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IDENTIFICATION OF THE METABOLITES OF AN ANTITUMOR TRICYCLIC NUCLEOSIDE (NSC-154020)*

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Abstract—The metabolism of 3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphthylene (TCN, NSC-154020) was studied in human erythrocytes. With use of high performance liquid chromatography, three metabolites in addition to the major metabolite, TCN 5′-monophosphate, were detected. After isolation and purification of the metabolites, their structures were elucidated by spectrometric methods and incubations with cultured cells and catabolic enzymes. All resulted from the oxidation of TCN. Two are proposed to be α- and β-anomers of a D-ribofuranosyl nucleoside with a pyrimido[4,5-c]pyridazine-4-one base structure. The third metabolite is proposed to be the 5′-monophosphate of the β-anomer. A mechanism for the oxidation of TCN is presented, but the exact biological oxidant involved remains unclear.

The tricyclic nucleoside 3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphthylene (TCN, NSC-154020) (Fig. 1) was synthesized by chemical modification of the naturally occurring 7-deazapurine nucleoside antibiotic toyocamycin [1]. Due to its unusual structure and activity against certain experimental tumors *in vivo*,‡ TCN was selected for further development by the National Cancer Institute. Uptake and phosphorylation by cells have been shown to utilize the adenosine transport system and adenosine kinase [2, 3]. The resulting product, TCN 5′-monophosphate (5′-MP), accumulates intracellularly and is apparently the cytotoxic metabolite. Another metabolite, putatively also a phosphate, was detected in varying amounts in extracts of H.Ep.-2 cells in one of the studies [3].

While studying the kinetics of TCN uptake and metabolism by preserved human erythrocytes, we detected several metabolites in addition to TCN 5′-MP. The structures of these new metabolites have been elucidated with the aid of mass spectrometry, high performance liquid chromatography (h.p.l.c.), u.v. and i.r. spectroscopy, and incubation experiments with catabolic enzymes and cultured wild type or variant Chinese hamster ovary (CHO) cells. In this paper, evidence to support the structure assignment of these metabolites and a mechanism for their formation are presented.

MATERIALS AND METHODS

Materials. [^{14}C-methyl]TCN ([^{14}C]TCN, 6.7 mCi/mmole) and unlabeled TCN were supplied by the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute. Radiolabeled TCN was 98 per cent radiochemically pure when analyzed with h.p.l.c. (see below). After recrystallization in hot methanol, unlabeled TCN was 98 per cent chromatographically pure. PCS, a phase combining scintillation mixture, was obtained from Amersham, Arlington Heights, IL. Alkaline phosphatase (EC 3.1.3.1) of calf intestine and 5′-nucleotidase (EC 3.1.3.5) of *Crotalus adamanteus* venom were from the Sigma Chemical Co, St. Louis, MO. Human erythrocytes, preserved 5–40 days in citrate phosphate-dextrose, were obtained from The Institute of Hemotherapy, Houston, TX.

High performance liquid chromatography. A Waters model ALC-204 liquid chromatograph (Waters Associates, Milford, MA), equipped with two model 6000A pumps, a U6K injector and a model 660 solvent programmer, was used for all analyses. The detector was a Varian Vari-Chrom (Varian Associates, Palo Alto, CA). Peaks were

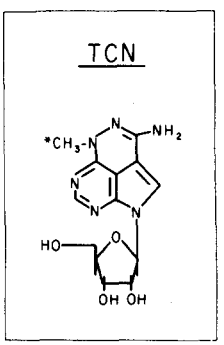

Fig. 1. Structure of NSC-154020, with the star indicating the position of the [^{14}C]-label.

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‡ Unpublished screening data, Drug Development Branch, Division of Cancer Treatment, National Cancer Institute.

electronically integrated with a Varian CDS-111 integrator and printed out on a Varian model 9176 recorder. Analytical and preparative separations were achieved on 3.9 mm × 30 cm and 7.8 mm × 30 cm μBondapak C_{18} columns (Waters Associates). For analytical separations a procedure previously developed by us (System 1 [4]) was used. Briefly, a 30-min concave gradient (curve 10 on the solvent programmer) from Buffer A (0.06 M K_2HPO_4 + 0.04 M KH_2PO_4, adjusted to pH 6.0 with H_3PO_4) to Buffer B (0.06 M K_2HPO_4 + 0.04 M KH_2PO_4 in 25% methanol, adjusted to pH 6.0 before methanol addition) was run at 1 ml/min. A faster method (System 2) used the same concave gradient programmed over 20 min from 20% Buffer B to 100% Buffer B at 2 ml/min. For desalting and purification, metabolites collected from the analytical column were concentrated *in vacuo* and applied to the preparative column. The mobile phase was 25% aqueous methanol at 3 ml/min (System 3). Some metabolites were recycled several times if necessary for improved chromatographic resolution. The absorbance of all effluent was monitored at 292 nm, the absorbance maximum of TCN. One metabolite could not be desalted with preparative h.p.l.c., and therefore was partially desalted by mixing with charcoal, washing with water, and eluting with ethanol–NH_4OH–water (65:2:33, by vol.).

