Studies on the Biological Action of Malononitriles

II. Distribution of Rhodanese (Transulfurase) in the Tissues of Normal and Tumor-bearing Animals and the Effect of Malononitriles Thereon*

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INTRODUCTION

The enzyme rhodanese, which catalyzes the formation of thiocyanate from cyanide and thiosulfate, is of interest to the cancer problem for reasons to be mentioned below. The administration of nitriles causes an increase in the thiocyanate level of the blood and in its urinary excretion. In seeking an explanation for the growth-retarding effect of certain substituted malononitriles and the lack of effect of others (4), it appeared logical to us to study the relations of this enzyme to tumors and the effect of the malononitriles thereon.

Mendel, Rudney, and Bowman (7) reported that rat tissues (including malignant tumors) which exhibit a high aerobic glycolysis contain negligible rhodanese activity, while tissues in which glycolysis is suppressed by oxygen have high rhodanese activity. The low rhodanese activity of tumor tissues has been reaffirmed by Rosenthal (8, 10), and low values were also found in many tissues with a normal aerobic metabolism. The above reports appeared only as abstracts without details or numerical values. Himwich and Saunders (5), in an investigation of the tissues of normal dogs, found disagreement between their results and those of the above-mentioned authors. On this point it should be mentioned that Rosenthal has noted that there are quite pronounced species differences in the activity of rhodanese of homologous tissues (8).

Mendel et al. (7), moreover, have suggested that, in tissues exhibiting aerobic glycolysis, the lack of rhodanese would permit an accumulation of cyanide which could account for the total or partial disappearance of the Pasteur effect. This hypothesis is of great potential interest for the field of cancer chemotherapy, as it suggests the possibility of altering the metabolic pattern of neoplastic tissue by appropriate inhibitors of the enzymes of glycolysis and respiration, an approach which has to some extent been exploited (1). The above hypothesis suggested that, through the administration of sufficient cyanide or nitriles, cellular respiration could be poisoned to a degree that would bring the metabolism of the tumor cell to a halt. It should be mentioned that a variety of alternate explanations have been offered to account for the lack or the inhibition of the Pasteur effect (8).

Nevertheless, it appeared worth while to explore the relation of rhodanese to the biological activity of the nitriles and, more generally, the significance of this enzyme for the cancer problem.

The questions raised by the discussion given above are: (a) Is the rhodanese activity of tumors really so low as to be totally insignificant? (b) Does continuous administration of malononitriles exert a noticeable influence on the rhodanese activity of the tissues of normal and tumor-bearing animals?

To obtain information on the several points raised, the distribution of rhodanese activity in the tissues of normal and tumor-bearing animals and the influence of malononitrile administration on the enzyme activity have been investigated. In addition, an endeavor has been made to explore the relation of this enzyme to the neoplastic process. The results obtained show that, while the rhodanese content of tumors, in general, is small, it is by no means inconsequential in comparison to other metabolically active tissues.

* This work was supported in part by grants from the National Cancer Institute, United States Public Health Service.
† U.S. Public Health Special Fellow, 1948–50.

Received for publication February 19, 1952.
MATERIALS AND METHODS

The tissues of normal and tumor-bearing strains, A, CSH, and C57 mice, and Long-Evans, Slonaker, and Sprague-Dawley rats were used. The tumors used were mouse carcinomas LCS, CSH-S, E 0771, mouse sarcomas S37, 6CSH-ED, and myeloid leukemia (Bar Harbor C1498), rat carcinomas 256 (Walker), and hepatomas produced by acs dye feeding. The mice were kept under identical conditions to those described in the previous paper (4). Both sexes were employed, as no significant difference in the rhodanese content of their tissue was detectable. In support of the observation by Rosenthal et al. (11), we found that the weight and age of the animals were more critical, since changes in the diet of the animals considerably influenced the enzyme content. All rats were kept on a Purina Laboratory Chow diet and used within a narrow range of weight.

