INHIBITORS OF PROTEIN SYNTHESIS: EFFECT ON THE LEVELS OF DEOXYCYTIDYLATE DEAMINASE, THYMIDYLATE SYNTHETASE, AND THYMIDINE KINASE IN REGENERATING RAT LIVER*

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We reported previously that a sharp rise in deoxycytidylate deaminase and thymidylate synthetase activities occurs in rat liver 14 to 18 hours after partial hepatectomy (Maley and Maley, 1960). As indicated in a preliminary report (Maley and Maley, 1961a), the elevation of these enzymes could be impaired by the prior injection of ethionine, suggesting that the increase in enzyme activity was associated with the synthesis of new enzyme molecules and not with the activation of pre-existing molecules. A more exacting study of the events leading to the increase in deaminase and synthetase activities was thus undertaken by the use of inhibitors capable of blocking or altering protein synthesis at specific steps.

RESULTS AND DISCUSSION

Deoxycytidylate deaminase is present at low, but measurable levels in normal adult rat liver (Maley and Maley, 1961b and c), the activity of which is presented in Fig. 1. Twenty-eight hours after partial hepatectomy, this level is increased by 3- to 4-fold. That the increase in deaminase activity results from newly synthesized enzyme molecules is supported by the data presented in Fig. 1. As anticipated from the earlier studies, ethionine markedly inhibited the ap-

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pearance of the enzyme when injected immediately after the operation, with lesser inhibition at the 5- and 14-hour intervals. Somewhat similar results were obtained with p-fluorophenylalanine. Since the average of 2 rats was used for the data at some time intervals, strict comparisons cannot be made. However, the inhibitory trend is made more significant by the fact that there are at least 8 rats for each group of inhibitors. At the 24-hour period, the inhibition by p-fluorophenylalanine was markedly diminished, which is in general accord with the results of Fujioka et al. (1963). These authors demonstrated a delay in the synthesis of what appears to be the messenger RNA fraction by p-fluorophenylalanine, when given from 0 to 5 hours after partial hepatectomy. Since there is little difference between the 0-, 5-, and 12-hour injections, our results appear to be more in the nature of an inhibition than a delay in synthesis. However, much higher amounts of p-fluorophenylalanine were used in our experiments, which makes a valid comparison with their results impossible. Whether the inhibition in the deaminase increase results from the synthesis of an altered enzyme containing p-fluorophenylalanine in place of the natural analogue, or from the impairment of messenger RNA synthesis, is presently unknown.

The data obtained with actinomycin[1] indicate that the information for deaminase synthesis is still not present at the 14-hour period. Recent experiments with actinomycin injected 16 hours after partial hepatectomy revealed the deaminase to be restored to the control regenerating liver level at 28 hours, suggesting that the information for deoxycytidylate deaminase transcription is not available in its entirety or in a functional form until 14 to 16 hours after partial hepatectomy.

While actinomycin prevents the transcription of genetic information (Reich et al., 1962; Hurwitz et al., 1962; Goldberg and Rabinowitz, 1962), puromycin disrupts the translation of this information by pre-

[1] We would like to express our appreciation to Merck, Sharp and Dohme for the actinomycin that was used in these experiments.