Erythrocyte studies. Erythrocytes were washed three times with 5 vol. of phosphate-buffered saline (PBS, 8.1 g NaCl, 0.22 g KCl, 1.14 g Na_2HPO_4, and 0.27 g KH_2PO_4 per liter, pH 7.4) and centrifuged at 12,000 *g* for 10 min at 4° in a Sorvall RC2-B refrigerated centrifuge. The cell pellet was suspended in PBS such that erythrocytes were 40% by volume. Before TCN or metabolite administration, the cells were incubated for 10 min in a 37° water bath with constant shaking at 120 cycles/min. Incubations were followed by centrifugation and two washings with 3 vol. of cold PBS. The cell pellet was extracted for 5 min on ice with 2.5 vol. of 0.56 N perchloric acid (PCA). The insoluble material was removed by centrifugation, and the supernatant fraction was collected and neutralized with 10 N KOH. The $KClO_4$ was discarded after centrifugation and the resulting supernatant fraction was analyzed directly.

CHO studies. CHO cells, grown as monolayers in 75 cm^2 flasks (Lux Scientific Corp., Newbury Park, CA), were maintained in exponential growth at 37° in McCoy's Modified 5a medium (Grand Island Biological Co., Grand Island, NY) containing penicillin and streptomycin and supplemented with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA) that had been dialyzed against 40 vol. of PBS over several days in our laboratory. The atmosphere was 5% CO_2 in humid air. After enumeration with a model ZBI electronic particle counter (Coulter Electronics, Inc., Hialeah, FL), 2×10^6 cells in 10 ml of medium were placed in each flask. The next day, the medium was replaced with 10 ml of fresh medium containing the compound to be tested. After incubation, the cells were harvested with trypsin and collected by centrifugation. After washing with 3 ml of cold PBS, the cell pellet was suspended in 0.6 ml of water and extracted for 5 min on ice with 0.4 ml of 1.0 N PCA. The supernatant fraction was removed after centrifugation and combined with that obtained from washing the insoluble matter with 0.5 ml of 0.4 N PCA. The combined extracts were finally neutralized with 10 N KOH, centrifuged to remove the $KClO_4$, and analyzed directly.

Chemical oxidation of TCN. Crystalline TCN was suspended in 30% aqueous hydrogen peroxide and incubated for 16 hr at 37° with constant shaking at 120 cycles/min. The mixture was filtered through Whatman No. 1 paper to remove residual TCN. TCN-related products were separated from the hydrogen peroxide by chromatographing on a 20 cm × 1.5 cm column of XAD-2 resin (Rohm & Haas, Philadelphia, PA). After the column was washed with 300 ml of water, the adsorbed compounds were eluted with 300 ml of methanol, evaporated to dryness under reduced pressure, and purified with preparative h.p.l.c.

Infrared and ultraviolet spectroscopy. Infrared spectra from individual compounds incorporated into KBr pellets were recorded with a Perkin–Elmer model 727B i.r. spectrophotometer. Ultraviolet spectra were obtained from neutral aqueous solutions with a Cary 14 recording spectrophotometer.

Mass spectrometry. Mass spectral data were obtained from a Finnigan model 3300F GC–MS system interfaced with an Incos 2300 data system. Samples were introduced either by gas chromatographic or solid probe inlet. Samples introduced by the gas chromatographic inlet were chromatographed on a column (1.5 m × 2 mm) packed with 3% OV-17 on 90/100 Anakrom Q. Mass spectra were obtained by electron impact ionization at 70 eV, scanning the selected mass range every 2 sec for g.c. samples and every 4 sec for solid probe samples.

