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KINETICS OF AMIDOPHOSPHORIBOSYLTRANSFERASE IN INTACT TUMOR CELLS

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SUMMARY

A method for measuring amidophosphoribosyltransferase (5-phosphoribosylamine:pyrophosphate phosphoribosyltransferase, EC 2.4.2.14) activity in intact Ehrlich ascites tumor cells is described. It depends on the measurement of the initial rate of glutamine-dependent utilization of intracellular 5-phosphoribosyl-1-pyrophosphate (*P*-ribose-*PP*). This utilization of *P*-ribose-*PP* is due solely to amidotransferase activity, as it can be inhibited completely by 6-methylmercaptopurine ribonucleoside-5′-phosphate.

Apparent Michaelis constants for amido phosphoribosyltransferase assayed in tumor cell extracts were 2 mM for glutamine and 1 mM for *P*-ribose-*PP*. In intact tumor cells, 2.5 mM intracellular *P*-ribose-*PP* gave half-maximal activity. Concentrations of glutamine in incubation media of greater than 1 mM gave maximal amidotransferase activity in intact cells. The apparent maximum amidotransferase activity was the same in intact tumor cells and cell extracts (approx. 0.30 μmole/min per ml packed cells).

INTRODUCTION

Enzyme action and regulation have been most studied in cell-free preparations of varying degrees of purity. However, the degree to which information gained from such studies is applicable to the action and regulation of enzymes in intact cells is still under investigation. For example, the concentrations of substrates, products and effectors normally used in the study of isolated enzymes are often significantly different from known or expected physiological concentrations. In addition, the enzyme concentrations used in enzyme studies are usually several orders of magnitude lower than those calculated for intact cells or whole tissues [1]. These problems have stimulated several studies of enzyme kinetics and other metabolic processes in intact cells. Some of these studies have shown that the kinetic properties of several enzymes are similar

Abbreviations: MeMPR (6-methylmercaptopurine ribonucleoside), 6-methylthio-9-β-D-ribofuranosylpurine; MeMPR-*P*, MeMPR 5′-phosphate; $S_{0.5}$, substrate concentration giving 50% of maximal velocity.

in intact cells and cell extracts [2–4], although other studies have found that the properties of certain enzymes in intact cells differ significantly from the properties of the same enzymes in cell extracts [5, 6]. We report here the details of a method for studying amidophosphoribosyltransferase (EC 2.4.2.14) activity in intact Ehrlich ascites tumor cells together with some data on the properties of this enzyme in cell extracts. Amidophosphoribosyltransferase is of particular interest because it catalyzes the first irreversible reaction specific to the pathway of purine biosynthesis de novo:

P-ribose-*PP* + glutamine + H_2O → ribosylamine 5-phosphate + glutamate + PP_i,

and because numerous studies of its properties in cell extracts and partially purified preparations have been made in relation to its possible role in the regulation of this pathway (chapter 5 of ref. 7 contains an extensive review).

MATERIALS AND METHODS

Sources of most of the materials used have been reported previously [8, 9]. L-[U-^{14}C]Glutamine (<200 Ci/mole; New England Nuclear Corp.) and unlabeled L-glutamine were mixed to obtain a 20-mM stock solution with a specific activity of 0.5 Ci/mole. This solution was repurified weekly by passage through a Dowex-1 chloride column to remove glutamate and pyrrolidone 5-carboxylate. Stock solutions of 5-phosphoribosyl-1-pyrophosphate (*P*-ribose-*PP*) (sodium salt; Sigma Chemical Co.) were assayed either using adenine phosphoribosyltransferase (EC 2.4.2.8) and radioactive adenine [10], or spectrophotometrically using the orotidine 5′-phosphate phosphoribosyltransferase (EC 2.4.2.10) reaction [11]. These assays showed that the commercial *P*-ribose-*PP* was approximately 80% pure by weight. 5-Azaorotate was obtained from Dr W. N. Kelley and from Drug Research and Development, National Cancer Institute, Bethesda, Md.

Methods of tumor cell preparation have been described previously [8, 9]. Packed cell volume was measured by centrifuging cells for 6 min at maximum speed in an International Clinical Centrifuge. Suspensions of Ehrlich ascites tumor cells (2.5%, v/v; 2 ml) were incubated with shaking at 37 °C in modified Krebs–Ringer medium containing 25 mM phosphate, pH 7.4 [8].

