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# BIOCHEMICAL PROPERTIES OF THE NUCLEOSIDE OF 3-AMINO-1,5-DIHYDRO-5-METHYL-1,4,5,6,8-PENTAAZAACENAPHTHYLENE (NSC-154020)*

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**Abstract**—A study of 3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphthylene (NSC-154020), a "tricyclic" nucleoside with activity against certain experimental tumors, was undertaken to determine if it differed in biochemical properties from structurally related 7-deazapurine nucleosides with established biological activity, such as sangivamycin. In cultured L1210 cells, [$^{14}$C-methyl]-NSC-154020 was converted to a single metabolite with the properties of a 5'-monophosphate as shown by (a) similarity to AMP in migration on paper chromatograms and in retention time when subjected to high pressure liquid chromatography (h.p.l.c.) on an ion exchange column and (b) conversion to a compound with the properties of NSC-154020 upon treatment with alkaline phosphatase or 5'-nucleotidase. In cultured H.Ep.-2 cells, the principal metabolite of NSC-154020 was also the monophosphate. H.Ep.-2 cells contained in addition a variable amount of a second metabolite which also had the retention time (on h.p.l.c. analysis) of a monophosphate and which was converted by the action of alkaline phosphatase or 5'-nucleotidase to a compound that migrated like NSC-154020 upon chromatography in three solvent systems. This second metabolite is as yet unidentified. In crude extracts of L1210 cells, addition of adenosine or 6-(methylthio)purine ribonucleoside decreased the phosphorylation of NSC-154020. NSC-154020 was a substrate for adenosine kinase 110-fold purified from H.Ep.-2 cells; the $K_m$ was 215 μM and the $V_{max}$ was 1.8 times greater than that of adenosine. No $^{14}$C from labeled NSC-154020 was found in the polynucleotides of either H.Ep.-2 cells or L1210 cells grown for 24 hr in the presence of the labeled nucleoside. Several different studies failed to reveal any selective sites of action for NSC-154020. In cultured L1210 cells it inhibited synthesis of DNA, RNA and protein and reduced ribonucleotide pools, but with little selectivity. The incorporation of [$^{14}$C]formate into polynucleotides was inhibited more severely than that of hypoxanthine; this is evidence for a blockade of purine synthesis *de novo*, an effect also produced by many other analogs of purines and nucleosides. Sangivamycin produced generally similar inhibitions of incorporation of formate and hypoxanthine. However, the cytotoxicity of NSC-154020 and sangivamycin to L1210 cells was not prevented or reversed by 5-amino-4-imidazolecarboxamide (AIC), adenine, guanine, hypoxanthine, uridine, or AIC + uridine; therefore, inhibition of *de novo* synthesis of purine and pyrimidine nucleotides is not a primary site of action of these compounds. Although the loci of action of NSC-154020 are not yet defined, the fact that it is not metabolized to polyphosphates indicates that its mechanism of action probably differs significantly from those of the related compounds, tubercidin and sangivamycin, which are converted to polyphosphates and incorporated into RNA and DNA.

In the course of a study of nucleosides of 7-deazapurines, Schram and Townsend [1] synthesized an unusual nucleoside with the structure shown in Fig. 1. This compound, which has been given the trivial name "tricyclic nucleoside" and the NSC number 154020, is related structurally to certain naturally occurring, biologically active 7-deazapurine nucleosides [2], particularly sangivamycin. NSC-154020 is of interest because of its novel structure and its activity against certain experimental tumors.† Since this compound contains a tricyclic ring system and is therefore not a true purine nucleoside analog, it was of interest to see if it differed in biochemical properties from other analogs of purine nucleosides. We report here data indicating biochemical behavior typical of adenosine analogs. A preliminary report of some of these results has been presented [3]. After our paper had been submitted, a paper by Plagemann [4] appeared which reports generally similar results on the metabolism and mechanism of action of NSC-154020. Points of difference and similarity in his results and ours are noted in Discussion.

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† V. H. Bono, personal communication of animal tumor screening results obtained by the Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

## MATERIALS AND METHODS

*Materials.* [$^{14}$C-methyl]NSC-154020 (6.7 mCi/mmole) was obtained from the Division of Cancer Treatment, National Cancer Institute, which was also the source of sangivamycin (NSC-65346). [8-$^{14}$C]-hypoxanthine, sodium [$^{14}$C]formate, [$^{3}$H-methyl]-thymidine, [5-$^{3}$H]uridine, and [4,5-$^{3}$H]leucine were obtained from New England Nuclear, Boston, MA.

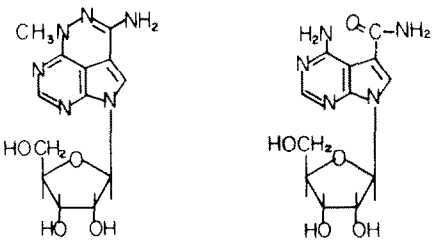


Fig. 1. Structure of 3-amino-1,5-dihydro-5-methyl-1-β-D-ribofuranosyl-1,4,5,6,8-pentaazaacenaphthylene (NSC-154020) and sangivamycin (NSC-65346).

