A Biochemical and Autoradiographic Study of the in Vivo Utilization of Tritiated Thymidine in Regenerating Rat Liver

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SUMMARY

The utilization of tritiated thymidine as a measure of DNA synthesis in 21-hr regenerating rat liver under specific experimental conditions has been evaluated by autoradiographic and biochemical methods. A rapid disappearance of the labeled thymidine from the blood in the 1st 10 min was accompanied by a rapid incorporation of the radioactivity into the acid-soluble fraction and DNA of the liver cells. Two min after administration, label was found in TMP, TDP, TTP, and DNA. A relatively constant ratio of these labeled precursor components in ASF during the 1st hr after injection suggests that equilibrium was reached immediately after administration.

The predominant biologic action in liver following administration is the rapid destruction of thymidine. Only 2 min after injection, 1/3 of the labeled thymidine in the ASF of the liver cells had been degraded to T and BAIB. This marked destruction is also shown by the fact that only 1.2% of the injected thymidine is incorporated into newly formed DNA. All the thymidine is removed from the blood within 1 hr. The results indicate that, under the specific experimental conditions, TdR-3H can be used as an index for DNA synthesis in individual cells as well as in the total cell population within 1 hr after administration.

Radioactive thymidine has been extensively utilized as a precursor in studies of DNA synthesis. It has been shown in in vivo and in vitro systems that thymidine is phosphorylated to its nucleotides, TMP, TDP, and TTP and that TTP is polymerized with triphosphates of deoxyribothymidine, deoxyadenosine, and deoxyguanosine into DNA (1, 3, 4, 6, 19, 23, 25, 26, 28, 31). However, the kinetics of incorporation of radioactive thymidine vary with different biologic test systems and different experimental conditions. It has been necessary to assess the reliability of tritiated thymidine as a measure of DNA synthesis in a particular biologic test system under specific experimental conditions and to evaluate its usefulness for other related problems, such as the biologic effects of radiation on the biosynthetic pathway of DNA and on the replication of DNA.

This report is concerned with the utilization of tritiated thymidine as a measure of DNA synthesis in 21-hr regenerating rat liver, the time of the maximum rate of DNA synthesis in the 1st cycle of hepatocytes synthesizing DNA after partial hepatectomy (8, 16, 20).

1 Supported in part by United States Atomic Energy Commission Contract AT(40-1)-2889, American Cancer Society Grant E309, and USPHS Grant GM10754.

2 The following abbreviations are used: DNA, deoxyribonucleic acid; ASF, acid-soluble fraction; T, thymidine; TdR, thymidine; TMP, TDP, TTP, thymidine mono-, di-, triphosphates; BAIB, β-aminoisobutyric acid; ETAA, ethano-ammonium acetate; AmBu, ammonia-isobutyric acid; Form-EtAc, formic acid-ethyl acetate.

Received for publication August 10, 1964; revised June 9, 1965.

MATERIALS AND METHODS

Male, Lewis strain rats weighing approximately 200 gm each were used. The animals were maintained under standard conditions for 1 week before the experiment.

