

Report Title: Effect of Octopirox, Piroctone Acid, and the Iron Chelate of Octopirox on DNA, RNA, and Protein Synthesis in L5178Y Mouse Lymphoma Cells and Syrian Hamster Embryo Cells in Culture

Test Type: Genotoxicity Study

Conducting Laboratory and Location: P&G Miami Valley Laboratories, Biological Testing Facility, Cincinnati, OH

Test Substance(s): G0539 – Octopirox in ethanol

Species: Mouse Lymphoma Cells; Syrian Hamster Embryo Cells

Test Conditions: DNA, RNA and protein synthesis measured.

Results: 2 hr exposure to OP or PA inhibited DNA but not RNA or protein synthesis in both cell lines. Inhibition of DNA synthesis by OP persisted for at least 24 hrs.

The iron chelate of Op had no effect on the rates of DNA, RNA or protein synthesis in L5178Y mouse lymphoma cells indicating inhibition of DNA synthesis by OP is associated with its ability to chelate ferric iron.

Study #: R&DD Report: VE-1083, YE-1305, YE-711, YE-1159

Report Date: 3/25/92

Accession #: 36915

APPROVAL:
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RESEARCH AND DEVELOPMENT DEPARTMENT REPORT
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Retention Limit: Permanent

Notebook #VE-1083
#YE-1305
#YE-711
#YE-1159

Work Done: 7/11/89 to 7/26/90

Effect of Octopirox, Piroctone Acid, and the Iron Chelate of Octopirox on DNA, RNA, and Protein Synthesis in L5178Y Mouse Lymphoma Cells and Syrian Hamster Embryo Cells in Culture

SUMMARY

Octopirox, piroctone acid, and the iron chelate of Octopirox were tested to determine their effect on the rates of DNA, RNA, and protein synthesis in L5178Y mouse lymphoma cells and Syrian hamster embryo cells in culture. A 2-hour exposure to either Octopirox or piroctone acid inhibited DNA synthesis, but not RNA or protein synthesis in both cell lines. The inhibition of DNA synthesis by Octopirox persisted for at least 24 hours. The iron chelate of Octopirox had no effect on the rates of DNA, RNA, or protein synthesis in L5178Y mouse lymphoma cells.

INTRODUCTION

The objective of these investigational experiments was to determine the effect of Octopirox, piroctone acid, and the iron chelate of Octopirox on the rates of DNA, RNA, and protein synthesis in L5178Y mouse lymphoma cells and Syrian hamster embryo cells in culture.

MATERIALS AND METHODS

Materials

The chemical structure of piroctone acid is shown in Fig. 1. Octopirox is the monoethanolamine salt of piroctone acid.

Octopirox was received from Beauty Care Division of Procter & Gamble on Divisional Request Document BYO856 under the Test Substance Identification Number G0539. Piroctone acid was received from Beauty Care Division of Procter & Gamble coded as BX724--Lot #2. The iron Octopirox chelate was synthesized as a 3:1 molar ratio of Octopirox to ferric iron, by slowly dripping acidified FeCl_3 into a solution of Octopirox in absolute ethanol. With time a dark red precipitate was formed. The supernatant was decanted, the precipitate washed once with excess absolute ethanol, then with water and dried. The dried powder was sent to Galbraith Laboratories (P.O. Box 51610, Knoxville, TN 37950-1610) for elemental analysis. The detailed procedure for the synthesis and the results of that analysis are in P&G Notebook VE-1083 p. 44-48.

All radiolabeled materials were from Amersham. Methyl- ^3H Thymidine (SP Act 82 Curies/mmmole) was used to determine rates of DNA synthesis. It was received from MVL radiochemistry under the identification number BPO-7754. 5,6 ^3H Uridine (SP Act. 48 Curies/mmmole) was used to determine the rates of RNA synthesis. It was received from MVL radiochemistry under the identification number BPO-7753. L-4,5 ^3H Leucine (SP Act. 69 Curies/mmmole) and L-3,4(n) ^3H Valine (SP Act. 26 Curies/mmmole) were used to determine the rates of protein synthesis. They were received from MVL radiochemistry under the identification numbers BPO-7752 for Leucine and BPO-7768 for Valine. This information is in the Materials Section of P&G Notebook YE-1305.

All other chemicals were analytical reagent grade and were received from commercial sources.

Cells and Culture Conditions

L5178Y TK \pm 3.7.2C mouse lymphoma cells were obtained from Dr. Donald Clive, Burroughs-Wellcome Company, Research Triangle Park, NC. Cells were maintained as suspension cultures in Fischer's medium for leukemic cells of mice (Gibco) supplemented with 10% horse serum (Gibco), sodium pyruvate, penicillin-streptomycin, and Pluronic F68 as described by Clive et al. (1979).

Cultures of Syrian hamster embryo (SHE) cells were received from Gary Kerckaert (Procter & Gamble). Preparation of the cells is recorded in Procter & Gamble Notebook VE-1387, p. 97 and 191. A detailed description of the isolation and culturing of SHE cells appears in LeBoeuf and Kerckaert (1986).

Methods

Determination of Rate of DNA, RNA, and Protein Synthesis in Either L5178Y Mouse Lymphoma Cells or SHE Cells in Culture

The following is a description of the methods used to determine the rates of DNA, RNA, or protein synthesis in either L5178Y mouse lymphoma cells or SHE cells in culture. Because of the investigative nature of these experiments, different volumes of cells, cell densities, and amounts of radiolabeled precursors were used in the various experiments. However, because each study has its own control the data are comparable from one study to the next.

Mouse lymphoma cells were grown overnight to a density of $1 - 2 \times 10^6$ cells/ml. The next morning the cells were adjusted to 3×10^5 cells per ml in $F_{10}P$ and 10 ml portions were added to 50 ml centrifuge tubes (Corning). The test material (Octopirox, piroctone, or iron Octopirox) was weighed, dissolved in absolute ethanol, diluted to the appropriate concentrations, and added to the cells. The centrifuge tubes were gassed with 5% CO_2 in air and placed on a roller drum in a $37^\circ C$ incubator. The cultures were rotated at 80 rpm for 2 hours. After the 2-hour incubation, approximately $1 \mu Ci$ of 3H thymidine (for DNA synthesis) or $1 \mu Ci$ of 3H uridine (for RNA synthesis) or $1 \mu Ci$ each of 3H leucine and 3H valine (for protein synthesis) was added to the appropriate tubes. For each assay, the control tubes received an equivalent amount of ethanol. The tubes were placed back on the roller drum for approximately 15 minutes and then for each test concentration one ml samples were placed into 6 microfuge tubes. The cells were pelleted at 14,000 rpm for 1 minute, the media was decanted, the cells in half of the tubes were resuspended in cold $F_{10}P$ in order to determine total uptake of the radiolabel. The cells in the other 3 tubes were resuspended in ice-cold 0.4M Perchloric acid (PCA) to determine the amount of the radiolabel incorporated into DNA. The PCA-treated cells were allowed to sit at room temperature while the $F_{10}P$ treated cells were resuspended and repelleted at 14,000 rpm for 1 minute. The $F_{10}P$ medium was decanted, the cell pellet was resuspended in 0.5 ml of water and the contents transferred to a scintillation vial. The PCA-treated cells were then pelleted at 14,000 rpm for 1 minute, resuspended in 0.5 ml of water and transferred to scintillation vials. Beckman Ready-Solv™/CP (Fullerton, CA) was added and the radioactivity was determined with a Beckman L55801 liquid scintillation spectrometer. The counts were corrected for quenching with Beckman's external standard H# method and are expressed as dpm.

