Enzyme, Nitrogen, and DNA Concentrations in Sarcoma 180 in Mice Treated with 6-Mercaptopurine*

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SUMMARY

The concentrations of homogenate-nitrogen, of DNA, and the activities of various nucleotidases, adenosine deaminase, cathepsin and lactic dehydrogenase have been studied in homogenates of Sarcoma 180 from nontreated mice, from mice given injections of 6-carboxymethylcellulose in saline, and from mice treated with 6-mercaptopurine suspended in saline containing 6-carboxymethylcellulose.

Statistically significant increases in the activities of all the nucleotidases, adenosine deaminase, and cathepsin have been observed in tumor homogenates of mice treated with 6-mercaptopurine. Statistically significant decreases in tumor weight, homogenate-nitrogen, DNA, and lactic dehydrogenase have been observed in the same homogenates.

The tumor homogenates of the controls receiving 6-carboxymethylcellulose in saline showed only increases in adenosine deaminase and cathepsin which, however, did not reach the activity levels attained in homogenates of mice treated with 6-mercaptopurine.

The significance of the findings is discussed.

Although the enzymic patterns of tumor growth have been the subject of many investigations (18), the processes of regression or arrested growth, whether spontaneous or induced by chemotherapy, have received relatively little attention. The study of most spontaneously regressing tumors is fraught with technical difficulties, since the number of such regressions is usually unpredictable, thus making unavailable sufficient material for systematic investigations. Studies on the spontaneously regressing Flexner-Jobling carcinoma, which shows spontaneous regression in about 35 per cent of all transplants, revealed definite changes in the enzymic pattern and, consequently, in the metabolism of the regressing tumors (8–10). Within the past few years the effects of the administration of alpha peltatin (26), acetylpodophyllotoxin derivatives (27), chloroethyamine derivatives (11), ethyleneimine derivatives (15–18), colchicine (1), and of 6-mercaptopurine (21) on glucose utilization, over-all glycolysis, DPN content, and a few other metabolic aspects have been investigated. Among the chemotherapeutic agents studied so far in their effect on tumor metabolism, 6-mercaptopurine occupies an important position because of its growth-inhibitory effect on both human and animal tumors (22) with relatively few toxic side effects (4).

This study is the first of a series in which a systematic effort will be made to elucidate the metabolic changes induced in tumors by administration of 6-mercaptopurine to the host. Sarcoma 180 has been chosen as the experimental tumor, since its histology is well known, treatment of the host with 6-mercaptopurine induces no necrosis in the tumor, nor do tumors of nontreated hosts contain necrotic portions, provided they are collected...
not later than the 9th day after transplantation (5).¹

MATERIALS AND METHODS
Randomly selected female Swiss albino mice (HaICR²) weighing between 18 and 29 gm. served as experimental animals and also as carriers of the tumor line. The tumor line was maintained by bilateral implantation of tumor fragments of equal size; the site of implantation was the subcutaneous ventro-lateral region of the host mice. It was customary to harvest these tumors on the 7th day after implantation. Twenty-six fragments, each approximately 5 mg. wet weight, were regularly prepared from one tumor and transplanted into the right ventro-lateral region of as many animals.

Thirteen of the tumor-bearing mice received daily treatment with 6-MP for 7 successive days, beginning at 24 hours after tumor implantation (Group 3). The daily dose was 75 mg/kg of body weight, suspended in 1.0 ml. of saline containing 0.5 per cent of CMC.³ This dose was divided into 2 parts of 0.5 ml.; one was injected regularly in the morning of each day and the other 7–8 hours later. A second group of thirteen mice (Group 2), serving as controls to the 6-MP-treated group, received comparable injections of CMC in saline only. The tumors from all mice were harvested regularly between the 7th and 9th day following implantation. Three tumors from each group were selected at random for histologic examination; the remainder were employed for the biochemical assessments of the present study. Another control group consisted of mice given implants of tumor. These mice received no injections of any kind (Group 1). These animals were also sacrificed between the 7th and 9th day after implantation and their tumors removed to ascertain that the various parameters under study did not vary significantly from normal values established for Sarcoma 180 as a preliminary study to the present one. A final control group (Group 1a) consisted of nontreated mice with tumors that were removed between the 4th and 5th day after implantation. The average weight of these younger tumors approximated that of the tumors from the 6-MP-treated mice at the end of 7 days of treatment. The younger tumors were studied to assess the influence of tumor weight on the various parameters.

