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IN VIVO INACTIVATION BY ACIVICIN OF CARBAMOYL-PHOSPHATE SYNTHETASE II IN RAT HEPATOMA*

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Abstract—The antitumor drug acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, *in vivo* irreversibly inactivated carbamoyl-phosphate synthetase II (glutamine-dependent) (EC 6.3.5.5), the first and rate-limiting enzyme of *de novo* pyrimidine nucleotide biosynthesis, in transplantable rat hepatoma and host liver. With two injections of 0.5 mg acivicin per 100 g body weight to one group and two injections of 5 mg to another group, enzyme activity decreased to 20 and 1% in hepatoma and to 99 and 31% in liver respectively. Aspartate carbamoyltransferase (EC 2.1.3.2) activity was not affected. Acivicin *in vitro* selectively inactivated glutamine-dependent activity of the synthetase II from the hepatoma and liver, with an inactivation constant (K_{inact}) of 90 μM and a minimum inactivation half-time (T) of 0.7 min. The inactivation velocity with 10 μM acivicin was 5.0-fold stimulated by 2 mM MgATP and 18.4-fold by 2 mM MgATP plus 16.7 mM bicarbonate. MgATP at 0.5 mM caused half-maximum stimulation of the inactivation velocity. Under *in vitro* conditions, L-glutamine (1 mM) protected the enzyme from inactivation by 10 μM acivicin. The synthetase activity was protected *in vitro* by 6 mM concentrations of glycine (84%), L-glutamate (59%) and L-aspartate (51%) and by 0.5 mM UTP (35%) from inactivation by 20 μM acivicin. The results are compatible with the suggestion that acivicin is an active site-directed affinity analog of L-glutamine.

Previous work in this laboratory showed that the activities of four key enzymes involved in glutamine-dependent amidations in purine metabolism (amidophosphoribosyltransferase, EC 2.4.2.14 [1] and GMP synthetase, EC 6.3.5.2 [2]) and in pyrimidine biosynthesis (carbamoyl-phosphate synthetase II, EC 6.3.5.5 [3] and CTP synthetase, EC 6.3.4.2 [4]) were increased in hepatomas and in other tumors. Based on the postulate that the increase of these key enzyme activities conferred a selective growth advantage to cancer cells, marking these enzymes as potential targets for chemotherapy [5, 6], investigations were started utilizing various L-glutamine antagonists. Acivicin‡ was isolated from fermentation broth [7] and was characterized as an antibiotic with antitumor action [8]. The agent irreversibly inhibited the activities of L-glutamine amidotransferases from *Escherichia coli, Serratia marcescens*, L5178Y leukemia, and fetal rat liver [9–11]. Investigations in this laboratory showed that two injections of acivicin (for a total of 10 mg/100 g body wt) into rats carrying transplantable hepatoma 3924A decreased the specific activities in the tumor of amidophosphoribosyltransferase, CTP synthetase and synthetase II to 50, 1 and 1%, respectively, of those of the control, saline-injected rats.§ Since we succeeded in abolishing 99% of the activity of the synthetase II in the hepatoma, it became of particular interest to elucidate the mechanism of action of acivicin on this enzyme.

Synthetase II (glutamine-dependent) is the first and rate-limiting enzyme of *de novo* pyrimidine biosynthesis [3, 12], and it is a regulatory enzyme of this pathway in normal liver [13] and in hepatoma 3924A.‖ The specific activity of synthetase II was elevated significantly in a series of chemically induced, transplantable hepatomas and the increase positively correlated with the growth rate of the tumors [3]. In this report we provide evidence that acivicin injection *in vivo* irreversibly inactivated the glutamine-dependent activity of synthase II in the hepatoma. *In vitro* incubation with acivicin of the purified enzyme from the tumor also brought about a rapid and irreversible inactivation of synthetase II. Acivicin action was selective in that the ammonia-dependent activity of the enzyme and the activity of aspartate carbamoyltransferase (EC 2.1.3.2) were not affected. In an analogy to the mechanism of action of acivicin for anthranilate synthetase and glutamate synthase from *S. marcescens* [11], we postulate that acivicin is an active site-directed affinity analogue of L-glutamine which chelates the synthetase II, achieving irreversible inactivation.

