Enzymatic Phosphorylation of the Antiherpetic Agent 9-[(2,3-Dihydroxy-1-propoxy)methyl]guanine

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The antiherpetic agent 9-[(2,3-dihydroxy-1-propoxy)methyl]guanine (iNDG) is phosphorylated by HSV1 thymidine kinase, and its phosphorylated products inhibit DNA polymerase activity. iNDG exists in two enantiomeric forms, each with a primary and a secondary hydroxyl; thus, a number of possibilities for preferential phosphorylation exist, which were explored in this study. HSV1 thymidine kinase phosphorylates the primary hydroxyl of both the *R* and the *S* isomers of iNDG. This was established by comparison with analogues in which either the primary or the secondary hydroxyl was replaced by fluorine or hydrogen and also by a study of the NMR spectrum of the monophosphate. GMP kinase phosphorylates the *R* and the *S* monophosphates to the respective diphosphates. Further phosphorylation, however, is much more efficient with the *S* than with the *R* isomer. Furthermore, (*S*)-iNDG triphosphate is a more potent inhibitor of HSV1 DNA polymerase than (*R*)-iNDG triphosphate. These differences in the biochemical specificities of the two isomers account for the observed higher antiviral potency of (*S*)-iNDG as compared to that of (*R*)-iNDG.

Our synthesis of acyclic nucleoside analogues with antiviral activity has led to the recently described 2′NDG (**3** in Figure 1)[1-4] and to its linear isomer 9-[(2,3-dihydroxy-1-propoxy)methyl]guanine [(±)-iNDG; **1** in Figure 1].[5-7] The former was found to be phosphorylated by HSV1 thymidine kinase to the monophosphate and this, in turn, by cellular enzymes to the triphosphate, which is a potent inhibitor of the virus-induced DNA polymerase.[9,10] Since iNDG exhibits an antiviral activity comparable to that of acyclovir[11] in tissue culture and superior to that of acyclovir in vivo,[6] we conducted a similar investigation on the phosphorylation of iNDG.

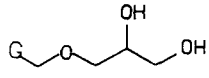
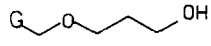
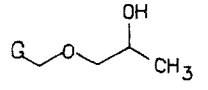
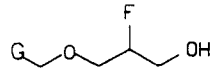
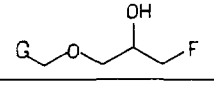
In contrast to acyclovir which has only one hydroxyl group in its side chain (**2** in Figure 1) and to 2′NDG which has two equivalent primary hydroxyls, iNDG has one primary and one secondary hydroxyl and exists in two enantiomeric forms *S* and *R* (**1a** and **1b**, respectively, in Figure 1) due to the asymmetry at carbon 2 of the 2,3-dihydroxypropyl group. It was, therefore, of interest to investigate (a) whether phosphorylation by the viral thymidine kinase involved the primary or the secondary hydroxyl, (b) whether both enantiomers were substrates for the viral and cellular kinases, (c) whether the respective triphosphates (if indeed both were made) were inhibitory to the viral DNA polymerase, and (d) whether the two enantiomers had similar antiviral activities.

Synthesis. Compound **4** was prepared by a published procedure.[12] The syntheses of analogues **5-7** are outlined in Scheme I. In each case the requisite chloromethyl ether (**11**, **17**, **23**) was prepared with the hydroxyl substituent masked as a benzyl ether. The 9-alkylated purines (**13**, **18**, **25**) were made by reaction of the chloromethyl ethers with the sodium salt of 2-amino-6-(benzyloxy)purine (**12**)[13,14] or with the highly moisture-sensitive compound tris(trimethylsilyl)guanine (**24**).[6,15-17] In the final step, the benzyl protecting groups were removed by catalytic hydrogenolysis in the presence of *p*-toluenesulfonic acid[6,17] (for **5** and **6**) or by transfer hydrogenation[18] (for **7**). The NMR and UV spectra of **5-7** were consistent with their assignments as N^9-alkylated guanine derivatives.

Results

Site of Phosphorylation. Two different approaches were used to determine whether the primary or the secondary hydroxyl was the site of phosphorylation of iNDG by the viral thymidine kinase. One approach involved a comparison with analogues in which either the primary or the secondary hydroxyl was replaced by fluorine or hy-

Table I. Phosphorylation of HSV1 Thymidine Kinase and Antiviral Activity of (±)-iNDG and Analogues

compd	struct	% phosphoryln[a] by HSV1 TK	antiviral act. ED_{50}, μg/mL
1		59	3-6
	G = guanin-9-yl		
4		88	50
5		5	100
6		42	25
7		4	NA[c]

[a] Percent total nucleoside converted to monophosphate by TK (see Results). [b] 50% inhibition of viral CPE: HSV1 (Schooler) in MRC-5 cells.[6] [c] NA = not active (ED_{50} > 100 μg/mL).

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(8) Abbreviations used: HSV1, herpes simplex virus type 1; TK, thymidine kinase; DTT, dithiothreitol; ACV, acyclovir, 9-[(2-hydroxyethoxy)methyl]guanine; 2′NDG, 2′-nor-2′-deoxyguanosine, 9-[(1,3-dihydroxy-2-propoxy)methyl]guanine; iNDG, iso-2′NDG, 9-[(2,3-dihydroxy-1-propoxy)methyl]-guanine.

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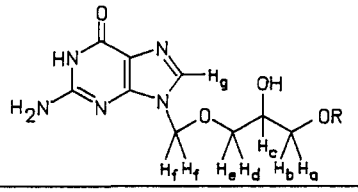
Table II. ^{1}H NMR Spectra of (±)-iNDG and Its Enzymatically Formed Monophosphate



R	chemical shifts in D_2O, δ						
	H_a	H_b	H_c	H_d	H_e	H_f	H_g
H	3.52[a] (dd, 1 H)	3.57[a] (dd, 1 H)	3.84 (dddd, 1 H)	3.58[a] (dd, 1 H)	3.67[a] (dd, 1 H)	5.52 (s, 2 H)	7.96 (s, 1 H)
PO_3^{2-}	3.84 (m, 2 H)		3.98 (m, 1 H)	3.64 (dd, 1 H)	3.72 (dd, 1 H)	5.54 (s,[b] 2 H)	7.98 (s, 1 H)

R	coupling constants, Hz							
	J_{ab}	J_{ac}	J_{ax}[c]	J_{bc}	J_{bx}[c]	J_{cd}	J_{ce}	J_{de}
H[d]	11.5	6.3		4.7		6.5	3.7	10.2
PO_3^{2-}[e]						6.6	3.7	10.5
PO_3^{2-}[f]	11.0	6.0	10.0	5.0	9.7	6.4	3.7	10.7

[a] Assignments of chemical shifts for the H_a-H_b pair vs. the H_d-H_e pair are tentative but are consistent with the pattern of coupling constants before and after phosphorylation. [b] Incipient nonequivalence is evidenced by a pair of weak satellite lines 12 Hz on each side of the unresolved δ 5.54 signal. [c] Coupling to phosphorus. [d] See footnote *a* regarding assignments. [e] Measured. [f] Simulation parameters.

