THE SYNTHESIS OF SOME TRITIUM-LABELLED
TERMINATORS
OF DNA SYNTHESIS

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SUMMARY

2',3'-Dideoxythymidine, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine-5'-phosphonate, thymidine-5'-phosphonate, labelled with tritium in the carbohydrate residue; thymidine-5'-methylphosphonate labelled in the methyl of the phosphonate group and 2',3'-dideoxyadenosine labelled in the base and/or carbohydrate residue were prepared by hydrogenolysis with tritium gas. The reaction of solid state catalytic hydrogenation was studied and tritium-labelled azydothymidine, azydothymidine-5'-phosphonate, acyclovire, acyclovire-5'-phosphonate were synthesized. A number of general approaches to the synthesis of tritium-labelled terminators of DNA synthesis were formulated.

KEY WORDS   *2',3'-Dideoxythymidine, acyclovire, 2',3'-dideoxyadenosine, azydothymidine, thymidine-5'-methylphosphonate, thymidine-5'-phophosphonate, 2',3'-dideoxycytidine-5'-phosphonate,*

INTRODUCTION

The interest in different terminators of DNA has been growing of late due to the search for inhibition approaches to the development of retroviruses, including the human immunodeficit virus[1],[2]. Different terminators of DNA synthesis labelled by radionuclides[3], and particularly by tritium[4-6], provide a necessary tool for investigations of this kind.

Since DNA synthesis terminators are analogous of nucleotides, traditional methods of synthesis of tritium labelled components of nucleic acids can be used for the synthesis of these tritium labelled compounds. These are mainly the reactions of

hydrogenolysis[7,8] and isotopic exchange[9] in solution with tritium gas. The application of the hydrogenolysis reaction with tritium requires the synthesis of special precursors and, consequently, is a rather complicated procedure. The number of potential inhibitors of retroviruses continues to grow. In connection with the above described facts, the elaboration of common approach to the production of tritium labelled terminators of DNA synthesis seems to be urgent. Therefore, parallel to the hydrogenolysis reaction we studied the solid state synthesis reaction of tritium labelled compounds of the class described. We have previously shown that solid state reaction provide an effective means of synthesis of tritium labelled nucleic acid components[10].

In this study, the synthesis of tritium labelled terminators of DNA synthesis by using hydrogenolysis with tritium gas is described. 2',3'-Dideoxythymidine, 2',3'-dideoxyadenosine, 2',3'-dideoxycytidine-5'-phosphonate, thymidine-5'-phosphonate, were labelled with tritium in the carbohydrate residue; thymidine-5'-methylphosphonate with tritium in the methyl group of the phosphonate moiety, and 2',3'-dideoxyadenosine labelled in the base and/or in the carbohydrate residue.