MATERIALS AND METHODS

Materials. Hepatoma 3924A was maintained as a bilateral, subcutaneous transplant in male inbred rats (ACI/N) and has a growth rate of 30 days to reach a diameter of 1.5 cm. Maintaining and killing

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‡ Abbreviations: acivicin, L-(αS,5S)-α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid; carbamoyl-P, carbamoyl-phosphate; synthetase I, carbamoyl-phosphate synthetase (ammonia); synthetase II, carbamoyl-phosphate synthetase (glutamine-hydrolyzing); PRPP, 5-phosphoribosyl 1-pyrophosphate; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid.

§ N. Prajda, M. S. Lui, T. Aoki and G. Weber, unpublished data.

‖ T. Aoki, H. P. Morris and G. Weber, *J. biol. Chem.*, in press.

the animals and excising the tumors and livers were as described in Refs. 14 and 15. Acivicin was a gift from the Upjohn Co., Kalamazoo, MI. For *in vivo* studies acivicin was dissolved in 0.9% NaCl solution, and the drug (0.5 or 5 mg/100 g body wt) or the solvent (controls) was injected intraperitoneally twice (at 0 and 16 hr) into rats bearing the hepatoma. Two hours after the second injection, the host liver and neoplasms were removed for the preparation of synthetase II.

The liver and hepatoma synthetase II was purified from the 105,000 *g* supernatant fraction of the tissue homogenate by ammonium sulfate and hydroxylapatite fractionation and gel filtration on Sephadex G-25 [3]. This procedure eliminated interference with the synthetase II assay by synthetase I, an enzyme involved in urea production. For the host liver and hepatoma from the control rat, the specific activities of the purified enzyme in the Sephadex G-25 fractions were 304 and 808 units (nmoles/hr) per mg protein respectively. The partially purified enzymes were also used for studies on *in vitro* inactivation by acivicin. To measure synthetase II activities in the soluble supernatant fractions, we used the recoveries of the aspartate carbamoyltransferase activities at each step of the purification, since synthetase II is present as a multifunctional protein together with carbamoyltransferase [13, 16].

Enzyme assays. Glutamine-dependent activity of carbamoyl-P synthetase II was measured as described previously [3, 13]. To assay the NH_3-dependent activity, L-glutamine was replaced by 10 mM NH_4Cl in the standard reaction mixture. Aspartate carbamoyltransferase activity was determined as reported [13].

Enzyme inactivation. In vitro inactivation by acivicin of carbamoyl-P synthetase II was carried out at 22° in a solution containing 50 mM potassium Hepes buffer (pH 7.0), 10 mM ATP, 15 mM $MgCl_2$, 16.7 mM $KHCO_3$, 7.5% (v/v) dimethylsulfoxide, 2.5% (w/v) glycerol, 1 mM dithiothreitol, the enzyme and acivicin. Inactivation was initiated by addition of the enzyme, and at the specified intervals an aliquot (0.1 ml) of the inactivation mixture was transferred to the second mixture (0.2 ml) to determine the remaining activity of the synthetase II. The combined mixture (0.3 ml) consisted of the standard reaction mixture described previously [3]. Appropriate control experiments were run to measure the net inactivation of the enzyme by acivicin.

RESULTS AND DISCUSSION

In vivo *inactivation by acivicin of synthetase II in host liver and hepatoma* 3924A. Acivicin injections (0.5 or 5 mg/100 g body wt) decreased synthetase II activities to 99 and 31% in the liver and to 20 and 1% in the hepatoma (Table 1). It is noteworthy that at either dose of acivicin the decrease in the enzymic activity was more marked in the hepatoma than in the host liver. Carbamoyltransferase activities were not changed.

The following observations indicate an irreversible inactivation *in vivo* of the synthetase II by acivicin. The activity was not restored during purification of the enzyme involving (a) extensive dilutions (1:1000) at four steps and (b) gel filtration of the enzyme–acivicin complex. Neither (c) addition of a high concentration of L-glutamine (50 mM) to the assay mixture nor (d) freezing (in liquid nitrogen) and thawing of the complex resulted in recovered enzyme activity.