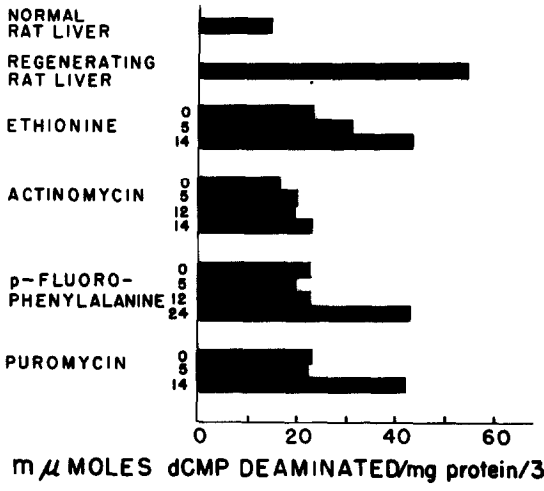

Figure 1. Influence of protein synthesis inhibitors on the deoxycytidylate deaminase increase in regenerating liver. Male rats (125-150 g) were partially hepatectomized (Higgins and Anderson, 1931) between 8:30 and 9:30 A.M. The livers were removed 28 to 29 hours later and a cell-free supernatant fraction was prepared by centrifuging a 30 per cent isotonic KCl homogenate for 1 hour at 105,000 X g. The inhibitors were injected intraperitoneally at the indicated hour, given on the ordinate, and in the following dosages: p-fluoro(DL)phenylalanine, 30 mg; (DL)-ethionine, 100 mg; actinomycin D, 50 μg; puromycin, 10 mg. Deoxycytidylate deaminase was determined by assay 3 (Maley and Maley, 1964), but the addition of dCTP to stabilize the enzyme was not necessary with freshly prepared extracts. Protein was determined by the method of Lowry et al. (1951). The data are expressed as average values from the following number of rats in each case: normal, 2; regenerating liver, 20; ethionine, 0 time, 4; 5-hour, 2; 14-hour, 2; p-fluorophenylalanine, 0 time, 6; 5-hour, 4; 12-hour, 2; 24-hour, 2; puromycin, 0 time, 6; 5-hour, 2; 14-hour, 2; actinomycin, 0 hour, 7; 5-hour, 4; 12-hour, 4; 14-hour, 2.

venting the completion of peptide chains (Yarmolinsky and de la Haba, 1959; Nathans and Lipmann, 1961; Allen and Zamecnik, 1962) and has been utilized in numerous experiments to demonstrate new protein synthesis. It was likewise utilized in the experiments reported in Fig. 1. As indicated, the synthesis of deoxycytidylate deaminase is sharply curtailed by this drug. Thus, the data presented in Fig. 1 suggest rather strongly that the increase in deoxycytidylate deaminase activity following partial hepatectomy is due to the synthesis of new enzyme protein, in accord with the accepted views on protein synthesis (Zubay, 1963).

In contrast to the above results, the anomalous effect of the inhibitors on the appearance of thymidylate synthetase activity requires a somewhat different explanation. As seen in Fig. 2, both ethionine and p-fluorophenylalanine inhibit the increase in synthetase activity which in itself is rather striking. With the former analogue, similar

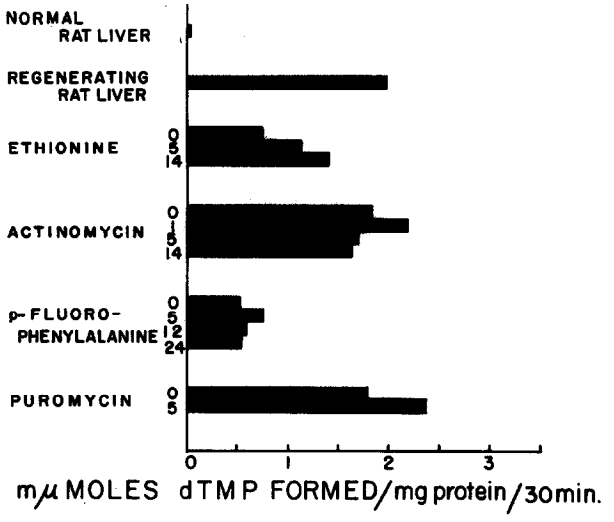

Figure 2. Influence of protein synthesis inhibitors on the appearance of thymidylate synthetase in regenerating liver. Aliquots of 0.2 ml from the supernatant fractions described in Fig. 1 were used for the synthetase assay (Maley and Maley, 1960). The sensitivity and accuracy of the assay were increased by the addition of a known amount of carrier thymidine to the Dowex 1-formate - Dowex 50-H^+ eluates and isolation of the desired product by paper chromatography in secondary butanol. The data are expressed as average values from the following number of rats in each case: normal, 4; regenerating liver, 20; ethionine, 2 at each time interval; p-fluorophenylalanine, 0 time, 6; 5-hour, 2; 12-hour, 4; 24-hour, 2; actinomycin, 0 time, 12; 1-hour, 4; 5-hour, 4; 14-hour, 4.

results to those of the deaminase were obtained, but with the latter, a marked inhibition was encountered, even when this analogue was given 24 hours after partial hepatectomy. Addition of p-fluorophenylalanine to the reaction mixtures did not inhibit the synthetase. Even more surprising was the finding that little or no inhibition of the synthetase

increase was encountered in actinomycin[2] or puromycin-treated animals. These results were obtained with the same liver extracts that demonstrated a marked inhibition of deaminase activity by the above-mentioned antibiotics.

