Studies on the Nature of the Regulation by Purine Nucleotides of Adenine Phosphoribosyltransferase and of Hypoxanthine Phosphoribosyltransferase from Ehrlich Ascites-Tumour Cells

By A. W. MURRAY*
Department of Biochemistry and Nutrition, University of New England, Armidale, New South Wales, Australia

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1. The progress curves of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase activity plotted against 5-phosphoribosyl pyrophosphate concentration were hyperbolic in nature. The inhibition of the former enzyme by AMP and GMP and of the latter enzyme by IMP and GMP showed completely competitive characteristics. 2. The effect of temperature on the reaction of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase was examined. The energy of activation of the former enzyme decreased at temperatures greater than 27° and that of the latter enzyme at temperatures greater than 23°. For each enzyme, the change in the heat of formation of the 5-phosphoribosyl pyrophosphate–enzyme complex at the critical temperature was approximately equal to the change in the energy of activation but was in the opposite direction. The inhibitor constants with both enzymes in the presence of nucleotides varied in different ways with temperature from the Michaelis constants for 5-phosphoribosyl pyrophosphate indicating that different functional groups were involved in binding substrates and inhibitors. 3. ATP was found to stimulate adenine-phosphoribosyltransferase activity at concentrations less than about 250μM and to inhibit the enzyme at concentrations greater than 250μM. The stimulation was unaffected by 5-phosphoribosyl pyrophosphate concentration but the inhibitory effect could be overcome by increasing concentrations of this compound. At low concentrations ATP reversed the inhibition of adenine phosphoribosyltransferase by AMP and GMP to an extent dependent on their concentration. 4. The properties of adenine phosphoribosyltransferase changed markedly on purification. Crude extracts of ascites-tumour cells had Michaelis constants for 5-phosphoribosyl pyrophosphate and adenine 75 and six times as high respectively as those obtained with purified enzyme. ATP had no stimulatory effect on activity of the purified enzyme or on that of crude extracts heated 15min. or longer at 55°. 5. It is suggested that at low concentrations ATP is bound to an 'activator' site which is separate from the substrate binding site of adenine phosphorytransferase and that at high concentrations ATP competes with 5-phosphoribosyl pyrophosphate at the active site of the enzyme.

The studies of Gerhart & Pardee (1962, 1963, 1964) and Gerhart & Schachman (1965) have clarified considerably the allosteric nature of the control of pyrimidine nucleotide biosynthesis by CTP and ATP in bacterial systems. Control of purine nucleotide metabolism has been studied in less detail although it is likely that 'feedback' inhibition occurs (see Mager & Magasanik, 1960; Wyngaarden & Greenland, 1963). Nierlich & Magasanik (1965) have shown that inhibition by purine nucleotides of the first step of purine biosynthesis has a mechanism differing in some aspects from that involved in the control of pyrimidine nucleotide biosynthesis.

It is likely that the purine phosphoribosyltransferases have a physiological significance in some specialized animal tissues by converting purine bases into nucleotide material by direct reaction with PRPP† (Murray, 1966*a*). Mechanisms regulating the activity of these enzymes are therefore of interest. It has recently been shown that the products (AMP, IMP and GMP) of the reactions

* Present address: School of Biological Sciences, Flinders University of South Australia, Adelaide, South Australia.

† Abbreviations: PRPP, 5-phosphoribosyl pyrophosphate; thio-IMP, 6 thioinosine 5′-phosphate.

catalysed respectively by adenine phosphoribosyltransferase, hypoxanthine phosphoribosyltransferase and guanine phosphoribosyltransferase from Ehrlich acsites-tumour cells inhibit the respective transferases by competing with PRPP (Murray, 1966*b*). In the present paper the nature of these inhibitions has been examined in more detail. Studies on the effect of temperatures on the reaction of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase from ascites-tumour cells established that different functional groups were involved in binding PRPP and the nucleotide inhibitors to the enzymes. ATP was found to stimulate the reaction of adenine phosphoribosyltransferase at low concentrations and to inhibit it at high concentrations. The enzyme could be selectively desensitized to the stimulatory effect of ATP by controlled heat treatment. In addition low concentrations of ATP overcame the inhibition of adenine phosphoribosyltransferase by AMP and GMP to an extent dependent on the concentration of these inhibitors. On the basis of these results it is postulated that adenine transferase has an 'activator' site binding ATP at low concentrations which is separate from the active site of this enzyme.

EXPERIMENTAL

Substrates. Samples of radioactive and non-radioactive purines were similar in purity to those described before (Murray, 1966*b*). 6-Mercapto[8-^{14}C]purine, obtained from California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A., contained no contaminants that could be detected after chromatography in the propanol–aq.NH_3 system and in the butanol–propionic acid system described by Atkinson & Murray (1965*a*). PRPP was purified as described before (Murray, 1966*b*).