The animals were killed by a blow on the head, the tissues were rapidly excised, put on ice, homogenized with 10 times their weight of distilled water, and filtered through cheese cloth. The homogenates were kept at 5° C. and were worked up within 12 hours of the preparation. We found that additions of the components in the following order gave good results, name-

RESULTS

The first experiments were performed to determine the effect in vitro of a number of nitriles on rhodanese activity. The results are shown in Table 1. In carrying out these experiments, amounts of the nitriles equivalent to the cyanide in experiment 2 were added and then incubated for the time intervals shown in the table. Thiosulfate and cyanide were then added, the enzyme mixtures were incubated for an additional 15 minutes, and the amount of thiocyanate formed was determined. The results show that none of the nitriles tested replaced cyanide as a substrate for the rhodanese in the homogenate enzyme system. Furthermore, these nitriles had no inhibitory effect on the enzyme, when compared to the incubations with either the enzyme and buffer, or the enzyme, buffer, and thiosulfate. In fact, it appears plau-

TABLE 1

THE EFFECT OF NITRILES ON RHODANESE

<table>
<thead>
<tr>
<th>No.</th>
<th>Inhibitor</th>
<th>Quantity† (mg.)</th>
<th>Time: 15'</th>
<th>60'</th>
<th>90'</th>
<th>120'</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.25</td>
<td>0.490</td>
<td>0.270</td>
<td>0.179</td>
<td>0.129</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>2.05</td>
<td>0.790</td>
<td>0.805</td>
<td>0.655</td>
<td>0.410</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.65</td>
<td>0.600</td>
<td>0.446</td>
<td>0.258</td>
<td>0.218</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.05</td>
<td>0.677</td>
<td>0.397</td>
<td>0.207</td>
<td>0.311</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>1.50</td>
<td>0.666</td>
<td>0.510</td>
<td>0.385</td>
<td>0.580</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Na2SO4</td>
<td>0.686</td>
<td>0.570</td>
<td>0.300</td>
<td>0.260</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Na2SO4+KCN</td>
<td>1.150</td>
<td>1.230</td>
<td>1.235</td>
<td>1.235</td>
<td></td>
</tr>
</tbody>
</table>

† Assays were performed in systems composed of 0.2 ml. of homogenate in 7.9 ml. phosphate buffer (pH 7.4) + nitrile (0.005 ml. were used as a control). The amounts of CN group per dose of inhibitor administered were all equivalent to 50 ¿ill of CN~.

‡ Homogenate + buffer only.

§ 50¿ill = 5.80 mg.

ly: 0.5 ml. of filtered tissue homogenate, 7.9 ml. phosphate buffer (pH 7.4), 0.5 ml. 0.1 M sodium thiosulfate, and 0.5 ml. 0.1 M potassium cyanide. The mixture was shaken for different time intervals (15 min.-9 hr.) at 37.5° C. The enzymatic activity was stopped at the desired time interval by adding 10 ml. of ferric nitrate solution, prepared according to the specifica-

TABLE 1. In carrying out these experiments, the components in the following order gave good results, namely that the nitriles might to some extent protect the enzyme against oxidation by tying up some of the trace metals. Furthermore, cyanide caused no inhibition of rhodanese in the homogenates, contrary to previous reports (5).

The other possibility, that the nitriles may have competitively displaced the cyanide, was ruled out in a series of experiments which showed that even twice the cyanide equivalent of the nitriles had no inhibitory effect on rhodanese. All the attempts to produce thiocyanate release from the nitriles in vitro either in homogenates or in tissue slices failed. These results made it necessary to turn to the intact animal to study the catabolism of the nitriles and the possible influence of the catabolic products on rhodanese.

The similarities between neoplastic and embryonic tissues led us to perform experiments to establish the time relations of the development of rhodanese activity in the growing embryo and, simultaneously, the effect of pregnancy on the con-
tent of this enzyme in the liver and kidney of the rat, particularly as no data were found in the literature. The results are shown in Table 2. It is interesting that the enzyme values in liver and kidney are both below the nonpregnant control averages of about 28 and 22 mg CN⁻ converted/gm of tissue, respectively, although it might be expected that pregnancy would provoke an increased response to detoxification mechanisms. The rhodanese activity of the whole embryo is of the same order of magnitude as found for tumor tissue, and when the initial shock of the treatment subsided, the difference in food intake became almost negligible.