The kinetic parameters of synthetase II were compared in the purified hepatoma enzyme fractions from untreated (control) and acivicin-treated (0.5 mg/100 g body wt) rats. Apparent K_m values for L-glutamine and MgATP were 26 μM and 2.2 mM, respectively, for both hepatoma enzymes; these values are comparable to those reported for normal rat liver and hepatoma 3924A [3]. Upon acivicin treatment, the maximum velocity was decreased to 21% (90.9 to 18.7 nmoles/hr per mg protein), much the same as the decrease in the enzyme activity measured in the standard assay system (Table 1). These results suggest that the synthetase II activity remaining after acivicin treatment represented the enzyme free from inactivation *in vivo* by this drug.

Competitive inhibition of synthetase II by acivicin in vitro. Figure 1 shows double-reciprocal plots of saturation kinetics with L-glutamine for the liver and hepatoma synthetase II in the presence of different concentrations of acivicin. For both enzymes, inhibition by acivicin was competitive with respect to L-glutamine; the apparent K_i value for acivicin was

Table 1. Effect of acivicin injection on carbamoyl-P synthetase II and aspartate carbamoyltransferase activities in host liver and hepatoma 3924A

Tissues	Acivicin doses mg/100 g body wt	Protein mg/g tissue	Protein % of control	Synthetase II nmoles/hr/mg protein	Synthetase II % of control	Carbamoyltransferase nmoles/hr/mg protein	Carbamoyltransferase % of control
Host liver	0	103.3 ± 2.2*	100	10.8 ± 0.4*	100	546 ± 66*	100
	0.5	101.0 ± 1.0	98	10.7 ± 1.0	99	642 ± 26	118
	5	102.2 ± 5.0	99	3.4 ± 0.8	31†	619 ± 59	113
Hepatoma 3924A	0	55.9 ± 1.1	100	88.4 ± 5.3	100	4170 ± 420	100
	0.5	53.2 ± 0.8	95	17.7 ± 1.1	20†	4400 ± 570	106
	5	47.7 ± 0.7	85†	1.2 ± 0.1	1†	4090 ± 230	98

* Mean ± S.E.M. of three rats in each group.
† Significantly different from control ($P < 0.05$ in Student's *t*-test).

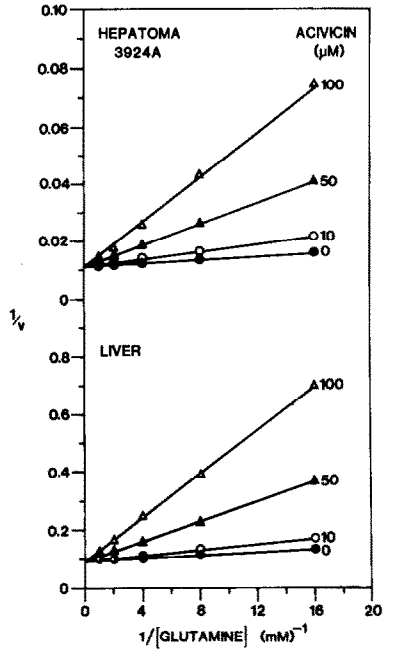

Fig. 1. Inhibition of synthetase II activity by acivicin. Activities were measured in the standard assay system with added acivicin and varied concentrations of L-glutamine. Units for velocity: nmoles/hr per mg protein.

7 μM, which is comparable to that observed for anthranilate synthetase [11].

In vitro *inactivation by acivicin of synthetase II of host liver and hepatoma* 3924A. When 10 μM acivicin and 1 mM L-glutamine were present together in the assay, nearly 100% of the enzyme activity remained during a 15-min incubation (Fig. 1). In the absence of L-glutamine, however, incubation of the enzyme with 10 μM acivicin for 15 min resulted in a remaining activity of 28% of the control (Fig. 2), indicating the presence of a qualitative difference between the two experiments. Incubation of the liver and hepatoma synthetase II with acivicin resulted in time-dependent inactivation of the glutamine-dependent activities of the enzymes (Fig. 2). Acivicin had little effect on the NH_3-dependent activities of the enzymes (Fig. 2).