Inhibitors. The barium salt of thio-IMP (Montgomery & Thomas, 1961) was purified as described by Atkinson, Morton & Murray (1963). Samples of AMP, ADP, GMP and IMP were of similar purity to those described before (Murray, 1966*b*).

Assay of purine-phosphoribosyltransferase activity. Extracts were prepared from Ehrlich ascites-tumour cells after 7 days of tumour growth as described by Murray (1966*a*). Conditions of assay with radioactive adenine, hypoxanthine and guanine have been described (Murray, 1966*b*) and similar assays were carried out with 6-mercapto-[8-^{14}C]purine (specific activity 4·36 μc/μmole). Procedures for calculation of kinetic parameters have been described by Atkinson & Murray (1965*a*).

Purification of adenine phosphoribosyltransferase. Adenine phosphoribosyltransferase in extracts of Ehrlich ascites-tumour cells was purified essentially as described by Hori & Henderson (1965). The final preparation had a specific activity 43 times that of the original extracts and catalysed the formation from adenine of 210 mμmoles of AMP/min./mg. of protein at 25°. This preparation was stored at −15° in 1·5M-$(NH_4)_2SO_4$ and dialysed with stirring against 500 vol. of 5mM-tris (adjusted to pH 7·8 with HCl) at 4° for 1 hr. immediately before use. The purified enzyme could be stored for at least 6 weeks without loss of activity.

RESULTS

Nature of the kinetics of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase in crude extracts of ascites-tumour cells. Plots of the velocity of both adenine and hypoxanthine phosphoribosyltransferase against the concentration of PRPP showed the expected hyperbolic curves (Fig. 1) when assays were carried out at either 25° or at 32°. Previous studies had shown that AMP and GMP competed with PRPP in the reaction catalysed by adenine phosphoribosyltransferase and that IMP and GMP competed with PRPP in the reaction catalysed by hypoxanthine phosphoribosyltransferase (Murray, 1966*b*). Further experiments established that this inhibition is of a completely competitive nature. Thus the

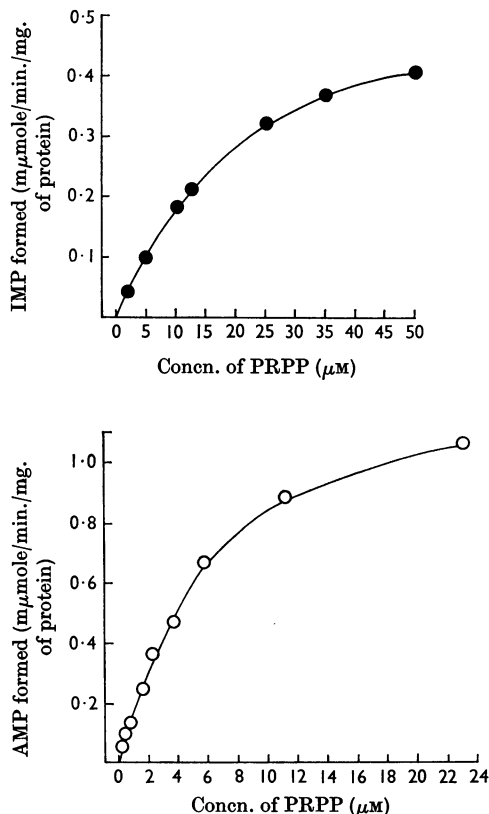

Fig. 1. Plots of the activity of adenine (○) and hypoxanthine (●) phosphoribosyltransferase in the presence of varying concentrations of PRPP. Assays were carried out at 25° and pH 7·8 with a crude extract from Erhlich ascites-tumour cells (see the Experimental section).

inhibition of either transferase by any nucleotide was completely overcome in the presence of excess of PRPP. Similarly, high concentrations of the nucleotide inhibitors resulted in 100% inhibition of each transferase. In the presence of 11·2 μM-PRPP, adenine phosphoribosyltransferase was completely inhibited by 3mM-AMP and by 12mM-GMP and hypoxanthine phosphoribosyltransferase by 8mM-IMP and by 8mM-GMP. These concentrations of nucleotides do not necessarily represent the lowest concentrations of inhibitor that caused 100% inhibition in each case. The high concentrations of nucleotides used in these experiments interfered with the binding of radioactive AMP and IMP by the disks of DEAE-cellulose paper used in the assay of transferase activity (see Atkinson & Murray, 1965a). The reaction products were separated by chromatography on Whatman no. 1 paper with 5% (w/v) disodium hydrogen phosphate as solvent. The areas corresponding to AMP and IMP were cut from the paper and radioactivity was estimated directly by liquid-scintillation counting as described before (Murray, 1966a).

