

NUCLEOTIDES—III*

SYNTHESIS AND EFFECTS WITH CELLS IN CULTURE OF ^{131}I -LABELED 5-iodo-2'-DEOXYURIDINE-5'-PHOSPHATE†

ALEXANDER HAMPTON, E. GAMBETTA HAMPTON and MAXWELL L. EIDINOFF

Divisions of Nucleoprotein Chemistry and Biophysics,
Sloan-Kettering Institute for Cancer Research,
Sloan-Kettering Division of Cornell University Medical College, New York

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Abstract—Iodination of 2'-deoxyuridine-5'-phosphate has given a 60 per cent yield of ^{131}I -labeled 5-iodo-2'-deoxyuridine 5'-phosphate. This nucleotide was significantly less effective than 5-iodo-2'-deoxyuridine as an exogenous source of 5-iodouracil in the deoxyribonucleic acid of human cervical carcinoma cells, strain H.Ep. 1, growing in culture. 5-Iodo-2'-deoxyuridine-5'-phosphate was dephosphorylated by the horse serum present in the culture medium and also by H.Ep. 1 cells in a protein-free medium.

5-iodo-2'-DEOXYURIDINE is of interest as a biological growth inhibitor^{1, 2} and as an agent with chemotherapeutic effect in human neoplastic disease.^{3, 4} 5-Iodo-2'-deoxyuridine markedly inhibits the conversion of thymidine-5'-phosphate to thymidine-5'-diphosphate and thymidine-5'-triphosphate in mouse L5178Y ascites cells *in vitro*,⁵ and it has been suggested⁵ that this inhibition may be mediated by biosynthesized 5-iodo-2'-deoxyuridine 5'-phosphate. This nucleotide has been obtained by enzymic hydrolysis of deoxyribonucleic acid from *Escherichia coli* grown with 5-iodouracil⁶ and from mammalian cells grown in culture with 5-iodo-2'-deoxyuridine.^{2, 7} The present communication describes a convenient chemical synthesis of ^{131}I -labeled 5-iodo-2'-deoxyuridine 5'-phosphate and a study of its effectiveness as an exogenous source for the incorporation of 5-iodouracil into deoxyribonucleic acid of cells in culture.

Treatment of 2'-deoxyuridine with a mixture of iodine (0.9 mole equivalent) and nitric acid (2.3 equivalents), initially at pH 0, has given a 56 per cent yield of 5-iodo-2'-deoxyuridine.¹ This procedure involves regeneration of iodine by interaction of hydriodic and nitric acids. For its application to the iodination of deoxyuridine-5'-phosphate, minimization of hydrolytic formation of inorganic phosphate was desirable, and conditions were used in which the pH, initially at 0.3, could be expected to increase to 0.6 during the course of the reaction: the rate of acid hydrolysis of simple monoalkyl phosphates is known to be minimal at pH 0.5 to 0.75.⁸ A relatively small amount of inorganic phosphate was formed during the reaction. A nucleotide barium salt was isolated and found by paper chromatographic and electrophoretic analysis to contain only two ultraviolet-absorbing components; of these, the major compound corresponded in properties to 5-iodo-2'-deoxyuridine-5'-phosphate,

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while the minor one was similar to 1.5 per cent as much 2'-deoxyuridine-5'-phosphate. The ratio of 5-iodouracil to bound phosphorus in the barium salt was 1:1.3, presumably due to the presence therein of phosphorylated deoxyribose derivatives. Purification of 0.3-m-mole quantities was conveniently accomplished by paper chromatography. The product (yield, 60 per cent) was homogeneous toward paper chromatography and electrophoresis; it had a ratio of 5-iodouracil to deoxyribose to phosphorus of 1:1.0:1.1, and was converted to 5-iodo-2'-deoxyuridine by 5'-phosphomonoesterase. The ultraviolet-absorption characteristics of the nucleotide were closely similar to those of 5-iodo-2'-deoxyuridine, and its specific activity, as expected, was about one-half that of the ^{131}I -containing iodine used in the synthesis. Separation from 2'-deoxyuridine 5'-phosphate is readily accomplished also by column chromatography on the formate-form of an anion-exchange resin, and data for stepwise-elution are given in the Experimental section.

Actively dividing human cervical carcinoma cells, strain H.Ep.1,⁹ were exposed for 2 days in culture to equimolar concentrations of ^{131}I -labeled 5-iodo-2'-deoxyuridine and its 5'-phosphate, respectively. Replacement of thymine in deoxyribonucleic acid by 5-iodouracil was 25 per cent less with the nucleotide as exogenous source than with the nucleoside. Similar relative incorporations of 5-iodouracil occur in tissues of the rat when the same two compounds are administered *in vivo*.¹⁰