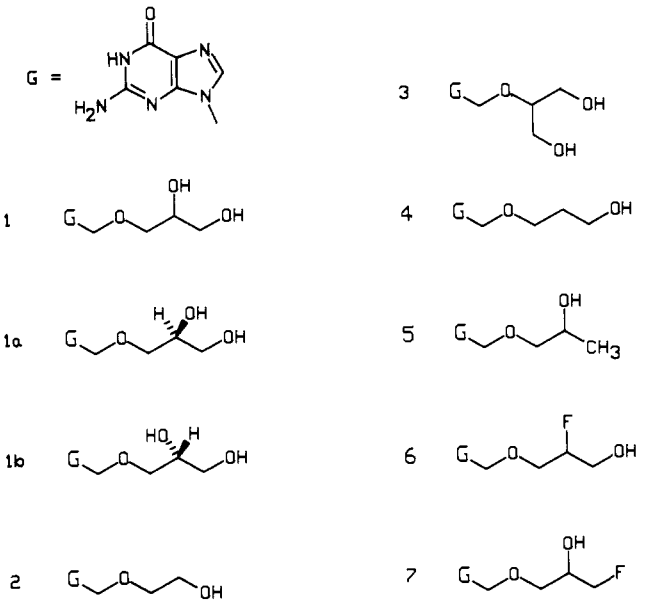


Figure 1. Structures of nucleoside analogues used in this study.

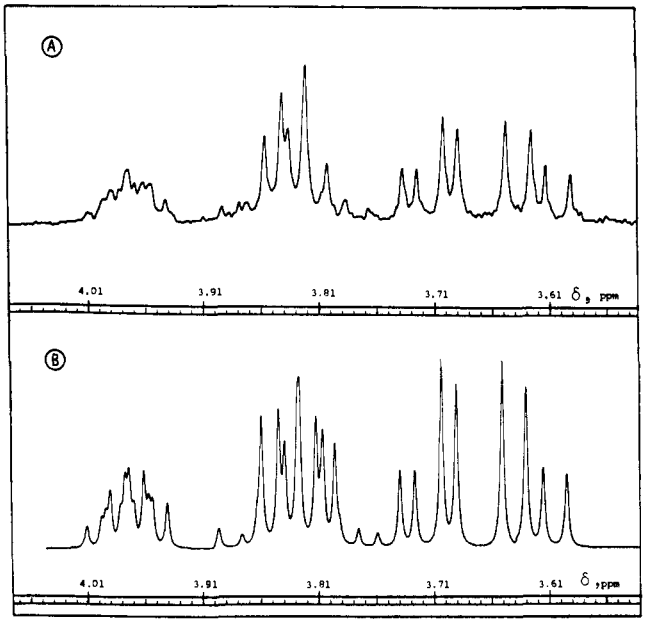


Figure 2. Comparison of observed and simulated ^{1}H NMR spectra: A, expanded spectrum (D_2O) of glyceryl moiety of enzymatically formed iNDG monophosphate; B, simulated spectrum of glyceryl moiety of the primary monophosphate of iNDG using parameters described in Table II and the text.

drogen. The other was a study of the NMR spectrum of iNDG monophosphate obtained by phosphorylation with HSV1 thymidine kinase. Racemic compounds were used in both of these studies.

The four analogues used for the comparison (Figure 1, **4–7**) were assayed for their capacity to act as substrates for the HSV1 TK and for their antiviral activity in cell culture. The results are shown in Table I. Compound **4** was previously reported by Keller et al.[19] to be a very good substrate for HSV1 TK (better than ACV) and to have antiviral activity. Our results are in general agreement with these observations, although in our cell culture infection system the antiviral activity of compound **4** was lower than that reported by Keller et al. A similar pattern was obtained with compound **6**: it was a good substrate for HSV1 TK and had antiviral activity. However, **5** and **7** were poor substrates for HSV1 TK and had little or no antiviral activity (Table I). Since the compounds with primary hydroxyls (**4**, **6**) are good substrates while those with secondary hydroxyls (**5**, **7**) are not, one can conclude

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Table III. Inhibition of HSV and HeLa DNA Polymerases[a]

inhibitor	% inhibn		inhibitor	% inhibn	
	HSV1 polymerase	HeLa α polymerase		HSV1 polymerase	HeLa α polymerase
ACV	86	37	(S)-iNDG	54	10
2'NDG	60	17	(R)-iNDG	14	0
(±)-iNDG	36	6			

[a] The compounds were subjected to "combined phosphorylation" and the products tested for inhibitory activity as described in the Experimental Section.

that the primary hydroxyl is the most likely site of phosphorylation also for iNDG.

In the NMR study the monophosphate of (±)-iNDG was synthesized enzymatically with HSV1 TK and purified by preparative HPLC. The 300-MHz ^1H NMR spectral data of this compound in D_2O are shown in Table II. A detailed analysis including spectral comparisons and simulation studies was performed in order to establish whether the primary or secondary hydroxyl was the site of phosphorylation.

Phosphorylation causes two spectral perturbations: a downfield shift of roughly 0.2–0.4 ppm for protons attached to the derivatized site[20] together with an increased number of lines of the displaced signal due to three-bond coupling with the phosphorus. Coupling between phosphorus and protons four bonds removed is generally very weak and often is not observed. Comparison of the spectrum of (±)-iNDG with that of the phosphorylated product revealed that signals associated with one methylene have been shifted downfield from approximately 3.5–3.6 to 3.84 ppm after derivatization and show an increase in complexity. In contrast, the methine multiplet in the phosphorylated product parallels its counterpart in (±)-iNDG, in both the number of lines and full width of the multiplet. Simluation studies (Figure 2; Table II) indicated that an almost perfect match of the methine pattern results from only the four vicinal coupling constants obtained from the two methylene signals. A reasonable match for the CH_2OP multiplet can be generated from the following parameters: $J(gem)$ = 11 Hz, $J(vic)$ = 6, 5 Hz, $J(POCH_2)$ = 10, 9.7 Hz and a geminal chemical shift separation of 10 Hz. It was therefore confirmed by these studies that enzymatic phosphorylation of 1 had occurred on the terminal, primary hydroxyl.