Plots of the percentage inhibition of adenine phosphoribosyltransferase against the concentration of AMP or GMP, or of hypoxanthine phosphoribosyltransferase against the concentration of IMP or GMP, also showed hyperbolic relationships when assays were carried out at either 25° or 32°.

Table 1. *Effect of temperature on the inhibition of adenine phosphoribosyltransferase by purine nucleotides*

Assays were carried out in the presence of 55 μM-[8-^{14}C]-adenine as described in the Experimental section. The concentration of PRPP was varied within the range 5·6–33·6 μM.

Temperature	K_m (μM)	V (mμmoles/min./mg. of protein)	Inhibitor	K_i (μM)
16·5°	9·1	1·01	AMP	12·5
			GMP	188
			thio-IMP	342
20·5	9·2	1·57	AMP	13·1
			GMP	209
			thio-IMP	338
25	8·0	2·50	AMP	10·5
			GMP	168
			thio-IMP	351
29·5	8·3	3·18	AMP	10·9
			GMP	167
			thio-IMP	335
35·5	4·8	4·02	AMP	18·5
			GMP	154
			thio-IMP	357
37·2	3·8	4·38	AMP	22·9
			GMP	156
			thio-IMP	348

Effect of temperature on the inhibition of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase in crude extracts of ascites-tumour cells by purine nucleotides. The effect of varying the temperature over the range 16·5–37·2° on the kinetic parameters of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase are shown in Table 1 and Table 2 respectively. The maximum velocities were derived by extrapolation. In no case was the maximum velocity in the presence of inhibitor significantly different from that in its absence, and the inhibitor constants (K_i) were calculated from the expression for competitive inhibition (see Dixon & Webb, 1964, p. 315). Samples of the extract from ascites-tumour cells that were incubated at 37·2° for 10min., cooled to 25° and then assayed, showed no loss of adenine-phosphoribosyltransferase or of hypoxanthine-phosphoribosyltransferase activity, indicating that there was no inactivation of either enzyme at the highest temperature used.

In Fig. 2 the effect of temperature on the maximum initial velocity of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase is shown. The energies of activation, derived from the Arrhenius equation (see Dixon & Webb, 1964, p. 141) are indicated on the graphs. The activation energy for the adenine transferase at first remained constant (17300cal./mole) with increasing temperature, but at a temperature near 27° there was a sharp change to a lower activation energy (6100cal./mole). Similar changes were seen

Table 2. *Effect of temperature on the inhibition of hypoxanthine phosphoribosyltransferase by purine nucleotides*

Assays were carried out in the presence of 92 μM-[8-^{14}C]-hypoxanthine as described in the Experimental section. The concentration of PRPP was varied within the range 11·2–67·2 μM.

Temperature	K_m (μM)	V (mμmoles/min./mg. of protein)	Inhibitor	K_i (μM)
16·5°	28	0·090	IMP	92
			GMP	30·6
			thio-IMP	843
19·6	26	0·328	IMP	87·5
			GMP	20·3
			thio-IMP	680
25	24·1	0·740	IMP	72
			GMP	19·8
			thio-IMP	650
29·2	8·3	1·04	IMP	71
			GMP	17·9
			thio-IMP	625
35·5	2·7	1·52	IMP	23·6
			GMP	5·7
			thio-IMP	161

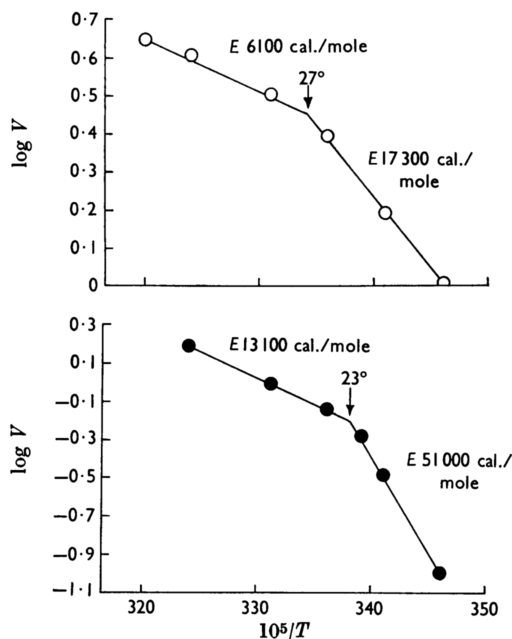

Fig. 2. Effect of temperature on the maximum velocity of adenine (○) and hypoxanthine (●) phosphoribosyltransferase. The maximum velocities were derived by extrapolation (see Tables 1 and 2). The energies of activation are indicated on the graphs.

