# Effect of Bredinin on the Primary Culture of Fetal Mouse Cells and Adult Mouse Lung Cells

Shinsaku Nakagawa,* Takao Hama and Tadanori Mayumi[1]

*Faculty of Pharmaceutical Sciences, Kobe-Gakuin University, 518, Ikawadani, Nishi-ku, Kobe 651–21, Japan.   Received March 27, 1992*

The effects of bredinin on the primary culture of fetal mouse cells (fetal cells) and adult mouse lung cells (lung cells) were compared. Bredinin inhibited the growth of both cells, and this inhibition was found to be caused, at least in part, by the inhibition of the S phase and/or the transition from the G1 to S phase of the cell cycle. Bredinin inhibited both DNA and RNA synthesis without affecting protein synthesis. However, the inhibitory effect of bredinin differed between the two cell lines; the fetal cells were more sensitive than the lung cells, and bredinin inhibited DNA synthesis 100 times more potently in the fetal cells. The inhibition of DNA synthesis by bredinin in the fetal cells was gradually lowered by *in vitro* aging of the fetal cells to a level similar to that in the lung cells. There was no difference in the rate of incorporation of bredinin into the cells between the fetal cells and the lung cells. When fetal tissue was used as an enzyme source, bredinin was converted to bredinin 5′-monophosphate (BMP), but when lung tissue was used, bredinin was not converted. This is in agreement with the finding that bredinin has selective toxicity on fetuses *in vivo* but is hardly toxic to adult cells, which suggests the involvement of BMP in the selective toxicity of bredinin on the fetus.

**Keywords**   bredinin; bredinin 5′-monophosphate; cell growth; DNA synthesis; primary cultured cell; cell cycle; mouse fetus

Bredinin (4-carbamoyl-1-$\beta$-ribofuranosylimidazolium-5-olate), an immunosuppressive agent, is an imidazole nucleoside isolated from the culture supernatant of *Eupenicillium brefeldianum* M-2166.[2−5] Bredinin has been reported to exert its cytotoxic action on L5178Y cells by blocking the pathway from xanthosine 5′-monophosphate (XMP) to guanosine 5′-monophosphate (GMP).[6]

On the other hand, in *in vivo* experiments in which bredinin was given to pregnant rats, it had no effect on the mother, but had selective toxicity and teratogenicity on the fetus.[7,8] This indicates that bredinin has some selective effect on fetal cells, causing selective toxicity.

In the present study, the cell specificity in the response to bredinin was examined using fetal cells and lung cells derived from adults.

## Materials and Methods
**Materials**   The reagents used in the present experiments were obtained from the following sources: Ham's F-12 medium, thymidine-free Ham's F-12 medium and Dulbecco's modified Eagle's medium (DMEM) from Nissui Seiyaku Co. (Japan); fetal calf serum (FCS) from Gibco; [methyl-³H]thymidine ([³H]TdR, 43 Ci/mmol), [2-¹⁴C]uridine (58 mCi/mmol) and L-[4,5-³H]leucine (130 Ci/mmol) from Amersham; Balb/3T3 cells and CHO-K1 cells from Dainipponseiyaku (Japan). All other chemicals were reagent-grade compounds from commercial sources. Bredinin, bredinin 5′-monophosphate (BMP) and [2-¹⁴C]bredinin (9.06 mCi/mmol) were provided from Toyo Jozo, Co., Ltd.

**Primary Culture**   Six- to 8-week-old ICR mice were obtained from Clea Japan, Inc. (Japan). The mouse fetuses were obtained on day 14 of gestation. The fetuses and the lungs from adult mice were washed three times with $Ca^{2+}$–$Mg^{2+}$-free Dulbecco's phosphate-buffered saline (PBS(−)), chopped to fine pieces and treated with a solution containing 0.25% trypsin and 0.05% collagenase in PBS(−) at 37 °C for 20 min. To obtain the primary cultured cells, the dissociated cells were seeded and cultured for 24 h in 15 ml of Ham's F-12 medium containing 10% FCS in 100 mm plastic dishes. The cells were washed three times with PBS(−) and kept at 37 °C for 10 min in a solution containing 0.25% trypsin in PBS(−). The detached cells were resuspended in Ham's F-12 medium containing 10% FCS and used in the test.