Estimation of P-ribose-PP

P-Ribose-*PP* was estimated by a modification of the method of Henderson and Khoo [10]. Samples of tumor cell suspension (100 μl) were transferred to small plastic tubes (12 mm × 75 mm, Falcon Plastics) in a boiling water bath and heated for 20 s for optimum extraction of *P*-ribose-*PP*; the tubes were then cooled in crushed ice. These extracts were then incubated at 37 °C for 30 min with 20 μl [8-^{14}C]adenine (1 mM; 5.0 Ci/mole) and 10 μl of crude Ehrlich ascites tumor cell extract containing adenine phosphoribosyltransferase activity (this was prepared as described by Henderson and Khoo [10] and the extract was further dialyzed for 16 h against 1 mM EDTA before use). The conversion of *P*-ribose-*PP* to [^{14}C]adenine nucleotides was effectively complete under these conditions. Protein was removed by adding 25 μl of ice-cold 8 M formic acid followed by centrifugation.

The [^{14}C]nucleotide products of the assay were separated from unused [^{14}C]-

adenine by descending paper chromatography of 100 μl portions of the assay mixture and 20 μg each of AMP, ADP, ATP and IMP on Whatman 3 MM paper in 1-propanol–30% (w/w) ammonia–water (60:30:10, v/v/v). The nucleotide spots were located under ultraviolet light, cut out, and their radioactivities measured at 55% counting efficiency in a liquid scintillation counter [8].

Nucleotide concentrations

Cell extracts were prepared by a slight modification of the method of Bagnara and Finch [12]. Samples of tumor cell suspension (200 μl) were transferred to small plastic tubes containing 10 μl of 8.4 M $HClO_4$ at 0–4 °C. After thorough mixing, the acid extraction was continued for 10–20 min at 0–4 °C and the suspended cell material was removed by centrifugation in the cold. An aliquot of the supernatant was neutralized with a solution of 3.6 M KOH–0.8 M $KHCO_3$ as previously described [12], and L-ascorbic acid was then added to the neutralized extract to a final concentration of 1 mM. The nucleotides in 20-μl samples of the neutralized extract were separated and individual nucleotide concentrations measured by high-pressure liquid chromatography using a Varian-Aerograph LCS-1000 chromatograph as described by Brown [13].

Assay of amidophosphoribosyltransferase in cell-free extracts

This assay was based on the *P*-ribose-*PP*-dependent hydrolysis of [^{14}C]glutamine [14, 15]. Ehrlich ascites cells were washed once with saline, resuspended in 5 vol. of 50 mM Tris–HCl (pH 8.0) containing 0.25 M sucrose and 1 mM $MgCl_2$, and broken by sonication. Cell debris was removed by centrifugation at 27 000 × *g* for 30 min. Assay mixtures contained 80 mM Tris–HCl (pH 8.0), 2 mM *P*-ribose-*PP*, 4 mM $MgCl_2$, 4 mM [^{14}C]glutamine and enzyme extract in a final volume of 250 μl. [^{14}C]Glutamate formation was measured by either of two methods. In one, 50-μl aliquots were spotted directly onto DEAE-cellulose paper discs, dried quickly under a heat lamp, and then processed as described by Martin [16]. In the second procedure, 50-μl aliquots were mixed with 20 μl of 0.2 M EDTA (pH 8.0), and chromatographed (descending) on Whatman No. 1 paper in ethanol–*tert*-butanol–formic acid–water (12:4:1:3, by vol.). Blank reactions lacked *P*-ribose-*PP*. Glutamate production was linear with respect to amount of added protein. The *P*-ribose-*PP*-independent glutaminase activity was less than 15% of the total activity.

RESULTS

Amidophosphoribosyltransferase activity in intact cells

The method described here for measuring amidophosphoribosyltransferase activity in intact Ehrlich ascites tumor cells is based on the utilization of *P*-ribose-*PP* which occurs upon addition of glutamine to the incubation medium. Suspensions of Ehrlich ascites tumor cells in glucose–salts medium are particularly suitable for this assay because intracellular glutamine concentrations are very low [17], and glutamine synthesis is slow [18]. In addition, *P*-ribose-*PP* is synthesized from glucose under these conditions and accumulates in the cells (Fig. 1). When exogenous glutamine is added, the concentration of *P*-ribose-*PP* decreases [19–21], and this is due to its increased utilization for purine biosynthesis de novo [18, 22, 23] (see also below). The

