[Jpn. J. Parasitol., Vol. 43, No. 5, 384–390, October, 1994]

Growth Inhibition by Acivicin and Allopurinol, Singly and in Combination, of *Crithidia fasciculata* and Antagonistic Effects of Nucleotide Precursors

TAKASHI AOKI

(Accepted for publication; December 28, 1994)

Abstract

The fifty percent inhibition concentration (IC_{50}) for acivicin that yielded the 50% growth inhibition of logarithmically growing *Crithidia fasciculata* in a serum-free medium was 1.5 μM and the value for allopurinol was 10 μM. Acivicin (20 μM) inhibited the growth by 86%, and this inhibition was antagonized by 10 μM each of cytidine plus guanosine; guanosine or deoxyguanosine alone was slightly antagonistic. At 10 μM allopurinol, 200 μM each of uracil, uridine, cytidine or deoxycytidine canceled the inhibition of protozoan growth. Coadministration of acivicin and allopurinol resulted in a synergistic growth inhibition, and this inhibition was protected by the simultaneous addition of cytidine, guanosine, and hypoxanthine. These results are consistent with the view that acivicin inhibits GMP and CTP synthetases and that allopurinol interferes with de novo pyrimidine synthesis in *C. fasciculata*.

Key words: *Crithidia fasciculata*, growth inhibition, acivicin, allopurinol, de novo pyrimidine synthesis, purine salvage synthesis

Introduction

Previous work in this laboratory demonstrated that acivicin [L-(α*S*,5*S*)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid], an L-glutamine antagonist, entailed a marked growth inhibition on *Crithidia fasciculata* in a serum-free medium (Aoki and Oya, 1988). The compound brought about an intracellular inactivation of L-glutamine-dependent activity of the protozoan carbamoyl-phosphate synthetase II (CPS II), the first key enzyme of de novo pyrimidine nucleotide biosynthesis. However, it was not clear whether this enzyme was responsible, as a principal target in acivicin action, for the protozoan growth inhibition. This study was thus aimed at the search for target(s), elucidating the mechanism of *Crithidia* growth inhibition. Growth inhibition by allopurinol was also analyzed.

Department of Parasitology, Juntendo University School of Medicine, Hongo, Bunkyo-ku, Tokyo 113, Japan.
青木　孝（順天堂大学医学部寄生虫学教室）

Materials and Methods

Materials

Crithidia fasciculata (ATCC 11745, *Anopheles* strain) was originally obtained from American Type Culture Collection and maintained in subcultures in an undefined medium LIT (Aoki and Oya, 1987a) and then in a serum-free medium GIT (Aoki and Oya, 1988). GIT was purchased from Wako Pure Chemical Industries (Osaka). The composition of GIT, in which the purified growth factor of bovine serum replaced fetal calf serum, was described previously (Kudo *et al.*, 1987). The medium also contained 21 mM bicarbonate, 2.5 mM L-glutamine, 0.16 mM L-aspartate, 0.25 mM glycine, 1.5 μM thymidine, 15 μM hypoxanthine, and 6.0 μM folic acid as purine and pyrimidine precursors. The GIT was proven free of microorganisms including mycoplasmas by the manufacturer.

Bases, nucleosides, and nucleotides were obtained from Nakarai Chemicals (Kyoto), Tokyo Kasei Industries (Tokyo), Sigma Chemical Co. (U.S.A.) or Boehringer Mannheim (Germany). Acivicin was a generous gift from Dr. George Weber, Indiana University School of Medicine,

Indianapolis, U.S.A. Allopurinol was purchased from Nakarai Chemicals. These compounds were dissolved in GIT and sterilized through Millipore membrane filter (0.22 μm).

Cell culture

Logarithmically growing organisms were inoculated with an initial cell density of 5–8×10^4/ml into GIT (5.0 ml) in a 25-cm^2 plastic flask (Corning-Iwaki Glass, Tokyo). The flasks were tightly screw-capped and incubated at 27°C for 7 h. Then, various compounds were added to the media to assess the inhibition of cell growth or antagonism against the growth inhibition. The flasks were further incubated at 27°C for 40 h and the cell densities were determined as described (Aoki and Oya, 1988).

