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Inhibition of phosphoribosyl pyrophosphate amidotransferase from Ehrlich ascites-tumour cells by thiopurine nucleotides*

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INHIBITION of the growth of tumour cells by the thiopurines 6-mercaptopurine and 6-methylthiopurine ribonucleoside is often attributed to the inhibitory effects of their nucleotide metabolites on phosphoribosyl pyrophosphate (PRPP) amidotransferase with consequent blocking of the primary pathway of purine nucleotide biosynthesis.[1–4] This conclusion is based on indirect experiments in which incorporation of radio active precursors such as glycine and formate into purines or purine precursors is inhibited when intact tumour cells are exposed to 6-mercaptopurine or 6-methylthiopurine ribonucleoside. Further indirect support for this view has come from studies on the inhibition of PRPP amidotransferase from pigeon liver by 6-thioinosine 5′-phosphate.[5, 6]

This paper describes the extraction and assay of PRPP aminotransferase from Ehrlich ascites-tumour cells and its inhibition by 6-thioinosine 5-phosphate, 6-methylthiopurine 5′-phosphate and 6-thioguanosine 5′-phosphate.

Ehrlich ascites-tumour cells were collected 10 days after inoculation and washed with 0·9% NaCl. The cells were suspended in 1·2 vol of 0·1 M ammonium citrate (pH 5·0) and homogenized for 30 sec with an Ultra Turrax homogenizer. After centrifuging at 9000 *g* for 15 min the supernatant was adjusted to pH 6 with 1 M Na_3PO_4, stored at 4° and used for enzyme assays within 2 hr.

PRPP amidotransferase activity was measured at 30° in reaction mixtures containing 1·02 mM L-glutamine (98 nc)–0·3 mM PRPP–5·5 mM $MgCl_2$–0·5 mM dithiothreitol–25 mM Tris (Cl^-, pH 8·0); reactions were started with 0·025 ml of enzyme to give a final volume of 0·1 ml. After the required time (usually 15 min) the reaction was stopped by addition of 0·2 ml of 2 mM L-glutamate in 80% (v/v) propan-2-ol at 1°. Portions (0·05 ml) of each supernatant were applied 10 cm from one end of a 1 × 43 cm strip of Whatman 3 MM paper and subjected to electrophoresis in 0·05 M borate (Na^+, pH 8·5) for 20 min at 50 V/cm in an apparatus similar to that described by Gross.[7] Staining of test strips with 0·1% ninhydrin–0·5% acetic acid in acetone showed that glutamine and glutamate were completely separated. Zones 5·5 cm (cathodic)–5·5 cm (anodic) and 5·5 cm–16·5 cm (anodic) from the origin contained glutamine and glutamate respectively with no overlap. Unstained papers were dried and radioactivity in the glutamine and glutamate zones was measured by liquid scintillation counting. Control assays were carried out without PRPP and the control rate was subtracted to give the net 'PRPP-dependent' rate. Alternatively chromatography in isopropanol–99% formic acid–water (40:2:10, by vol.) was used to separate glutamate (R_f 0·27) and glutamine (R_f 0·12) Assays carried out using electrophoresis or chromatography gave comparable results.

The specific activity of PRPP amidotransferase in the crude extracts was about 0·6 n-mole glutamate formed/min/mg of protein with a total activity of about 15 n-moles/min/g of packed cells. The initial rate of L-glutamate formation was maintained for at least 15 min, with net conversion of up to 20 per cent of the glutamine.

The effects of increasing concentrations of 6-thioinosine 5′-phosphate,[8] 6-thioguanosine 5′-phosphate[9] and 6-methylthioinosine 5′-phosphate[10] on the reaction rate are shown in Fig. 1; concentrations of nucleotides required for 50 per cent inhibition were $1·6 \times 10^{-3}$, 4×10^{-4} and 5×10^{-5} M, respectively. Similar patterns of inhibition were obtained when endogenous nucleotides were removed by making a slurry of the crude extract with Sephadex G-25 and then centrifuging to obtain a concentrated protein solution.

Growth of the Ehrlich cells used in these experiments during 10 days in female Swiss albino mice was 2·6 ± 0·6 g of packed cells in saline-injected controls (6 animals) and 1·9 ± 0·5 g (6 animals) in mice injected daily with 40 mg/kg body wt. of 6-mercaptopurine; values found for controls and mice injected with 20 mg/kg body wt. of 6-methylthioinosine were 1·6 ± 0·5 (6 animals) and 0·15 ± 0·01 g (6 animals) of packed cells, respectively.

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6-Methylthioinosine has been shown previously to inhibit the growth of a number of types of tumour cells[1, 3] and is rapidly converted into 6-methylthioinosine 5′-phosphate in sensitive cells; levels of this nucleotide as high as 1 mM were found in Ehrlich ascites-tumour cells following exposure to the nucleoside, probably through the action of adenosine kinase.[9] Inhibition of growth by 6-methylthioinosine has been attributed to inhibition of PRPP amidotransferase by its nucleotide in sensitive cells; resistance in cells that form the nucleotide was attributed to modification of PRPP amidotransferase making it insensitive to 6-methylthioinosine 5′-phosphate.[11] The results presented here show that 6-methylthioinosine 5′-phosphate, at levels that are attained during chemotherapy, is an effective inhibitor of the amidotransferase.