The rates of DNA, RNA, and protein synthesis in SHE cells were determined as described above except SHE cells were grown as monolayers in T-25 flasks. The test materials were added to the flasks for 2 hours, followed by one of the radiolabeled precursors (3H thymidine, 3H uridine, or 3H valine and leucine) for 15 minutes. In the time-course experiment, the cells were removed by trypsinization and collected on filters (Whatman GFD) and extracted with 0.4M PCA. The filters were placed in scintillation vials and the amount of incorporated radiolabel determined by liquid scintillation spectrometry as described above. In the study to determine the rate of DNA, RNA, and protein synthesis, the cells were removed by scraping, collected by centrifugation, extracted with 0.4 M PCA, resuspended in water, and transferred to scintillation vials.

RESULTS

The first set of experiments were designed to determine the length of time incorporation of the radiolabeled precursors into DNA, RNA, or protein was linear in L5178Y mouse lymphoma cells or SHE cells. These data are found in Notebook YE-1305 experiments K and P. In the experiment with the L5178Y mouse lymphoma cells, $5 \mu Ci$ of either 3H thymidine, 3H uridine,

or ^3H valine and ^3H leucine were added to a Corning 50 ml centrifuge tube containing 5.0 ml of F_{10}P medium with 9.5×10^5 cells/ml. The cultures were placed on the roller drum and 1 ml samples removed hourly. The rates of incorporation were determined as described in the Methods Section. The results are shown in Table I and Fig. 2. The rate of incorporation of each labeled precursor remain linear for two hours.

In the experiment with the SHE cells, the cells were received in T-25 flasks with 5 ml of medium. 5 μCi of the labeled precursors were added to the respective flasks and they were placed back in the incubator. At hourly intervals, flasks were removed and the rates of incorporation of the labeled precursors were determined as described in the Methods Section. The results are shown in Table II and Fig 3. Incorporation remained linear for at least 4 hours. It was decided that a 15 minute pulse with the radiolabeled materials would provide sufficient incorporation for inhibition studies and be well within the linear time-range.

Table III and Fig. 4 show the effect of Octopirox on DNA, RNA, and protein synthesis in L5178Y mouse lymphoma cells. The data are from Experiment M^c, Notebook YE-711. From Fig. 4 it is clear that Octopirox inhibits DNA but not RNA or protein synthesis in L5178Y mouse lymphoma cells in culture. The inhibition of DNA synthesis was repeated (Experiment P, YE-711), and these results are shown in Table IV and Fig. 5. These data confirm the observation in Table III and Fig. 4 that Octopirox inhibits DNA synthesis in L5178Y mouse lymphoma cells in culture.

Table V and Fig. 6 show the effect of piroctone acid on the rates of DNA, RNA, and protein synthesis in L5178Y mouse lymphoma cells. These data are from Experiment N, Notebook YE-711. From Table V and Fig. 6 it is clear that piroctone acid inhibits DNA, but not RNA or protein synthesis in L5178Y mouse lymphoma cells in culture. It is probable that the 0.3 and 1.0 μM data points were mislabeled while transferring either the cultures to the microfuge tubes or the PCA extracts to the scintillation vials. This was confirmed in a repeat experiment (Experiment Q, Notebook YE-711). The data are shown in Table VI and Fig. 7.

The effect of Octopirox on the rates of DNA, RNA, and protein synthesis in SHE cells in culture is shown in Table VII and Fig. 8. These data are from Exp. S, Notebook YE-711. From Table VII and Fig. 8, it is clear that Octopirox inhibits DNA synthesis, but not RNA or protein synthesis in SHE cells in culture.

The data in Figs. 4 and 5 clearly demonstrate that Octopirox inhibits DNA synthesis in L5178Y mouse lymphoma cells after only 2 hours of exposure. The next set of experiments show longer-term effects of Octopirox on these cells in culture. Table VIII and Fig. 9 show that the rates of DNA synthesis in cells 24 hours after a 2-hour exposure to levels of Octopirox which inhibit DNA synthesis are roughly half that of the controls. In this experiment, the cells were exposed for 2 hours to increasing concentrations of Octopirox and the rate of DNA synthesis determined. After 2 hours half of the cells were used to determine the rate of DNA synthesis, the other half were collected by centrifugation, washed twice with F_{10}P , then resuspended in F_{10}P and placed on the roller drum overnight. The next morning the cell densities were determined and the cultures then exposed to ^3H thymidine for 15 minutes to determine the rate of DNA synthesis in each culture. In these experiments it was necessary to express the data as $\text{dpm}/10^5$ cells, since there was significant growth overnight. The data are from Experiment G, Notebook YE-1159.

Since Octopirox is an exceptionally strong chelator of ferric iron, it was of interest to determine if the inhibition of DNA synthesis induced by Octopirox was related to its iron-binding capacity or possibly to a chelate that may have formed during the exposure. To test this possibility the iron chelate of Octopirox was synthesized. The binding constant data indicated the chelate would be a 3:1 molar ratio of Octopirox to ferric iron. Elemental analysis

data (Notebook VE-1083, p. 48) indicate the 3:1 molar ratio chelate was the species formed. L5178Y mouse lymphoma cells were exposed for 2 hours to increasing concentrations of the iron chelate as described in Methods. In this experiment, we also examined the longer-term toxicity of iron-Octopirox as described above. The rates of DNA synthesis after the 2-hour exposure and 24 hours later are shown in Table IX and Figure 10. It is clear that the iron chelate of Octopirox has no effect on DNA synthesis in L5178Y mouse lymphoma cells in culture at either time point when tested at equal molar concentrations of Octopirox which totally inhibit DNA synthesis in these cells. These data are from Experiment I, Notebook YE-1159.

CONCLUSIONS

Octopirox and piroctone acid inhibit DNA synthesis in L5178Y mouse lymphoma and SHE cells in culture.

The inhibition of DNA synthesis by-in-large persists for more than 24 hours after a 2-hour exposure to Octopirox. However, this may be due to the experimental procedure employed. Octopirox is not very soluble in water. Since, in these experiments, the cells were exposed to Octopirox then collected by centrifugation, it is likely the insoluble Octopirox pelleted with the cells. Subsequent washing and resuspension in aqueous media only repeated the collection of Octopirox with the cell pellet. This is only a possibility. There are no data to support it.

The inhibition of DNA synthesis by Octopirox appears to be associated with its ability to chelate ferric iron, since the iron chelate does not affect DNA synthesis in L5178Y mouse lymphoma cells.

Neither Octopirox nor piroctone acid affect the rates of RNA or protein synthesis in L5178Y mouse lymphoma or SHE cells.

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REFERENCES

Clive, D., K.O. Johnson, J.F.S. Spector, A.G. Batson, and M.M.M. Brown, Mutation Research, 1979, 59: 61-108.

LeBoeuf, R.A., and G.A. Kerckaert, Carcinogenesis, 1986, 7: 1431-1440.

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Table I

Time-course for rates of synthesis of DNA, RNA, and protein in L5178Y mouse lymphoma cells. Rates of DNA, RNA, and protein synthesis were determined by measuring the incorporation of ^3H thymidine, ^3H uridine, or ^3H leucine and ^3H valine, respectively, into PCA extracted cells.

