present experiments C1 in serum was subjected only to the forces of diffusion, whereas in the cited studies (*6, 7*) additional stresses on the molecule, either electrical or centrifugal, were imposed. Since our earlier work has indicated that C1 has a tendency to dissociate (*7*), we think that our current studies, which indicate no free C1 subunits, are more representative of the status of C1 in normal serums. It is, however, quite likely that free C1 subunits will be found in serums from patients with various diseases, and some evidence for this has been obtained by Laurell *et al.* (*6*). The techniques presented here will permit a careful analysis of this possibility.

R. J. ZICCARDI, N. R. COOPER
Department of Molecular Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037

References and Notes

1. I. H. Lepow *et al.*, *J. Exp. Med.* **117**, 983 (1963); H. J. Müller-Eberhard and H. G. Kunkel, *Proc. Soc. Exp. Biol. Med.* **106**, 291 (1961); M. M. E. De Bracco and R. M. Stroud, *J. Clin. Invest.* **50**, 838 (1971); K. Sakai and R. M. Stroud, *J. Immunol.* **110**, 1010 (1973).
2. M. A. Calcott and H. J. Müller-Eberhard, *Biochemistry* **11**, 3443 (1972).
3. R. J. Ziccardi and N. R. Cooper, *J. Immunol.* **116**, 496 (1976).
4. G. Valet and N. R. Cooper, *ibid.* **112**, 339 (1974).
5. G. B. Naff, J. Pensky, I. H. Lepow, *J. Exp. Med.* **119**, 593 (1964).
6. A.-B. Laurell, U. Mårtensson, A. G. Sjöholm, *Acta Pathol. Microbiol. Scand. Sect. C* **84**, 455 (1976).
7. R. J. Ziccardi and N. R. Cooper, *J. Immunol.* **118**, 2047 (1977).
8. A.-B. Laurell and U. Mårtensson, *Acta Pathol. Microbiol. Scand. Sect. B* **82**, 585 (1974).
9. R. J. Ziccardi and N. R. Cooper, *J. Exp. Med.*, in press.
10. This is publication No. 1363 from Scripps Clinic. This work was supported by PHS grants AI 07007 and CA 14692 from NIH. R.J.Z. is the recipient of an NIH fellowship award 1 F32 AI 05400-01. We thank J. Harper Ziccardi for outstanding technical assistance and C. Davis for excellent secretarial services.

18 July 1977; revised 14 November 1977

Diurnal Rhythm: Effects on Hepatic Regeneration and Hepatic Regenerative Stimulator Substance

Abstract. *The effect of a controlled lighting schedule on the activity of a weanling rat liver extract that stimulates DNA synthesis in regenerating adult rat liver, and on the response of the test animals to the extract, has been investigated. Both activity of the extract and endogenous DNA synthesis in the weanling animals follow the same distinct diurnal rhythm. Reversal of the lighting schedule reverses the rhythm of endogenous DNA synthesis but activity of the extract no longer correlates with the peak of DNA synthesis. Diurnal rhythm also has a striking effect on DNA synthesis in the regenerating test animal, but the extract increases DNA synthesis to the same relative degree, regardless of the time of day the hepatectomy is performed.*

The mammalian liver possesses a remarkable capacity for hypertrophy and hyperplasia in response to a variety of stimuli (*1*). However, the mechanism or mechanisms that control the growth spurt have remained elusive (*2*).

An extract of weanling rat liver can stimulate incorporation of [methyl-^{3}H]-thymidine ([^{3}H]Tdr) into the DNA of regenerating adult rat liver (*3*). This hepatic regenerative stimulator substance (SS) was demonstrable in adult rat liver only following partial hepatectomy, when it appeared prior to the first wave of DNA synthesis and remained demonstrable for 3 days after the hepatectomy. The SS was heat-stable and acid-labile, was not dialyzable, and had a molecular weight of about 10,000 (*3, 4*).

Both normal and regenerating rodent liver display distinct diurnal rhythms of mitotic activity and [^{3}H]Tdr incorporation into DNA (*5*). Barbiroli and Potter (*6*), by controlling the periods of light and food availability, have demonstrated the existence of at least two different regulatory systems for DNA synthesis in the regenerating rat liver. Synthesis was maximal 23 hours after hepatectomy, regardless of the time of day hepatectomy was performed. However, this phenomenon was superimposed on a constant underlying rhythm of endogenous DNA synthesis that was entrained to the light: dark and feeding schedule.