In very advanced hepatoma tissues the rhodanese activity became significantly lowered. It is possible that the values for hepatoma are misleadingly low, as the necrotic parts were not removed prior to analysis. No rhodanese activity ever was found in any of the necrotic tissue of any of the rat or mice tumors investigated.

The above results, which made it evident that the azo dye treatment resulted in somewhat lowered tissue content of rhodanese, raised the question as to how transplanted tumors would influence the tissue levels of this enzyme. To this end the tissues of normal and Walker carcinoma 256-

The significance of rhodanese for the neoplastic process was studied by determining the changes in rhodanese activity of the tissues of Sprague-Dawley rats at different stages of advancing malignancy induced by azo dye feeding (3'-methyl-4-dimethylaminoazobenzene) and also by estimation of the rhodanese content of the tissues of normal and tumor-bearing mice.

The results, up to 3 months of azo dye feeding, are shown in Table 3. The livers of the azo dye-fed animals were carefully examined, and the unaffected and diseased portions were separated and analyzed individually. Two of the 3-month animals showed cirrhotic foci but no distinct areas of hepatoma even after 3 months of feeding. The rhodanese activity of the tissues of these animals did not differ from that of the controls, but the enzyme content of the cirrhotic parts of the liver was lowered slightly.

The animals which developed hepatomas showed decreased rhodanese levels. The lower values of the azo dye-fed animals during the early stages might be due to the initial lowered food intake. Later on,

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**TABLE 2**

<table>
<thead>
<tr>
<th>AGE OF EMBRYO (WEEKS)</th>
<th>WHOLE EMBRYO (AV. mg CN⁻ converted/gm tissue)</th>
<th>EMBRYO LIVER (AV. mg CN⁻ converted/gm tissue)</th>
<th>KIDNEY PREG NANT RAT</th>
<th>KIDNEY PREG NANT RAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.39 ± 0.18</td>
<td>2.00 ± 0.12</td>
<td>14.00 ± 2.13</td>
<td>14.50 ± 2.07</td>
</tr>
<tr>
<td>3</td>
<td>1.49 ± 0.18</td>
<td>2.55 ± 0.05</td>
<td>24.30 ± 2.74</td>
<td>22.50 ± 2.65</td>
</tr>
<tr>
<td>at birth</td>
<td>1.65 ± 0.13</td>
<td>14.50 ± 2.70</td>
<td>24.03 ± 2.73</td>
<td>18.50 ± 1.04</td>
</tr>
</tbody>
</table>

* Each group represents tissues and progeny of five female rats of an average weight of 300 gm. Enzyme determinations of the embryos were on the whole embryos including the placenta.

**TABLE 3**

<table>
<thead>
<tr>
<th>PERIOD DYE ADMINISTRATION</th>
<th>AV. WT. ANIMALS</th>
<th>BRAIN (AV. mg CN⁻ converted/gm tissue)</th>
<th>TESTES</th>
<th>ADRENALS</th>
<th>KIDNEY (AV. mg CN⁻ converted/gm tissue)</th>
<th>LIVER (AV. mg CN⁻ converted/gm tissue)</th>
<th>LIVER TUMOR AREAS</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>20 (4)</td>
<td>0.47 ± 0.10</td>
<td>0.70 ± 0.29</td>
<td>0.96</td>
<td>6.18 ± 1.90</td>
<td>8.44 ± 1.40</td>
<td>8.44 ± 1.40</td>
</tr>
<tr>
<td>Control</td>
<td>100 (4)</td>
<td>1.39 ± 0.34</td>
<td>1.71 ± 0.53</td>
<td>1.70</td>
<td>15.00 ± 2.16</td>
<td>20.70 ± 2.56</td>
<td>20.70 ± 2.56</td>
</tr>
<tr>
<td>5</td>
<td>200 (4)</td>
<td>0.60 ± 0.40</td>
<td>1.56 ± 0.58</td>
<td>3.68</td>
<td>14.00 ± 4.19</td>
<td>19.50 ± 3.92</td>
<td>19.50 ± 3.92</td>
</tr>
<tr>
<td>Control</td>
<td>200 (4)</td>
<td>0.60 ± 0.19</td>
<td>1.46 ± 0.14</td>
<td>3.02</td>
<td>16.00 ± 0.85</td>
<td>23.00 ± 3.00</td>
<td>23.00 ± 3.00</td>
</tr>
<tr>
<td>12</td>
<td>250 (4)</td>
<td>1.19</td>
<td>1.70</td>
<td>2.70</td>
<td>28.70</td>
<td>28.70</td>
<td>28.70</td>
</tr>
<tr>
<td>Control</td>
<td>270 (4)</td>
<td>0.80 ± 0.09</td>
<td>1.70 ± 0.33</td>
<td>3.00</td>
<td>17.00 ± 2.54</td>
<td>29.50 ± 5.76</td>
<td>29.50 ± 5.76</td>
</tr>
</tbody>
</table>

