Numerous studies have been undertaken to determine the relationships between RNase activity and cancer, and a review concerning these relationships has been published (14). An understanding of the role of RNase in growth processes has been complicated by demonstrations that there is a multiplicity of RNases (14) and of intracellular inhibitors for RNase (9, 11) in animal tissues. Furthermore, the recent discovery that some samples of RNA contain impurities that interfere with the determination of RNase and RNase inhibitor activities (3, 18, 21) has made it clear that the RNase inhibitor is more widely distributed and occurs in considerably greater amounts than heretofore suspected.

With the use of technics modified to allow complete expression of RNase inhibitor activity, a study was made of RNase and RNase inhibitor activities in several transplantable rat hepatomas (17). Shortman (22) has measured RNase and RNase inhibitor activities in regenerating rat liver.

To complement these studies, RNase inhibitor and latent RNase (l-RNase) activities were determined with modified assay conditions during the administration of 2-acetamidofluorene (AAF) to rats. A preliminary examination of inhibitor activity during AAF feeding has indicated a rise in the inhibitor level (13).

**MATERIALS AND METHODS**

Animals and diets.—Male rats of the Charles River strain, weighing an average of 73 gm at the start of the experiment, were divided into 2 groups of 100 each. The experimental group was fed a complete synthetic diet (16) containing 12% casein and 0.06% AAF (dry weight basis). Control animals were pair-fed the diet without carcinogen. Water was allowed ad libitum. The feeding of these diets was continued for 20 weeks, after which both the control and the AAF-fed rats were placed on Purina rat pellets; the control animals were again pair-fed against the AAF-fed group.

At various time intervals up to Week 12, 6 drug-fed and 6 control rats were randomly selected for RNase inhibitor and l-RNase assays. Following this, 1–4 AAF-fed and 2–6 control rats were selected for assays.
Chart 1.—Top, specific RNase inhibitor activity (based on DNA of homogenate) in the livers of rats fed 2-acetamidofluorene and pair-fed controls. Bottom, the same, based on N content of the supernatant fraction. The brackets indicate the S.E. of the mean. Where they are omitted, not enough values were available to calculate this. The open circles with a line represent values obtained on liver nodules. Open circles, AAF-fed; closed circles, control.

Chart 2.—Top, specific latent RNase activity (based on DNA of homogenate) in the livers of rats fed 2-acetamidofluorene and pair-fed controls. Bottom, the same, based on N content of the supernatant fraction. The brackets indicate the S.E. of the mean. Where they are omitted, not enough values were available to calculate this. The closed circles with a line represent values obtained on liver nodules.

Assays.—RNase inhibitor activity was determined as previously described (25) with the following modifications. The incubation mixtures contained 1 ml of 0.029 M Veronal-acetate buffer (VA buffer), pH 7.8, 0.15 ml of 0.05 M ethylenediaminetetraacetic acid (EDTA) adjusted to pH 7.8, and unlabeled RNA. The volumes of water that were added to the mixtures were sufficient to bring the total incubation volume to 3.0 ml. The assays were performed with 2 different volumes of supernatant fraction, which were sufficient to bring the RNase activity to 20% and 75% inhibition of RNase. The reaction was terminated by adding 3.0 ml of 1 N HCl in 76% ethanol containing 0.5% lanthanum chloride. Correction blanks were applied, and inhibitor activity units were defined as previously described (15) (see also “Results”).

Latent RNase activity was determined as previously described (15) with the following modifications. The incubation mixtures contained 1 ml of 0.029 M VA buffer, pH 7.8, 0.40 ml of water, 0.20 ml of 0.01 M p-chloromercuribenzenesulfonic acid (CMBS) adjusted to pH 7.8, 1.0 ml of 1% RNA, and 0.40 ml of the same liver supernatant fraction used in the inhibitor assay. The reaction was terminated as described above. For unit definition, the 1-RNase activity was compared with the activity of
0.030 μg crystalline pancreatic RNase, which was assayed under identical conditions. One unit of 1-RNase activity is equivalent to the RNase activity given by 1 μg of crystalline bovine pancreatic RNase assayed under the same conditions.

DNA was extracted from the rat liver homogenates essentially by the method of Schneider (20) and measured colorimetrically by the procedure of Dische as modified by Burton (1). Nitrogen was determined by a standard micro-Kjeldahl method.