Preparation of tumors homogenates.—The animals were killed by cervical dislocation; the tumors were excised, weighed, washed briefly in ice-cold saline, and dissected to remove macroscopically visible blood vessels. The tumor slices were again briefly washed in saline, freed quickly from any adhering moisture by being pressed between filter paper, weighed on a torsion balance to within 1 mg., and immediately transferred to glass homogenizing tubes immersed in crushed ice. Homogenization was effected in saline in a polytetrafluoroethylene (Teflon) homogenizer. The time required for homogenization did not exceed 1 minute. The homogenates were then filtered through a thin layer of glass wool to remove macroscopically visible pieces of nonhomogenizable material, mostly connective tissue.⁴ The homogeneity of the filtrates was ascertained by the fact that aliquots were found to contain equal amounts of nitrogen estimated as described below. The time lapse between homogenization and incubation with the substrate was not allowed to exceed 15 minutes.

Assay of enzyme activity.—Catheptic activity was estimated by determination of tyrosine assayed in the NPN fraction after incubation of the homogenates with a preparation of purified horse blood hemoglobin. The method of assay and the preparation of the hemoglobin solution have been described (8). Two ml. of hemoglobin were mixed with 2 ml. of acetate buffer, pH 3.5, and 1 ml. of homogenate was added. The incubation time was 15 minutes for homogenates of Groups 2 and 3 and was extended to 30 minutes for those of Group 1 and 1a. Homogenates were prepared of 1 part tissue (wet weight) and 30 volumes of saline.

Determination of ATPase.—ATPase was determined as previously described (9), but EDTA was added to a final concentration of 0.001 M, and the MgCl₂ concentration increased to 0.2 ml. of a 0.0984 M solution in a total of 3.2 ml. of reaction mixture.⁵ Homogenates were prepared of 1 part tissue (wet weight) and 30 volumes of saline.

¹ The following abbreviations will be used: 6-MP for 6-mercaptopurine; CMC for 6-carboxymethylcellulose; ATP and ATPase, respectively, for adenosine triphosphate and adenosine triphosphatase; DNA for deoxyribonucleic acid; NPN for nonprotein nitrogen; homogenate-N for homogenate nitrogen; Pₐ for orthophosphate; EDTA for ethylenediaminetetraacetic acid. The term "CMC-treated" implies I.P. injections of 6-carboxymethylcellulose in saline; the term "6-MP-treated" indicates that the mice received I.P. injections of 6-mercaptopurine in saline containing 6-carboxymethylcellulose.

² Millerton Research Farm, Inc.
³ 6-MP in the dose administered is insoluble at, or near, neutrality. CMC has been used to stabilize the finely dispersed suspensions of the compound.

⁴ In all instances where EDTA was added the optimum Mg²⁺ concentration was established.
1 part tissue (wet weight) in 40 volumes of saline.

**Determination of adenosine-5'-phosphatase.**—Adenosine-5'-phosphatase was determined by assay of the Pi liberated in a mixture consisting of 20 μmoles of adenosine-5'-phosphoric acid (Sigma) dissolved in 0.6 ml. of water and adjusted to pH 7.5; 1.20 ml. of 0.038 M diethyl barbiturate buffer, pH 7.5; 0.2 ml. of a 0.0984 M MgCl₂ solution; 0.2 ml. of a 0.016 M EDTA solution; and 0.6 ml. of water. After a 1-minute pre-incubation time, 0.4 ml. of homogenate was added to make a final volume of 3.2 ml. The homogenate was prepared from 1 part tissue (wet weight) and 30 volumes of saline. The temperature of incubation was 37° C., and the incubation time 30 minutes. The reaction was terminated by addition of 0.8 ml. of a 50 per cent trichloroacetic acid solution.

**Determination of inosine-5'-phosphatase.**—Inosine-5'-phosphatase was determined by the Pi liberated in a mixture similar to that described above for adenosine-5'-phosphate except that 9 μmoles of the tetrahydrate of the disodium salt of inosine-5'-phosphate (Sigma) served as substrate and 0.30 ml. of 0.0984 M MgCl₂ was used.

