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Use of Isotopically Chiral [4′-^{13}C]Penciclovir and ^{13}C NMR To Determine the Specificity and Absolute Configuration of Penciclovir Phosphate Esters Formed in HSV-1- and HSV-2-Infected Cells and by HSV-1-Encoded Thymidine Kinase

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ABSTRACT Penciclovir is a potent antiherpesvirus agent which is highly selective due to its phosphorylation only in virus infected cells. Phosphorylation of one of the hydroxymethyl groups of penciclovir (PCV) creates a chiral centre leading to the possible formation of (*R*)- and (*S*)-enantiomers. The absolute configuration and stereospecificity of the PCV-phosphates produced in cells infected with herpes simplex viruses types 1 and 2 (HSV-1 and HSV-2), as well as by HSV-1-encoded thymidine kinase, were determined using isotopically chiral [4′-^{13}C]PCV precursors and ^{13}C NMR spectroscopy of the isolated metabolites. The absolute configuration of penciclovir-triphosphate (PCV-TP) produced in HSV-1-infected cells was shown to be *S* with an enantiomeric purity of greater than 95%. However, in contrast to HSV-1-infected cells in which none of the (*R*) enantiomer was detected, about 10% of (*R*)-PCV-TP was produced in HSV-2-infected cells. Phosphorylation of PCV by HSV-1-encoded thymidine kinase was found to give 75% (*S*)- and 25% (*R*)-PCV-monophosphate. The proportion of the (*S*)-isomer appears to be amplified in the subsequent phosphorylations leading to the triphosphate.

KEY WORDS: chiral stereospecificity, phosphorylation, MRC-5 cells, herpesvirus

Penciclovir (Fig. 1, PCV) is a highly selective antiherpesvirus agent which is formed following oral administration of famciclovir (see preceding paper). In uninfected human cells, there is virtually no phosphorylation of penciclovir. In complete contrast, the herpesvirus-encoded thymidine kinase phosphorylates penciclovir to the monophosphate, which is then phosphorylated further to the di- and triphosphate by cellular enzymes.[1,2] Phosphorylation of one of the hydroxymethyl groups of PCV creates a chiral centre leading to the possible formation of (*R*)- and (*S*)-enantiomers. As the (*S*)-enantiomer (Fig. 1) is analogous to the natural nucleotide, it may be expected that this enantiomer would be formed preferentially.

In order to determine the stereospecificity and absolute configuration of the PCV phosphates produced, isotopically chiral PCV was synthesised with ^{13}C incorporation in one of the hydroxymethyl groups, namely compounds **1a** and **1b** (Fig. 1).[3] ^{13}C NMR spectroscopy of the PCV-phosphates isolated from the appropriate biological systems could then be used to determine whether the phosphate was adjacent to the ^{13}C label in the metabolites and thereby establish the stereospecificity of phosphorylation of PCV. Part of this work has been reported briefly.[4]

MATERIALS AND METHODS

Radiochemicals

[6-^{3}H]Thymidine (74 GBq/mmol) was obtained from Amersham International Plc., Little Chalfont, United Kingdom.

Preparation of [4′-^{13}C]PCV

The synthesis of [4′-^{13}C]PCV has been described previously.[3] Initially, racemic [4′-^{13}C]PCV was prepared. Compound **1a**, enantiomeric purity >99%, was prepared with unlabelled compound **1** by adding an equal amount of unlabelled intermediate during synthesis. Similarly, compound **1b**, enantiomeric purity 85%, was prepared with **1** but with the addition of three parts of unlabelled intermediate.

Penciclovir-Triphosphate (PCV-TP) Formation and Isolation

The methodology was similar to that described previously.[1,2] In brief, confluent MRC-5 cells (ca. 1×10^8) were

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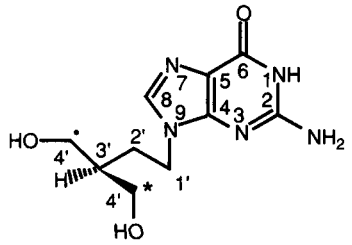
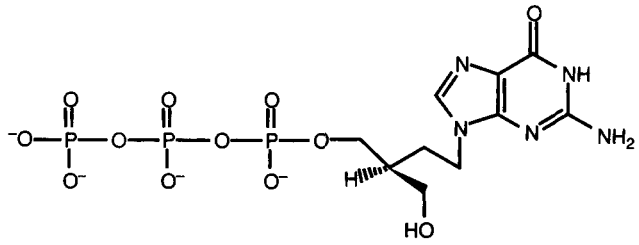