Yeast RNA was obtained from Schwarz BioResearch, Inc., (Mount Vernon, N.Y.), lot No. 6080.

RESULTS

Throughout the experimental period the inhibition of RNase by increasing volumes of supernatant fraction, for both the control and the AAF-fed groups, gave typically S-shaped curves, as previously indicated (15). As described by Roth (15), semilog plots relating the extent of inhibition of different amounts of RNase by different supernatant fraction volumes (usually 4 different volumes) were made for both AAF-fed and control animals at various times throughout the experimental period. Generally, all such plots resulted in a family of straight lines, which had identical slopes within a family. From a semilog plot, which was composed from a number of individual plots, units of RNase inhibitor activity were assigned to the various percent inhibition values.

To be certain that maximum RNase inhibitor and 1-RNase activities were obtained throughout the experimental period under the given assay conditions, increased concentrations of EDTA and CMS were included in the assays at intervals during this period. There were, however, no significant differences in inhibitor and 1-RNase activity values under these conditions.

The results of the assays for RNase inhibitor and 1-RNase activities during AAF administration are expressed on the basis of both DNA in the whole homogenate and N in the supernatant fraction. In addition, total activities have been tabulated as units/liver. Data have also been included on the activities in the liver nodules produced by feeding AAF. These results are represented in Charts 1–3.

The tissue parameters, the liver wet weight, and the ratios of supernatant fraction N to DNA and of DNA to liver wet weight, have been tabulated in Table 1.

**RNase inhibitor activity.**—When the rats were placed on the synthetic diet, there was an initial drop in the specific activity of RNase inhibitor in both the control and the AAF-fed animals (Chart 1). This drop, which was appreciable, can probably be ascribed to the shift from a natural to a synthetic diet. After the initial drop, the level of RNase inhibitor remained relatively constant, at a low level, from Week 3 to 17 in the control animals. In the AAF-fed rats, however, from Week 3 through week 17 RNase inhibitor activity increased, when compared with the controls, and reached a peak at about Week 12. This peak activity was equal to a 100% increase over the control group. Whether the maximum activity occurred earlier or later than Week 12 was not determined, since the assays were performed at widely spaced intervals. During the period in which AAF feeding was discontinued (Weeks 20–42), specific RNase inhibitor activity of the drug-fed rats decreased to values lower than, or similar to, those of the control group. The inhibitor activity in the liver nodules, at least one of which was a hepatoma, was not greatly different from that of adjacent liver or of the livers of the control group (Chart 1). The results were generally similar whether calculations were based on DNA or N.

Control rats throughout the experimental period maintained a general constancy of specific RNase inhibitor activity with the exception of the initial drop in activity during the first few weeks of feeding. Based on either DNA or N, this constancy was apparent to Week 30; however, in the last 10 weeks the control inhibitor activity, calculated on a DNA basis, rose markedly to a value almost double that of Week 30, although it is to be noted that this increase was not apparent when an N

5 Livers and AAF-induced nodules from the groups of Weeks 39 and 41 were submitted for histopathologic examination. One nodule had mild cholangitis and a large circumscribed hepatoma, whereas the 2 other nodules and the livers of the AAF-fed group had hyperplastic cholangitis with cyst formation. Control livers had evidence of mild cholangitis and hydropic degeneration.
TABLE 1  
RELATIONSHIP OF TISSUE WEIGHT, DEOXYRIBONUCLEIC ACID (DNA), AND NITROGEN (N) IN LIVERS OF 2-ACETAMIDOFUORENE-FED (AAF) RATS AND PAIR-FED CONTROLS