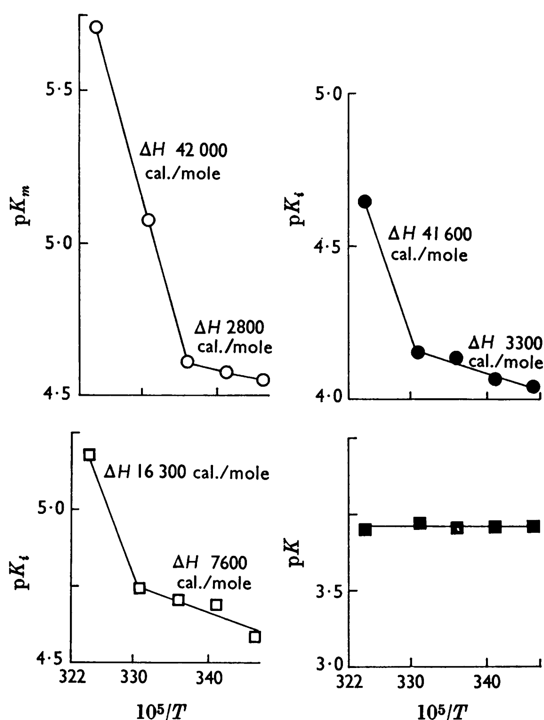

Fig. 3. Effect of temperature on the K_m of PRPP (○) and the K_i of IMP (●), GMP (□) and thio-IMP (■) with hypoxanthine phosphoribosyltransferase. The values were taken from Table 2 and the heats of formation (ΔH) of the PRPP–enzyme complex and of the inhibitor–enzyme complexes are indicated on the graphs.

with hypoxanthine phosphoribosyltransferase with the activation energy changing from 51000cal./mole to 13100cal./mole. With this enzyme the critical temperature was near to 23°. Several cases have been reported with other enzymes where a sharp transition was found between two linear regions of the Arrhenius plot (for references see Massey, Curti & Ganther, 1966).

Plots of pK_m (i.e. $-\log K_m$) of PRPP reacting with hypoxanthine phosphoribosyltransferase and pK_i (i.e. $-\log K_i$) in the presence of IMP, GMP and thio-IMP are shown in Fig. 3 (the data are taken from Table 2). The approximate heats of formation (ΔH) of the PRPP–enzyme complex and of the inhibitor–enzyme complexes are indicated. For calculation of the ΔH values it was assumed that the Michaelis constants obtained were equivalent to K_s, the equilibrium constant of the dissociation of the enzyme–substrate complex into free enzyme and substrate (see Dixon & Webb, 1964, p. 54). There is no evidence at present to decide if this is, in fact, the case, From Figs. 2 and 3 it can be seen that the change in activation energy at 23° is accompanied by an opposite change in the apparent heat of combination of hypoxanthine phosphoribosyltransferase with PRPP. Thus the ΔH value below this temperature was found to be 2800cal./mole and above this temperature to be 42000cal./mole. The difference in ΔH values above and below 23° is 39200 cal./mole, which is similar to the difference in the energy of activation calculated from the change in maximum velocity with temperature (37900cal./mole). Massey (1953) found a similar situation with the reaction of fumarase at low pH values. The combination of both IMP and GMP with hypoxanthine phosphoribosyltransferase at different temperatures (see Fig. 3) shows a similar relationship to the combination of PRPP with this enzyme. However, the critical temperature indicating a change in the heat of combination of enzyme with inhibitor is approximately 29° with both nucleotides, compared with 23° for the PRPP–enzyme combination. Experiments were also carried out to determine the effect of thio-IMP on the reaction between 6-mercaptopurine and PRPP catalysed by extracts of ascites-tumour cells (see Atkinson & Murray, 1965a). Thio-IMP was found to be a competitive inhibitor of PRPP. At each of the

temperatures studied the values of K_i were not significantly different from the values of this parameter found for the inhibition of hypoxanthine phosphoribosyltransferase by IMP.

