Inhibition of Purine Phosphoribosyltransferases of Ehrlich Ascites-Tumour Cells by 6-Mercaptopurine

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1. The formation of adenosine 5'-phosphate, guanosine 5'-phosphate and inosine 5'-phosphate from [8-14C]adenine, [8-14C]guanine and [8-14C]hypoxanthine respectively in the presence of 5-phosphoribosyl pyrophosphate and an extract from Ehrlich ascites-tumour cells was assayed by a method involving liquid-scintillation counting of the radioactive nucleotides on diethylaminoethylcellulose paper. The results obtained with guanine were confirmed by a spectrophotometric assay which was also used to assay the conversion of 6-mercaptopurine and 5-phosphoribosyl pyrophosphate into 6-thioinosine 5'-phosphate in the presence of 6-mercaptopurine phosphoribosyltransferase from these cells. 2. At pH 7-8 and 25º the Michaelis constants for adenine, guanine and hypoxanthine were 0-9 μM, 2-9 μM and 11-0 μM in the assay with radioactive purines; the Michaelis constant for guanine in the spectrophotometric assay was 2-6 μM. At pH 7-9 the Michaelis constant for 6-mercaptopurine was 10-9 μM. 3. 25 μM-6-Mercaptopurine did not inhibit adenine phosphoribosyltransferase. 6-Mercaptopurine is a competitive inhibitor of guanine phosphoribosyltransferase (Kₐ 4-7 μM) and hypoxanthine phosphoribosyltransferase (Kₐ 8-3 μM). Hypoxanthine is a competitive inhibitor of guanine phosphoribosyltransferase (Kₐ 3-4 μM). 4. Differences in kinetic parameters and in the distribution of phosphoribosyltransferase activities after electrophoresis in starch gel indicate that different enzymes are involved in the conversion of adenine, guanine and hypoxanthine into their nucleotides. 5. From the low values of Kₐ for 6-mercaptopurine, and from published evidence that ascites-tumour cells require supplies of purines from the host tissues, it is likely that inhibition of hypoxanthine and guanine phosphoribosyltransferases by free 6-mercaptopurine is involved in the biological activity of this drug.

In many recent investigations of the mechanism of action of the antimetabolite 6-mercaptopurine the inhibitory activity of its metabolite, 6-thioinosine 5'-phosphate, has been emphasized (for reviews see Montgomery et al. 1963; Brockman, 1963; Brockman & Anderson, 1963). 6-Thioinosine 5'-phosphate was shown to be a competitive inhibitor of inosine 5'-phosphate dehydogenase and adenylsuccinate lyase from Ehrlich ascites-tumour cells, and a non-competitive inhibitor of adenylosuccinate synthetase from these cells (Atkinson, Morton & Murray, 1963, 1964b). Little attention has been given to 6-mercaptopurine as an inhibitor of enzymes. Carter (1959) found that 6-mercaptopurine inhibited the conversion of guanine into GMP by GMP–pyrophosphate phosphoribosyltransferase (guanine phosphoribosyltransferase; EC 2.4.2.-) from Escherichia coli.

This paper describes kinetic studies of the conversion of adenine, guanine and hypoxanthine into their 5'-phosphoribosyl derivatives (AMP, GMP and IMP) by adenine phosphoribosyltransferase (EC 2.4.2.7), guanine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) from Ehrlich ascites-tumour cells. The results show that 6-mercaptopurine is a competitive inhibitor of phosphoribosyl transfer from 5'-phosphoribosyl pyrophosphate to guanine and hypoxanthine but causes no significant inhibition of the reaction with adenine.

EXPERIMENTAL
Substrates and inhibitors

Purines. A sample of guanine from British Drug Houses Ltd. contained about 0-1 mol.prop. of adenine. A 0-5% solution of this material in boiling 3-6 x H₂SO₄ was filtered and the guanine sulphate, which crystallized on cooling, was
converted into the silver salt of guanine with Ag₂SO₄. The guanine was freed of silver by extraction into 1-5 N HCl and precipitated by adjustment of the solution to pH 7 with NaOH. After being washed with water and with ethanol the guanine was dried; it was found to be free of contaminants which absorbed light at 254 µm on chromatography in propan-2-ol-aq. 1-4 N-NH₃ (85:16-5, v/v; Rₐ 0-45) or in freshly mixed butan-1-ol-propionic acid-water (2:1:1:1-4; by vol.; Rₐ 0-48).