<u>Time (hr)</u>	<u>dpm</u>		
	<u>DNA Synthesis</u>	<u>RNA Synthesis</u>	<u>Protein Synthesis</u>
1	177,077	143,465	30,885
2	403,524	287,189	53,019
3	454,467	363,741	91,398
4	505,747	397,924	117,574

Table II

Time-course for rates of synthesis of DNA, RNA, and protein in SHE cells. Rates of DNA, RNA, and protein synthesis were determined by measuring the incorporation of ^3H thymidine, ^3H uridine, or ^3H leucine and ^3H valine, respectively, into PCA extracted cells.

<u>Time (hr)</u>	<u>dpm</u>		
	<u>DNA Synthesis</u>	<u>RNA Synthesis</u>	<u>Protein Synthesis</u>
1	10,758 \pm 449 ^(a)	8,581 \pm 897	7,178 \pm 343
2	33,328 \pm 2,271	20,006 \pm 85	6,173 \pm 390
3	48,446 \pm 3,487	35,701 \pm 3,114	9,531 \pm 896
4	78,350 \pm 8,405	47,033 \pm 7,230	11,594 \pm 362

^(a) mean of 4 values \pm standard deviation

Table II

The effect of Octopirox on the rates of DNA, RNA, and protein synthesis in L5178Y mouse lymphoma cells in culture. Rates of DNA, RNA, and protein synthesis were determined by measuring the incorporation of ^3H thymidine, ^3H uridine, or ^3H leucine and ^3H valine, respectively, into PCA extracted cells.

Concentration of Octopirox (μM)	dpm		
	<u>DNA Synthesis</u>	<u>RNA Synthesis</u>	<u>Protein Synthesis</u>
0 (control)	8,047 \pm 664 ^(a)	3,035 \pm 581	1,909 \pm 360
10	946 \pm 213	2,649 \pm 210	1,787 \pm 271
3	964 \pm 381	2,863 \pm 430	1,582 \pm 215
1	6,721 \pm 376	2,462 \pm 190	1,579 \pm 740
0.3	8,243 \pm 371	2,777 \pm 1,086	2,327 \pm 1,126
0.1	7,937 \pm 1,579	3,574 \pm 346	2,535 \pm 576

^(a) mean of 3 values \pm standard deviation

Table IV

The effect of Octopirox on the rate of DNA synthesis in L5178Y mouse lymphoma cells in culture. Rate of DNA synthesis was determined by measuring the incorporation of ^3H thymidine into PCA extracted cells.

Concentration of Octopirox (μM)	<u>dpm</u> <u>DNA Synthesis</u>
0 (control)	7,511 \pm 343 ^(a)
10	135 \pm 26
3	192 \pm 27
1	176 \pm 29
0.3	7,863 \pm 740
0.1	8,125 \pm 281

^(a) mean of 3 values \pm standard deviation

Table V

The effect of piroctone acid on the rates of DNA, RNA, and protein synthesis in L5178Y mouse lymphoma cells in culture. Rates of DNA, RNA, and protein synthesis were determined by measuring the incorporation of ^3H thymidine, ^3H uridine, or ^3H leucine and ^3H valine, respectively, into PCA extracted cells.

<u>Concentration of Piroctone Acid (μM)</u>	<u>dpm</u>		
	<u>DNA Synthesis</u>	<u>RNA Synthesis</u>	<u>Protein Synthesis</u>
0 (control)	7,126 \pm 962 ^(a)	1,187 \pm 103	797 \pm 59
10	112 \pm 6	820 \pm 20	615 \pm 72
3	115 \pm 7	685 \pm 39	411 \pm 34
1	3,829 \pm 190	853 \pm 59	664 \pm 116
0.3	126 \pm 10	828 \pm 43	594 \pm 73
0.1	7,166 \pm 380	995 \pm 203	699 \pm 11

^(a) mean of 3 values \pm standard deviation

Table VI

The effect of piroctone acid on the rate of DNA synthesis in L5178Y mouse lymphoma cells in culture. Rate of DNA synthesis was determined by measuring the incorporation of ^3H thymidine into PCA extracted cells.

<u>Concentration of Piroctone Acid (μM)</u>	<u>dpm</u> <u>DNA Synthesis</u>
0 (control)	9,841 \pm 2,040 ^(a)
10	208 \pm 14
3	400 \pm 111
1	193 \pm 44
0.3	12,580 \pm 3,418
0.1	10,948 \pm 747

^(a) mean of 3 values \pm standard deviation

Table VII

The effect of Octopirox on the rates of DNA, RNA, and protein synthesis in SHE cells in culture. Rates of DNA, RNA, and protein synthesis were determined by measuring the incorporation of ^3H thymidine, ^3H uridine, or ^3H leucine and ^3H valine, respectively, into PCA extracted cells.

Concentration of Octopirox (μM)	dpm		
	<u>DNA Synthesis</u>	<u>RNA Synthesis</u>	<u>Protein Synthesis</u>
0 (control)	9,001	737	401
10	344	456	409
3	580	352	458
1	3,511	599	350
0.3	8,835	611	226
0.1	6,300	602	595

Table VIII

Long-term effect on the rate of DNA synthesis in L5178Y mouse lymphoma cells by a 2-hour exposure to Octopirox. The rate of DNA synthesis was determined by measuring the incorporation of ^3H thymidine into PCA extracted cells.

Concentration of Octopirox (μM)	dpm/ 10^5 Cells	
	<u>DNA Synthesis (Day 1)</u>	<u>DNA Synthesis (Day 2)</u>
0 (control)	1,641 \pm 175 ^(a)	1,199 \pm 96
10	78 \pm 4	401 \pm 44
3	68 \pm 25	517 \pm 82
1	2,634 \pm 235	1,183 \pm 122
0.3	2,214 \pm 154	1,292 \pm 151
0.1	1,792 \pm 281	1,137 \pm 99

^(a) mean of 3 values \pm standard deviation

Table D

Long-term effect on the rate of DNA synthesis in L5178Y mouse lymphoma cells by a 2-hour exposure to the iron chelate of Octopirox. The rate of DNA synthesis was determined by measuring the incorporation of ^3H thymidine into PCA extracted cells.

Concentration of the Iron Chelate of Octopirox (μM)	dpm/ 10^5 Cells	
	<u>DNA Synthesis (Day 1)</u>	<u>DNA Synthesis (Day 2)</u>
0 (control)	1,182 \pm 207 ^(a)	935 \pm 104
10	1,147 \pm 205	923 \pm 81
3	1,240 \pm 225	863 \pm 277
1	1,249 \pm 141	930 \pm 22
0.3	1,350 \pm 63	1,004 \pm 146
0.1	1,062 \pm 262	812 \pm 127

^(a) mean of 3 values \pm standard deviation

Figure 1

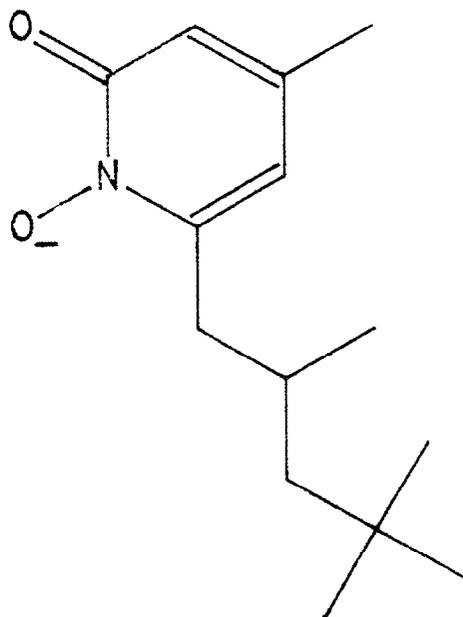


Fig. 1. The chemical structure of piroctone acid.
Octopirox is the monoethanolamine salt of piroctone acid.