Alkaline phosphatase from *Escherichia coli*, 5′-nucleotidase from *Crotalus adamanteus* venom, and adenosine deaminase from calf intestine were from Sigma Chemical Co., St. Louis, MO. Soluene 350 was obtained from Packard Instruments, Inc., Downers Grove, IL.

*High pressure liquid chromatography.* Analyses by high pressure liquid chromatography (h.p.l.c.) were performed at ambient temperature with a Waters Associates (Milford, MA) model 202 apparatus and a Partisil-10 SAX anion exchange column (Whatman, Inc., Clifton, NJ), 4.6 mm i.d. × 25 cm. A linear gradient (40 min) from 5 mM $NH_4H_2PO_4$, pH 2.8, to 750 mM $NH_4H_2PO_4$, pH 3.7, was used. The flow rate was 2 ml/min, and detection of eluted materials was accomplished by measure of u.v. absorbance at 254 or 280 nm. Integration of peak areas was made with a Hewlett–Packard model 3380A digital electronic integrator, and quantitation for ribonucleotide pool studies was achieved using the peak area–nmol relationships obtained with known ribonucleoside monophosphates.

*Cell cultures.* The cell cultures used were a human epidermoid carcinoma line (H.Ep.-2) established initially by Moore *et al.* [5] and a line of mouse leukemia L1210. H. Ep.-2 cells were grown as monolayers or in suspension culture in SRI-14 medium [6], and L1210 cells were grown in Fischer's medium [7] in suspension cultures, either without agitation (referred to as "stationary" cultures) or with agitation with a magnetic stirrer (referred to as "spinner" cultures). Penicillin and streptomycin were present in all cell culture media.

*Metabolic studies.* For study of the metabolism of NSC-154020, the labeled nucleoside was added to suspension cultures of H.Ep.-2 cells ($2 \times 10^5$ ml cells/ml) and to spinner cultures of L1210 cells ($7 \times 10^5$ cells/ml) at a final concentration of 1.6 μM (0.01 μCi/ml). At 4 or 24 hr, the cells were harvested, washed free of medium with 0.85% saline solution, and then extracted with cold 0.5 N $HClO_4$. This extract, after precipitation of $HClO_4$ by the addition of KOH, was subjected to chromatography on Partisil-10 SAX columns, as described above, or to paper chromatography as described in Fig. 2.

The effects of NSC-154020 on the synthesis of macromolecules were determined in cultures of L1210 cells by the use of labeled leucine, uridine and thymidine. NSC-154020 was added to stationary cultures at concentrations of 0.01, 0.03 or 0.09 μM, representing multiples of the $ID_{50}$ value (0.03 μM) that had been determined previously; the $ID_{50}$ is the concentration required to inhibit by 50 per cent the proliferation of a culture over a 48-hr period. The labeled precursors ([4,5-$^3$H]L-leucine, sp. act. 5.0 Ci/m-mole; [5-$^3$H]uridine, sp. act. 28 Ci/m-mole; [$^3$H-methyl]-thymidine, sp. act. 7 Ci/m-mole) were added 0.5 hr thereafter to give a final concentration of 2 μCi/ml. After 4 hr, duplicate samples were taken and chilled in an ice bath and the cells were then separated by centrifugation. The cells were washed three times with 0.85% saline solution and dissolved in 2.5 ml of 0.1% sodium lauryl sulfate solution containing carrier DNA to give a final concentration of 50 μg DNA/ml. For determination of the radioactivity in total nucleic acids or protein, 1.0 ml of cold 20% (w/v) TCA was added to 1 ml of the lauryl sulfate solution; the resulting precipitate was collected by filtration on Whatman glass fiber filters and washed with 10% TCA and then with 80% ethanol. When [$^3$H]leucine was the precursor, the $^3$H content of this residue was a measure of protein synthesis; when labeled thymidine or uridine was the precursor, the radioactivity of the residue was a measure of incorporation into RNA + DNA. For determination of the radioactivity, the filters were placed in 1.0 ml Soluene 350 and incubated overnight, after which toluene scintillator was added, and the sample was assayed. To determine incorporation into DNA and RNA individually, 0.1 ml of 4 N NaOH was added to 1 ml of the lauryl sulfate solution and the solution was incubated overnight at 37°. This solution was neutralized with 0.1 ml of 4 N HCl, and 0.3 ml of cold 50% TCA was added. The resulting precipitate of DNA was then collected by filtration and counted as described above. Radioactivity of RNA was determined as the difference between the total TCA-insoluble activity and the alkali-stable radioactivity. These procedures are a modification of those of Hershko *et al.* [8].

For determination of the effects of NSC-154020 or sangivamycin on the utilization of formate and hypoxanthine, L1210 cells in culture ($10^6$cells/ml) were treated with inhibitor 1 hr before addition of the labeled compound. At 4 hr thereafter the cells were harvested, washed, and extracted with hot 80% ethanol. The ethanol extract was subjected to two-dimensional chromatography on paper, first in phenol–water (72%, v/v) and then in a solvent consisting of equal volumes of 93.4% aqueous 1-butanol and 44% aqueous propionic acid. Radioactive areas were located by autoradiography. The residue from the ethanol extraction was assayed directly for radioactivity. These procedures have been described in more detail elsewhere [9].