Partial hepatectomies were performed under ether anesthesia by the method of Higgins and Anderson (17). Fifty μe of tritiated thymidine (5-methyl-TdR-3H) with a specific activity of 3 c/mmole in 1 ml of normal saline solution were injected i.v. at 21 hr after the operation, and the animals were sacrificed at 2, 10, and 30 min and at 1, 3, and 5 hr after administration by opening the chest cage and excising the heart. Cardiac blood samples were collected at 2, 5, 10, and 30 min; at 1, 3, and 12 hr; and at 1, 2, and 7 days. The distribution of radioactivity in the thymidine and thymine present in the blood plasma at different time intervals was determined by paper chromatography and tritium counting. A modified form of the Schneider procedure was used for extraction of DNA and the acid-soluble fraction (21, 29). The DNA content of the extract was determined by the diphenylamine method (9).
The acid-soluble fraction extracted from about 0.4 gm of rat liver was neutralized with 7N KOH at 2°C, and the precipitate was removed by centrifugation. The supernatant was concentrated by freeze-drying. The residue was then dissolved in a small volume of 10% isopropanol (pH 7). The sample was chromatographed unidirectionally on a Whatman No. 1 paper strip (2 inches x 26 inches) in the following solvent systems (13, 30): (a) ETAA—90% ethanol and 1 m ammonium acetate containing 0.1 m Versene (70:30); (b) AmBu—isobutyric acid, water, ammonia (sp. gr. 0.880) and 0.1 m Versene (100:55.8:4.2:1.6); and (c) Form-EtAc—ethyl acetate, formic acid, and water (70:20:10). The radioactive spots were characterized by the use of unlabeled markers of T, TdR, TMP, TDP, and TTP. The position of the spots was located under ultraviolet light (2537 Å), and the spots were eluted with 0.1 N HCl. The amount of radioactivity present in individual spots was determined by liquid scintillation counting.

Aliquots of the same liver samples used in the biochemical analyses were also used in the autoradiographic studies. Squashes made from random samples of finely minced liver were fixed by freeze substitution. DNA was stained by the Feulgen technic prior to application of AR-10 stripping film (21).

RESULTS

Tritium activity in the blood plasma.—Chart 1 summarizes the tritium activity in the blood measured at different time intervals after injection of the labeled thymidine. Two min after administration, 12.6% of the total tritiated thymidine injected was estimated to be in the blood. The radioactivity diminished as time after injection increased. One hr after administration, 6.6% of the tritium activity remained in the circulating blood, and 4.9% was present after 1 day.

Essentially, all labeled thymidine disappeared from the blood by 1 hr after injection. The remaining activity was present in thymine or in other forms. The distribution of tritium activity in thymidine and its degradation products in the blood plasma determined chromatographically is also shown in Chart 1. The rate of disappearance of tritiated thymidine from the blood was 0.4 μc/min in the 1st 10 min and 10 times less for the period between 10 min and 1 hr (Chart 5).

Tritium activity in the acid-soluble fraction of regenerating liver cells.—An inverse relationship between the disappearance of tritium activity from the acid-soluble frac-

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**TABLE 1a**

<table>
<thead>
<tr>
<th>TIME AFTER INJECTION</th>
<th>NO. OF ANIMALS</th>
<th>T*</th>
<th>TdR</th>
<th>X</th>
<th>TMP</th>
<th>TDP</th>
<th>TTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min</td>
<td>12</td>
<td>0.221 (0.44)</td>
<td>0.046 (0.09)</td>
<td>1.129 (2.26)</td>
<td>0.296 (0.6)</td>
<td>0.102 (0.2)</td>
<td>0.039 (0.08)</td>
</tr>
<tr>
<td>10 min</td>
<td>10</td>
<td>0.259 (0.52)</td>
<td>0.080 (0.16)</td>
<td>0.970 (1.94)</td>
<td>0.391 (0.78)</td>
<td>0.175 (0.35)</td>
<td>0.109 (0.22)</td>
</tr>
<tr>
<td>30 min</td>
<td>10</td>
<td>0.182 (0.36)</td>
<td>0.061 (0.12)</td>
<td>0.702 (1.40)</td>
<td>0.288 (0.85)</td>
<td>0.112 (0.22)</td>
<td>0.068 (0.14)</td>
</tr>
<tr>
<td>1 hr</td>
<td>10</td>
<td>0.106 (0.21)</td>
<td>0.019 (0.04)</td>
<td>0.489 (0.98)</td>
<td>0.144 (0.29)</td>
<td>0.053 (0.11)</td>
<td>0.033 (0.07)</td>
</tr>
<tr>
<td>3 hr</td>
<td>10</td>
<td>0.011 (0.02)</td>
<td>0.002 (0.005)</td>
<td>0.033 (0.07)</td>
<td>0.019 (0.04)</td>
<td>0.010 (0.02)</td>
<td>0.008 (0.02)</td>
</tr>
<tr>
<td>5 hr</td>
<td>8</td>
<td>0.010 (0.02)</td>
<td>0.002 (0.004)</td>
<td>0.017 (0.03)</td>
<td>0.006 (0.01)</td>
<td>0.003 (0.006)</td>
<td>0.003 (0.006)</td>
</tr>
</tbody>
</table>

