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Mechanism of Action of 1-β-D-Ribofuranosyl-1,2,4-Triazole-3-Carboxamide (Virazole), A New Broad-Spectrum Antiviral Agent

(purine and pyrimidine precursors/IMP dehydrogenase/Virazole 5′-phosphate/guanase/ 1,2,4-triazole-3-carboxamide)

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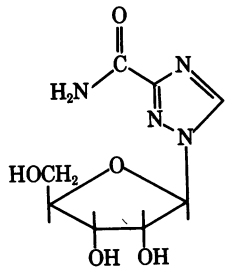
ABSTRACT **The antiviral activity of the synthetic nucleoside, Virazole (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), against measles virus in Vero cell cultures was substantially reversed by xanthosine, guanosine, and to a slightly lesser extent by inosine. Virazole 5′-phosphate was subsequently found to be a potent competitive inhibitor of inosine 5′-phosphate dehydrogenase (IMP:NAD$^+$ oxidoreductase, EC 1.2.1.14) isolated from *Escherichia coli* (K_m = 1.8 × 10^{-5} M) with a K_i of 2.7 × 10^{-7} M. Guanosine 5′-phosphate (GMP) was a competitive inhibitor of this enzyme with a K_i of 7.7 × 10^{-6} M. Virazole 5′-phosphate was similarly active against IMP dehydrogenase isolated from Ehrlich ascites tumor cells, with a K_i of 2.5 × 10^{-7} M. The K_m for this enzyme was 1.8 × 10^{-5} M, and the K_i for GMP was 2.2 × 10^{-4} M. These results suggest that the antiviral activity of Virazole might be due to the inhibition of GMP biosynthesis in the infected cell at the step involving the conversion of IMP to xanthosine 5′-phosphate. This inhibition would consequently result in inhibition of the synthesis of vital viral nucleic acid.**

The synthesis and development of a broad spectrum antiviral agent has been a challenging task because of the intimate association of virus replication and biochemical processes of the host cell. In addition, such an agent must inhibit a step in the process of virus replication that is common to a wide variety of RNA and DNA viruses (1). The synthesis and broad spectrum antiviral activity of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (I, Virazole) have recently been reported (1,2).



I

Studies concerning the effect of Virazole on the uptake of radioactive precursors into the nucleic acid fraction of RK-13 cell cultures infected either with herpes type 1 (DNA) virus (3) or vesicular stomatitis virus (4) have demonstrated Virazole to be a potent inhibitor of both RNA and DNA synthesis in these cells. This inhibition was accompanied by almost complete cessation of the production of infectious virus particles. These results suggested that the inhibition of virus replication might occur at an early step in the virus infective process that leads to the *de novo* synthesis of viral nucleic acid. The present report describes studies directed toward determination of the primary site of inhibition of the viral nucleic acid synthesis.

Abbreviations: AICA, 4-Aminoimidazole-5-carboxamide; AICAR, 5-Amino-1-β-D-ribofuranosylimidazole-4-carboxamide.