<table>
<thead>
<tr>
<th>Week</th>
<th>Group</th>
<th>No. of samples</th>
<th>Liver wt. (gm)</th>
<th>Supernatant N/DNA (mg/mg)</th>
<th>DNA/liver wt. (mg/mg)</th>
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<tr>
<td>0</td>
<td>Control</td>
<td>6</td>
<td>3.57</td>
<td>4.60 ± 0.16*</td>
<td>1.95 ± 0.04</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>6</td>
<td>3.78</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAF-fed</td>
<td>6</td>
<td>2.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>6</td>
<td>4.03</td>
<td>3.72 ± 0.21</td>
<td>2.37 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>AAF-fed</td>
<td>6</td>
<td>3.61</td>
<td>3.52 ± 0.04</td>
<td>2.43 ± 0.03</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>5</td>
<td>4.64</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAF-fed</td>
<td>6</td>
<td>5.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
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<td>5.14</td>
<td>4.17 ± 0.17</td>
<td>2.00 ± 0.08</td>
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<tr>
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<td>6</td>
<td>5.87</td>
<td>3.64 ± 0.21</td>
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<tr>
<td>12</td>
<td>Control</td>
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<td>4.73</td>
<td>5.00 ± 0.25</td>
<td>2.03 ± 0.09</td>
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<tr>
<td></td>
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<td>6</td>
<td>7.08</td>
<td>4.73 ± 0.24</td>
<td>1.96 ± 0.12</td>
</tr>
<tr>
<td>17</td>
<td>Control</td>
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<td>5.53</td>
<td>3.86 ± 0.15</td>
<td>2.36 ± 0.09</td>
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<td></td>
<td>AAF-fed</td>
<td>4</td>
<td>6.17</td>
<td>3.71 ± 0.14</td>
<td>2.33 ± 0.16</td>
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<td>AAF feeding discontinued</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>25a</td>
<td>Control</td>
<td>2</td>
<td>7.40, 8.00</td>
<td>4.44, 4.46</td>
<td>2.76, 2.76</td>
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<td></td>
<td>AAF-fed</td>
<td>1</td>
<td>11.9</td>
<td>2.49</td>
<td>5.04</td>
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<tr>
<td>30</td>
<td>Control</td>
<td>2</td>
<td>10.22, 10.65</td>
<td>5.41, 4.50</td>
<td>2.11, 2.11</td>
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<td></td>
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<td>1</td>
<td>8.75</td>
<td>3.41</td>
<td>3.10</td>
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<tr>
<td>39c</td>
<td>Control</td>
<td>6</td>
<td>12.11</td>
<td>9.41 ± 0.17</td>
<td>1.17 ± 0.02</td>
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<tr>
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<td>3</td>
<td>28.7, 45.8, 13.1</td>
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<tr>
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<td>AAF-fed nodule</td>
<td>2</td>
<td>2.0</td>
<td></td>
<td></td>
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<tr>
<td>41c</td>
<td>AAF-fed liver</td>
<td>2</td>
<td>30.30, 20.81</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAF-fed nodule</td>
<td>1</td>
<td>4.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* S.E. of the mean.
* Both AAF-fed rats and controls were placed on pellets without AAF during Week 21 for the remainder of the experimental period. Controls were pair-fed against the AAF-fed rats.
* Among the samples of livers and adjoining nodules submitted for histopathologic examination, Nodule 2, Week 39, was determined to be a hepatoma. See histopathologic report.
* The listed weight includes that due to the liver plus the nodule.

basis was used (Chart 1). The reason for this increase in the control group is not known.

The total liver activities of RNase inhibitor (Chart 3) during the period of drug feeding followed a pattern similar to that given by the specific activity values, namely, increased activities with a peak during Weeks 8–17 in the AAF-fed group when compared with the controls, which maintained a general constancy of activity. At Week 25 the total RNase inhibitor activity in the control livers rose to a value 3-fold higher than at Week 17 and then remained at this level for the remainder of the experimental period. Total RNase inhibitor activities in the AAF-fed rats remained essentially at a plateau through Weeks 12–30 and appeared to be considerably elevated at Week 41, the final period of measurement, which probably reflected the increase in liver weight.

Activity of 1-RNase.—Activity values of 1-RNase were more variable among the individual rats within each tested group, as indicated by the relatively large standard errors of the mean (Chart 2). This scatter of activities and fluctuations within the experimental period necessitates some caution in an interpretation of these results. In general, it appears that during the period of AAF administration, specific 1-RNase activity in AAF-fed rats is lower than that in the control group. Within the period in which AAF feeding was discontinued, the 1-RNase activity was generally similar, both for the livers and resultant nodules of the AAF-fed group and for the livers of the control group. Activities based on N were slightly higher for the AAF-fed group in some cases; however, these results are based on only a few samples. This is particularly true of the point at Week 25 in Chart 2 for the AAF-fed group, since it actually represents only 1 rat and may very well be an aberrant value.

Throughout the experimental period, the control rats appeared to maintain a general constancy of specific 1-RNase activities, with the exception, again, of Weeks 30–39, in which the values, on a DNA (but not N) basis, showed a marked increase.

