*Biochemical Pharmacology*, Vol. 38, No. 22, pp. 4045–4051, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00


# INHIBITION OF TWO ENZYMES IN *DE NOVO* PURINE NUCLEOTIDE SYNTHESIS BY TRICIRIBINE PHOSPHATE (TCN-P)*

E. COLLEEN MOORE,†‡ ROBERT B. HURLBERT,§ GERRY R. BOSS and STEVE P. MASSIA†

† Pharmacology Section, Medical Oncology Department, and § Department of Biochemistry and Molecular Biology, The University of Texas M.D. Anderson Hospital and Tumor Institute at Houston, Houston, TX 77030; and ‖ Department of Medicine, University of California, San Diego, La Jolla, CA 92037, U.S.A.

(*Received* 26 *May* 1987; *accepted* 2 *November* 1988)

**Abstract**—We previously reported that triciribine (tricyclic nucleoside, TCN, NSC-154020), after phosphorylation in cultured CCRF-CEM human leukemic lymphoblasts inhibited *de novo* purine nucleotide synthesis, GTP more than ATP [Moore *et al. Biochem. Pharmac.* **38**, 4037 (1989)]. To determine the enzymes inhibited, triciribine phosphate (TCN-P, NSC-280594) was tested in dialyzed extracts of the cells. A new assay for glycinamide ribotide (GAR) synthesis was based on incorporation of [$^{14}$C]glycine into GAR as a ribose-containing compound retained on boronyl gel columns. Glutamine, phosphoribosyl pyrophosphate (PRPP), ATP and glycine were required for the two-step sequence of glutamine: amidophosphoribosyltransferase (EC 2.4.2.14) and phosphoribosylamine-glycine ligase (EC 6.3.4.13). When PRPP was near the normal intracellular concentration (0.1 mM), 1.2 mM TCN-P inhibited GAR synthesis by 71–95%. To permit separate assay of the ligase step, 6-diazo-5-oxo-L-norleucine was used to inhibit amidophosphoribosyltransferase and phosphoribosylamine (PRA) was supplied *in situ* by chemical reaction of ribose-5-phosphate and ammonia (as ammonium acetate). The ligase was not inhibited by TCN-P. Thus, TCN-P inhibits amidophosphoribosyltransferase; it acts as an analog of the purine nucleotides which regulate this first committed step of *de novo* purine biosynthesis by an allosteric feedback mechanism. The measured intracellular concentration (0.1 mM) of PRPP was not changed in cells treated with TCN. IMP dehydrogenase (EC 1.1.1.205), the first *de novo* step committed to guanosine nucleotide synthesis, was also tested. It was inhibited by TCN-P, competitively with IMP, 66% at 1.2 mM TCN-P and 8 μM IMP. The degree of inhibition of these two enzymes was sufficient to account for the effects on purine nucleotide biosynthesis observed in intact cells treated with TCN.

Triciribine phosphate, also called tricyclic nucleoside phosphate (TCN-P¶, NSC-280594) (Fig. 1), is an experimental antineoplastic agent which is undergoing clinical trials at this and other institutions [1–4]. It is dephosphorylated in plasma to the nucleoside TCN [5], which is transported in cells and rephosphorylated by adenosine kinase [5–8]; cells lacking adenosine kinase are resistant to TCN and to TCN-P [5].

Cells exposed to TCN and accumulating TCN-P intracellularly are inhibited in growth, cloning efficiency, and incorporation of precursors into RNA, DNA, and protein [6–9] and into purine nucleotides [10].

Incorporation of formate into formylglycinamide ribotide (FGAR) was inhibited strongly by TCN, showing that inhibition of *de novo* purine synthesis occurred at this or an earlier step in the pathway [10]. Incorporation of hypoxanthine into ATP was stimulated as would be expected, while incorporation of hypoxanthine into GTP was inhibited, indicating an additional site of inhibition between IMP and GTP [10].

We report here new experiments with TCN-P, using extracts from CCRF-CEM human leukemic lymphoblasts, designed to determine the exact sites of inhibition. We used a convenient new assay procedure for 5-phosphoribosylglycinamide (GAR) formation which measured incorporation of labeled glycine into ribose-containing compounds (presumably only GAR) retained on boronyl gel columns. The chemical generation *in situ* of the unstable intermediate 5-phosphoribosylamine (PRA) was used to dissociate effects of TCN-P on synthesis of phosphoribosyl pyrophosphate (PRPP), PRA, and GAR. The assay tests whether TCN-P inhibits the first committed enzyme of the *de novo* pathway, glutamine: amidophosphoribosyltransferase (EC 2.4.2.14), which is subject to feedback inhi-

* Supported by Grant CA 34204 from the National Cancer Institute, United States Public Health Service, and by institutional funds.

‡ Corresponding author: Pharmacology-Box 52, The University of Texas M.D. Anderson Hospital and Tumor Institute at Houston, 1515 Holcombe Blvd., Houston, TX 77030.