[8-14C]Guanine and [8-14C]adenine, from California Corp. for Biochemical Research, contained no contaminants which could be detected by spectroscopy or radioautography after chromatography in the systems described above.

[8-14C]Hypoxanthine was prepared from [8-14C]adenine by deamination. Sodium nitrite (30 mg.) in about 1 ml. of water was added slowly to a solution of [8-14C]adenine (3 mg.; 50 µCi) in 0-5 ml. of 2 N-acetic acid. After 3 hr. at 40° the solution was diluted with 10 ml. of water, brought to pH 8 with 2 N-NaOH and passed through a column (7 cm. x 1 cm.) of Amberlite CG-120 (H⁺ form). The column was washed with 20 ml. of water and a mixture of hypoxanthine and adenine was then eluted with 15 ml. of aq. 17 N-NH₃. The residue obtained on evaporation of the eluate was dissolved in water (5 ml.) and the solution was again evaporated. This procedure was repeated once more to remove NH₃. After electrophoresis as a band 20 cm. wide on Whatman 3MM paper in 0-05 M-formate (NH₄)₂SO₄ pH 3-5 the hypoxanthine was obtained free of adenine by elution with 40 ml. of water. On radioautography and measurement of the radioactivity of the adenine and hypoxanthine separated in this way it was found that 85-90% of the adenine had been deaminated.

[14C]Adenine and [14C]hypoxanthine (California Corp. for Biochemical Research) and 6-mercaptopurine (Sigma Chemical Co.) contained no detectable impurities when examined by spectrophotometry or by chromatography in the systems described above.

5-Phosphoribosyl pyrophosphate. Analysis of the magnesium salt with orotic acid phosphoribosyltransferase (Kornberg, Lieberman & Simms, 1955a) confirmed the analysis (70% corrected for hydration) reported by the manufacturers (Pabst Laboratories).

Preparation of extracts for measurement of purine-phosphoribosyltransferase activities

Extracts for assays with radioactive purines. Extracts were prepared from Ehrlich ascites cells as described by Atkinson et al. (1963) except that 10 mM-tris (Cl⁻, pH 7-8) was used instead of phosphate buffer. The supernatant obtained on centrifuging at 20000 g was further clarified by centrifuging at 100000 g for 1 hr. at 2° and dialysed against 50 vol. of 10 mM-Tris (Cl⁻, pH 7-8) for 24 hr. at 2°. The supernatant obtained on centrifuging the dialysis residue at 20000 g for 15 min. (dialysed first extract'; Table 1) was used in assays of conversion of radioactive purines into their nucleotides with PRPP.*

Extracts for spectrophotometric assays. The supernatant obtained on centrifuging at 100000 g as described above (first extract'; Table 1) was mixed with 1 ml. of 5% (w/v) streptomycin sulphate/40 mg. of protein (calculated from E₂₆₀₅₄ and E₂₆₀₄₄; Warburg & Christian, 1942). After

* Abbreviations: PRPP, 5'-phosphoribosyl pyrophosphate; thio-IMP, 6-thioinosine 5'-phosphate.

Table 1. Properties of fractions obtained in the purification of 6-mercaptopurine phosphoribosyltransferase from Ehrlich ascites-tumour cells

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (ml.)</th>
<th>E₂₆₀₅₄</th>
<th>Protein (mg.)</th>
<th>E₂₆₀₄₄</th>
<th>Net formation of thio-IMP (mµmoles/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First extract</td>
<td>48</td>
<td>0-64</td>
<td>549</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Dialysed first extract</td>
<td>52</td>
<td>0-66</td>
<td>530</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Streptomycin supernatant</td>
<td>60</td>
<td>0-85</td>
<td>343</td>
<td>170</td>
<td></td>
</tr>
<tr>
<td>First heated supernatant</td>
<td>58</td>
<td>0-88</td>
<td>160</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td>7-0</td>
<td>1-40</td>
<td>60</td>
<td>106</td>
<td></td>
</tr>
<tr>
<td>Dialysed enzyme</td>
<td>8-5</td>
<td>1-60</td>
<td>52</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Second heated supernatant</td>
<td>6-5</td>
<td>1-61</td>
<td>35</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

* Calculated from E₂₆₀₅₄ and E₂₆₀₄₄ (Warburg & Christian, 1942).