Phosphorylation of the Enantiomers. The two enantiomers, (R)-iNDG and (S)-iNDG, were prepared from chiral intermediates.[6] The two isomers were subjected to phosphorylation by incubating with a crude extract of HSV1-infected HeLa cells, supplemented with purified HSV1 TK, GMP kinase, and creatine kinase. Under these "combined phosphorylation" conditions, 2'NDG is converted rapidly and almost quantitatively to the triphosphate.[9] At regular time intervals, samples of the incubation mixtures of the two enantiomers were analyzed by HPLC and the amounts of mono-, di-, and triphosphate present were determined. The results are illustrated in Figures 3 and 4. (S)-iNDG is rapidly phosphorylated to the monophosphate, and this is in turn converted to the di- and eventually to the triphosphate (Figure 2). After 3 h of incubation, 85% of the compound is found as the triphosphate. In the case of the (R)-iNDG (Figure 3), phosphorylation to the monophosphate appears to occur almost as fast as with the S isomer. Diphosphate formation also appears to proceed with a rate not very different from that observed with the S isomer. The rate of triphosphate formation, however, is much slower: only 16%

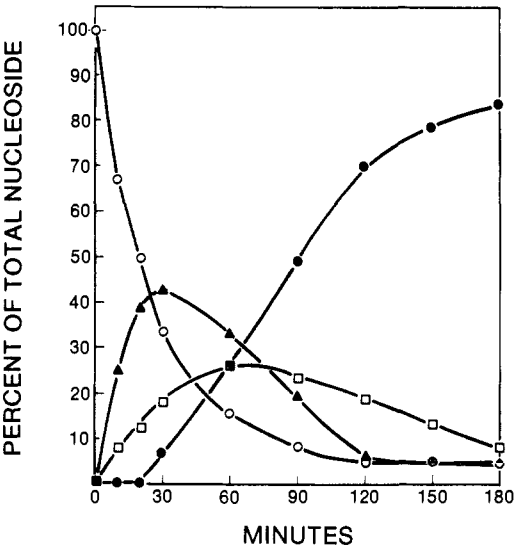

Figure 3. Time course of (S)-iNDG phosphorylation under the "combined phosphorylation" conditions described in Biochemical Methods: ○, nucleoside analogue; ▲, monophosphate; □, diphosphate; ●, triphosphate.

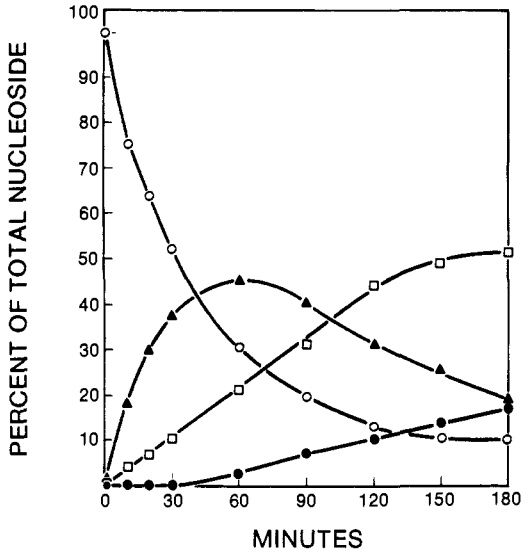

Figure 4. Time course of (R)-iNDG phosphorylation. Conditions and symbols are as in Figure 3.

of the (R)-iNDG is converted to the triphosphate after 3 h of incubation. This results in the accumulation of the diphosphate and monophosphate (49% and 23% respectively, after 3 h of incubation).

Inhibition of the DNA Polymerases. The capacity of the triphosphates of the two enantiomers to inhibit HSV and cellular DNA polymerases was determined by removing aliquots of the phosphorylation assay mixtures at the end of the 3-h incubation period and adding them to a DNA polymerase assay system. A crude extract of HSV1-infected HeLa cells served as the source of enzyme in this system, and the viral polymerase was assayed in the presence of 0.1 M $(NH_4)_2SO_4$ while the cellular polymerases

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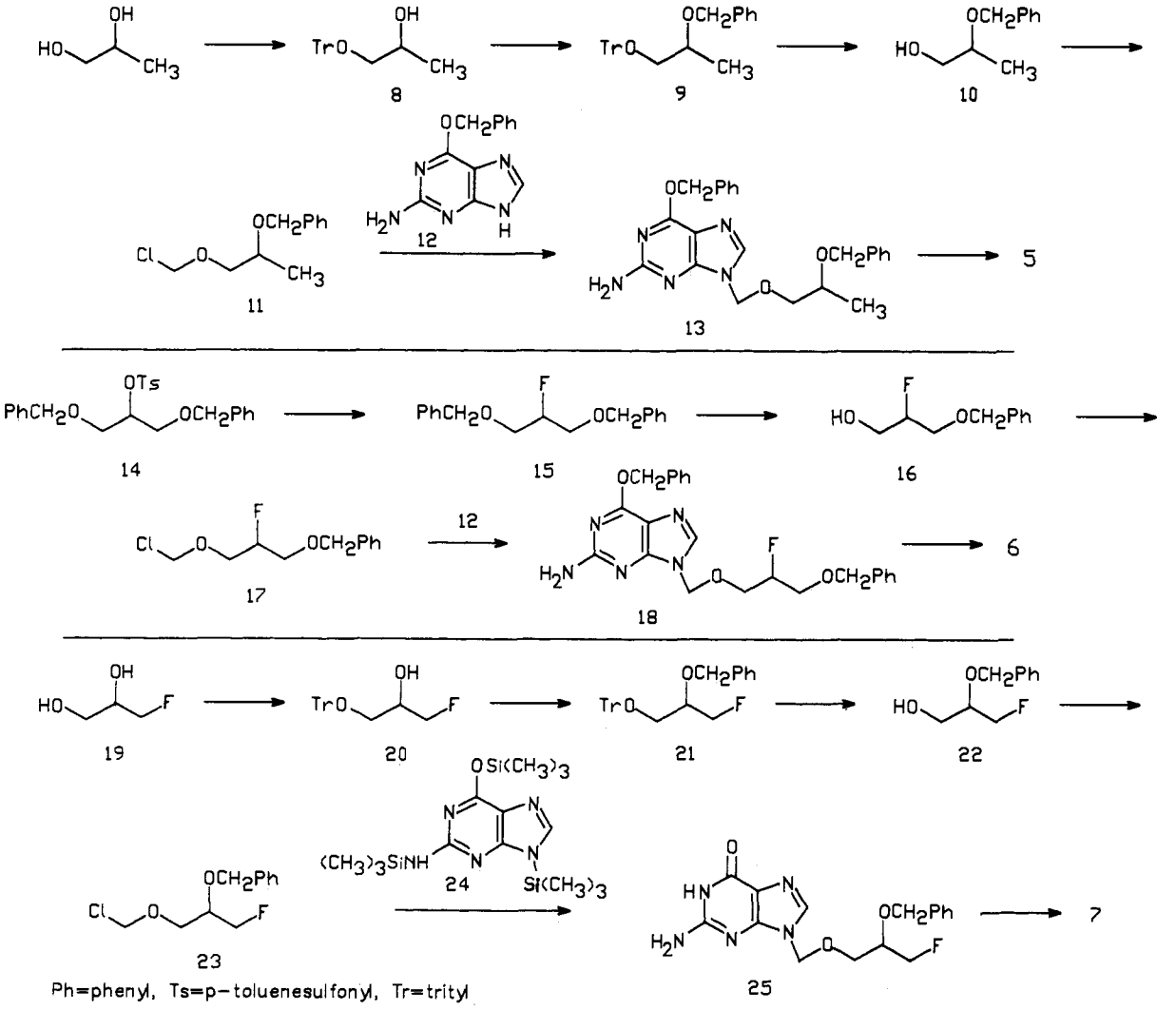
Scheme I