¶ Abbreviations: TCN, tricyclic nucleoside, 6-amino-4-methyl-8-(β-D-ribofuranosyl)pyrrolo[4,3,2-de]-pyrimido-[4,5-c]-pyridazine, triciribine (NSC-154020); TCN-P, the 5′-phosphate of TCN (NSC-280594); PRPP, 5-phosphoribosyl-1-pyrophosphate; PRA, 5-phosphoribosyl 1-amine; GAR, 5-phospho-β-D-ribofuranosylglycinamide; FGAR, 5-phospho-β-D-ribofuranosyl-αN-formylglycinamide; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DON, 6-diazo-5-oxo-L-norleucine; MMPRP, 6-methylthio-IMP, the nucleotide of 6-methylmercaptopurine; $MgAc_2$, magnesium acetate; KAc, potassium acetate; and $NH_4Ac$, ammonium acetate.

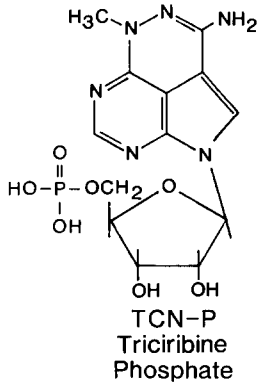


Fig. 1: Structure of TCN-P.

bition by purine nucleotides and analogs [11], or the second enzyme of the pathway, GAR synthetase (phosphoribosylamine-glycine ligase, EC 6.3.4.13). Preliminary reports have appeared [12, 13].

We also tested IMP dehydrogenase (EC 1.1.1.205) directly; this enzyme is the first step of the *de novo* branch after IMP which leads to guanine nucleotides. It was judged a more likely site of inhibition of GTP synthesis than the next step, amination of XMP, because no XMP was observed to accumulate in the cells treated by TCN.

## MATERIALS AND METHODS

*Materials.* [$^{14}$C]Glycine, 104 mCi/mmol, was obtained from Schwartz Bioresearch (now ICN, Irvine, CA). [8-$^{14}$]IMP, 61 mCi/mmol, was from the Amersham Corp. (Arlington Heights, IL). AffiGel 601 (boronyl polyacrylamide beads) and Dowex-1 ion exchange resin were obtained from BioRad Laboratories (Richmond, CA). PEI-cellulose sheets were obtained from Brinkmann Instruments (Westbury, NY); some lots gave poor separation of IMP and XMP and were not usable (all had been stored at 4° for more than a year). PRPP, 6-diazo-5-oxo-L-norleucine (DON), and other biochemicals were from the Sigma Chemical Co. (St Louis, MO); the concentration of PRPP was calculated with corrections for its age and the decomposition rate given by Sigma. Hydrofluor scintillation fluid was from National Diagnostics (Sommerville, NJ). Methylmercaptopurine ribonucleoside phosphate (MMPRP) was a gift from L. L. Bennett, Jr, of Southern Research Institute (Birmingham, AL). TCN-P was obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute. CCRF-CEM human leukemic lymphoblasts were originally obtained from William Plunkett (University of Texas, M.D. Anderson Hospital).

*Enzyme extract.* The cell extract was prepared from CCRF-CEM cells grown in suspension culture in RPMI 1640 medium (Gibco, Grand Island, NY), by a method based on that of Oates *et al.* [14]. Log phase cells ($2.3 \times 10^8$ in 400 ml) were collected, taken up in 2.2 ml of buffer (0.25 M sucrose, 1.0 mM $MgAc_2$, 5.0 mM KAc, 10 mM HEPES buffer, pH 7.4), and lysed by freezing and thawing three times. The lysate was centrifuged for 8 min in an Eppendorf microcentrifuge, and then for 1 hr at 100,000 *g*. The supernatant fraction was dialyzed for 24 hr against three changes of 50 mM HEPES, 1.0 mM $MGAc_2$, 5.0 mM KAc. The resulting 1.7 ml of dialyzed extract was frozen in small portions and stored at −80°. The GAR-synthesizing activity was stable for several months while frozen, but decreased by about 25% on the first freezing and thawing. It was mostly lost if the extract was frozen a second time.

*Assay of synthesis of GAR by amidophosphoribosyltransferase and GAR synthetase.* The formation of GAR from PRPP, [$^{14}$C]glycine, glutamine,