Dephosphorylation of 5-iodo-2'-deoxyuridine 5'-phosphate occurred during its incubation with cells in culture: after 1 day the concentration of the nucleotide in the medium decreased to 25 per cent of the initial value, and after 2 days dephosphorylation was essentially complete. The horse serum present in the medium possessed significant dephosphorylating activity, but incubation of the nucleotide with washed H.Ep.1 cells in a protein-free medium effected dephosphorylation at a comparable rate. Phosphatase activity of intact cells of *E. coli* in protein-free media has been reported for the 5'-phosphates of deoxyuridine, deoxycytidine and thymidine,¹¹ and for the 5'-phosphates of uridine, cytidine and adenosine¹² with some normal and neoplastic mammalian tissues. Location of the phosphatase activity at the cell surface is indicated for *E. coli*,¹¹ Ehrlich carcinoma cells¹² and rat liver cells.¹³ That 5-iodo-2'-deoxyuridine-5'-phosphate is less effective than the parent nucleoside as a source of intracellular 5-iodouracil is consistent with evidence, summarized elsewhere,¹² that phosphomonoesters penetrate natural membranes with difficulty.

H.Ep.1 cells were incubated with ^{131}I -labeled 5-iodo-2'-deoxyuridine and its 5'-phosphate for varying periods of time (Table 1), following which they were incubated in the absence of these compounds until 2 days after the outset of the experiment. Variations observed in 5-iodouracil-content of the deoxyribonucleic acid were in rough agreement with those expected of an asynchronously growing culture with a generation-time of the order of 40 hr.⁷ For example, the iodouracil content was about 100-fold greater after 48 hr than after 1 hr of exposure of the cells to the iodouracil-derivatives. The effectiveness of the nucleotide as a source of iodouracil in deoxyribonucleic acid was consistently 60–80 per cent that of the nucleoside, even with the shorter incubation periods (1 to 5 hr) in which extracellular dephosphorylation of the nucleotide was presumably less extensive. It is possible that during these short periods nucleotide molecules became bound by electrostatic forces to the cell surface, later to serve as a source of intracellular 5-iodouracil during growth in the medium lacking 5-iodouracil derivatives.

EXPERIMENTAL

5- ^{131}I -2'-deoxyuridine-5'-phosphate

A mixture of sodium 2'-deoxyuridine-5'-phosphate tetrahydrate* (220 mg; 0.519 m-mole), iodine- ^{131}I (118 mg; 0.464 m-mole; specific activity, 8.65 mc/m-mole), 1 N HNO_3 (2.08 ml) and chloroform (1 ml) was warmed under reflux for 4 hr. The mixture was diluted with water to 30 ml and extracted with ether (3×2 ml) to

TABLE 1. 5-iodo-2'-DEOXYURIDINE AND ITS 5'-PHOSPHATE AS SOURCES OF 5-iodouracil IN THE DNA OF H.Ep.1 CELLS *in vitro*

Hours of cell exposure to sources of 5-iodouracil	Hours of incubation after removal of sources of 5-iodouracil	% Replacement of DNA thymine by 5-iodouracil*		Relative effectiveness of sources (A/B)
		A Nucleotide as source	B Nucleoside as source	
1	47	0.17	0.24	0.71
2	46	0.30	0.39	0.77
5	43	0.50	0.60	0.83
27	21	7.63	13.7	0.56
48	0	23.9	34.5	0.69

* Averages from duplicate experiments. The method of determination is given under Experimental.

remove excess of iodine. The pH was adjusted to 7.5 with 1 N LiOH and aqueous barium acetate (0.8 ml of 2 M) was added. After 1 hr at 25° , barium phosphate was removed by centrifugation and washed twice with a small volume of water. Two volumes of ethanol were added to the combined supernatant fractions. After 2 hr at 2° , the white precipitate was collected by centrifugation, washed with ethanol-water (3:1; 2×3 ml), and dried over NaOH at 15 mm. It was dissolved in water (10 ml) and insoluble solid was removed and washed with water (2×2 ml). Addition to the supernatant fluids of 2 vols of ethanol gave 297 mg of crude nucleotide barium salt.† An aqueous solution (10 ml) of this product was evaporated across twelve strips of Whatman 3 MM filter paper (width 46 cm) and subjected to descending chromatography for 36 hr with *n*-butanol-acetic acid-water (5:2:3). In ultraviolet light, the desired nucleotide was visible as a dark band 33 cm from the origin; traces of a dark band of unidentified material and of deoxyuridylic acid were located 37.5 and 28 cm, respectively, from the origin. Spectrophotometric analysis of eluted portions of the chromatogram showed the amount of deoxyuridylic acid present was 1.5 per cent that of 5-iodo-2'-deoxyuridylic acid. The portions of paper containing the latter were pulped in 600 ml of water and the paper was collected by filtration and washed with 300 ml of water. The combined filtrates were concentrated under reduced pressure to about 40 ml, the pH was adjusted to 7.5 with 1 N LiOH, and the barium salt of the nucleotide was precipitated, as described above, by the addition, in succession, of

* Purchased from the Sigma Chemical Company, St. Louis, Missouri.

