Peptidase Activity in the Thymus of a Normal and a Leukemic Strain of Mice during Growth and Aging*

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A characteristic of the normal thymus gland is its rapid growth during the early stages of life followed by a progressive involution as the animal ages. On the other hand, in the Akr strain of mice a marked hyperplasia of this gland usually occurs in those animals which develop lymphatic leukemia. In this strain the thymus is believed to be the primary site of leukemogenesis (6). In the following study one metabolic aspect of the thymus, namely, the rate at which thymus homogenates hydrolyze the tripeptide glycylglycylglycine, was examined. The object was to find out (a) how the peptidase activity per unit weight of thymus varies throughout the life span of normal mice, (b) whether the peptidase activity of the thymus from the leukemic strain of mice differs from that of normal mice at comparable ages, and (c) whether a difference exists between the peptidase activity of the glands from the leukemic strain of mice which develop leukemia and those which do not.

Preliminary to this a study of the characteristics of the hydrolysis of triglycine by thymus homogenates was undertaken.

METHODS

The study was performed on 63 normal Rockland all-purpose (RAP) mice from 12 to 427 days of age, and on 49 mice of a leukemic strain (Akr) from 18 to 376 days of age. Both male and female mice were used. The animals were kept in a temperature-controlled environment (80°F ± 3°F) and received Purina Fox Chow ad libitum. All mice were apparently healthy, with the exception of one animal of the RAP strain and twelve animals of the Akr strain which were acutely ill with spontaneous lymphatic leukemia, as judged by abnormally enlarged lymphatic organs.

The mice were decapitated, and each thymus was removed, cleaned, weighed on a microtorsion balance, and placed in a homogenizing tube containing isotonic sodium chloride—0.03 M sodium phosphate solution (pH 7.0). The gland was then homogenized with a motor-driven glass pestle (9) for 2 minutes, care being taken to avoid tissue remnants. The tissue concentration of the homogenate was 25 mg/ml. Peptidase activity was measured by the micro-method of Grassmann and Heyde (7), with the use of triglycine (GGG; obtained from Hoffmann-La Roche) as substrate. This substrate was chosen because of the ease with which the hydrolysis can be measured quantitatively, because of the comparatively fast rate at which it is hydrolyzed by the tissue, and because of its relative cheapness. The reaction mixture consisted of 0.2 ml. of tissue suspension, 1 ml. of 0.1 M substrate, pH 7.0, 0.8 ml. distilled water, and 0.01 ml. toluene. Thus, the final tissue concentration was 2.5 mg/ml, the substrate concentration, 0.05 M. Hydrolysis was carried out in triplicate at 38°C and measured by repeated titrations of 0.2-ml. samples with 0.01 N alcoholic KOH (final concentration 90 per cent alcohol). The pH was checked with a glass electrode at the beginning and end of the incubation and remained at pH 7.0 within less than 0.1 unit, owing to the buffering power of the substrate. Unless otherwise stated results are expressed as per cent hydrolysis of the substrate during the 1st hour of incubation, assuming the splitting of one peptide linkage only.

For the purpose of determining the characteristics of hydrolysis of triglycine by thymus homogenates preliminary experiments were carried out on thymus suspensions from the normal RAP strain in which the components of the reaction mixture and the incubation times were varied as described below.

The following methods of statistical analysis were utilized: t test (11) and correlation analysis (12). A probability less than 0.05 was considered statistically significant.

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RESULTS

Characteristics of the hydrolysis of triglycine by thymus suspensions.—The course of hydrolysis of triglycine with time at concentrations of 0.05 M and lower was studied and found to follow first order kinetics. In Chart 1 the course of hydrolysis in reaction mixtures of various substrate and tissue concentrations is shown. The values for the (curves 4 and 3), and of substrate without tissue, in buffer and in distilled water (curves 6 and 5), were also set up. In the complete reaction mixture hydrolysis proceeded beyond the point which would account for the complete splitting of one bond of the tripeptide. However, when the values for the enzyme and substrate controls were subtracted, hydrolysis did not go beyond 100 per cent.