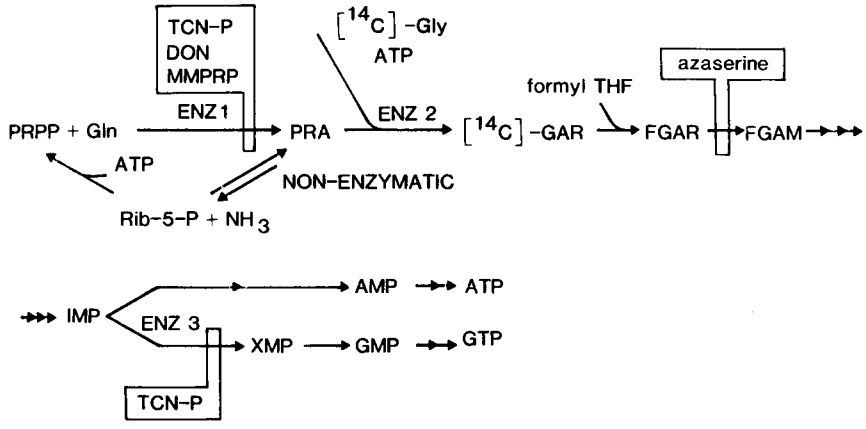


Fig. 2. Partial pathway of purine synthesis *de novo*. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; Gln, glutamine; MMPRP, 5-phosphoribosylmethylmercaptopurine; PRA, 5-phosphoribosylamine; GAR, 5-phosphoribosylglycinamide; FGAR, 5-phosphoribosylformylglycinamide; FGAM, 5-phosphoribosylformylglycinamidine; and DON, 6-diazo-5-oxo-L-norleucine. Enzyme 1: amidophosphoribosyltransferase; Enzyme 2: GAR synthetase; and Enzyme 3: IMP dehydrogenase.

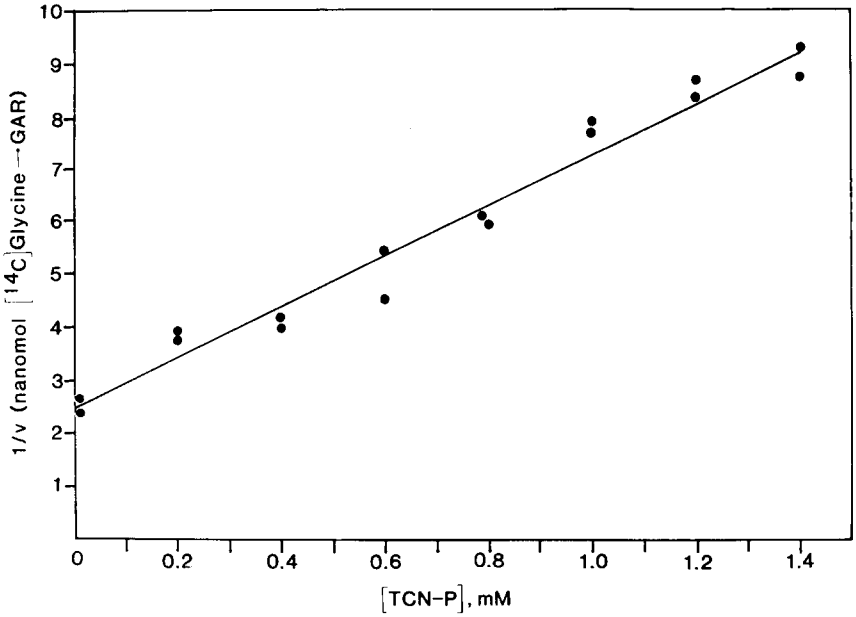


Fig. 3. Inhibition of GAR synthesis from PRPP and glutamine by TCN-P (Dixon plot). The reaction mixture was the same as in Table 1 but with 20 μl enzyme and incubated for 15 min. Abscissa, TCN-P concentration (mM) in the reaction mixture. Ordinate, reciprocal of activity (nmol glycine converted to GAR in 15 min).

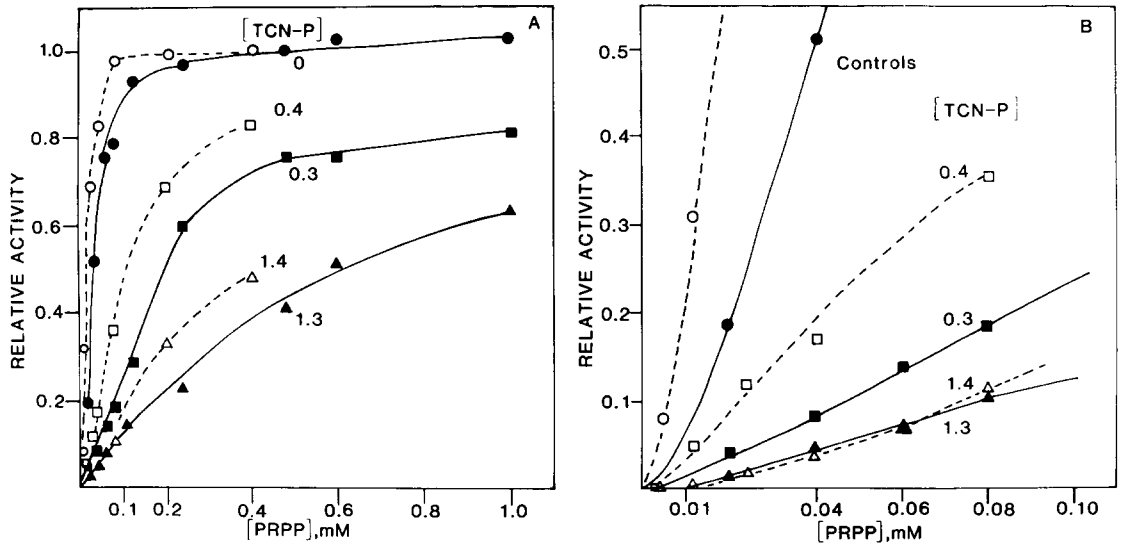


Fig. 4. Effect of TCN-P and PRPP concentrations on GAR synthesis. The standard reaction mixture was used, with 0.4 mM [$^{14}$C]glycine (14,900 dpm/nmol), and the PRPP concentration was varied using an equimolar mixture of PRPP and $MgAc_2$. The total volume was 100 μl and the enzyme volume 20 μl. Broken lines: Experiment 1, enzyme preparation 1. Solid lines: Experiment 2, enzyme preparation 2. The TCN-P concentration (mM) is shown for each curve. Results for each experiment are expressed as a fraction of the activity at 0.4 or 0.48 mM PRPP, 1.15 nmol GAR formed in 15 min for experiment 1 and 0.72 nmol for experiment 2. Panel B shows the results at low PRPP concentrations on an expanded scale.