All determinations of radioactivity were made with a Packard liquid scintillation spectrometer equipped with an external standard.

*Reversal studies.* The effectiveness of various compounds in preventing or reversing the cytotoxicity of NSC-154020 was determined in stationary cultures of L1210 cells. NSC-154020 was present at a concentration which gave cytostasis for a 48-hr period. Candidate reversal agents were added at the same time as NSC-154020. Cells were counted in a Coulter counter 24 and 48 hr after addition of the compounds.

*Enzyme assays.* NSC-154020 was assayed as a substrate for adenosine kinase, partially purified from H.Ep.-2 cells [10], and for bovine intestinal adenosine deaminase. Details of the kinase assay are given in Fig. 4. Activity as a substrate for adenosine deaminase was determined by observation of any changes in absorption at 292 nm (the absorption maximum for NSC-154020) occurring when the enzyme and substrate were incubated at pH 7.5.

## RESULTS

*Metabolism of NSC-154020.* Soluble extracts of L1210 cells grown in the presence of [$^{14}$C]NSC-154020 for 18 hr contained no residual substrate; almost all of the $^{14}$C was present as a single metabolite with an $R_f$ value of ~0.15 in the solvent system used (Fig. 2). This $R_f$ value is about the same as that of AMP in this solvent. When the cell extract was treated with either alkaline phosphatase or 5'-nucleotidase prior to chromatography, the only radioactive compound noted was one with the same $R_f$ value as NSC-154020. When the initial cell extract was subjected to h.p.l.c., all of the radioactivity was eluted as a single peak with a retention time about the same as that of AMP (Fig. 3).

In H.Ep.-2 cells (Figs. 2 and 3) the principal metabolite of NSC-154020 was a compound with the properties of a monophosphate, as judged by the same criteria as those used for the characterization of the metabolite from L1210 cells. In these cells there was, in addition, a substance with a lower $R_f$ value in the butanol–propionic acid solvent (Fig. 2). This metabolite disappeared when the extract was treated with phosphatase or nucleotidase, and radioactivity appeared at the $R_f$ value of NSC-154020. The radioactivity also migrated to the $R_f$ value of NSC-154020 in two other solvent systems: isobutyric acid–concentrated $NH_4OH$–$H_2O$ (57:4:39, v/v), $R_f$ 0.68; and 0.1 M sodium phosphate, pH 2.8–solid $(NH_4)_2SO_4$–1-propanol (100:60:2, v/w/v), $R_f$ 0.06. Thus, this second metabolite also appears to be a phosphate. Its $R_f$ value is in the range of those of ADP and ATP. When the cell extract was subjected to h.p.l.c. (Fig. 3), most of the $^{14}$C was eluted as a single peak with the retention time of AMP; a small amount of $^{14}$C was eluted as a second peak with a retention time of about 9 min, which is less than that of the range of the diphosphates of the normal nucleosides. No radioactivity was present at the retention times expected for di- and triphosphates; nor was there any evidence for residual substrate, which would not have been retained and would therefore be eluted at the solvent front. The amount of this second metabolite relative to the major one varied widely between experiments. The experiment shown in Fig. 2 is representative of those in which the larger amounts were found, and that in Fig. 3 shows one in which considerably smaller amounts were present. Experiments similar to these, in which the exposure period to [$^{14}$C]-NSC-154020 was 4 hr instead of 18 hr, have also been performed with both H.Ep.-2 cells and L1210 cells with results similar to those reported above.

*Evidence that adenosine kinase catalyzes the phosphorylation of NSC-154020.* Since NSC-154020 is related structurally to other adenosine analogs, it was considered probable that adenosine kinase was the enzyme responsible for its phosphorylation. To obtain evidence on this point, adenosine and 6-(methylthio)-purine ribonucleoside (a known substrate for adenosine kinase [10]) were studied as competitors with NSC-154020 for phosphorylation by enzymes present in crude extracts of L1210 cells. The results are shown in Table 1. Both adenosine and 6-(methylthio)purine ribonucleoside decreased the formation of the monophosphate of NSC-154020. 6-(Methylthio)purine ribonucleoside was the more effective inhibitor. This is probably because a significant amount of the adenosine was deaminated by the crude cell extracts, whereas 6-(methylthio)purine ribonucleoside, although its $K_m$ value for the kinase reaction is much greater than that of adenosine [10], is not a substrate for enzymes degrading purine nucleosides [11]. These results indicate that adenosine kinase probably is the enzyme responsible for the phosphorylation of NSC-154020. This compound was therefore assayed as a substrate for adenosine kinase partially purified from H.Ep.-2 cells. As shown in Fig. 4, NSC-154020