* T, thymine; TdR, thymidine; TMP, thymidine monophosphate; TDP, thymidine diphosphate; TTP, thymidine triphosphate; X, % of the tritium activity was found in BAIB, and the rest was distributed into 5-hydroxymethyl uridine, 5-hydroxymethyl uracil, and other unidentified spots.

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TABLE 1b

<table>
<thead>
<tr>
<th>TIME AFTER INJECTION</th>
<th>Av. % OF TOTAL cpm IN LIVER SAMPLES</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T3</td>
</tr>
<tr>
<td>2 min</td>
<td>12.1 ± 1.5</td>
</tr>
<tr>
<td>10 min</td>
<td>13.8 ± 2.5</td>
</tr>
<tr>
<td>30 min</td>
<td>13.0 ± 0.5</td>
</tr>
<tr>
<td>1 hr</td>
<td>11.6 ± 1.1</td>
</tr>
<tr>
<td>3 hr</td>
<td>14.8 ± 2.4</td>
</tr>
<tr>
<td>5 hr</td>
<td>22.6 ± 3.9</td>
</tr>
</tbody>
</table>

* The abbreviations are defined in the footnote to Table 1a.

during the 1st 10 min, a rapid decline in the next 50 min, and a continuous decrease at a slower rate after 1 hr. However, the relative proportion of radioactivity present in all these components at various time periods after injection appeared to be reasonably constant during the 1st hr (Table 1b). Approximately ⅓ of the total radioactivity was found in thymine and "Compound X." Compound X had a migration rate greater than that of thymidylic acid and slower than that of thymine in both EiAA and AmBu solvent systems. Further analysis of Compound X in a separate experiment showed that about ⅓ of its radioactivity was β-aminoisobutyric acid. Four ultraviolet light-absorbing spots, each containing a small amount of radioactivity, were resolved from this compound in Form-EtAc solvent system. Detailed analysis of Compound X will be reported separately.

In the level of thymine nucleotides, about 60% of the activity was observed in TMP, 25% in TDP, and 15% in TTP. The total tritium activity in all these components decreased with time, and only a trace amount of radioactivity has been detected in chromatograms of 5-hr samples.

Incorporation of labeled thymidine into newly formed DNA.—The grain counts per nucleus are a measure of the relative rate of incorporation of the labeled nucleotides into DNA of single cells. The percentage of labeled cells is an index of the fraction of the total cells engaged in DNA synthesis at a specific time. Approximately half the liver cells synthesizing DNA were labeled during the
The rapidity of the clearance rate of the injected TdR-3H from the blood of an hepatectomized rat was found to be similar to the results reported by Hughes et al. (18) in mouse and by Rubini et al. (27) in man. All these results indicate that the availability of the injected TdR-3H in the circulating blood lasts only a short while; it is limited primarily to the 1st 30–60 min after administration. The percentage of injected TdR-3H available in the total fluid at comparable time intervals is, in all probability, greater than that in circulating blood since equilibration among the plasma, the interstitial fluid, and total body water should occur rapidly (see “Addendum”). The small amount of tritium activity in TdR and its nucleotides determined in the ASF of 1-hr samples should reflect the upper limit of the availability time of TdR-3H in the total circulating body fluids.