† This material did not crystallize from water and could not be purified economically by fractional precipitation from water by addition of ethanol.

barium acetate and ethanol. The product was collected, washed and dried as previously, and dissolved in 40 ml of water. Traces of insoluble solid were removed and the supernatant material was percolated through a column (10 cm) of 12 ml of Dowex 50 (sodium form) ion-exchange resin. The resin was washed with water (about 40 ml) until the absorbancy of the effluent at 288 $m\mu$ decreased to 0.25.

The solution of nucleotide sodium salt contained 5-iodouracil, deoxyribose,^{14, 15} and total phosphorus¹⁶ in the ratio 1:1.0:1.1. 5-Iodouracil was determined spectrophotometrically, with the assumption that the molar absorbancy index of the nucleotide was the same (7750 at 288 $m\mu$ and pH 2) as that of 5-iodo-2'-deoxyuridine; 2'-deoxycytidine-5'-phosphate (California Corporation for Biochemical Research) was used as a deoxyribose standard. The spectrophotometric assay indicated that the solution contained 134 mg (60 per cent yield) of 5-iodo-131-2'-deoxyuridine 5'-phosphate (specific activity 4.3 mc/m-mole). In 0.01 N HCl, it had an ultraviolet absorption maximum at 288 $m\mu$, A_{250}/A_{260} 0.64, A_{280}/A_{260} 1.85; 5-iodo-2'-deoxyuridine has the same maximum, and A_{250}/A_{260} 0.62, A_{280}/A_{260} 2.05. Paper electrophoresis of the product at a gradient of 18 V/cm (0.05 M borate buffer, pH 9.1) revealed a single component, mobility 8.0 cm/hr; 2'-deoxyuridine-5'-phosphate, run simultaneously, migrated at the rate of 7.4 cm/hr. The product was treated with crude snake venom phosphomonoesterase and the mixture was analysed with the butanol-acetic acid system: the spot (R_f 0.42) corresponding to the nucleotide was replaced by a single spot (R_f 0.75) corresponding to 5-iodo-2'-deoxyuridine.

Column chromatography of premixed components on Dowex 1 (formate) ion-exchange resin (height, 20 cm; diameter, 1.5 cm) gave the following order of elution: 5-iodo-2'-deoxyuridine (after passage of 80 ml of 0.05 M HCOONH_4 -0.005 M HCOOH), 2'-deoxyuridine-5'-phosphate (500 ml of the same eluant) and 5-iodo-2'-deoxyuridine-5'-phosphate (400 ml of 0.1 M HCOONH_4 -0.1 M HCOOH).

Exposure of H.Ep.1 cells in culture to ^{131}I -labeled 5-iodo-2'-deoxyuridine and its 5'-phosphate

The cells were grown on Petri plates in Eagle's medium containing 10 per cent horse serum and used after several cell divisions had occurred. The culture procedures and the method of measuring replacement of thymine by 5-iodouracil in deoxyribonucleic acid have been detailed.⁷ Cells were incubated in triplicate in media 56 μM with respect to the above iodouracil derivatives. The specific activity (^{131}I) of the nucleoside was 0.215 $\mu\text{C}/\mu\text{mole}$, that of the nucleotide 0.42 $\mu\text{C}/\mu\text{mole}$; these levels of radioactivity are less than 5 per cent of those which influence incorporation of 5-iodo-2'-deoxyuridine into deoxyribonucleic acid in this system.⁷ After 50 hr the media were removed and the cells were washed three times with 0.5% glucose prior to isolation⁷ and analysis of the deoxyribonucleic acid. The nucleoside effected 30.9, 34.2 and 31.5 per cent replacement of thymine by 5-iodouracil, while use of the nucleotide caused replacements to the extent of 24.3, 25.5 and 24.9 per cent, respectively. In all experiments, 90 per cent of the total radioactivity of the cells was precipitated with the deoxyribonucleic acid.

The results of Table 1 were obtained by procedures similar to the above. The medium contained 56- μM nucleoside or nucleotide, and was removed from different cultures after incubation for 1, 2, 5 and 27 hr respectively. The cells were washed twice with saline before addition of the normal medium and further incubation.

Dephosphorylation of 5-iodo-2'-deoxy e-5'-phosphate in vitro

Solutions containing 25 μg of the nucleotide per ml in the media given below were incubated for 24 hr and then analysed by chromatography in butanol-acetic acid-water (5:2:3). Use of a paper strip scanner and crystal scintillation counter showed that with Eagle's medium all the ^{131}I -activity was located at the R_f of 5-iodo-2'-deoxyuridine-5'-phosphate, but with Eagle's medium containing 10 per cent horse serum or with saline containing 10 per cent horse serum, 60–80 per cent of the radioactivity was at the R_f of 5-iodo-2'-deoxyuridine and the remainder at the R_f of the 5'-phosphate.

Solutions of iododeoxyuridine-5'-phosphate in the above-described three media were incubated with H.Ep.1 cells for 24 hr. In all cases, 60–80 per cent of the radioactivity then corresponded to 5-iodo-2'-deoxyuridine and the remainder to unchanged nucleotide. The 5'-phosphate of the nucleoside was no longer detectable after incubation for 48 hr with H.Ep.1 cells in Eagle's medium and 10 per cent horse serum.

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