Penciclovir (PCV)

(1) • = ^{12}C, * = ^{12}C
(1a) • = ^{12}C, * = ^{13}C
(1b) • = ^{13}C, * = ^{12}C



(*S*)-Penciclovir-triphospate
[(*S*)-PCV-TP]

Fig. 1. Structures.

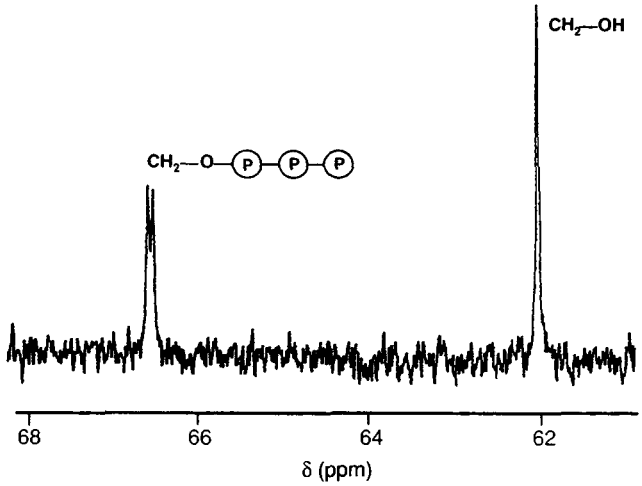


Fig. 2. ^{13}C NMR spectrum of penciclovir-triphosphate isolated after racemic [4′-^{13}C]penciclovir was incubated with HSV-1-infected MRC-5 cells.

infected with HSV-1 strain SC16 at 0.01 PFU/ml. At 24 h postinfection, cells were put into fresh medium containing 100 μ*M* [4′-^{13}C]PCV (**1a**) and incubated for a further 24 h. The intracellular phosphates were extracted with ethanol:water: phosphate buffered saline (PBS) (50:40:10) and purified by HPLC on a Waters NovaPak C_{18} column with 1 m*M* heptyltriethylammonium phosphate as ion-pairing agent in the elution buffer. The fraction corresponding to synthetic (racemic) PCV-TP was collected and the sample evaporated to dryness. Similarly, MRC-5 cells were infected with HSV-2 strain MS for 18 h, incubated with [4′-^{13}C]PCV (**1a**) for 18 h, and the phosphates extracted and isolated. In each case, the yield of [4′-^{13}C]PCV-TP was about 0.6 mg.

In comparable experiments, [4′-^{13}C]PCV (**1b**, enantiomeric purity 85%) was incubated with HSV-1- and HSV-2-infected MRC-5 cells and the corresponding PCV-TPs isolated.

Preparation of HSV-1-Encoded Thymidine Kinase (TK)

Confluent BHK21 TK^- cells were infected with HSV-1 SC16 at 3 PFU/cell. After 1 h, the cells were incubated in growth medium (Glasgow's MEM containing 10% newborn calf serum and 10% tryptose phosphate broth) for 18 h at 37°C. The cells were washed twice and harvested in cold PBS. The pelleted cells were resuspended in 25 m*M* Tris-HCl pH 7.8, 1 m*M* dithiothreitol, 10% (v/v) glycerol, and 0.6% (v/v) Nonidet P40. The following procedures were carried out at 0 to 5°C. The cell suspension was vortexed to rupture the cells, the homogenate centrifuged at 14,000 rpm for 5 min to remove cell debris, and the supernatant containing HSV-1 TK collected. The TK was precipitated by ammonium sulphate (fraction from 20% to 50%), redissolved in 25 m*M* Tris-HCl pH 7.8, 1 m*M* dithiothreitol, 10% (v/v) glycerol, desalted on a Sephadex-G25 PD10 column (Pharmacia Ltd., Milton Keynes, United Kingdom), and loaded onto a TK-affinity column prepared according to Lee et al.[5] HSV-1 TK was eluted from the column with 300 μ*M* Tris-HCl pH 7.8, 2 m*M* dithiothreitol, 10% (v/v) glycerol, and 500 μ*M* thymidine. Fractions were assayed for TK activity, and those with high levels of TK were combined. Bovine serum albumin (final concentration 200 μg/ml) was added and the TK was desalted on Sephadex-G25 PD10.