**Determination of adenosine deaminase.**—Adenosine deaminase was determined spectrophotometrically by the method of Kaplan, Colowick, and Ciotti (19). To 2.95 ml. of phosphate buffer, pH 7.5, containing 0.218 μmoles of adenosine (Pabst) 0.05 ml. of tumor homogenate was added diluted as described for adenosine-5'-phosphatase. The decrease in optical density at 265 μm was observed every 30 seconds after the first 30-second interval for a total of 4 minutes. The temperature of incubation was 37.5° C.

**Lactic dehydrogenase.**—Lactic dehydrogenase was estimated by the method of Meister (30). One part tissue (wet weight) was homogenized with 40 volumes of saline, filtered through glass wool, and then again diluted 50-fold.

**Determination of DNA.**—DNA was extracted from the tumor tissues essentially by the method of Schneider (23). Equal volumes of 10 per cent trichloroacetic acid and glass wool-filtered homogenate, prepared by homogenizing 1 part tumor tissue with 20 volumes of saline, were mixed and heated for 20 minutes at 98° C. DNA standards in 5 per cent trichloroacetic acid were heated at the same time. DNA was determined in the supernatants according to Brody's modification of Dische's method (3). Color development at 25° C. was allowed to proceed for 30 minutes, after which it reached a maximum, and the color remained unchanged for at least 20 additional minutes. DNA concentration has been expressed as μg DNA/ml of glass wool-filtered homogenate.⁴

**Determination of nitrogen.**—Nitrogen was assayed in aliquots of the glass wool-filtered homogenates used for enzyme assays by micro-digestion with sulfuric acid and H₂O₂ followed by nesslerization of aliquots. Determinations were done in duplicate, with a Coleman Junior spectrophotometer.

**Measurement of enzyme activity.**—All measurements of enzyme activities are based on the initial zero order time interval of the reactions. Preliminary experiments provided assurance that reaction velocity was directly proportional to the homogenate concentration. The enzyme activities, with the exception of cathepsin, were uniformly assayed in the pH range of 7.4–7.5. At this pH glycolysis proceeds at maximum rate. The role of the above enzymes in the over-all glycolysis by Sarcoma 180 homogenates is at present under investigation.

**RESULTS**

The histopathologic effect of intraperitoneal administration of 6-MP to the host on Sarcoma 180 (5) has been re-examined and confirmed.⁷ The principal characteristic cytologic change consisted predominantly of giant, multinuclear cells with both nuclear and cytoplasmic vacuolization. These cytologic changes appear to persist until resorption of the tumor is complete or growth is resumed. Histologic examination of the tumors from mice treated with 6-MP or of control tumors revealed no necrosis; connective tissue was found only in the peripheral portion of the sarcomas. When tumors of nontreated mice were collected in the earlier phases of tumor growth (between the 4th and 5th day after implantation, Group 1a) the original transplant was still clearly discernible; this portion was discarded. Macroscopic examination revealed poor vascularization of tumors of 6-MP-treated mice (Group 3). About 1 per cent of the tumors of the control groups (Groups 1, 1a, and 2) were so thoroughly vascularized that a clear-cut separation of tumor tissue from macroscopically visible blood vessels could not be effected. Such tumors were rejected.

Since CMC was used as a vehicle for 6-MP and the tumors of the 6-MP treated animals (Table 1, Group 3)⁸ were considerably lower in weight than those of the control groups (Groups 1, 1a, and 2) since CMC was used as a vehicle for 6-MP treated mice (Group 3), pools of two or three tumors were used for each homogenate specimen.

⁴The authors are indebted to Dr. L. F. Cavalieri for a generous sample of highly purified DNA which was used as standard.

⁷The authors are greatly indebted to Dr. Stephen Sternberg for the histologic examination of the tumor specimen.

⁸Because of the low weights of the tumors of Group 1a and Group 3, pools of two to three tumors were used for each homogenate specimen.
those of the nontreated control groups (Table 1, Groups 1 and 2), it was necessary to evaluate the effect of i.p. injections of CMC on the one hand, of tumor weight on the other, on the parameters studied. The influence of CMC on the parameters of homogenates from tumors harvested between the 7th and 9th day is presented in Table 1, Group 2. As compared to the parameters of the tumor homogenates from tumors of comparable weight of nontreated mice (Group 1), Group 2 showed no significant changes except in cathepsin and adenosine deaminase levels, which were both increased (P in both instances <0.01).