We have examined the relationship of diurnal rhythms to the presence of SS in the donor animal and the responsiveness of the test animal. Male Sprague-Dawley rats were used as donors (50- to 60-g weanlings) and test animals (170- to 190-g adults). All experimental animals were caged in a windowless, air-conditioned room with lighting controlled by a timer to provide darkness from 7:00 p.m. to 7:00 a.m. For some experiments, lighting was reversed to provide darkness from 7:00 a.m. to 7:00 p.m. Food and water were freely available, and all animals were acclimatized to one of the lighting regimens for 7 to 10 days before experimentation.

To determine any diurnal variations in the presence of SS, it was prepared at 3-hour intervals over a 24-hour period from donor weanling rats. A modified extraction method produced a more purified and stable extract than that previously reported (*3*). Livers were removed from donor animals and rapidly cooled in iced saline. The pooled livers were homogenized in 0.9 percent NaCl (35 percent, weight to volume) in a chilled Omnimixer (Sorvall). The homogenate was heated at 65°C for 15 minutes in a water bath and centrifuged at 27,000*g* for 10 minutes. The supernatant solution, designated SS, was lyophilized and stored at −20°C until assayed for stimulator activity.

The assay uses 170- to 190-g male Sprague-Dawley rats, housed under the same "normal" conditions (darkness from 7:00 p.m. to 7:00 a.m.) as test animals. Between 1:00 and 2:30 p.m., the left-lateral lobe of the liver was removed from the test animals under light ether anesthesia (about 34 percent hepatectomy). Lyophilized SS was reconstituted to its original volume with water, and at 4 to 6 hours after the hepatectomy, 5 ml was injected intraperitoneally into the test animals. At 23 hours after the hepatectomy, the animals were killed, the livers were removed, and [^{3}H]Tdr incorporation into liver DNA of each rat was determined in vitro in a tissue slice assay (*3*). Liver DNA was extracted and quantified by measuring absorbance at 260 nm (A_{260}) (*7*). Incorporation of [^{3}H]Tdr was determined by adding a portion of the purified DNA to Aquasol (New England Nuclear) and assaying the radioactivity in a scintillation spectrometer. Data are expressed as disintegrations per minute (dis/min) per *A* unit of DNA. Control animals underwent an identical operative procedure except for the actual removal

Table 1. Diurnal variation in response to partial hepatectomy and injection with hepatic regenerative stimulator substance (SS). Data are expressed as means ($\bar{X}$) and standard errors of the mean (S.E.M.) and were analyzed with *t*-tests.

Time of operation	Incorporation of [^{3}H]Tdr (dis/min per *A* unit of DNA)						*P*
	Saline injection			SS injection			
	N	$\bar{X}$	S.E.M.	*N*	$\bar{X}$	S.E.M.	
9:00 to 11:00 a.m.	12	3860	555	16	9633	1658	< .01
1:30 to 3:00 p.m.	56	804	56	86	1994	210	< .01

0036-8075/78/0310-1082$00.50/0

of the liver and were injected intraperitoneally with 5.0 ml of 0.9 percent NaCl.

Normal endogenous DNA synthesis in the weanling rat liver was also determined by tissue slice assay of incorporation of [^{3}H]Tdr into DNA of livers prepared from weanlings killed at 3-hour intervals over a 24-hour period. The weanling rats demonstrated a biphasic incorporation of [^{3}H]Tdr into liver DNA with maximum incorporation during the dark phase from 12:00 midnight to 9:00 a.m. (Fig. 1A). When the lighting sequence was reversed, thymidine incorporation was reversed with DNA uptake rising sharply at 12:00 noon and remaining increased until 9:00 p.m. (Fig. 1B).

The SS extracted from these animals showed a similar diurnal rhythm. Under normal lighting conditions extracts prepared from animals between midnight and 9:00 a.m. gave more than a twofold stimulation of [^{3}H]Tdr incorporation, whereas no activity was measurable at other times (Fig. 1C). Reversing the light:dark cycle for 6 to 8 days caused the SS diurnal rhythm to disappear and SS to be detectable over the entire 24 hours (Fig. 1D). Further acclimatization of the donor rats to the reversed lighting for a total of 14 to 18 days resulted in another diurnal pattern (Fig 1E), with maximum SS activity between 6:00 a.m. and 3:00 p.m. and no SS activity between 6:00 p.m. and 3:00 a.m.

