Biochem. J. (1984) **220**, 707–716
Printed in Great Britain

Selective inhibition of alanine aminotransferase and aspartate aminotransferase in rat hepatocytes

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(*Received 16 December 1983/Accepted 7 March 1984*)

1. Experiments were conducted with intact rat hepatocytes to identify inhibitors and incubation conditions that cause selective inhibition of alanine aminotransferase or aspartate aminotransferase. Satisfactory results were obtained by preincubating cells with L-cycloserine or L-2-amino-4-methoxy-*trans*-but-3-enoic acid in the absence of added substrates. 2. When cells were incubated for 20 min with 50 μM-L-cycloserine, alanine aminotransferase activity was decreased by 90%, whereas aspartate aminotransferase was inhibited by 10% or less. On subsequent incubation, synthesis of glucose and urea from alanine was strongly inhibited, but glucose synthesis from lactate was unaffected. 3. L-2-Amino-4-methoxy-*trans*-but-3-enoic acid (400 μM) in hepatocyte incubations caused 90–95% inactivation of aspartate aminotransferase, but only 15–30% loss of alanine aminotransferase activity. After preincubation with the inhibitor, glucose synthesis from lactate was almost completely blocked; with alanine as the substrate, gluconeogenesis was unaffected, and urea synthesis was only slightly decreased. 4. By comparison with preincubation with inhibitors, simultaneous addition of substrates (alanine; lactate plus lysine) and inhibitors (cycloserine; aminomethoxybutenoic acid) resulted in smaller decreases in aminotransferase activities and in metabolic rates. 5. Other compounds were less satisfactory as selective inhibitors. Ethylhydrazinoacetate inactivated the two aminotransferases to similar extents. Vinylglycine was almost equally effective in blocking the two enzymes *in vitro*, but was a very weak inhibitor when used with intact cells. Concentrations of DL-propargylglycine (4 mM) required to cause at least 90% inhibition of alanine aminotransferase in hepatocytes also caused a 16% decrease in aspartate aminotransferase. 6. When tested *in vitro*, alanine aminotransferase was, as previously reported by others, more sensitive to inhibition by amino-oxyacetate than was aspartate aminotransferase, but in liver cell incubations the latter enzyme was more rapidly inactivated by amino-oxyacetate.

This study was undertaken to identify inhibitors and incubation conditions that will cause selective inactivation of AlaAT or AspAT in isolated rat hepatocytes. These two enzymes have important roles in hepatic nitrogen metabolism and gluconeogenesis, and many experiments aimed at elucidating those roles have been conducted with aminotransferase inhibitors that are structurally related to the amino acid substrates. However, it is often not recognized that such inhibitors may react with a variety of pyridoxal phosphate-dependent enzymes. For example, in addition to several aminotransferases (listed by John *et al.*, 1978), amino-oxyacetate has been reported to inhibit glutamate decarboxylase and histidine decarboxylase (Roberts & Simonsen, 1963; Leinweber, 1968), alanine racemase (Free *et al.*, 1967) and cystathionase (EC 4.4.1.1; Beeler & Churchich, 1976). Similarly, L-cycloserine has been shown to inhibit six different aminotransferases (Azarkh *et al.*, 1960; Wong *et*

Abbreviations used: AlaAT, alanine aminotransferase (EC 2.6.1.2); AspAT, aspartate aminotransferase (EC 2.6.1.1); AMB, L-2-amino-4-methoxy-*trans*-but-3-enoic acid.

al., 1973). For metabolic studies with intact cells or more complex systems, such broad reactivity raises the possibility that observed effects might be due to actions other than that for which an inhibitor's use was intended.

In spite of reacting with multiple enzymes, it is likely that the concentration of an aminotransferase inhibitor causing inactivation of any one enzyme will be different from those required for inactivation of other catalysts. Therefore we sought to achieve selective inhibition of AlaAT and AspAT by identifying the inhibitor that acts most strongly against each enzyme and by testing for the lowest concentration that causes inactivation with minimal effect on the other enzyme. This paper describes results with six aminotransferase inhibitors and shows that with two of those, L-cycloserine and AMB, selective inactivation of AlaAT and AspAT can be obtained in metabolic experiments with hepatocytes. Since the problem of multiple sites of action is not limited to aminotransferase inhibitors, the approach described may be useful with other classes of metabolic inhibitors. A preliminary account of this work has been presented (Kerich *et al.*, 1983).