The variation with temperature of the K_m of PRPP and the K_i of AMP with adenine phosphoribosyltransferase are shown in Fig. 4 (the data are taken from Table 1). The K_i of GMP and of thio-IMP have not been plotted as there is no apparent trend in the values found over the temperature range studied. There was an opposite and approximately equal change in the heat of combination of PRPP with enzyme accompanying the change in activation energy as was found with hypoxanthine phosphoribosyltransferase; with the adenine transferase the critical temperature is near 27° in each case. The difference in ΔH values above and below 27° was 11 300 cal./mole and the difference in the energy of activation was 11 200 cal./mole. However, plots of pK_i against $1/T$ (see Fig. 4)

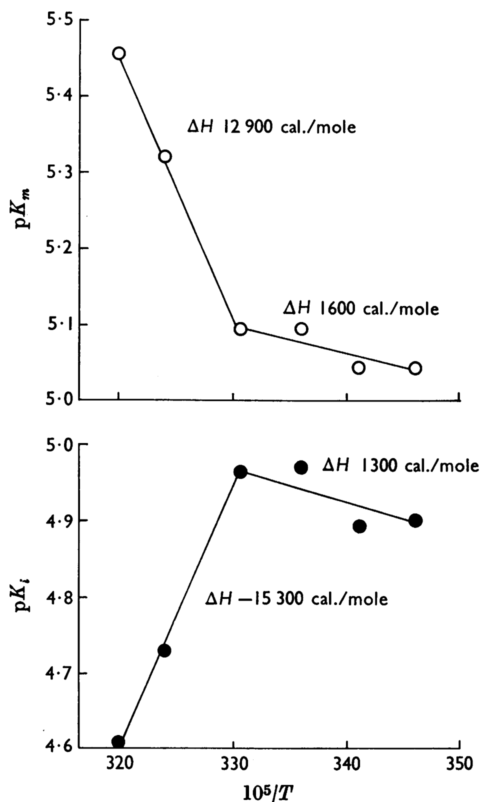

Fig. 4. Effect of temperature on the K_m of PRPP (○) and the K_i of AMP (●) with adenine phosphoribosyltransferase. The values were taken from Table 1 and the heats of formation (ΔH) of the PRPP–enzyme complex and of the AMP–enzyme complex are indicated on the graphs.

showed that the effect of temperature on AMP binding by adenine phosphoribosyltransferase is different from its effect on PRPP binding by this enzyme. Thus at 29° there is a decrease in the heat of combination of AMP with enzyme corresponding to an increase in K_i'. The change is such that at temperatures greater than 29° the combination of enzyme with AMP is exothermic (ΔH −15 300 cal./mole). The combination of both adenine and hypoxanthine phosphoribosyltransferase with all other nucleotide inhibitors and with PRPP are endothermic reactions over the range of temperatures studied.

Effect of combinations of nucleotides on the activity of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase. Experiments were carried out to determine the effect of combinations of the nucleotide inhibitors on the reaction of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase. In the presence of 47 μM-PRPP adenine transferase was inhibited 58, 12 and 40% by 1·25 mM-AMP, -GMP and -ADP respectively. In the presence of AMP + GMP, AMP + ADP and ADP + GMP (each nucleotide at a concentration of 1·25 mM) the reaction was inhibited 62, 65 and 44% respectively. Thus each of the combinations tested resulted in greater inhibition than that obtained with the same concentration of either nucleotide alone. The increase in inhibition was not great but the results were reproducibly obtained in a series of experiments. Similar effects were observed over a range of nucleotide concentrations from 0·125 to 0·31 mM. Similarly, in the presence of 5 μM-PRPP, hypoxanthine phosphoribosyltransferase was inhibited 58% in the presence of 31 μM-GMP, 82% in the presence of 82 μM-IMP and 86% in the presence of 31 μM-GMP + 82 μM-IMP.

Stimulation and inhibition of adenine phosphoribosyltransferase by ATP. At low concentrations of ATP the reaction of adenine phosphoribosyltransferase in the crude preparation was found to be stimulated. In Fig. 5 the effect of varying concentrations of ATP on the activity of this enzyme at 25° in the presence of a constant concentration (7 μM) of PRPP is shown. Maximum stimulation of enzyme activity was found with an ATP concentration between 40 and 50 μM. Similar stimulation by ATP was obtained by using a crude extract that had been dialysed against 1000 vol. of water for 24 hr. (2°). Concentrations of ATP greater than about 250 μM resulted in inhibition of the adenine transferase. The stimulation and inhibition was quantitatively similar when assays were carried out at 33°. Fig. 5 also shows the effect of ATP on a preparation of adenine phosphoribosyltransferase purified 43-fold (see the Experimental section). No stimulation was observed over the range of ATP concentrations indicated. In addition no stimulation was induced

by reducing the ATP concentration to 3·1 μM and the PRPP concentration to 0·52 μM. Preliminary experiments indicated that the K_m of the purified enzyme with PRPP (measured at 25°) was approx. 0·10 μM compared with 7·5 μM obtained with the crude extract. The K_i values for AMP and ATP were 0·32 and 12·4 μM respectively, compared with 7·5 and 395 μM obtained with the crude extract. These determinations were carried out by varying the PRPP concentration from 0·104 to 1·04 μM. Concentrations of adenine phosphoribosyltransferase were adjusted to obtain the formation of about 0·02 mμmole of AMP/2 min. at 25° with the lowest concentration of PRPP used. The assays involved the measurement of low amounts of radioactivity and the values of K_m and K_i are only accurate to within about 15%. By comparison the K_m for adenine obtained with crude extracts (0·63 μM; see also Atkinson & Murray, 1965a,b) was decreased to a value of 0·13 μM with the purified enzyme.