Assay for TK Activity

A sample (2 μl) was incubated at 37°C for 30 min with 25 m*M* NaF, 5 m*M* $MgCl_2$, 50 m*M* Tris-HCl pH 7.5, 0.1 m*M* dithiothreitol, 2.5 m*M* ATP. The reaction was started by adding [^3H]thymidine (2 μl) (total assay volume 10 μl) and stopped by addition of 500 m*M* Tris-HCl (40 μl). The samples were spotted onto DEAE-filtermats (type 1205-405, Pharmacia-LKB) which were washed 3 times in 1 m*M* ammonium formate before drying and scintillation counting.

PCV-Monophosphate Formation and Isolation

[4′-^{13}C]PCV (**1a**) (100 μ*M*) was incubated at 37°C for 20 h with 25 m*M* NaF, 5 m*M* $MgCl_2$, 50 m*M* Tris-HCl pH 7.5, 0.1-m*M* dithiothreitol, 2.5 m*M* ATP, and purified TK (from above). The incubation solution was chromatographed on DEAE-Sepharose column equilibrated in 0.1 *M* ammonium formate and [4′-^{13}C]PCV-monophosphate was eluted with 0.2 *M* ammonium formate. After repeated freeze drying to remove ammonium formate, [4′-^{13}C]PCV-monophosphate was purified by HPLC on a Phase Sep C_{18}/C_3NH_2 column using isocratic elution (150 m*M* K_2HPO_4/KH_2PO_4 pH 6.8, 6% MeOH). The fraction corresponding to synthetic (racemic) PCV-monophosphate was collected.

