Metabolic Inhibitors and Nucleotide Turnover

I. Inhibition of Uptake of Radiophosphate in Rat Liver and Hepatoma Slices

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In chemotherapeutic studies on various types of cancer, it has been observed that a few compounds related in structure to the purines and pyrimidines have a carciolytic effect on certain tumors. It is of considerable interest to elucidate the mechanisms by which these effects are produced. Toward this end investigations have been carried out in this laboratory on the effect of a purine analog, 2,6-diaminopurine, and of a pyrimidine nucleoside analog, 5-aminouridine, on the incorporation of labeled nucleic acid precursors into the ribonucleic acids of slices of rat liver and of a transplantable rat hepatoma. 2,6-Diaminopurine has been shown to prolong the life of leukemic mice (3, 10) and to be selectively toxic for tissue cultures of tumor tissue (1, 12). Stock and Sugiura (reported by Visser [16]) have shown that 5-aminouridine inhibits some types of tumor growth in rodents, and it has also been established that this compound is a metabolic inhibitor in Neurospora (9). In this paper are described the effects of these compounds on the incorporation of P32 into gross tissue fractions and into the individual ribonucleotides.

MATERIALS AND METHODS

Animals.—The rats used in this study were the grey Irish variety, strain A X C. A strain of hepatoma 1 was maintained in these rats by serial intraperitoneal transplants at intervals of 3-6 weeks. Tumors were allowed to grow to a size of 10-20 gm. before being used experimentally.

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Incubations.—Animals were killed by cervical dislocation and the desired tissue removed and sliced with a Stadie-Riggs tissue slicer (11) into slices 0.5 mm. in thickness. The slices were blotted, and portions weighing approximately 600 mg. (wet weight) were placed in 25-ml. Erlenmeyer flasks containing 2 ml. of Krebs-Ringer-Bicarbonate buffer (15) fortified with 0.1 per cent glucose. Inhibitor compounds were added as indicated. After a 15-minute equilibration period with 95 per cent O2-5 per cent CO2 gas mixture, 5-25 mc. of P32PO4 in 1 ml. of the same buffer were added and the vessels shaken at 120 2-cm. strokes per minute in the incubator for 4 hours at 37° C.

Inhibitors.—2,6-Diaminopurine 3 and 5-aminouridine (8) were dissolved in buffer and added to the incubation vessels at a final concentration of 6.7 DIM.

Fractionation.—The tissue was fractioned according to the procedure described by Rafelson et al. (7), obtaining the trichloroacetic acid-soluble, lipid-free residue (TPP), the phospholipid fraction (LP), and the inorganic phosphate fraction (IP). The pentosenucleic acid present in the TPP fraction was partially purified and hydrolyzed to yield a mixture of the four ribonucleotides (18).

Isolation of nucleotides.—Individual nucleotides were isolated from the mixture by ionophoresis on filter paper at pH 3.7, ionic strength 0.07, and eluted according to the methods previously reported (18).

Analysis.—The LP fraction was digested by a micro-Kjeldahl method and hydrolyzed to orthophosphate. The total phosphorus in the LP and IP fractions was determined according to a modification of the method of Fiske and Subbarow (18).

* The P32PO4 was obtained on allocation from the Atomic Energy Commission, Oak Ridge, Tenn.

† Kindly supplied by Dr. G. B. Brown, Sloan-Kettering Institute, New York, N.Y., and Miss K. Fukuhara, University of Southern California, Los Angeles, Calif.
The phosphorus content of each of the ionophoretically separated nucleotides was calculated from the observed ultraviolet absorption measured in a Beckman DU spectrophotometer by means of the published molar extinction coefficients (6).

**Determination of radioactivity and expression of results.**—Aliquots of each fraction were evaporated in copper planchets and counted either with a shielded mica end-window or with an internal gas flow Geiger counter. All samples were counted for a time sufficiently long to reduce the counting error to values negligible in comparison to the observed variation between replicate samples. Radioactivity results for control samples are reported values (less than 0.01). The extent of incorporation of $^{32}$P into both liver and hepatoma during the first 4 hours was proportional to the time of incubation, as found also by Williams et al. (19) with mouse tumors. This indicates a stable metabolic system and an adequate supply of oxidizable substrate.

Preparations of liver and hepatoma slices were incubated in the absence of inhibitor, in the presence of each inhibitor singly, and in the presence of both inhibitors simultaneously. The results are summarized in Tables 1 and 2.

**Control incubations.**—It was observed (Table 1) that the incorporation of $^{32}$P into uridylic acid was greater than that into cytidylic acid, and the incorporation into adenylc acid was greater than that into guanylic acid, an observation made repeatedly by other investigators (2, 4, 13, 14).

**Effects of inhibitors.**—The effects of inhibitors on $^{32}$P uptake are shown in Table 2. The presence of 5-aminouridine caused a significant and fairly uniform reduction of incorporation into all the components determined for each tissue. The effect on the liver fractions was in each case (excepting uridylic acid) greater than that on the hepatoma fractions.