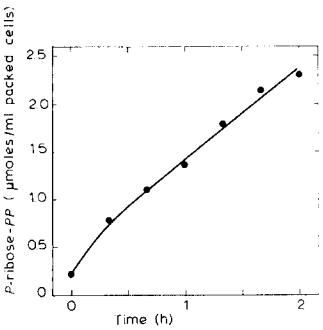

Fig. 1. Synthesis of *P*-ribose-*PP* in Ehrlich ascites tumor cells. Tumor cells (2.5% suspension, v/v) were incubated in modified Krebs–Ringer medium containing 25 mM phosphate and 5.5 mM glucose.

rate of the glutamine-dependent utilization of *P*-ribose-*PP* should therefore be a direct measure of the amidophosphoribosyltransferase activity of these cells. This method was used previously in semi-quantitative measurements of amidotransferase activity [19–21], but the rate of this reaction was not accurately determined in those studies.

Experiments were first conducted to establish suitable conditions under which the initial rate of glutamine-dependent *P*-ribose-*PP* utilization could be measured, and to establish that amidophosphoribosyltransferase was indeed the enzyme responsible for this process.

Initial rate of P-ribose-PP utilization

Fig. 2 shows the time course of *P*-ribose-*PP* utilization following addition of 2 mM glutamine (this concentration was chosen because it supports maximal rates of purine biosynthesis de novo in these cells [22]). Concentrations of *P*-ribose-*PP* decrease rapidly during the first min after addition of glutamine, then more slowly but linearly for the next 5 min; the rate of *P*-ribose-*PP* utilization subsequently further

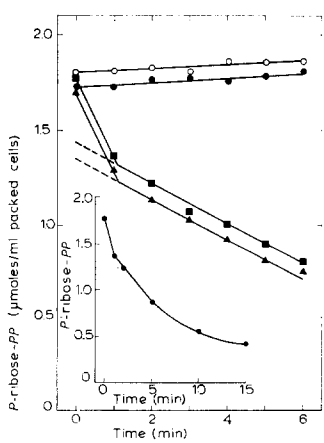

Fig. 2. Glutamine-dependent utilization of *P*-ribose-*PP*. Tumor cells were incubated as described in Fig. 1 for 60 min, after which the medium was made 1 mM in glycine (●), 2 mM in glutamine (▲), or 1 mM in glycine plus 2 mM in glutamine (■); control medium (○).

decreases, perhaps due either to low *P*-ribose-*PP* concentrations or to product inhibition by glutamate. These results also show that glycine, which is a substrate of the second reaction of the purine biosynthetic pathway, has no effect on the glutamine-dependent rate of *P*-ribose-*PP* utilization. The biphasic nature of the curve of *P*-ribose-*PP* utilization during the first 6 min following addition of glutamine will be discussed further below.

Inhibition of P-ribose-PP utilization

To determine if the glutamine-dependent utilization of *P*-ribose-*PP* was, in fact, due entirely to amidophosphoribosyltransferase activity, we incubated Ehrlich ascites tumor cells with 6-methylmercaptopurine ribonucleoside (MeMPR), a purine nucleoside analogue which is converted to the 5′-monophosphate form by adenosine kinase [24–27]. The phosphorylated compound is a potent inhibitor of purine biosynthesis de novo [20, 28, 29], an effect which is believed to be due to its inhibition of amidophosphoribosyltransferase activity [30, 31].

Fig. 3 shows that incubation of cells with MeMPR completely prevents

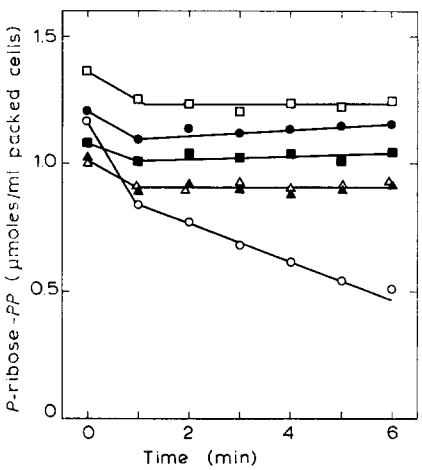

Fig. 3. Inhibition of *P*-ribose-*PP* utilization. Tumor cells were incubated as described in Fig. 1 for 30 min, after which MeMPR was added to final concentrations of 0 (○), 10 μM (▲), 25 μM (△), 50 μM (■), 75 μM (●) or 100 μM (□). Incubations were then continued for 30–60 min, glutamine was then added (2 mM, final concentration), and *P*-ribose-*PP* concentrations were measured.