Figure 2

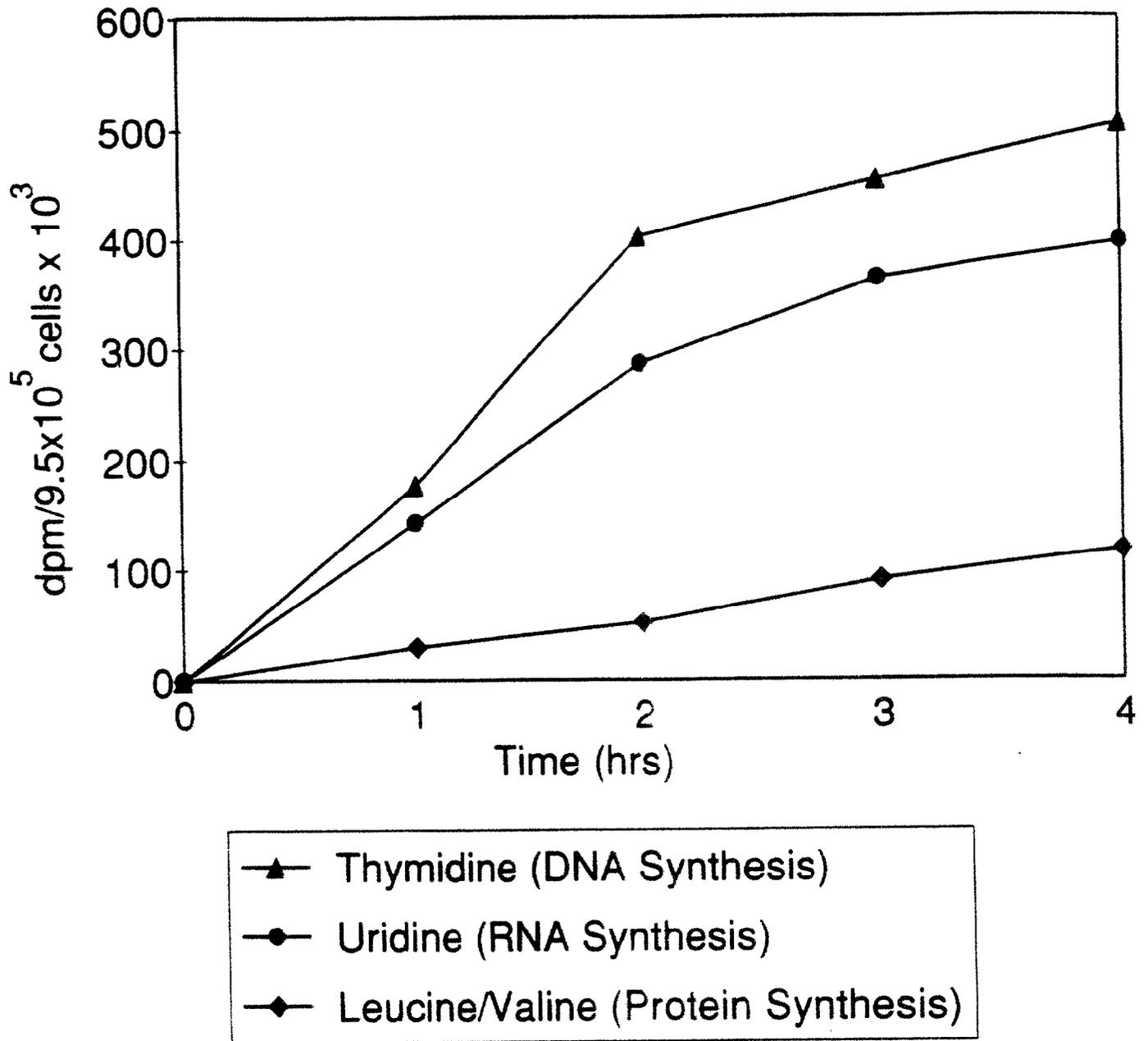


Fig. 2. Time-course for rates of synthesis of DNA, RNA, and Protein in L5178Y Mouse Lymphoma Cells.