The presence of 2,6-diaminopurine caused a significant reduction of incorporation into all the components of each tissue. In liver, this compound caused a greater effect on the purine nucleotides as relative specific activity (RSA), this being defined as the counts/min/μg of phosphorus for each of the fractions expressed as the percentage of the counts/min/μg of phosphorus in the IP fraction. Results for samples containing inhibitors are expressed as per cent inhibition, i.e.,

$$\text{RSA of control} \times 100$$

All values are the mean of four incubation samples.

**RESULTS**

Preliminary studies established the fact that in experiments in which $^{32}$P was added immediately prior to precipitation with trichloroacetic acid, the TPP and LP fractions had very low RSA was greater than that into cytidylic acid, and the incorporation into adenylc acid was greater than that into guanylic acid, an observation made repeatedly by other investigators (2, 4, 13, 14).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Phospholipid</th>
<th>Adenylic acid</th>
<th>Guanylic acid</th>
<th>Uridylic acid</th>
<th>Cytidylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>1.32±0.10</td>
<td>1.54±0.12</td>
<td>1.04±0.08</td>
<td>2.18±0.04</td>
<td>1.48±0.26</td>
</tr>
<tr>
<td>Hepatoma</td>
<td>5.79±0.08</td>
<td>1.52±0.05</td>
<td>1.05±0.05</td>
<td>1.36±0.10</td>
<td>1.15±0.05</td>
</tr>
</tbody>
</table>

* Counts/min/μg of P in fraction divided by counts/μg of IP times 100.
† Each value is the mean of four replicate incubation tubes.
‡ Standard error of the mean.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Phospholipid</th>
<th>Adenylic acid</th>
<th>Guanylic acid</th>
<th>Uridylic acid</th>
<th>Cytidylic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>AU (liver)</td>
<td>59±2‡</td>
<td>61±9</td>
<td>55±5</td>
<td>58±7</td>
<td>68±6</td>
</tr>
<tr>
<td>AU+DAP (liver)</td>
<td>71±5</td>
<td>87±4</td>
<td>87±2</td>
<td>69±8</td>
<td>88±5</td>
</tr>
<tr>
<td>DAP (liver)</td>
<td>10±2</td>
<td>47±13</td>
<td>60±6</td>
<td>50±10</td>
<td>35±15</td>
</tr>
<tr>
<td>AU (hepatoma)</td>
<td>24±5</td>
<td>57±3</td>
<td>51±5</td>
<td>48±6</td>
<td>50±7</td>
</tr>
<tr>
<td>AU+DAP (hepatoma)</td>
<td>77±5</td>
<td>86±2</td>
<td>70±3</td>
<td>76±3</td>
<td>69±3</td>
</tr>
<tr>
<td>DAP (hepatoma)</td>
<td>66±4</td>
<td>77±2</td>
<td>64±4</td>
<td>59±7</td>
<td>57±6</td>
</tr>
</tbody>
</table>

* Each value is the mean of four replicate incubation tubes.
† AU = 6.7 mM 5-aminouridine; DAP = 6.7 mM 2,6-diaminopurine. AU + DAP = 6.7 mM 5-aminouridine and 6.7 mM 2,6-diaminopurine.
‡ (RSA of controls – RSA of inhibited) X 100.
§ RSA of controls.
than on the pyrimidine nucleotides or the phospholipids. In hepatoma, diaminopurine was a powerful inhibitor of incorporation into adenyl acid and was a more effective inhibitor of all fractions in this tissue than in those of liver.

It was further observed that, when the two inhibitors were present simultaneously, they caused a greater reduction of incorporation than that caused by either compound alone.

**DISCUSSION**

The greater sensitivity of hepatoma to diaminopurine is well correlated with the observation that diaminopurine prolongs the life of leukemic mice (8, 10) and is selectively toxic for tissue cultures of tumor tissue (1, 19). The difference in the behavior of the two tissues toward aminouridine is also suggestive of a differential effect on liver and hepatoma, with liver being the more sensitive. Interpretation must await results of further cancer screening tests with this compound.

The two antagonists displayed little or no specificity with reference to their "parent" compounds. Thus, aminouridine inhibited P32O4 incorporation into the pyrimidine nucleotides and phospholipids as effectively as it did that into the pyrimidine nucleotides. Likewise, diaminopurine was an effective inhibitor of incorporation into pyrimidine nucleotides and phospholipids. In this case, however, it inhibited still more effectively the incorporation into the purine nucleotides, particularly adenyl acid. This lack of uniformity seems to indicate a twofold action of diaminopurine, as previously suggested by Hitchings and co-workers (5). These observations will be amplified and the implications discussed more fully in a subsequent publication (17).

**SUMMARY**

The purine analog, 2,6-diaminopurine, and the pyrimidine nucleoside analog, 5-aminouridine, have been shown to reduce the incorporation of radioactive phosphate into the phospholipids and the ribonucleic acid nucleotides of slices of rat liver and a rat hepatoma. 5-Aminouridine appeared somewhat more effective in liver than in hepatoma, while 2,6-diaminopurine was more effective in hepatoma. Inhibition by aminouridine was nearly uniform in all fractions in both tissues, while that by diaminopurine was greater in the purine nucleotides in liver and in adenyl acid in hepatoma.

Differences between the metabolism of liver and hepatoma slices have been observed. Furthermore, purine and pyrimidine analogs have each been shown to inhibit incorporation into both purine and pyrimidine nucleotides, as well as that into phospholipids.

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