 1963, p. 40). In addition it appears that the contribution by injected thymidine must be small in comparison with the contribution *de novo* or the pathway *de novo* must be subject to increasing negative feedback as the amount of injected thymidine is increased (Maley & Maley, 1963).

Using Quastler's four-factor model for thymidine metabolism and making several simplifying assumptions, Cleaver (1967, p. 76) presents equations for 'trace' and 'flooding' labelling of DNA, with and without end-product inhibition. For the flooding condition incorporation of radioactive thymidine into DNA was proportional to specific radioactivity, as intuitively expected, but for trace labelling without end-product inhibition incorporation was proportional to the product of the amount of thymidine used and its specific radioactivity, that is to the total number of μcuries injected.

We have found that when the [^{14}C]orotic acid incorporation results from Fig. 3(b) (as ordinate) are plotted against [^3H]thymidine incorporation results from Fig. 3(a), the experimental points fall on either of two lines (not shown), one having a slope of 0.47 (and a correlation coefficient of 0.89) and the other having a slope of 0.20 (and a correlation coefficient of 0.94). The points forming the line with greater slope correspond to regenerating livers at 24, 48 and 72 h following surgery and killed at 18:00 h, that is during the rapid proliferative phase of regeneration. Those points forming the line with lesser slope correspond to regenerating livers at 120, 168 and 336 h following surgery, after liver cell proliferation has been essentially completed. The values for rats killed at 06:00 h, 36 h after surgery, fall on the line with lesser slope.

In growing rats at about 28, 35 and 48 days of age a similar situation is seen with corresponding plots of the results listed in Table 1. At the time of day

(18:00h) when thymidine kinase and thymidine incorporation are greatest, a slope of 0.44 (correlation coefficient of 0.91) is obtained and at 06:00h a slope of 0.18 (correlation coefficient of 0.95) is obtained. Our results are not conclusive on this point since fluctuations in the pyrimidine ribonucleotide pools may be occurring but the slopes of the plots of orotic acid versus thymidine incorporation into DNA suggest that the pathway *de novo* makes a proportionally greater contribution to the thymidylate pool during active proliferation than at other times; stated another way, the salvage pathway may assume a greater relative importance when growth is essentially completed.

Discussion

The use of controlled feeding schedules and the planning and interpretation of regenerating liver experiments in terms of diurnal rhythms is yielding new insights into this old experimental problem. The interaction of the biological clock (which is entrained to a diurnal rhythm by the controlled feeding and lighting schedule) with the anabolic processes leading to hyperplasia results in a stepwise replacement of liver mass following partial hepatectomy.

Few of the regenerating liver experiments reported have yielded results that can be reinterpreted with respect to the diurnal rhythms shown to be operating. Serious misinterpretations of the regenerative process have resulted from attempts to avoid diurnal rhythms. For example, when the results for rats killed at 20:00h or 00:00h in Figs. 4(a), 4(c) and 4(e) are plotted according to Bucher *et al.* (1964), biphasic curves compatible with the one they reported for weanling rats are obtained. In attempting to avoid diurnal variations in DNA labelling by killing at a constant time of day and operating around the clock, Bucher *et al.* (1964) made the unwarranted assumption that the physiological state of the animal in terms of its oscillating systems at the time of operation and during the subsequent events required for regeneration did not affect the response obtained. Instead of avoiding the diurnal variation, it was simply obscured and the curves obtained from such results could not represent the path by which the liver is regenerated in a single rat. The results for thymidine kinase and other enzymes involved in DNA synthesis in regenerating liver (Labow *et al.*, 1969) and the results indicating a delaying effect of hydrocortisone on precursor incorporation into DNA of regenerating liver (Rizzo *et al.*, 1971) were obtained by killing at a constant time of day and so also fail to describe sequential changes during liver regeneration.

