Inhibition of Purine Phosphoribosyltransferases from Ehrlich Ascites-Tumour Cells by Purine Nucleotides

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1. The purine bases adenine, hypoxanthine and guanine were rapidly incorporated into the nucleotide fraction of Ehrlich ascites-tumour cells *in vivo*. 2. The reaction of 5′-phosphoribosyl pyrophosphate with adenine phosphoribosyltransferase from ascites-tumour cells (K_m 6·5–11·9 μM) was competitively inhibited by AMP, ADP, ATP and GMP (K_i 7·5, 21·9, 395 and 118 μM respectively). Similarly the reactions of 5′-phosphoribosyl pyrophosphate with both hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase (K_m 18·4–31 and 37·6–44·2 μM respectively) were competitively inhibited by IMP (K_i 52 and 63·5 μM) and by GMP (K_i 36·5 and 5·9 μM). 3. The nucleotides tested as inhibitors did not appreciably compete with the purine bases in the phosphoribosyltransferase reactions. 4. It was postulated that the purine phosphoribosyltransferases of Ehrlich ascites-tumour cells may be effectively separated from the adenine nucleotide pool of these cells.

There is considerable evidence that the activities of enzymes involved in purine nucleotide biosynthesis and interconversion are controlled to some extent by 'feedback' inhibition and by product inhibition. Thus bacterial IMP dehydrogenase is inhibited by GMP and by GDP, and GMP reductase by IMP, AMP and ATP (Mager & Magasanik, 1960). Similarly adenylosuccinate synthetase, which is involved in the conversion of IMP into AMP, is inhibited by AMP, adenylosuccinate, GMP and GDP (Wyngaarden & Greenland, 1963). In addition, the enzyme 5′-phosphoribosyl pyrophosphate amidotransferase, which catalyses the first step of new purine biosynthesis, is inhibited by both AMP and GMP (Nierlich & Magasanik, 1965).

It has been suggested that adenine phosphoribosyltransferase and hypoxanthine phosphoribosyltransferase are essential enzymes in the purine nucleotide metabolism of rapidly growing tissues (Murray, 1966). It was therefore decided to establish the effect of the two immediate products (AMP and IMP) and of metabolically closely related compounds on these enzyme systems. In the present paper the effects of AMP, ADP, ATP, IMP and GMP on adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase in extracts of Ehrlich ascites-tumour cells have been studied. AMP, ADP and GMP were found to be powerful competitive inhibitors of PRPP* in the reaction catalysed by adenine phosphoribosyltransferase. IMP and GMP competed with PRPP in the reactions catalysed by both hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase. On the basis of these inhibitions and the results of experiments showing the rapid incorporation of purine bases into their 5′-phosphoribosyl derivatives *in vivo* it is suggested that in Ehrlich ascites-tumour cells the adenine nucleotide pool may be metabolically separated from the purine phosphoribosyltransferases.

EXPERIMENTAL

Purine bases and nucleotides. Samples of radioactive and non-radioactive purines were those described by Murray (1966). [8-^{14}C]Guanine, obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., contained no contaminants that could be detected by spectrophotometry or by radioautography after chromatography in the propanol–aq. NH_3 system and in the butanol–propionic acid system described by Atkinson & Murray (1965*a*). Samples of AMP, ADP, ATP, IMP and GMP were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. On electrophoresis in 0·05 M-sodium citrate buffer, pH 4·2, the sample of ADP used was found to contain 0·05 mol.prop. of AMP. The resultant concentration of AMP in assays showing the inhibition of adenine

* Abbreviation: PRPP, 5′-phosphoribosyl pyrophosphate.

phosphoribosyltransferase by ADP was 12·7 μM. The presence of this concentration of AMP in assays containing PRPP at 18·6 μM caused less than 4% inhibition of adenine phosphoribosyltransferase. The other nucleotide samples contained no contaminants that could be detected under ultraviolet light after electrophoresis as described above.