MATERIALS AND METHODS

1-β-D-Ribofuranosyl-1,2,4-triazole-3-carboxamide was synthesized by Dr. J. T. Witkowski (International Chemical and Nuclear Corp. Nucleic Acid Research Institute, Irvine, Calif.) (1). Virazole 5′-phosphate was prepared by phosphorylation of the unprotected nucleoside with phosphoryl chloride in trimethylphosphate (5) as follows. A solution of 3.0 ml of phosphoryl chloride (32.6 mmol) in 60 ml of trimethylphosphate was cooled to 0°, and 2.44 g of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide (10.0 mmol) was added with stirring. The mixture was protected from moisture and was stirred for 5–8 hr at 0° until phosphorylation was complete, as shown by thin-layer chromatography of a hydrolyzed aliquot on silica gel with acetonitrile–0.1 N aqueous ammonium chloride (7:3) as developer. The plate was visualized with a spray of methanol–sulfuric acid–anisaldehyde (18:2:1) and then with heating at 110°. After the phosphorylation was complete, the solution was poured into 100 ml of water at 0°, and the pH was adjusted to 2.0 with 2 N sodium hydroxide. The solution was extracted with two 150-ml portions of chloroform to remove trimethylphosphate. The aqueous solution was applied to a column of activated charcoal (60 g), and the column was washed with water until the eluate was salt free. The nucleotide was eluted with a solution of ethanol–water–concentrated ammonia (10:10:1). The solvent was removed under reduced pressure, and the residue was dissolved in dilute ammonia. Ethanol was added to the solution, and the resulting precipitate was collected. The product was purified by reprecipitation of the aqueous solution with ethanol to give the ammonium salt of Virazole 5′-phosphate as a monohydrate (1.90 g, 53%). *Anal.* Calcd for $C_8H_{16}N_5O_8P \cdot H_2O$: C, 26.74; H, 5.05; N, 19.50. Found; C, 26.78; H, 5.23; N, 19.68.

Virazole 5′-phosphate was obtained by passage of a solution of the ammonium salt through a column of Bio-Rad AG

50W-X2 (H). Elution with water afforded the nucleotide. The solvent was removed under reduced pressure, and the product was crystallized from ethanol to provide pure Virazole 5′-phosphate: mp 162–164° dec; $[\alpha]_D^{25°} - 26.9°$ (*c* 1.0, H_2O). *Anal.* Calcd for $C_8H_{13}N_4O_8P$: C, 29.62; H, 4.04; N, 17.28. Found: C, 29.78; H, 4.18; N, 17.11.

The following nucleosides and nucleotides were obtained from ICN Nutritional Biochemicals Corp.: adenosine, deoxyadenosine, guanosine, deoxyguanosine, guanosine 5′-phosphate (GMP), cytidine, uridine, thymidine, 5-amino-1-β-D-ribofuranosylimidazole-4-carboxamide (AICAR), AICAR 5′-phosphate, xanthosine, xanthosine 5′-phosphate (XMP), orotidine, hypoxanthine, inosine, and inosine 5′-phosphate (IMP). [^{3}H]Virazole (46.5 Ci/mol) was obtained from ICN Chemical and Radioisotope Division. *Escherichia coli* alkaline phosphatase (chromatographically pure) was obtained from Worthington Biochemical Corp., Freehold, N.J.

Reversal Studies. The *in vitro* antiviral activity of Virazole and the reversal of this activity by nucleosides and nucleotides were studied in Vero cells infected with measles virus in the following manner. 60-mm Culture plates containing Vero cells grown to confluency were infected with 0.2 ml of measles virus containing about 250 plaque-forming units. After absorption of the virus for 90 min, 5 ml of an agar overlay was added, containing 0–500 μg of Virazole per ml, with and without 200 μg of the reversal agent per ml. The agar overlay consisted of Eagle's minimum essential medium supplemented with 0.1% $NaHCO_3$, 2% fetal-bovine serum, and 0.9% agar. After incubation at 37° for 4 days in a humidified CO_2 incubator, the cells were nourished with 2 ml of agar overlay containing 0.01% neutral red, and the number of plaque-forming units was determined.

Guanine Deaminase (Guanine Aminohydrolase, EC 3.5.4.3). Guanine deaminase was prepared from rat liver by the procedure of Kalckar (6). The 40–60% ammonium sulfate fraction was used as the enzyme source. The enzyme was assayed by the method of Roush and Norris (7). This method measures the decrease in absorbance at 245 nm due to the conversion of guanine to xanthine. The initial velocity is expressed as the mol of xanthine formed/min.