Table 1. Effects of adenosine and 6(methylthio)purine ribonucleoside on the phosphorylation of [$^{14}$C-methyl]NSC-154020 in extracts of L1210 cells*

| Compound added | Concn (mM) | Phosphorylation of NSC-154020 (per cent of control) |
|---|---|---|
| None | | 100 |
| Adenosine | 1 | 86 |
| | 10 | 50 |
| | 100 | 0 |
| 6(methylthio)purine ribonucleoside | 1 | 79 |
| | 10 | 0 |
| | 100 | 0 |

* The extracts were crude supernatants (100,000 *g*) from cultured L1210 cells. The reaction mixture consisted of the following in a final volume of 1.0 ml: [$^{14}$C-methyl]-NSC-154020, 1 mM; ATP 2.5 mM; $MgCl_2$ 0.25 mM, potassium phosphate buffer, pH 7.0, 50 mM; and adenosine or 6(methylthio)purine ribonucleoside at the indicated concentrations. The inhibitors and [$^{14}$C]NSC-154020 were added simultaneously, and the reaction was terminated after 30 min by immersion in a boiling water bath. The amount of nucleotide formed was determined by the method described in Fig. 4. In the controls 14 per cent of the substrate was phosphorylated.

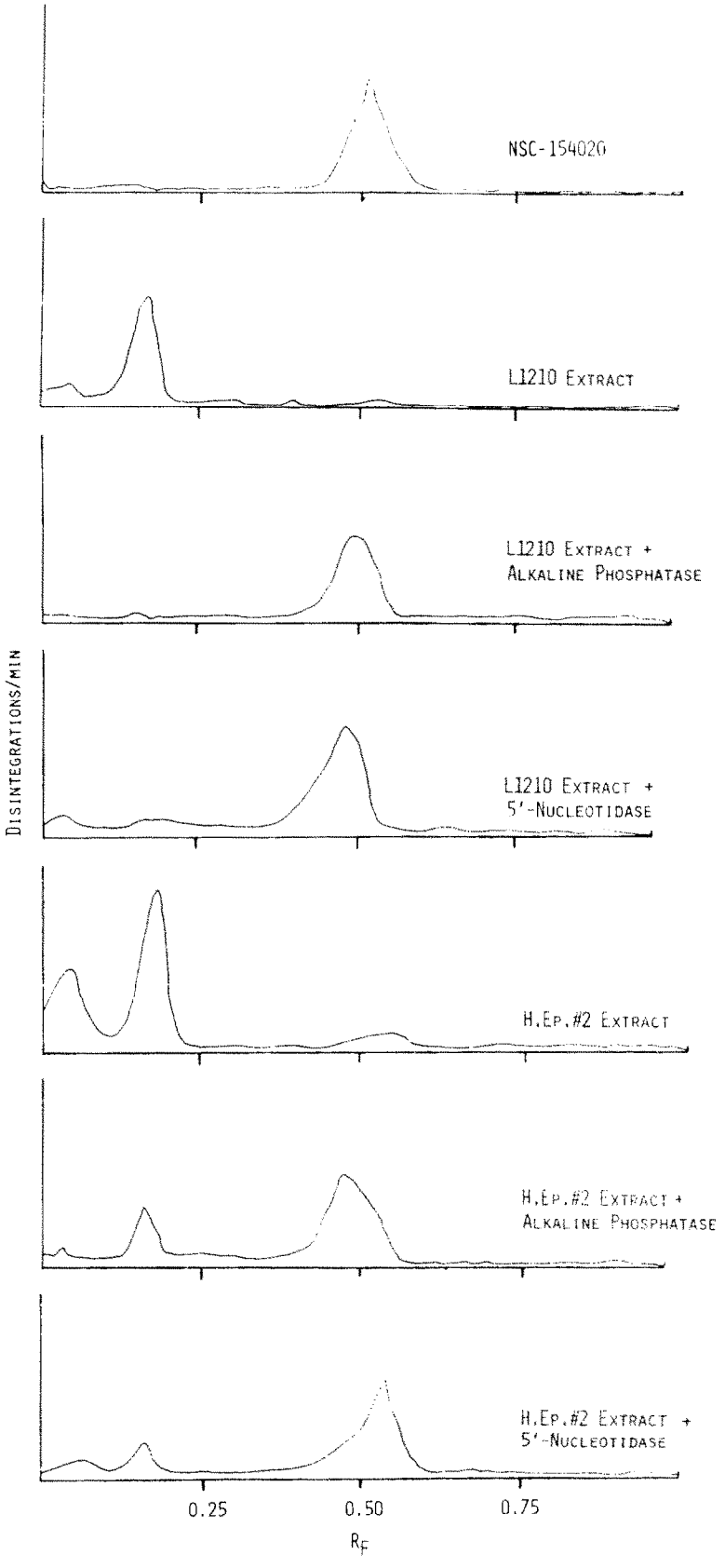


Fig. 2. Metabolism of NSC-154020 by L1210 cells and H.Ep.-2 cells. Cells in suspension cultures were grown in the presence of [$^{14}$C-methyl]NSC-154020 for 18 hr, after which a cold 0.5 N $HClO_4$ extract was prepared as described in the text. The extract was neutralized with KOH, and $KClO_4$ was removed by centrifugation. One portion of this extract was chromatographed on paper in a solvent consisting of equal parts of 93.8% butanol and 44% aqueous propionic acid, and the resulting chromatogram was scanned for $^{14}$C in a Packard model 2701 chromatogram scanner. Another portion of the extract was treated with alkaline phosphatase for 90 min, after which the reaction was stopped by immersion in a boiling water bath and subjected to chromatography as described above. A third portion of the extract was treated with 5'-nucleotidase overnight prior to chromatography and then analyzed as described above. The plot at the top shows the results when [$^{14}$C-methyl]NSC-154020 was chromatographed alone.