Measurement of purine-phosphoribosyltransferase activities

Assays with radioactive purines. Solutions containing 0-25 µmoles of PRPP, 10 µmoles of MgCl₂ and 40 µmoles of tris, adjusted to pH 7-8 with HCl, were mixed with the phosphoribosyltransferase preparation (about 0-45, 1-8 and 3-6 mg. of protein for assays with adenine, guanine and hypoxanthine respectively). After 5 min. at 25° a solution of [8-14C]adenine, [8-14C]guanine or [8-14C]hypoxanthine (specific activities: 2-85, 0-28 and 0-25 µCi/µmole respectively) in 0-01-0-15 ml. was added to give the concentration of purine needed for the kinetic studies, in a final volume of 1-4 ml. Guanine was added as a solution (0-01-0-15 ml) in 0-01 N-HCl and with this purine the reaction mixtures contained 100 µmoles of tris buffer. When non-radioactive hypoxanthine or 6-mercaptopurine was used as inhibitor these were added to the enzyme and buffered PRPP 10 sec. before the radioactive purines. Reactions were stopped after 5 min. at 1° the suspension was centrifuged at 10000 g for 10 min. at 2°. The supernatant ('streptomycin supernatant'; Table 1) was kept at 55-60° for 3 min., cooled to 2° and centrifuged as before ('first heated supernatant'; Table 1). Protein which precipitated between 0-3 and 0-7 saturation of (NH₄)₂SO₄ was dissolved in 3 mx-tris (Cl⁻, pH 7-8). The solution ('(NH₄)₂SO₄ fraction'; Table 1) was dialysed for 16 hr. against 300 vol. of 3 mx-tris (Cl⁻, pH 7-8). The dialysis residue ('dialysed enzyme'; Table 1) was heated rapidly to 70°, kept at this temperature for 2-5 min., cooled to 2°, and centrifuged at 60000 g for 30 min. The supernatant ('second heated supernatant'; Table 1) was used for spectrophotometric assays of guanine phosphoribosyltransferase and 6-mercaptopurine phosphoribosyltransferase. After 3 weeks at −15° this fraction had 80% of its initial activity with 6-mercaptopurine.
the required period at 25° by dilution with 50 ml. of water at 1°. The reaction tubes were rinsed twice with 5 ml. portions of cold water and the combined solutions were passed through 4 cm. disks of DEAE-cellulose paper in a Millipore filtration apparatus (Millipore Filter Corp., Bedford, Mass., U.S.A.). Filtration was completed within 30 sec. of the initial dilution. Zero-time samples were obtained by adding the radioactive purines after the cold water. The filtrates were passed through the filters twice more and the disks of paper were then washed with 50 ml. of 4 mM-NH2HCO3 and with water (10 x 150 ml.). The paper disks were dried without heating for about 2 hr. and the radioactive nucleotide on the paper was measured by liquid-scintillation counting (cf. Wang & Jones, 1959; Sherman, 1963). Disks were immersed in 10 ml. of 0-3% 2,5-diphenyloxazole-0-02% p-bis-2,5-diphenyloxazolylbenzene in toluene and counted with a photomultiplier (EM 19514S) attached to a N530F scaler (Ecko Electronics Ltd.). The counting efficiency of this system, measured with [8-14C]ATP that was applied to the paper as described above, was 55 ± 2%. Enzymic activities are expressed as μmole