Ph=phenyl, Ts=p-toluenesulfonyl, Tr=trityl

were assayed without any added salt. As shown in Table III, there was considerably more inhibition of the HSV1 DNA polymerase by the phosphorylated derivatives of (*S*)-iNDG than by those of (*R*)-iNDG.

Figure 5 illustrates the results of another experiment in which the purified triphosphates of (*S*)- and (*R*)-iNDG were compared as inhibitors of purified HSV1 DNA polymerase. At all three concentrations tested, the *S* isomer had higher inhibitory activity than the *R*.

Discussion

Both approaches used to investigate whether the primary or secondary hydroxyl group of iNDG was the site of phosphorylation by HSV1 TK, the comparison with analogues and the NMR study, indicated that the primary hydroxyl was selectively phosphorylated. This answer could not have been predicted since, in the absence of the constraints of the furanose ring, the side chain has considerably more conformational flexibility and can be folded in such ways as to bring either the primary or the secondary hydroxyl in a position equivalent to that occupied by the 5′-hydroxyl of thymidine, a natural substrate of TK.

The antiviral activity of the compounds compared in Table I generally correlates with the presence of a primary hydroxyl capable of phosphorylation. This correlation is not quantitative, but this is not surprising, since phosphorylation to the monophosphate is not a sufficient condition for antiviral activity.

It is possible that mono-, di-, and triphosphates with the phosphate(s) on the secondary hydroxyl could arise in the incubation mixtures, not by the action of TK but by phosphate migration from the primary hydroxyl. It is

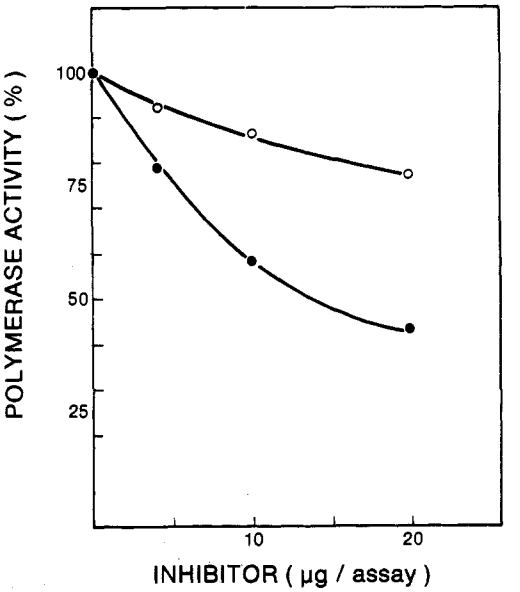


Figure 5. Inhibition of HSV1 DNA polymerase by purified (*R*)- and (*S*)-iNDG triphosphates. Assay conditions are in the Experimental Section: ○, (*R*)-iNDG-TP; ●, (*S*)-iNDG-TP. A 0.5-unit sample of HSV1 DNA polymerase (phosphocellulose fraction) was used for each 100-µL assay. The 100% value (without inhibitor) was 125 pmol of dTMP incorporated in 1 h.

unlikely that such migration could take place at the monophosphate level under the mild conditions of the phosphorylation experiments (37 °C, pH 7.5). However, at the triphosphate level and to some extent at the diphosphate level, there is a real possibility of formation of a cyclic phosphate diester via a nucleophilic attack of the vicinal hydroxyl and the displacement of phosphate or pyrophosphate. The opening of this cyclic diester, either chemical or enzymatic, could give rise to a monophosphate on the primary or secondary hydroxyl or a mixture of both. Experiments designed to examine the presence and proportion of secondary triphosphates in the phosphorylation mixtures of (*R*)- and (*S*)-iNDG are in progress. As far as the observed differences between the two enantiomers are concerned, they could be the result of a stereochemical effect on the rate(s) of one or more of the several reactions involved.

In the present set of experiments we could only compare the rates at which the mono-, di-, and triphosphate accumulate in the case of each enantiomer. Judging from the initial slopes of the respective curves in Figures 2 and 3, monophosphate is produced at a higher rate from (*S*)-iNDG than from (*R*)-iNDG, and the same is true for diphosphate production, but the differences are not very pronounced. On the other hand, the rate of triphosphate production is considerably higher with (*S*)-iNDG than with (*R*)-iNDG. The net result is the accumulation of triphosphate as the main product (85%) in the case of (*S*)-iNDG and the disphosphate (49%) in that of (*R*)-iNDG. This appears to be contrary to the experience with conventional nucleosides, where the third phosphorylation step is the least specific.[21]

In the DNA polymerase inhibition experiment performed at the end of the "combined phosphorylation", neither the triphosphate nor the enzymes were purified, which made the system resemble more closely the in vivo situation. Under these experimental conditions, however, it is not possible to ascertain whether the lower inhibition by (*R*)-iNDG is due to the lower concentration of the triphosphate or also to an inherently lower inhibitory activity. The experiment of Figure 5 indicates that the (*R*)-iNDG triphosphate is a poorer inhibitor than (*S*)-iNDG triphosphate even at equimolar concentration. Thus, a combination of these two factors, faster accumulation of the triphosphate and higher inhibitory activity of the latter against the viral DNA polymerase, presumably accounts for the higher antiherpetic activity of (*S*)-iNDG. Indeed, as reported by Ashton et al.,[5,6] (*S*)-iNDG exhibits an activity comparable to that of ACV in cell culture and superior to that of ACV in vivo.