IMP Dehydrogenase (IMP:NAD$^+$ Oxidoreductase, EC 1.2.1.14)·E. coli B. 50 g of *E. coli* B were washed in 500 ml of buffer containing 10 mM Tris·HCl (pH 7.8), 10 mM $MgAc_2$, 60 mM KCl, and 7 mM 2-mercaptoethanol. The cells were suspended in 100 ml of the same buffer containing 2 mM 2-mercaptoethanol. They were broken in a French pressure cell at 12,500 psi and treated with 2 μg/ml of DNase for 1 hr at 0°. The lysate was centrifuged at 30,000 × *g* for 30 min. The supernatant (S-30) was centrifuged again at 144,000 × *g* for 90 min. This supernatant (S-144) was treated with 2% protamine sulfate (4 ml/100 ml of S-144) for 45 min at 0°, and the precipitate was removed by centrifugation. The supernatant was then treated with ammonium sulfate to 40% saturation, and the pellet from this fraction was dissolved in 5 mM Tris·HCl (pH 7.8)–7 mM 2-mercaptoethanol. This fraction was stored at −70°.

IMP dehydrogenase was assayed by the spectrophotometric method outlined by Magasanik (8). The reaction was performed in a quartz cell with a 1-cm path containing 100 μmol of Tris·HCl (pH 8.5), 5 μmol of reduced glutathione, 100 μmol of KCl, 2.5 μmol of NAD$^+$, 0.02–0.1 ml of enzyme, and disodium IMP at the indicated concentrations, in a total volume of 3.0 ml. The reactions were performed at 25° and started by the rapid addition of NAD$^+$ or IMP. They were followed for 5–10 min by measurement of the increase in absorbance at 340 nm due to the oxidation of NAD$^+$ and were measured against a blank containing no added IMP. The initial velocity of the reaction was expressed as the change in absorbance per min during the first 5 min of the reaction.

IMP Dehydrogenase from Ehrlich Ascites Tumors. A preparation of IMP dehydrogenase was obtained from Ehrlich ascites tumor cells by a slight modification of the procedure of Anderson and Sartorelli (9). The ascites fluid from 50 mice was pooled, and the cells were removed by centrifugation and washed by suspending three times in an equal volume of 0.9% NaCl. The cells were broken by sonication for 30 sec in 100 ml of 0.1 M Tris·HCl (pH 8.0) with a Branson sonifier. The suspension was clarified by centrifugation at 10,000 × *g* for 20 min, and then centrifuged at 105,000 × *g* for 2 hr. This supernatant (S-105) was treated with 2% protamine sulfate (4 ml/100 ml of S-105) for 30 min at 0°, and the precipitate was removed by centrifugation. The protamine sulfate supernatant was treated with ammonium sulfate, and the material precipitating between 20 and 40% saturation was removed and dissolved in 0.1 M Tris·HCl (pH 8). This sample was dialyzed for 2 hr against 1 liter of 0.01 M Tris·HCl and then stored frozen at −70° before use.

The enzyme was again assayed by the spectrophotometric method outlined previously. Reactions contained in a final volume of 2.5 ml: 250 μmol of Tris·HCl (pH 8.0), 250 μmol of KCl, 1.25 μmol of NAD$^+$, 0.05 ml of enzyme, and disodium IMP at the indicated concentrations. The reaction was run at 37° and was started by the addition of IMP. Initial rates were again followed for 15–20 min and expressed as the change in absorbance at 340 nm per min.

In Vivo Metabolism of [^{3}H]Virazole in Mouse Liver. Two mice were administered 200 μCi each of [^{3}H]Virazole in 0.3 ml of saline solution by oral gavage. After 2 hr, the animals were killed. The livers were removed and washed with saline solution. The two livers were pooled and minced in 5 ml of cold 5% perchloric acid and homogenized in a Kontes glass homogenizer. The perchloric acid-soluble fraction was neutralized to pH 7 with KOH, and the insoluble material was centrifuged down. The supernatant was chromatographed on previously coated, cellulose F thin-layer chromatography plates (Brinkmann Instruments, Westbury, N.Y.) in isopropanol–ammonia–H_2O (7:1:2). Radioactive components were detected by dividing the plates into 0.5 × 1 cm sections. The sections were scraped into scintillation vials and counted in toluene-based scintillation fluid (Tolu-scint, ICN Chemicals Radioisotopes, Irvine, Calif.).