Figure 3

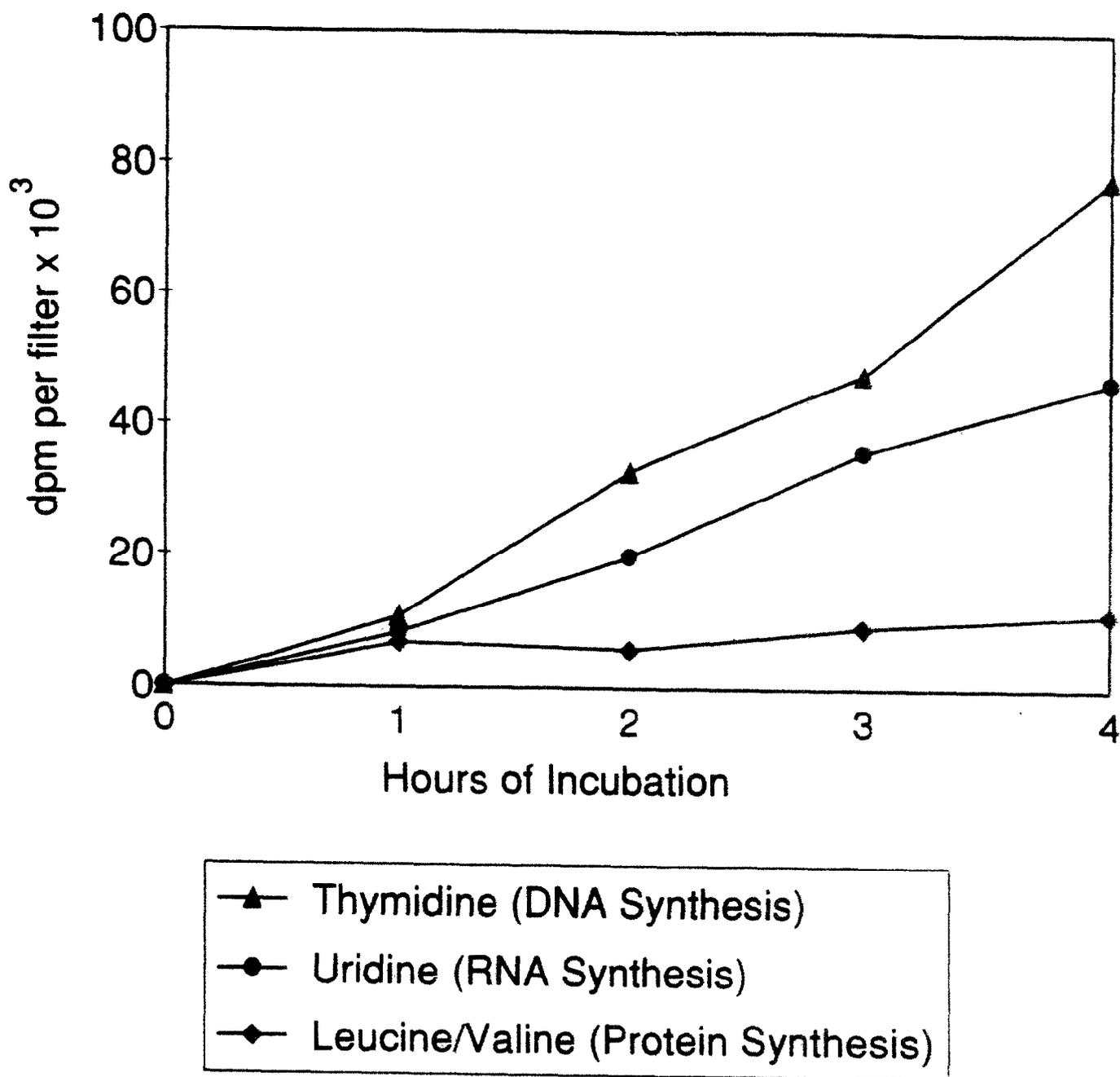


Fig. 3. Time-course for rates of synthesis of DNA, RNA, and Protein in SHE cells.

Figure 4

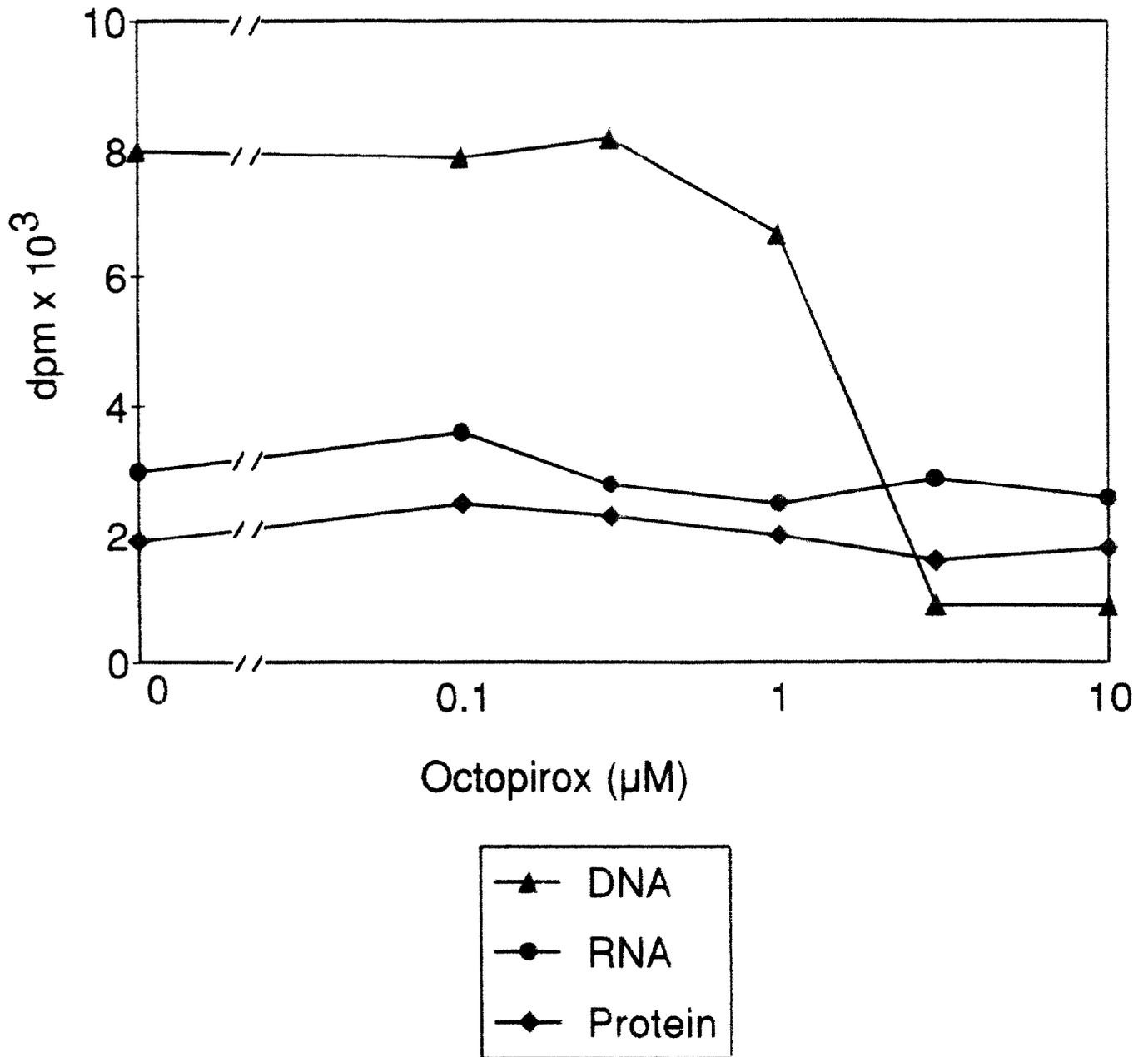


Fig. 4. The effect of Octopirox on the rates of DNA, RNA, and Protein synthesis in L5178Y Mouse Lymphoma Cells in culture.