**Examination of Cell Growth-Inhibiting Activity**   CHO-K1 and Balb/3T3 cells were seeded with $1 \times 10^4$ cells/35 mm dish, primary cultured cells were seeded with $1 \times 10^5$ cells/35 mm dish and cultured in Ham's F-12 medium containing 10% FCS for 24 h. The medium was replaced with fresh medium containing bredinin. After 42 h of cultivation, the cells were counted by a Coulter electronic particle counter (Coulter Electronic Inc., Fine Particle Group, Hialeah, Fla.). The percentage of cultured cells that survived under various treatments was determined by comparing the cell number with

those obtained under control conditions. The growth rate was calculated using the following equations[9]:

$$\text{growth rate (\%)} = \frac{\begin{array}{c}\log(\text{number of cells cultured with bredinin})/ \\ (\text{number of cells at time 0})\end{array}}{\begin{array}{c}\log(\text{number of cells in control culture})/ \\ (\text{number of cells at time 0})\end{array}} \times 100$$

**Effect of Bredinin on the Synthesis of DNA, RNA and Protein**   Fetal mouse cells and adult lung cells, $1 \times 10^5$ cells/35 mm dish, were seeded in thymidine-free Ham's F-12 medium containing 10% FCS and cultured for 36 h. Bredinin as dissolved in thymidine-free Ham's F-12 medium containing 10% FCS, at the designated concentration, and after adjustment to pH 7.4 at 37 °C, the culture solution was replaced. Then, [³H]TdR and [2-¹⁴C]uridine at the final concentration of 0.2 mCi/ml, and L-[4,5-³H]leucine at the final concentration of 4 mCi/ml were added and the cells were pulse-labeled for 1, 2, 4 or 8 h. These cells were washed in saline three times. After dissolution by the addition of 1 ml of 0.2 N NaOH, the radioactivity was counted with a liquid scintillation counter (Tri-carb 460CD, Packard).

**Analysis of Cell Cycle**   Balb/3T3 cells were planted in DMEM containing 10% FCS, $5 \times 10^4$ cells/35 mm dish, and cultured for 3 d. The culture solution as replaced with DMEM containing 0.1% dialyzed FCS, and the cells were cultured for 1 d to arrest them in the G1 phase. The cell cycle was started by replacing the medium with DMEM containing 2% dialyzed FCS with or without bredinin added. This time was designated as time 0. The cultures were pulse-labeled at 4-h intervals for 28 h with [³H]TdR to make the final concentration 0.2 mCi/ml. These cells were washed with physiological saline three times and the incorporated radioactivity was counted by the above methods.

**Examination of the Incorporation of Bredinin into the Cell**   The cells were seeded with $1 \times 10^6$ cells/60 mm dish and cultured for 24 h. [2-¹⁴C]Bredinin (10 $\mu$M) was dissolved in Ham's F-12 medium containing 10% FCS and adjusted to pH 7.4 at 37 °C and then the culture medium was replaced (time 0). After the cells were pulse-labeled for 0.5, 1, 2 and 4 h, the cells were washed three times with physiological saline, and the radioactivity was counted.

The cell volume was determined with a Coulter Counter and the amount of bredinin incorporated was shown by the amount of bredinin per unit cell volume.