The time–course of inactivation of the hepatoma synthetase II by different concentrations of acivicin demonstrated first-order kinetics up to 90% inactivation of the enzyme activity (Fig. 3A). The plots of inactivation half-time vs 1/(acivicin concentration) exhibited a linear relationship (saturation kinetics) (Fig. 3B), theoretically consistent with formation of a reversible intermediate preceding irreversible inactivation as suggested by Meloche [17] and Tso *et al.* [11]. Kinetic parameters for acivicin inactivation of the enzyme were calculated from the linear plots, based on the equation [17]:

$$\text{Inactivation half-time} = T\,K_{inact}/[I] + T,$$

where [I] is the acivicin concentration, T, which can be calculated from the intercept of the ordinate, is the minimum inactivation half-time at infinite concentration of inactivator, and K_{inact} is the inactivation constant. For acivicin the inactivation constant was 90 μM and the minimum inactivation half-time was 0.7 min under the conditions used.

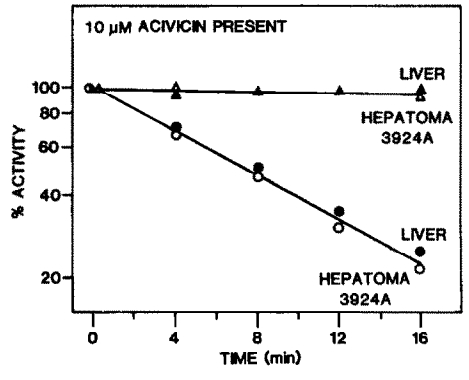

Fig. 2. Inactivation of synthetase II by acivicin. Inactivation by 10 μM acivicin was carried out as described in Materials and Methods. Glutamine-dependent activity was measured for liver (●) and hepatoma (○) in the standard assay, and NH_3-dependent activity for liver (▲) and hepatoma (△) in the system in which 1 mM L-glutamine was replaced by 10 mM NH_4Cl. Enzymes: 2.1 units (6.8 μg protein) for liver and 11 units (14 μg protein) for hepatoma 3924A.

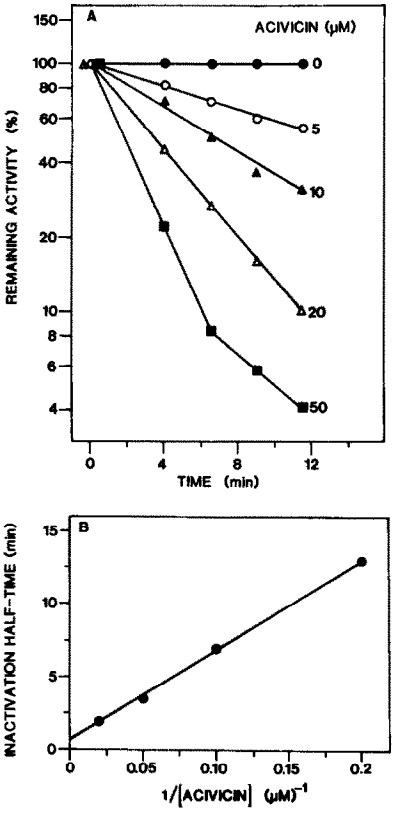

Fig. 3. Effect of acivicin concentration on inactivation of synthetase II. Inactivation of the hepatoma 3924A enzyme was carried out as described in Materials and Methods except for the addition of the different concentrations of acivicin, as indicated. Key: (A) time–course of inactivation by different concentrations of acivicin; and (B) the plot of inactivation half-time vs 1/[acivicin]. Enzyme: 11 units (14 μg protein).