Spectrophotometric assays. Enzyme was added to blank and test cells (path length 1 cm.) containing MgCl2 (20 μmole), guanine (0-045 μmole), 6-mercaptopurine (0-044 μmole) and tris (100 μmole, adjusted to pH 7-8 with HCl). After equilibration for 4 min. in the thermostatic cell-holder of a Beckman DK-2A spectrophotometer the reaction was started by addition of 0.1 ml. of 5 mM-PRPP to the test cell (final volume of cell contents, 2-5 ml.). Changes of extinction were recorded at 255 μw and 260 μw at 25°. Initial rates of increase were maintained for at least 30 min. but the rates were usually measured for 10 min. The rate of conversion of guanine into GMP was calculated by the use of 3-6 x 1013 as the net change of molecular extinction coefficients at 255 μw (Pabst Laboratories, 1961). The rate of formation of thio-IMP from 6-mercaptopurine was calculated by the use of 5-4 x 1013 as the net change of extinction coefficients of 320 μw (from measured extinction coefficients of 18-0 x 1013 for the purine and 23-4 x 1013 for its nucleotide at pH 7-8). When mixtures of guanine and 6-mercaptopurine were examined the interference due to extinction changes at 255 μw arising from the reaction 6-mercaptopurine → thio-IMP and at 320 μw arising from the reaction guanine → GMP were negligible (Δm320μm, 0-02 and Δm320μm, 0-01 respectively).

Calculation of kinetic parameters. Michaelis constants (Km), inhibitor constants (Ki) and extrapolated maximum velocities in the absence (V) and presence (Vp) of inhibitors, together with their coefficients of variation, were calculated as described previously (Atkinson et al. 1964b; cf. Wilkinson, 1961).

Analysis of the products formed in the presence of the purine-phosphoribosyltransferase preparation

Identification of the products formed from radioactive purines. Solutions containing 40 μmole of tris, 8 μmole of MgCl2, 0-5 μmole of PRPP and 17-4 μmole of [8-14C]adenine, 64-5 μmole of [8-14C]guanine or 166 μmole of [8-14C]hypoxanthine, adjusted to pH 7-8 with HCl, were kept at 25° for 4 min. after addition of 'dialysed first extract' (cf. Table 1; 1-0, 3-6 and 7-2 mg. of protein respectively in the final volume of 1-2 ml.). The specific activities of the purines were the same as in the kinetic experiments and the final protein concentrations were twice those used in the corresponding kinetic experiments. The test solutions and controls without PRPP were treated with trichloroacetic acid; the acid-soluble fraction was isolated as described before (Atkinson et al. 1963) and two equal portions (approx. 40% of the total fraction) from each reaction mixture were chromatographed on Whatman 3MM paper in the butan-1-ol–propionic acid–water system described above and in butan-1-ol–acetic acid–water (20:3:7, by vol.). Guanine, guanosine, GMP, hypoxanthine, inosine, IMP, adenine, adenosine and AMP were chromatographed as reference compounds. The purines were detected with a low-pressure mercury lamp and radioactive compounds by radioautography for 8 days. The radioactive activity in the products was measured by liquid-scintillation counting as described above (see the Results and Discussion section). Disks of DEAE-cellulose used in the assay of hypoxanthine phosphoribosyltransferase were extracted with acetone and with ether and radioactive material was eluted with M-NH2HCO3. After removal of NH2HCO3 under reduced pressure the residue was chromatographed in isobutyric acid–aq. 0-19 M-NH3 (661:339, v/v). This system separates IMP (Rf 0-21) and AMP (Rf 0-48).

Tests for cleavage of AMP, IMP and GMP. In separate experiments the reaction mixtures containing PRPP and [8-14C]adenine or [8-14C]hypoxanthine were treated with trichloroacetic acid after 4 and 8 min. respectively. The acid-soluble fractions, after removal of trichloroacetic acid as before, were subjected to electrophoresis on Whatman 3MM paper (17 cm. wide) in 0-05 M-formate (NH4+, pH 3-5) at 17 v/cm. for 3 hr. at 2° and the [8-14C]AMP and [8-14C]IMP were located by comparison with reference compounds and eluted with 15 ml. of water. After removal of ammonium formate by evaporation the [8-14C]AMP (5-2 μmole; 21-7 μc) and [8-14C]IMP (4-3 μmole; 0-99 μc) were treated with 'dialysed first extract' (cf. Table 1) of ascites-tumour cells in solutions containing 4 μmole of MgCl2, 0-5 μmole of PRPP, 25 μmole of tris (Cl-, pH 7-8) and non-radioactive adenine (16 μmole) or non-radioactive hypoxanthine (98 μmole). The reaction mixture with AMP contained 0-26 mg. of protein in a final volume of 1-25 ml. and was kept at 25° for 4 min. before addition of 1 ml. of 10% trichloroacetic acid. The reaction mixture with IMP contained 2-6 mg. of protein in a final volume of 1-7 ml. and was kept at 25° for 6 min. before addition of 1 ml. of 10% trichloroacetic acid. After electrophoresis of the acid-soluble fractions as described above the distribution of radioactivity in regions corresponding to AMP, adenosine, adenine, IMP and (inosine + hypoxanthine) was measured by scintillation counting. To test for the presence of hypoxanthine and inosine in the region containing AMP and of AMP in the region containing hypoxanthine the corresponding pieces of paper were freed of scintillator by elution with 5 ml. of acetone and 5 ml. of ether; purine derivatives were then eluted with 10 ml. of water. The residues obtained on evaporation of the aqueous eluates were chromatographed in the butanol–acetic acid–water system described above and the distribution of radioactivity was examined as before. When a solution containing 4 μmole of MgCl2, 100 μmole of tris (Cl-, pH 7-8) and 70 μmole of GMP or 62 μmole of thio-IMP was mixed with 'dialysed first extract' (Table 1; 0-82 mg. of protein) in a final volume of 3 ml at 25° the rates of change
of $E_{255\text{nm}}$ or $E_{280\text{nm}}$ respectively were less than 0.001/min. in a cell of 1 cm. path length.

