Biochemical Pharmacology, Vol. 21, pp. 3257–3265. Pergamon Press, 1972. Printed in Great Britain.

PROLONGATION OF THE LAG PERIOD PRECEDING THE ENHANCEMENT OF THYMIDINE AND THYMIDYLATE KINASE ACTIVITY IN REGENERATING RAT LIVER BY 5-AZACYTIDINE

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(*Received* 1 *May* 1972; *accepted* 7 *July* 1972)

Abstract—The administration of 5-azacytidine to rats 1 hr after partial hepatectomy leads to a complete inhibition of thymidine and thymidylate kinase in 24 hr-regenerating liver and to a prolongation of the lag period preceding their increased activity. While 5-azacytidine does not affect DNA synthesis in the liver of sham-operated animals, complete inhibition of its formation in 24 hr-regenerating liver was observed. Inhibition of liver protein synthesis following 5-azacytidine has a different time course and is in agreement with the degradation of liver polyribosomes. Maximal polyribosome degradation and the greatest inhibition of protein synthesis in regenerating liver occur 2–8 hr after 5-azacytidine administration. Decreased thymidine and thymidylate kinase activity in relation to the restored proteosynthetic capacity of the liver after 5-azacytidine administration is discussed.

5-AZACYTIDINE possesses considerable biological activity[1] which is primarily associated with its incorporation into different types of liver ribonucleic acids.[2] We supposed that, utilizing the inhibitory properties of this compound,[3–5] it would be of interest to study the processes taking place in the liver after partial hepatectomy. Regenerating liver is characterized by a specific sequence of metabolic reactions,[6–8] and a number of compounds have been used recently with a similar purpose.[9–13]

In a manner analogous to enzyme induction, liver regeneration is mediated by short-lived RNA molecules synthesized soon after partial hepatectomy.[14,15] On account of this similarity and in view of the known effect of 5-azacytidine on enzyme induction,[3,16] alterations of enzymes taking part in DNA formation during liver regeneration have been investigated. The synthesis of DNA is accomplished by different enzyme systems among which thymidine and thymidylate kinases, which are responsible for the synthesis of thymidine 5′-phosphates, are especially important.[17,18]

MATERIALS AND METHODS

Chemicals. Thymidine 5′-monophosphate, thymidine 5′-triphosphate and adenosine 5′-triphosphate were supplied by CalBiochem, Los Angeles. 5-Azacytidine was synthesized in this Institute. Thymidine-2-^{14}C, thymidine-2-^{14}C 5′-monophosphate (24 μCi/μmole) and L-valine-U-^{14}C (39 μCi/μmole) were obtained from the Institute for Research, Production, and Uses of Radioisotopes, Prague.

Post-mitochondrial liver fraction. For the experiments, groups of female Wistar rats (170–180 g) kept under standard conditions were used. Partial hepatectomy (66 per

cent) was carried out under light ether narcosis.[19] The animals were killed by decapitation, bled and the excised livers were homogenized with cooling in a glass homogenizer. A tight-fitting Teflon pestle was used with 3 vol. of ice-cold 0·025 M Tris–HCl buffer (pH 7·5), containing 5×10^{-3} M $MgCl_2$ and $2{\cdot}5 \times 10^{-2}$ M KCl. The homogenate was centrifuged (10,000 *g*, 20 min, 3°), and the defatted supernatant fraction was used for the determination of enzyme activity.