PRPP. The magnesium salt of PRPP was obtained from the Pabst Laboratories, Milwaukee, Wis., U.S.A. This material was purified by anion-exchange chromatography on a column of DEAE-cellulose (10 cm. × 1·2 cm.^{2} for 100 mg. of PRPP) with a linear gradient (0–1·0 M) of NH_4HCO_3. Elution of PRPP from the column was followed by the orcinol procedure (Mejbaum, 1939). Fractions containing PRPP were combined, and 4 ml. of 0·5 M-$MgCl_2$ was added, followed by 3 vol. of ethanol (−15°). After standing overnight at −15° the magnesium salt of PRPP was collected by centrifugation (at −15°) and washed twice at −5° with cold ethanol and twice with ether at room temperature. The magnesium salt was dissolved in cold water and passed through a column (5 cm. × 1·2 cm.^{2}) of Amberlite CG-120 (Na^+ form) at 4°. The column was washed with 10 ml. of cold water and the effluent fractions were combined. The solution of the sodium salt of PRPP was stored at −15° (recovery 82%). Analysis of the purified PRPP with orotic acid phosphoribosyltransferase (Kornberg, Lieberman & Simms, 1955) showed that the material was 85% pure. In experiments for the measurement of kinetic parameters concentrations of PRPP were calculated from determinations of the amount of enzymically active material present.

Test for the incorporation in vivo *of purine bases into the acid-soluble and nucleic acid nucleotides of Ehrlich ascites-tumour cells.* Female Swiss mice (49–56 days old) were inoculated with about 10^8 Ehrlich ascites-tumour cells. On the seventh day after inoculation mice were intraperitoneally injected with either [8-^{14}C]adenine (2·5 μmoles; 5 μC), [8-^{14}C]hypoxanthine (2·5 μmoles; 1·9 μC) or [8-^{14}C]-guanine (0·4 μmole; 2 μC). After 1 hr. the mice were killed by decapitation, the tumour cells collected by centrifugation (5 min. at 2000 *g*) and washed twice with 10 ml. of 0·9% NaCl. The fractions soluble in cold trichloroacetic acid were prepared as described by Atkinson & Murray (1965*b*). The extracts were chromatographed on Whatman 3MM paper in butan-1-ol–acetic acid–water (20:3:7, by vol.). The nucleotides, which remained on the origin (see Atkinson, Morton & Murray, 1963), were eluted with 50 ml. of water and the eluates were evaporated to dryness. The residues were transferred to glass hydrolysis tubes (4 cm. × 0·25 cm.^{2}) with a known volume (approx. 0·25 ml.) of N-HCl. The tubes were sealed and completely immersed in a boiling-water bath for 1 hr. Samples (0·04–0·20 ml.) were applied as bands to Whatman no. 1 paper and chromatographed in 5% (w/v) Na_2HPO_4 saturated with 3-methylbutan-1-ol. After location of the purines under ultraviolet light, areas containing adenine (R_F 0·42), hypoxanthine (R_F 0·61) and guanine (remained on the origin) were cut from the paper. Radioactivity associated with these areas was determined by liquid-scintillation counting as described previously (Murray, 1966).

The residues after extraction of the tumour cells with cold acid were suspended in 6 ml. of 5% (w/v) trichloroacetic acid and heated at 90° for 15 min. After centrifuging, the precipitates were washed twice with 5 ml. portions of 5% trichloroacetic acid and the combined supernatants and washings were extracted three times with 40 ml. portions of ether. After evaporation to dryness the residues were suspended in 2 ml. of N-HCl and the radioactivity associated with adenine and guanine was determined after chromatography as described above.

Assay of purine-phosphoribosyltransferase activities. Extracts were prepared from Ehrlich ascites-tumour cells after 7 days of tumour growth as described previously (Murray, 1966). Conditions of assay with radioactive adenine and hypoxanthine have been described (Murray, 1966) and similar assays were carried out with [8-^{14}C]-guanine (specific activity 0·88 μC/μmole). Procedures for calculation of kinetic parameters were those used by Atkinson & Murray (1965*a*). In experiments to determine the Michaelis constants of PRPP in the reactions catalysed by adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase determinations were carried out at five concentrations of PRPP (range 4·7–56·2 μM for the first two enzymes and 4·9–58·8 μM for guanine phosphoribosyltransferase). Concentrations of adenine, hypoxanthine and guanine were kept constant at 53, 99 and 66 μM respectively.