Expression of results

The results were expressed as the average cell density of two flasks for each compound tested. Variations of cell densities in these two flasks were less than 2% of the preliminarily determined average of five flasks. The degree of growth inhibition by acivicin or allopurinol could be estimated using an equation (Sato *et al.*, 1983),

$$\% \text{ of Control} = \frac{\text{Cells/ml (treated)} - \text{Cells/ml (seeded)}}{\text{Cells/ml (control)} - \text{Cells/ml (seeded)}} \times 100$$

However, the seeded cell densities were always less than 3% of the lowest treated cell densities so that we adopted the following simple equation to estimate the inhibition of cell growth,

$$\% \text{ of Control} = \frac{\text{Cells/ml (treated)}}{\text{Cells/ml (control)}} \times 100$$

where both the cell densities were measured at 40 h after drug administration.

Results

Conditions of cultivation

Five different lots of GIT, lot numbers V006, V007, W101, W102, and 0107, were tested for their potentials to support the growth of *C. fasciculata*. Doubling times of the cells were approximately 5.2, 6.5, 4.5, 5.0, and 5.4 h for V006, V007, W101, W102, and 0170, respectively. Long time cultivation for 47 h, at which the growth reached the late exponential phase in the control culture, resulted in the more accurate cell counts than the shorter time cultivation for 23 h (Fig. 1). Therefore, a batch of GIT (V007) which yielded the slowest growth was chosen and used in this study to set the appropriate timings of inoculation, drug addition, and cell counting at 0, 7, and 47 h, respectively.

Growth inhibition by acivicin or allopurinol and effect of nucleosides and bases

Figure 1 depicts the time course of growth inhibition by different concentrations of acivicin. Forty hours after addition of 0.63, 1.3, 2.5, 5.0, 10, and 20 μM acivicin, cell counts were decreased to 64, 52, 41, 25, 16, and 10% of the control, respectively, yielding the 50% inhibition concentration (IC_{50}) of 1.5 μM. Similar experiment with use of allopurinol resulted in IC_{50} value of 10 μM (data not shown).

A series of combinations of acivicin and bases or nucleosides were added to the GIT cultures of *C. fasciculata* to measure the degree of rescue effect of these nucleotide precursors on the growth inhibition by acivicin (Table 1). Acivicin (20 μM) inhibited the growth by 86%, and this inhibition was antagonized by 10 μM each of cytidine plus guanosine. Cytidine plus deoxyguanosine also protected the protozoan cells from growth inhibition. Combinations of guanosine (or deoxyguanosine) with either of uridine, cytosine or deoxycytidine, each of which did not affect the growth inhibition, appeared to entail a little antagonism. Guanosine or deoxyguanosine alone also slightly antagonistic. Higher concentration (100 μM) of guanosine antagonized more markedly the growth inhibition by lower concentration (10 μM) of acivicin (Aoki and Oya, 1988). Organic bases, singly and in combination, had no effect on the growth inhibition by acivicin (Table 1).

Since allopurinol is an analog of a purine base, hypoxanthine, we expected that purine bases or nucleosides would antagonize the growth inhibition by allopurinol of *C. fasciculata* cells. However, the inhibited growth with 10 μM allopurinol (IC_{50}) was not recovered by any of 10 μM purine bases and nucleosides (data not shown). Marked increase in the concentration of these compounds to 200 μM conferred on hypoxanthine and adenosine some protection against the inhibited growth (Table 2). On the contrary, high concentrations of a pyrimidine

