Nucleic Acid Metabolism in Regenerating Rat Liver
VI. Soluble Enzymes Which Convert Thymidine to Thymidine Phosphates and DNA*†

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The presence of a multienzyme system which incorporates thymidine into DNA in the soluble fraction of regenerating rat liver homogenates (5, 6) raises the question of the biological significance of this enzyme system. The rate of DNA synthesis in vivo as a function of time after partial hepatectomy has been studied in several laboratories, with a variety of isotopic substrates (1, 11-13, and references cited). To examine the relation between DNA synthesis in vivo and activity of the soluble enzyme system, a technic for studying these phenomena simultaneously in the same animal was used, following the plan previously used to correlate DNA synthesis in vivo and in tissue slices (13). The results have shown that the appearance of the enzyme system is correlated with the appearance of DNA synthesis in vivo, but that enzymatic activity continues to increase over the period 24-48 hours after hepatectomy, whereas DNA synthesis in vivo decreases.

MATERIALS AND METHODS

Male albino rats weighing 140-180 gm. were partially hepatectomized and fed ad libitum. At the desired time after hepatectomy each animal received 1 umole of orotic acid-6-C14, specific activity 1 µc/µmole. Exactly 2 hours later the animal was sacrificed by decapitation and the liver removed and placed in ice-cold saline; 20 per cent homogenates were prepared with the use of the buffered sucrose homogenization medium described previously (6). Nuclei were removed by centrifuging homogenates for 10 minutes at 600 X g, and the specific activity of the DNA isolated from them was determined by the usual procedure (13). The nuclear supernatant fraction was then centrifuged for 60 min. at 40,000 r.p.m. (= 105,000 X g) in the Spicio preparative centrifuge. Although the clear supernatant fraction contained radioactive uridine nucleotides, these did not interfere with the polymerase assays (see below).

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of total radioactivity present as phosphorylated compounds, and this was used as a measure of thymidine kinase.

**Assay for polymerase.**—Routine incubations contained 0.1 ml. DNA (500 µg.), 0.2 ml. supernatant fraction, and 0.2 ml. of a substrate mixture. The substrate mixture was prepared to contain in the final incubation mixture: ATP, 5 mM; Mg++, 5 mM; 3-phosphoglycerate, 6 mM; Tris: HCl, pH 8.0, 40 mM; dAMP, dGMP, dCMP, 0.1 mM; H3-thymidine (89 µc/amole), 0.06 mM. DNA was isolated as described previously (12). An aliquot was plated, and an aliquot was taken for calorimetric analysis (7). The specific radioactivity of DNA after 60 minutes of incubation is proportional to enzyme activity (6) in this assay. Although protein determinations were performed (9) on the supernatant fraction from each animal, the agreement between samples was within the limit of the precision of the method, and therefore specific activities are not corrected for small variations in protein concentration. Protein concentration of the supernatant fraction as prepared here averaged 19 mg/ml. When supernatant fractions containing radioactive pyrimidine nucleotides (from the arctic acid injection) were used, suitable controls were run. Such controls contained all the components listed for the enzyme assay except H3-thymidine. In all cases the radioactivity incorporated into DNA was negligible. This was not unexpected, since even if the conversion of ribotide to deoxyribotide demonstrated by Grossman (10) were operating in such supernatants, the folic-dependent methylation of deoxyuridine (2, 8, 16) would probably be ineffective in the reaction system employed.

All radioactivity determinations were performed in windowless flow counters (6). Since DNA plates from enzyme incubations all contained essentially the same amount of DNA, no correction for self-absorption has been made. C14 determinations on DNA labeled in vivo after injection of labeled orotic acid were corrected for self-absorption in the usual way (18).

**RESULTS**

The capacity of regenerating liver supernatant fraction to form thymidine nucleotides is shown in Chart 1. These data were obtained from a series of paper chromatograms such as the representative one depicted in Chart 2. A similar study with supernatant fraction from normal liver indicated insignificant formation of phosphorylated compounds from thymidine of the specific activity employed in these experiments. The appearance and disappearance of the thymidine kinase are further demonstrated in Chart 3, which shows representative chromatograms on supernatant fraction assays for normal, 24-hour regenerating, and completely regenerated (3-week) rat livers. The
chromatograms of Chart 3 are part of a larger study designed to find out whether a sequential induction of enzymes was occurring, i.e., could thymidine kinase be shown to increase significantly before the polymerizing enzyme appeared? The complete study is presented in Table 1, which contains results of kinase and polymerase assay on the same supernatant fraction. These results indicate the simultaneous increase of two of the enzymes, thymidine kinase and polymerase, required for the incorporation of thymidine into DNA. Although finer discrimination in methodology and timing might detect some difference, this conclusion is valid within the limits of the present study. Studies with H-TMP will be helpful in assaying the activity of enzymes that phosphorylate thymidylic acid. The earlier study of Hecht, Potter, and Herbert (14) indicated that TMP is phosphorylated by cytoplasmic fraction from regenerating liver.

It is of some interest at this point to mention that the enzymes which phosphorylate dAMP, dGMP, and dCMP are readily detectable in supernatant fractions from normal as well as regenerating rat livers. It is also noteworthy that the specific activities (µmoles product/hour/mg protein) of these enzymes are much greater than the corresponding thymidine-converting enzymes (4).