and ATP was dependent on the sequential activities of amidophosphoribosyltransferase and GAR synthetase (Fig. 2). These enzyme activities were measured in a reaction mixture containing, in a 50–100 μl volume, 80 mM HEPES, pH 8.0, 5.0 mM $MgAc_2$, 1.0 mM ATP (2.0 mM in some early experiments), 6.0 mM glutamine, [$^{14}$C]glycine (sp. act. and concentration as noted in the legends), 0.12–0.16 mM PRPP, and 10–30 μl of cell extract per 50 μl reaction volume. Incubation was for 15 or 20 min at 37° [15].

*Assay of GAR synthetase independently.* To measure the synthesis of GAR separately, it was necessary to generate the unstable intermediate PRA from

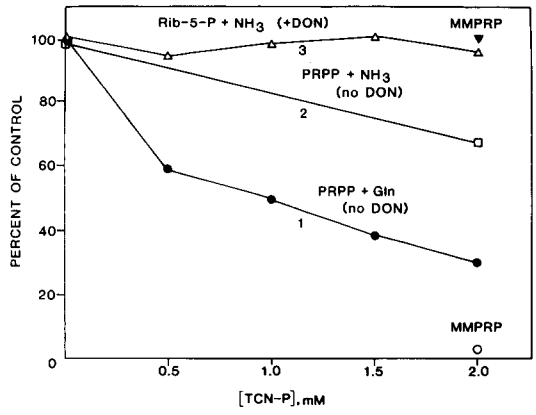


Fig. 5. Effects of TCN-P, DON and MMPRP on GAR synthesis with PRA generated enzymatically or chemically. The same amount of enzyme extract (20 μl in 100 μl total volume) was used for all conditions. Each inhibited point was calculated as a percentage of its own no-TCN-P control. Curve 1 (●): PRPP 0.40 mM, glutamine 6.0 mM, and glycine 0.23 mM (sp. act. 6,400 dpm/nmol), untreated enzyme, control activity 1.13 nmol in 15 min. Curve 2 (□): as Curve 1 except $NH_4Ac$, 25 mM, instead of glutamine, control activity 0.79 nmol. Curve 3 (△): ribose-5-phosphate and $NH_4Ac$ with glycine 0.03 mM (sp. act. 36,600 dpm/nmol), treated enzyme (20 mg DON/ml, 3° overnight and 37° for 7 min), control activity 0.20 nmol. Points (○) and (▼): MMPRP, 2 mM, instead of TCN-P, conditions of curves 1 and 3 respectively. Activity of DON-treated enzyme with PRPP plus glutamine or $NH_4Ac$ was zero.

ribose-5-phosphate and $NH_3$ in the reaction mixture, as described by Nierlich and Magasanik [16] and Nierlich [17]. To eliminate any possible interference from PRPP synthesized in the reaction mixture, the enzyme extract was treated with the irreversible inhibitor DON to inactivate the amidophosphoribosyltransferase. Treatment of the extract with 20 mg/ml DON overnight at 4° and for 7 min at 37° destroyed all activity with PRPP. The reaction mixture for GAR synthetase [17] contained 80 mM HEPES, pH 8.0, 5.0 mM $MgAc_2$, 1.0 mM ATP, 0.06–0.40 mM [$^{14}$C]glycine, 12 mM ribose-5-phosphate, 25 mM $NH_4Ac$, and 10–25 μl of DON-treated cell extract, in a total volume of 100 μl. It was incubated for 15 min at 37°.

*Chromatography on boronate gel columns.* GAR formation in both reactions was measured as the amount of label incorporated into ribose-containing compounds. We added to each reaction mixture 1.0 ml of Buffer A (0.05 M *N*-methylmorpholinium acetate, pH 9.1, containing 0.05 M $MgAc_2$ and 0.025 M KAc) and applied it to a column of AffiGel 601 equilibrated with Buffer A (1.0 cm diameter × 2.5 cm, in a tube fitted with a stopcock and a No. 25 needle at the outlet to control the flow rate and with a filter paper disc tamped firmly down over the gel). The columns were washed with 6.0 ml of Buffer A in three portions, followed by 3.0 ml of 0.02 M *N*-methylmorpholinium acetate, pH 9.1. The unreacted labeled glycine passed through; the ribose-containing product was eluted with 5.0 ml of 0.25 M $NH_4Ac$, pH 5, and counted (4.0 ml in 10 ml of Hydrofluor). The yield of GAR was calculated from the stated specific activity of the glycine; only relative values within experiments were desired for our purposes. Zero time or zero enzyme blanks were included in each experiment; the blank values (usually about 0.03 nmol GAR for a 50-μl reaction mixture) were subtracted from the results.