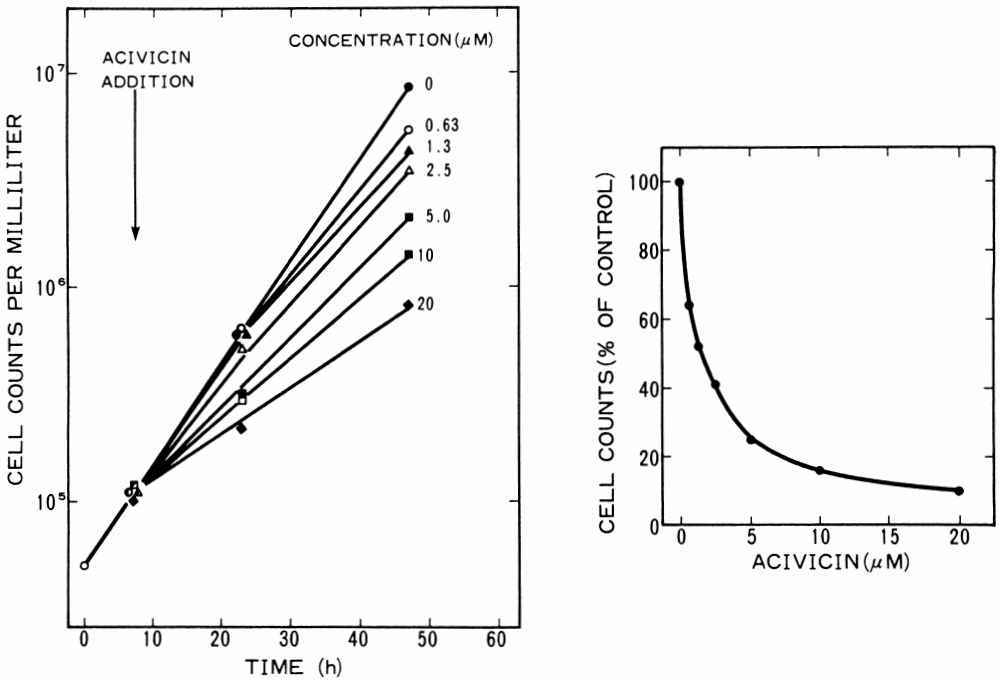

Fig. 1 Effect of different concentrations of acivicin on the growth of *Crithidia fasciculata* in a serum-free medium GIT. Left: the number of cells/ml was plotted as a function of time of cultivation at 27°C. Right: the number of cells/ml (% of control) at 47 h of cultivation was plotted as a function of acivicin concentration.

Table 1 Effect of nucleosides and bases on acivicin-inhibited growth of *C. fasciculata**

Compound	Cell counts/ml		Compound	Cell counts/ml	
	$\times 10^{-6}$	% of control		$\times 10^{-6}$	% of control
None (control)	7.10	100	Acivicin plus:		
Acivicin	0.99	13	U + C	0.71	10
			U + A	0.72	10
			U + G	0.80	11
Acivicin plus:			C + A	1.00	14
Uracil (U)	0.82	11	C + G	1.00	14
Cytosine (C)	0.96	13	A + G	0.93	13
Adenine (A)	0.91	12	UR + CR	0.73	10
Guanine (G)	1.00	14	UR + AR	0.61	9
Uridine (UR)	0.72	9	UR + GR	1.62	23
Cytidine (CR)	0.69	9	CR + AR	0.71	10
Adenosine (AR)	0.84	11	CR + GR	7.10	100
Guanosine (GR)	1.30	17	AR + GR	1.37	19
Deoxycytidine (CdR)	0.84	11	C + GR	1.42	20
Deoxyguanosine (GdR)	1.30	17	C + GdR	1.56	22
			CR + G	0.92	13
			CR + GdR	6.82	96
			CdR + G	1.14	16
			CdR + GR	2.34	33
			CdR + GdR	1.63	23

*The concentration of acivicin was 20 μM and that of compounds added was 10 μM.

base (uracil) and nucleosides (uridine, cytidine, and deoxycytidine) were highly antagonistic to the growth inhibition by allopurinol. In general, nucleosides were more markedly antagonistic to the growth inhibition by acivicin and by allopurinol than bases (Tables 1 and 2).

Table 2 Effect of nucleosides and bases on allopurinol-inhibited growth of *C. fasciculata*

Compound added	Cell counts/ml	
	$\times 10^{-6}$	% of control
None (control)	3.7	100
10 μM Allopurinol	1.6	43
10 μM Allopurinol plus:		
200 μM Adenine	1.9	51
200 μM Guanine	1.4	38
200 μM Hypoxanthine	2.5	68
200 μM Adenosine	2.7	73
200 μM Guanosine	2.0	54
200 μM Inosine	2.0	54
200 μM Deoxyguanosine	1.4	38
200 μM Uracil	3.5	95
200 μM Cytosine	1.9	51
200 μM Uridine	4.1	111
200 μM Cytidine	4.1	111
200 μM Deoxycytidine	4.3	116

Growth inhibition by acivicin plus allopurinol and effect of nucleosides and bases

Table 3 shows the effect of combinations of acivicin and allopurinol on the growth of *C. fasciculata*. The concentrations of these compounds were lowered to less than IC_{50} values, since the growth inhibition by higher concentration of acivicin or allopurinol was as closer to saturation (Fig. 1) as to mask the net combination effect of these agents. The values listed in Table 3 as "calculated" were determined by the method of Sato *et al.* (1983) to evaluate whether the combination of two drugs brought about a synergistic inhibition of cell growth. When the "calculated" value was larger than the observed cell growth, therefore, the combination yielded a synergistic inhibition. At the fixed concentration of 3.0 μM allopurinol, additions of varied concentrations of acivicin (0.25–1.0 μM) clearly indicated synergistic growth inhibition (Table 3). At the fixed concentration of 0.4 μM acivicin, additions of different allopurinol concentrations (2.0–8.0 μM) also gave similar synergism (data not shown).