The solid state catalytic hydrogenation (SCH) of compounds suggested as DNA synthesis terminators was studied. Azydothymidine (12), azydothymidine-5'-phosphonate (13), acyclovire (14), acyclovire-5'-phosphonate (15), and 2',3'- dideoxy-2',3'-didehydrothymidine (1) were all used. The latter compound was studied as a precursor for the synthesis of 2',3'-dideoxythymidine. The effect of different catalysts and temperature and solid state components ratio on the yield and tritium incorporation into the final product were studied. These experiments were carried out with tritium-protium (1:1000) mixture. Finally the synthesis of the labelled compounds were performed with carrier-free tritium gas(>96%).
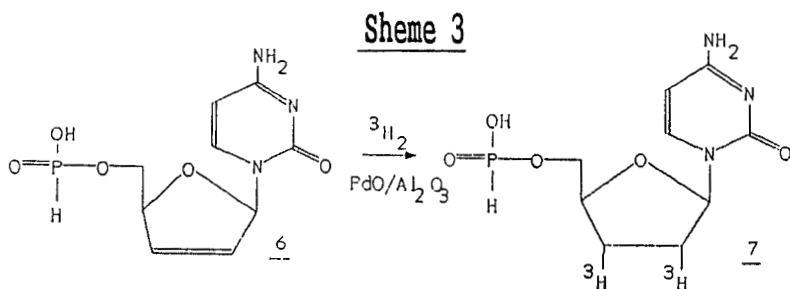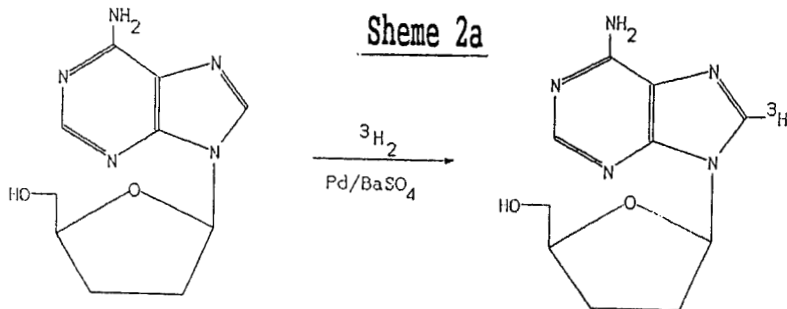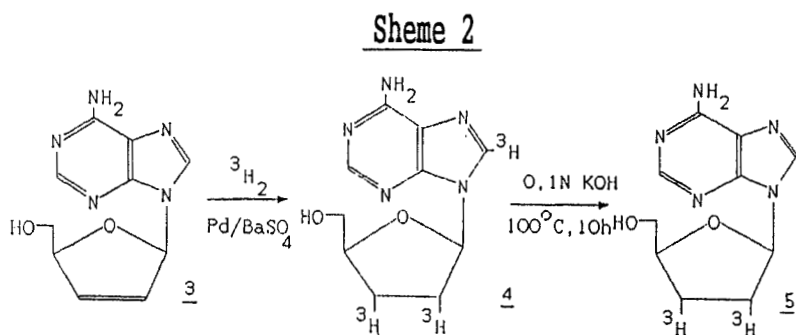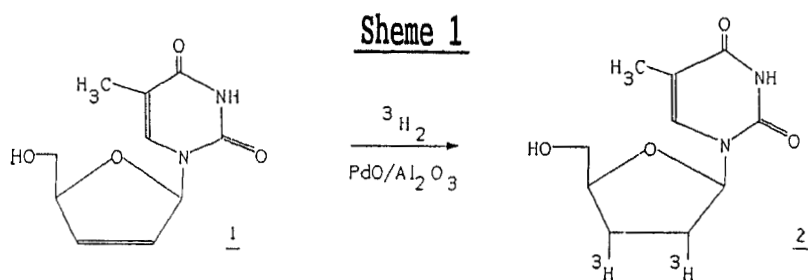
RESULTS AND DISCUSSION

2',3'-Dideoxy-2',3'-didehydrothymidine(1), 2',3'-dideoxy-2',3'-didehydroadenosine (3), 2',3'-dideoxy-2',3'- didehydro cytidine -5'-phosphonate (6), thymidine-5'-iodiummethylphosphonate (8), 2'-bromothymidine-5'-phosphonate (10) served as precursors of the synthesis for the hydrogenolysis with tritium. Schemes 1-5 show the precursors and tritium-labelled products.
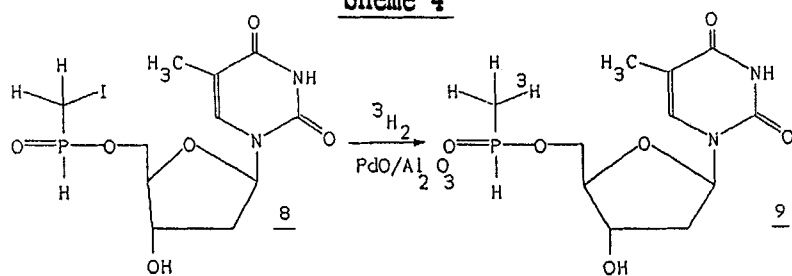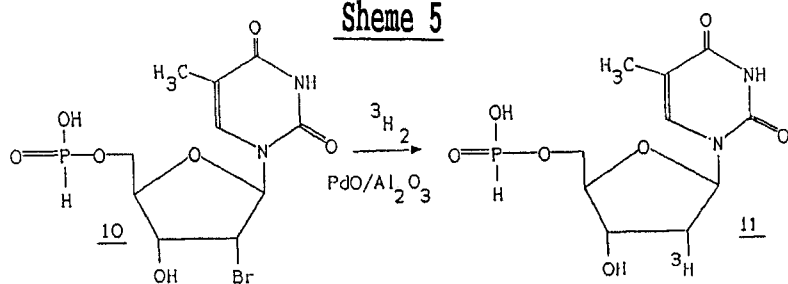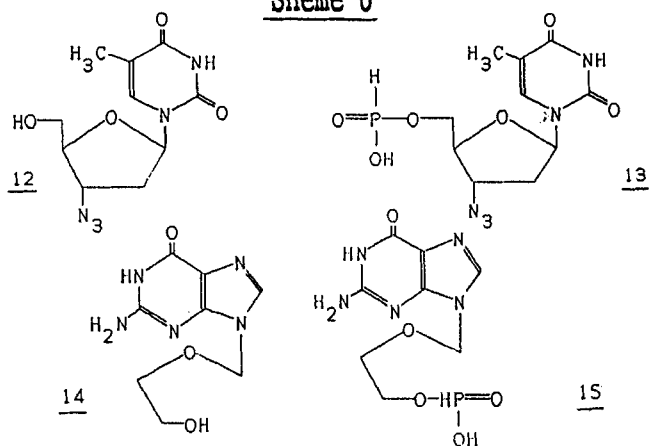
The reduction of the 2',3'-double bond (3) (Scheme 2) was accompanied by tritium isotope exchange with the C-8(H) of adenine. This fact was proved by the abnormally high specific activity of the compound (73.2 Ci/mmol) corresponding to the incorporation of about 3 atoms into the molecule. In the ^{3}H-NMR spectrum a signal at 8.25 ppm corresponding to tritium in the adenine C-8 position was registered. After re-exchange at C-8, the preparation (5) was obtained, containing tritium in the nucleoside portion of the molecule.