Trimethylsilylation. Small samples (50–200 μg) were trimethylsilylated by adding 100 μl of pyridine and 10 μl of *N,O*-bis(trimethylsilyl) acetamide, followed by heating at 80° for 15 min. Deuteriotrimethylsilyl derivatives were prepared by substituting 10 μl of d_{18}-*N,O*-bis(trimethylsilyl) acetamide in the same procedure. Two microliters of the resulting solution was transferred to a capillary sample cup and inserted into the mass spectrometer using the solid probe insertion.

Permethylation. Samples (50–500 μg) were dissolved in 100 μl of dry dimethylsulfoxide (DMSO), and 100 μl of a 1.0 M solution of sodium methylsulfinylmethide in DMSO [5] was added, followed by 20 μl of methyl iodide or d_3-methyl iodide. The reaction mixture was quenched by the addition of 1.5 ml of water. The permethylated product was extracted into 0.3 ml of methylene chloride which was washed twice with 1.5 ml of water, separated, and evaporated to dryness. The derivatives were introduced into the mass spectrometer by either direct probe or the gas chromatographic inlet.

Radiochemical assay. Radioactivity was determined with a Packard model 2650 liquid scintillation spectrometer with an automatic external standard for correction of quenching. Aqueous samples (0.2 to 1.0 ml) were mixed with 11 ml of PCS before determinations. When fractionating cell extracts by h.p.l.c., each fraction was collected for 30 sec, mixed with PCS, and counted directly.

Enzyme studies. Assays with alkaline phosphatase

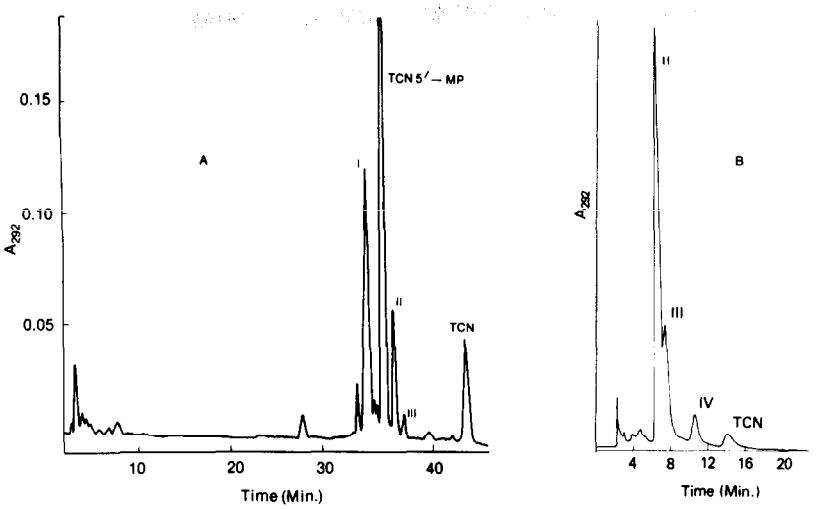

Fig. 2. Chromatographic (h.p.l.c.) separation of TCN metabolites from erythrocytes using System 1 [4] (A) and of hydrogen peroxide-generated TCN products using System 3 (see Materials and Methods) (B).

were carried out in 0.05 M Tris buffer (pH 8.6) containing 5.0 mM $MgCl_2$, and those with 5′-nucleotidase were carried out as described previously [6].

RESULTS

Erythrocyte studies. After incubation of TCN with human erythrocytes, 50 μl of the PCA soluble fraction was chromatographed to give the separation shown in Fig. 2A. In repeated experiments peaks I–III were always present. When the incubation was carried out with [^{14}C]TCN, all of these components were radioactive. With preserved erythrocytes, the concentrations of components II and III were a maximum at 5–15 min after 1:0 M TCN administration and, then, declined slightly or remained constant as the incubation proceeded for 2 hr. The kinetics of formation of I were similar to those of TCN 5′-MP, both of which increased in concentration throughout the incubation period. The combined concentrations of metabolites I, II and III after a given period of incubation were variable and depended primarily upon the age of the preserved blood; smaller amounts of all three resulted when the erythrocytes from freshly drawn blood were used, whereas the largest amounts were produced when the blood had been preserved for 40 days or more. The amount of I, relative to II and III, decreased with increasing blood storage time, as did TCN 5′-MP relative to TCN. Incubation of erythrocytes with TCN at 50 μM or below caused more than 90 per cent of the intracellular radioactivity to appear as I or TCN 5′-MP. Under these conditions TCN 5′-MP formation was favored with both freshly drawn and preserved erythrocytes, with relatively more I formed with preserved cells.