Electrophoresis of purine phosphoribosyltransferases in starch gel

A portion of 'dialysed first extract' (Table 1) was subjected to electrophoresis in starch gel as described by Atkinson, Jackson & Morton (1964a); the initial pH of the system was 9-5 and the concentration of tris was 78 mM. Purine phosphoribosyltransferases were detected by placing DEAE-cellulose paper soaked in PRPP, MgCl$_2$, tris chloride and the appropriate radioactive purine in contact with the cut surface of the gel for 1 hr. at 25° and then removing radioactive compounds other than nucleotides by washing the papers. [8-14C]Adenine (17.7 m$\mu$moles; 50-5 mCi) and [8-14C]hypoxanthine (212 m$\mu$moles; 53 mCi) were applied to the paper as solutions containing 0.4 m$\mu$ mole of PRPP, 8 m$\mu$ moles of MgCl$_2$ and 20 m$\mu$ moles of tris (Cl-, pH 7-8) in a final volume of 2.9 ml. For [8-14C]guanine (129 m$\mu$moles; 36 mCi) the solution contained an extra 80 m$\mu$ moles of tris chloride. The papers were washed with water (200 ml.), 4 mM-NH$_4$HCO$_3$ (200 ml.) and again with water (4 x 200 ml.), and were dried. The distribution of radioactivity in 5 mm. x 15 mm. segments of the papers was measured by scintillation counting and is shown in Fig. 1.

![Graph](image)

**Fig. 1.** Distribution of adenine phosphoribosyltransferase (○), guanine phosphoribosyltransferase (●) and hypoxanthine phosphoribosyltransferase (▲) activities of an extract from Ehrlich ascites-tumour cells after electrophoresis in starch gel. For conditions of electrophoresis and details of the assays see the Experimental section.

A qualitative confirmation of these results was obtained by radioautography of duplicate strips.

RESULTS AND DISCUSSION

Assay of purine phosphoribosyltransferases. The spectrophotometric assay of guanine phosphoribosyltransferase and 6-mercaptopurine phosphoribosyltransferase (Cartor, 1959) was satisfactory for studies of the conversion of these two purines, alone or in admixture, into their nucleotides in the presence of a partially purified fraction ('second heated supernatant'; Table 1) from Ehrlich ascites-tumour cells. Both purines were converted quantitatively into their nucleotides and the $K_m$ of guanine and $K_i$ of 6-mercaptopurine (acting as a competitive inhibitor of the conversion of guanine into GMP) did not differ significantly from those measured in assays with radioactive guanine (Table 2). In the conditions of the assay there was no change in $E_{280\text{nm}}$ when guanine or GMP was mixed with the enzyme in the absence of PRPP nor was there a detectable change in $E_{280\text{nm}}$ with 6-mercaptopurine or thio-IMP without PRPP; these results indicate the absence of interference by guanase or by enzymes which catalyse the cleavage of the glycosidic linkage in GMP or thio-IMP. In the experiments with radioactive purines, chromatographic and electrophoretic examination of the products formed from [8-14C]hypoxanthine and PRPP in the presence of 'dialysed first extract' from the ascites-tumour cells showed quantitative conversion of the purine into nucleotide. The only radioactive product on the DEAE-cellulose disks in experiments with hypoxanthine was IMP. With [8-14C]guanine the only radioactive product detected was GMP in the conditions of the kinetic experiments, but with higher concentrations of protein (3-6 mg. in 1.2 ml. of reaction mixture; for details see the Experimental section) about a tenth as much guanosine as GMP was formed. No guanosine was detected in the absence of PRPP. With twice the concentration of protein used for kinetic experiments 5% as much adenosine as AMP was formed from [8-14C]adenine in the presence of PRPP. Half this quantity of adenosine was formed in the absence of added PRPP.