Our results demonstrate a diurnal rhythm of DNA synthesis in weanling rat liver and its close relationship to the cycle of light and dark. Under normal lighting conditions, the SS content of the weanling liver followed a pattern similar to that of the endogenous DNA synthesis. The parallelism and timing of SS presence and DNA synthesis in rapidly growing weanling rats and the appearance of SS in the liver of adult rats prior to the increase in DNA synthesis induced by a partial hepatectomy (*3*) suggest a causative role. However, changing the light:dark cycle readily reversed the diurnal rhythm of DNA synthesis in the weanling but did not have clear-cut effects on SS activity. Thus it seems unlikely that SS exerts a controlling influence on normal DNA synthesis, although it clearly stimulates regenerative growth (*3*).

Since liver regeneration has a diurnal periodicity, it was necessary to examine the possibility that the test animal might respond differently to SS at different times of the day. To answer this question, SS was prepared between 8:00 and 8:30 a.m. lyophilized, and frozen. It was tested in adult rats hepatectomized between 9:00 and 11:00 a.m. or 1:30 and 3:00 p.m. (Table 1).

There was a substantial decrease in incorporation of [^{3}H]Tdr in the afternoon experiments, the SS-injected animals having a mean value only one-half that of the morning controls. However, when compared with appropriate afternoon control animals, a 2.5-fold stimulation was again produced, comparable to that produced by SS injection of the morning animals. This substantial difference in values was obtained despite a mere 2- to 3-hour difference in the time of hepactectomy; it could easily have been misinterpreted as an inhibitory effect rather than a stimulatory one had inappropriate controls been used. Had experiments been carried out in both morning and afternoon, with no regard to time of hepatectomy, a wide range of overlapping values would have obscured any effect. Similar-