Thymidine and thymidylate kinase assay. The incubation mixtures contained 0·04 M Tris–HCl buffer (pH 7·4) and 0·1 ml of liver postmitochondrial fraction corresponding to 25 mg of liver tissue in a total volume of 0·5 ml. For the determination of thymidine kinase, the reaction mixture contained 5×10^{-5} M thymidine-2-^{14}C and $2{\cdot}5 \times 10^{-3}$ M adenosine 5′-triphosphate with $1{\cdot}25 \times 10^{-3}$ M $MgCl_2$; incubation was carried out at 37°. Thymidylate kinase was assayed at 40° in a reaction mixture containing 5×10^{-5} M thymidine-2-^{14}C 5′-monophosphate and 2×10^{-3} M adenosine 5′-triphosphate with equimolar Mg^{2+} ions. Aliquots of incubation mixtures, withdrawn during the linear course of respective enzyme reactions, were separated by chromatography on Whatman No. 1 paper with appropriate standards in a solvent system composed of isobutyric acid–ammonium hydroxide–water (66:1·5:33). Radioactive spots were cut out and assayed with a Packard liquid scintillation spectrometer in 10 ml of scintillation fluid [4 g 2,5-diphenyloxazole, 0·15 g *p*-bis-(2-[4-methyl-5-phenyloxazolyl])-benzene in 1 l. of toluene]. The activity of enzymes is expressed as millimicromoles of thymidine 5′-monophosphate or of thymidine 5′-di- and tri-phosphates formed during a 10-min incubation period.

Synthesis of liver deoxyribonucleic acids. Thymidine-2-^{14}C (2 μCi/0·5 μmole/animal) or deoxycytidine-2-^{14}C (0·2 μCi/0·2 μmole/animal) was administered i.p. to groups of 3–6 female rats at different time intervals after partial hepatectomy or sham operation. The excised livers were homogenized in 5 vol. of ice-cold 0·2 M $HClO_4$. After centrifugation (5000 *g*, 10 min, 2°), the precipitates were extracted three times with 0·2 M $HClO_4$ and subjected to alkaline hydrolysis (1 M KOH, 16 hr, 20°). After neutralization with 10% $HClO_4$, the hydrolysates were centrifuged and sediments evaporated to dryness. Hydrolysis of DNA in the sediment was carried out for 1 hr with 70% $HClO_4$ at 100°; after neutralization, the supernatant fractions were separated chromatographically using Whatman No. 3 paper in a solvent system composed of 1-butanol–acetic acid–water (10:1:3). Separated thymine and cytosine spots were eluted and rechromatographed in a solvent system composed of isopropyl alcohol–ammonium hydroxide–water (7:1:2). Spectroscopically pure bases were estimated with a spectrophotometer (Unicam SP 700) and the radioactivity was assayed in a Packard liquid scintillation spectrometer.

Isolation and sucrose density gradient centrifugation of liver polyribosomes. Livers were homogenized ten times in a glass homogenizer with a tight-fitting Teflon pestle with 3 vol. of ice-cold 0·25 M sucrose containing 0·025 M Tris–HCl buffer (pH 7·5), $2{\cdot}5 \times 10^{-2}$ M KCl and 5×10^{-3} M $MgCl_2$. Isolation of polyribosomes following centrifugation of the homogenate (10,000 *g*, 20 min, 3°) was carried out as described elsewhere.[20] The supernatant fraction was adjusted to 1·25 per cent with respect to sodium deoxycholate, left for 10 min at 2°, and layered over a discontinuous gradient, consisting of 2·0 and 1·3 M ice-cold sucrose in the homogenization buffer. Centrifugation was carried out at 39,000 *g* for 130 min at 4°. Polyribosomes were washed, suspended in the same buffer and layered on a precooled 10–40% sucrose density

gradient. Centrifugation was performed with a Spinco ultracentrifuge (rotor SW 41, 4°, 41,000 rev/min for 80 min or rotor SW 25, 4°, 63,000 rev/min for 3 hr). Polyribosomal profiles were obtained by measuring the absorbancy with a Unicam SP 700 spectrophotometer or with a Beckman spectrophotometer with automatic recording.

Labeling of liver proteins in vivo. L-Valine-U-^{14}C (4 μCi/50 nmoles/animal) was administered i.p. 1 hr prior to killing to groups of 3–5 animals. Excised livers were homogenized with 7 vol. of cold 0·14 M KCl; the homogenate was centrifuged (10,000 *g*, 20 min, 3°) and 3 ml of the supernatant fraction was added to 3 ml of ice-cold 12% $HClO_4$. The precipitate was repeatedly washed with 5 ml of 6% $HClO_4$ to remove free amino acids, and proteins free of lipids were dissolved in 98% formic acid. The radioactivity of aliquots was assayed with a Packard scintillation spectrometer. Synthesis of liver proteins is expressed as dis./min. of valine-^{14}C incorporated into total proteins of S_3-supernatant fraction corresponding to 1 g of liver tissue.