P-ribose-*PP* utilization in the period from 1 to 6 min after addition of glutamine, but only partially inhibits this process during the first min. Thus although amidophosphoribosyltransferase may be solely or mainly responsible for the second phase of *P*-ribose-*PP* utilization (1–6 min), these results suggest the possibility that other processes may contribute to *P*-ribose-*PP* utilization during the first min following addition of glutamine. This point had to be studied further.

The initial rapid rate of P-ribose-PP utilization

One possible explanation for the insensitivity of the initial (0–1 min) rapid rate

of *P*-ribose-*PP* utilization to inhibition by MeMPR was that glutamine may stimulate pyrimidine biosynthesis de novo via the carbamyl phosphate synthetase reaction, and thereby increase *P*-ribose-*PP* utilization through the conversion of orotate to orotidine 5′-phosphate; MeMPR is not believed to inhibit pyrimidine biosynthesis de novo [32]. Attempts were therefore made to inhibit this route of *P*-ribose-*PP* utilization by incubating cells with 1 mM 5-azaorotate, an inhibitor of orotate phosphoribosyltransferase activity in several intact mammalian cells [33–35]. Fig. 4 shows, however, that

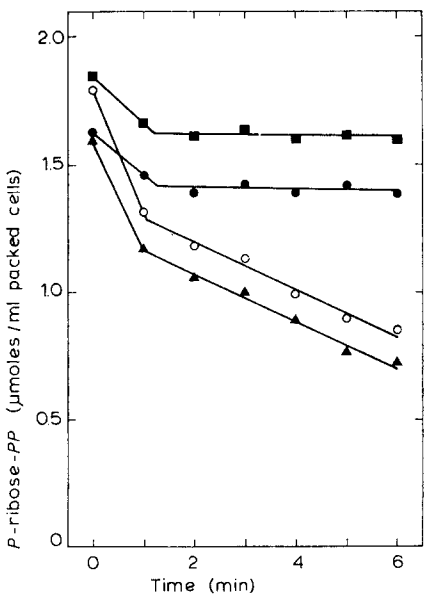

Fig. 4. Effect of 5-azaorotate on *P*-ribose-*PP* utilization. Tumor cells were incubated as described in Fig. 1 for 30 min, after which the medium was made 100 μM in MeMPR (■), 1 mM in 5-azaorotate (▲), or 100 μM in MeMPR plus 1 mM in 5-azaorotate (●); control medium (○). Incubations were continued for 30 min, glutamine was then added (2 mM, final concentration), and *P*-ribose-*PP* concentrations were measured.

5-azaorotate had no effect on the rate of *P*-ribose-*PP* utilization in either control or MeMPR-treated cells. These results show that under the conditions used, the initial rapid decrease in *P*-ribose-*PP* concentration is probably not due to stimulation of pyrimidine biosynthesis by added glutamine.

Another possible cause of the MeMPR-insensitive initial rapid decrease in *P*-ribose-*PP* concentration upon addition of glutamine is that there is an initial "burst" of amidophosphoribosyltransferase activity, due at least in part to enzyme-bound *P*-ribose-*PP*, and that the prior binding of this substrate in effect protects the enzyme inhibition by MeMPR-*P* for a brief period of amidotransferase activity. Thus in the experiments of Figs 3 and 4, cells were incubated with glucose, leading to accumulation of *P*-ribose-*PP* and presumably to its binding to the amidotransferase, before MeMPR was added. To study this hypothesis, cells were first incubated with MeMPR to allow synthesis of MeMPR-*P* at low *P*-ribose-*PP* concentrations; glucose was then