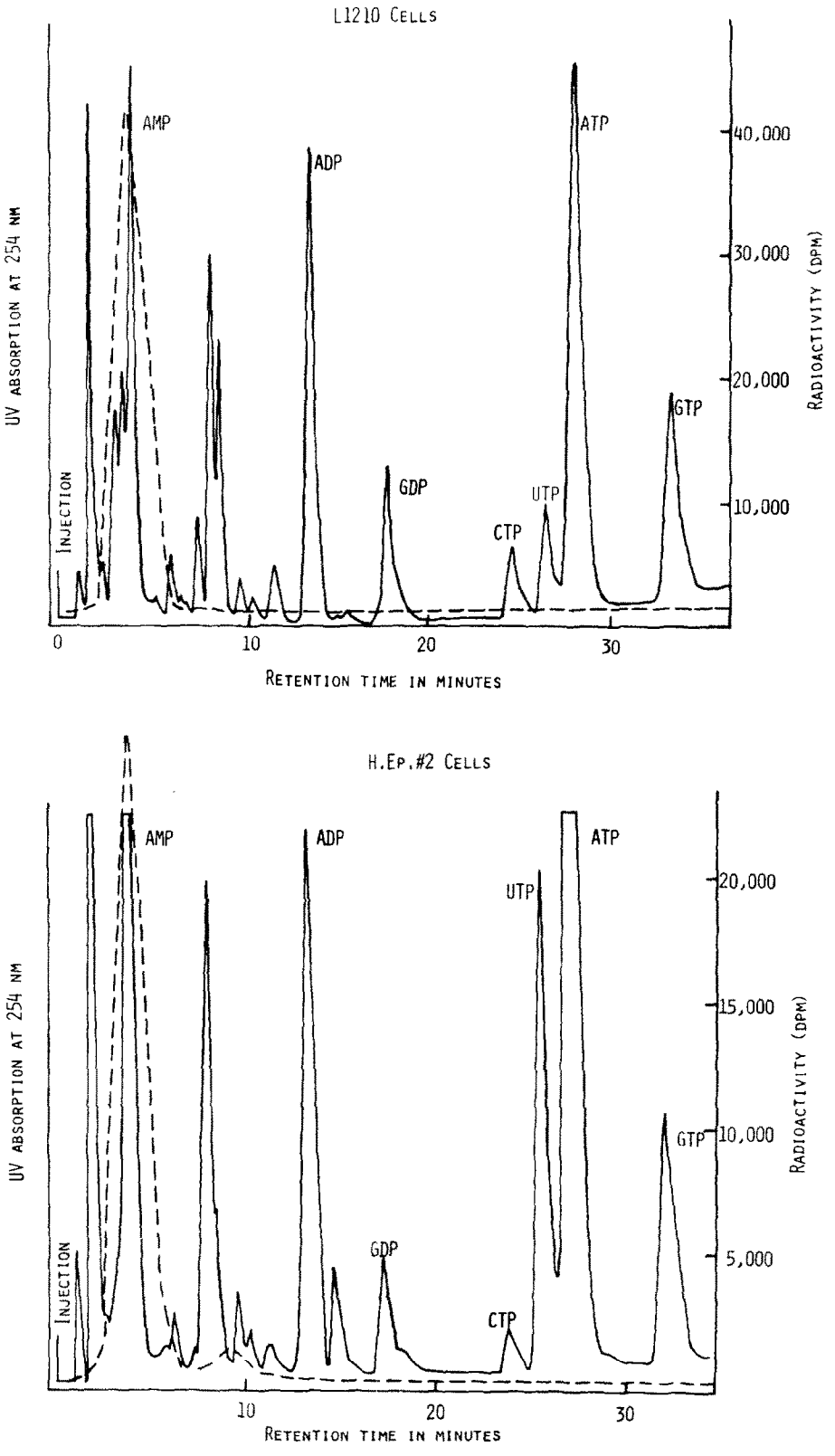


Fig. 3. High pressure liquid chromatography of extracts of L1210 cells and H.Ep.-2 cells grown in the presence of [$^{14}$C-methyl]NSC-154020. The cells were grown and an $HClO_4$ extract was prepared as described in Fig. 2. A portion of this extract (25 $\mu$l, representing $1 \times 10^7$ to $2.5 \times 10^7$ cells) was subjected to chromatography on a Partisil-10 SAX column as described in the text. Samples were taken at 1-ml intervals for radioassay. Key: (———) u.v.; and (·····) radioactivity. The u.v. detector-integrator was attenuated so that $A_{254} = 0.128$ (full-scale deflection).