Phosphate had no effect on the rate of hydrolysis of triglycine by tissue. The values of the enzyme control in buffer could therefore be substituted for the values of the enzyme control in water. The pH in the latter sample was too low to permit enzyme activity. During the first 6 hours of incubation the incomplete reaction mixtures did not hydrolyze, obviating the necessity for running enzyme and substrate controls in short-term experiments.

Chart 1c illustrates the results of one experi-
ment in which the activity of aliquots of the same tissue suspension was measured at two different concentrations of substrate. At the concentration of 0.025 M, the absolute rate of hydrolysis was twice as high as at 0.0125 M, the per cent hydrolysis per unit time being the same, in accord with first order kinetics. However, in several other experiments in which the effect of different initial substrate concentrations was tested on aliquots of the same tissue suspension, there was no direct proportionality between the hydrolysis rate and the initial substrate concentration, as would be expected from a first order reaction. This is shown in Chart 2, in which the amount hydrolyzed during the first hour has been plotted against initial substrate concentration. Even for concentrations below 0.05 M the hydrolysis rate increased less rapidly than the substrate concentration, with one exception (curve through points marked + in Chart 2; the course of hydrolysis with time at the two substrate concentrations in this experiment is shown in Chart 1c). Thus, in a reaction mixture the rate of hydrolysis was proportional to the concentration of unhydrolyzed substrate at any time (since first order curves were obtained, see Chart 1), but when the same tissue preparation was added to different substrate concentrations, the rates of hydrolysis were not proportional to the initial substrate concentration. The reason for this is not clear.1

1 With human plasma, on the other hand, the rate of hydrolysis of triglyceride in the presence of 0.001 M cobalt was found by Mrs. R. Richer in our laboratory to be directly proportional to the initial substrate concentration at concentrations between 0.02 and 0.08 M and with 0.4 ml plasma/ml reaction mixture.

At substrate concentrations above 0.05 M and with 2.5-5 mg tissue/ml, there was a further slowing down of increase in rate (Chart 2). However, at a tissue concentration of 2.5 mg/ml, the rate still increased with increase in substrate concentration up to 0.28 M. At higher substrate concentrations it was not possible to dissolve the substrate. In one experiment the effect of substrate concentration was tested at a low tissue concentration, 1.5 mg/ml. Substrate concentration had little or no effect on the initial rate of hydrolysis with this low tissue concentration (Chart 2; see curve labeled 1.5 mg/ml), but again a decrease in rate occurred with time (Chart 1a).

The activity at various substrate concentrations was also found not to be in consistent agreement with the Michaelis-Menten equation as judged from a Lineweaver-Burk (8) plot of the data.

It was thought that increasing the substrate concentration might, by increasing the tonicity of the solution, inhibit the liberation of enzyme from cellular structures and thus slow down the increase in rate with increasing substrate concentration. However, a thymus suspension prepared by homogenizing the tissue in 10 volumes of saline-phosphate and then diluting 1:10 with distilled water showed the same activity as the same tissue diluted with saline-phosphate: 21 and 20 per cent, respectively, of 0.05 M GGG were hydrolyzed in 1 hour at a final tissue concentration of 2.5 mg/ml reaction mixture. Reaction mixtures to which thymus homogenate suspended in saline-phosphate had been added were examined microscopically, and no cells were found.

The rate of hydrolysis was proportional to the tissue concentration at concentrations up to at least 5.6 mg/ml (Table 1).

The pH optimum for the hydrolysis of triglyceride by thymus suspensions was determined in two experiments and was found to be around pH 7.0 (Chart 8).