Phosphomonoesterase Digestion of Neutralized Mouse-Liver Extracts. Aliquots of the neutralized liver extracts from mice treated with [^{3}H]Virazole were treated with *E. coli* bacterial alkaline phosphatase (orthophosphoric monoester phosphohydrolase). This enzyme removes terminal monesterified phosphate groups from both ribo- and deoxyribonucleotides. 100 μl of the liver extract was incubated together with 25 μl of 1 M ammonium formate (pH 9.2) and 5 μl of alkaline phosphatase (1.8 units) for 90 min at 38°. An aliquot of this reaction was then chromatographed as described above.

TABLE 1. *Effect of orotodine, inosine, xanthosine, and guanosine on anti-measles virus activity of Virazole in Vero cell cultures**

Type of compound added	Total plaque-forming units at Virazole concentration (μg/ml)			
	500	100	20	0
Virazole	0	3	176	213
Virazole + orotidine	0	16	191	210
Virazole + inosine	0	125	166	177
Virazole + xanthosine	53	211	215	215
Virazole + guanosine	29	168	196	202

* Orotidine, inosine, xanthosine, or guanosine were incorporated in the agar overlay at a concentration of 200 μg/ml, along with 0, 20, 100, or 500 μg/ml of Virazole. The agar overlay was added onto the cultures after 1–1.5 hr of virus adsorption.

RESULTS

The inhibition by Virazole of measles virus replication, as measured by plaque formation in Vero cell cultures, was substantially reversed by guanosine or xanthosine and to a slightly lesser extent by inosine (Table 1). Adenosine, deoxyadenosine, deoxyguanosine, cytidine, uridine, thymidine, orotidine, AICAR, and AICAR 5′-phosphate were all essentially inactive as reversal agents. This reversal of the antiviral activity of Virazole by guanosine and related compounds suggested that Virazole interfered with some step in the GMP biosynthetic pathway. Moreover, it suggested that Virazole might be recognized as an analog of guanosine or of a guanosine precursor. Indeed, the enzyme guanine aminohydrolase, which converts guanine to xanthine, was purified from rat liver and found in our laboratories to be inhibited by the base of Virazole, 1,2,4-triazole-3-carboxamide (10). The kinetics of the inhibition are nearly competitive, but of the mixed type. The K_i calculated on the basis of purely competitive kinetics was about 4×10^{-5} M (Fig. 1). 4-Aminoimidazole-5-carboxamide (AICA), which is a competitive inhibitor of this enzyme (11), had a similar K_i of 3.7×10^{-5} M in our assay.

The effective reversal of Virazole's antiviral activity by xanthosine and guanosine, compared with the less effective reversal by inosine, drew particular attention to the step in the GMP biosynthetic pathway involving the conversion of IMP to XMP as a possible site of antiviral action. The

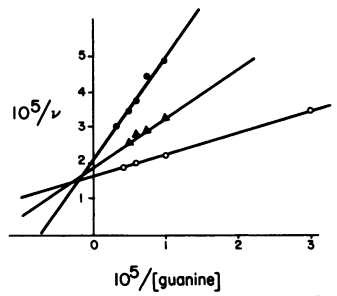


FIG. 1. Inhibition of guanine deaminase activity by 1,2,4-triazole-3-carboxamide (see *Methods*). Initial velocity was measured at 245 nm. The inhibitor concentrations were: no inhibitor (○); 1.5×10^{-5} M (▲); 1.9×10^{-5} M (●).