Modulation of acivicin inactivation of synthetase II in vitro. Ligands of synthetase II enhanced or inhibited the inactivation by acivicin of the hepatoma enzyme (Table 2). The relative inactivation velocity was calculated by 1/(inactivation half-time) [17]. Free ATP (2 mM) was slightly inhibitory for inactivation velocity. However, 2 mM ATP plus 7 mM $MgCl_2$, that would be approximately equivalent to 2 mM MgATP (a substrate of the synthetase II) plus free 5 mM Mg^{2+} (an essential activator of the enzyme) [18], enhanced the inactivation velocity 5-fold. In the presence of 5 mM Mg^{2+} and 16.7 mM $KHCO_3$ (substrate), the inactivation velocity was stimulated 18.4- or 21.5-fold by 2 mM or 10 mM MgATP respectively. Free 5 mM Mg^{2+} and 16.7 mM $KHCO_3$ stimulated the inactivation velocity by 47 and 43% respectively. The combination of these ligands had an additive effect on the inactivation velocity (106% stimulation). By contrast, L-glutamine (1 mM) protected the enzyme from inactivation by acivicin (Table 2).

Plots of relative inactivation velocity vs MgATP concentrations exhibited a slightly sigmoidal curve at low concentrations of MgATP (Fig. 4). However, at higher concentrations of MgATP, the double-reciprocal plots were linear. The MgATP concentration required for the half-maximal stimulation of the inactivation velocity was 0.5 mM; this was close to the tissue content of ATP (1.0 mM) in hepatoma 3924A [19].

The mechanism of action of acivicin on liver and hepatoma synthetase II is in line with that of the bacterial glutamine amidotransferases, anthranilate synthetase and glutamate synthase [11], with respect to the selective inactivation of the glutamine-dependent activity (Fig. 2), saturation kinetics (Fig. 3), and protection from inactivation by L-glutamine (Table 2). The ligand chorismate was required for rapid inactivation of the glutamine-dependent anthranilate synthetase activity by acivicin, diagnostic for affinity labeling (alkylation of a cysteine residue) of the glutamine-binding site of the enzyme [11]. Likewise, the ligands MgATP, bicarbonate and

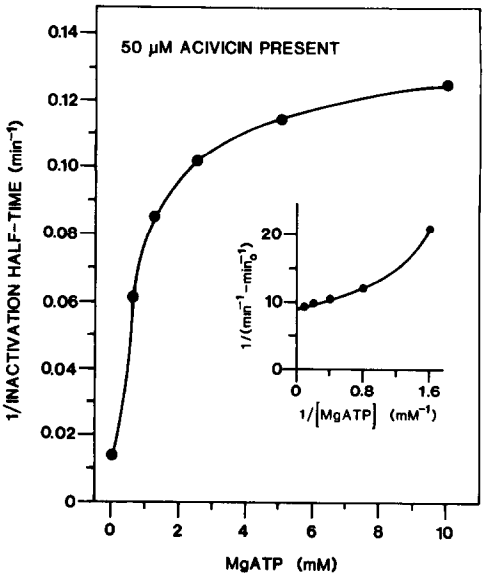

Fig. 4. Effect of MgATP concentration on inactivation by acivicin of synthetase II. Inactivation by 50 μM acivicin of the hepatoma 3924A enzyme was studied as described in Table 2, except for the additions of different concentrations of MgATP and free Mg^{2+} at 5 mM. Key: min_0^{-1}: minimum relative inactivation velocity in the absence of MgATP (5 mM Mg^{2+} present). Inset: Double-reciprocal plot of $1/s$ vs $1/v$, where v is the inactivation velocity in the presence of MgATP minus the inactivation velocity without MgATP.

Mg^{2+} were required for maximum inactivation of the mammalian synthetase II by acivicin (Table 2 and Fig. 4). These results suggest that acivicin is an active site-directed affinity analogue of L-glutamine for the liver and hepatoma synthetase II. Studies on injection of acivicin in mice showed a decrease in activity of synthetase II in Lewis lung tumor; however, the authors interpreted the results as enzyme inhibition [20]. In the present studies, synthetase II was deter-

Table 2. Effect of ligands on inactivation by acivicin of synthetase II*

Additions	Inactivation half-time (min)	Inactivation velocity (% of control)
None (control)	140	100
2 mM ATP	170	82
5 mM $MgCl_2$	95	147
16.7 mM $KHCO_3$	98	143
2 mM ATP, 7 mM $MgCl_2$	28	500
16.7 mM $KHCO_3$, 5 mM $MgCl_2$	68	206
2 mM ATP, 7 mM $MgCl_2$, 16.7 mM $KHCO_3$	7.6	1840
10 mM ATP, 15 mM $MgCl_2$, 16.7 mM $KHCO_3$	6.5	2150
1 mM L-Glutamine	(infinite)†	(negligible)