Figure 5

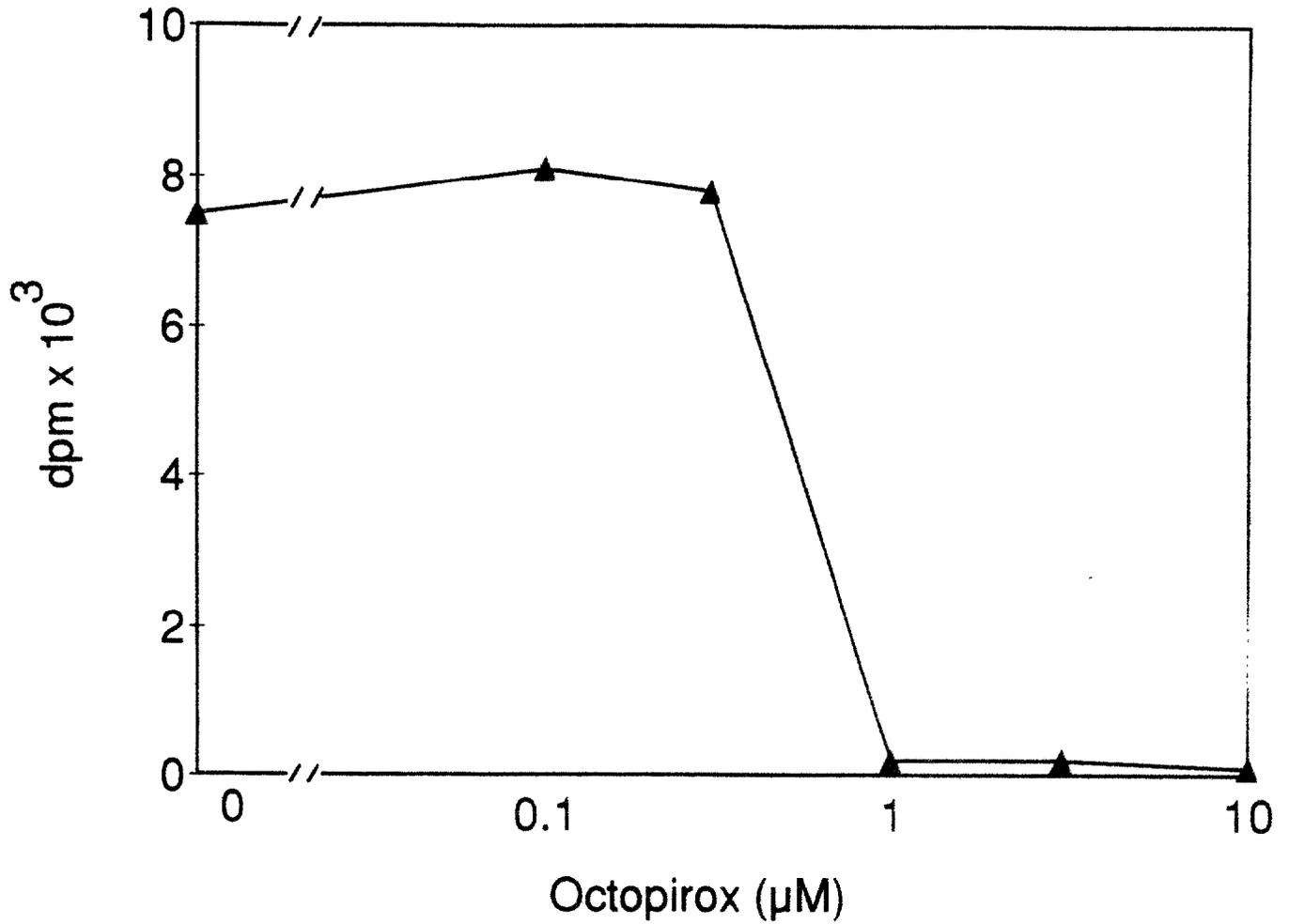


Fig. 5. The effect of Octopirox on the rate of DNA synthesis in L5178Y Mouse Lymphoma Cells in culture.

Figure 6

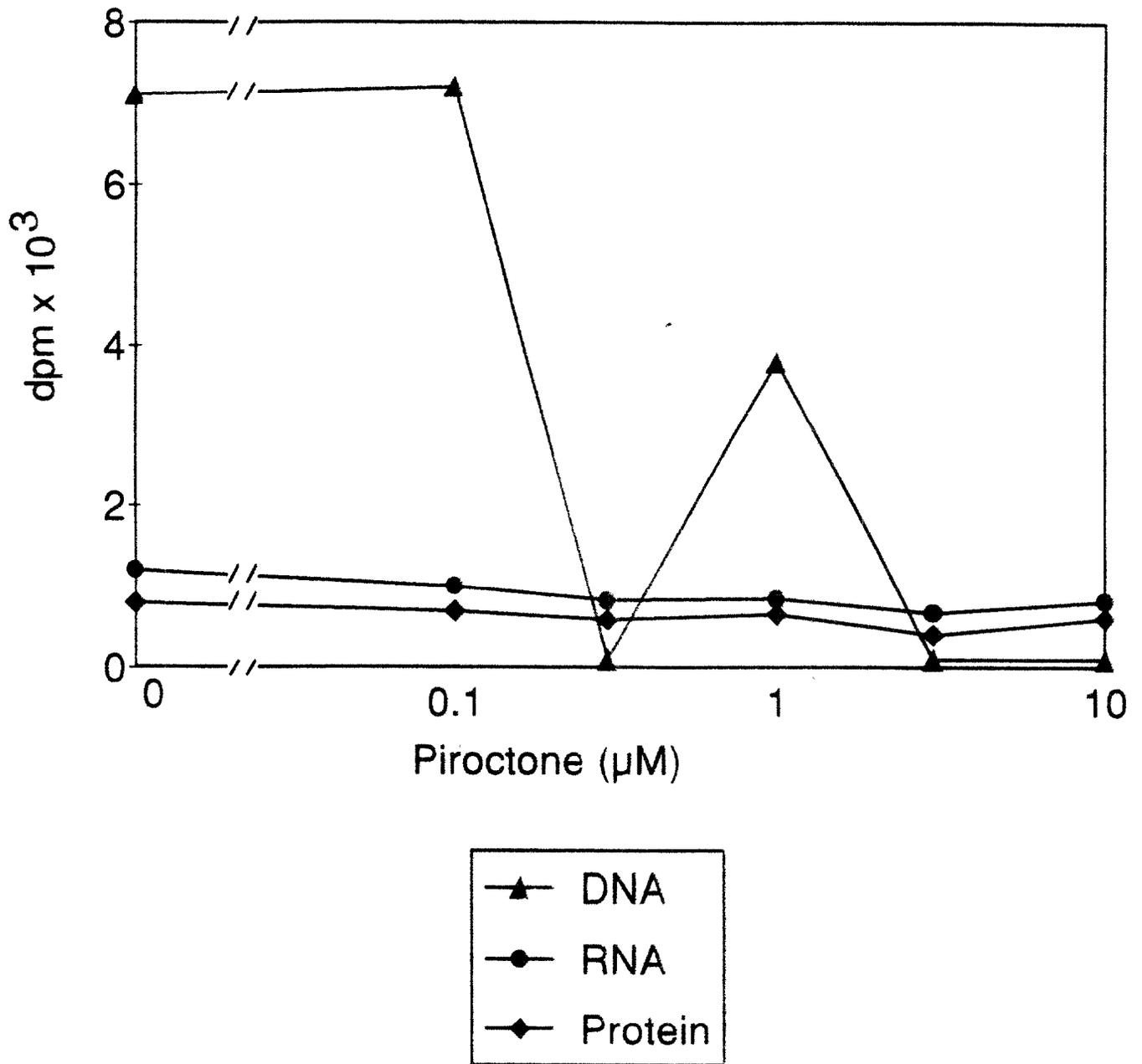


Fig. 6. The effect of Piroctone acid on the rates of DNA, RNA, and Protein synthesis in L5178Y Mouse Lymphoma Cells in culture.