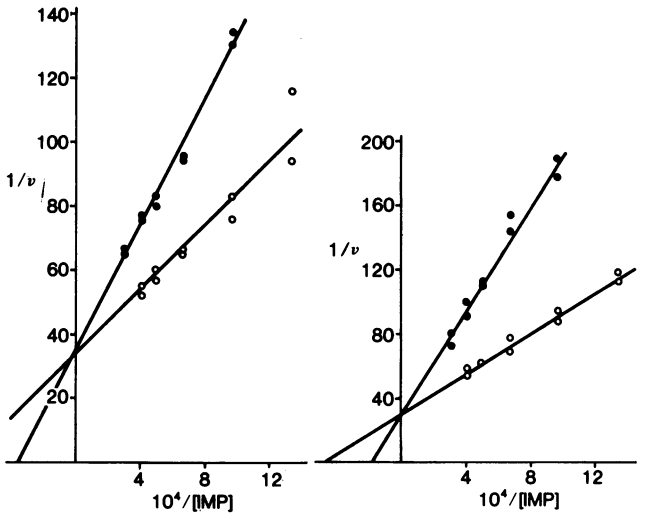


FIG. 2 (*left*). Inhibition of *E. coli* IMP dehydrogenase activity by Virazole 5′-phosphate (see *Methods*). Initial velocity was measured at 340 nm. No inhibitor (○); 1.7×10^{-7} M (●).

FIG. 3 (*right*). Inhibition of *E. coli* IMP dehydrogenase by GMP. Reaction conditions were the same as in Fig. 2. No inhibitor (○); 1.4×10^{-4} M (●).

enzyme involved in this step, IMP dehydrogenase, is readily purified from bacteria (12, 13), where it is present at much higher concentrations than in normal mammalian tissues (14). Thus, the *E. coli* enzyme provided a convenient source as a model for studying the effect of Virazole 5′-phosphate on this step in the biosynthesis of GMP. Virazole itself was examined and found not to be inhibitory to this enzyme at millimolar concentrations. However, Virazole 5′-phosphate was a powerful competitive inhibitor of the enzyme (Fig. 2), with a K_i of 2.7×10^{-7} M. It was 50–100 times more effective than GMP (Fig. 3), a known feedback inhibitor of this reaction with a K_i of 7.7×10^{-5} M in our assay. Similar data were obtained with the IMP dehydrogenase isolated from Ehrlich ascites cells (Figs. 4 and 5). This enzyme had the same K_m as the *E. coli* enzyme, 1.8×10^{-5} M. The respective K_i values obtained with the Ehrlich ascites enzyme were: Virazole 5′-phosphate, 2.5×10^{-7} M, and GMP, 2.2×10^{-4} M. These data indicate that the two enzymes are rather similar with regard to their interaction with Virazole 5′-phosphate, and that the more active bacterial enzyme could be used for any further studies with Virazole 5′-phosphate and its derivatives.

Evidence that Virazole can be converted to the 5′-phosphate *in vivo* was obtained by administration of radioactive [^{3}H]Virazole to mice. Neutralized acid extracts were prepared from mouse livers, a primary source of nucleoside kinase activity, 2 hr after oral administration of 200 μCi of [^{3}H]Virazole per mouse. 6 μCi, or about 3% of the administered radioactivity, was recovered in these extracts. Cellulose thin-layer chromatography of these extracts produced two radioactive components in about equal amounts (Fig. 6*a*); one component coincident with Virazole (Fig. 6*d*) and a second component near the origin. The slow component was eliminated by phosphomonoesterase treatment, indicating a phosphorylated species, with a concomitant increase in the amount of the fast component (Fig. 6*c*).

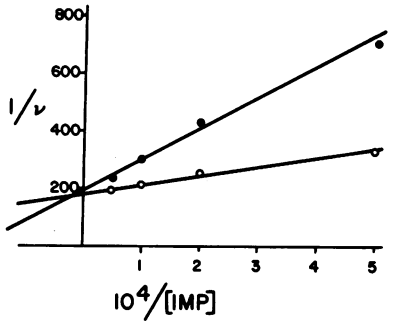


FIG. 4. Inhibition of Ehrlich ascites IMP dehydrogenase by Virazole 5′-phosphate. Initial velocities were measured at 340 nm. No inhibitor (○); 6.0×10^{-7} M (●).