When 4.3 μM-[8-14C]IMP was treated, in conditions similar to those used in kinetic studies, with 'dialysed first extract', PRPP and non-radioactive hypoxanthine (for details see the Experimental section), 4% of the nucleotide was converted into hypoxanthine and no inosine was detected. When 5-2 μM-[8-14C]AMP was allowed to react with the 'dialysed first extract' in the presence of PRPP and non-radioactive adenine the upper limits for conversion of the nucleotide into IMP, adenosine and adenine were 0-8, 0-4 and 0-2% respectively. From
Table 2. Inhibition of purine phosphoribosyltransferases by 6-mercaptopurine

Values in parentheses are the coefficients of variation of the means.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>$K_m$ (µM)</th>
<th>$K_p$ (µM)</th>
<th>$K_i$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>7.8</td>
<td>0.89 (0.04)</td>
<td>1.69 (0.01)</td>
<td>6-Mercaptopurine</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>7.8</td>
<td>11.0 (0.08)</td>
<td>0.66 (0.02)</td>
<td>6-Mercaptopurine</td>
</tr>
<tr>
<td>Guanine</td>
<td>7.8</td>
<td>2.5 (0.11)</td>
<td>1.21 (0.02)</td>
<td>6-Mercaptopurine</td>
</tr>
<tr>
<td>B*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Guanine</td>
<td>7.8</td>
<td>3.3 (0.05)</td>
<td>0.80 (0.01)</td>
<td>Hypoxanthine</td>
</tr>
<tr>
<td>C*</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-Mercaptopurine</td>
<td>7.9</td>
<td>10.9 (0.04)</td>
<td>1.82 (0.01)</td>
<td>6-Mercaptopurine</td>
</tr>
</tbody>
</table>

Different enzyme preparations were used for experiments A, B and C.

Assay with radioactive substrate.

Spectrophotometric assay.

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converted into materials which were bound to DEAE-cellulose; with PRPP the formation of bound products (shown by the chromatographic analysis to be 5'-phosphoribosyl derivatives) was linear for a sufficient period (Fig. 2) to permit calculation of initial rates in the presence or absence of 6-mercaptopurine or of $[^{14}C]$hypoxanthine.

With adenine the mean rate was measured from values at 0, 0.5, 1 and 2 min.; with guanine and hypoxanthine the mean rate was measured from values at 0, 1, 2 and 4 min. On prolonged incubation the rate of conversion of guanine into GMP and of hypoxanthine into IMP in the presence of 6-mercaptopurine increased. In these conditions much of the 6-mercaptopurine had been converted into thio-IMP, as discussed by Carter (1959). With each of the radioactive purines the activity of the corresponding purine phosphoribosyltransferase was proportional to the concentration of protein in the reaction mixture (Fig. 3).

Kinetic studies with the purine phosphoribosyltransferases. The reciprocal of the rate of conversion of each purine into its 5'-phosphoribosyl derivative was a linear function of the reciprocal of the concentration of that purine. Table 2 lists Michaelis constants and extrapolated maximum velocities for conversion of adenine, guanine and hypoxanthine into AMP, GMP and IMP (measured by the assay with radioactive purines). It also lists the values found, by the spectrophotometric assay, for conversion of guanine and 6-mercaptopurine into GMP and thio-IMP.

