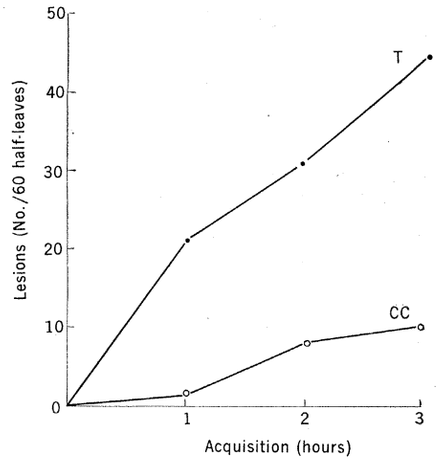

Fig. 2. Effect of length of acquisition period on transmission expressed as number of lesions produced by aphids that had previously probed on tobacco leaves covered with purified tobacco mosaic virus (5 mg/ml); *T*, aphids reared on tobacco; *CC*, aphids reared on Chinese cabbage. Thirty aphids were placed on each half-leaf.

dropped in the center of a test half-leaf. Half-leaves were placed on rings as described above. After 4 hours on the test leaves, aphids were killed by saturating the atmosphere in the petri dish with Thiodane. Lesions were counted after 3 days, and initially all lesions were indexed to see whether they were caused by TMV.

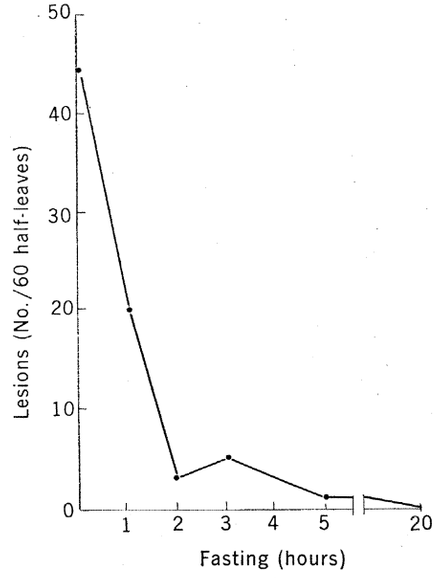

Fig. 3. Effect of fasting on virus transmission expressed as number of lesions produced by aphids that were starved for various periods after having stayed for 3 hours on tobacco leaves covered with purified tobacco mosaic virus (5 mg/ml). Thirty aphids were placed on each half-leaf.

For studying retention of TMV, aphids were left for 3 hours on leaves that were sprayed with purified TMV (5 mg/ml). They were placed in plastic bottles, starved for various periods, and then placed on healthy leaves as described.

Two controls were used. First, for each half-leaf receiving viruliferous aphids, another half-leaf was treated similarly except for receiving no aphids. This should monitor the level of background contamination inherent in the system. Second, 1200 aphids that had stayed on virus-covered (5 mg/ml) leaves for 3 hours were anesthetized, and their stylets were cut off; the insects were then transferred to test leaves. This should indicate whether aphids carry TMV on their tarsi and inoculate TMV by walking over the leaf surface.

We showed that *M. persicae* can acquire TMV from virus-covered leaves, carry it to healthy leaves, and inoculate the virus. Lesions on *N. glutinosa* developed randomly over the leaf surface and only rarely in areas where aphids had been dropped. Transmission was noticeable after aphids had probed on leaves covered with 0.01 mg of purified TMV per milliliter, and it increased in efficiency up to 1 mg/ml (Fig. 1). Aphids could also acquire TMV from leaves covered with crude extracts (frozen infected tissue extracted without buffer in an Erweka juice extractor) producing an average of 16 lesions per 60 leaves. Efficiency of transmission depended on period of acquisition and previous feeding history of the aphids (Fig. 2). Aphids from *Brassica pekinensis* were less efficient vectors than aphids reared on tobacco; they were more restless, and began to drop off after 4 to 5 hours on tobacco leaves. Aphids could retain TMV for several hours after they had left the leaves, but transmission decreased after 2 to 3 hours of fasting (Fig. 3). No lesions developed in the controls. Aphids whose stylets had been cut off were less mobile than normal aphids, but their mobility increased greatly after application of insecticide.