The total activities of 1-RNase during the entire experimental period followed a pattern similar to that given by the specific activity values, namely, increased activities with a peak during Weeks 8–17 in the AAF-fed group when compared with the controls, which maintained a general constancy of activity. At Week 25 the total RNase inhibitor activity in the control livers rose to a value 3-fold higher than at Week 17 and then remained at this level for the remainder of the experimental period. Total RNase inhibitor activities in the AAF-fed rats remained essentially at a plateau through Weeks 12–30 and appeared to be considerably elevated at Week 41, the final period of measurement, which probably reflected the increase in liver weight.

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Throughout the experimental period, the control rats appeared to maintain a general constancy of specific 1-RNase activities, with the exception, again, of Weeks 30–39, in which the values, on a DNA (but not N) basis, showed a marked increase.

The total activities of 1-RNase during the entire experimental period followed a pattern similar to that given by the specific activity values, except that there was an increase in total activities of both the control and the AAF-fed groups after cessation of AAF feeding (Chart 3).

Tissue parameters.—In agreement with previous reports (4, 5, 7, 8, 13, 23), the AAF-fed rats had a progressive liver weight increase with retarded body weight gain; both parameters are compared with the control group (body weights are not tabulated in this report) and are illustrated in Table 1.

Both the AAF-fed and the control groups followed a similar pattern in the ratio of supernatant fraction N to DNA during the period of drug administration; the values for the AAF-fed group were generally below those of the
control group. The ratio dropped initially and then increased by Week 12 to a level slightly higher than, or approximately equal to, that at the start of the experimental period. Thereafter, a drop of this ratio was observed at Week 17. With AAF feeding discontinued, the drug-fed group showed a further decline in the ratio at Week 25, whereas the control group showed a ratio increase, which continued to the end of the experimental period.

During AAF administration, both the AAF-fed and the control groups had very similar liver DNA to wet weight ratios, with the exception of Week 8, in which the ratio of the control group was significantly lower than that of the AAF-fed group. The control group ratio at that week was the same as the value at the start of the experimental period. For both groups the ratio values at Weeks 3 and 17 were higher than the initial value, and at Week 12 their value was similar to this initial value. It is of interest to note that previous studies have revealed a moderately increased peak of DNA to wet weight ratios for the livers of AAF-fed rats at a period of 6–7 weeks after commencement of AAF ingestion (4, 8).

At the conclusion of AAF feeding, both groups initially increased the DNA to wet weight ratios over the values at Week 17 of AAF feeding. This initial increase in ratio after the cessation of AAF administration has been previously observed by Griffin et al. (4). After this increase the ratios for both groups decreased; the ratio for the control group was significantly lower at the end of the experimental period than at the beginning.

In a preliminary experiment (13), which was run much earlier and which utilized older assay methods, an increase in RNase inhibitor in the AAF-fed rats was observed from Week 4 through Week 8 and reached 59% during this period. These results in general confirmed the present experiments. This earlier experiment was terminated at Week 8.

**DISCUSSION**

Since it was desirable to relate RNase inhibitor and 1-RNase activities to 2 different units of reference, the measurements of these activities were made on the basis of N and of DNA. The latter reference was chosen in view of reports that, during the preneoplastic period, the livers of AAF-fed rats showed either a relatively similar amount of DNA/average nucleus (2, 8, 19, 24) or a slight fluctuation and decrease in this amount (6) when compared with controls. At later times during administration of AAF, the DNA content/average nucleus has been reported to rise (24), whereas in the AAF-induced hepatoma, the content was not significantly different from controls (2, 8, 19). This similarity between the control and the AAF-fed groups, especially in the early stages of AAF feeding, might then allow the comparison of RNase inhibitor and 1-RNase activities to be made on a generally equivalent cellular basis, provided the extent of ploidy and the number of binucleate cells are similar for both the control and the AAF-fed groups throughout AAF administration. To the authors’ knowledge, no studies have been made on the latter cellular changes during AAF administration; hence any comparison on a cellular basis must be made with some reservation.

To aid in the interpretation of the data on an N basis, the extent of change of supernatant N for the AAF-fed group, compared with controls, was determined with DNA as the basis of reference (Table 1). The data on supernatant N to DNA ratios show that, in general, the AAF-fed group had slightly less supernatant N/unit of DNA. This decrease in the N to DNA ratio might then reflect a slight increase in specific RNase inhibitor and 1-RNase activities, which would not necessarily be due to a net change in the absolute amount of these constituents.