The columns were regenerated by washing with 4 ml each of 0.5 M $NH_4Ac$–0.05 M EDTA, 5.0 $NH_4Ac$, water, 2 portions of 0.1 M $Na_2B_4O_7$ (with stirring; the gel swells), and water. They were stored in 0.02 M $NaN_3$ and could be reused many times, provided they were not exposed for long periods to high pH, which caused discoloration. Before the next use, the columns were cycled through $NH_4Ac$, borate, and water, and equilibrated with Buffer A. This procedure was developed for the separation of $^{32}$P-labeled ribo- and deoxyribonucleotides.* With strict adherence to the protocol, the highly labeled glycine substrate was separated completely and 90–96% of the ribonucleotide was recovered.

*Measurement of PRPP in cells.* The cells were incubated with or without 100 μM TCN for 2 hr; they were then extracted and the PRPP was determined enzymatically, as described previously [18].

*Assay of IMP dehydrogenase.* IMP dehydrogenase activity was measured by an adaptation of the method of Leyva *et al.* [19]. The same extract of CEM cells was used, diluted in dialysis buffer so that 0.7 to 1.2 μl was added in a volume of 10 or 15 μl. The reaction mixture contained 20 mM HEPES, pH 7.4, 0.5 mM $MgAc_2$ (partly from the enzyme buffer), ethanol (from labeled substrate) up to 0.12%, 0.5 M KCl, 1.5 mM NAD, 3.0 mM dithiothreitol, and 0.005 to 0.05 mM [$^{14}$C]IMP (61 μCi/μmol), in a total volume of 50 μl. After incubating for 20 or 30 min at 37°, the tubes were chilled and 50 μl of cold 100% ethanol and 10 μl of a carrier mixture containing 50 μM each of IMP and XMP were added. The samples were centrifuged for 2 min in an Eppendorf microcentrifuge, and 10-μl aliquots were spotted on washed PEI-cellulose sheets and chromatographed with 1.2 M LiCl. The XMP and IMP spots were cut out and eluted for 2 hr in 2 ml of 2 M ammonium formate, pH 5.0, which was counted in 10 ml of Hydrofluor. Yield was calculated as the dpm in XMP divided by the sum of the dpm in IMP and XMP and multiplied by the picomoles of IMP in the reaction mixture. A no-enzyme blank (1.03% of substrate) was subtracted. An unidentified spot between IMP and XMP contained 7.2% of the radioactivity on the chromatogram, but did not change with enzyme or TCN-P; it was ignored in the calculations.

## RESULTS

*Effect of TCN-P on GAR synthesis.* The enzyme preparation gave good GAR-synthesis activity with PRPP, ATP, glycine, and glutamine as substrates.

* With the assistance of Barbara Moon, Karen Wassick and Helen Chew.

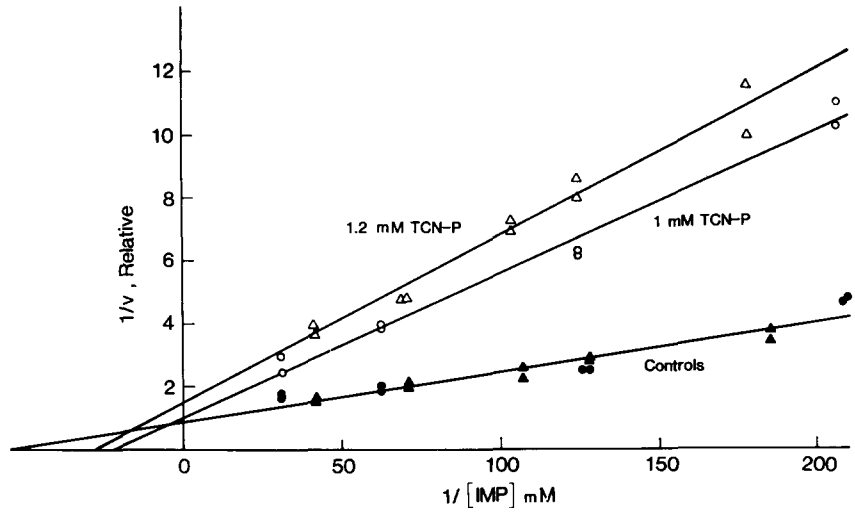


Fig. 6. Inhibition of IMP dehydrogenase by TCN-P (Lineweaver–Burk plot). Two experiments were combined by expressing velocity (pmol XMP formed in 30 min) as a fraction of the apparent maximum velocity in each experiment (81 and 114 pmol respectively). The apparent $K_m$ was 0.0167 mM. The lines were fitted by the unweighted least-square method.