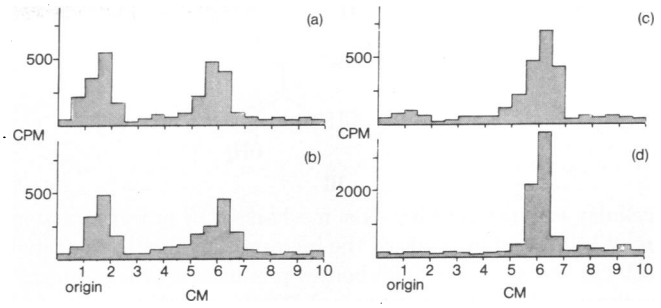
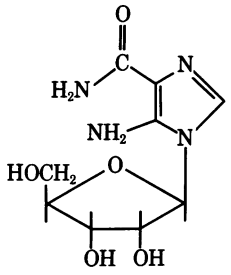


FIG. 6. Cellulose thin-layer chromatography of neutralized, acid-soluble, liver extracts from mice treated with [^{3}H]Virazole (see *Methods*). (*a*) No incubation; (*b*) 38°, 90 min; (*c*) 38°, 90 min with phosphomonoesterase; (d) [^{3}H]Virazole.

DISCUSSION

Virazole (**I**), has a structure that is apparently similar to the purine biosynthetic precursor AICAR (**II**); however, neither



II

AICAR nor its 5′-phosphate was able to reverse Virazole's antiviral activity. Indeed, AICAR 5′-phosphate was essentially inactive as an inhibitor of IMP dehydrogenase. Furthermore, the studies with guanine aminohydrolase indicate that the base moieties of Virazole and guanine are recognized as being similar to this enzyme, and in addition, x-ray crystallographic studies indicate that Virazole appears to have a structure in the crystal state that is similar to guanosine (15). The reversal of Virazole's antiviral activity by guanosine and guanosine precursors demonstrated here, together with the potent inhibition of IMP dehydrogenase by Virazole 5′-phosphate, suggest that the antiviral effect of Virazole may be a result of the inhibition of GMP synthesis at the step involving the conversion of IMP to XMP. The inhibition of IMP dehydrogenase by Virazole 5′-phosphate suggests that this compound is recognized enzymatically as an analog of IMP or perhaps GMP, rather than AICAR 5′-phosphate. This inhibition of IMP dehydrogenase could then result in a concomitant inhibition of viral RNA and/or DNA synthesis. Some preliminary results with RK-13 cells infected with herpes virus have also shown a reversal of the antiviral activity of Virazole by guanosine and xanthosine (unpublished results) in this DNA virus, suggesting that the mechanism of antiviral action of Virazole may be the same in both RNA and DNA virus infections.

In studies of the catalytic site of IMP dehydrogenase from *A. aerogenes* (16), Hampton described a mechanism for the binding of IMP involving the oxygen at carbon-6 and the nitrogen-1 position of the molecule, based on the higher affinity of this enzyme for IMP and GMP than for the ribonucleotide of unsubstituted purine. His proposed structure is illustrated in Fig. 7*a*. We envisage a similar interaction of the enzyme with the carboxamide group of Virazole 5′-phosphate (illustrated in Fig. 7*b*). The hydrogen that bonds to the keto group would then be supplied by some group from the enzyme, while the hydrogen on the amide nitrogen would bond to a proton acceptor on the enzyme. A consideration of the antiviral activities of several synthetic nucleosides related to Virazole (17) indicates that the structure–antiviral activity correlation of these compounds is consistent with the hydrogen bonding requirements outlined in Fig. 7*b*.