The effect of tumor weight on the various parameters was established as follows: tumors of nontreated mice were harvested between the 4th and 5th day after transplantation (Table 1, Group 1a) when their weights fell within the range of the 8-day-old tumors of the 6-MP-treated mice (Table 1, Group 3). The homogenate-N and DNA concentration, as well as the inosine-5'-phosphatase activity of the former tumor homogenates, was significantly lower (P = 0.02 for homogenate-N, <0.01 for DNA and for inosine-5'-phosphatase) than in the tumor homogenates from nontreated mice collected between the 7th and 8th day (Table 1, Group 1). The ATPase, adenosine-5'-phosphatase, lactic dehydrogenase, adenosine deaminase, and cathepsin activities showed no significant change.

The following parameters of the tumor homogenates of Group 3, Table 1 (6-MP-treated mice) showed highly significant decreases as compared with the CMC controls (Table 1, Group 2): tumor weight, homogenate-N, DNA, and lactic dehydrogenase (P in all instances <0.01). In contrast, highly significant increases were obtained for cathepsin, ATPase, adenosine-5'-phosphatase, inosine-5'-phosphatase, and adenosine deaminase (P in all instances <0.01). Statistical evaluation showed that the DNA and homogenate-N concentration and the lactic dehydrogenase activity were significantly lower, and all the other enzyme activities significantly higher in Group 3 than in Group 1a, the nontreated group with comparable tumor weights (P for DNA = 0.02, for all other parameters <0.01). Table 2 shows that the homogenate-N increased with the weights of the sarcomas.

**DISCUSSION**

The parameters studied refer solely to that part of the tumor tissue extractable by a 1-minute homogenization. The connective tissue could not be investigated, owing to its failure to disintegrate, as reported above. From the available references concerned with the composition and metabolism of connective tissue of various types and origins (2, 24) it appears unlikely that the connective tissue portion of the tumor plays a significant part in its over-all metabolism. The portion of the homogenizable tissue nitrogen increases in the course of tumor growth; this is shown by the ratio of µg.

**TABLE 1**

**EFFECT OF 6-MP ON TUMOR WEIGHT, DNA, AND ENZYME PARAMETERS**

<table>
<thead>
<tr>
<th></th>
<th>NONTREATED</th>
<th>CMC-TREATED</th>
<th>6-MP TREATED</th>
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<tbody>
<tr>
<td></td>
<td>Group 1</td>
<td>Group 1a</td>
<td>Group 2</td>
</tr>
<tr>
<td>Tumor weight (mg)</td>
<td>652 ± 179 (40)</td>
<td>155 ± 68 (119)</td>
<td>710 ± 210 (142)</td>
</tr>
<tr>
<td>Homogenate-N (µg/ml)§</td>
<td>688 ± 54 (14)</td>
<td>566 ± 66 (14)</td>
<td>620 ± 45 (9)</td>
</tr>
<tr>
<td>DNA (µg/ml)§</td>
<td>184 ± 30 (14)</td>
<td>137 ± 25 (14)</td>
<td>160 ± 23 (9)</td>
</tr>
<tr>
<td>Cathepsin</td>
<td>392 ± 80 (17)</td>
<td>290 ± 114 (10)</td>
<td>1980 ± 508 (97)</td>
</tr>
<tr>
<td>ATPase</td>
<td>1860 ± 381 (23)</td>
<td>1840 ± 172 (10)</td>
<td>2090 ± 645 (10)</td>
</tr>
<tr>
<td>Adenosine-5'-phosphatase</td>
<td>162 ± 65 (15)</td>
<td>127 ± 37 (15)</td>
<td>176 ± 57 (90)</td>
</tr>
<tr>
<td>Inosine-5'-phosphatase</td>
<td>132 ± 34 (15)</td>
<td>88 ± 7 (11)</td>
<td>114 ± 32 (19)</td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>84 ± 18 (15)</td>
<td>94 ± 15 (6)</td>
<td>115 ± 19 (17)</td>
</tr>
<tr>
<td>Lactic dehydrogenase</td>
<td>1180 ± 127 (16)</td>
<td>1040 ± 90 (18)</td>
<td>1060 ± 157 (22)</td>
</tr>
</tbody>
</table>