RESULTS

Prolongation of the lag period preceding the enhancement of thymidine and thymidylate kinase activity. A single dose of 5-azacytidine 1 hr after partial hepatectomy results in a complete inhibition of thymidine kinase activity in 24 hr-regenerating liver.[21] The data in Table 1 indicate that 5-azacytidine also depresses thymidylate kinase. The inhibitory effect is much less pronounced when the drug is given during the phase of maximal enzyme activity, i.e. at 24 hr of liver regeneration. Contrary to 5-azacytidine, the dietary

TABLE 1. LIVER THYMIDINE AND THYMIDYLATE KINASE ACTIVITY AFTER ADMINISTRATION OF 5-AZACYTIDINE TO PARTIALLY HEPATECTOMIZED RATS*

Regeneration (hr)	5-Azacytidine administration (hr)	Thymidine kinase (nmoles ±S.E.)	(%)	Thymidylate kinase (nmoles ±S.E.)	(%)
Sham-operated		2·62 ± 0·45	100	0·97 ± 0·15	100
Sham-operated	1	2·83 ± 0·38	108	0·93 ± 0·21	95
24		17·21 ± 1·92†	657	3·48 ± 0·65†	356
24	1	2·41 ± 0·22†	92	0·90 ± 0·11†	93
48		12·12 ± 2·14‡	465	7·04 ± 1·21‡	723
48	24	9·75 ± 1·68‡	374	5·65 ± 0·98‡	582

* 5-Azacytidine was administered i.p. to groups of five rats at the dose level of 10 μmoles/100 g at 1 or 24 hr after partial hepatectomy.
† $P < 0·002$.
‡ $P < 0·05$.

administration of tryptophan 24 hr after partial hepatectomy prolongs the duration of the enhanced thymidine and thymidylate activity.[22] The simultaneous administration of 5-azacytidine with tryptophan equally prevents the enhancing effect of this amino acid; no significant change of thymidine 5′-nucleotidase activity after 5-azacytidine administration alone or in combination with tryptophan has been observed.[22]

The time course of thymidine and thymidylate kinase activities during liver regeneration is shown in Fig. 1, A and B. A single dose of 5-azacytidine 1 hr after partial hepatectomy results in a prolongation of the lag period preceding the enhanced

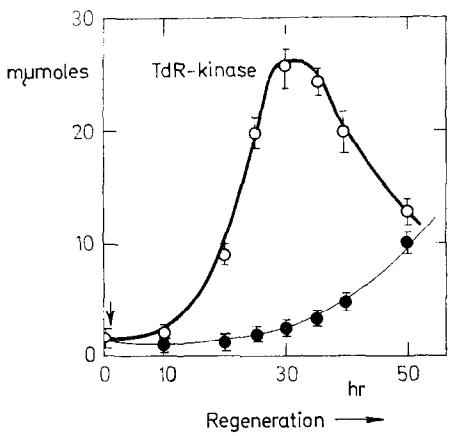

FIG. 1(A)

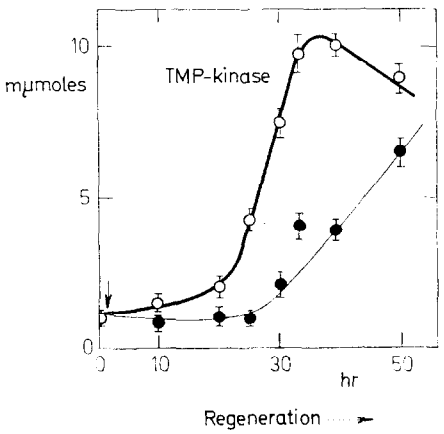

FIG. 1(B)