6-Thioinosine 5′-phosphate was a much poorer inhibitor of PRPP amidotransferase although the nucleotide is formed in Ehrlich cells after 6-mercaptopurine treatment and its inhibitory effect on this enzyme is the most widely quoted basis for the inhibitory action of 6-mercaptopurine against mammalian cells.[4] However, 6-mercaptopurine was not an effective inhibitor of the growth of the Ehrlich ascites-tumour cells used in these studies and results are needed with enzyme isolated from cells showing greater sensitivity to this drug.

As with PRPP amidotransferase from pigeon liver,[5] the regulatory properties of enzyme from Ehrlich cells are readily modified on storage and purification, and studies of inhibitory effect should be carried out with freshly-prepared enzyme.

The direct assay described here is convenient for use with enzyme of low specific activity in extracts from small samples of cells. Conventional spectrophotometric assays were not sensitive enough for use with such material. The method is of potential use for tests of drug resistance in clinical material.

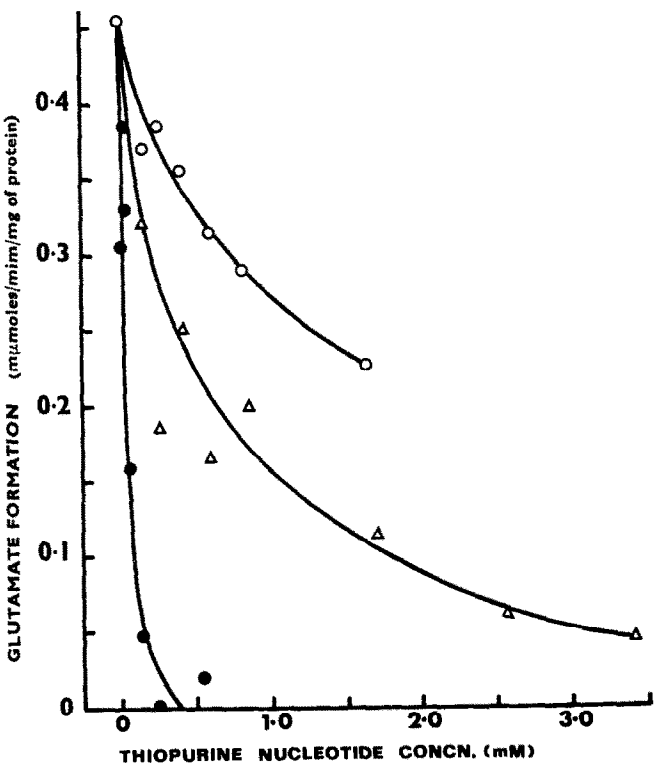

FIG. 1. Effects of increasing concentrations of 6-thioinosine 5′-phosphate (○), 6-thioguanosine 5′-phosphate (△) and 6-methylthioinosine 5-phosphate (●) on the activity of PRPP amidotransferase. Standard assays were carried out as described in the text.

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Simultaneous estimation of 5-hydroxytryptamine and 5-hydroxyindol-3-acetic acid in rat brain

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SEVERAL methods have been described for the separate estimation of tissular 5-hydroxytryptamine (5-HT) and 5-hydroxyindol-3-acetic acid (5-HIAA). According to the method of Bogdanski *et al.*[1] 5-HT is extracted into butanol and its native fluorescence measured after activation in 3 N HCl. Vanable[2] published a more specific and sensitive method in which ninhydrine is employed to form a fluorescent compound with 5-HT. The procedure described by Snyder[3] is based on the latter method. For the estimation of 5-HIAA several methods are based on extraction with diethyl-ether followed by quantitative estimation.[4, 5] Recently, Giacalone and Valzelli[6] proposed the use of butyl acetate for the extraction of the acid, followed by spectrofluorometric assay.

The method described below allows the simultaneous estimation of 5-HT and 5-HIAA in the same brain extract. It combines a slightly modified Snyder[3] procedure with the method of Giacalone and Valzelli[6] for the estimation of 5-HT and 5-HIAA respectively, and is based on the observation of Dreux,[7] who, in the course of urinary 5-HT determinations, eliminates 5-HIAA by extraction into ethyl acetate.

MATERIALS AND METHODS

Reagents. Butyl acetate (chromat. grade) is washed successively with N NaOH, N HCl and twice with distilled water. Phosphate buffer: 0·1 M (KH_2PO_4 + Na_2HPO_4) pH 6·5, to which 0·1% ascorbic acid is added just before use. Borate buffer: 0·5 M (H_3BO_3 + NaOH) pH 10. Butanol is washed successively with (2 N NaOH, distilled water, 2 N HCl, distilled water), twice, distilled water, and then saturated with borate buffer and NaCl. Heptane is washed successively with (2 N NaOH, distilled water, 2 N HCl, distilled water), twice. Ninhydrine solution: 0·1 M ninhydrine in 0·1 ml phosphate buffer pH 6·5.