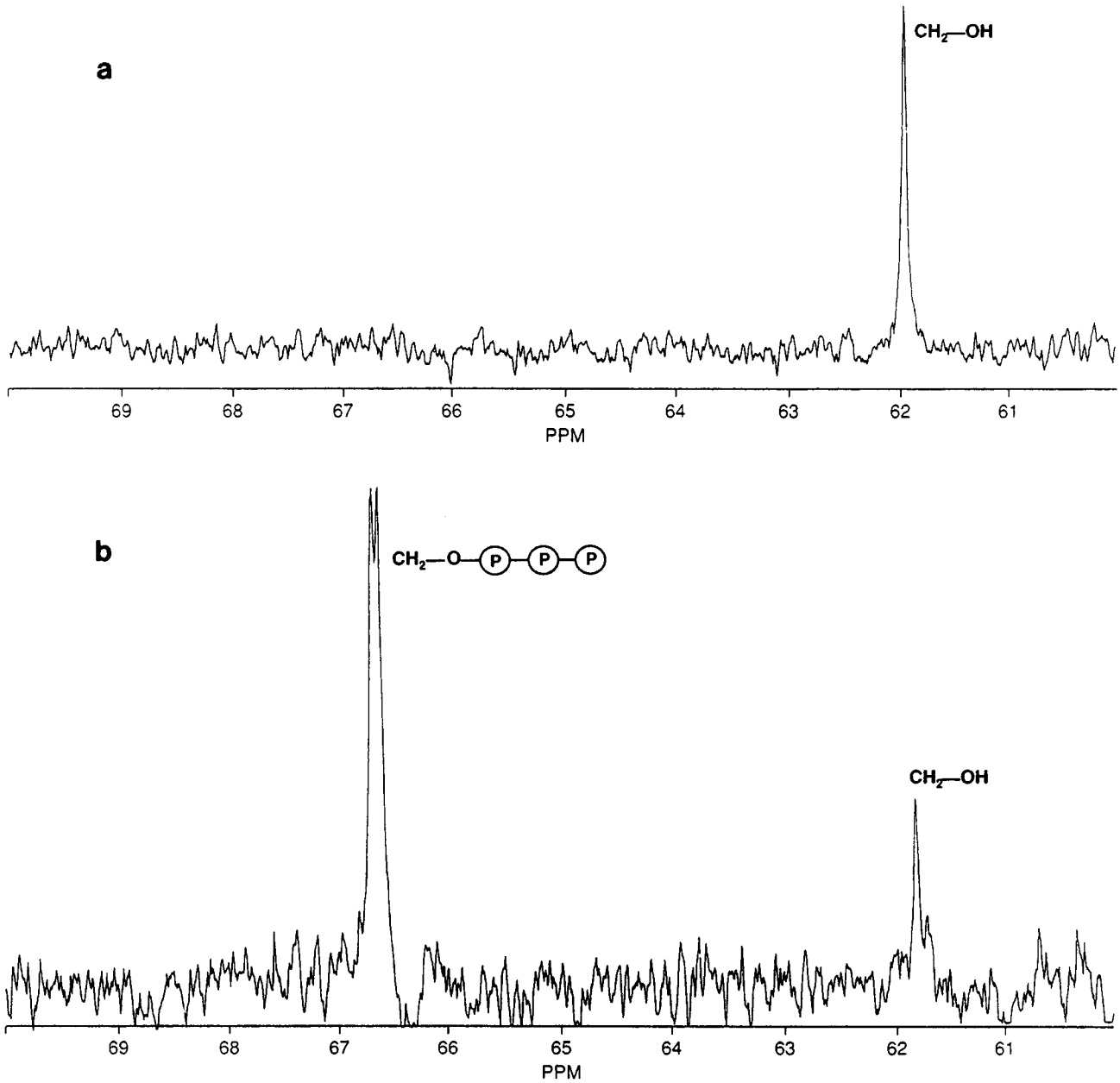


Fig. 3. ^{13}C NMR spectra of penciclovir-triphosphate isolated after (a) [4′-^{13}C]penciclovir (**1a**) and (b) [4′-^{13}C]penciclovir (**1a**/**1b**:15/85) were incubated with HSV-1-infected MRC-5 cells. (Adapted from Jarvest et al.[4], with the permission of the Royal Society of Chemistry.)

^{13}C NMR Spectroscopy

Proton-decoupled ^{13}C NMR spectra of the samples of PCV phosphate esters were recorded as D_2O solutions on a Bruker AM400 NMR spectrometer using standard software. The ^{13}C NMR chemical shifts were referenced relative to an external standard of dioxan. Peak ratio determination was achieved via peak height analysis or more accurately by instrumental integration. In addition to the ^{13}C NMR resonances of the PCV-phosphates, the ^{13}C NMR spectra also contained resonances of heptyltriethylammonium phosphate from the HPLC buffer. However, these ^{13}C NMR resonances were not in the spectral region of interest and hence did not interfere with the reported analyses.

RESULTS

In a preliminary experiment, [4′-^{13}C]PCV-TP was isolated after incubating racemic [4′-^{13}C]PCV with HSV-1-infected cells. The ^{13}C NMR spectrum (Fig. 2) showed two resonances, a singlet at 62.0 ppm and a doublet ($^2J_{COP}$ = 5.9 Hz) at 66.6 ppm. When isotopically chiral [4′-^{13}C]PCV **1a** was incubated with HSV-1 infected cells, the ^{13}C NMR spectrum (Fig. 3a) of the isolated [4′-^{13}C]PCV-TP (ca. 0.6 mg) showed