Previous work has shown that aphids can acquire and release TMV, but no transmission was demonstrated (*3*, *4*). Natural transmission of TMV has been claimed, but several of these claims could not be substantiated (*1*, *2*).

We conclude that the transmission we obtained did result from the probing or feeding of aphids on the test leaves (or both) because aphids without stylets did not produce lesions, and the transmission was less efficient with aphids not adapted to tobacco.

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9 April 1969

Irreversible Inhibition of Nuclear Exoribonuclease by Thymidine-3′-Fluorophosphate and p-Haloacetamidophenyl Nucleotides

Abstract. *Exoribonuclease purified from Ehrlich ascites tumor cell nuclei and in intact HeLa cell nuclei is irreversibly inactivated by low concentrations of* p-*bromo- and* p-*iodoacetamidophenyl nucleotides and by thymidine-3′-fluorophosphate. Iodoacetate, bromoacetate, and thymidine-5′-fluorophosphate do not affect the enzyme. Although* p-*haloacetamidophenyl nucleotides inactivate ribonucleic acid polymerase of isolated HeLa cell nuclei, thymidine-3′-fluorophosphate does not affect the activity of this enzyme in vitro.*

Inhibitors that form covalent bonds with enzymes at their catalytic or regulatory active sites (active-site-directed irreversible enzyme inhibitors) are important tools for investigating the structure and function of many enzymes (*1*). In conjunction with studies on the cellular function and molecular mechanism of action of a nuclear exoribonuclease that selectively destroys single-stranded or rapidly labeled RNA (*2*), we have synthesized a new series of *p*HAP (*3*) nucleotides which irreversibly inhibit this enzyme. The aim of these studies is to develop new agents which will be specific and potent

enough to aid investigation of the destruction of RNA in the cell nucleus in vivo, by selectively blocking intranuclear destruction of newly synthesized, rapidly labeled RNA. We report here that *p*HAP nucleotides inactivate purified exoribonuclease extracted from Ehrlich ascites tumor cell nuclei (*2*) and the enzyme in intact nuclei isolated from HeLa cells (see cover) grown in suspension culture (*4*). The nuclear exoribonuclease of HeLa cells and Ehrlich ascites tumors is also inactivated by T3F but not by T5F.

Compounds used in enzyme inactivation studies are shown in Figs. 1 and 2. The 3′-*p*HAP nucleotides (Fig. 1A) were prepared (*5*) from thymidine-3′-*p*-nitrophenyl phosphate (*6*) by reduction of the nitro group (hydrogenation with palladium-on-carbon catalyst), followed by treatment with the appropriate haloacetic anhydride. The nucleoside-3′,5′-di-*p*HAP-phosphates (Fig. 1B) were prepared by treating thymidine with di-*p*-nitrophenyl phosphorochloridate (*7*), followed by mild alkaline hydrolysis which yielded thymidine-3′5′-di-*p*-nitrophenyl phosphate, which in turn was then hydrogenated and treated with a haloacetic anhydride to yield the desired compounds. The dinucleotide thymidylyl (3′→ 5′)thymidine-3′,5′-di-*p*IAP phosphate (Fig. 1C) was made in an analogous fashion from thymidylyl(3′→ 5′) thymidine (*8*). The compounds T3F and T5F (Fig. 2) were made by the method of Wittmann (*9*). All compounds were homogeneous in at least two paper chromatographic systems and gave appropriate analyses for carbon, hydrogen, nitrogen, phosphorus, and halogen.