Coadministration of acivicin and allopurinol (10 μM each) demonstrated a marked inhibition of cell growth by 84%, and this inhibition was partially antagonized by 10 μM each of cytidine plus guanosine (Table 4); the remaining inhibition would be due to the inhibitory effect of allopurinol. However,

Table 3 Synergistic growth inhibition of *C. fasciculata* cells by acivicin and allopurinol

Concentration (μM)		Cell counts/ml		Calculated
Acivicin	Allopurinol	$\times 10^{-6}$	% of control	a × b*/100 (%)
0		4.8	100	
0.25		5.0	104	
0.50		4.5	94	
0.75		4.2	88	
1.00		3.7	77	
0	3.0	4.5	94	
0.25	3.0	3.7	77	98
0.50	3.0	3.2	67	88
0.75	3.0	3.1	65	83
1.00	3.0	2.9	60	72

*a×b represents $(1-i_a) \times (1-i_b)$, where i_a and i_b are the fractional growth inhibitions caused by acivicin and allopurinol, respectively.

Table 4 Growth inhibition by acivicin and allopurinol of *C. fasciculata* cells and effect of nucleosides and bases

Compound added	Cell counts/ml	
	$\times 10^{-6}$	% of control
None (control)	4.6	100
10 μM Acivicin plus 10 μM allopurinol	0.74	16
plus cytidine & guanosine (10 μM each)	2.6	57
plus 200 μM adenosine	0.87	19
plus 200 μM hypoxanthine	0.87	19
plus cytidine & guanosine, and 200 μM adenosine	1.0	22
plus cytidine & guanosine, and 200 μM hypoxanthine	4.8	104
plus cytidine & guanosine, and 200 μM uracil	3.3	72
plus cytidine & guanosine, and 200 μM cytosine	3.5	76
plus cytidine & guanosine, and 200 μM uridine	1.8	39
plus cytidine & guanosine, and 200 μM cytidine	4.0	87
plus cytidine & guanosine, and 200 μM deoxycytidine	3.2	70
Cytidine & guanosine (10 μM each)	5.5	120
200 μM Adenosine	4.3	93
200 μM Hypoxanthine	4.9	107

pyrimidine base and nucleosides which protected the cells from the growth inhibition by allopurinol, in combination with cytidine plus guanosine, did not show a complete restoration of cell growth to the control level, indicating that the complete rescue could not be attained by the sum of individual rescues against acivicin and allopurinol. Instead, a combination of cytidine, guanosine, and hypoxanthine achieved the nearly complete antagonism against the growth inhibition by two drugs (Table 4), although hypoxanthine alone did not completely antagonize the inhibition by allopurinol (Table 2). Interestingly, the addition of adenosine to cytidine plus guanosine cancelled the rescue effect gained by the latter two nucleosides (Table 4).

Discussion

Trypanosomatids are devoid of the de novo purine biosynthetic pathway (Hammond and Gutteridge, 1984), and thus may possess three targets for acivicin (see Fig. 2), GMP synthetase, CTP synthetase, and CPS II, in purine and pyrimidine nucleotide biosynthesis. These enzymes catalyze the conversions from XMP to GMP, from UTP to CTP, and from bicarbonate, L-glutamine, and ATP to carbamoyl-phosphate, respectively. Since all three enzymes utilize the amido nitrogen of L-glutamine in their catalyses (Zalkin, 1985a; Zalkin, 1985b; Kaseman and Meister, 1985) and acivicin inactivates L-glutamine-dependent activity of *Crithidia* CPS II by affinity labeling (Aoki and Oya, 1987b; 1988), the drug may also act as an affinity analog of L-glutamine on GMP and CTP synthetases in *C. fasciculata*.