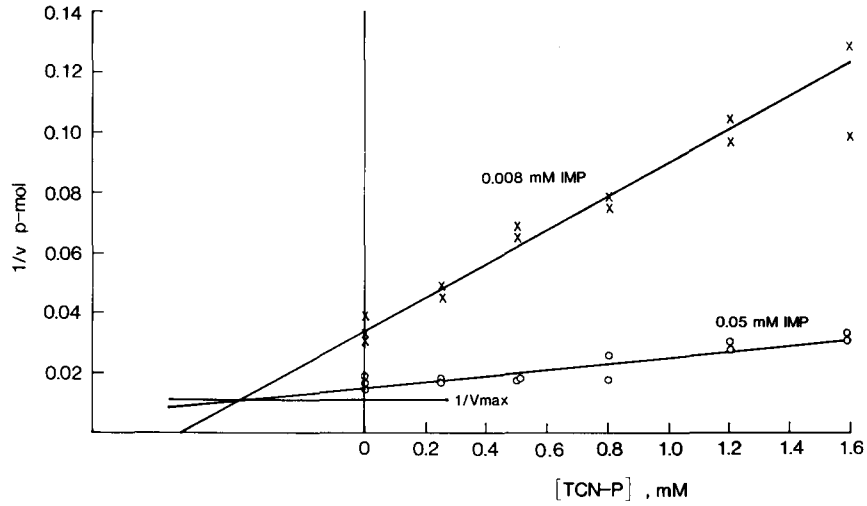


Fig. 7. Inhibition of IMP dehydrogenase by TCN-P (Dixon plot). Ordinate: reciprocal of the activity expressed as pmol XMP formed in 20 min by 1.19 μl enzyme. The lines were fitted by the least-squares method; one point (0.008 mM IMP, 1.6 mM TCN-P) was omitted from the calculation. The maximum velocity in this experiment was estimated from the control velocity at 0.05 mM IMP and the control line of Fig. 6.

The optimum PRPP concentration was 0.16 to 0.34 mM; higher concentrations were inhibitory. When additional magnesium equal to the PRPP was added, the PRPP optimum was 0.2 to 0.6 mM, and there was less inhibition at higher concentrations (data not shown). Because we found the intracellular PRPP concentration in CCRF-CEM cells to be 0.1 mM (see below), we used 0.12 or 0.16 mM for most experiments. The activity was linear with time up to at least 20 min. The curve of activity versus enzyme concentration was sigmoid as might be expected for a two-enzyme reaction; it was near-linear in the range from 10 to 30 μl extract in 50 μl total volume for one batch of enzyme or in 100 μl for another. The activity was dependent on all three substrates (Table 1) and was inhibited almost completely under these conditions by 1.8 mM TCN-P. The activity was higher with 1 mM ATP than with 2 mM; the effects of varying the glycine or glutamine concentration were not studied. Figure 3 shows that the inhibition was almost linear when the inverse activity was plotted against [TCN-P]; the 50% inhibitory concentration in this experiment (with 0.12 mM PRPP) was 0.5 mM. Another experiment with a different enzyme preparation gave 0.4 mM.

Inhibition by TCN-P varied with PRPP concentration and also among enzyme preparations (Fig. 4). At 3 mM PRPP there was no inhibition by 2 mM

Table 1. Substrate requirements for GAR synthesis

| Reaction mix | Product (dpm) |
|---|---|
| Complete | 23,870 |
| Zero time | 648 |
| Omit PRPP | 503 |
| Omit ATP | 1,009 |
| Omit glutamine | 930 |
| Omit PRPP, ATP and glutamine | 800 |
| Complete plus 1.8 mM TCN-P | 810 |

The complete mixture contained 0.04 mM [$^{14}$C]glycine (sp. act. 0.1 $\mu$Ci/nmol), 0.12 mM PRPP, 2.0 mM ATP, and 5.0 mM glutamine, with HEPES buffer, 5.0 mM $MgAc_2$, and 10 $\mu$l enzyme extract in a total volume of 50 $\mu$l. Incorporation of label into ribose-containing compounds, retained by AffiGel 601, was measured after incubation for 12 min at 37°. Each value is the average of duplicates.

TCN-P. As shown in Fig. 4, however, the degree of inhibition by 0.4 mM TCN-P with the less sensitive enzyme preparation ranged from 83% at 0.05 mM PRPP to 27% at 0.4 mM. The more sensitive enzyme preparation was inhibited to a similar degree by 0.3 mM TCN-P. The activity versus PRPP concentration plots showed the sigmoidicity characteristic of the regulatory enzyme amidophosphoribosyltransferase, and the curves were displaced towards higher PRPP concentrations in the presence of TCN-P. At concentrations of PRPP from 0.02 to 0.12 mM in the control and 0.1 to 0.5 mM in the TCN-P-inhibited samples, the Lineweaver–Burk double-reciprocal plots approached straight lines with a quasi-competitive relationship between PRPP and TCN-P (not shown). Because of the sigmoid curves the calculation of actual $K_m$ and $K_i$ values is not possible.