The level of specific RNase inhibitor activity during AAF administration showed a considerable increase, with the activity reaching a maximum (100% greater than controls) at about Week 12. Within the period in which drug feeding was discontinued, the activity level decreased to values lower than, or similar to, those of the control group. This decrease appears to be related to the absence of AAF feeding; however, a positive correlation of this response cannot be made with this data since this decrease may have been a continuance of the decline of inhibitor activity noted in the latter weeks (Week 17) of drug administration. The scarcity of experimental animals did not allow a separation into a group of animals on continued maintenance of the drug and a 2nd group on withdrawal of the drug. The specific activity values in the adjoining liver nodules, at least one of which was a hepatoma, were similar to or slightly lower than those in the experimental and control groups. Consequently, it would appear that inhibitor activity is not appreciably changed from activity in normal liver in this primary AAF-induced hepatoma.

The data on 1-RNase specific activities suggest a depression of these activities in the AAF-fed group during the period of drug administration. One implication of these findings is that the RNase capable of being inactivated by the RNase inhibitor, presumably alkaline RNase, may be decreased in the supernatant fraction during drug feeding. Further assessment of these results must await additional studies on the 1-RNase system and its correlation with RNase inhibitor.

The significance of these changes in the RNase-inhibitor system is unclear, mainly because of a dearth of reports on the interrelations in this system under conditions of AAF administration. Roth (13) has reported a 50% decrease in alkaline RNase activity in the mitochondrial fraction in the livers of AAF-fed rats. The acid RNase level under these conditions has been reported to show little change (13) or some increase (10) in the mitochondrial fraction. RNA levels were reported to decrease in the mitochondrial and microsomal fractions and to exhibit an increase in the nuclear and supernatant fractions of the livers of AAF-fed rats (10).

In addition to the cited results in the AAF system, RNase inhibitor activity was measured in some transplantable hepatomas and in regenerating rat liver. Roth et al. (17) found no correlation between RNase inhibitor activity and the proliferation rates of the hepatomas, but suggested that RNase inhibitor may bear a direct relationship to the rate of turnover of RNA (12). With the observation of a moderate increase in RNase inhibitor levels in regenerating rat liver, Shortman (22) speculated that...
that the inhibitor may function in a "tooling-up" process for hyperactive protein synthesis and in an accumulation of RNA by the cell.

Laird and Barton (6, 7) reported on changes in the rate of cellular proliferation during AAF administration. There was no significant change in the total number of liver cells during the early weeks of AAF administration; thereafter, the total number of cells increased at a constant rate during the period of carcinogen administration. This hyperplasia under conditions of AAF administration has been observed by many investigators. When the drug was discontinued, the cellular proliferation rate stopped after a short delay and did not resume, at least in the next 10 weeks, during which tests were made. The level of specific RNase inhibitor activity during the administration of AAF is in some instances associated with these rate changes. RNase inhibitor activity does not rise in the early weeks of AF administration, coincident with the period prior to the onset of hyperplasia; thereafter, RNase inhibitor increases at about the time of the commencement of hyperplasia. However, there is a decline in the inhibitor level before AAF discontinuation when the increased rate of hyperplasia is reported to be constant. This last observation and the low level of specific inhibitor activity in the induced hepatoma would suggest a lack of correlation between the proliferation rate and the inhibitor level.

During the administration of AAF there was a large mortality. Thus, a difficulty in the evaluation of some of these is the possibility that the liver may be responding to the toxic nature of the drug. The induction of at least 1 hepatoma among the 5 histologically evaluated AAF-fed rats implies that the conditions of administration of AAF were sufficient for the induction of hepatomas. The period of AAF administration may then be considered to correspond to a preneoplastic period.

Although at the present time little correlation can be made with the observed changes in the liver during AAF administration, it is important to note the substantial increase and subsequent decrease in the level of RNase inhibitor under these conditions. With further studies of the changes during AAF administration and also of interrelationships in the RNase-inhibitor systems, the significance of these changes may become apparent. This system with this increase in the inhibitor level may, in fact, serve to evaluate further the role of RNase inhibitor in cell metabolism.

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Ribonuclease Inhibitor and Latent Ribonuclease in Rat Liver during Feeding of 2-Acetamidofluorene

Robert J. Wojnar and Jay S. Roth


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