The results of three experiments in which the effect of various metal ions on the activity of thymus suspensions was examined are summarized

\[\text{TABLE 1} \]

<table>
<thead>
<tr>
<th>TISSUE CONCENTRATION (mg/ml)</th>
<th>(\mu\text{mol H}^+) GROUPS LIBERATED DURING 1ST HOUR</th>
<th>Total</th>
<th>Per mg tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.4</td>
<td>7.5</td>
<td>0.7</td>
<td>2.7</td>
</tr>
<tr>
<td>8.8</td>
<td>30.2</td>
<td>3.5</td>
<td>3.0</td>
</tr>
<tr>
<td>5.0</td>
<td>53.5</td>
<td>3.5</td>
<td>2.4</td>
</tr>
</tbody>
</table>
in Table 2. In the presence of cobalt the rate of hydrolysis is increased, and more \( \text{NH}_3^+ \) groups are liberated than can be accounted for by assuming complete hydrolysis of one bond of the tripeptide. Cobalt is known to activate glycylglycine dipeptidase activity (10). Manganese and magnesium had, if anything, a slightly inhibitory effect.

In comparison with previous findings on the behavior of tripeptidase the following points may be mentioned. The hydrolysis of triglycine by mouse thymus suspensions prepared by homogenizing in saline-phosphate is affected by pH and by metal ions in a similar way as is the hydrolysis of triglycine by human plasma, leukocytes, and erythrocytes (13). On the other hand, the activities of purified tripeptidase preparations of calf thymus and of horse erythrocytes show optima around pH 8 and are not affected by cobalt ions (5, 1). Nevertheless, since in our thymus preparations little or no hydrolysis occurred beyond the point which accounts for the complete splitting of one bond of the tripeptide, it is reasonable to assume that, in the absence of cobalt, mainly tripeptidase activity was measured.

**TABLE 2**

**EFFECT OF METAL IONS ON THE HYDROLYSIS OF TRIGLYCINE BY THYMUS SUSPENSIONS**

(0.05 M GGG; pH 7.0; total volume, 2 ml.; Co\(^{++}\) and Mn\(^{++}\) concentration, 0.001 M; Mg\(^{++}\), 0.01 M)

<table>
<thead>
<tr>
<th>TISSUE CONC. (mg/ml)</th>
<th>HOURS</th>
<th>CONTROL</th>
<th>CO(^{++})</th>
<th>Mn(^{++})</th>
<th>Mg(^{++})</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>3</td>
<td>54</td>
<td>75</td>
<td>104</td>
<td>106</td>
</tr>
<tr>
<td>2.5</td>
<td>5</td>
<td>41</td>
<td>64</td>
<td>104</td>
<td>106</td>
</tr>
<tr>
<td>=</td>
<td>24</td>
<td>104</td>
<td>133</td>
<td>106</td>
<td>106</td>
</tr>
<tr>
<td>6</td>
<td>1</td>
<td>50</td>
<td>62</td>
<td>42</td>
<td>40</td>
</tr>
<tr>
<td>=</td>
<td>3</td>
<td>87</td>
<td>129</td>
<td>87</td>
<td>82</td>
</tr>
<tr>
<td>=</td>
<td>24</td>
<td>109</td>
<td>108</td>
<td>104</td>
<td>97</td>
</tr>
</tbody>
</table>

**CHART 3.**—Effect of pH on the hydrolysis rate; 0.05 M GGG; 5 mg tissue/ml; total volume, 2 ml. Similarly marked values were obtained with aliquots of the same tissue suspension.

**CHART 4.**—Relation between body weight and age: \( \times \) = normal RAP mice; \( \times' \) = RAP mouse with active lymphatic leukemia; \( \bigcirc \) = apparently healthy Akr mice; \( \bullet \) = Akr mice with active lymphatic leukemia; \( x^7, O^7 \) = pregnant mice. The numeral beside a point indicates the number of animals with that value.
With thymus suspensions the rate of hydrolysis of triglycine decreased with time in accord with first order kinetics. First order rates have also been observed with purified horse erythrocyte tripeptidase (1). For purified preparations of calf thymus, on the other hand, zero order kinetics have been reported (5).