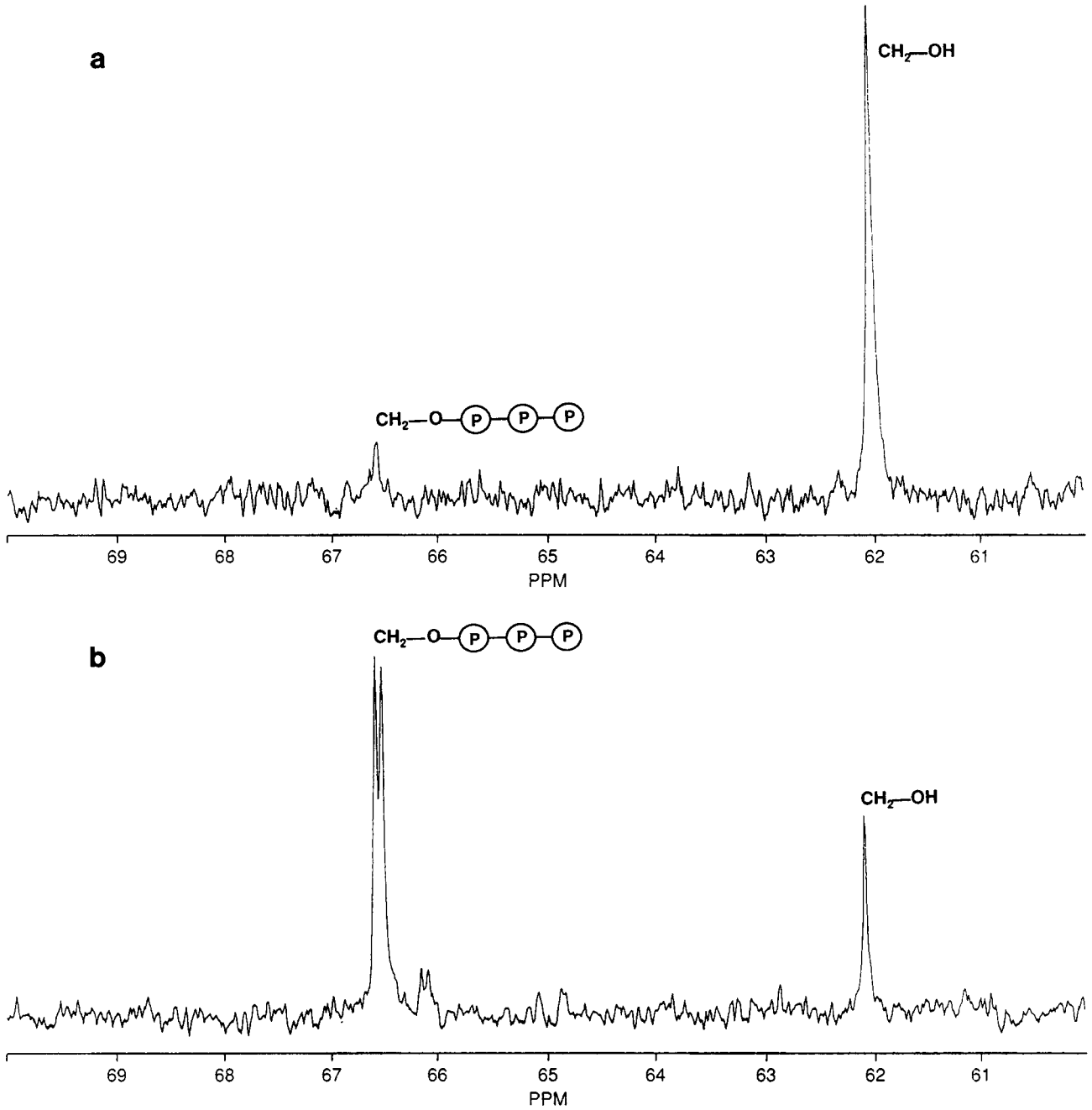


Fig. 4. ^{13}C NMR spectrum of penciclovir-triphosphate isolated after **(a)** [4′-^{13}C]penciclovir (**1a**) and **(b)** [4′-^{13}C]penciclovir (**1a/1b**:15/85) were incubated with HSV-2-infected MRC-5 cells.

only a singlet resonance at 62.0 ppm. No doublet resonance at ca. 66.6 ppm was observed. However, due to the signal-to-noise limits of the ^{13}C NMR spectrum, a peak ratio at least 95:5 was assigned. When the 85% enantiomerically pure [4′-^{13}C]PCV **1b** was incubated with HSV-1 infected cells, the ^{13}C NMR spectrum (Fig. 3b) of the isolated [4′-^{13}C]PCV-TP showed two resonances: a major resonance at 66.6 ppm (doublet, $^{2}J_{COP}$ = 5.9 Hz) and a minor resonance at 61.8 ppm. No peak ratio was calculated from this spectrum since the enantiomeric purity of the chiral precursor was only 85% and hence made quantitation more difficult. In addition, the signal-to-noise ratio in the ^{13}C NMR spectrum was worse than that achieved in the ^{13}C NMR spectrum of the [4′-^{13}C]triphosphate derived from **1a.**