**Conversion of Bredinin to Bredinin 5′-Monophosphate**   The fetus and the mother's lungs were removed on day 14 of gestation and washed well with physiological saline. Then they were homogenized in 0.1 M Tris–HCl buffer (pH 7.4), and centrifuged at $27000 \times g$ for 45 min at 4 °C. The supernatant after dialysis against the same buffer at 4 °C was used as the enzyme source. [2-¹⁴C]Bredinin (20 $\mu$M, 9.06 mCi/mmol) was incubated with 4 mM ATP, 1.5 mM $MgCl_2$ and enzyme (83 mg protein/ml) in 0.1 M Tris–HCl buffer (pH 7.4) at 37 °C. After 180-min incubation, the reaction was stopped by the addition of perchloric acid. The supernatant was neutralized with KOH and subjected to separate of bredinin and BMP

with a HPLC equipped with a Lichrosorb $NH_2$ column. The mobile phase consisted of acetonitrile : 0.1 M phosphate buffer (pH 7.4) = 2 : 1 (v/v) and flow rate was 1 ml/min.

## Results and Discussion

Administration of bredinin to pregnant rats causes selective toxicity and teratogenicity on the fetus, while having little effect on the mother. This suggests the possibility that the selective toxicity on the fetal cells is caused by a cell specific action of bredinin. The effect of bredinin on the cell specificity was examined using fetal cells and adult cells.

Figure 1 shows the effect of bredinin on the proliferation of various cells. The data revealed a dose-dependent inhibitory effect in all the cell lines examined containing CHO-K1, Balb/3T3, lung cells and fetal cells. However, the inhibitory effect of bredinin on the proliferation of cells differed with the cell, and the fetal cell had shown the greatest sensitivity. The 50% cell growth-inhibiting concentration of bredinin was about 1 μM for fetal cells and 5 μM for adult lung cells.

The effect of bredinin on the synthesis of DNA, RNA

and protein was examined (Fig. 2). The addition of 10 μM bredinin inhibited the synthesis of both DNA and RNA in both the fetal cells and lung cells, but not the protein synthesis in both cells at least for 8 h of culture. The protein synthesis until 8 h after the addition of bredinin may be synthesized from the endogenous m-RNAs. Sakaguchi et al. reported that bredinin inhibited the incorporation of thymidine and uridine, but not leucine, into macromolecules in L5178Y cells.[10] Therefore, the inhibition of cell proliferation by bredinin is considered to be caused by the inhibition of the synthesis of DNA and RNA. In the lung cells, the syntheses of DNA and RNA were not inhibited until a 4-h culture, but in the fetal cells, they were inhibited almost completely within a 1-h culture period. The means that the sensitivity to bredinin markedly differed between the fetal cells and lung cells.

Figure 3 shows a comparison between the two cells for the dose dependency of the inhibitory effect of bredinin on DNA synthesis at the 8th h of culture, revealing that the fetal cells have greater sensitivity than the lung cells. The bredinin showed a similar inhibitory effect on both the cell proliferation and DNA synthesis in fetal cells. However, in the lung cells, the inhibitory effect of bredinin on cell proliferation was stronger than that on DNA synthesis. Therefore, the inhibitory effect of bredinin on cell proliferation could not be explained only by the inhibition
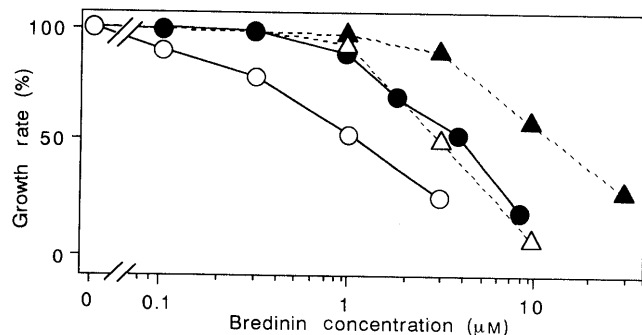


Fig. 1. Effect of Bredinin on Proliferation of Various Cells

Various cells were cultured with or without bredinin. ○, fetal cell; ●, lung cell; △, Balb/3T3 cell; ▲, CHO-K1 cell.