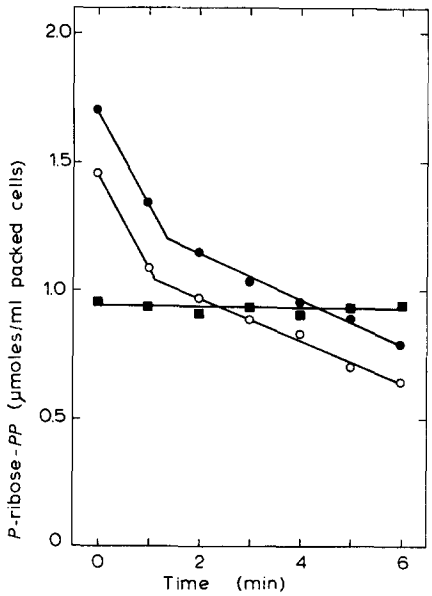

Fig. 5. Effect of MeMPR at low *P*-ribose-*PP* concentration. Tumor cells were incubated for 60 min in modified Krebs–Ringer phosphate medium containing 5.5 mM glucose (○), or 5.5 mM glucose plus 1 mM pyruvate (●); or cells were incubated for 30 min in the same medium containing no glucose plus 1 mM pyruvate and 100 μM MeMPR followed by addition of glucose (5.5 mM, final concentration) and incubation for a further 60 min (■). Glutamine was added (2 mM, final concentration) and *P*-ribose-*PP* concentrations were measured.

added to initiate *P*-ribose-*PP* synthesis*. Fig. 5 shows that under these conditions, addition of glutamine did not lead to *P*-ribose-*PP* utilization. Whether or not the "burst" hypothesis is correct, these results show that the glutamine-dependent utilization of *P*-ribose-*PP* is, within the limits of detection of our methods, due solely to amidophosphoribosyltransferase. Because the results above suggest that the initial "burst" of amidophosphoribosyltransferase activity is not typical of the rate of reaction of this enzyme in cells, the initial rate of the amidotransferase reaction was measured in the period from 1 to 6 min after addition of glutamine.

Effect of substrate concentration on amidophosphoribosyltransferase activity

The effect of substrate concentration on the rate of amidophosphoribosyltransferase in intact tumor cells was studied next. Fig. 6 shows the relationship between the initial rate of the amidotransferase reaction and the concentration of glutamine initially present in the incubation medium at a constant, relatively high, concentration of *P*-ribose-*PP*. The rate measured reaches a maximum at glutamine concentrations greater than 1 mM, and a final concentration of 2 mM was used in

*If cells are incubated with MeMPR in glucose-free medium and glucose is added after 30 min, the rate of *P*-ribose-*PP* synthesis is much slower than when MeMPR and glucose are present together. However, if pyruvate is present during the incubation with MeMPR in the absence of glucose, the subsequent synthesis of *P*-ribose-*PP* from glucose proceeds at the normal rate. The bases of these phenomena are not known, but pyruvate itself does not support *P*-ribose-*PP* synthesis, nor does it affect the rate of glutamine-dependent *P*-ribose-*PP* utilization.

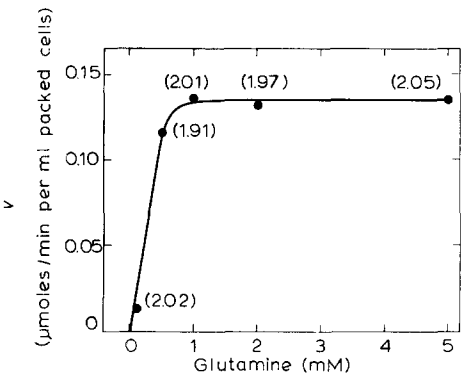

Fig. 6. Effect of glutamine concentration on amidophosphoribosyltransferase activity. Tumor cells were incubated as described in Fig. 1 for 90 min and the rate of the amidotransferase reaction was measured after addition of various concentrations of glutamine to the incubation medium. Figures in parenthesis show the initial *P*-ribose-*PP* concentration in μmoles/ml packed cells.

subsequent experiments. The actual intracellular concentration of glutamine in these experiments is not known, and is difficult to measure due to its rapid metabolism [36]; in addition it seems likely that glutamine is actively transported [37] and hence intracellular concentrations may be greater than those in the medium.

The relationship between the rate of the amidotransferase reaction (at constant glutamine concentration) and *P*-ribose-*PP* concentration is shown in Fig. 7. For this experiment, the intracellular concentration of *P*-ribose-*PP* was varied by incubating cells for various times in Krebs–Ringer phosphate medium containing 5.5 mM glucose (see Fig. 1)*. The results gave a linear double-reciprocal plot with an $S_{0.5}$ for *P*-ribose-*PP* of approximately 2.5 mM in cell water, and an apparent maximum velocity of 0.29 μmole/min per ml packed cells. The initial concentration of *P*-ribose-*PP* was taken as the value of the intercept of the linear portion of the *P*-ribose-*PP* utilization curve extrapolated to zero time (see Fig. 2).