Our data obtained in this study support an idea about the order of degree of susceptibility to acivicin of these *Crithidia* enzymes to be GMP synthetase ≥ CTP synthetase > CPS II. This view enables us to understand that simultaneous addition of cytidine and guanosine, which might by-path the inhibition of CTP and GMP synthetase activities (Fig. 2), could result in the recovery from the growth inhibition. Strong interference of acivicin with GMP synthetase would suppress the synthesis of RNA (GMP → GDP → GTP → RNA) and DNA (GMP → GDP → dGDP → dGTP → DNA) in the absence of purine salvage substrates. The postulated highest sensitivity of this enzyme to acivicin is consistent with the fact that addition of guanosine or

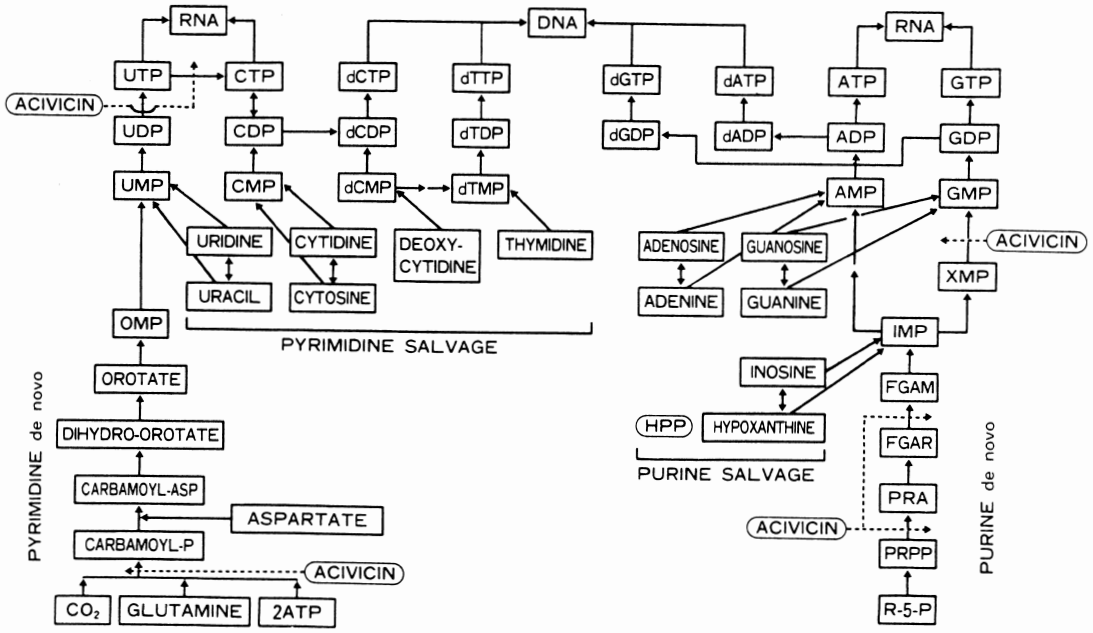

Fig. 2 Biosynthetic pathways for nucleotides and nucleic acids in mammalian tissues. Five possible targets for acivicin are indicated. Trypanosomes lack the purine de novo biosynthetic pathway and easily incorporate allopurinol (HPP).

deoxyguanosine afford a partial protection against the growth inhibition (Aoki and Oya, 1988 and this work). The inhibition by acivicin of CTP synthetase may also suppress the DNA synthesis (CTP → CDP → dCDP → dCTP → DNA) (Fig. 2), and thus the addition of deoxycytidine and guanosine protected, in a certain extent, the *Crithidia* cells from the growth inhibition (Table 1).

It was difficult to gain a recovery from growth inhibition by allopurinol (Table 2). The very high concentration of pyrimidine base and nucleosides antagonized the growth inhibition, in accordance with the previous report that allopurinol-riboside monophosphate strongly inhibited in vitro the activity of orotidylate (OMP) decarboxylase, the enzyme that catalyzes the last step of de novo UMP synthesis, in *C. fasciculata* extract (Dewey and Kidder, 1973). Thus, the primary target of allopurinol in *Crithidia* growth inhibition may be the de novo pyrimidine pathway, while possible target(s) in purine synthesis are not clear.

Interestingly, coadministration of cytidine, guanosine, and adenosine resulted in no antagonism against the growth inhibition by acivicin plus allopurinol (Table 4). It would be plausible that addition of allopurinol strongly inhibits the de novo pyrimidine biosynthesis and that acivicin inhibits the activity of CPS II. Such a simultaneous inhibition would synergistically deplete UDP and UTP pools, yielding a marked unbalance between pyrimidine and purine pools for nucleic acid synthesis. Addition of adenosine to this situation would bring about further imbalance of nucleic acid precursors, producing much more growth inhibition. As a result, the rescue effect by cytidine plus guanosine against acivicin would be apparently hidden. On the other hand, hypoxanthine, the physiological substrate, would compete with allopurinol for the site of entry or transporter of the cell membrane and decrease the uptake of allopurinol, reducing the inhibition of de novo pyrimidine synthesis, and eventually cytidine plus guanosine succeed to antagonize the growth inhibition by acivicin. Namely, cytidine, guanosine, and hypoxanthine, all together, antagonized the growth inhibition by acivicin plus allopurinol.