Fig. 3. Effect of Bredinin on DNA Synthesis of Primary Cultured Cell

Cells were cultured with bredinin and labeled with [³H]TdR (0.2 μCi/ml) for 8 h. ●, fetal cells; ▲, lung cells.
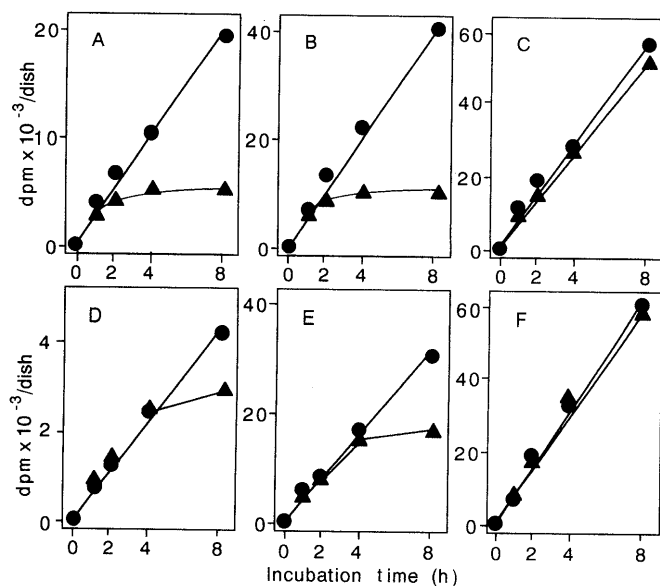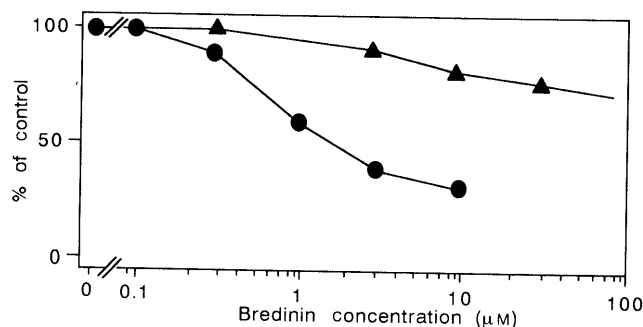


Fig. 2. Effect of Bredinin on DNA, RNA and Protein Synthesis of Fetal Mouse Cells and Adult Mouse Lung Cells

Fetal cells (A, B, C) and lung cells (D, E, F) were cultured with (▲) or without (●) bredinin (10 μM) for various periods. [³H]TdR (A, D; 0.2 μCi/ml), [2-¹⁴C]uridine (B, E; 0.2 μCi/ml) and L-[4,5-³H]leucine (C, F; 4 μCi/ml) were added at 0 time.
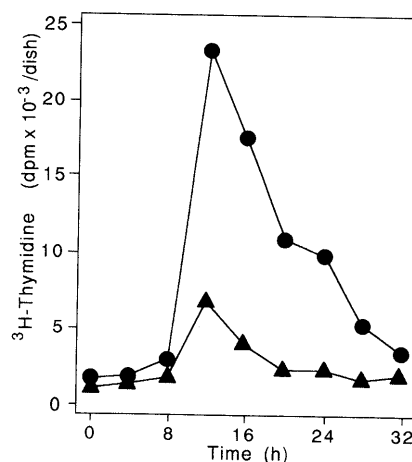


Fig. 4. Effect of Bredinin on DNA Synthesis of Balb/3T3 Cell Arrested in G1 Phase

Balb/3T3 cells were arrested in G1 phase. Medium was changed to DMEM containing 2% dialyzed FCS at 0 time. The cells were labeled with [³H]TdR for 4 h at each period. ●, control; ▲, bredinin (5 μM).

of the synthesis of DNA and RNA. The other cause for inhibition of cell proliferation by bredinin has not yet been elucidated.