Figure 7

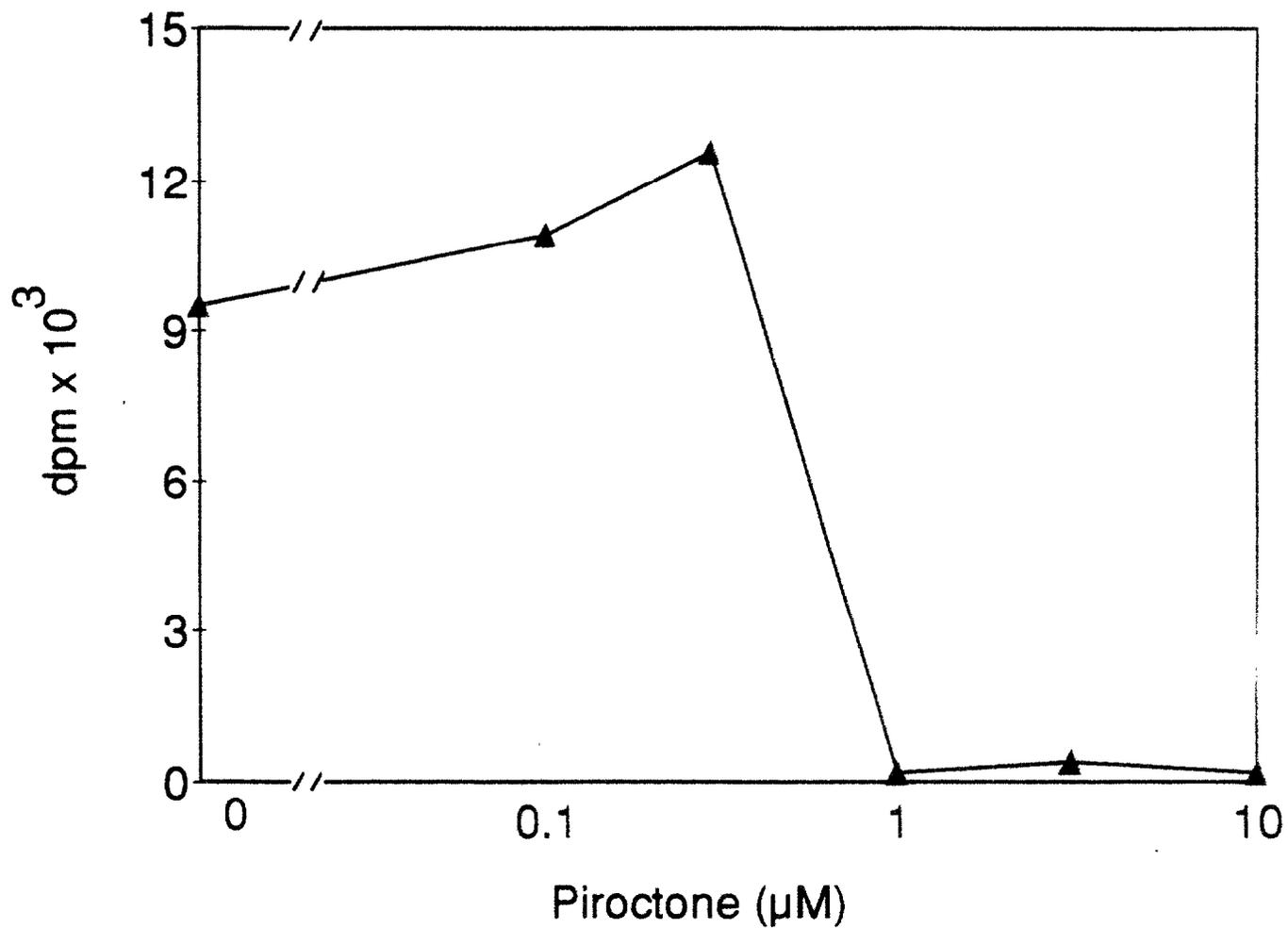


Fig. 7. The effect of Piroctone acid on the rate of DNA synthesis in L5178Y Mouse Lymphoma Cells in culture.

Figure 8

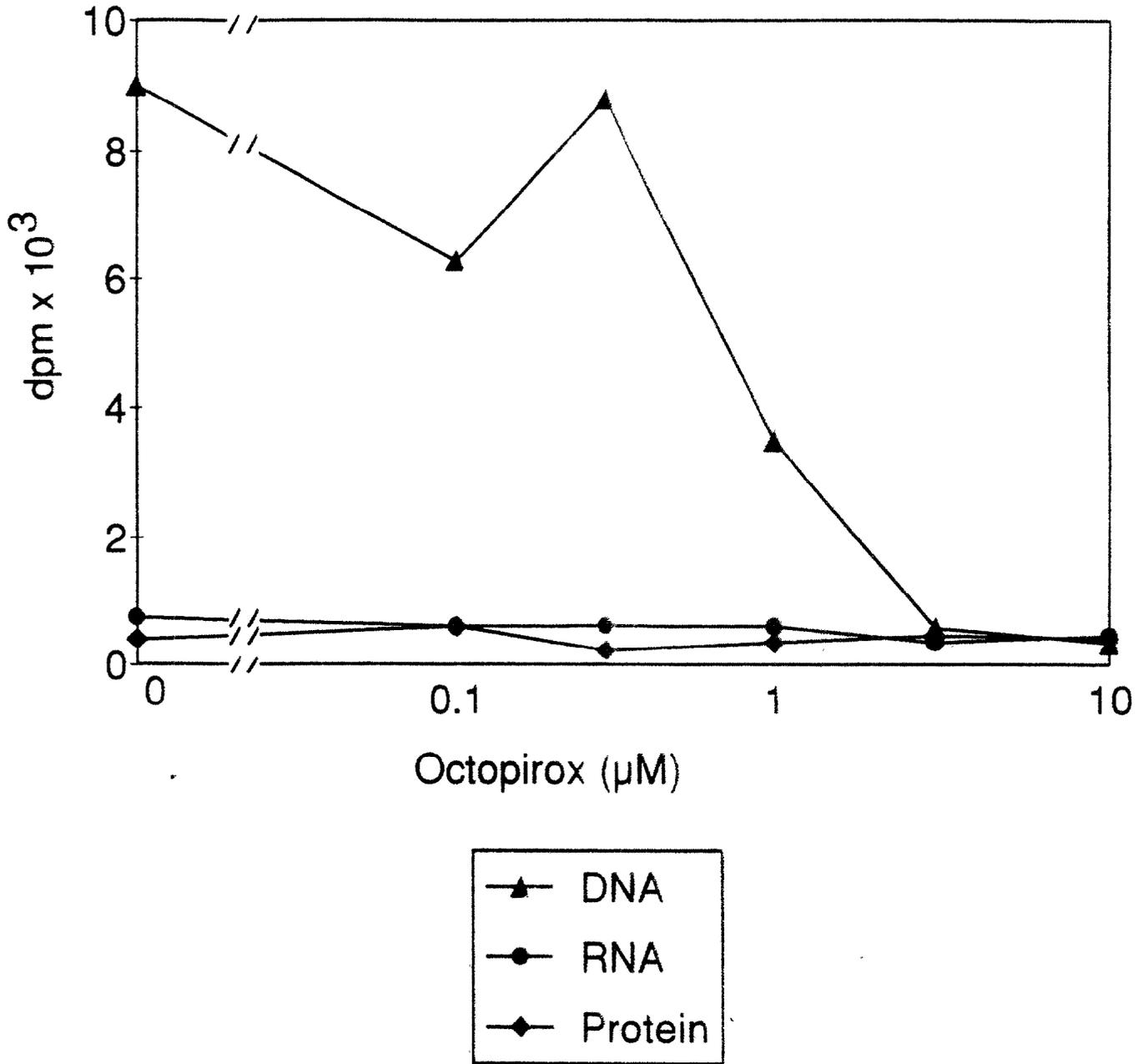


Fig. 8. The effect of Octopirox on the rates of DNA, RNA, and Protein synthesis in SHE Cells in culture.