Spectrometric analysis. From our studies of [^{14}C]TCN metabolism by human erythrocytes, it readily became apparent that the ratios of absorbance at 292 nm to dpm of I, II or III were much higher than that of TCN or TCN 5′-MP. The observation suggested that the base portion of TCN had been modified. After isolation and purification of II and III by h.p.l.c., a u.v. spectrum of each was obtained and compared with that of TCN (Fig. 3). The metabolites had virtually identical u.v. spectra, with absorbance maxima at 235, 284 and 360 nm. Metabolite I, which eluted near the solvent front with preparative h.p.l.c. and, therefore, had to be desalted with charcoal, gave a less defined u.v. spectrum that was generally similar to that of II. The i.r. spectrum of II (not shown), exhibited strong absorbance at 1600 cm^{-1}, indicating the presence of a carbonyl group, which is absent with TCN. An insufficient amount of III was obtained for an i.r. spectrum.

Chemical oxidation of TCN. The presence of a carbonyl in II suggested that TCN may be oxidized by human erythrocytes. In an attempt to reproduce this oxidation, TCN was reacted with aqueous hydrogen peroxide. After removal of hydrogen peroxide and lyophilization, the reaction products were chromatograhically separated using System 3 (Fig. 2B). In repeated experiments, the amount of II

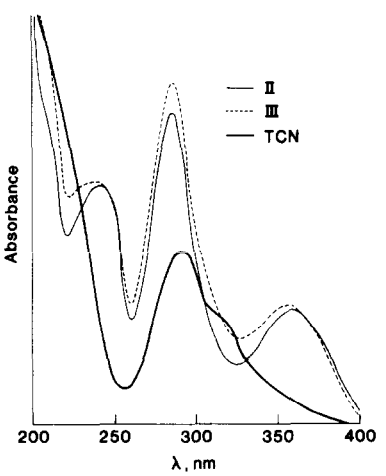

Fig. 3. Ultraviolet spectra of II, III and TCN in PBS (pH 7.4).

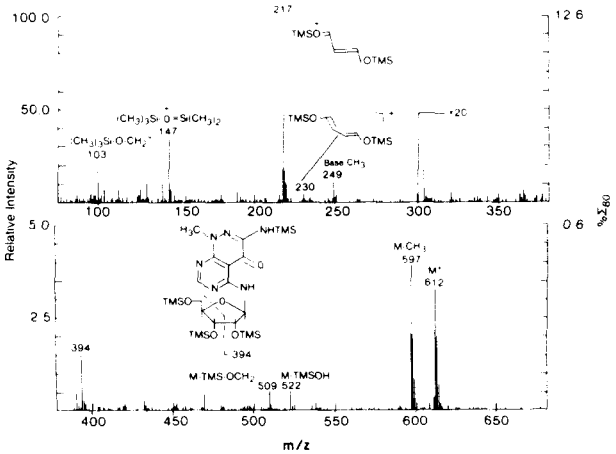

Fig. 4. Mass spectrum and major mass spectral fragment ions of the trimethylsilyl (TMS) derivative of II. The corresponding derivative of III gave an identical spectrum.

produced was always much greater than III. In addition to II and III, a new component, IV, was produced. All were radioactive when [^{14}C]TCN was used. That products II and III from the hydrogen peroxide oxidation are identical to those produced by erythrocytes is supported by their (1) h.p.l.c. retention times in three solvent systems; (2) u.v. spectra, and (3) mass spectra (see below). In addition, component IV had a u.v. spectrum similar to those of II and III.

Mass spectral analysis. Mass spectra of the trimethylsilylated derivatives of II and III were identical, exhibiting apparent molecular ions at *m/z* 612 and fragment ions at *m/z* 103, 147, 217, 394 and 597 (Fig. 4). The *m/z* values of these molecular ions, combined with those for the corresponding d_9-trimethylsilyl derivatives (at *m/z* 648), indicate that four trimethylsilyl groups had been added and that the underivatized molecular weights of II and III were 324, four mass units greater than TCN. These spectra also show that the ribose group was unchanged but do not indicate how the base portion had been modified.

The mass spectrum of underivatized IV (Fig. 5) shows a molecular ion at *m/z* 192, consistent with the molecular weight of the aglycone of II and III. Fragment ions at *m/z* 164, 150, 122, 95 and 68 correspond to losses of hydrogen cyanide, cyanamide, and carbon monoxide, indicative of an aromatic nitrogen heterocycle possessing a carbonyl.