Four of the most important factors that may influence the incorporation of labeled TdR into DNA are: (a) the amount of labeled TdR administered; (b) the enzymes involved in the biosynthesis of DNA; (c) the degradation of the labeled TdR; and (d) the de novo synthesis of DNA precursors by the rat. The evidence available at present indicates that the addition of a large quantity of nonlabeled TdR to a system has relatively little effect on the rate of incorporation of labeled TdR into DNA. This was specifically shown by Gerber (15) in perfused regenerating rat liver and by Nygaard and Potter (22) in the thymus of rat. Gerber found that about 30% of the TdR present in the perfusate was degraded during the flow through the liver at concentrations of approximately 4 μg/100 ml. When thymidine at a concentration of 8 mg/100 ml was added to the perfusate, about 6 mg/hr/liver was degraded. The increase in the TdR concentration by a factor of 2000 reduced the rate of incorporation of labeled TdR into DNA by a factor of 200.

The powerful catabolic reaction of thymidine in regenerating liver was also demonstrated in this study; only 2 min after administration, 3/4 of the 3H in the ASF of the liver cells was found in T and BAIB.

Nygaard and Potter (22) found little or no effect on the efficiency of the incorporation of the injected 10 μM TdR-2-14C into DNA of thymus by varying “carrier level” from 1 to 50 μM. But the reduction of the “carrier level” from 50 μM to 1 μM resulted in a 3- to 6-fold increase in the efficiency of incorporation of labeled TdR into DNA of spleen and intestines. They suggest that this is the result of the smaller pool size in the spleen and intestines and, hence, of the lesser dilution of the precursor. The work of Gerber and the results of this study suggest that regenerating liver is more like thymus than spleen and intestines.

Crone and Itzhaki (11) used orotic acid-14C as a measure of DNA and ribonucleic acid synthesis in regenerating liver. They found that no reduction in labeling of DNA occurred after the addition of varying amounts of unlabeled cytidine.

Sequential increase in both the concentration of the thymine nucleotides and the enzyme activity involved in the stepwise phosphorylation of thymidine in regenerating liver are known (5, 7, 8, 10, 23, 31).
activity is reached approximately 48 hr after partial hepatectomy, and a decrease in both enzyme activity and the concentration of labeled precursors follows during the next 24 hr. In principle, the ratios of the concentrations of the 3 thymine nucleotides observed under specific experimental conditions simply reflect the over-all results of the activity of these kinases and the degradation enzymes involved. The relative proportions of labeled TMP (56%), TDP (25%), and TTP (19%) observed in 21-hr regenerating liver are comparable to the findings of Weissman et al. (31), whose data were obtained at 20 hr after partial hepatectomy. The consistency of the relative proportions of these precursor components during the 1st hr after injection of TdR-3H suggests that equilibrium between the labeled and nonlabeled thymine nucleotides was reached immediately after the injection.

The autoradiographic and biochemical data shown in Table 2 and Charts 2 and 4 indicate that the rate of uptake in individual cells, measured autoradiographically, is more rapid than that in the total population of cells. The maximum total incorporation of labeled thymidine in individual cells and in the total cell population is reached in 1 and 3 hr, respectively, after TdR-3H administration; however, the general characteristics of the 2 curves are similar. A number of experiments carried out in this laboratory have demonstrated the correlation between the 2 methods in assessing changes in rates of DNA synthesis.

The differences may be the result of the basic differences in the 2 methods of analysis. Autoradiography measures the relative rate of DNA synthesis in individual cells. The biochemical results give an average value of DNA synthesis for the total cell population. The fraction of cells synthesizing DNA is, therefore, diluted by the non-synthesizing cells since the results are expressed as specific activity, cpm/mg DNA. The more likely explanation of the difference is related to an inherent problem in the autoradiographic technic. The autoradiographs are exposed for a length of time to give an average of 25-30 grains/nucleus. When the grain counts/nucleus and the percentage of labeled cells are counted, all cells with less than 5 grains are excluded. This would introduce an error of 10-15% in the autoradiographic data. The occurrence of the maximum uptake at 3 hr in the biochemical data, as opposed to 1 hr in the autoradiographic data, might be explained by a small continued increase in DNA synthesis in some cells that is excluded from the autoradiographic results. The chromatographic data would also support this possibility since small amounts of labeled thymine nucleotides are still present in the ASF 1 hr after injection.