Figure 9

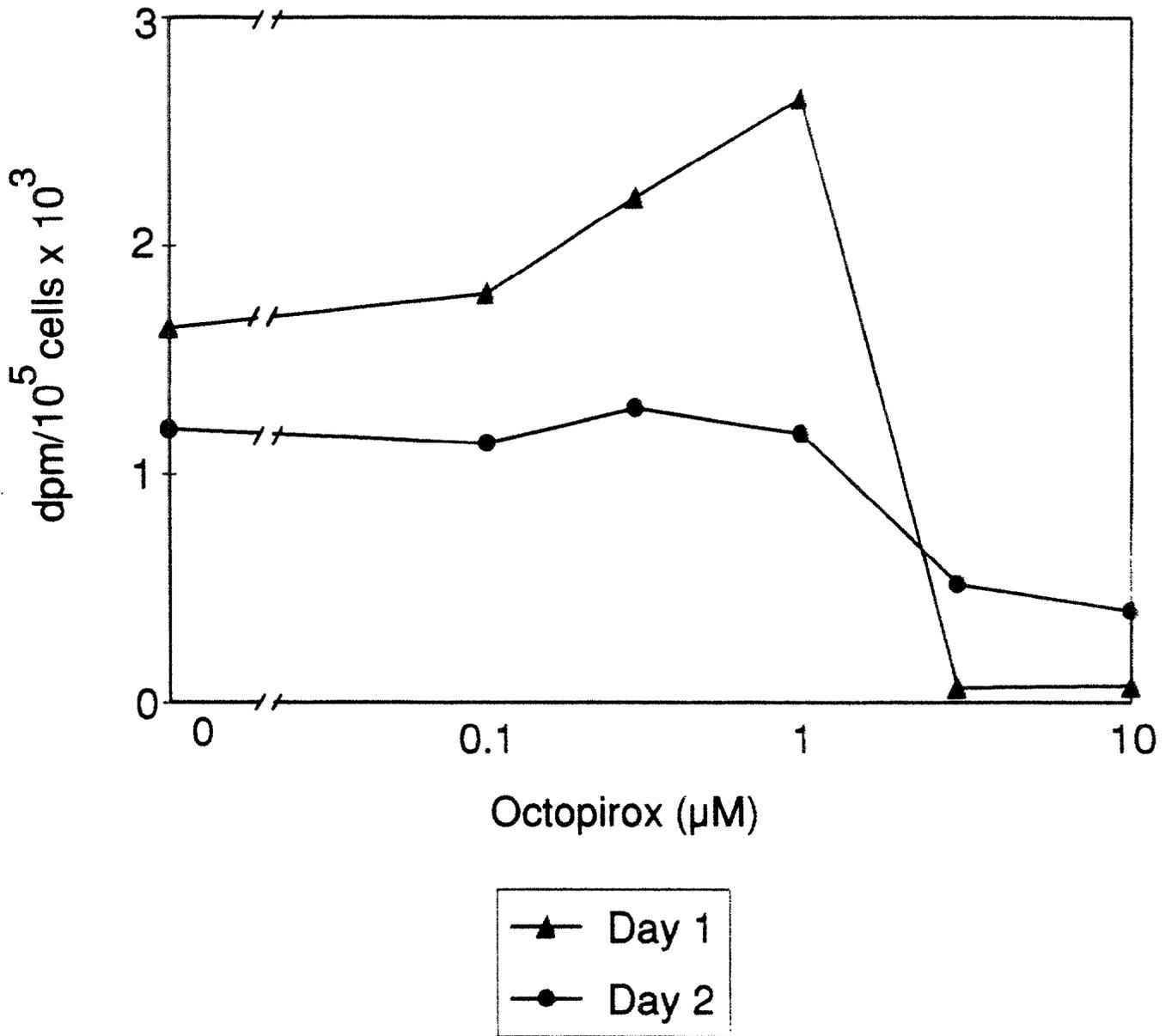


Fig. 9. Long-term effect on the rate of DNA synthesis in L5178Y Mouse Lymphoma Cells by a 2-hour exposure to Octopirox.

Figure 10

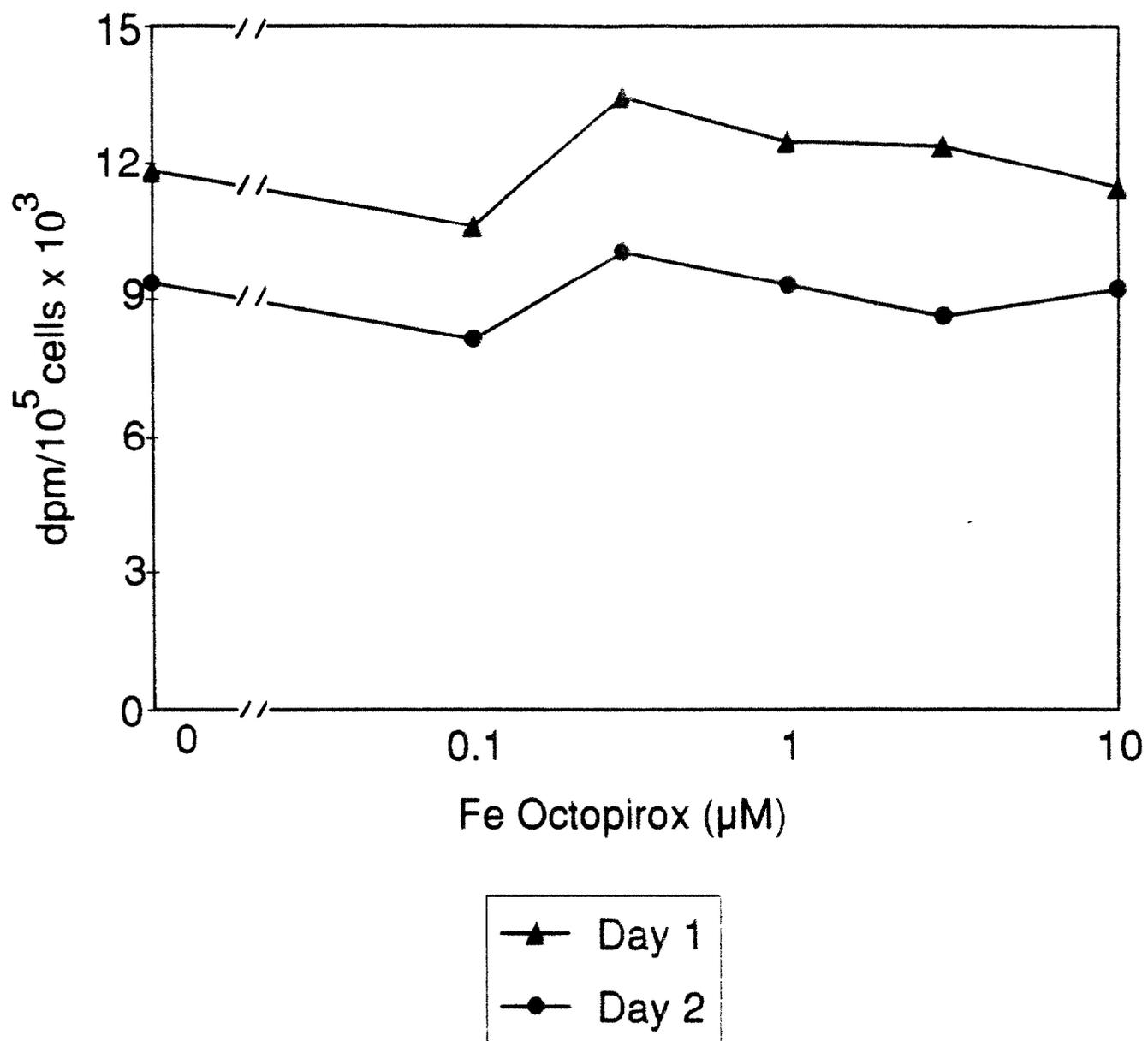


Fig. 10. Long-term effect on the rate of DNA synthesis in L5178Y Mouse Lymphoma Cells by a 2-hour exposure to the iron chelate of Octopirox.