* The azo dye-treated animals were obtained through the courtesy of Dr. A. Clark Griffin, Dept. of Chemistry, Stanford University, Stanford, Calif.

† Figures in parentheses represent number of animals analyzed statistically.

‡ The adrenals of the animals were pooled.

§ Cirrhosis.

# Well developed hepatoma.
bearing Slonaker rats were examined on animals in which the weights and the diet were carefully equalized. The Walker carcinoma was preferred, because of not only its good response to chemotherapeutic agents, but also its rapid growth and abundant vascularization. The latter made it the tumor of choice for the study of the supply of cyanide and the draining-off of thiocyanate in tumors.

Table 4 contains the results obtained with tumors after 2-3 weeks of growth. The trend toward lower rhodanese values in the tissues of the Walker 256 carcinoma-bearing rats is unmistakable. Nevertheless, the results must be accepted with caution until it is established more conclusively that the effect observed was actually a function of the malignancy.

Rats, because of their size, were convenient animals for the enzyme and detoxication studies. However, since mouse tumors were used in the experiments investigating the growth-retarding effect of malononitriles, it was important to estimate the rhodanese content of the tissues of normal and tumor-bearing mice. Table 5 shows the tabulated values of the rhodanese activity of the different strains of normal and tumor-bearing mice. In contrast to the observations with rats, the transplanted tumors of the mice did not appreciably influence the rhodanese content of the tissues analyzed. The efficiency of the cyanide conversion of the tumors was not much lower than that of either the brain or the spleen, and, in the case of the tumor LCS-A, it was even higher than the values for the brain and spleen of the control animals.

The above results bring us back to our first question and the specific problem of whether the rhodanese content of the tumors is really insignificant. In certain types it is decreased, impaired perhaps, but it is not insignificant. If one considers that most of these tumors grow to 3-5 gm., then their power to convert cyanide into thiocyanate becomes considerable. The specific activity per gram of tumor weight was, no doubt, even higher at the early period of the growth when the necrotic parts of the tumor did not as yet cause much dilu-
onic tissue. Even though the liver of a 2-week-old embryo is so small that manipulation under a binocular is necessary, on the basis of its rhodanese activity it could transform close to 2 μg of cyanide/mg/15 minutes. This is not insignificant when it is considered that an average adult rat excretes between 60 and 160 μg. of thiocyanate daily, which, at a most conservative estimate, corresponds to not less and perhaps more than 10 per cent of the total thiocyanate content of the rat body.

In order to answer our second question regarding the release of cyanide from the malononitriles as being responsible for the growth-retarding effect, the rhodanese content of animals treated with malononitriles was investigated.

The results of these observations are tabulated in Table 6. This table indicates that neither malononitrile nor p-nitrobenzalmalononitrile had any significant effect on the rhodanese content of the tissues of the CSH-S-bearing mice. The values of the control animals in this series agree well with those of the CSH animals given in Table 5.