Inhibitors (in solutions adjusted to pH 7·8) were added in a volume of 0·05 ml. 15 sec. before the addition of radioactive purine base. In kinetic experiments the formation of nucleotide was measured at 0, 1, 2 and 4 min. at each concentration of substrate.

RESULTS

Incorporation in vivo *of* [8-^{14}C]*adenine,* [8-^{14}C]-*hypoxanthine and* [8-^{14}C]*guanine into the purine nucleotides of Ehrlich ascites-tumour cells.* At 1 hr. after the intraperitoneal injection of [8-^{14}C]-adenine (2·5 μmoles; 5 μC) into mice (7 days after inoculation with ascites-tumour cells) 48 ± 10% of the injected radioactivity was incorporated into acid-soluble or nucleic acid nucleotide material (mean of five animals; for details of the fractionation procedure see the Experimental section). Of this radioactivity over 99% was associated with adenine nucleotides and only trace amounts with hypoxanthine and guanine nucleotides and with nucleic acid adenine. About 42% of the radioactivity was recovered in the nucleotide fraction of tumour cells after 1 hr. when 0·25 μmole of adenine (5 μC) was intraperitoneally administered to mice.

Similarly 1 hr. after injection of [8-^{14}C]hypoxanthine (2·5 μmoles; 1·9 μC) 27 ± 8% of the injected radioactivity was associated with hypoxanthine nucleotides (presumably IMP), 20% with adenine nucleotides and 8% with guanine nucleotides. No radioactivity was detectable in the nucleic acid fraction. Under similar conditions 20 ± 7% of injected [8-^{14}C]guanine (0·4 μmole; 2 μC) was incorporated into nucleotide material. No radioactivity was associated with adenine or hypoxanthine nucleotides, and only traces of radioactivity with nucleic acid guanine.

Rapid incorporation of [8-^{14}C]adenine, [8-^{14}C]-hypoxanthine and [8-^{14}C]guanine into the nucleotide fraction of Ehrlich ascites-tumour cells *in vitro* has been reported previously (Paterson & Hori, 1962).

Kinetic studies with the purine phosphoribosyltransferases. The reciprocal of the rate of conversion of either adenine, hypoxanthine or guanine into its 5′-phosphoribosyl derivative was a linear function of the reciprocal of the concentration of PRPP. Table 1 lists Michaelis constants and extrapolated maximum velocities for the incorporation of the 5′-phosphoribosyl moiety into nucleotide material in the presence of an excess of each of the purine bases. The maximum velocities with adenine, hypoxanthine or guanine as substrate were similar to those reported by Atkinson & Murray (1965*a*,*b*). The values found for the Michaelis constant of PRPP with adenine phosphoribosyltransferase from Ehrlich ascites-tumour cells ranged from 6·5 to 11·9 μM. The Michaelis constant for PRPP with this enzyme from yeast has been found to be 140 μM and with that from ox liver to be less than 40 μM (Flaks, 1963). The values found for the Michaelis constants of PRPP with hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase ranged from 18·4 to 31 μM and from 36·7 to 44·2 μM respectively.