The catalytic heterogeneous isotope exchange reaction was used for the introduction of tritium into the purine portion of 2',3'-dideoxyadenosine (Scheme 2a).

The product was subjected to acid hydrolysis in order to determine the specificity of tritium incorporation. The heterocyclic base was isolated and its specific activity determined. These experiments showed that 97-98% of the tritium was localized within the nucleoside portion of the molecule in compounds (2), (5), (11). In compound (7), about 93% of the tritium was contained in the nucleoside part of the molecule; a parallel reaction of tritium isotope exchange with the pyrimidine base, particularly with the proton at C-6 of cytosine[11] , probably takes place in the reaction proceeding according to Scheme 3.

Scheme 6 shows the compounds used for studying the SCH reaction.

Sheme 1

$$\xrightarrow[\text{PdO/Al}_2\text{O}_3]{^3\text{H}_2}$$

1   **2**

Sheme 2

$$\xrightarrow[\text{Pd/BaSO}_4]{^3\text{H}_2}$$

$$\xrightarrow[100°\text{C}, 10\text{h}]{0,1\text{N KOH}}$$

3   **4**   **5**

Sheme 2a

$$\xrightarrow[\text{Pd/BaSO}_4]{^3\text{H}_2}$$

Sheme 3

$$\xrightarrow[\text{PdO/Al}_2\text{O}_3]{^3\text{H}_2}$$

6   **7**

Sheme 4

$$8 \xrightarrow[\text{PdO/Al}_2\text{O}_3]{^3\text{H}_2} 9$$

Sheme 5

$$10 \xrightarrow[\text{PdO/Al}_2\text{O}_3]{^3\text{H}_2} 11$$
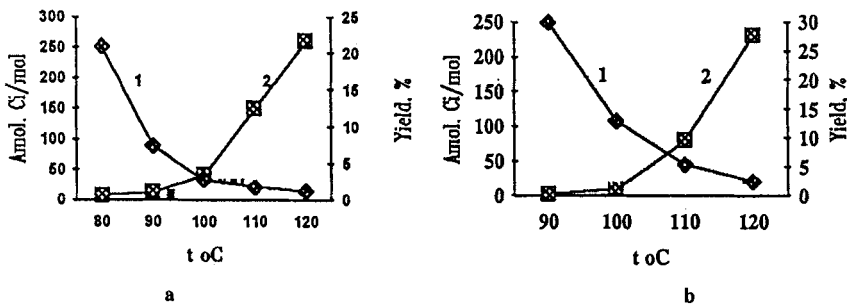
Sheme 6

12   13

14   15

a

b

Figure 1. Dependence on temperature of chemical yield (1) and A_{mol} (2) of ^{3}H-Azidothymidine in reaction of SCH with tritium on Pd/CaCO$_3$ (a) and Pd/BaSO$_4$ (b).