* Inactivation by 10 μM acivicin of synthetase II of hepatoma 3924A was conducted in reaction mixtures (pH 7.0) containing 50 mM potassium Hepes, 7.5% (v/v) dimethylsulfoxide, 2.5% (w/v) glycerol, 1 mM dithiothreitol, enzyme (11 units; 14 μg protein) and additions as indicated. Inactivation half-times were calculated as in Fig. 3. Inactivation velocity was calculated as 1/(inactivation half-time).

† Under the conditions used, the inactivation half-time was too large to be determined and, thus, inactivation velocity was negligible.

mined by direct assay of the purified enzyme, making it possible to identify the decreased activity after acivicin treatment as a result of the inactivation of the enzyme.

It was suggested that, on the same polypeptide chain of synthetase II, partial reactions of L-glutamine hydrolysis and of NH_3-dependent carbamoyl-P synthesis provided the overall reaction for glutamine-dependent carbamoyl-P synthesis, and that the binding of MgATP and Mg^{2+} to the site of NH_3-dependent carbamoyl-P synthesis produced a conformational change in synthetase II resulting in activation of the glutamine-utilizing site [21]. The present results are consistent with this view and support the concept that the partial reactions for overall carbamoyl-P synthesis may be regulated in a coordinate manner by MgATP, Mg^{2+} and bicarbonate [21]. Acivicin [with a high affinity (K_i) of 7 μM] is thus a useful tool for elucidating the detailed mechanisms of partial and overall reactions of carbamoyl-P synthesis.

Inactivation *in vitro* by acivicin of liver and hepatoma carbamoyl-P synthetase II was inhibited by various compounds (Table 3). An allosteric feedback inhibitor (UTP), a product (L-glutamate) and competitive inhibitors (glycine and L-alanine) [21], and L-aspartate decreased the inactivation velocity to 65, 41, 16, 73 and 49% of the control respectively. The UTP concentration used was similar to that (0.3 mM) observed in hepatoma 3924A [19]. MgATP was required for rapid inactivation (Table 2 and Fig. 4); UTP inhibited synthetase II activity by decreasing the affinity of the enzyme for MgATP [22]. Therefore, the inhibition by UTP of inactivation velocity may be attributable to the decreased affinity for MgATP. PRPP had little stimulatory effect on the inactivation velocity (Table 3).

Since rapidly growing hepatoma 3924A would be active in metabolizing the substrate, product, and competitive inhibitors of the synthetase II, the tumor might have lowered concentrations of these metabolites, comparable to those in the host liver. L-Glutamine concentration in liver is around 2 mM [23]. In a tumor transplanted subcutaneously in the mouse, the concentration of L-glutamine was 0.6 mM whereas it was 2.7 mM in the host liver [10]. Higher concentrations of L-glutamine and glycine or a combination of these metabolites in the host liver might account for the lesser degree of inactivation *in vivo* by acivicin of synthetase II in host liver than in hepatoma 3924A (Table 1). Alterations in the concentrations of L-glutamine and acivicin in our model system are under investigation. The more extensive inactivation of synthetase II in the tumor (Table 1) might provide selective toxicity against hepatoma cells. Inactivation of synthetase II by acivicin was also observed in our *in vitro* studies in extracts of human colon adenocarcinomas (apparent $K_i = 4$ μM, $K_{inact} = 0.1$ mM, $T = 0.7$ min).

In vivo inactivation by acivicin of the tumor synthetase II (Table 1) should result in a greatly reduced capacity of the *de novo* pathway for supplying pyrimidine nucleotide precursors to cancer cells. Eighteen hours after acivicin injection (5 mg/100 g body wt) the CTP pool decreased to approximately 18% in hepatoma 3924A.* The increased activities of the enzymes of salvage pyrimidine biosynthesis (uridine kinase, uridine phosphorylase and uracil phosphoribosyltransferase) in the hepatoma [15] also provide to cancer cells an enhanced capability of pyrimidine supply. Agents that block the salvage uridylate synthesis in combination with acivicin treatment should heighten the inhibition of UTP biosynthesis.