FIG. 1. Thymidine (A) and thymidylate (B) kinase activities of regenerating livers of 5-azacytidine-treated rats. 5-Azacytidine (●) or saline (○) was administered to groups of 7–12 rats 1 hr after partial hepatectomy at the dose level of 10 μmoles/100 g, and the animals were killed at different phases of regeneration. The activities of thymidine and thymidylate kinase are expressed (±S.E.) as millimicromoles of phosphorylated 5×10^{-5} M thymidine-2-^{14}C or thymidine-2-^{14}C 5′-monophosphate during a 10-min period of incubation.

activity of both enzymes. The duration of this period varies in relation to the dose level and the timing of 5-azacytidine administration; in general, it is not longer than 30 hr for thymidine kinase or 35–40 hr for thymidylate kinase. Figure 2, A and B, shows the lag period preceding the onset of enhanced activity of the enzymes under investigation by the repeated administration of 5-azacytidine. The peak of the induced enzyme activity at later stages of regeneration is greatly decreased.

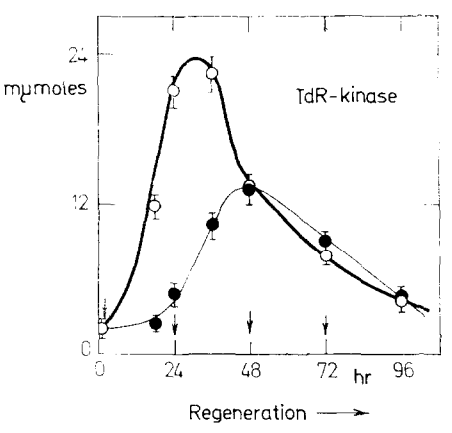

FIG. 2(A)

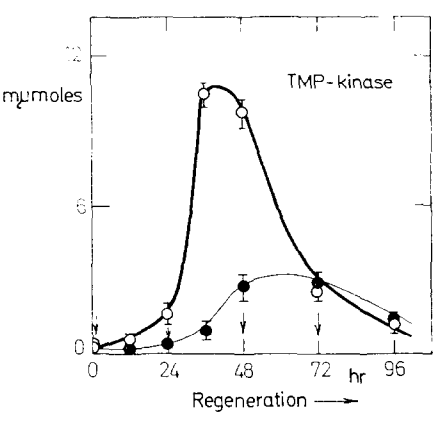

FIG. 2(B)

FIG. 2. Effect of repeated administration of 5-azacytidine on thymidine (A) and thymidylate (B) kinase activities of regenerating rat liver. 5-Azacytidine (●) or saline (○) was administered to groups of 6–9 rats at the dose level of 5 μmoles/100 g at 1 hr and then repeatedly at 24-hr intervals after partial hepatectomy (arrows). The animals were killed at different stages of regeneration and the enzyme activities (millimicromoles ±S.E.) were determined in cell-free liver extracts.

The catabolic activity of thymidine phosphorylase and thymidine 5′-nucleotidase is not increased under similar conditions (unpublished), which suggests that the lag period preceding the increase of thymidine and thymidylate kinase activity is due to an effect of 5-azacytidine on enzyme synthesis.

Inhibition of DNA synthesis in regenerating liver by 5-azacytidine. In accordance with its effect on the activity of thymidine and thymidylate kinases, 5-azacytidine administration results in a strong depression of the uptake of thymidine into liver DNA (Table 2). The inhibitory effect of 5-azacytidine on DNA synthesis was considerably less when the drug was administered at later stages of regeneration after DNA formation had already started. The decreased incorporation of deoxycytidine into DNA in regenerating livers indicates that not only the pathway of thymidine 5′-phosphate synthesis is inhibited (Table 2), even though the decrease of the intracellular pool of thymidine 5′-phosphates could result in the diminished incorporation of deoxycytidine into liver DNA. The administration of 5-azacytidine does not affect DNA synthesis in the liver of sham-operated animals.