The assay with radioactive adenine was particularly suitable for measurement of the very low Michaelis constant (0.89 ± 0.04 µM) of this purine. Kornberg, Lieberman & Simms (1955b) found no variation in activity of adenine phosphoribosyltransferase from yeast with adenine concentrations above 45 µM, nor did Flaks, Erwin & Buchanan (1957) find an increased rate with the enzyme from these results it is unlikely that any serious interference with the kinetic measurements resulted from hydrolysis or phosphorolysis of bonds in the nucleotides formed during the assays.

In the absence of added PRPP, $[^{8-14}C]$adenine, $[^{8-14}C]$guanine or $[^{8-14}C]$hypoxanthine was not

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Fig. 2. Synthesis of purine nucleotides by an extract of Ehrlich ascites-tumour cells in the presence of PRPP and 12.4 µM-adenine (○), 46 µM-guanine (●) or 132 µM-hypoxanthine (▲). For details of protein concentrations and conditions of the assays with radioactive purines see the Experimental section.
with a partially purified fraction from the ascites-tumour cells 6-mercaptopurine was again a competitive inhibitor of conversion of guanine into GMP. The value of $K_i$ (5-1 ± 1-9 μΜ) did not differ significantly from that found in the assay with radioactive guanine. The Michaelis constant for conversion of 6-mercaptopurine into thio-IMP was 10-9 ± 0-44 μΜ with the partially purified fraction, and the extrapolated maximum rate (1-82 ± 0-2 μMoles/min./mg. of protein) was 23% of that with guanine. In this experiment the final pH was 7-9 while the final pH in the other kinetic experiments was 7-8. It was unlikely that the increase of 0-1 pH unit would cause large changes in the kinetic parameters.

The Michaelis constants for conversion of 6-mercaptopurine and hypoxanthine into their 5'-phosphoribosyl derivatives do not differ significantly from each other or from the $K_i$ of 6-mercaptopurine when it acts as a competitive inhibitor of the latter reaction (Table 2). These parameters do differ significantly ($P < 0-05$) from the $K_i$ values of 6-mercaptopurine and hypoxanthine when these purines act as competitive inhibitors of conversion of guanine into GMP. From these results, and from the different distributions of adenine, guanine and hypoxanthine phosphoribosyltransferases on electrophoresis in starch gel (Fig. 1), it is likely that different enzymes are involved in the conversion of guanine and hypoxanthine into GMP and IMP respectively. It has been shown previously that different enzymes convert adenine and hypoxanthine into their nucleotides in extracts from yeast (Kornberg et al. 1955b) and liver (Flaks et al. 1957).

With 'dialysed first extract' from Ehrlich ascites-tumour cells it was found that the rate of conversion of a mixture of 17-4 μM-[8-14C]adenine and of 64-5 μM-[8-14C]guanine into nucleotides (124 counts/sec./mg. of protein after incubation for 4 min. with PRPP; for details see the Experimental section) was not significantly less than the sum of the rates observed with [8-14C]adenine (114 counts/sec./mg. of protein) and [8-14C]guanine (9-4 counts/sec./mg. of protein) when these purines were tested separately in the assay with PRPP. Similarly, with a less active preparation, the rate of conversion of a mixture of 26-5 μM-[8-14C]adenine and 142 μM-[8-14C]hypoxanthine into nucleotides (108 counts/sec./mg. of protein) was as great as the sum of the rates observed when the purines were tested separately (86 and 22-2 counts/sec./mg. of protein respectively).

There is some evidence that Ehrlich ascites-tumour cells can convert purines, derived from the tissues of the host, into nucleotides and nucleic acids (Henderson & LePage, 1959; Smellie, Thomson, Goutier & Davidson, 1956; Kimball & LePage, 1963). It is not known to what extent this is an
essential feature of growth of these cells, but Ehrlich ascites-tumour cells (Smellie et al. 1956), like the cells of bone marrow (Lajtha & Vane, 1958), convert [14C]formate very inefficiently into purine nucleotides in vitro, and it has been suggested that both types of cell depend on a supply of purines from the liver. From the low values of $K_i$ reported here it is evident that 6-mercaptopurine is a potent competitive inhibitor of the conversion of hypoxanthine and guanine into their nucleotides. In cells which use the 'salvage' pathway of nucleotide biosynthesis from free purines (Kornberg, 1957) this inhibition is of potential importance in the control of growth by 6-mercaptopurine.

REFERENCES