Amidophosphoribosyltransferase activity in cell-free extracts

A double-reciprocal plot of glutamine concentration versus amidotransferase activity is shown in Fig. 8; *P*-ribose-*PP* concentration was 2 mM. Under these conditions the apparent K_m for glutamine was 2 mM.

Amidotransferase assays using 2 mM *P*-ribose-*PP* (Fig. 8) are linear for at least 45 min by which time almost all of the *P*-ribose-*PP* has been depleted. However, with lower initial concentrations of *P*-ribose-*PP* (as required for study of the effect of *P*-ribose-*PP* concentration on amidotransferase activity), the results are complicated by the presence in crude tumor cell extracts of a phosphatase or pyrophosphatase that acts on *P*-ribose-*PP*, and the assays are linear for only a few min. The reaction velocities for these assays have been estimated by drawing tangents to these curves at zero

* Intracellular adenine and guanine nucleotide concentrations remain unchanged during incubation of Ehrlich ascites cells for up to 3 h in modified Krebs-Ringer phosphate medium containing 5.5 mM glucose. It is therefore unlikely that the amidotransferase reaction might be affected by changing concentrations of potential nucleotide feedback effectors under the conditions of this experiment.

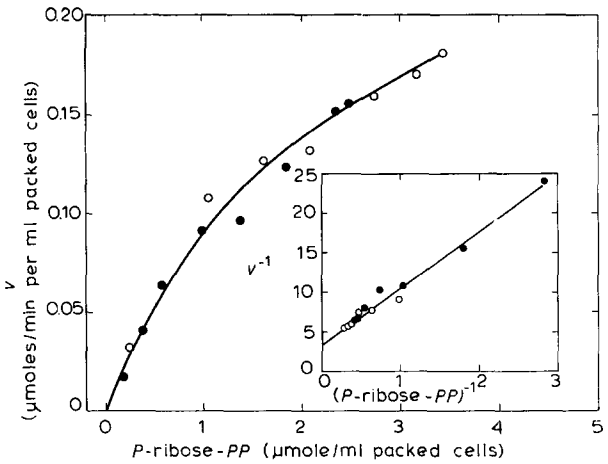

Fig. 7. Effect of *P*-ribose-*PP* concentration on amidophosphoribosyltransferase activity. Tumor cells were incubated as described in Fig. 1 for various times (i.e. the cells had accumulated various concentrations of *P*-ribose-*PP*) after which glutamine was added (2 mM, final concentration) and the rate of the amidotransferase reaction was measured. Open and closed symbols show data from separate experiments. Inset: Double-reciprocal plot of these results.

time. This destruction of *P*-ribose-*PP* means that *P*-ribose-*PP* concentrations available to the amidotransferase will be less than the nominal amounts added.

Fig. 9 shows a plot of *P*-ribose-*PP* concentration versus amidotransferase activity at a glutamine concentration of 2 mM. The concave nature of the double reciprocal plot (see inset) is probably due to destruction of *P*-ribose-*PP* as mentioned above. From the values of reaction velocity measured at high *P*-ribose-*PP* concentrations, the $S_{0.5}$ for *P*-ribose-*PP* was calculated to be approx. 1.0 mM. The $S_{0.5}$ and K_m values for *P*-ribose-*PP* and glutamine respectively are similar to the corresponding values reported for the amidotransferase from adenocarcinoma 755 [30] and human placenta [15]. From Figs 8 and 9, the apparent maximum velocities at saturating con-

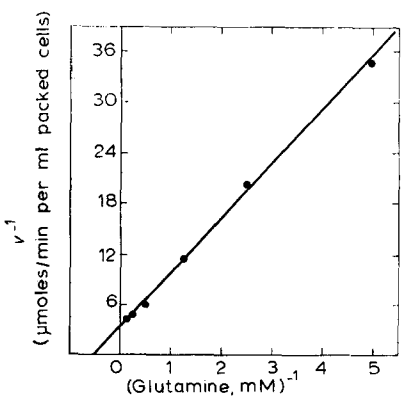

Fig. 8. Double-reciprocal plot of initial velocity of amidophosphoribosyltransferase against glutamine concentration. *P*-Ribose-*PP* concentration was 2 mM.