Experimental Section

Proton NMR spectra were obtained with a Varian T-60A, XL-200, or SC300 spectrometer, using tetramethylsilane as internal standard. Key: s = singlet, d = doublet, AB q = AB quartet pattern (center), dd = doublet of doublets, dm = doublet of multiplets, d quint = doublet of quintets, ddd = doublet of doublets of doublets, br = broad, v = very, Tr = trityl, Ar = aryl, Pu = purinyl. UV spectra were determined on a Perkin-Elmer Lambda 5 spectrophotometer. Mass spectra were obtained with a Varian MAT 731 instrument using fast atom bombardment (FAB). Melting points (uncorrected) were determined in open capillary tubes with a Thomas-Hoover apparatus. Preparative HPLC separations were carried out on a Waters Prep 500 instrument using Prep Pak silica gel cartridges. Compounds showed satisfactory purity by TLC (Analtech silica gel GF plates) and/or analytical HPLC (Varian Model 5060 liquid chromatograph) in the indicated solvent systems.

(±)-2-(Benzyloxy)-1-(trityloxy)propane (9). To a solution of 31.8 g (0.10 mol) of (±)-1-(trityloxy)-2-propanol (8)[22] in 100 mL of dry DMF was added 4.4 g (0.11 mol) of NaH (60% in oil), and the mixture was stirred at ambient temperature under N_2 as H_2 evolution proceeded. After 2 h, the mixture was cooled in an ice bath, and 17.3 mL (19.0 g, 0.15 mL) of benzyl chloride was added dropwise. After completion of the addition, the mixture was stirred at room temperature for 3 days and then added to 800 mL of ice H_2O and extracted with 600 mL of ether. The ethereal solution was washed with 3 × 500 mL of H_2O, filtered, and concentrated to give a yellow-orange oil. Purification by column chromatography on silica gel (elution with hexane containing a gradient of 5–10% ethyl acetate) afforded 22.9 g (56%) of very viscous, colorless oil: TLC in 9:1 hexane–ethyl acetate; 60-MHz ^{1}H NMR ($CDCl_3$) δ 1.16 (d, J = 7 Hz, 3 H, CH_3), 3.10 (m, 2 H, CH_2OTr), 3.7 (m, 1 H, OCH), 4.53 (s, 2 H, $ArCH_2$), 7.2 (m, 20 H, ArH). Anal. ($C_{29}H_{28}O_2$) C, H.

(±)-2-(Benzyloxy)-1-propanol (10). A mixture of 20.4 g (0.050 mol) of **9**, 180 mL of glacial acetic acid, and 120 mL of H_2O was stirred at 90–95 °C for 4 h and then cooled and filtered to remove precipitated trityl alcohol. Concentration of the filtrate in vacuo gave a yellow oil, which was found by TLC (9:1 hexane–ethyl acetate) to contain some acetylated product. Consequently, the oil was dissolved in 70 mL of methanol and treated dropwise with methanolic 1 M sodium methoxide until strongly basic. After stirring overnight at room temperature, the solution was neutralized with acetic acid and evaporated in vacuo. The residue was taken up in ether, filtered, and again concentrated to give a yellow oil. Chromatography on a silica gel column (elution with 90:10 and then 80:20 hexane–ethyl acetate) yielded 7.49 g (90%) of colorless oil: 60-MHz ^{1}H NMR ($CDCl_3$) δ 1.19 (d, J = 6 Hz, 3 H, CH_3), 2.1 (vbr s, 1 H, OH), 3.4–3.9 (m, 3 H, $CHCH_2$), 4.59 (AB q, 2 H, $ArCH_2$), 7.36 (s, 5 H, ArH). This compound has been prepared previously by different routes.[23,24]

(±)-2-(Benzyloxy)-1-(chloromethoxy)propane (11). A mixture of 1.66 g (10 mmol) of **10**, 0.30 g (10 mequiv) of paraformaldehyde, and 5 mL of methylene chloride was stirred at 0 °C under protection from moisture as a stream of HCl gas was bubbled through it. After 3 h, the resulting solution was purged with a stream of N_2 to remove excess HCl and then dried with Na_2SO_4 and filtered. Concentration of the filtrate in vacuo gave 2.01 g (93%) of colorless residual oil: 60-MHz ^{1}H NMR ($CDCl_3$) δ 1.19 (d, J = 6 Hz, 3 H, CH_3), 3.4–3.9 (m, 3 H, $CHCH_2$), 4.54 (s, 2 H, $ArCH_2$), 5.36 (s, 2 H, OCH_2Cl), 7.24 (s, 5 H, ArH).

(±)-2-Amino-6-(benzyloxy)-9-[[2-(benzyloxy)-1-propoxy]methyl]purine (13). A suspension of 1.77 g (7.3 mmol) of 2-amino-6-(benzyloxy)purine (12)[13,14] in 18 mL of dry DMF was warmed until all of the solid dissolved and then cooled and treated with 0.29 g (7.3 mmol) of NaH (60% in oil). Stirring under N_2 at ambient temperature was continued for 30 min until H_2 evolution was complete. Then, a solution of 2.01 g (≥8.4 mmol based on 90% minimum purity) of 11 in 2 mL of DMF was added. The mixture was stirred at room temperature overnight and then filtered and concentrated in vacuo. Column chromatography of the residue on silica gel (elution with ethyl acetate) gave 0.72 g (24%) of a colorless viscous oil: 200-MHz ^{1}H NMR ($CDCl_3$) δ 1.16 (d, J = 7 Hz, 3 H, CH_3), 3.54 (m, 2 H, OCH_2CH), 3.68 (m, 1 H, OCH), 4.59 (AB q, 2 H, $CHOCH_2Ar$), 4.93 (br s, 2 H, NH_2), 5.52, 5.60 (s, each 2 H, NCH_2O and Pu-6-OCH_2Ar), 7.2–7.5 (m, 10 H, ArH), 7.74 (s, 1 H, C^8-H).