Inhibition of IMP dehydrogenase as a mechanism of antiviral action has been implicated for the antibiotic mycophenolic acid (**III**) (18), a compound also active against both RNA and DNA viruses *in vitro* and possessing antitumor activity (19, 20). The antiviral activity of **III** is also reversed by guanosine. The inhibition of a normal

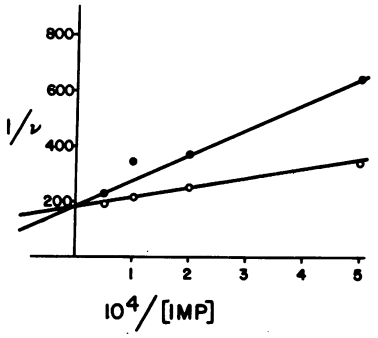


FIG 5. Inhibition of Ehrlich ascites IMP dehydrogenase activity by GMP. The reaction conditions were the same as in Fig. 4. No inhibitor (○); 4.0×10^{-4} M (●).

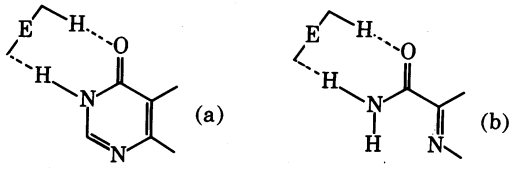
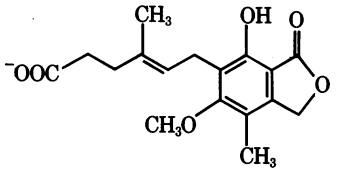


FIG. 7. Proposed interaction of IMP dehydrogenase (*E*) with (*a*) IMP, according to Hampton (16) and (*b*) with Virazole 5′-phosphate.



III

cellular enzyme activity as a mechanism of antiviral action may be viewed in terms of the increased need of the infected cell for certain of the metabolites produced by these enzyme activities. The synthesis of viral RNA and DNA in infected cells puts an increased demand on the cell for the overall synthesis of nucleotides. With herpes virus infections, for example, there is a particular increase in enzyme activities associated with GMP and cytidine 5′-phosphate (CMP) biosynthesis (21); thus inhibition of the GMP or CMP biosynthetic pathways should be particularly effective in preventing the synthesis of herpes viruses that contain a DNA with a very high (68%) G+C content (22). Indeed, Virazole is very effective against type 1 herpes simplex virus, both in cell culture and *in vivo* (1).

The inhibition of IMP dehydrogenase activity as a plausible mechanism for the antiviral action of Virazole *in vivo* is, of course, dependent on the conversion of this drug to the 5′-phosphate. The data presented here demonstrate that Virazole can be phosphorylated *in vivo*. In addition, some preliminary studies have shown Virazole to be a substrate for a nucleoside kinase isolated from rat liver. Other recent studies on the uptake of radioactive Virazole in mice by administration of subtoxic doses both intraperitoneally and orally have been reported. The recovery of both radioactivity and antiviral activity from serum indicated that concentrations of the drug may reach as high as 0.1–1 mM. At these drug concentrations, only a small fraction of Virazole would have to be converted to the 5′-phosphate to attain effective concentrations of the actual inhibitor (23).

We do not know whether Virazole is active because of selective inhibition of a viral-specific IMP dehydrogenase or whether it inhibits the IMP dehydrogenase of the host cell that may normally be stimulated upon virus infection. In either event, such inhibition of IMP dehydrogenase in the virus-infected cell could be lethal to the viral progeny, but may be of little consequence to the resting adult host cell. It is tempting to speculate that the inhibition of IMP dehydrogenase in the host cell at the time of infection is sufficient to inhibit the early viral RNA synthesis, which is necessary for the coding of vital viral-induced enzymes. The answers to these questions remain to be elucidated. It is clear, however, that Virazole should serve as a useful tool in future study of the molecular biology of mammalian virus infection.

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