The effect of varying the PRPP concentration on both the stimulation and the inhibition by ATP of adenine phosphoribosyltransferase in crude extracts is shown in Table 3. It is apparent that the

Table 3. Effect of concentration of 5-phosphoribosyl-pyrophosphate on the stimulation and inhibition of adenine phosphoribosyltransferase by ATP

Assays were carried out in the presence and absence of either 31 or 450 μM-ATP at 25° as described in the Experimental section.

Concn. of PRPP (μM)	Percentage stimulation by 31 μM-ATP	Percentage inhibition by 450 μM-ATP
0·52	46	—
1·0	49	54
2·6	45	27
5·2	46	13
10·4	34	9·5
15·6	32	—

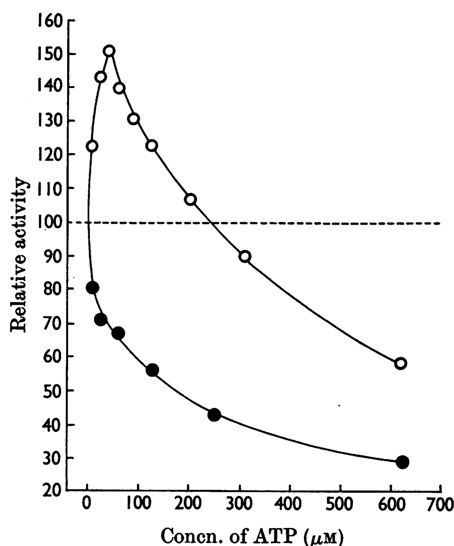

Fig. 5. Effect of ATP concentration on the reaction of adenine phosphoribosyltransferase in crude ascites-tumour-cell extracts (○) and on the reaction of purified adenine phosphoribosyltransferase (●). The purified enzyme was prepared as described in the Experimental section and had a specific activity 43 times that of the crude extract. Assays were carried out as described in the Experimental section in the presence of 7 μM-PRPP. The crude extract catalysed the formation of 1·6 mμmoles of AMP/min./mg. of protein (assays contained 0·1 mg. of protein) in the absence of ATP under these assay conditions. Details of the activity of the purified enzyme are given in the Experimental section. The enzymic rates are expressed as a percentage of the rates in the absence of ATP.

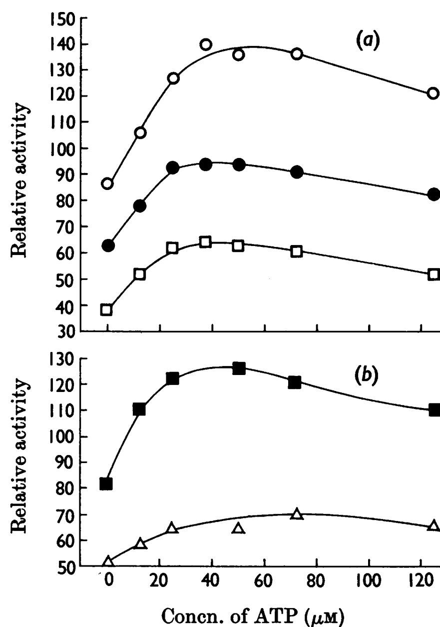

Fig. 6. Effect of ATP concentration on the inhibition of adenine-phosphoribosyltransferase activity in cell-free extracts of ascites-tumour cells by AMP (a) and by GMP (b). Assays were carried out as described in the Experimental section in the presence of 4·7 μM-PRPP, together with 6·2 μM-AMP (○), 31 μM-AMP (●), 65 μM-AMP (□), 62 μM-GMP (■) or 310 μM-GMP (△) and varying concentrations of ATP. The enzyme rates are expressed as a percentage of the rate in the absence of both ATP and nucleotide inhibitor.

PRPP concentration has little effect on the stimulation of this enzyme over the range 0·52–15·6 μM-PRPP. However, higher concentrations of PRPP tend to overcome the inhibitory effect of ATP (see also Murray, 1966*b*).