Preparation and properties of purified exoribonuclease have been described (*2*). The method for isolating intact HeLa cell nuclei (*4*) was the same as that used for the preparation of Ehrlich tumor cell nuclei (*2*), except that the HeLa cells were first treated with 0.01*M* NaCl and 0.001*M* potassium phosphate, *p*H 6.8, before being lysed with Triton N-101 detergent. The isolated HeLa nuclei were free of cytoplasmic contamination, as determined both by phase and electron microscopy (*4*). Degradation of poly A by purified exoribonuclease to yield 5′-AMP was done under standard conditions (*2*). When poly A was degraded under similar conditions by isolated HeLa nuclei, the product was almost exclusively 5′-AMP. Only traces of oligonucleotides were detectable in the reaction mixture (either in early or late stages of digestion) by thin-layer chromatographic analysis (*2*). Thus the nuclear exoribonuclease described in a variety of mouse tissues (*2*) also exists in HeLa nuclei, where it is the principal agent which degrades poly A at *p*H 7.4. Although inorganic phosphate (P_i) stimulated the conversion of poly A to 5′-AMP, the reaction was not dependent on P_i, nor did we find any evidence that adenosine-5′-diphosphate is either a transient intermediate or final product of the reaction.

Table 1. Inactivation (percent) of purified nuclear exoribonuclease by preliminary incubation at 25°C with *p*HAP nucleotides. Structures of compounds are shown in Fig. 1.

Figure	Compound	Concentration of inhibitor during preliminary incubation*					
		1×10^{-3} Molar		1×10^{-4} Molar		1×10^{-5} Molar	
		Exp. A	Exp. B (control)†	Exp. C	Exp. D (control)†	Exp. E	Exp. F (control)†
1A	R=H	None	None				
1A	R=$COCH_3$	None	None				
1A	R=$COCH_2Cl$	15	None				
1A	R=$COCH_2Br$	25	None	None‡	None‡	None	None
1A	R=$COCH_2I$	45	6	12‡	None‡	None	None
1B	R and R′=H			None	None		
1B	R and R′=$COCH_3$			None	None		
1B	R and R′=$COCH_2Cl$			None	None		
1B	R and R′=$COCH_2Br$			48	9	11	None
1B	R and R′=$COCH_2I$			51	8	15	None
1C	R and R′=$COCH_2I$			58§	10§	28	None
	Bromoacetate	None	None				
	2-Bromoacetamide	10	None	None‡	None‡	None	None
	Iodoacetate	None	None				
	2-Iodoacetamide	12	None	None‡	None‡	None	None

* The concentration of inhibitor during the final incubation of enzyme with substrate at 37°C was one-tenth of the concentration during preliminary incubation at 25°C. † The inhibitor was added to enzyme (preliminary incubation without inhibitor) immediately after the addition of substrate. The resultant concentration of inhibitor during the final incubation of enzyme with substrate at 37°C was thus identical for any pair of figures (in experiments A versus B, in C versus D, in E versus F) comparing the effects of preliminary incubation without and with inhibitors. ‡ The preliminary incubation of the enzyme with inhibitor was at $2 \times 10^{-4}M$. § The preliminary incubation of the enzyme with inhibitor was at $5 \times 10^{-5}M$.