a

b

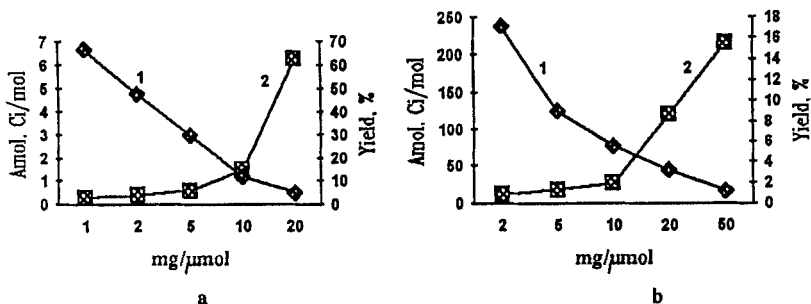**Figure 2.** Dependence on solid state components ratio of chemiccal yield (1) and A_{mol} (2) of ^{3}H-Azidothymidine in reaction of SCH with tritium a:Pd/BaCO$_3$,90 ^{o}C, 30 min; b:Pd/BaSO$_4$,120 ^{o}C, 30 min.

Fig.1 shows the dependence on temperature of the chemical yield and specific activity (A_{mol}) of azydothymidine (12) produced in the reaction of SCH with tritium. The data presented show that the yield of the final compound decreases and its A_{mol} grows with temperature growth. More degradation of compound (12) was observed when using Pd/CaCO$_3$. The dependence of the chemical yield and A_{mol} of tritium-labelled (12) on the solid state components ratio is given in Fig.2. These results demonstrate that A_{mol} of the final compound grows and its yield decreases with the increase in the reaction mixture of the catalyst's total mass fraction. Varying the nature of palladium catalyst, the temperature of the

reaction and components ratio in the solid state makes it possible to vary the yield and A_{mol} of the final compound. To achieve the higher A_{mol} values, the use of a barium sulfate catalyst is preferable. With the use of this catalyst, the maximum yield is achieved with a catalyst-compound ratio of 10 mg/µmol.

Table 1 shows the results obtained by studying the SCH reaction of azydothymidinephosphonate (**13**). As in the case of (**12**), the largest yield of (**13**) was observed for a catalyst based on barium sulfate.

Table 1. Solid State Catalytic Hydrogenation of
Azydothymidinephosphonate. The Catalyst is $Pd/BaSO_4$

t^oC	Yield, %	A_{mol}, Ci/mol
60	53	1.1
80	43	3.2
100	45	3.3
120	49	4.0
140	45	4.7
160	20	19.0
180	traces	---- -

Table 2 shows results for the effect of various catalysts and the temperature on the yield and A_{mol} of acyclovire (**14**) obtained in the SCH reaction. These results show that the nature of the palladium catalyst carrier has no effect on the value of A_{mol} and, in particular, on the yield of (**14**). As in previous experiments, the A_{mol} increase in accompanied by a decrease in the final compound yield. This effect is mostly observed when the reaction temperature is raised above 240^oC.

Table 2. Solid State Catalytic Hydrogenation of Acyclovire

t^oC	Catalyst	Yield,%	A_{mol}, Ci/mol
160	$Pd/CaCO_3$	73.0	47
160	$Pd/BaSO_4$	71.0	41
180	$Pd/CaCO_3$	67.0	68
180	$Pd/BaSO_4$	65.0	50
200	$Pd/CaCO_3$	78.0	135
200	$Pd/BaSO_4$	77.0	120
220	$Pd/CaCO_3$	47.0	210
220	$Pd/BaSO_4$	52.0	193
240	$Pd/CaCO_3$	7.7	435
240	$Pd/BaSO_4$	6.2	415

Similar studies were carried out with acyclovirephosphonate (<u>15</u>), (Table 3). In this case, tritium incorporation into (<u>15</u>) hardly increase, while the yield considerably decreases.

Table 3. Solid State Catalytic Hydrogenation of
        Acyclovirephosphonate. The Catalyst is Pd/CaCO$_3$.

$t^\circ C$	Yield, %	A_{mol}, Ci/mol
180	27.0	189
200	16.1	197
220	9.8	200

The influence of reaction conditions on the solid state catalytic hydrogenation of compound (<u>1</u>) to (<u>2</u>) is shown in Table 4. The increase in tritium incorporation into the final compound is accompanied a decrease in its degradation.

Table 4. Synthesis of Tritium Labelled 2',3'-Dideoxythymine.

Catalyst	Conditions		Yield, %	A_{mol},Ci/mol
	$t^\circ C$,	min.		
Pd/BaSO$_4$	120	30	54.0	134
Pd/CaCO$_3$	120	30	56.0	144
Pd/CaCO$_3$	130	30	12.5	237
Pd/CaCO$_3$	140	30	7.0	360
Pd/CaCO$_3$	150	30	5.2	434
Pd/Al$_2$O$_3$	20	120	90.0	123
	(liquid state)			

Table 5 shows some features of the synthesis of the above mentioned preparations when carrier-free tritium gas is employed. Table 6 shows the tritium distribution in acyclovire (<u>14</u>), according to ^{3}H-NMR data; over 70% of the tritium is incorporated into the end methylene group of the nucleoside portion of the molecule.