TABLE 2. EFFECT OF 5-AZACYTIDINE ON DNA SYNTHESIS IN LIVERS OF SHAM-OPERATED AND PARTIALLY HEPATECTOMIZED RATS*

Regeneration (hr)	5-Azacytidine administration (hr)	Control (dis./min/μmole ±S.E.)	5-Azacytidine-treated (dis./min/μmole ±S.E.)	(%)
Thymidine-2-^{14}C				
Sham-operated	1	1424 ± 260	1596 ± 385	112
Sham-operated	22		1474 ± 270	103
24	1	52,080 ± 7060	426 ± 65	(0·8)
24	22		27,503 ± 2470	53
Deoxycytidine-2-^{14}C				
24	1	1265 ± 230	28 ± 4	(2·2)

* Thymidine-2-^{14}C (2 μCi/0·5 μmole/animal) or deoxycytidine-2-^{14}C (0·2 μCi/0·2 μmole/animal) was administered i.p. 2 hr before killing to groups of 4–5 rats. 5-Azacytidine was given i.p. at a single dose of 10 μmoles/100 g at 1 or 22 hr after operation. DNA synthesis is expressed as dis./min. per micromole of thymine-2-^{14}C or cytosine-2-^{14}C isolated from liver DNA. Control = 100 per cent.

Repeated administration of 5-azacytidine leads at 35–40 hr after partial hepatectomy to a complete block of DNA synthesis normally occurring in regenerating liver (Fig. 3). The duration of inhibition cannot be prolonged by a higher dosage or by a further administration of the drug, due to its toxic effects. Single injection of 5-azacytidine 1 hr after partial hepatectomy results in the increased incorporation of the label into the DNA of 48 hr-regenerating liver as compared with the control (Fig. 3).

Polyribosome degradation and inhibition of protein synthesis. The administration of 5-azacytidine *in vivo* leads to the degradation of liver polyribosomes.[4,23] The degradation sets in shortly after the drug administration and is maximal at 2–8 hr thereafter. A typical distribution profile of polyribosomes in 20 hr-regenerating liver 2 hr after the administration of 5-azacytidine is shown in Fig. 4. The ratio of polyribosomes to the fraction of disomes 1 hr after 5-azacytidine administration in relation to the time course of liver regeneration is shown in Fig. 5. It should be remembered that the

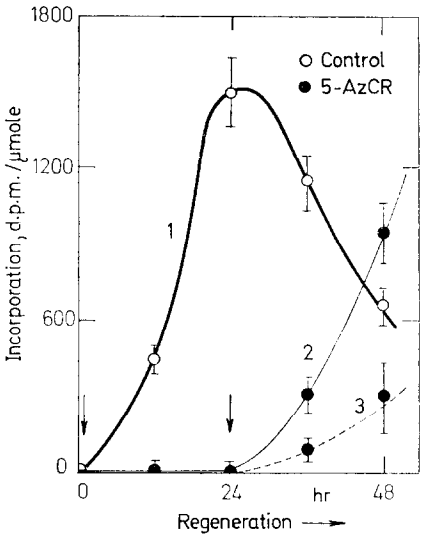

FIG. 3. Inhibition of DNA synthesis in regenerating rat liver by 5-azacytidine. 5-Azacytidine was administered i.p. (10 μmoles/100 g) to groups of 3–4 rats at 1 hr (2) or at a dose level of 20 μmoles/100 g at 1 and 24 hr (3) after partial hepatectomy (arrows); controls (1) received 0·9% NaCl solution. Deoxycytidine-2-^{14}C (0·2 μCi/0·2 μmole/animal) was administered at different stages of liver regeneration 2 hr prior to killing. The incorporation of the label into DNA is expressed as a specific radioactivity of cytosine-2-^{14}C isolated from liver DNA in dis./min. per micromole ±S.E.

polyribosome distribution is also affected by the process of regeneration itself as well as by conditions of stress.[24]

Decreased incorporation of valine into hepatic proteins of S_3-fraction after 5-azacytidine in comparison to control regenerating liver is in agreement with polyribosome degradation (Fig. 5). The maximal depression of amino acid incorporation