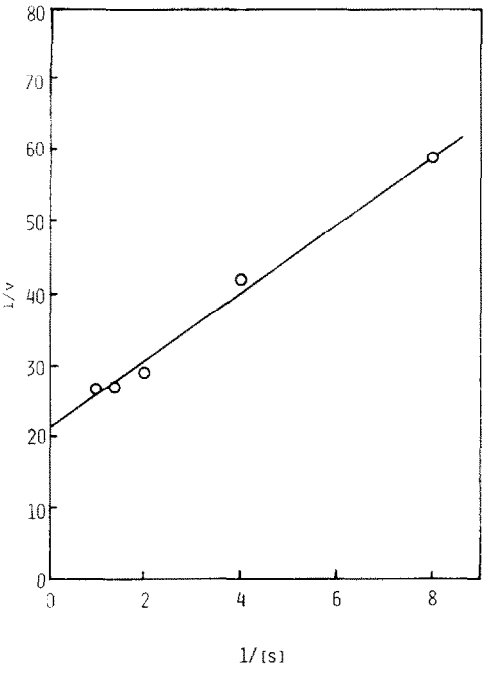


Fig. 4. Lineweaver–Burk plot for determination of kinetic constants for the phosphorylation of NSC-154020 by adenosine kinase. The incubation mixture contained, in a final volume of 0.2 ml, NSC-154020 at the indicated concentrations, enzyme 110-fold purified from H.Ep.-2 cells [10]; ATP-$\gamma$-[$^{32}$P], 2.5 mM; $MgCl_2$, 0.25 mM; and potassium phosphate buffer, 50 mM, pH 7.0. After incubation at 25° for 30 min, the reaction was stopped by immersion in a boiling water bath. The reaction mixture was subjected to chromatography on paper in butanol–propionic acid (see Fig. 2), after which the paper strips were assayed in a Packard model 7201 radiochromatogram scanner to determine the amount of nucleotide formed [10]. Values for the ordinates are the reciprocals of the reaction rate in nmoles/min; those for the abscissas are reciprocal substrate concentrations (mM).

was a good substrate for this enzyme; the $K_m$ was 215 $\mu$M and the $V_{max}$ was 167 nmoles/min/mg; the corresponding values for adenosine were 1.8 $\mu$M and 94 nmoles/min/mg.

*Inactivity of NSC-154020 as a substrate for adenosine deaminase.* NSC-154020 may be regarded both as an $N^6$-substituted adenosine analog and as a 7-deazapurine nucleoside, and as such would not be expected to be a substrate for adenosine deaminase because (a) 7-deazaadenosine derivatives are not deaminated by this enzyme [2], and (b) [$N^6$]methyladenosine is at best a poor substrate [12, 13]. Nevertheless it was conceivable that the tricyclic ring system might possess some unusual property with respect to this enzyme, which, if it acted upon this substrate, would open one of the rings by rupture of the bond between the $N$-5 atom and the pyrimidine moiety. Therefore, NSC-154020 was assayed as a substrate. Incubation of NSC-154020 at pH 7.5 with a large excess of bovine intestinal deaminase produced no change in the u.v. absorption spectrum; these results indicate that, as expected, NSC-154020 is not a substrate for the deaminase.

*Effects of NSC-154020 on ribonucleotide pools.* NSC-154020 at a concentration of 0.3 $\mu$M caused a decrease in pools of ribonucleoside di- and triphosphates; raising the concentration to 1.5 $\mu$M produced no further decrease in the pool sizes (Table 2). The pools of adenine nucleotides were decreased by a smaller percentage than those of nucleotides of guanine, uracil or cytosine. Pools of monophosphates are not shown because of the presence of the monophosphate of NSC-154020, which is not separated from other monophosphates and therefore interferes with their quantitation.

*Effects on synthesis of macromolecules.* The effects of NSC-154020 on macromolecular synthesis were determined at three concentrations and after two periods of exposure to the drug (Table 3). Short-term exposure to NSC-154020 produced inhibition of incorporation of leucine, uridine and thymidine without any marked selectivity, except perhaps at the highest concentration, which produced a greater effect on leucine than on the other precursors. After 16 hr, all three processes had recovered from inhibition caused by the lowest concentration. At the intermediate concentration, there was a selective effect on incorporation of uridine; and at the highest concentration, incorporation of all three precursors was strongly inhibited.