Bredinin has been shown to suppress incorporation of [³H]TdR into cellular DNA. It is well known that most cells in cultures of confluent or serum starved mouse 3T3 stay in the early G1 or G0 stage of the cell cycle, and an addition of serum to these quiescent cells stimulates their growth, leading to DNA synthesis and cell division.[11,12] The site of action of bredinin on the cell cycle was analyzed using Balb/3T3 cells as shown in Fig. 4. The addition of 2% FCS stimulated the serum-starved cells to transit from the G1 to S phase. Bredinin showed a suppressive effect on the transition from the quiescent to the growing state. The cell growth was inhibited about 70% at 5 $\mu$M of bredinin concentration. The DNA synthesis in Balb/3T3 cells appeared to begin after an 8 h lag period both in the presence and absence of bredinin. The rate of the DNA synthesis was markedly inhibited when bredinin was present in the culture, but bredinin did not shift the point where cells enter into the S phase. This suggests that the cell growth-inhibiting effect of bredinin was due, at least in part, to the inhibition of the DNA synthesis in S phase and/or the transition from the G1 to the S phase. There has been no direct evidence in which bredinin shows the inhibitory effect on the entry into the S phase or whether it effects the rate of DNA synthesis in the S phase.

The cells were aged *in vitro*, and the inhibitory effect of bredinin on DNA synthesis was compared at various ages of the cells (Fig. 5). Figure 5A, B, C shows the time course for the uptake of [³H]TdR into the cells in the absence or presence of bredinin. As shown in Fig. 5D, the sensitivity to the inhibitory effect decreased with the advance in the population doubling level (PDL) of the primary fetal cells, and the inhibition pattern of the 6 PDL fetal cells was

similar to that of the lung cells shown in Fig. 2D. Inhibition of DNA synthesis at the 8th h of the culture of 6 PDL and 10 PDL fetal cells was almost the same and the level was also similar to that of the lung cells. This shows that the inhibitory effect of bredinin on DNA synthesis decreased with the aging of the cells.

Whether the cell specificity for the inhibitory effect of bredinin was caused by a difference in the incorporation of bredinin into the cells was examined (Fig. 6). The uptake of bredinin in the fetus (0 PDL), 10 PDL fetal cells, and lung cells reached a plateau after 2 h of culture. Thus, the difference in the inhibitory effect of bredinin between the cells was confirmed not to be due to a difference in the amount of bredinin incorporated into the cells. Usually, nucleosides are incorporated into the cell by a transporter located on the cell surface.[13] Paul[14] and Cass[15] reported that nitrobenzilthioinosine specifically inhibited the nucleoside transporter. The treatment with nitrobenzilthioinosine decreased the growth inhibitory effect of bredinin (data not shown). Therefore, the cell specificity of bredinin is not considered to be due to the difference in the amount of bredinin in the cell caused by a difference in the transporter. The radioactivity of bredinin incorporated into the cell was not detectable in the acid insoluble fraction (protein, DNA and RNA molecules) (data not shown).

According to studies on the action mechanism of bredinin, Hisazumi *et al.* reported that bredinin did not inhibit inosine 5'-monophosphate dehydrogenase or GMP synthetase, but that BMP had a strong inhibitory effect.[16] Therefore, we examined the cell specificity in the conversion of bredinin