The inactivation of exoribonuclease by *p*HAP nucleotides was found to be dependent on preliminary treatment of the enzyme with the inhibitors (Table 1). In experiments A, C, and E, purified enzyme [50 μl of solution, containing 0.5 unit of enzyme (from Ehrlich tumor) dissolved in a mixture containing 0.1*M* tris-HCl (*p*H 8.0), DTT, 0.001*M*, and BSA, 2 mg/ml] was first incubated at 25°C for 2 hours with inhibitors in 0.1*M* tris-HCl, *p*H 8.0 (50 μl). Substrate solution (900 μl) was then added to give a final reaction mixture containing 0.005*M* poly A, (adenine equivalent, randomly labeled with 0.2 μc of ^3H in the adenine moiety); 0.1*M* tris-HCl, *p*H 7.4; 0.002*M* $MgCl_2$; 0.025*M* potassium phosphate, *p*H 7.4; 0.0003*M* DTT; and BSA, 200 μg/ml. The final reaction mixture was then incubated at 37°C for 20 minutes, and the reaction was stopped by addition of 1 ml of ice-cold 0.8*M* perchloric acid. The tubes were centrifuged at 0°C for 30 minutes at 1900*g*, and radioactivity present in the supernatant was measured in a liquid scintillation counter. In the respective control experiments (B, D, and F, Table 1), the enzyme was first incubated at 25°C for 2 hours without inhibitor; substrate solution was then added, this being followed immediately by addition of inhibitor and incubation at 37°C for 20 minutes. The enzyme retained complete activity during the preliminary incubation at 25°C without inhibitor. Table 1 (experiments A, C, and E) shows that when *p*HAP nucleotides were preincubated with the tumor enzyme they inactivated exoribonuclease; the respective control experiments (B, D, and F) show that the inhibition of the enzyme by these agents was dependent on the preliminary incubation. In contrast, the degree of inhibition of exoribonuclease caused by a competitive inhibitor, such as adenylate tetranucleotide terminated with a 2′,3′-cyclic phosphate group, ApApApA > p (*2*), was independent of preliminary incubation. The *p*-chloroacetamidophenyl nucleotides were much weaker inhibitors than the corresponding bromo- and iodo- compounds, and the *p*-aminophenyl and *p*-acetylaminophenyl analogs did not cause any irreversible inhibition at concentrations at which the bromo- and iodoacetamidophenyl nucleotides were potent. The inactivation of the enzyme

by a bromoacetyl or iodoacetyl group was dependent on the affinity of the enzyme for the nucleotide ligand to which the inactivating group was attached (Table 1). Thus, bromoacetate, 2-bromoacetamide, iodoacetate, or 2-iodoacetamide were extremely weak inactivators; however, suitable placement of bromoacetyl or iodoacetyl groups on a nucleotide ligand made these extremely potent inactivating groups. It has been shown (*2*) that increasing the chain length (and negative charge) of a homologous series of reversible oligonucleotide inhibitors increases the binding affinity of such reversible inhibitors for the enzyme. Similarly (Table 1, experiments A, C, and E), the ability of an iodoacetyl group to inactivate exoribonuclease was greatly enhanced by increasing the negative charge on the nucleotide ligand to which the iodoacetyl group was attached.

Results similar to those in Table 1 were also obtained when the effects of *p*IAP nucleotides on the exoribonuclease of intact HeLa cell nuclei were studied. When 10^7 nuclei were preincubated at 37°C for 30 minutes at *p*H 7.0 in 0.25 ml of 0.32*M* sucrose containing 0.002*M* $MgCl_2$, 0.002*M* tris-HCl, and 0.001*M* potassium phosphate, and then assayed under standard conditions for their ability to degrade poly A to 5′-AMP, it was found that they lost only 10 percent of their original enzyme activity due to preliminary incubation alone. However, when these nuclei were incubated at 37°C for 30 minutes in the above sucrose solution, containing also $1 \times 10^{-4}M$ TDI, and then assayed, there was a 58 percent inhibition of activity (over and above the loss of activity due to preliminary incubation alone). In contrast, the enzyme activity in the control nuclei [to which the drug was added after the addition of substrate, immediately before the final incubation (the protocols of these experiments followed those of experiments B, D, and F in Table 1)] was inhibited less than 10 percent. Preliminary incubation of thymidine-3′,5′-di-*p*-acetamidophenyl phosphate (1×10^{-4} *M*) with HeLa cell nuclei did not cause any inactivation of exoribonuclease, whereas iodoacetate and iodoacetamide (2×10^{-4} *M*) caused less than 10 percent inactivation.