* M. S. Lui and G. Weber, unpublished data.

REFERENCES

1. N. Prajda, N. Katunuma, H. P. Morris and G. Weber, *Cancer Res.* **35**, 3061 (1975).
2. T. J. Boritzki, R. C. Jackson, H. P. Morris and G. Weber, *Biochim. biophys. Acta* **658**, 102 (1981).
3. T. Aoki and G. Weber, *Science* **212**, 463 (1981).
4. H. Kizaki, J. C. Williams, H. P. Morris and G. Weber, *Cancer Res.* **40**, 3921 (1980).
5. G. Weber, *New Engl. J. Med.* **296**, 486 (1977).
6. G. Weber, *New Engl. J. Med.* **296**, 541 (1977).
7. D. G. Martin, D. J. Duchamp and C. G. Chidester, *Tetrahedron Lett.* 2549 (1973).
8. G. L. Neil, A. E. Berger, B. K. Bhuyan, C. L. Blowers and S. L. Kuentzel, *Adv. Enzyme Regulat.* **17**, 375 (1979).
9. D. A. Cooney, H. N. Jayaram, J. A. Ryan and V. H. Bono, *Cancer Chemother. Rep.* **58**, 793 (1974).

Table 3. Effect of metabolites on inactivation by acivicin of synthetase II*

Additions	Final concn (mM)	Inactivation half-time (min)	Inactivation velocity (% of control)
None (control)		3.2	100
UTP	0.5	4.9	65
PRPP	0.1	2.7	118
L-Glutamate	6.0	7.8	41
L-Aspartate	6.0	6.5	49
Glycine	6.0	20.4	16
L-Alanine	6.0	4.4	73

* Inactivation by 20 μM acivicin of synthetase II of hepatoma 3924A was measured as described in Materials and Methods except for the additions noted; the concentrations of ATP and $MgCl_2$ in the inactivation mixtures were reduced to 2 and 7 mM respectively. Inactivation half-times were calculated from plots as in Fig. 3. Enzyme: 11 units (14 μg protein).

10. H. N. Jayaram, D. A. Cooney, J. A. Ryan, G. Neil, R. L. Dion and V. H. Bono, *Cancer Chemother. Rep.* **59**, 481 (1975).
11. J. Y. Tso, S. G. Bower and H. Zalkin, *J. biol. Chem.* **255**, 6734 (1980).
12. M. Tatibana and K. Shigesada, *Adv. Enzyme Regulat.* **10**, 249 (1972).
13. M. Mori and M. Tatibana, *Meth. Enzym.* **51**, 111 (1978).
14. G. Weber, E. Olah, M. S. Lui, H. Kizaki, D. Y. Tzeng and E. Takeda, *Adv. Enzyme Regulat.* **18**, 3 (1980).
15. G. Weber, T. Shiotani, H. Kizaki, D. Tzeng, J. C. Williams and N. Gladstone, *Adv. Enzyme Regulat.* **16**, 3 (1978).
16. P. F. Coleman, D. P. Suttle and G. R. Stark, *Meth. Enzym.* **51**, 121 (1978).
17. H. P. Meloche, *Biochemistry* **6**, 2273 (1967).
18. M. Tatibana and K. Shigesada, *J. Biochem., Tokyo* **72**, 537 (1972).
19. R. C. Jackson, M. S. Lui, T. J. Boritzki, H. P. Morris and G. Weber, *Cancer Res.* **40**, 1286 (1980).
20. T. W. Kensler, L. J. Reck and D. A. Cooney, *Cancer Res.* **41**, 905 (1981).
21. M. Mori and M. Tatibana, *Biochim. biophys. Acta* **483**, 90 (1977).
22. M. Tatibana and K. Shigesada, *J. Biochem., Tokyo* **72**, 549 (1972).
23. J. B. Wyngaarden and E. W. Holmes, Jr., *Ciba Fdn Symp.* **48**, 43 (1977).