These results suggested a primary effect on amidophosphoribosyltransferase, the first of the two enzymes involved. However, in order to determine whether the second enzyme, GAR synthetase, was inhibited, we measured its activity separately. Phosphoribosylamine is too labile to be available as a reagent. Attempts to accumulate PRA by preincubation of PRPP and glutamine with cell extract in the absence of ATP and glycine were unsuccessful; others have reported that the compound is extremely labile. We therefore adopted Nierlich's method [16–17] of generating PRA by a spontaneous chemical reaction of ribose-5-phosphate and ammonia ($NH_4Ac$) *in situ* with the other components of the GAR synthetase reaction mixture, ATP and labeled glycine.

Our experiments were complicated by the fact that amidophosphoribosyltransferase can also use ammonia as a substrate in place of glutamine; at the high concentration of $NH_4Ac$ required, this reaction was active. Although the activity with PRPP plus glutamine could be destroyed easily by the irreversible inhibitor DON (2 mg/ml), prolonged pretreatment with 17–25 mg/ml DON was necessary to destroy the activity with $NH_3$. The activity of the DON-treated extract, with PRA generated from ribose-5-phosphate and $NH_3$, measured only GAR synthetase; it was only 0.1 to 0.4 of that with the two-step reaction from PRPP and glutamine at the same level of untreated enzyme. The activity was linear with the amount of cell extract up to 25 $\mu$l in 100 $\mu$l total volume (not shown), but was probably limited by the low levels of PRA generated and other suboptimal reaction conditions. The activity before treatment by DON was 20% higher, which may represent additional PRA available from endogenous PRPP (generated from ATP and ribose-5-phosphate).

The effect of TCN-P on the various reactions is illustrated by Fig. 5. The GAR-synthesizing activity with chemical generation of PRA (DON-treated enzyme, representing only GAR synthetase) was completely unaffected by 2 mM TCN-P, and also by 2 mM MMPRP (methylmercaptopurine riboside phosphate), a known feedback inhibitor of amidophosphoribosyltransferase. The activity dependent on PRA generated enzymically from PRPP plus glutamine was inhibited by TCN-P and even more strongly by MMPRP; it was also destroyed completely by the treatment with DON. The activity with PRPP plus $NH_3$ was also inhibited by TCN-P, but not as strongly as the reaction utilizing glutamine.

*Effect of TCN-P on intracellular PRPP synthesis.* The PRPP concentration in control CCRF-CEM cells and cells treated with 100 $\mu$M TCN for 2 hr was $109 \pm 12$ pmol/$10^6$ cells and $104 \pm 11$ pmol/$10^6$ cells respectively (mean $\pm$ SD of three independent experiments). Since the average cell volume, determined by Coulter counter, was approximately 1 $\mu$l/$10^6$ cells, the intracellular PRPP concentration was about 0.1 mM. There was no significant change in cell volume after 2 hr of TCN treatment.

*Effect of TCN-P on IMP dehydrogenase activity.* IMP dehydrogenase activity was linear with the amount of cell extract up to 2 $\mu$l and with time to 20 min (data not shown). Figure 6 shows an apparent $K_m$ for IMP of approximately 17 $\mu$M; the maximum velocity was 3.6 to 4.2 pmol/min/$\mu$l enzyme. The reaction was inhibited by TCN-P (Figs. 6 and 7). The inhibition was strongly dependent on the concentration of IMP, as shown in Figs 6 and 7. At 8 $\mu$M IMP, 1.2 mM TCN-P inhibited enzyme activity by 66%, while at 50 $\mu$M IMP it inhibited only by 39%. Although a Lineweaver–Burk plot (Fig. 6) did not conclusively demonstrate competitive inhibition, a Dixon plot in a separate experiment (Fig. 7) showed the inhibition lines crossing at $1/v = 1/V$, which indicates a competitive relationship between IMP and TCN-P [20]. The $K_i$ was 0.4 mM.

## DISCUSSION

We have shown that TCN-P inhibited the first committed step on the *de novo* pathway to purine nucleotides, amidophosphoribosyltransferase. This regulatory enzyme is feedback-inhibited by a number of purine nucleotides and analogs thereof, including methylthio-IMP [21]. The mechanism of inhibition by these nucleotides is an allosteric modification of PRPP binding. One indication of this effect is an altered sigmoid character of the activity versus PRPP concentration curves, with displacement toward higher concentrations of PRPP [11, 21, 22]. We

found these curves with different batches of enzyme to be sigmoid and also variable (Fig. 4) as noted by others [21], with an apparent increase in sigmoidicity in the presence of TCN-P. The data suggest that TCN-P is interacting with an allosteric site.

Inhibition of other steps in the pathway appears unlikely. The second enzyme, GAR synthetase, was shown directly not to be inhibited. The synthesis of PRPP is also probably not sensitive, since we found that intact cells exposed to TCN were not depleted of PRPP, and pyrimidine nucleotide concentrations in TCN-treated intact cells were also normal or increased [10]. We were unable to obtain enough formate incorporation in the cell extracts to determine whether the conversion of GAR to FGAR was inhibited. However, the inhibition of amidophosphoribosyltransferase by 1.2 mM TCN-P in five experiments with three enzyme preparations (including those of Figs. 3 and 4), at 0.12 or 0.16 mM PRPP, approximating the intracellular concentration, was 71–95%. Whole cells treated with 200 $\mu$M TCN, which in other experiments generated 1 to 1.5 mM TCN-P intracellularly, were inhibited 83% in FGAR synthesis or 79% in ATP synthesis [10]. Thus, the inhibition of amidophosphoribosyltransferase alone is sufficient to account for the overall inhibition of adenosine nucleotide synthesis in CCRF-CEM cells.