The complete curve demonstrating the correlation between enzymatic incorporation of thymidine and orotic acid incorporation is illustrated in Chart 4. It is apparent that the forms of these two curves are remarkably different in certain respects. The most striking difference is in the period of 24–30 hours, when DNA synthesis measured by orotic acid incorporation is decreasing and enzyme activity for thymidine is increasing. Aside from the period of 24–30 hours, there is a remarkable parallelism between the two curves, including the period of initial rise (18–24 hours), the period of secondary fluctuations (30–50 hours, cf. 12), and the final period of decline to resting values.

That the polymerizing enzyme is actually increasing is shown in Table 2, in which the time points were obtained by the method described above, which starts with thymidine, and by an assay (Method III, [6]) which utilizes preformed deoxynucleoside triphosphates and therefore measures polymerase independently of kinases. The increase in polymerase activity is observed by both methods. It is noteworthy that the actual level of polymerase in normal liver or early regeneration is appreciably higher than is indicated by the assay which utilizes thymidine. Since this finding concerns the early phase of regeneration, it does not affect conclusions which concern the simultaneous increase of enzyme activity and DNA synthesis, and this correlation at later times of regeneration.

**DISCUSSION**

The major objective of this investigation was to observe the activity of the soluble polymerizing enzyme during a period when the rate of DNA synthesis
synthesis is undergoing change. The result shows that the enzyme (system) appears in the supernatant fraction at the time DNA synthesis begins and constitutes reasonable evidence that the two phenomena are physiologically related. The relation between enzyme activity and rate of DNA synthesis beyond the time of simultaneous appearance remains unclear. The close correlation of detailed correlation observed in this study. The previous correlation was found when both parameters were determined at a cellular level, and the present study compares cellular (controlled) DNA synthesis with enzymatic (uncontrolled) activity. Perhaps whole homogenate and cell fractionation studies will be helpful in further resolution of this issue. The conclusions to be drawn from the present investigations are somewhat limited by the fact that only one cell fraction was examined in detail. The methods developed and the activity levels observed in the supernatant fraction do, however, provide a base-line for further detailed studies on other cell fractions. Cell fractions which contain appreciable phosphatase, pyrophosphatase, and DNAase activity will require more elaborate methodology. Some of the details presented in this communication are concerned primarily with the demonstration of polymerizing activity in the supernatant fraction and should not at this time be considered as establishing the site of DNA synthesis as it occurs in the cell. In particular, the low level of thymidine kinase in soluble fraction from normal animals is a subject for further research, since a rather large percentage of normal whole homogenate incubations has resulted in the appearance of thymidine nucleotides. Our earlier speculation (3) that enzyme activity may reflect the total capacity for DNA synthesis (cells synthesizing DNA and cells having synthesized DNA and still retaining this enzymatic capacity), whereas in vitro incorporation measures only the activity of cells actually synthesizing DNA during the interval of exposure to isotope, remains as a useful working hypothesis. Although the sudden increase in activity of two enzymes of DNA synthesis cannot immediately be interpreted as enzyme induction, it is tempting to speculate that such might be the case. Further studies, including demonstration of new protein formation, will be required in making this decision. Procedures for obtaining purified enzyme (4) will be necessary for such studies.

The crude supernatant fraction of 48-hour regenerating liver has a specific activity of approximately 0.2 mmoles H\textsuperscript{3}thymidine incorporated/mg protein/hour. By the assumption that equimolar proportions of each nucleotide are incorporated into DNA, the total supernatant fraction has a maximum capacity to synthesize about 30 \( \mu \)g of DNA per hour. In the intact rat, liver regeneration results in synthesis of approximately 10 mg of DNA in 5 days (Chart 7, Paper I). It is suggested that the supernatant enzyme has a capacity approximating DNA synthesis in vivo sufficient closely to be the enzyme responsible for DNA synthesis.

### Table 2

**Demonstration of Polymerase Appearance by Use of Kinase-Independent Assay**

<table>
<thead>
<tr>
<th>Hours post-operative</th>
<th>Kinase-dependent assay</th>
<th>Kinase-independent assay</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>370</td>
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<td>10</td>
<td>240</td>
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<tr>
<td>72</td>
<td>7,950</td>
<td>12,600</td>
</tr>
</tbody>
</table>

All data are specific activity of DNA after 60 minutes' incubation at 38°C.

*In the kinase-dependent assay thymidine is utilized as substrate. In the kinase-independent assay TTP is utilized as substrate and is not limited by the rate of conversion of thymidine to TTP.*

1. A technic for comparing DNA synthesis in regenerating rat liver in vivo and in cell-free supernatant fractions from the same animal in vitro has been devised.

2. Methods for evaluating the activity of enzymes phosphorylating thymidine are presented.

3. Enzymatic incorporation of thymidine into DNA was found to appear at the same time (18 hours after operation) as DNA synthesis in vitro but continued to increase over the period of 18–30 hours while DNA synthesis in vivo was decreasing (24–30 hours).

4. The enzymes which convert thymidine to thymidine nucleotides appear at about the same time as the polymerizing enzymes.

^1 Unpublished experiments, Miss Anne Brumm.
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