Permethylation of II and III gave products with molecular ions at *m/z* 394. This indicates that five methyl groups were present, presumably three on the ribose and two on the primary amine. However, permethylation of the aglycone, IV, using methyl iodide and d_3-methyl iodide, gave products whose spectra exhibit molecular ions at *m/z* 248 and 260 and correspond to the addition of four methyl groups. That four methyl groups were added instead of the expected three suggests that the ribose of II and III was bonded to an exocyclic secondary amine which became a second primary amine in the aglycone. The accumulated mass spectral data presented here, and their interpretation, support the pyrimido[4,5-c]pyridazine-4-one structure proposed for these metabolites.

Metabolism of II, III and IV. Products II, III and IV from the hydrogen peroxide oxidation of TCN and [^{14}C]TCN were isolated using preparative h.p.l.c., with II and III requiring recycling of the effluent three times before each was at least 95 per cent free of the other. Unlabeled II, III and IV were each incubated for 1 hr with erythrocytes, while 2–4 × 10^6 dpm of each of labeled II, III and IV was incubated for 4 hr with CHO cells. Radioactive components in chromatograms of CHO cell extracts corresponded only to u.v.-absorbing components in chromatograms of erythrocyte extracts. Typical chromatograms of erythrocyte extracts are shown in Fig. 6. Neither III nor IV was metabolized, while II was converted exclusively to I. I, generated from II in erythrocytes, was collected, concentrated *in vacuo*, and partially desalted with charcoal. After

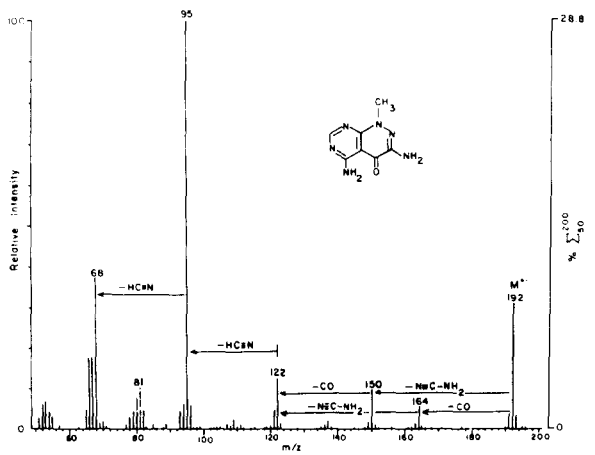

Fig. 5. Mass spectrum of underivatized IV.

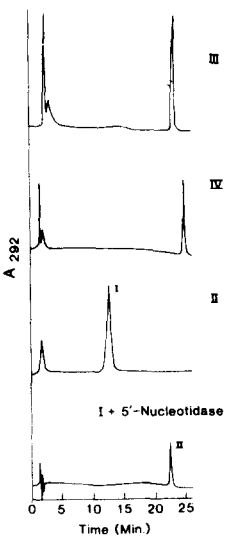

Fig. 6. Chromatographic separations of PCA extracts from incubations of (from top) III, IV and II with erythrocytes using System 2 (see Materials and Methods). Neither III nor IV was metabolized. II was metabolized exclusively to I, which was converted back to II by 5′-nucleotidase.

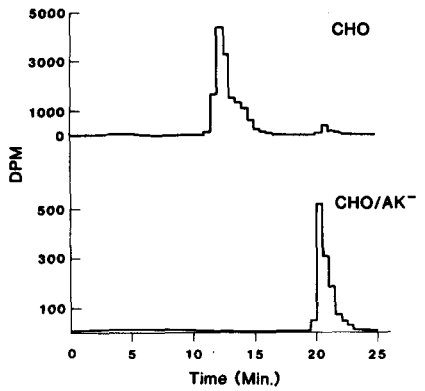

Fig. 7. Radiochromatogram of the PCA extracts from incubation of II with CHO or CHO/AK⁻ cells using System 2. With CHO cells, II is converted to I which accumulates intracellularly. The amounts of unchanged II accumulated within each cell line are approximately equal (note the difference in the scale).

drying and redissolving in water, I was incubated for 30 min with either 1 unit of 5′-nucleotidase or 1 unit of alkaline phosphatase. The results were identical. A chromatogram of the PCA extract of the 5′-nucleotidase experiment is shown in the lower panel of Fig. 6. Since each enzyme converted I to II, I is most likely the 5′-MP of II. The enzyme responsible for phosphorylation of II is probably adenosine kinase (EC 2.7.1.20), since a variant CHO cell line deficient in this enzyme activity (CHO/AK⁻), which fails to phosphorylate TCN, also failed to metabolize II (Fig. 7). Because di- and triphosphates elute much earlier than monophosphates in our h.p.l.c. system [4], and no early eluting radioactivity was observed, I, as with TCN 5′-MP, appears not to be a substrate of any of the monophosphate kinases.