Increasing age has been reported to delay the regeneration of liver in rats (Bucher *et al.*, 1964); this effect is now seen as an effect of age on the time of onset of the first wave of DNA synthesis following

partial hepatectomy at some, but perhaps not all, times of day. Any re-investigation of the effect of age on liver regeneration should examine not only the timing and height of the first wave of DNA synthesis but the number of waves required to restore the liver mass. DNA cannot be used as a basis for comparison when its concentration is changing. Our method of expressing results per liver per 100g body wt. allows one to see and evaluate the changes that occur at longer intervals of time than is possible when concentration expressions or total values are used.

The processes that bring a liver cell up to the S phase (Howard & Pelc, 1953) appear to require at least 12h for completion; if this time-interval is satisfied before a certain critical time in the light period is reached, the cell will enter S phase immediately. If this time-interval is not satisfied until after the critical time, the cell will delay entry into S phase until the 'gate' (Edmunds & Funch, 1969) again permits DNA synthesis, near the onset of darkness. Delayed entry should result in increased synchrony and a shorter period of DNA synthesis for this cell cycle only. This is consistent with our results but these are by no means conclusive on this point.

An earlier model (Barbiroli & Potter, 1971) postulated two groups or lots of cells. 'The duplication of the first of the liver cells has a high priority' and these cells respond to the operation with a peak of thymidine incorporation 23h after the operation, independent of the timing of the operation. Under certain operating times a second lot of cells responds to the operation, resulting in a second peak of thymidine incorporation into DNA that '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'. Non-replicating cells are depicted by Gelfant & Smith (1972) as being G₁-blocked or G₂-blocked, with the proportion of cells in the second category increasing with aging. The release from the G₁ block may result in the first peak of thymidine incorporation and a delayed release from the G₂ block or delays because of controls on the subsequent mitosis and G₁ phase of the cycle would be consistent with the liver cells of somewhat older rats in the Barbiroli & Potter (1971) model responding near the end of the dark period with thymidine incorporation, irrespective of the timing of the operation.

We thank Professor Eulyn L. Jensen, Dairy Science Department, for assistance in statistical analysis, M. Lichtenstein, C. Dorn and M. Padway for maintaining the feeding regimens, M. Lichtenstein and M. Mecikalski for assistance with the chemical assays and K. Hilmer for processing the radioautographs and for counting cells labelled by radioactivity. Financial support was provided in part by Department Grant CA-07175 and by Training Grant T01-CA-5002 from the National Cancer Institute.