Fig. 6.   Time Course of Bredinin Uptake by Fetal Cells and Lung Cells

Cells were cultured with 10 $\mu$M [2-¹⁴C]bredinin for various intervals. ○, fetal cells (0 PDL); ●, fetal cells (10 PDL); △, lung cells.
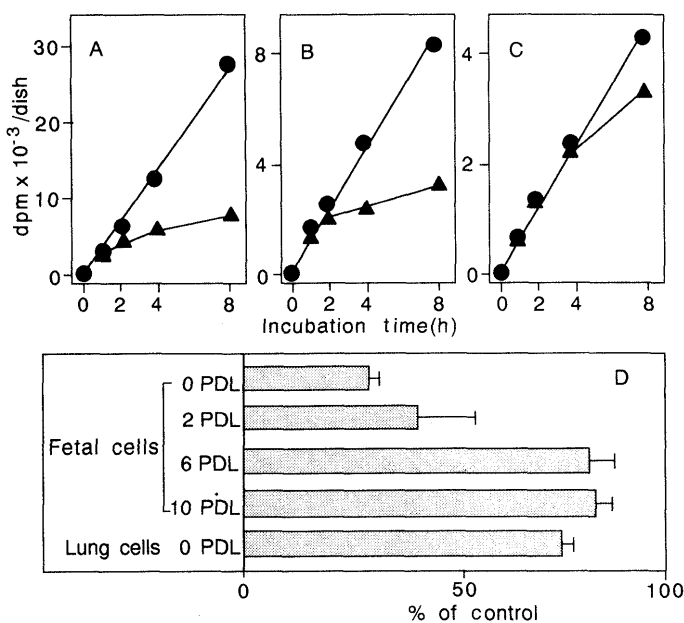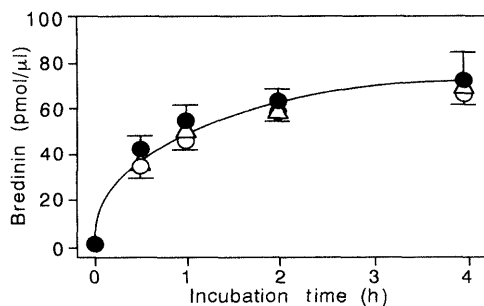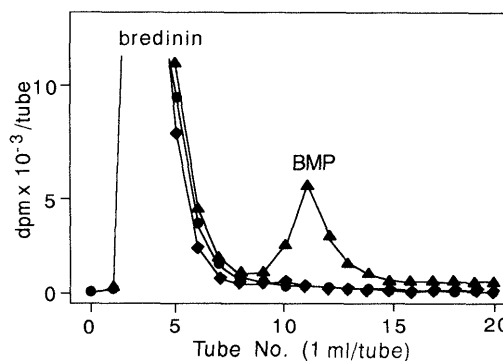
Fig. 5.   Effect of Bredinin on DNA Synthesis of Fetal Cells and Lung Cells

A, B, C; fetal cells were cultured with (▲) or without (●) bredinin (10 $\mu$M) for various periods. [³H]TdR (0.2 $\mu$Ci/ml) was added at 0 time. A; 0 PDL, B; 2 PDL, C; 6 PDL, D; Cells were cultured with bredinin (10 $\mu$M) and pulsed with [³H]TdR (0.2 $\mu$Ci/ml) for 8 h.

Fig. 7.   HPLC Chromatogram of [2-¹⁴C]Bredinin Metabolites Catalyzed by Enzymes from Fetus or Adult Lung

[2-¹⁴C]Bredinin (20 $\mu$M, 9.06 Ci/mol) was incubated for 3 h with ATP (4 mM), MgCl₂ (1.5 mM), and enzyme (27000 × $g$ supernatant, 83 mg protein/ml). ●, control (enzyme-free); ▲, fetus; ◆, adult lung.