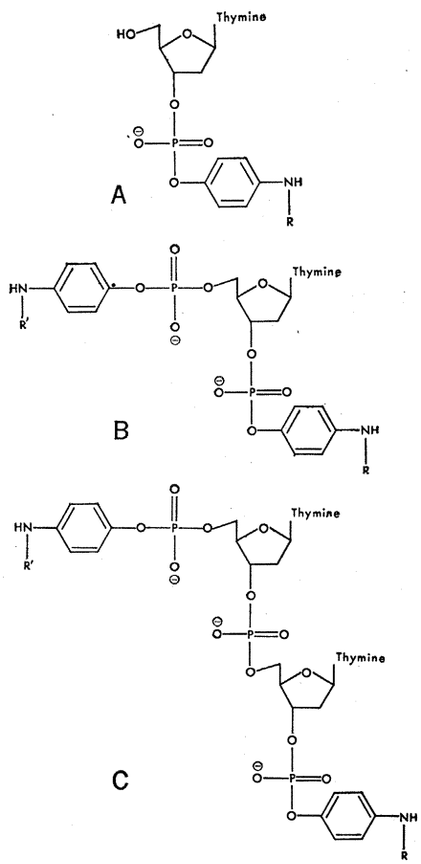

Fig. 1. Structures of *p*-haloacetamidophenyl nucleotides.

The isolated HeLa nuclei can also be used to assay the effects of nuclease inhibitors on RNA polymerase activity. Nuclei were preincubated at 37°C for 15 minutes (in the same sucrose solution used previously, with 0.001*M* DTT added) and then assayed for RNA polymerase (*10*) in the presence of 0.4*M* ammonium sulfate. The reaction was dependent on the addition of all four ribonucleoside-5′-triphosphates and was inhibited by actinomycin D. When TDI ($1 \times 10^{-4}M$) was preincubated with HeLa nuclei, it inactivated RNA polymerase by 49 percent (over and above the 15 percent loss of activity due to preliminary incubation without inhibitor). In contrast, neither 0.01*M* T3F nor T5F caused any inactivation of HeLa nuclear RNA polymerase during preliminary incubation, nor did T5F (0.01*M*) inactivate HeLa nuclear exoribonuclease. However, T3F did inactivate HeLa exoribonuclease by 57 and 38 percent when preincubated (at 37°C for 30 minutes) at 1×10^{-2} and $5 \times 10^{-3}M$, respectively, with nuclei. Similar results were obtained with Ehrlich tumor enzyme. The inhibition of exoribonuclease by T3F was dependent on preincubation, because in the appropriate controls (the protocols were again like those of experiments B, D, and F, Table 1) addition of T3F to the reaction mixture after addition of substrate caused only 13 and 5 percent loss of activity, respectively (*11*). Thus, the results obtained with T3F show that it is possible for an irreversible inhibitor to inactivate exoribonuclease, without inactivating RNA polymerase, in HeLa cell nuclei.

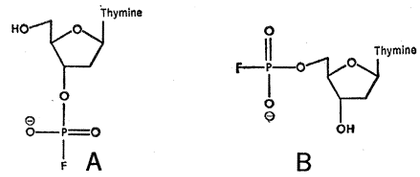

Fig. 2. Thymidine-3′-fluorophosphate (A) and 5′-fluorophosphate (B).

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3. The abbreviations used are: *p*HAP, *p*-haloacetamidophenyl; *p*IAP, *p*-iodoacetamidophenyl; T3F, thymidine-3′-fluorophosphate; T5F, thymidine-5′-fluorophosphate; poly A, polyadenylic acid; 5′-AMP, adenosine-5′-monophosphate; DTT, dithiothreitol; BSA, bovine serum albumin; TDI, thymidine-3′,5′-di-*p*-iodoacetamidophenylphosphate (Fig. 1B, R and R′ = $COCH_2I$); tris, tris(hydroxymethyl)aminomethane.
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