To evaluate further the effectiveness of these inhibitors, their influence on the appearance of thymidine kinase was studied, since its activity is also enhanced during liver regeneration (Bollum and Potter, 1959; Weissman et al., 1960). The activity of this enzyme was measured in the same extracts used to determine the deaminase and synthetase activities. As seen in Table I, the results obtained with thymidine kinase are consistent with the deaminase data, but not with those obtained for the synthetase. As indicated, a partial reversal of p-fluorophenylalanine inhibition could be effected by L-phenylalanine. Similar results were also obtained with the deaminase, but not consistently with the synthetase. Other enzymes that are elevated by partial hepatectomy and would appear to fall in the same class as deoxycytidylate deaminase and thymidine kinase, are DNA polymerase (Guidice and Novelli, 1963) and RNA polymerase (Tsukada and Lieberman, 1964) since their synthesis is also blocked by actinomycin. The synthesis of the latter enzyme is also impaired by p-fluorophenylalanine.

The reason for the lack of effectiveness of actinomycin and puromycin in preventing the synthesis of thymidylate synthetase is not apparent at the present time, and, although numerous proposals can be advanced for the resistance of the synthetase to inhibition, none appears to be all-inclusive. If anything, the above findings do indicate that the use of inhibitors of protein synthesis can lead to anomalous results, particularly inhibitors with a wide spectrum of selectivity.

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[2] The average thymidylate synthetase activity for 30 rats was 1.98 mμmoles dTMP formed per mg per 30 minutes with a standard deviation of the mean of ± 0.13. The average activity for 16 rats injected at zero time with actinomycin was 1.68 ± 0.11. In no case, where 4 or more rats were used (Fig. 2), was the standard deviation greater than 20% of the average value.

TABLE I

INHIBITION OF THYMIDINE KINASE APPEARANCE IN PARTIALLY HEPATECTOMIZED RATS

EXPERIMENTAL CONDITIONS*	ENZYME ACTIVITY (mμmoles/mg/10 min)
24-hr control (6)	4.83 ± 0.37**
Plus p-fluoro(DL)phenylalanine (4)	0.26 ± 0.048
Plus p-fluoro(DL)phenylalanine (4) and (L) phenylalanine+	1.26 ± 0.44
Plus puromycin (4)	0.22 ± 0.03
28-hr control (4)	9.30 ± 0.47
Plus actinomycin D (2)	0.20

* Inhibitors injected at 0 time, except for puromycin which was given at 0, 6, 12 hrs. For dosage, see Fig. 1.

** Standard deviation of the mean.

+ 15 mg injected at 0, 6, 12 hrs.

Influence of protein synthesis inhibitors on the appearance of thymidine kinase in regenerating rat liver. The enzyme was assayed by measuring the phosphorylation of thymidine-2-^{14}C. The reaction mixture contained the following compounds in μmoles: thymidine-2-^{14}C, 0.1 (2.56×10^6 cpm/μmole); ATP, 5.0; $MgCl_2$, 5.0; NaF, 15; Tris buffer, pH 8.0, 50; 0.2 ml of the extracts described in Fig. 1. The final volume was 0.5 ml. After incubation at 37° for 30 minutes the reaction was stopped with 2.0 ml of 1 N perchloric acid. The acidified solution was heated for 30 minutes at 100°, cooled in ice and neutralized with 50 per cent KOH. The centrifuged solution was passed over a Dowex 1-formate column (3 cm X 1 cm) and the column eluted as follows: 35 ml of 0.01 N formic acid, 20 ml of 1.0 N formic acid and 40 ml of 4.0 N formic acid. The latter eluate contained the desired thymidylic acid-2-^{14}C, and was used as a measure of thymidine kinase activity. The figures in parentheses indicate the number of rats used to calculate the data.

SUMMARY

The increase in deoxycytidylate deaminase and thymidine kinase activities following partial hepatectomy of rat liver, was greatly impaired by the intraperitoneal injection of ethionine, p-fluorophenylalanine, actinomycin, and puromycin. However, the elevation of thymidy-

late synthetase, while inhibited by the amino acid analogues, was only slightly, if at all, affected by actinomycin and puromycin.

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