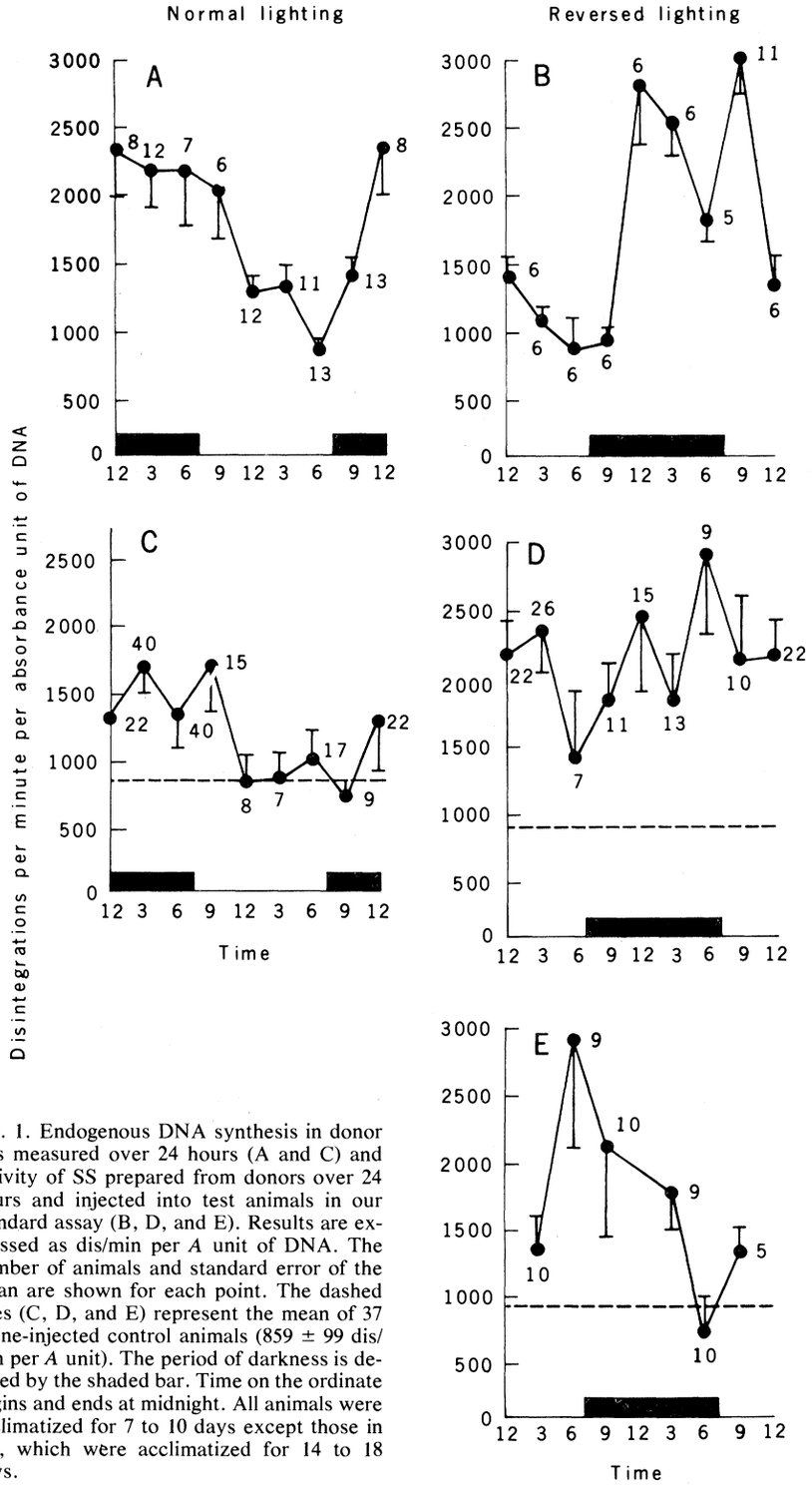

Fig. 1. Endogenous DNA synthesis in donor rats measured over 24 hours (A and C) and activity of SS prepared from donors over 24 hours and injected into test animals in our standard assay (B, D, and E). Results are expressed as dis/min per *A* unit of DNA. The number of animals and standard error of the mean are shown for each point. The dashed lines (C, D, and E) represent the mean of 37 saline-injected control animals (859 ± 99 dis/min per *A* unit). The period of darkness is denoted by the shaded bar. Time on the ordinate begins and ends at midnight. All animals were acclimatized for 7 to 10 days except those in (E), which were acclimatized for 14 to 18 days.

ly, as shown by the first set of experiments, we would have been led to believe that there was no stimulatory factor present had we not prepared our SS early in the morning.

The time of injection after hepatectomy is also important. Our earlier work (*3*) showed a time lag between injection of SS and the appearance of its effect. The alternative interpretation, assumed by Llanos in his work with mice (*8*), that the absence of an effect is due to the different time of day at which the injection is given, does not appear to operate in these experiments since the same stimulation was produced regardless of the time of day.

Variations in the donor animals must also be carefully controlled. Not only is the time of preparation of the SS from the donor important (as shown in this report) but also, with increasing age of the donor animals, the stimulatory effect disappears (*3*).

The problem of determining what so elegantly controls liver regeneration has been intriguing and elusive. The necessity of using a live animal model produces additional variables, difficult to control and not present in classical cell-free biochemical studies or in work with tissue culture, isolated perfused organs, or isolated cell suspensions. Failure to appreciate and to control adequately some of the readily accessible variables has led to much of the confusion and dispute in this literature (*1, 2*). Diurnal rhythm affects a variety of liver functions (*9*) including endogenous and regenerative growth (*5, 6*) and must be carefully regarded in studies of these phenomena.

DOUGLAS R. LABRECQUE*
ALAN FEIGENBAUM
NICHOLAS R. BACHUR
Baltimore Cancer Research Center, Baltimore, Maryland 21211

References

1. N. L. R. Bucher, *Int. Rev. Cytol.* **15**, 245, (1963).
2. ———, *N. Engl. J. Med.* **277**, 686 and 738 (1967); K. Weinbren, *Gastroenterology* **37**, 657 (1959).
3. D. R. LaBrecque and L. A. Pesch, *J. Physiol. (London)* **248**, 273 (1975).
4. ———, N. R. Bachur, *Gastroenterology* **66**, 728 (1974).
5. J. J. Jaffee, *Anat. Rec.* **120**, 935 (1954); B. Jackson, *ibid.* **134**, 365 (1959); C. P. Barnum, C. D. Jardetsky, F. Halberg, *Tex. Rep. Biol. Med.* **15**, 134 (1957); J. M. Echave Llanos, M. E. Aloisso, M. Souto, R. Balduzzi, J. M. Surur, *Virchows Arch. B* **8**, 309 (1971).
6. B. Barbiroli and V. R. Potter, *Science* **172**, 738 (1971).
7. K. Weinbren and E. Woodward, *Br. J. Exp. Pathol.* **45**, 442 (1964).
8. J. M. Echave Llanos, in *Control of Cellular Growth in Adult Organisms*, H. Teir and T. Rytomaa, Eds. (Academic Press, London, 1967), p. 209.
9. E. F. Baril and V. R. Potter, *J. Nutr.* **95**, 228 (1968); V. R. Potter, E. F. Baril, M. Watanabe, E. D. Whittle, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **27**, 1238 (1968); H. A. Hopkins, R. J. Bonney, P. R. Walker, J. D. Yager, Jr., V. R. Potter, *Adv. Enzyme Regul.* **11**, 169 (1973); K. Letnansky, *FEBS Lett.* **32**, 270 (1973).