Comparison of the hydrolysis of triglycine by suspensions of thymus from the normal (RAP) and the leukemic (Akr) strain of mice.—Before discussing the results on the peptidase activity of the thymus glands, some characteristics of the two animal colonies used in this work will be described.

Chart 4 shows the relation between body weight and age in the normal and the leukemic strain. At all age levels mice of the leukemic strain weighed significantly less than the RAP strain, the difference being highly significant after 2 months of age (P < 0.001). In both colonies the body weight increased rapidly with increasing age until the animals were about 2 months old. Thereafter, the normal strain continued to increase in weight with increasing age (0.02 > P > 0.01), but in the leukemic strain a gradual and insignificant decrease in body weight occurred (P > 0.10). The difference between these two body weight-age correlation coefficients was statistically significant (0.05 > P > 0.02). There was no significant difference between the body weights of Akr animals which had developed leukemia and those of apparently normal Akr animals (0.8 > P > 0.7).

Chart 5 shows the relation between thymus weight and age in both colonies. In the RAP (normal) mice the thymus weight increased with age until the animals were about 1 month old (P < 0.001), after which a gradual but statistically significant involution of the gland occurred (0.05 > P > 0.02). The growth and involution of the gland were also statistically significant when related to the body weight in the two age groupings (0.01 > P > 0.001). In the leukemic strain the thymus weight during the 1st month varied between 18 and 93 mg., but no significant correlation between age and thymus weight was apparent (P > 0.10), possibly because the age range in this strain was narrower, 18-30 days as compared to 12-30 days in the RAP strain. However,
the thymus weight did increase in a statistically significant manner with the body weight in the Akr animals (P < 0.001). In Akr animals 1 month old and older, no significant correlation existed between thymus weight and age or body weight (P > 0.10). This was owing to a marked enlarge-

Table 3 and in Charts 6 and 7. In the RAP and Akr strains, there was no statistically significant change in the peptidase activity of the growing gland (P > 0.10), possibly because the age range studied did not include animals under 12 days of age. In the RAP strain a significant decrease

<table>
<thead>
<tr>
<th>Age grouping</th>
<th>RAP strain</th>
<th>Akr strain</th>
<th>Diff.</th>
<th>Significance of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>All ages</td>
<td>(62) 15.8 ± 0.63*</td>
<td>(49) 17.2 ± 0.59</td>
<td>5.4</td>
<td>0.01 &gt; P &gt; 0.001</td>
</tr>
<tr>
<td>18-30 days</td>
<td>(10) 15.1 ± 0.45</td>
<td>(17) 17.8 ± 0.55</td>
<td>2.7</td>
<td>0.01 &gt; P &gt; 0.001</td>
</tr>
<tr>
<td>6 months and over</td>
<td>(25) 12.6 ± 0.66</td>
<td>(92)† 17.5 ± 1.20</td>
<td>4.9</td>
<td>0.01 &gt; P &gt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(15)† 17.3 ± 1.53</td>
<td>(12)§ 17.3 ± 1.53</td>
<td>Diff.</td>
<td>0.01 &gt; P &gt; 0.001</td>
</tr>
</tbody>
</table>

Figures in parentheses indicate number of mice.
* Standard error.
† Mice with leukemia.
§ Mice without leukemia.

Table 3—Average Peptidase Activity of Thymus Suspensions from the RAP (Normal) and the Akr (Leukemic) Strain of Mice

Per cent hydrolysis of 0.05 M GGG during 1st hour; 2.5 mg tissue/ml

The results on the peptidase activity of the thymus of normal and leukemic mice are shown

occurred during the period when thymic involution takes place (P < 0.001). In the leukemic strain, the peptidase activity showed no statistically significant change with age, even if values for the activity of thymus suspensions from the Akr mice with acute leukemia were excluded (P > 0.10). There was a highly significant difference between the peptidase activity of the glands from the RAP and the leukemic strain (Table 3). The difference was most noticeable in animals over 6 months of age, when Akr mice
develop acute lymphatic leukemia, but was also evident during the 1st month when the mice show no apparent manifestations of the disease. The highest peptidase values in the Akr strain were obtained from glands of animals which did not develop leukemia (Chart 7). There was no statistically significant difference, however, between the peptidase activity of the glands from these animals and from those which did develop lymphatic leukemia (Table 3; 0.9 > P > 0.8).