The effects of NSC-154020, at concentrations higher than those of the experiments of Table 3, on the incorporation of formate and hypoxanthine into the acid-insoluble fraction are shown in Fig. 5. The incorporation of formate was inhibited by 0.3 $\mu$M NSC-154020 whereas that of hypoxanthine was not. At a higher concentration of drug, the incorporation of hypoxanthine was also inhibited, but not as much as that of formate. Figure 5 also shows similar data for sangivamycin, which was more inhibitory than

Table 2. Effects of NSC-154020 on ribonucleotide pools of cultured L1210 cells*

| | Nucleotide (nmoles/$10^9$ cells) | | | | | |
|---|---|---|---|---|---|---|
| | ADP | ATP | GDP | GTP | UTP | CTP |
| Control | 800 | 1300 | 410 | 560 | 750 | 120 |
| NSC-154020 (0.3 $\mu$M) | 230 | 790 | 70 | 220 | 190 | 30 |
| NSC-154020 (1.5 $\mu$M) | 250 | 840 | 70 | 270 | 190 | 50 |

* Cells in spinner cultures were exposed to NSC-154020 at the indicated concentrations after which an $HClO_4$ extract was prepared and subjected to chromatography on a Partisil SAX column. See text and Fig. 3 for details. The results shown for a concentration of NSC-154020 of 0.3 $\mu$M are those of a single experiment; those for 1.5 $\mu$M are the averages of three experiments.

Table 3. Effects of NSC-154020 on the synthesis of macromolecules in L1210 cells*

| Precursor | Concn of NSC-154020 ($\mu$M) | Incorporation (% of control) 0.5 hr† | 16 hr† |
|---|---|---|---|
| [4,5-$^3$H]leucine | 0.01 | 78 | 121 |
| | 0.03 | 52 | 80 |
| | 0.09 | 22 | 16 |
| [5-$^3$H]uridine | 0.01 | 89 | 111 |
| | 0.03 | 52 | 41 |
| | 0.09 | 56 | 8 |
| [$^3$H-methyl]thymidine | 0.01 | 83 | 106 |
| | 0.03 | 63 | 99 |
| | 0.09 | 43 | 7 |

* To L1210 cells in culture, NSC-154020 was added at the indicated concentrations, and 0.5 or 16 hr thereafter $^3$H-labeled precursors (thymidine, uridine or leucine) were added. Cells were harvested 4 hr after addition of the labeled compounds, and crude macromolecular fractions were isolated and assayed for radioactivity. See text for details.

† Time to which cells were exposed to NSC-154020 prior to addition of the labeled precursor.

NSC-154020 and which showed similarly a greater effect on the incorporation of formate than on the incorporation of hypoxanthine. Analysis of the soluble fractions of these cells by two-dimensional paper chromatography followed by autoradiography showed an essentially complete exclusion of $^{14}$C from purine nucleotides when [$^{14}$C]formate was the precursor and no specific changes in content of $^{14}$C in any spots when [$^{14}$C]hypoxanthine was the precursor.

*Attempted reversal of cytotoxicity.* NSC-154020 at a concentration of 0.15 $\mu$M produced cytostasis of L1210 cells over a period of 48 hr, and its effects were not prevented or reversed by presence in the medium of the following compounds at the indicated concentrations ($\mu$M): 5-amino-4-imidazolecarboxamide (AIC), 120; adenine, 19; hypoxanthine, 150; uridine, 80; and AIC, 120 + uridine, 80. These negative results are not shown.

## DISCUSSION

The metabolism of NSC-154020 in cell cultures is simple in that only one (or two) metabolites are formed and in that there was no incorporation into polynucleotides. The only metabolite that was found in L1210 cells, and the principal metabolite in H. Ep.-2 cells, was a compound with the properties of a 5′-monophosphate, a finding consistent with the activity of NSC-154020 as a substrate for adenosine kinase. The identity of the second metabolite found in H. Ep.-2 cells is as yet not established. This compound has the properties of a monophosphate, but is separable from the presumed 5′-monophosphate. It apparently is not a 2′- or 3′-phosphate because of its activity as a substrate for 5′-nucleotidase. That it probably is not a phosphate of a ring-altered metabolite of NSC-154020 is shown by the fact that treatment with phosphatase or nucleotidase converted it to a compound indistinguishable from NSC-154020 by chromatography on paper in three solvent systems. It is possible that it is a complex of the 5′-phosphate of NSC-154020 with a substance present in H.Ep.-2 cells but not in the L1210 cells. No evidence was found in any of the experiments of a compound with the expected chromatographic properties of the aglycone of NSC-154020; this indicates that this agent is not acted upon by nucleoside phosphorylases or hydrolases. Plagemann [4], in similar studies with Novikoff hepatoma cells, mouse L-cells, HeLa cells, and H.Ep.-2 cells, found the monophosphate to account for more than 90 per cent of the intracellular radioactivity present after growth in the presence of [$^{14}$C]NSC-154020, and also noted the absence of any degradation products; the presence of a second metabolite in H.Ep.-2 cells was not mentioned.

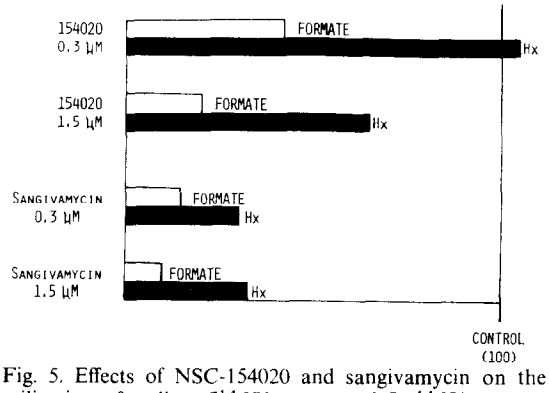


Fig. 5. Effects of NSC-154020 and sangivamycin on the utilization of sodium [$^{14}$C]formate and [8-$^{14}$C]hypoxanthine by L1210 cells. The inhibitors were added 1 hr before the labeled compounds and the cells were harvested 4 hr after addition of the labeled compounds. The acid-insoluble fraction was isolated and assayed for $^{14}$C as described in the text.