In the presence of 254 μM-AMP and 53 μM-adenine the apparent Michaelis constant for PRPP was increased from 11·9 μM to 416 μM. The extrapolated maximum velocity in the absence of AMP (2·20 mμmoles/min./mg. of protein) was not significantly different from the maximum velocity in the presence of this compound (2·27 mμmoles/min./mg. of protein). AMP is therefore a competitive inhibitor of PRPP in the adenine-phosphoribosyltransferase reaction (K_i 7·5 μM). Similarly GMP, ADP and ATP are competitive inhibitors of PRPP in this reaction (K_i 118, 21·9 and 395 μM respectively). When the last two nucleotides were incubated for 2 min. in the assay system in the absence of PRPP, no detectable ADP or AMP was formed from ATP and only a trace (less than 1%) of AMP was formed from ADP. The reaction products were separated by electrophoresis in 0·05 M-sodium citrate buffer, pH 4·2. Thus the inhibitions observed with ATP and ADP were not due to the presence of phosphatases in the cell extracts converting these compounds into AMP. The presence of 166 μM-IMP had no inhibitory effect on the reaction catalysed by adenine phosphoribosyltransferase. The kinetic parameters associated with these inhibitions are summarized in Table 1. In the presence of 288 μM-PRPP and 0·65 μM-adenine the reaction was not significantly inhibited by 166 μM-IMP, 255 μM-ADP or 255 μM-ATP. Therefore none of these compounds compete with adenine under the conditions described. Under the same conditions the reaction was inhibited 5·8% by 255 μM-GMP and 3·2% by 259 μM-AMP.

The reaction of PRPP with both hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase was competitively inhibited by IMP (K_i 52 μM and 63·5 μM respectively; see Table 1) and by GMP (K_i 36·5 and 5·9 μM respectively; see Table 1). AMP did not compete with PRPP in the reaction catalysed by either enzyme. In the presence of 288 μM-PRPP and either 1·45 μM-hypoxanthine or 1·62 μM-guanine, neither hypoxanthine phosphoribosyltransferase nor guanine

Table 1. *Inhibition of purine phosphoribosyltransferases by purine nucleotides*

Assays were carried out in the presence of 53 μM-[8-^{14}C]adenine, 99 μM-[8-^{14}C]hypoxanthine or 66 μM-[8-^{14}C]-guanine as described in the Experimental section. The concentration of PRPP was varied in the range 4·7–58·8 μM. Values in parentheses are the coefficients of variation of the means.

Enzyme	K_m (μM)	$V_{max.}$ (mμmoles/min./mg. of protein)	Inhibitor	Concn. of inhibitor (μM)	K_p (μM)	V_p (mμmoles/min./mg. of protein)	K_i (μM)
Adenine phosphoribosyltransferase	11·9 (0·10)	2·20 (0·02)	AMP	254	416 (0·09)	2·27 (0·03)	7·5 (0·14)
	7·3 (0·05)	2·34 (0·01)	ADP	254	93 (0·06)	2·40 (0·02)	21·9 (0·08)
	7·3 (0·05)	2·34 (0·01)	ATP	254	12·0 (0·03)	2·32 (0·01)	395 (0·15)
	8·0 (0·07)	2·15 (0·04)	IMP	166	8·2 (0·08)	2·20 (0·03)	—
	6·5 (0·15)	2·06 (0·06)	GMP	256	20·6 (0·12)	1·99 (0·03)	118 (0·28)
Hypoxanthine phosphoribosyltransferase	18·4 (0·04)	0·62 (0·04)	IMP	166	77 (0·06)	0·58 (0·03)	52·0 (0·09)
	31 (0·05)	0·59 (0·03)	GMP	126	138 (0·06)	0·61 (0·04)	36·5 (0·10)
	25·3 (0·04)	0·58 (0·01)	AMP	259	24·5 (0·07)	0·58 (0·02)	—
Guanine phosphoribosyltransferase	36·7 (0·09)	0·82 (0·02)	GMP	126	810 (0·12)	0·94 (0·10)	5·9 (0·16)
	44·2 (0·10)	0·76 (0·05)	IMP	166	160 (0·10)	0·80 (0·05)	63·5 (0·19)
	40·0 (0·08)	0·74 (0·05)	AMP	259	41·3 (0·05)	0·76 (0·04)	—

phosphoribosyltransferase was inhibited by 259 μM-AMP. Under these conditions hypoxanthine phosphoribosyltransferase was inhibited by 6 and 5% and guanine phosphoribosyltransferase by 24 and 14% in the presence of 255 μM-GMP or 178 μM-IMP respectively.