On the basis of the experimental data obtained, a number of general approaches to the synthesis of tritium labelled terminators of DNA synthesis can be formulated. The hydrogenolysis of specially synthesized precursors with tritium gas is very convenient for the production of preparations specifically labelled by tritium. If the specificity of tritium incorporation

is not particularly good or the synthesis of precursor is difficult, the SCH reaction with tritium gas seems to provide the most convenient method. It is important that this be realized when developing a common procedure.

Table 5. Synthesis of Tritium-Labelled Terminators of DNA Synthesis.

Compound	Synthesis Conditions	A_{mol}, Ci/mmol	Final Compound Yield, %
2',3'-Dideoxythymidine	Hydrogenolysis	51.2	46.0
2',3'-Dideoxythymidine	Pd/CaCO$_3$, 120°C	66.2	52.1
2',3'-Dideoxyadenosine(4)	Hydrogenolysis	73.2	79.8
2',3'-Dideoxyadenosine(5)	Re-exchange at C-8	52.4	80.3
[8-^{3}H]2'3'-Dideoxyadenosine	Isotope exchange	23.2	81.0
2',3'-Dideoxy-cytidinephosphonate(7)	Hydrogenolysis	59.1	74.3
Thymidinemethyl-phosphonate(9)	Hydrogenolysis	17.2	70.3
Thymidine-phosphonate(10)	Hydrogenolysis	18.5	71.5
Azydothymidine(12)	Pd/BaSO$_4$ 120°C	30.0	3.2
Azydothymidine-phosphonate(13)	Pd/BaSO$_4$ 120°C	6.3	69.8
Acyclovire(14)	Pd/CaCO$_3$ 120°C	124.0	8.1
Acyclovire phosphonate(15)	Pd/CaCO$_3$ 120°C	56.5	27.6

Table 6. Tritium Distribution in Acyclovire (from ^{3}H-NMR)

δ ppm	Position	Relative content of tritium, %	^{3}H atoms per molecule
3.48 3,46	2',3'	74.07	3.15
5.34	1'	11.46	0.49
7.82	8	14.47	0.62

EXPERIMENTAL

UV absorption spectra were registered on a SF-16 spectrometer. Tritium NMR spectra were registered in D$_2$O by using an AS 250 Bruker NMR spectrometer. Radioactivity was determined on a liquid scintillation counter. Catalysts 5% Pd/CaCO$_3$ and 5% Pd/BaSO$_4$ from Fluka", 5% PdO/Al$_2$O$_3$ from St. Petersburg. The preliminary purification of labelled nucleosides was performed by chromatography on G-10 Sephadex (16x900 mm), eluent water, 25 ml/h. Final purification was performed by HPLC. Table 7 shows the

isolation conditions of tritium labelled compounds. Acyclovire and acyclovirephosphonate were obtained from Ivanovsky Institute of Virology (Prof. G.Galegov's laboratory).

Table 7. HPLC of Tritium-Labelled Terminators of DNA Synthesis.

Compound	Column	Mobile phase	Retention Time, min.
1	Separon SGX C 18	7% MeCN in 0.1 M TEAB	8.13
2	7 µm, 3.3x150 mm	pH 7.0 0.5 ml/min	9.89
3	Nucleosil 120-5	5% MeCN in 0.1 M TEAB	12.00
4,5	C 18 4.6x250 mm	pH 7.0 1.0 ml/min	18.50
6	Separon SGX C 18	2% MeCN in 0.1 M TEAB	12.10
7	7 µm, 3.3x150 mm	pH 7.0 0.5 ml/min	10.90
11			11.50
14	Separon SGX C 18	2% MeCN in 0.1 M TEAB	7.38
15	7 µm, 3.3x150 mm	pH 7.0 0.5 ml/min.	8.87
12	Separon SGX C 18	10% MeCN in 0.1 M	16.10
	5 µm, 3.3x150 mm	TEAB pH 7.0 0.5 ml/min.	
13	Nucleosil 120-5	10% MeCN in 0.1 M TEAB	8.01
	C 18 4.6x250 mm	pH 7.0 1.0 ml/min.	