**DISCUSSION**

The intriguing hypothesis of Mendel and his associates (7) attributes the partial or total abolition of the Pasteur effect in some tissues with high aerobic glycolysis to the accumulation of cyanide. They reason that this accumulation of cyanide is the result of the practical absence of rhodanese activity in those tissues. In brain, spleen, and ileal mucosa, where rhodanese occurs in sufficient quantities, they argue that glycolysis is suppressed in oxygen almost to "the maximum anticipated by the Meyerhof quotient," whereas in tumors the aerobic glycolysis would not be affected because of the presence of enough cyanide to inhibit most of the respiration. These arguments gain some support through the observations of Warburg (18) that cyanide favors aerobic glycolysis of cells and that embryonic tissues can become malignant through the anoxia caused by cyanide administration to tissue cultures. A serious objection against attributing the lack of the Pasteur effect in tumors to an insufficiency of the rhodanese content arises from the observation made by Rosenthal (9, 10) and the work reported here.

If cyanide is to inhibit cellular function to a significant degree, without causing death of the animal, then the total amount of cyanide produced cannot exceed 26-70 μg/kg/15 min. Of course, it is known that in vivo death would be due to the rapid poisoning of the cytochrome system of the brain. This again does not signify that in vivo the rhodanese would not be able to detoxify that lethal amount of cyanide.

We know that there is a small but steady rate of thiocyanate formation in the body. Also, it may

**TABLE 6**

**The Effect of Injection of Malononitrile on the Rhodanese Content of CSH-S-Bearing Mice**

<table>
<thead>
<tr>
<th></th>
<th>Av. wt. (gm.)</th>
<th>Tumor (Av. mg CN⁻ converted/gm tissue)</th>
<th>Liver</th>
<th>Kidney</th>
<th>Adrenals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malononitrile</td>
<td>25.0</td>
<td>0.79±0.08</td>
<td>10.92±1.05</td>
<td>3.15±0.92</td>
<td>1.50</td>
</tr>
<tr>
<td>p-Nitrobenzalmalononitrile</td>
<td>23.5</td>
<td>0.68±0.04</td>
<td>11.05±0.86</td>
<td>2.50±0.79</td>
<td>1.48</td>
</tr>
<tr>
<td>Control</td>
<td>20.0</td>
<td>0.58±0.13</td>
<td>9.91±1.18</td>
<td>3.59±0.85</td>
<td>1.65</td>
</tr>
</tbody>
</table>

* Duration of treatment was 16 days. Administered daily dose was 40 μg. of malononitrile and 160 μg. of p-nitrobenzalmalononitrile in 0.05 ml. asym. propylene glycol. These quantities are less than the previously employed therapeutic doses given in Table 3.

* Adrenals were pooled.

† The controls were CSH-S-bearing untreated animals.
presented in this paper, and also from other unpublished evidence, the authors feel that, as far as the tumors analyzed by them were concerned, the interesting hypothesis of Mendel and his associates cannot be supported. In this respect the conclusions reached agree with certain of Rosenthal's observations (9, 10).

The reasons for an anomalous Pasteur effect in tumors must, therefore, be due to other causes.

SUMMARY
1. Malononitriles did not inhibit the rhodanese activity in tissue homogenates, nor did they competitively displace the cyanide.
2. The rhodanese content of fetal tissue increased in amount with development and at birth. The rhodanese activity of the tissue of the mother was not significantly influenced by pregnancy.
3. Animals fed 3'-methyl-4-dimethylaminoazobenzene showed a slight decrease in the rhodanese activity coincident with the development of hepatoma. The hepatoma itself showed a significantly damaged rhodanese activity, while the surrounding unimpaired liver tissues continued functioning normally.
4. The Walker rat carcinoma 256 produced a general lowering of the rhodanese activity in the tissues, which could not be correlated with either loss of weight or a lowered food intake. This was not the case in the tissues of mice with transplanted tumors.
5. A detailed analysis of different mouse transplanted tumors and the tissues of both the normal and tumor-bearing mice of strains A, C3H, and C57 revealed that the tumors do not have less rhodanese content than their homologous tissues or of some of the vital organs. Liver and kidney, in comparison, possess a disproportionately high activity of rhodanese that well fits in with their function of being the prime sites of cyanide detoxification.
6. Administration of malononitrile and p-nitrobenzal malononitrile to C3H-S-bearing mice for a period of 16 days did not influence the rhodanese activity of the tissues or that of the tumors.

REFERENCES
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