When [4′-^{13}C]PCV (**1a**) was incubated with HSV-2 infected cells, the ^{13}C NMR spectrum (Fig. 4a) of the isolated [4′-^{13}C]PCV-TP (ca. 0.6 mg) showed two resonances: a major singlet resonance at 62.0 ppm and a minor resonance at 66.6 ppm expected to be a doublet but unresolved due to low signal-to-noise ratio. Peak height analysis yielded a peak ratio of ca. 90:10. When [4′-^{13}C]PCV (**1b**), with enantiomeric purity of only 85%, was incubated with HSV-2-infected cells, the

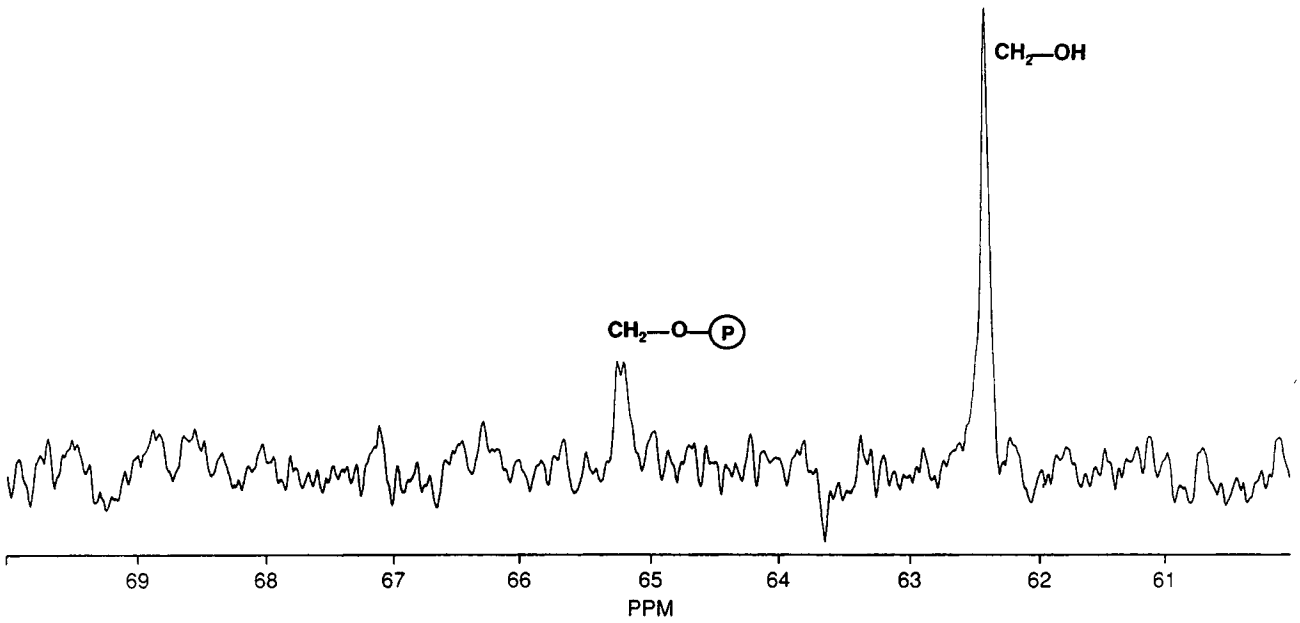


Fig. 5. ^{13}C NMR spectrum of penciclovir-monophosphate isolated after [4′-^{13}C]penciclovir (**1a**) was incubated with HSV-1-encoded thymidine kinase.

^{13}C NMR spectrum (Fig. 4b) of the isolated [4′-^{13}C]PCV-TP showed the same two signals but in this case the doublet ($^2J_{COP}$ = 5.9 Hz) at 66.5 ppm was the major signal. No peak ratio was calculated from this spectrum for the reasons given above. The ^{13}C NMR spectrum showed also a weak doublet at ca. 66.1 ppm. By comparison with data on unlabelled compounds, the signal was assigned to the carbon of the phosphorylated hydroxymethyl of [4′-^{13}C]penciclovir-diphosphate.