(±)-9-[(2-Hydroxy-1-propoxy)methyl]guanine (5). A mixture of 690 mg (1.64 mmol) of **13**, 313 mg (1.64 mmol) of *p*-toluenesulfonic acid monohydrate, 120 mg of 20% palladium hydroxide on carbon, and 10 mL of methanol was shaken overnight with H_2 at an initial pressure of approximately 3 atm. The mixture was diluted with 6 mL of H_2O, neutralized with 2.5 N NaOH, and partially evaporated in vacuo to remove most of the

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methanol. The remaining mixture was heated on a steam bath and then filtered through Celite. The precipitate that separated from the filtrate on cooling was isolated to give 198 mg (50%) of white crystals. The analytical sample was further recrystallized from H_2O: mp 265 °C dec; TLC in 80:20:2 chloroform–methanol–H_2O; reversed-phase analytical HPLC (Whatman ODS-3) in 9:1 H_2O–methanol; UV λ_{max}(pH 1) 255 nm (ϵ 12 500), 275 (sh, ϵ 8350), λ_{max}(pH 7) 252 nm (ϵ 12 400), 270 (sh, ϵ 8640), λ_{max}(pH 13) 265 (ϵ 11 100); 200-MHz ^{1}H NMR (Me_2SO-d_6) δ 0.97 (d, J = 6 Hz, 3 H, CH_3), 3.28 (m, 2 H, OCH_2CH), 3.67 (m, 1 H, OCH), 4.68 (d, J = 5 Hz, 1 H, OH), 5.36 (s, 2 H, NCH_2O), 6.53 (br s, 2 H, NH_2), 7.84 (s, 1 H, C^8-H), 10.60 (br s, 1 H, N^1-H); mass spectrum (FAB), m/e 240 (M + H^+). Anal. ($C_9H_{13}N_5O_3 \cdot 1.2H_2O$) C, H, N.

1,3-Bis(benzyloxy)-2-fluoropropane (15). A solution of 38.8 g (91 mmol) of 1,3-bis(benzyloxy)-2-propanol *p*-toluenesulfonate (**14**),[25] 170 mmol of tetrabutylammonium fluoride (from concentration of 170 mL of a 1 M solution in tetrahydrofuran), and 100 mL of acetonitrile was stirred at reflux under N_2 for 3 days. The solvent was removed by rotary evaporation, and the residual oil was partitioned between ether and H_2O. The ethereal layer was washed further with H_2O and then dried ($MgSO_4$) and concentrated in vacuo. Only partial purification of the residue was achieved by preparative HPLC (elution with 97:3 hexane–ethyl acetate). The crude product thus isolated was distilled, with the bulk of the material being collected at 168–170 °C (1 mm). Finally, the best fractions were combined and chromatographed on a column of silica gel (gradient elution with hexane containing 2–5% ethyl acetate) to yield 3.67 g (15%) of colorless oil: analytical HPLC (Whatman Partisil) in 95:5 hexane–ethyl acetate; 200-MHz ^{1}H NMR ($CDCl_3$) δ 3.72 (dd, J = 23, 5 Hz, 4 H, CH_2CHFCH_2), 4.59 (s, 4 H, $ArCH_2$), 4.84 (d quint, J = 48, 5 Hz, 1 H, CHF), 7.36 (s, 10 H, ArH).

(±)-3-(Benzyloxy)-2-fluoro-1-propanol (16). A mixture of 3.50 g (12.8 mmol) of **15**, 80 mg of 20% palladium hydroxide on carbon, and 35 mL of absolute ethanol was shaken with hydrogen at an initial pressure of approximately 3 atm. After 45 min, TLC (4:1 hexane–ethyl acetate) showed the monobenzyl product spot (R_f 0.18) to be somewhat stronger than the starting material spot (R_f 0.64) when visualized by UV light. At this time, the catalyst was removed by filtration through Celite, and the filtrate was concentrated in vacuo. The residue was chromatographed on a silica gel column (gradient elution, 95:5 to 75:25 hexane–ethyl acetate) to yield 1.26 g (54%) of colorless oil: 200-MHz ^{1}H NMR ($CDCl_3$) δ 1.94 (t, J = 6.5 Hz, 1 H, OH), 3.55 (dd, J = 22, 5 Hz, 2 H, CH_2OCH_2CHF), 3.90 (ddd, J = 23, 6.5, 4.5 Hz, 2 H, $CHFCH_2OH$), 4.62 (s, 2 H, $ArCH_2$), 4.76 (dm, J_{HF} = 48 Hz, 1 H, CHF), 7.38 (s, 5 H, ArH).

(±)-3-(Benzyloxy)-1-(chloromethoxy)-2-fluoropropane (17). This material, prepared from **16** by the method used to prepare **11**, was obtained in 87% yield as a colorless oil: 200-MHz ^{1}H NMR ($CDCl_3$) δ 3.65 (dd, J = 21.5, 4.5 Hz, 2 H, OCH_2CHF), 3.87 (dd, J = 22.5, 4.5 Hz, 2 H, OCH_2CHF), 4.53 (s, 2 H, $ArCH_2$), 4.78 (dm, J_{HF} = 48 Hz, 1 H, CHF), 7.29 (s, 5 H, ArH).

(±)-2-Amino-6-(benzyloxy)-9-[[3-(benzyloxy)-2-fluoro-1-propoxy]methyl]purine (18). Alkylation of 2-amino-6-(benzyloxy)purine (**12**)[13,14] with **17** by the method to prepare **13** gave a 36% yield of white crystals: mp 76–78 °C; TLC in ethyl acetate; 200-MHz ^{1}H NMR ($CDCl_3$) δ 3.64 (dd, J = 22, 4.5 Hz, 2 H, OCH_2CHF), 3.79 (dd, J = 23, 4 Hz, 2 H, OCH_2CHF), 4.54 (s, 2 H, CH_2OCH_2Ar), 4.74 (dm, J_{HF} = 44 Hz, 1 H, CHF), 4.91 (br s, 2 H, NH_2), 5.51, 5.59 (s, each 2 H, Pu-6-OCH_2Ar), 7.3–7.6 (m, 10 H, ArH), 7.74 (s, 1 H, C^8-H). Anal. ($C_{23}H_{24}FN_5O_3$) C, H, F, N.