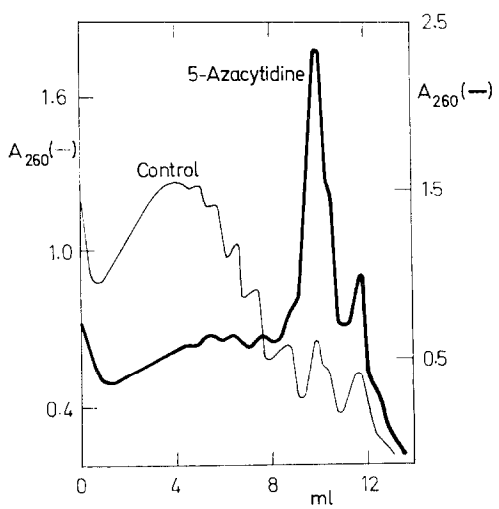

FIG. 4. Degradation of liver polyribosomes after the administration of 5-azacytidine. The drug was injected i.p. (10 μmoles/100 g) into two rats at 18 hr after partial hepatectomy 2 hr before killing. Distribution of liver polyribosomes from 5-azacytidine-treated and control animals was carried out using 10–40% sucrose density gradient centrifugation (41,000 rev/min, 3°, 80 min).

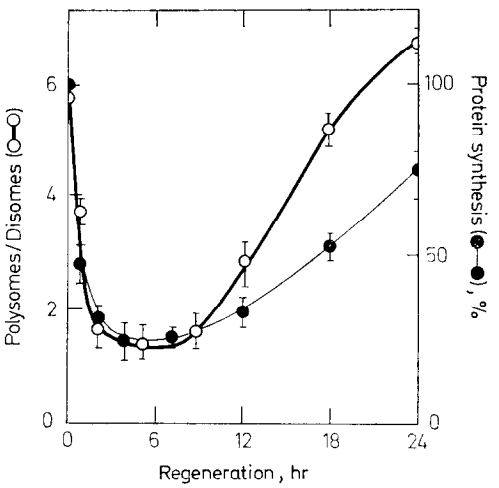

FIG. 5. Relationship between polyribosome degradation and depressed protein synthesis. 5-Azacytidine (10 μmoles/100 g) was administered i.p. to groups of six rats 1 hr after partial hepatectomy. At different phases of regeneration, two animals from each group were killed and the ratio of polyribosomes to disomes in their livers was determined (○). The remaining groups of animals received at the same time, 1 hr prior to killing, L-valine-U-^{14}C (4 μCi/0·05 μmole/animal). From the liver, S_3-supernatant fractions were prepared and by comparing with respective controls (36,600 ± 4200 dis./min/g of liver at zero time; 54,700 ± 6700 dis./min/g of liver at 24 hr regenerating liver) the decrease of valine incorporation into total proteins of the S_3-fraction was determined (●). Controls = 100 per cent.

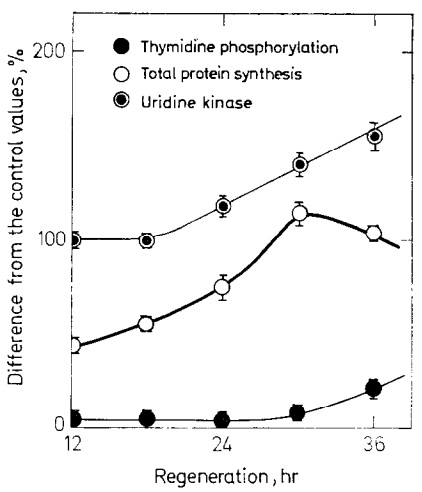

FIG. 6. Synthesis of total liver proteins and the activity of thymidine phosphorylating enzymes after 5-azacytidine administration. The drug was given i.p. to groups of 3–6 rats at a dose level of 10 μmoles/100 g 1 hr after partial hepatectomy. The animals were killed at different phases of liver regeneration 2 hr after L-valine-U-^{14}C (4 μCi/0·05 μmole/animal). The radioactivity of proteins (○) in S_3-supernatant liver fractions and the activity of thymidine phosphorylating kinases (●) were assayed as described in Methods; uridine kinase (⊙) was measured as described earlier.[27] The obtained values are expressed as per cent of respective controls = 100% (control protein synthesis at 12 hr of regeneration, 39,800 ± 5300 dis./min/g of liver; thymidine phosphorylation, 5·45 ± 0·64 nmoles; uridine kinase, 1·18 ± 0·12 μmoles/g of liver/hr).