In Fig. 6 the effect of increasing ATP concentrations on the inhibition of adenine phosphoribosyltransferase by different concentrations of either AMP or GMP is shown. It can be seen that the maximum stimulation obtainable in the presence of ATP became less as the concentration of AMP or of GMP was increased. Thus in the presence of 62 μM-GMP the maximum stimulation by ATP was 44% and in the presence of 310 μM-GMP it was 19%. Similarly in the presence of 6·2, 31 and 65 μM-AMP the maximum stimulation was 51, 32 and 26% respectively. Maximum stimulation of adenine phosphoribosyltransferase in the presence of each nucleotide inhibitor was obtained with about 40 μM-ATP, which is similar to the concentration of ATP giving maximum stimulation in the absence of inhibitor (see Fig. 5).

ATP had some inhibitory effect on the reaction of hypoxanthine phosphoribosyltransferase at all concentrations tested (range 10–150 μM) and no stimulation of the activity of this enzyme was observed.

Adenine phosphoribosyltransferase could be 'desensitized' to the stimulatory effect of ATP by controlled heat treatment. In Fig. 7 the effect of incubating the crude extract of ascites-tumour cells at 55° for various times before assaying at 25° on the stimulation of adenine phosphoribosyltransferase by ATP and on the activity of the enzyme in the absence of ATP is shown. The stimulatory effect of ATP was completely removed by a heat treatment of 15 min. or longer, and this was accompanied by a slight decrease in the activity of the enzyme. The inhibition of adenine phosphoribosyltransferase by 6·25 μM-AMP (85%), in the absence of ATP, was not affected by heat treatments of up to 30 min.

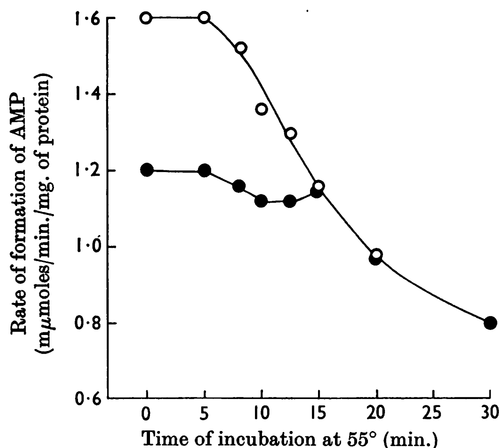

Fig. 7. Densensitization of adenine phosphoribosyltransferase to ATP stimulation by heat treatment. Samples of a crude extract from ascites-tumour cells were incubated for the indicated times at 55°, cooled, and assayed for adenine-phosphoribosyltransferase activity at 25° in the presence (○) and absence (●) of 31 μM-ATP. The PRPP concentration was 4·7 μM.

DISCUSSION

The results of these studies indicated that the rate of reaction of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase plotted against PRPP concentration gave the normal hyperbolic relationship expected from simple Michaelis–Menten kinetics. There was no evidence for a sigmoid-shaped curve, which would be expected if substrate bound at low concentrations influenced the binding of further substrate molecules (see Monod, Changeux & Jacob, 1963; Monod, Wyman & Changeux, 1965). In addition the nucleotide inhibitors showed completely competitive characteristics when tested with both enzymes. These initial studies suggested that both PRPP and the nucleotide inhibitors were bound at the same site on the enzyme surface. However, the results of further experiments indicated that this may be an over simplification. The K_m for PRPP with adenine phosphoribosyltransferase varied in a very different way from K_i for AMP with this enzyme when assays were carried out at different temperatures. Thus there was a rapid decrease in K_m and an increase in K_i at temperatures greater than about 27° showing that PRPP binding by adenine phosphoribosyltransferase is affected differently by temperature from AMP binding by this enzyme. The results do not necessarily mean that there are different sites for binding substrate and inhibitor but rather that AMP is bound by groups which may or may not be associated with the active centre of the adenine transferase but which have different heats of ionization from those binding AMP. The K_i for GMP and that for thio-IMP are unaffected by temperature over the range studied, indicating that binding of these compounds by adenine phosphoribosyltransferase may involve groups not involved in AMP and PRPP binding. Similar conclusions can be drawn from experiments showing the effect of temperature on the inhibition of hypoxanthine phosphoribosyltransferase by IMP, GMP and thio-IMP. The effect of temperature on the K_m for PRPP with both adenine and hypoxanthine phosphoribosyltransferase may indicate modification of the active sites of the enzymes, possibly by alteration of the protein conformation.