In the RAP strain one animal was found which had developed spontaneous lymphocytic leukemia. The peptidase activity of the thymus was 29 per cent, as high as the highest value obtained with the Akr strain (Chart 6, indicated by L). This was the only animal of the RAP strain ever found with this disease in our laboratory.

DISCUSSION

The decrease with age in the peptidase activity per unit weight of tissue of the thymus from normal mice is probably mainly due to a replacement of metabolically more active tissue with adipose material and connective tissue. In earlier work in this laboratory, a decrease with age was also observed in the oxygen uptake and peptidase activity of rat thymus (3). The decrease in metabolic activity corresponded to the decrease in the relative amount of cortical tissue of rat thymus as observed by Andreasen (2).

In the leukemic strain the peptidase activity of animals over 6 months of age was in several cases considerably above the level found at 1 month. These high values may indicate either a higher concentration of metabolically active tissue or the presence of a more active type of cell. Both explanations may apply, since histological studies on the thymus of mice with lymphogenous leukemia have revealed a diffuse infiltration of the gland by small, somewhat atypical lymphocytes, or by undifferentiated stem cells: "The normal structure is usually entirely obliterated and the histological picture is uniform, with tightly packed small round cells" (4).

It will be remembered that in the Akr strain high rates of hydrolysis were obtained with thymus glands of normal weight from non-leukemic mice, and conversely normal rates were also observed with enlarged glands from mice which had developed lymphocytic leukemia. The high rate of hydrolysis in "normal" Akr mice may be a manifestation of abnormal metabolic activity occurring before morphological changes are evident. Victor and Potter (14) have found that the glycolysis of lymph nodes from the leukemic C58 strain of mice increases with age and that this increase also occurs before morphological signs of leukemia are apparent. In normal strains a decrease in the glycolytic activity of lymph nodes with age was found by these authors. A pre-leukemic stage may already exist in 1-month-old Akr mice, since at this early age higher rates of hydrolysis by thymus suspensions were also obtained with Akr than with RAP mice. On the other hand, the different peptidase activity observed in 1-month-old Akr and RAP mice may be a strain difference bearing no relation to the leukemic process.

The normal rates of triglycine hydrolysis obtained in a number of animals with active leukemia were surprising. Further studies are needed to explain this behavior. It is conceivable that during the leukemic process the peptide-splitting activity of the thymus reaches a peak and finally decreases in the later stage of leukemia.

SUMMARY

1. The characteristics of hydrolysis of triglycine by thymus homogenates from normal mice were investigated. The course of hydrolysis with time proceeds according to first order kinetics. At tissue concentrations between 1 and 6 mg/ml, the rate of hydrolysis is proportional to the tissue concentration. The pH optimum lies around pH 7.0. In the presence of cobalt, the rate of hydrolysis is increased, and more than one bond of the tripeptide is split. Magnesium and manganese have, if anything, a slight inhibitory effect.

2. In normal Rockland (RAP) mice the peptidase activity of the thymus per unit weight of tissue, measured by the rate of hydrolysis of triglycine in vitro, decreases with the age of the animal.

3. The average peptidase activity of the thymus is significantly higher in Akr than in RAP mice. This difference was found to be greatest, and the highest values in the Akr strain were obtained, at the age period when most of the Akr mice spontaneously develop acute lymphocytic leukemia. However, in animals under 1 month old, the values of the Akr strain were also significantly higher than those of the normal (RAP) strain.

4. There is no significant difference between the peptidase activity of the thymus from Akr mice which had developed spontaneous lymphocytic leukemia and that of apparently healthy Akr mice. The activity with both types of Akr mice varied greatly and ranged from normal to about 2½ times the normal value.

5. The significance of these findings is discussed.
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