to BMP. Figure 7 shows the conversion of bredinin to BMP. The supernatant of the homogenate of fetus or adult lung was used as the enzyme source. The conversion of bredinin to BMP was clearly seen when the fetal tissue was used as the enzyme source, while the conversion to BMP was hardly seen when the adult lung was used as the enzyme source, or the enzyme source was excluded (control). This was in agreement with the fact that bredinin showed a selective toxicity, a teratogenetic effect and a marked inhibitory effect of proliferation on the fetus, and the cell specificity was suggested to be due to the formation of BMP, the active form of bredinin. In the adult lung, the kinase activity responsible for the phosphorylation of bredinin decreased and/or 5'-nucleotidase activity responsible for the dephosphorization of BMP increased. Koyama reported that bredinin resistant cells are attributed to defective adenosine kinase activity and, that bredinin is metabolized by adenosine kinase, which may phosphorylate to a toxic nucleotide, BMP, in sensitive cells.[17] Adenosine kinase activity and 5'-nucleotidase activity in fetal cells and lung cells were not examined. Sun reported also that 5'-nucleotidase activity in embryonic fibroblast cells increased during *in vitro* aging.[18] Therefore, 5'-nucleotidase activity may be lower in fetal cells than in lung cells.

These findings strongly suggest the relationship between the formation of BMP and the selective toxicity of bredinin on the fetus. Further studies on the enzyme responsible for the phosphorylation of bredinin, and comparison of the enzyme activity in various cell cultures should help to elucidate the selective toxicity of bredinin on the fetus.

**References and Notes**
1) Present address: *Faculty of Pharmaceutical Sciences, Osaka University, 1-6, Yamada-oka, Suita, Osaka 565, Japan.*
2) Y. Kobayashi, K. Matsumoto, Y. Hoshino and K. Hayano, *Teratology*, **10**, 87 (1974).
3) K. Mizuno, M. Tsujino, M. Takada, M. Hayashi, K. Atumi, K. Asano and T. Matuda, *J. Antibiot.*, **27**, 775 (1974).
4) H. Yoshioka, K. Nakatsu, M. Hayashi and K. Mizuno, *Tetrahedron Lett.*, **1975**, 4031.
5) M. Hayashi, T. Hirano, M. Yaso, K. Mizuno and T. Ueda, *Chem. Pharm. Bull.*, **23**, 245 (1975).
6) K. Sakaguchi, M. Tsujino, M. Yoshizawa, K. Mizuno and K. Hayano, *Cancer Res.*, **35**, 1643 (1975).
7) K. Okamoto, Y. Kobayashi, K. Yoshida, Y. Nozaki, Y. Kawai, H. Kawano, T. Mayumi and T. Hama, *Cong. Anoma.*, **18**, 227 (1978).
8) K. Okamoto, K. Hoshino, A. Yoshida, Y. Kawai, T. Mayumi and T. Hama, *Cong. Anoma.*, **20**, 359 (1980).
9) K. Sakaguchi, M. Tsujino, M. Hayashi, K. Kawai, K. Mizuno and K. Hayano, *J. Antibiot.*, **29**, 1320 (1976).
10) K. Sakaguchi, M. Tsujino, K. Mizuno, K. Hayano and N. Ishida *J. Antibiot.*, **28**, 798 (1975).
11) R. W. Holley and J. A. Kiernan, *Proc. Natl. Acad. Sci. U. S. A.*, **60**, 300 (1968).
12) R. Dulbecco, *Nature* (London), **227**, 802 (1970).
13) R. D. Berlin and J. M. Oliver, *Int. Rev. Cytol.*, **42**, 287 (1975).
14) B. Paaul, M. F. Chen and A. R. P. Paterson, *J. Med. Chem.*, **18**, 968 (1975).
15) Carol E. Cass, E. Dahlig, E. Y. Lau, T. P. Lynch and A. R. P. Paterson, *Cancer Res.*, **39**, 1245 (1979).
16) T. Hisazumi, M. Tsuda and T. Katsunuma, Abstracts of Papers, The 55th Annual Meeting of the Japanese Biochemical Society, Osaka, October 1982, p. 534.
17) H. Koyama and M. Tsuji, *Biochem. Pharmacol.*, **32**, 3547 (1983).
18) Alexander S. Sun, Bharat B. Aggarwal and Lester Packer, *Arch. Biochem. Biophys.*, **170**, 1 (1975).