* Present address: Veterans Administration Hospital, Iowa City, Iowa 52240.

26 September 1977

Enhancement of Bovine Pancreatic Ribonuclease Activity by Mercaptoethanol

Abstract. *Incubation of ribonuclease with 0.1*M *mercaptoethanol at* p*H 8.5 can increase the enzyme's hydrolytic activity toward cytidine 2',3'-monophosphate (cyclic CMP) under standard assay conditions. Cation-exchange chromatography of the ribonuclease-thiol reaction mixture revealed seven fractions. The fraction with the highest activity had an approximate tenfold decrease in the apparent Michaelis constant for cyclic CMP with respect to native ribonuclease. The enhanced activity is a metastable property since this fraction reverts back to the control activity and chromatographic behavior of native ribonuclease on standing in solution at room temperature.*

While developing a method for the selective ^{13}C enrichment of the *S*-methyl carbon of methionine-29 in bovine pancreatic ribonuclease for nuclear magnetic resonance studies, we unexpectedly prepared ribonuclease derivatives with enhanced activity toward cytidine 2',3'-monophosphate (cyclic CMP). The labeling reaction requires two steps: (i) the methylation of ribonuclease with $^{13}CH_3I$, which produces *S*-methylmethionine-29 ribonuclease (*1*), and (ii) the regeneration of the native methionyl residue via a nucleophilic displacement of one of the *S*-methyl groups by 2-mercaptoethanol (*2*). This reaction sequence results in a product that incorporates 50 percent of the ^{13}C label (*3*).

Unfortunately, ribonuclease contains four disulfide bonds which potentially could be reduced by the thiol leading to a disruption of the enzyme's tertiary structure. During trial experiments to determine what concentration of mercaptoethanol native ribonuclease would tolerate without a loss in activity, we observed an unexpected increase in ribonuclease activity.

Ribonuclease (Worthington RAF, phosphate free and monophoretic on gel electrophoresis) was incubated with mercaptoethanol (0.025 to 0.200*M*) in 0.2*M* phosphate at *p*H 8.5 and room temperature. The thiol concentration and enzyme activity were measured periodically as described in Fig. 1. With initial thiol concentrations below 0.1*M*, the activity remained at control levels for about 3 days before the increase in activity was observed (Fig. 1A). With greater than 0.1*M* thiol, we found an initial decrease in enzymatic activity which returned to and eventually increased above the control level.

We have repeated similar experiments seven times and, although the maximum activity reached (160 to 220 percent of control), the time to peak enhancement (10 to 19 days), and the residual thiol concentration at the activity maximum (0 to 40 percent of the initial level) varied, the general pattern of activity enhancement was always the same. This enhancement effect and the variability observed appeared to be a function of the degree of exposure of the samples to the air, which, in turn, controlled the rate of thiol loss via autoxidation.

The importance of sample exposure to the air is demonstrated in Fig. 1B and Fig. 2. No increase in activity was found in any of the samples incubated in syringes to exclude air (Fig. 1B). After 19 days of incubation, the thiol concentration decreased from 0.1 to 0.08*M* while the ribonuclease activity simultaneously decreased to 80 percent. In contrast, the 0.1*M* sample incubated for the same length of time in the test tubes (Fig. 1A) had a residual thiol concentration of 0.032*M* and a ribonuclease activity of 178 percent of control. If greater exposure to the air was ensured by incubating the samples in open beakers (Fig. 2), then maximum activity was generated in only 4 to 5 days. These data suggested that the oxidation of mercaptoethanol to diethanoldisulfide may be involved in the enhancement process. However, when diethanoldisulfide (0.05 to 0.50*M*) was included in the reaction mixtures in either the test tubes or the beakers (Fig. 2), the rate of thiol decrease, the time required for the generation of peak activity, and the degree of activity enhancement were much the same as observed in the absence of the disulfide. Apparently, either sufficient amounts of disulfide were generated by mercaptoethanol autoxidation or the decrease in thiol level per se rather than the simultaneous increase in the disulfide concentration was the cause of activity enhancement.

0036-8075/78/0310-1084$00.50/0