The method used and the results obtained in this

study would be useful for better understanding of metabolic pathways, e.g., the principal target of allopurinol in pathogenic trypanosomatids would be the incorporation of allopurinol into RNA, leading to the breakdown of abnormal RNA (Marr, 1991), whereas in *C. fasciculata* it would be the de novo pyrimidine pathway (this study). This may indicate the differences of allopurinol metabolism and of substrate specificity of OMP decarboxylase in *C. fasciculata* and pathogenic trypanosomatids. Our method would also provide a basis for possible chemotherapeutic modality, e.g., synergistic growth inhibition by two drug combinations (Table 3). As a prosperity in this line of investigation, we have recently succeeded in developing a culture system of host cells infected with *Trypanosoma cruzi* and in quantitatively determining the time course of parasite growth inside host cells (Nakajima-Shimada *et al.*, 1994). Allopurinol was quite effective in reducing the *T. cruzi* growth in this system (Aoki *et al.*, 1994).

Acknowledgements

The author thanks Miss H. Kuki for her technical assistance. A part of this work was supported by the Japan-US Medical Cooperative Program on Parasitic Diseases.

References

1) Aoki, T. and Oya, H. (1987a): Kinetic properties of carbamoyl-phosphate synthetase II (glutamine-hydrolyzing) in the parasitic protozoan *Crithidia fasciculata* and separation of the enzyme from aspartate carbamoyltransferase. Comp. Biochem. Physiol., 87B, 143–150.
2) Aoki, T. and Oya, H. (1987b): Inactivation of *Crithidia fasciculata* carbamoyl-phosphate synthetase II by the antitumor drug acivicin. Mol. Biochem. Parasitol., 23, 173–181.
3) Aoki, T. and Oya, H. (1988): Acivicin inhibits *Crithidia fasciculata* growth in a serum-free medium and inactivates carbamoyl-phosphate synthetase II *in vivo*. Comp. Biochem. Physiol., 90C, 391–396.
4) Aoki, T., Nakajima-Shimada, J. and Hirota, Y. (1994): Quantitative determination of *Trypanosoma cruzi* growth inside host cells in vitro and effect of allopurinol. *In* Purine and Pyrimidine Metabolism in Man, VIII, Ed. by A. Sahota and M. Taylor, Plenum Publishing Co., New York, N.Y., in press.
5) Dewey, V. C. and Kidder, G. W. (1973): The sites of action of allopurinol in *Crithidia fasciculata*. J. Protozool., 20, 678–682.
6) Hammond, D. J. and Gutteridge, W. E. (1984): Purine and pyrimidine metabolism in the Trypanosomatidae. Mol. Biochem. Parasitol., 13, 243–261.
7) Kaseman, D. S. and Meister, A. (1985): Carbamyl phosphate synthetase (glutamine-utilizing) from *Escherichia coli*. Methods Enzymol., 113, 305–326.
8) Kudo, T., Morishita, R., Suzuki (Seno), R. and Tachibana, T. (1987): A great improvement of fusion efficiency in mouse B cell hybridoma production by use of the new culture medium, GIT. Tohoku J. Exp. Med., 153, 55–66.
9) Marr, J. J. (1991): Purine analogs as chemotherapeutic agents in leishmaniasis and American trypanosomiasis. J. Lab. Clin. Med., 118, 111–119.
10) Nakajima-Shimada, J., Hirota, Y., Kaneda, Y. and Aoki, T. (1994): Quantitative determination of growth of amastigotes and trypomastigotes in an cultivation system of HeLa cells infected with *Trypanosoma cruzi*. J. Protozool. Res., 4, 10–17.
11) Sato, A., Carter, G. L., Bacon, P. E. and Cory, J. G. (1983): Effects of biochemical modulation of drug combinations directed at the ribonucleotide reductase site on leukemia L1210 cell growth in culture. Adv. Enzyme Regul., 21, 259–270.
12) Zalkin, H. (1985a): GMP synthetase. Methods Enzymol., 113, 273–278.
13) Zalkin, H. (1985b): CTP synthetase. Methods Enzymol., 113, 282–287.