The ^{13}C NMR spectrum (Figure 5) of [4′-^{13}C]PCV-monophosphate, isolated from the incubation of [4′-^{13}C]PCV (**1a**) with HSV-1-encoded thymidine kinase, showed two resonances: a major singlet resonance at 62.4 ppm and a minor doublet ($^2J_{COP}$ = 5.3 Hz) resonance at 65.2 ppm. Instrumental integration yielded a peak ratio about 75:25 for this sample.

DISCUSSION

Phosphorylation of one of the hydroxymethyl groups of PCV creates a chiral centre leading to the possible formation of (*R*)- and (*S*)-enantiomers. By using isotopically chiral [4′-^{13}C]PCV in the appropriate biological systems, the enantiomers of the isolated phosphates can be distinguished using ^{13}C NMR spectroscopy. Phosphorylation of the isotopically enriched hydroxymethyl of [4′-^{13}C]PCV will produce the enantiomer in which the ^{13}C label is two bonds removed from phosphorous and so will resonate as a doublet at ca. 66.5 ppm in the ^{13}C NMR spectrum. Conversely, the enantiomer produced as a result of phosphorylation at the unenriched hydroxymethyl group of [4′-^{13}C]PCV can be identified by the ^{13}C in this enantiomer resonating as a singlet at ca. 62.0 ppm.

The fact that both of the above resonances were observed in the ^{13} NMR spectrum of racemic [4′-^{13}C]PCV-TP, isolated after racemic [4′-^{13}C]PCV had been incubated with HSV-1-infected cells, showed that enantiomers both with and without the phosphate adjacent to the ^{13}C label had been produced. This spectrum was similar to that obtained with synthetic PCV-TP.[4] When isotopically chiral [4′-^{13}C]PCV (**1a**) was incubated in HSV-1-infected cells, the ^{13}C NMR spectrum of the isolated triphosphate showed a single resonance at 62.0 ppm, indicating that a single enantiomer had been produced. From this it was concluded that phosphorylation had occurred at the unenriched hydroxymethyl group of **1a**. Conversely, when isotopically chiral [4′-^{13}C]PCV (**1b**, enantiomeric purity 85%) was incubated in HSV-1-infected cells, the predominant resonance in the ^{13}C NMR spectrum of the isolated triphosphate was a doublet at 66.6 ppm indicating that the phosphate was adjacent to the ^{13}C label of this metabolite. Therefore these results indicate that the absolute configuration of PCV-TP produced in HSV-1-infected cells is *S* as shown in Figure 1. Furthermore, the ^{13}C NMR spectrum of the triphosphate derived from **1a** indicated that the enantiomeric purity was greater than 95%. The ^{13}C NMR spectrum of the triphosphate derived from **1b** showed also a minor resonance at 61.8 ppm indicating the presence of phosphate on an isotopically unenriched hydroxymethyl group. However, the peak ratio corresponded to the lower enantiomeric purity (85%) of the [4′-^{13}C]PCV precursor. Thus, only the (*S*)-enantiomer of PCV-TP was detected in HSV-1-infected cells.

When isotopically chiral [4′-^{13}C]PCV (**1a**) or (**1b**) were incubated with HSV-2-infected cells, the major peaks in the ^{13}C NMR spectra of the isolated triphosphates corresponded to those from the HSV-1 experiments. Therefore it was concluded that the major triphosphate produced in HSV-2 infected cells was (*S*)-PCV-TP (Fig. 1). However, about 10% of (*R*)-PCV-TP also was produced in HSV-2-infected cells as evident from the peak ratio determined for the ^{13}C NMR spectrum of the triphosphate derived from **1a**.