Since the concentration of the labeled catabolic products and phosphorylated thymine nucleotides detected in the precursor pool steadily fell with increasing time, reutilization of DNA-degraded products in the synthesis seems unlikely. Moreover, there seems to be little possibility of appreciable reutilization of the breakdown products of labeled DNA from cells from other organs, since negligible amounts of circulating TdR-3H or T-3H were found in the blood 1 hr after administration.

CONCLUSIONS

The utilization of tritiated thymidine as a measure of DNA synthesis in 21-hr regenerating rat liver under specific experimental conditions has been evaluated by autoradiographic and biochemical methods. A rapid disappearance of the labeled thymidine from the blood in the 1st 10 min was accompanied by a rapid incorporation of the radioactivity into the acid-soluble fraction and DNA of the liver cells. Two min after administration, label was found in TMP, TDP, TTP, and DNA. A relatively constant ratio of these labeled precursor components in ASF during the 1st hr after injection indicates that equilibrium was reached immediately after the administration. Both autoradiographic and biochemical data indicate that the maximum rate of TdR-3H incorporation into DNA occurred during the 1st 30 min after injection. This coincides with the time for the maximum disappearance of tritium activity from the ASF of the liver cells.

The predominant biologic action in liver following administration is the rapid destruction of thymidine. Only 2 min after injection, $\frac{1}{3}$ of the TdR-3H in the ASF of the liver cells had been degraded to T and BA1B. This marked destruction is also shown by the fact that only 1.2% of the injected TdR is incorporated into newly formed DNA. All the TdR is removed from the blood within 1 hr. The continuous labeling in DNA, from 1 to 3 hr after TdR-3H injection, may be accounted for by the utilization of the labeled TdR and its nucleotides remaining in the ASF of the liver cells at this time. The results indicate that under the specific experimental conditions TdR-3H can be used as an index for DNA synthesis in individual cells as well as in the total cell population within 1 hr after administration. The combined autoradiographic and biochemical methods used in the study of DNA synthesis in the total population of cells and of changes within this population have a wide potential use for a number of biologic and medical problems.

ACKNOWLEDGMENTS

We wish to acknowledge the assistance of Dr. F. W. Banghart in the statistical analysis of the data. The assistance of Miss S. S. Williams and Miss H. J. Forster is also gratefully acknowledged. We would also like to acknowledge the helpful suggestions of Drs. V. R. Potter and G. B. Gerber and their making available to us unpublished data for reference.

ADDENDUM

The calculations for the labeled TdR in the blood were based on the assumption that the plasma volume is 6.7 ml/100 gm of body weight (12). Estimates of the equilibration of labeled TdR in the different water components of the body were made on the assumption that $\frac{1}{3}$ of the body water is intracellular and $\frac{1}{3}$ extracellular. It was further assumed that the interstitial fluid contained $\frac{1}{3}$ and the plasma, $\frac{1}{3}$, of the total body water of the rat. It was estimated that 12.5% of the injected TdR was present in the plasma 2 min after administration. On the basis of these assumptions, 37.8% of the TdR would be in the interstitial fluid at this time. Approximately $\frac{1}{3}$ of the labeled TdR should, therefore, be in extracellular fluid and $\frac{1}{3}$ in the intracellular fluid 2 min after administration. Since the intracellular fluid is twice the volume of the extracellular fluid, these calculations suggest that equilibrium between intracellular and extracellular fluid is not reached within 2 min; however, these data and the data showing a continuing increase in uptake of labeled TdR in the ASF fraction of the liver cells for 10 min suggest that equilibrium is reached within 10 min.

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REFERENCES


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