DISCUSSION

We have presented evidence concerning the structures of three TCN metabolites produced *in vitro*. Results which indicate that two of the metabolites (II and III) are anomers of a bicyclic ribonucleoside include their identical u.v. mass spectra and their chromatographic resolvability. They do not interconvert when incubated with cells *in vitro* (Fig. 6).

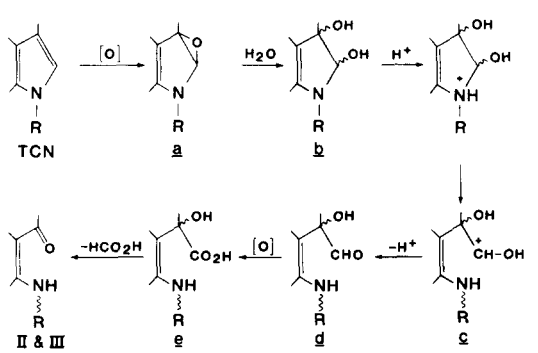

Fig. 8. Proposed mechanism for the oxidation of TCN.

The formation of the anomeric pair of metabolites II and III from TCN may involve the following possible sequence of reactions (Fig. 8). The epoxide (a) resulting from the oxidation of the 2,2′-double bond undergoes hydration to give the diol (b). Initial protonation of N^1 in (b) followed by ring opening leads to the formation of the protonated α-hydroxy aldehyde (c). Like in other ring-opening reactions of purine ribonucleosides, it is at this step that anomerization at the ribosyl C-1^1 occurs [7, 8]. Upon losing the proton from (c), the α-hydroxy aldehyde (d) is further oxidized to the α-hydroxy carboxylic acid(e), which affords II and III through the loss of a molecule of formic acid.

Metabolite II is assigned the β-D-ribofuranosyl configuration, since it was produced in higher quantities and was further metabolized by human erythrocytes and wild type CHO cells to metabolite I. A variant CHO cell line deficient in adenosine kinase activity failed to convert II to I. Most probably, therefore, adenosine kinase is the mediating enzyme. Since metabolite III was not metabolized *in vitro* and its mass spectrum is identical to that of II, it is assigned the α-D-ribofuranosyl configuration. Metabolite I is postulated to be the 5′-MP of II, since it was retained longer than most ribonucleoside di- and triphosphates with our h.p.l.c. system. Moreover, it was readily converted to II with either 5′-nucleotidase or alkaline phosphatase. Using the h.p.l.c. system described by Bennett *et al.* [3]. I eluted with a retention time similar to their unknown phosphate, while TCN co-eluted with metabolites II and III. The ribose moiety of II or III, as with TCN, was not cleaved by cells *in vitro*. When TCN was chemically oxidized with hydrogen peroxide, the aglycone of II or III (but apparently not that of TCN) was produced. The release of bases from nucleosides and nucleotides upon reaction with hydrogen peroxide has been observed by others [9]. This compound (IV) was not metabolized by either erythrocytes or CHO cells. To summarize, TCN *in vitro* can be converted to TCN 5′-MP by adenosine kinase or oxidized to II and III. II is metabolized to I, apparently also by adenosine kinase. A direct conversion of TCN 5′-MP to I has not been demonstrated.

Our observations that the oxidation of TCN by erythrocytes increased with preservation time, that exponentially growing CHO cells oxidized very little TCN, and that incubation of TCN with hydrogen peroxide produced metabolites seen *in vitro* suggest that the oxidation of TCN was brought about by intracellular hydrogen peroxide. However, this does not appear to be the exclusive mechanism, because we have observed that 40% suspensions of erythrocytes that have been preserved for 30–40 days were much more efficient at oxidizing TCN than 30% (8.8 M) hydrogen peroxide (data not shown). Further, TCN was oxidized to II and III by rat liver microsomes. Like other hydrophobic molecules, TCN is potentially a substrate for aniline hydroxylase activity of erythrocytes and for the cytochrome P-450-dependent mixed function oxygenases of hepatic microsomes, which are biochemically similar [10–12]. TCN metabolism *in vivo* and the biochemical effects of the metabolites remain to be studied.

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