References

- Adelstein, S. J., Baldwin, C. & Kohn, H. I. (1971) *Develop. Biol.* **26**, 537-546
- Barbason, H. & Lelievre, P. (1972) *C. R. Acad. Sci. Ser. D* **274**, 585-587
- Barbiroli, B. & Potter, V. R. (1971) *Science* **172**, 738-741
- Baril, E. F. & Potter, V. R. (1968) *J. Nutr.* **95**, 228-237
- Barnum, C. P., Jardetsky, C. D. & Halberg, F. (1957) *Tex. Rep. Biol. Med.* **15**, 134-147
- Beltz, R. E. (1962) *Arch. Biochem. Biophys.* **99**, 304-312
- Bonney, R. J., Hopkins, H. A., Walker, P. R. & Potter, V. R. (1973) *Biochem. J.* **136**, 115-124
- Bresnick, E. (1971) *Methods Cancer Res.* **6**, 347-397
- Bresnick, E. & Burleson, S. S. (1970) *Cancer Res.* **30**, 1060-1063
- Bresnick, E., Williams, S. S. & Mosse, H. (1967) *Cancer Res.* **27**, 469-475
- Bucher, N. L. R. (1963) *Int. Rev. Cytol.* **15**, 245-300
- Bucher, N. L. R. (1967a) *N. Engl. J. Med.* **277**, 686-696
- Bucher, N. L. R. (1967b) *N. Engl. J. Med.* **277**, 738-746
- Bucher, N. L. R. & Malt, R. A. (1971) *Regeneration of Liver and Kidney*, Little, Brown and Co., Boston
- Bucher, N. L. R., Swaffield, M. N. & DiTroia, J. F. (1964) *Cancer Res.* **24**, 509-512
- Burki, K., Schindler, R. & Pfenninger, M. (1971) *Cell Tissue Kinet.* **4**, 529-537
- Burton, K. (1956) *Biochem. J.* **62**, 315-322
- Cleaver, J. E. (1967) *Thymidine Metabolism and Cell Kinetics*, North-Holland Publishing Co., Amsterdam
- Deng, Q. & Ives, D. H. (1972) *Biochim. Biophys. Acta* **277**, 235-244
- Edmunds, L. N., Jr. & Funch, R. R. (1969) *Science* **165**, 500-503
- Gebert, R. A. (1967) Ph.D. Dissertation, University of Wisconsin, Madison
- Gelfant, S. & Smith, J. G. (1972) *Science* **178**, 357-361
- Grisham, J. W. (1962) *Cancer Res.* **22**, 842-849
- Halberg, F., Barnum, C. P., Siebert, R. H. & Bittner, J. J. (1958) *Proc. Soc. Exp. Biol. Med.* **97**, 897-900
- Harkness, R. D. (1957) *Brit. Med. Bull.* **13**, 87-93
- Hecht, L. I. & Potter, V. R. (1956) *Cancer Res.* **16**, 988-993
- Higgins, G. M. & Anderson, R. M. (1931) *Arch. Pathol.* **12**, 186-202
- Hopkins, H. A., Flora, J. B. & Schmidt, R. R. (1972) *Arch. Biochem. Biophys.* **153**, 845-849
- Howard, A. & Pelc, S. R. (1953) *Heredity Suppl.* **6**, 261-273
- Ives, D. H., Durham, J. P. & Tucker, V. S. (1969) *Anal. Biochem.* **28**, 192-205
- Jaffee, J. J. (1954) *Anat. Rec.* **120**, 935-954
- Kambara, T., Hisaka, M. & Eto, H. (1964) *Kumamoto Med. J.* **17**, 18-30
- Labow, R., Maley, G. F. & Maley, F. (1969) *Cancer Res.* **29**, 366-372
- Leduc, E. H. (1964) in *The Liver: Morphology, Biochemistry, Physiology* (Rouiller, C., ed.), vol. 2, pp. 63-89, Academic Press, New York
- Lee, K., Sun, S. & Miller, O. N. (1968) *Arch. Biochem. Biophys.* **125**, 751-757
- Lieberman, I. (1970) *In Vitro* **6**, 46-57
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Maley, F. & Maley, G. F. (1961a) *Cancer Res.* **21**, 1421-1426
- Maley, F. & Maley, G. F. (1961b) *Biochim. Biophys. Acta* **47**, 181-183
- Maley, G. F. & Maley, F. (1963) *Science* **141**, 1278-1279
- Mayfield, J. E. & Bonner, J. (1972) *Proc. Nat. Acad. Sci. U.S.* **69**, 7-10
- Morse, P. A. (1963) Ph.D. Dissertation, University of Wisconsin, Madison
- Potter, V. R. (1970) *Miami Winter Symp.* **2**, 291-313
- Potter, V. R., Baril, E. F., Watanabe, M. & Whittle, E. D. (1968) *Fed. Proc. Fed. Soc. Exp. Biol.* **27**, 1238-1245
- Rizzo, A. J., Heilpern, P. & Webb, T. E. (1971) *Cancer Res.* **31**, 876-881
- Rogers, A. W. (1967) *Techniques of Autoradiography*, Elsevier Publishing Co., London
- Schmidt, G. & Thannhauser, S. J. (1945) *J. Biol. Chem.* **161**, 83-89
- Schneider, W. C. (1945) *J. Biol. Chem.* **161**, 293-303
- Short, J., Brown, R. F., Husakova, A., Gilbertson, J. R., Zemel, R. & Lieberman, I. (1972) *J. Biol. Chem.* **247**, 1757-1766
- Taylor, A. T., Stafford, M. A. & Jones, O. W. (1972) *J. Biol. Chem.* **247**, 1930-1935
- Watanabe, M., Potter, V. R. & Pitot, H. C. (1968) *J. Nutr.* **95**, 207-227
- Younger, L. R., King, J. & Steiner, D. F. (1966) *Cancer Res.* **26**, 1408-1414