Hydrogenolysis. Precursor solution, the catalyst and the magnet mixer were placed in a 5 cm^3 glass reaction vial. The vial was connected to a system for gaseous tritium, the system evacuated and tritium gas introduced into the vial at P=300 mm Hg. The reaction was carried out at room temperature, the reaction mixture being stirred with the magnet mixer. Upon reaction termination, excess tritium was removed from the vial and the catalyst separated by filtration. The filtrate was neutralized with 1 N HCl to pH 7. Labile tritium was removed by evaporation at 35-37 ^{O}C. The remaining solid was dissolved in water, and the labelled compound was isolated.

Solid State Catalytic Hydrogenation. The solid state mixture of the initial compound and catalyst was placed in a 5 cm^3 glass reaction vial. The vial was connected to a system for gaseous tritium, and pumping out was performed to a pressure of

less than 10^{-3} mm Hg; 95-97% tritium gas or tritium-protium mixture (1:1000) was then introduced into the vial. The vial was thermostated at a chosen temperature and suitable time and then cooled; the excess of tritium was then removed from the vial. The reaction products were washed with water and the catalyst separated by filtration. Labile tritium was removed by evaporation of the filtrate to dryness and the final product was isolated.

$[2',3'-^3H]2',3'$-Dideoxythymidine (2). 2.3 mg of precursor (1) (10 µmol) in 0.2 ml of 0.4 N KOH and 50 mg of 5% PdO/Al_2O_3 were taken for reaction. The reaction was carried out over the course of 120 min. After chromatography on Sephadex G - 10 and HPLC, 236 mCi of $[2',3'-^3H]2',3'$-dideoxythymidine (46% yield, 51.3 Ci/mmol), were obtained. The acid hydrolysis showed about 3% of the tritium was in the adenine residue.

$[8-^3H]2',3'$-Dideoxyadenosine. 2.35 mg (10 µmol) of $2',3'$-dideoxyadenosine in 0.2 ml of 0.1 N KOH with 60 mg of 5% $Pd/BaSO_4$ were taken for reaction. The reaction was carried out over the course of 3 hrs. The final compound was isolated on Sephadex G-10. A product with a specific activity of 23 Ci/mmol was obtained, the yield - 81%.

A analysis by HPLC method showed that the compound was homogeneous. The retention time of the preparation coincided with the retention time of the standard, (18.5 min.). The ^3H-NMR spectrum showed the tritium was localized at position C-8.

$[2',3'-^3H]2',3'$-Dideoxycytidinephosphonate (7). 4.6 mg of precursor (6) (16.7 µmol) in 0.3 ml of 0.4 N KOH and 60 mg of 5% PdO/Al_2O_3 were taken for reaction. The reaction was carried out over the course of 120 min. The final compound was isolated by HPLC, with a A_{mol} of 59.1 Ci/mmol, the yield being 74.3%. Acid hydrolysis showed 7% of the tritium to be contained in the pyrimidine residue.

Thymidine[methyl-^{3}H]methylphosphonate (**9**). 5.0 mg of precursor (**8**) (11 µmol) in 0.3 ml of 1 N KOH and 50 mg of 5% PdO/Al$_2$O$_3$ were taken for dehalogenation. The reaction was carried out over the course of 120 min. After isolation by HPLC, (**9**) was obtained with a specific activity of 17.2 Ci/mmol, the yield being 70.3%. Acid hydrolysis showed 2% of tritium to be in the pyrimidine residue.

[2'-^{3}H]Thymidinephosphonate (**11**). 3.85 mg of precursor (**10**) (10 µmol) in 0.2 ml of 0.4 N KOH and 25 mg of 5% PdO/Al$_2$O$_3$ were taken for debromination. The reaction was carried out over the course of 120 min. After chromatography on Sephadex G-10 and HPLC, (**11**) was isolated with a A$_{mol}$ of 18.5 Ci/mmol, the yield being 71.5%. Acid hydrolysis showed less than 3% of tritium to be in the thymidine part of molecule.

Acid Hydrolysis. About 5 mCi of tritiated compound were diluted with 1 mg of unlabeled analog and dissolved in 1.0 ml of HCl solution. Pyrimidine compounds were hydrolyzed during 4 h in 1 N HCl at 100°C. Purine compounds were hydrolyzed in 0.1 N HCl for 1 h at 100°C. After hydrolysis , hydrochloric acid was removed by evaporation at 37°C. Hydrolysate was analyzed on Sephadex G-10. Peaks of individual bases were isolated and their A$_{mol}$ determined.

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