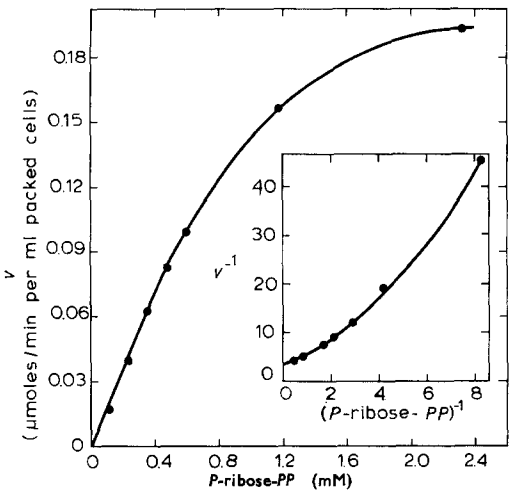

Fig. 9. Plot of initial velocity of amidophosphoribosyltransferase against *P*-ribose-*PP* concentrations. Glutamine concentration was 2 mM. Inset: Double-reciprocal plot of these results.

centrations of glutamine and *P*-ribose-*PP* respectively are 0.31 and 0.29 μmole/min per ml packed cells. These results are in close agreement with the apparent maximum activity observed in intact tumor cells (Fig. 7).

DISCUSSION

Of the several pathways which utilize *P*-ribose-*PP* in Ehrlich ascites tumor cells, the purine biosynthetic pathway appears to be the most important quantitatively. The demonstration that the glutamine-dependent utilization of *P*-ribose-*PP* in intact tumor cells is not affected by azaorotate suggests that the pyrimidine biosynthetic pathway does not utilize significant amounts of *P*-ribose-*PP* under our experimental conditions. Moreover, the observations that the glutamine-dependent utilization of *P*-ribose-*PP* is completely inhibited in cells incubated with MeMPR is evidence that this utilization of *P*-ribose-*PP* is caused solely by the amidophosphoribosyltransferase reaction. We conclude, therefore, that our method provides a specific measurement of the initial rate of the amidotransferase reaction in intact Ehrlich ascites tumor cells.

When the effect of *P*-ribose-*PP* concentration on amidotransferase activity in extracts was studied at a nonsaturating (2 mM) glutamine concentration (Fig. 9), the $S_{0.5}$ of approximately 1,0 mM for *P*-ribose-*PP* was 2.5-fold lower than the $S_{0.5}$ for this substrate calculated for the amidotransferase reaction assayed in the intact cell (Fig. 7). This difference between the properties of the enzyme in intact cells and in cell-free extracts may result from compartmentation of *P*-ribose-*PP* or permeability barriers within the cell, but it probably arises from the effects of high enzyme concentration [38]. Reeves and Sols [5], and Weitzman and Hewson [6], have also observed higher "K_m values" for enzymic reactions when they are assayed in intact cells compared with the value of K_m obtained for the same enzymic reaction assayed in extracts. Another factor which could affect the K_m value for *P*-ribose-*PP* is the concentration of phosphate ion. Kelley and coworkers [15] have shown that phosphate ion inhibits

the amidotransferase partially purified from human placenta by competition with *P*-ribose-*PP*. This effect of phosphate ion could contribute to the higher K_m observed for *P*-ribose-*PP* in intact cells because the incubation medium used contained 25 mM phosphate, while the amidotransferase activity in cell-free extracts was assayed in phosphate-free medium. However, the apparent maximum amidophosphoribosyltransferase activity in intact tumor cells agrees closely with the values obtained for the enzyme assayed in cell-free extracts.

Our results do not permit detailed analysis of the effect of glutamine concentration on amidophosphoribosyltransfrase activity in intact tumor cells. The probability that glutamine is actively transported into Ehrlich ascites cells [37], together with its rapid metabolism within the cell [36], makes accurate measurements of the intracellular concentration of this amino acid very difficult. It is clear, however, that initial concentrations of glutamine in the medium greater than 1 mM appear to saturate the amidophosphoribosyltransferase in the intact tumor cells as assayed by our method.

In conclusion, the method outlined in this paper for assay of amidophosphoribosyltransferase in intact Ehrlich ascites tumor cells offers a unique possibility to measure the rate of this enzyme under conditions similar to those commonly employed in studies of other aspects of purine metabolism in intact cells [8, 9].

ACKNOWLEDGEMENT

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