* Number of tumors or homogenate specimens are given in parentheses.
† Denotes statistically significant difference from Group 1.
‡ Denotes statistically significant difference from Group 1a.
§ Denotes statistically significant difference from Group 2.
# Estimated in glass wool-filtered homogenates of 1 part wet tissue in 20 volumes of saline.
The enzyme activities are expressed per mg. of homogenate-N as follows: catheptic activity as µg. of tyrosine liberated in 1 hour; ATPase, adenosine-5'-phosphatase, and inosine-5'-phosphatase as µg. of P1 liberated in 1 hour; adenosine deaminase and lactic dehydrogenase, respectively, as µg. of NH3 and of lactic acid formed in 1 minute.
homogenate-N/weight of tumor (mg.) (Table 2), and this indicates the relative decrease in connective tissue. Similar observations have been made with two rat sarcomas, where the amount of connective tissue was determined by histological technics (12). The increase in homogenate-N in the course of the growth of Sarcoma 180 in nontreated mice parallels the increase in DNA fairly well. Of the enzyme parameters only inosine-5'-phosphatase was a function of tumor weight, being significantly decreased in the younger tumors.

Comparison of tumor homogenates of 6-MP-treated mice with those of either nontreated or CMC-injected mice showed highly significant differences in all parameters. As has already been stated, the CMC control tumor homogenates had significantly higher adenosine deaminase and cathepsin levels than the nontreated controls but showed no changes in the other parameters, e.g.,

<p>| Table 2 |</p>
<table>
<thead>
<tr>
<th>Tumor Weight and Total Soluble Nitrogen Recovery of Tumors of Nontreated Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor weight (mg.)</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>155 ± 68</td>
</tr>
<tr>
<td>680 ± 250</td>
</tr>
<tr>
<td>1,240 ± 280</td>
</tr>
</tbody>
</table>

* In glass wool-filtered homogenates of 1 part wet tissue in 20 volumes of saline.

As the data that have been presented show, the significantly different values for all the parameters of the homogenates of the 6-MP-treated group cannot be explained on the basis that these tumors weigh less than those of the two control groups. Comparison with homogenates of tumors within the same weight range implanted into mice which received no subsequent treatment (Table 1, Group 1a) showed the same pattern of differences as compared with homogenates of tumors of greater weights (Table 1, Group 1).

Administration of 6-MP has been shown to inhibit the growth of the Flexner-Jobling carcinoma (25). Heidelberger and Keller (14) found that administration of 6-MP to rats carrying this tumor is associated with a diminished uptake of C14-labeled guanine and adenine into the DNA fraction of the tumor. Our data show that treatment of the host with 6-MP (Table 1, Group 3) not only results in tumors with a much lower weight average but is also associated with a lower DNA concentration in the tumor homogenates as compared with the control groups. However, the DNA assayed derives solely from the saline-extractable material of the tumor tissue, representing the nuclei and the cytoplasm, but not the connective tissue. It thus represents the major but not necessarily the total amount of DNA.

Increases in the activities of a variety of enzymes, notably of nucleotidases and of cathepsin, have been observed in homogenates of spontaneously regressing Flexner-Jobling carcinomas (8–10), and of cathepsin in extracts of dystrophic muscle tissues (28, 29). The significance of increased levels of nucleotidases, especially of ATPase or apyrase, has been studied in spontaneously regressing tumors and found to be associated with a shift from ADP rephosphorylation to ADP breakdown, terminating in the formation of inosine (10). The ensuing uncoupling of phosphorylation was evident in a decreased phosphate uptake and lactic acid production (9). It is of interest that a 50 per cent decrease in both oxygen uptake and glycolysis was observed in previous studies with tissue slices of Sarcoma 180 collected from mice treated with 6-MP (21). The possibility that a mechanism similar to the one reported above is operative in this instance is at present under investigation.

Lactic acid dehydrogenase has been so far the only enzyme studied in tumor homogenates of 6-MP-treated mice which showed decreased instead of increased activity. The significance of this observation on the over-all glycolysis by these homogenates will be assessed within the framework of a general study of the glycolysis and respiration of all four groups of homogenates.

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