We have also shown that TCN-P inhibited the first *de novo* step committed to formation of guanosine nucleotides, competitively with IMP and with a $K_i$ of 0.4 mM. The intracellular IMP of CCRF-CEM cells, either treated or untreated, was too low to detect readily by HPLC. Thus, the enzymic inhibition by TCN-P at lower IMP concentrations is the most relevant (for example 66% at 8 $\mu$M IMP and 1.2 mM TCN-P). This moderate degree of inhibition is in accord with our observations in intact cells; inhibition of GTP synthesis by TCN was less than other effects and quite variable [10]. For example, during labeling with hypoxanthine, the ratio of specific activities, GTP/ATP, was reduced 40% by TCN treatment.

The actual sensitivities of the two enzymes of TCN-P in intact cells would be difficult to predict. The sensitivity of the transferase is influenced by the concentrations of its substrates and of all the purine nucleoside monophosphates, which vary with the metabolic state and growth phase of the cells. Similarly, the intracellular effect of TCN-P on the dehydrogenase would depend greatly on the low concentrations of IMP, which would vary with the remaining degree of *de novo* synthesis of purine nucleotides as well as the amount of inosine scavenged from metabolism and the culture medium. The similar degrees of inhibition observed in our enzymic and whole-cell experiments [10] with TCN-P and TCN do indicate that TCN-P acts as an analog of purine nucleoside monophosphates, probably with only two sites of inhibition in purine nucleotide biosynthesis: amidophosphoribosyltransferase and IMP dehydrogenase.

## REFERENCES


1. G. Powis, P. J. Basseches, D. M. Kroschel, R. L. Richardson, M. J. O'Connell and L. K. Kvols, *Cancer Treat. Rep.* **70**, 359 (1986).
2. L. G. Feun, N. Savaraj, G. P. Bodey, K. Lu, B. S. Yap, J. A. Ajani, M. A. Burgess, R. S. Benjamin, E. McKelvey and I. Krakoff, *Cancer Res.* **44**, 3608 (1984).
3. A. Mittleman, E. S. Casper, T. A. Godwin, C. Cassidy and C. W. Young, *Cancer Treat. Rep.* **67**, 159 (1983).
4. R. B. Schilcher, C. D. Haas, M. K. Samson, J. D. Young and L. H. Baker, *Cancer Res.* **46**, 3147 (1986).
5. L. L. Wotring, G. W. Crabtree, N. L. Edwards, R. E. Parks, Jr. and L. B. Townsend, *Cancer Treat. Rep.* **70**, 491 (1986).
6. L. L. Bennett, Jr., D. Smithers, D. L. Hill, L. M. Rose and J. A. Alexander, *Biochem Pharmac.* **27**, 233 (1978).
7. P. G. W. Plagemann, *J. Natn. Cancer Inst.* **57**, 1283 (1976).
8. P. D. Schweinsberg, *Ph.D. Dissertation* University of Texas Graduate School of Biomedical Sciences at Houston (1981).
9. L. L. Wotring, J. E. Passiatore, J. L. Roti Roti, J. L. Hudson and L. B. Townsend, *Cancer Res.* **45**, 6355( 1985).
10. E. C. Moore, R. B. Hurlbert and S. P. Massia, *Biochem. Pharmac.* **38**: 4037 (1989).
11. J. F. Henderson, *Regulation of Purine Biosynthesis*, p. 166. American Chemical Society, Washington, D.C. (1972).
12. E. C. Moore, R. B. Hurlbert and S. P. Massia, *Proc. Am. Ass. Cancer Res.* **26**, 246 (1985).
13. E. C. Moore, R. B. Hurlbert, J. M. Ravel and S. Massia, *Proc. Am. Ass. Cancer Res.* **27**, 303 (1986).
14. D. C. Oates, D. Vannais and D. Patterson, *Cell* **20**, 797 (1980).
15. J. M. Lewis and S. C. Hartman, *Meth. Enzymol.* **51**, 171 (1978).
16. D. P. Nierlich and B. Magasanik, *J. Biol. Chem.* **240**, 366 (1965).
17. D. P. Nierlich, *Meth. Enzymol.* **51**, 179 (1978).
18. R. B. Pilz, R. C. Willis and G. R. Boss, *J. biol. Chem.* **259**, 2927 (1984).
19. A. Leyva, E. W. Holmes, Jr. and W. N. Kelley, *Biochem. Pharmac.* **25**, 527 (1976).
20. M. Dixon, *Biochem. J.* **55**, 170 (1953).
21. J. B. Wyngaarden, in *Current Topics in Cellular Regulation* (Eds. B. Horecker and E. Stadtman), Vol. 5, p. 135. Academic Press, New York (1972).
22. D. P. Nierlich and B. Magasanik, *J. biol. Chem.* **240**, 358 (1965).