occurs 2–8 hr after 5-azacytidine injection; 24 hr later, a small degree of inhibition persisted (25–35 per cent). While the degree of inhibition of the protein synthesis in the liver after 5-azacytidine decreases gradually during the regenerative process, reaching control values at 30–36 hr after the analogue (Fig. 6), thymidine phosphorylating enzymes are almost completely inhibited throughout this phase. On the contrary, uridine kinase exhibits an entirely different pattern and it rises steadily from 20 to 24 hr after the administration of the drug without any sign of impairment.[27]

DISCUSSION

During the course of liver regeneration, the activities of thymidine and thymidylate kinases rise considerably due to their *de novo* synthesis.[6,8,25] The rate of enzyme synthesis in regenerating liver is controlled by the processes of induction and repression, depending on the formation of liver nucleic acids. Since the synthesis of DNA in regenerating rat liver depends on the age of experimental animals,[26] young adult female rats have been used throughout the experiments.

It was of interest to know for how long different enzymes participating in DNA synthesis in regenerating liver may be blocked without causing serious toxic effects in experimental animals. Although the synthesis of enzymes necessary for the formation of DNA during liver regeneration are completely inhibited by 5-azacytidine at 20–24 hr after partial hepatectomy, uridine kinase activity at the same time is stimulated (Fig. 6). Consequently, regenerating liver does not synthesize DNA, while the formation of RNA is enhanced[27] and protein synthesis is affected only negligibly (Fig. 5).

The slightly different effect of 5-azacytidine on thymidine and thymidylate kinase is apparent from Figs. 1 and 2. It should be taken for granted that the sensitivity of enzyme systems towards 5-azacytidine should be distinct, since the half-life of template RNAs for various enzymes, as well as their turnover rates, is known to be different.[28–30] The lower inhibitory effect of 5-azacytidine administered 22 hr after partial hepatectomy on thymidine and thymidylate kinase activity (Table 1) can be explained by the fact that both enzymes or their template RNA's are synthesized early after partial hepatectomy, and are considerably more affected by the drug administered shortly after the operation.

The administration of 5-azacytidine results in impaired synthesis of DNA in the case of regenerating liver only (Table 2). Decreased DNA synthesis after 5-azacytidine has been observed during the earliest stages of sea-urchin development, followed later by the pronounced retardation of its morphological development.[31] It seems that in this case, as in regenerating liver, 5-azacytidine interferes primarily with RNA synthesis which precedes the onset of DNA replication.[32] 5-Azacytidine was also found to inhibit isoproterenol-stimulated DNA synthesis in mouse salivary glands[5] and the synthesis of DNA in L1210 cells cultivated *in vitro*.[33]

We did not find any relation between the degradation of liver polyribosomes and altered DNA formation in regenerating rat liver (Figs. 3 and 5). Similarly, degradation of hepatic polyribosomes and inhibition of protein synthesis are not associated with the depression of thymidine and thymidylate kinase synthesis. Enzyme activity is inhibited by 5-azacytidine 30–36 hr after the administration of the drug when the polyribosomal pattern is normal and the synthesis of liver proteins is restored (Fig. 6). The available data seem to indicate that the depression of liver regeneration after 5-azacytidine administration is only partially due to the degradation of polyribosomes

associated with the decreased synthesis of hepatic proteins. Complete inhibition of thymidine and thymidylate kinases at the time of restored protein synthetic capacity of the liver may best be explained by the effect of 5-azacytidine on the synthesis of RNA during the early stages of liver regeneration.

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