(±)-9-[(2-Fluoro-3-hydroxy-1-propoxy)methyl]guanine (6). Hydrogenolytic debenzylation of **18** under the same conditions used to prepare **5** gave a 58% yield of white crystals. The analytical sample was further recrystallized from H_2O: mp 240–242 °C; analytical reversed-phase HPLC (Whatman ODS-3) in 95:5 H_2O–methanol; UV λ_{max}(pH 1) 255 nm (ϵ 12 500), 275 (sh, ϵ 8420), λ_{max}(pH 7) 252 nm (ϵ 13 200), 270 (sh, ϵ 9410), λ_{max}(pH 13) 264 nm (ϵ 12 100); 200-MHz ^{1}H NMR (Me_2SO-d_6) δ 3.52 (dm, J_{HF} = 23 Hz, 2 H, OCH_2CHF), 3.67 (dm, J_{HF} = 25 Hz, 2 H, OCH_2CHF), 4.56 (dm, J_{HF} = 48 Hz, 1 H, CHF), 4.97 (t, J = 5.5 Hz, 1 H, OH), 5.38 (s, 2 H, NCH_2O), 6.55 (br s, 2 H, NH_2), 7.85 (s, 1 H, C^8-H), 10.57 (br s, 1 H, N^1-H); mass spectrum (FAB), m/e 258 (M + H^+). Anal. ($C_9H_{12}FN_5O_3 \cdot H_2O$) C, H, N. ($C_9H_{12}FN_5O_3 \cdot H_2O$) C, H, N.

(±)-1-Fluoro-3-(trityloxy)-2-propanol (20). A solution of 3.76 g (36 mmol, based on 90% purity) of (±)-3-fluoro-1,2-propanediol (**19**)[26] and 11.15 g (40 mmol) of trityl chloride in 50 mL of dry pyridine was stirred at room temperature under N_2 for 3 days. The mixture was worked up as described for **8**, and the oily product was chromatographed on a silica gel column (elution with hexane containing 1–15% ethyl acetate) to give 7.31 g (60%) of white crystals: mp 89.5–92 °C; TLC in 9:1 hexane–ethyl acetate; 200-MHz ^{1}H NMR ($CDCl_3$) δ 2.29 (d, J = 6 Hz, 1 H, OH), 3.29 (dd, J = 6, 1 H, 2 H, CH_2OTr), 4.02 (dm, J_{HF} = 19 Hz, 1 H, OCH), 4.51 (dm, J_{HF} = 48 Hz, 2 H, CH_2F), 7.3–7.5 (m, 15 H, ArH). Anal. ($C_{22}H_{21}FO_2$) C, H, F.

(±)-2-(Benzyloxy)-1-fluoro-3-(trityloxy)propane (21). To a mechanically stirred suspension of 0.92 g (23 mmol) of NaH (60% in oil) in 20 mL of dry DMF maintained under N_2 was added dropwise a solution of 7.06 g (21 mmol) of **20** in 15 mL of DMF. When evolution of H_2 had ceased, 3.60 mL (3.96 g, 31 mmol) of benzyl chloride was added. After the mixture was stirred overnight at room temperature, an additional 0.10 g of NaH and 0.5 mL of benzyl chloride were added, and stirring was continued for 1 h. The mixture was then added to 500 mL of ice H_2O and extracted with ether. The ethereal layer was washed twice with H_2O, dried ($MgSO_4$), filtered, and concentrated to dryness. Trituration of the residue with ether–petroleum ether afforded 6.42 g (72%) of white crystals: mp 95–97 °C; TLC in 9:1 hexane–ethyl acetate; 200-MHz ^{1}H NMR ($CDCl_3$) δ 3.28 (d, J = 5 Hz, 2 H, CH_2OTr), 3.79 (d quint, J = 19, 5 Hz, 1 H, OCH), 4.58 (dd, J = 48, 5 Hz, 2 H, CH_2F), 4.65 (s, 2 H, $ArCH_2$), 7.25–7.50 (m, 20 H, ArH). Anal. ($C_{29}H_{27}FO_2$) C, H, F.

(±)-2-(Benzyloxy)-3-fluoro-1-propanol (22). Detritylation of **21** according to the method used for the preparation of **10**, after column chromatography on silica gel (gradient elution with 97:3 to 80:20 hexane–ethyl acetate), gave a 60% yield of colorless oil: TLC in 4:1 hexane–ethyl acetate; 200-MHz ^{1}H NMR ($CDCl_3$) δ 1.91 (t, J = 6 Hz, 1 H, OH), 3.6–3.9 (m, 3 H, $CHCH_2O$), 4.56 (dm, J_{HF} = 54 Hz, 2 H, CH_2F), 4.69 (s, 2 H, $ArCH_2$), 7.39 (s, 5 H, ArH).

(±)-2-(Benzyloxy)-1-(chloromethoxy)-3-fluoropropane (23). By the method used to prepare **11**, this material was obtained as a colorless oil from **22** in 100% yield by weight with purity estimated at approximately 90%: 60-MHz ^{1}H NMR ($CDCl_3$) δ 3.6–4.0 (m, 3 H, including d, J = 4.5 Hz at δ 3.77, $CHCH_2O$), 4.50 (dd, J = 47, 4.5 Hz, 2 H, CH_2F), 4.60 (s, 2 H, $ArCH_2$), 5.43 (s, 2 H, OCH_2Cl), 7.27 (s, 5 H, ArH).

(±)-9-[[2-(Benzyloxy)-3-fluoro-1-propoxy]methyl]guanine (25). A solution of 3.5 mmol of freshly prepared tris(trimethylsilyl)guanine (**24**)[6,15-17] in 5 mL of dry xylene was stirred under N_2 in an oil bath at 120–125 °C as 1.00 g (approximately 3.9 mmol based on 90% estimated purity) of **23** was added dropwise over 25 min. Stirring under N_2 was continued at 125 °C for 8 h. Concentration of the solution in vacuo gave a residual oil, which was treated with 10 mL of 1-propanol and 3.5 mL of glacial acetic acid and heated to reflux for several min. Crystallization occurred on cooling, and this solid was isolated on a filter. By NMR, this material appeared to be contaminated with about 10% of the 7-alkylated isomer. Recrystallization from a mixture of 10 mL of 1-propanol and 4 mL of glacial acetic acid gave 380 mg (31%) of >98% 9-isomer as a white solid: mp 189–195 °C; TLC in 9:1 chloroform–methanol; 200-MHz ^{1}H NMR (Me_2SO-d_6) 3.5–3.8 (m, 3 H, $CHCH_2O$), 4.48 (dm J_{HF} = 48 Hz, 2 H, CH_2F), 4.56 (s, 2 H, $ArCH_2$), 5.38 (s, 2 H, NCH_2O), 6.56 (br s, 2 H, NH_2), 7.32 (m, 5 H, ArH), 7.86 s, 1 H, C^8-H), 10.62 (br s, 1 H, N^1-H).