The primary purpose of this study was to determine if NSC-154020 differed in biochemical properties from other known nucleoside analogs. NSC-154020 is related structurally to both $N^6$-substituted adenosine derivatives and to the 7-deazapurine nucleosides, and may be regarded as an $N^6$-substituted-7-deazaadenosine derivative in which the $N^6$-substituent is linked covalently to the 7-position. A comparison of the metabolism of NSC-154020 with that of related nucleoside analogs shows that it is in fact that which would be predicted. The three enzymes that act directly on adenosine and adenosine analogs are adeno-

sine kinase, adenosine deaminase, and purine nucleoside phosphorylase. Tubercidin and sangivamycin are substrates for adenosine kinase [10, 14] but not for the deaminase or phosphorylase [2]. With respect to these three enzymes, NSC-154020 exhibited the same behavior as tubercidin and sangivamycin, but it showed differences with respect to further metabolism of the monophosphates, since tubercidin and sangivamycin are converted to polyphosphates and incorporated into RNA and DNA [2, 15], whereas NSC-154020 is not. The behavior of NSC-154020 is also consistent with that of $N^6$-substituted adenosines. These derivatives, even some with bulky $N^6$-substituents, are substrates for adenosine kinase [10, 16, 17], but the $N^6$-substitution limits the formation of higher phosphates [18]. Presumably it is the $N^6$-substitution that prevents the formation of higher phosphates of NSC-154020; it is possible that the higher phosphates might be formed in human cells under conditions of prolonged incubation, as has been observed for some $N^6$-substituted adenosines [16].

Thus, insofar as its metabolism is concerned, NSC-154020 resembles known adenosine analogs. With respect to similarities in metabolic effects, less definite statements can be made because the mechanism of action of no adenosine analog is understood completely. Since the biological activity of NSC-154020 must be ascribed to the nucleoside itself or to its monophosphate, its metabolic effects should be less complex than those of nucleosides that are incorporated into polynucleotides, and it might therefore be useful to make a comparison between NSC-154020 and another nucleoside that is not readily converted to higher phosphates. Such a compound is 6-methyl-thiopurine ribonucleoside, which is also a substrate for adenosine kinase [11] but is not converted into higher phosphates except under unusual conditions [11, 19]. Both 6-(methylthio)purine ribonucleoside [11] and NSC-154020 (Fig. 5) inhibit *de novo* purine synthesis. This apparently is a primary site of action of 6-(methylthio)purine ribonucleoside because its cytotoxicity can be prevented by AIC or hypoxanthine [20]. However, this inhibition apparently is not the primary cause of the cytotoxicity of NSC-154020 because AIC or hypoxanthine provided no degree of reversal. Thus, although 6-(methylthio)-purine ribonucleoside and NSC-154020 are metabolized similarly, they do not share a common primary site of action.

Some evidence of a specific effect of NSC-154020 is that it decreased the pool sizes of UTP and CTP more than it did those of the purine nucleotides (see Table 2). This effect might be indicative of a blockade on the pyrimidine pathway and would not be totally unexpected in view of the observation [21] that adenosine itself is toxic as a result of a blockade of this pathway. However, effects on the pyrimidine pathway are not specific and probably are not a significant contribution to the activity of NSC-154020 because uridine failed to give any detectable degree of reversal. The fact that a combination of AIC and uridine also failed to reverse is indicative that concurrent blockade of both pyrimidine and purine pathways is not the mechanism of action. A combination of uridine and adenosine or hypoxanthine was also ineffective in preventing or reversing NSC-154020-induced inhibition of Novikoff hepatoma cells [4].

NSC-154020 did inhibit the incorporation of precursors into DNA, RNA and protein but with little or no specificity. Similar observations with Novikoff hepatoma cells were made by Plagemann [4], who pointed out that a study of this type does not clearly differentiate between inhibition of transport of the precursor and inhibition of metabolic pathways leading to the macromolecules.

Although the studies reported here and by Plagemann [4] have failed to reveal any NSC-154020-induced metabolic effect of sufficient magnitude and specificity to account for the high cytoxicity of this compound, adenosine and adenosine analogs are known to produce a variety of metabolic effects, many of which have not yet been examined as sites of action for NSC-154020. Among these are inhibition of synthesis of PRPP [22] and effects on pools of cyclic nucleotides [23, 24].

With respect to the question as to whether NSC-154020 represents a new type of structure with biological activity, no evidence so far obtained reveals any features of metabolism or metabolic effects that are not shared with certain other adenosine analogs. It appears, then, that NSC-154020 should be classified as another biologically active 7-deazapurine nucleoside.

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