However, there is no way of deciding from these experiments if a separate substrate binding site is unmasked at high temperatures with concomitant suppression of the binding site predominating at low temperatures. It is of interest that Arrhenius plots showing the relationship between the maximum velocity and temperature with both adenine and hypoxanthine phosphoribosyltransferase indicated a transition from a higher to a lower activation energy as the temperature was increased. Similar breaks in the Arrhenius plots of D-amino acid oxidase have been reported by Massey *et al.* (1966). These workers concluded that this enzyme could exist in two stable forms, which one depending on the temperature, with different energies of activation. Although no physical evidence is available it is possible that a similar temperature-dependent conformational change occurs with both adenine and hypoxanthine phosphoribosyltransferase.

The effects of combinations of nucleotides on the inhibition of adenine phosphoribosyltransferase and of hypoxanthine phosphoribosyltransferase are difficult to interpret. If a single site was involved in binding all inhibitors it would be expected that combinations of inhibitors would compete with one another for the inhibitor binding site. Thus equimolar concentrations of any two inhibitors would result in an inhibition in between that obtained with either compound alone. Combinations of AMP with GMP, AMP with ADP and ADP with GMP resulted in slightly greater inhibition of adenine phosphoribosyltransferase than was obtained with the individual nucleotides. This implies that at least some of the groups binding each of AMP, ADP and GMP are different but not that separate sites are involved in the binding of each compound. Similar conclusions can be drawn with regard to IMP and GMP inhibition of hypoxanthine phosphoribosyltransferase.

The ability of ATP to act as both a stimulator and an inhibitor of adenine phosphoribosyltransferase is of particular interest. The stimulatory effect was not overcome by increasing PRPP concentrations, indicating that ATP was not bound at the active site, although the inhibitory effect was reversed by increasing concentrations of PRPP. In addition, the finding that the ability of ATP to stimulate adenine phosphoribosyltransferase could be selectively removed by controlled heat treatment or by purification of the enzyme, provided direct evidence that ATP is bound at a separate site on the enzyme surface (see also Changeux, 1961; Gerhart & Pardee, 1962; Martin, 1962; Patte, Le Bras, Loviny & Cohen, 1963). These results suggest that at low concentrations ATP is bound at a separate 'activator' site with a high affinity for this compound and that at higher concentrations ATP competes with PRPP at the active site. However, the possibility of a separate 'inhibitor' binding site for ATP has not been excluded. The stimulation by ATP does not affect the affinity of PRPP for adenine phosphoribosyltransferase but acts by increasing the maximum velocity of the reaction (i.e. a 'positive V system' as defined by Monod *et al.* 1965). It is not clear why the extent of ATP stimulation in the presence of AMP and GMP should decrease as the concentration of nucleotide inhibitor is increased. A similar situation was observed with the decrease in the ability of L-valine to overcome the inhibition of L-threonine deaminase by increasing concentrations of L-isoleucine (Changeux, 1963). At low concentratons L-valine increased the activity in the absence of inhibitor and was assumed to act by increasing the affinity of the enzyme for the natural substrate (L-threonine) or by decreasing its affinity for L-isoleucine.

The large decrease observed in the K_m for PRPP with adenine phosphoribosyltransferase on purification implies an increase in the affinity of PRPP for the enzyme. This was accompanied by desensitization to stimulation by ATP. Gerhart & Pardee (1964) reported that aspartate carbamoyltransferase had a higher affinity for aspartate when the enzyme had been desensitized to the inhibitory effect of CTP. The desensitization resulted from a dissociation of the enzyme into catalytic and regulatory sub-units, and it has been suggested (Gerhart & Schachman, 1965) that the catalytic activity of the former sub-unit was inhibited when it was combined with the regulatory sub-unit. The results obtained here could similarly be explained by postulating that native adenine phosphoribosyltransferase is composed of catalytic and ATP-binding sub-unit(s), which are separated during purification. By analogy with aspartate carbamoyltransferase the dissociated catalytic sub-units would be expected to have a greater affinity for PRPP than the native enzyme.

Although the results have shown that PRPP and the nucleotide inhibitors (AMP and GMP) are bound by different functional groups on adenine phosphoribosyltransferase, there is no conclusive evidence that they are bound other than at the active site of the enzyme. None of the treatments tested has separated the inhibitory effects of the nucleotides from the catalytic activity of the enzyme. It is clear, however, that the ability of ATP both to stimulate and to inhibit the reaction of adenine phosphoribosyltransferase could be a mechanism controlling the activity of this enzyme *in vivo*.

The weak inhibition of both adenine and hypoxanthine phosphoribosyltransferase by thio-IMP indicates yet another site of action of this derivative of the growth inhibitor 6-mercaptopurine (for a review of the metabolic effects of thio-IMP see Brockman, 1965; Murray, 1966c). The relation, if

any, of these inhibitions to the mechanism of action of 6-mercaptopurine is not apparent.

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