(±)-9-[(3-Fluoro-2-hydroxy-1-propoxy)methyl]guanine (7). A mixture of 208 mg (0.60 mmol) of **25**, 200 mg of 20% palladium hydroxide on carbon, 6 mL of cyclohexene, and 3 mL of absolute ethanol was stirred at reflux under N_2. After 1 day, an additional 100 mg of palladium hydroxide catalyst was added, and stirring at reflux was continued for 5 h. The mixture was filtered while hot through Celite. The filter cake was resuspended in H_2O,

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heated on a steam bath, and again filtered. The combined filtrates were concentrated in vacuo to give a residue that solidified on trituration with acetone. Recrystallization from H_2O gave an 18% yield of off-white crystals: mp 253 °C dec; homogeneous by reversed-phase analytical HPLC (Whatman ODS-3) in 95:5 H_2O–methanol; UV λ_{max}(pH 1) 255 nm (ϵ 12800), 271 (sh, ϵ 8680), λ_{max}(pH 7) 251 nm (ϵ 14100), 268 (sh, ϵ 9840), λ_{max}(pH 13) 265 nm (ϵ 11300); 200-MHz ^{1}H NMR (Me_2SO-d_6) δ 3.4–3.5 (m, 2 H, $CHCH_2O$), 3.76 (dm J_{HF} = 21 Hz, 1 H, OCH), 4.32 (dm, J_{HF} = 48 Hz, 2 H, CH_2F), 5.26 (d, J = 6 Hz, 1 H, OH), 5.37 (s, 2 H, NCH_2O), 6.54 (br s, 2 H, NH_2), 7.85 (s, 1 H, C^8-H), 10.60 (br s, 1 H, N^1-H); mass spectrum (FAB), m/e 258 ($M + H^+$). Anal. ($C_9H_{12}FN_5O_3 \cdot 0.33H_2O$) C, H, F, N.

Biochemical Methods. HeLa cells (TC52) (10^6 cells/mL), infected by HSV1 (Patton strain) at a multiplicity of 10 and harvested 8 h later were prepared by Bethesda Research Laboratories. A crude extract of these cells was obtained by suspending the cells (135 mg/mL) in 0.01 M Tris-HCl, pH 7.5, 2 mM DTT; after 30 min at 4 °C an equal volume of a buffer containing 0.7 M KPO_4, pH 7.5, 0.5 mM DTT, 0.17% Nonidet P-40, and 28% glycerol was added. After another 30 min at 4 °C the lysate was centrifuged at 100000g for 1 h; the supernatant was the "crude extract".

The same crude extract, after dialysis against 20 mM Tris-HCl, pH 7.5, 3 mM DTT, and 10% glycerol, was used as the source for the isolation of the viral TK by affinity chromatography, following a published procedure.[27] The effluent from the affinity column was further processed, following the method of Weissbach et al.[28] to obtain purified HSV1 DNA polymerase (phosphocellulose fraction).

"**Combined phosphorylation**" was carried out at 37 °C in a final volume of 200 μL. The incubation mixture contained 50 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 5 mM ATP; 2 mM DTT; 2.5 mM NaF; 15 mM phosphocreatine; 5 units of creatine kinase; 100 μg of bovine serum albumin; 1 unit of HSV1 TK; 0.4 unit of GMP kinase; 25 μL of crude extract of HSV1-infected HeLa cells; and 40 μg of the nucleoside analogue, dissolved in 40 μL of 50% Me_2SO. At regular time intervals, a 10-μL sample of the incubation mixture was analyzed by HPLC, using a Micropac AX-10 column (Varian) and a linear gradient from 0.01 to 1 M KH_2PO_4 (adjusted to pH 3.0) in 7 min followed by 5 min at 1 M KH_2PO_4. The amounts of nucleoside and its mono-, di-, and triphosphate derivatives were determined by computation of the areas of the respective peaks using a VISTA 401 Data System (Varian).

For simple TK assays, the incubation mixtures contained the same components as in the "combined phosphorylation" except for the GMP kinase and the crude extract of HSV1-infected cells. Incubation was at 37 °C for 1 h.

For large-scale preparations of phosphorylated derivatives, the reaction mixtures were scaled up and the incubation was prolonged (18–24 h). The products were purified by preparative HPLC on Micropac AX-10 columns and desalted by DEAE chromatography with triethylammonium carbonate solutions, followed by lyophilization. The purified compounds gave a single peak in analytical HPLC and had the same UV spectrum as their unphosphorylated counterparts.

DNA Polymerase Inhibition. Samples (20 μL) of the "combined phosphorylation" incubation mixtures after 3 h of incubation at 37 °C were added to a new incubation mixture containing in a final volume of 100 μL 50 mM Tris-HCl buffer, pH 8; 5 mM $MgCl_2$; 1 mM DTT; 13 μg of bovine serum albumin; 30 μg of activated salmon sperm DNA; 0.05 mM each of the four deoxyribonucleoside triphosphates dATP, dGTP, dCTP, and dTTP, the latter labeled with ^{3}H in the 5-methyl (20 cpm/pmol); 10 μL of crude extract of HSV1-infected HeLa cells; and 0.1 M $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ was omitted when the cellular polymerases were assayed.[28] After 1-h incubation at 37 °C the reaction was stopped with 1 mL of 10% TCA, and the precipitates were collected on glass fiber filters (Whatman GFC) and washed three times with 5% TCA and once with ethanol. The radioactivity of the precipitates on the fiber disks was counted in Aquasol 2 (New England Nuclear).

The same incubation mixtures and assay conditions were used when the purified triphosphates of (*R*)- and (*S*)-iNDG were tested, except for the use of purified DNA polymerase instead of the crude extract of infected cells. In both the crude extract assay and the purified enzyme assay, velocities were proportional to enzyme concentration and product formation increased linearly with time of incubation under the conditions employed.

Nucleosides and nucleotides (other than those whose syntheses were described above), phosphocreatine, creatine kinase, and DTT were from Sigma. GMP kinase was from Boehringer-Mannheim. [^{3}H]Thymidine triphosphate came from New England Nuclear.

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