Incubation of isotopically chiral [4′-^{13}C]PCV (**1a**) with HSV-1-encoded thymidine kinase yielded [4′-^{13}C]PCV-monophosphate for which the ^{13}C NMR spectrum showed two

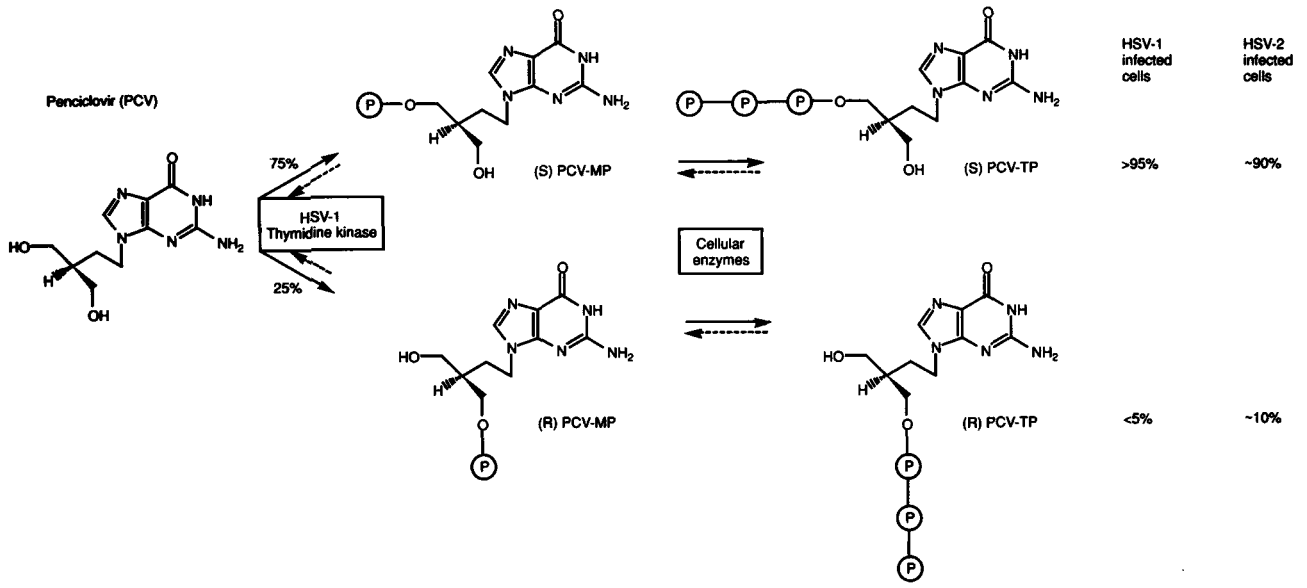


Fig. 6. Summary of penciclovir phosphorylation.

resonances. The major resonance was a singlet at 62.4 ppm and originated from the monophosphate enantiomer with the phosphate on the unenriched hydroxymethyl. The minor resonance was a doublet at 65.2 ppm ($^2J_{COP}$ = 5.3 Hz) and originated from the monophosphate enantiomer with the phosphate adjacent to the ^{13}C-enriched hydroxymethyl carbon. Therefore from the nature of these ^{13}C NMR resonances and their peak area ratio it was concluded that HSV-1 encoded thymidine kinase phosphorylated PCV to give about 75% of (*S*)-PCV-monophosphate and 25% of the (*R*)-enantiomer.

Phosphorylation of the structurally related acyclonucleoside, ganciclovir, by HSV-1 thymidine kinase is stereospecific.[6] These workers did not determine unambiguously the absolute configuration of the phosphate but inferred from structural comparisons that it was the (*S*)-enantiomer. Also they showed that the presumed (*S*)-mono- and diphosphates were more quickly phosphorylated by mammalian kinases than the other enantiomer. Therefore the enantiomeric purity may be expected to increase at each phosphorylation step leading to the triphosphate ester.

CONCLUSION

HSV-1-encoded thymidine kinase phosphorylated PCV preferentially to give 75% (*S*)- and 25% (*R*)-PCV-monophosphate. In HSV-1-infected cells, the absolute configuration of PCV-TP was *S* with an enantiomeric purity of greater than 95%. This is the enantiomer which is analogous to the natural nucleotide. However, in contrast to HSV-1-infected cells in which none of the (*R*)-enantiomer was detected, about 10% of (*R*)-PCV-TP was produced in HSV-2-infected cells. These results are summarised in Figure 6.

ACKNOWLEDGMENTS

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