DISCUSSION

It is apparent that each of the immediate products of the reactions catalysed by adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase from Ehrlich ascites-tumour cells (AMP, IMP and GMP respectively) inhibit the respective enzymes by competing with PRPP. However, as the reactions of the free purine bases with the phosphoribosyltransferases are not greatly inhibited by the nucleotide products, nucleotide synthesis could proceed in the presence of low purine concentrations provided that the PRPP concentration was high enough.

Henderson & Khoo (1965*a*) estimated that Ehrlich ascites-tumour cells contain about 0·3 μ-mole of PRPP/g. of packed tumour cells. If it is arbitrarily assumed that 50% of the weight of packed cells represent extracellular water, this would correspond to an intracellular PRPP concentration of 0·6mM. Under these conditions adenine phosphoribosyltransferase would be inhibited 50% by 0·37mM-AMP (i.e. about 0·18 μ-mole of AMP/g. of tumour cells). Overgaard-Hansen (1965) reported values of about 0·4–0·5, 0·5–0·6 and 4·2–4·8 μmoles of AMP, ADP and ATP respectively/ml. of Ehrlich ascites-tumour cells. As ADP is also an inhibitor of adenine phosphoribosyltransferase, it would be expected that this enzyme would be effectively inhibited *in vivo*. Paterson & Hori (1962) concluded that conversion of adenine into AMP in Ehrlich ascites-tumour cells was mainly catalysed by adenine phosphoribosyltransferase; thus the rapid incorporation of adenine into nucleotide material observed *in vivo* implies that a part or all of the adenine nucleotide pool is prevented from inhibiting adenine phosphoribosyltransferase, and may be separated from this enzyme *in vivo*. This conclusion assumes that the kinetics of AMP inhibition of adenine phosphoribosyltransferase at 37° and at 25° (the temperatures used in these studies) are comparable and that other cellular constituents do not affect the inhibition *in vivo*. The hypothesis is supported, however, by the observation by Henderson & Khoo (1965*b*) that in the presence of high concentrations of adenine and guanine the concentration of free PRPP in Ehrlich ascites-tumour cells was decreased to a low level but that ribonucleotide synthesis continued. However, until more is known of the intracellular concentrations and distribution of adenine nucleotides and PRPP in Ehrlich ascites-tumour cells the physiological implications of these inhibitions remain uncertain. Inhibition of hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase by IMP and GMP are not likely to be significant *in vivo* as the pool sizes of hypoxanthine and guanine nucleotides in Ehrlich ascites-tumour cells are very small (A. W. Murray, unpublished work).

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REFERENCES

Atkinson, M. R., Morton, R. K. & Murray, A. W. (1963). *Biochem. J.* **89**, 167.
Atkinson, M. R. & Murray, A. W. (1965*a*). *Biochem. J.* **94**, 64.
Atkinson, M. R. & Murray, A. W. (1965*b*). *Biochem. J.* **94**, 71.
Flaks, J. G. (1963). In *Methods in Enzymology*, vol. 6, p. 136. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Henderson, J. F. & Khoo, K. Y. (1965*a*). *J. biol. Chem.* **240**, 2349.
Henderson, J. F. & Khoo, K. Y. (1965*b*). *J. biol. Chem.* **240**, 2358.
Kornberg, A., Lieberman, I. & Simms, E. S. (1955). *J. biol. Chem.* **215**, 389.
Mager, J. & Magasanik, B. (1960). *J. biol. Chem.* **235**, 1474.
Mejbaum, W. (1939). *Hoppe-Seyl. Z.* **258**, 117.
Murray, A. W. (1966). *Biochem. J.* **100**, 664.
Nierlich, D. P. & Magasanik, B. (1965). *J. biol. Chem.* **240**, 358.
Overgaard-Hansen, K. (1965). *Biochim. biophys. Acta*, **104**, 330.
Paterson, A. R. P. & Hori, A. (1962). *Canad. J. Biochem. Physiol.* **40**, 181.
Wyngaarden, J. B. & Greenland, R. A. (1963). *J. biol. Chem.* **238**, 1054.