Experimental

Hepatocytes were isolated as described previously (Cornell *et al.*, 1973; Krebs *et al.*, 1974) from 2-day-starved male Wistar rats weighing 200–250g. ATP content in each preparation was measured as an index of cell quality (Cornell, 1983) and in all cases was 2.4–2.6 μmol/g wet wt. of cells. Hepatocyte incubations with 0.095–0.120g wet wt. of cells in 4ml were conducted at 38°C as described by Cornell *et al.* (1973), and, for metabolite measurements, acid extracts were made as described by Crow *et al.* (1978). When enzyme activities were to be determined, hepatocytes were rapidly separated from the incubation medium with the use of brominated hydrocarbons and the centrifuge tubes described in detail elsewhere (Cornell, 1980). The cell pellet was resuspended in Triton X-100 extraction medium (Janski & Cornell, 1980), 1ml/50mg wet wt. of cells, and kept on ice for 90min with three vortex-mixings during that period. This extraction releases the total cellular content of cytosolic and mitochondrial matrix enzymes. Hepatocyte wet weights were obtained from the measured dry weights and the conversion factor of 3.7 (Krebs *et al.*, 1974).

Amino-oxyacetate, DL-vinylglycine and DL-propargylglycine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ethyl hydrazinoacetate was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). AMB was given by Dr. W. E. Scott (Hoffman–LaRoche, Nutley, NJ, U.S.A.), as were some samples of L-cycloserine. Other samples of L-cycloserine, bought from Aldrich Chemical Co., were white and granular, whereas those obtained from Hoffman–LaRoche were pale-yellow powders. However, L-cycloserine from both sources gave equivalent results in experiments reported here. Enzymes for analytical purposes were obtained from Boehringer (Mannheim) Corp., Indianapolis, IN, U.S.A., and substrates and cofactors were from Boehringer or Sigma. Other chemicals were reagent-grade products.

All enzyme-activity measurements were made at 38°C. Throughout this paper, one unit of activity is defined as the amount of enzyme catalysing conversion of 1 μmol of substrate/min. AlaAT activity was measured as described by Bergmeyer & Bernt (1974), except that 2-oxoglutarate was present in both the reference and the assay cuvettes, and reaction was initiated by addition of 25mM-alanine to the mixture in the assay cuvette. Methods for determining AspAT and lactate dehydrogenase were as described by Janski & Cornell (1980); citrate synthase was assayed as described by Srere *et al.* (1963).

Glutamate and alanine were measured as described by Cornell *et al.* (1974); urea was measured by the procedure of Bernt & Bergmeyer (1974) and glucose by the method of Slein (1963). Aspartate was measured by the general procedure of Bergmeyer *et al.* (1974), modified to achieve shorter reaction times and more well-defined stable end points. The modified assay contained, in a total volume of 2ml, 0.5ml of 0.2M-potassium phosphate buffer, pH7.6, 12units of malate dehydrogenase (no. 127892, Boehringer Mannheim; 1200units/mg, 10mg/ml), 0.33 μmol of NADH, 10 μmol of 2-oxoglutarate, and cell extract containing 0.01–0.10 μmol of aspartate. Reaction was initiated by addition of 20 units of AspAT (no. 105554, Boehringer; 200units/mg, 10mg/ml) and was complete in 6–10min.

Results

L-*Cycloserine*

Cycloserine is a structural analogue of alanine, and previous studies with rat liver extracts (Azarkh *et al.*, 1960; Barbieri *et al.*, 1960) or isolated mitochondria (Dieterle *et al.*, 1978) have indicated that AlaAT is more sensitive than AspAT to inhibition by L-cycloserine. However, when used at concentrations of 1–5mM, as is common in metabolic studies (Parrilla *et al.*, 1974; Meijer *et al.*, 1975, 1978), L-cycloserine will inhibit

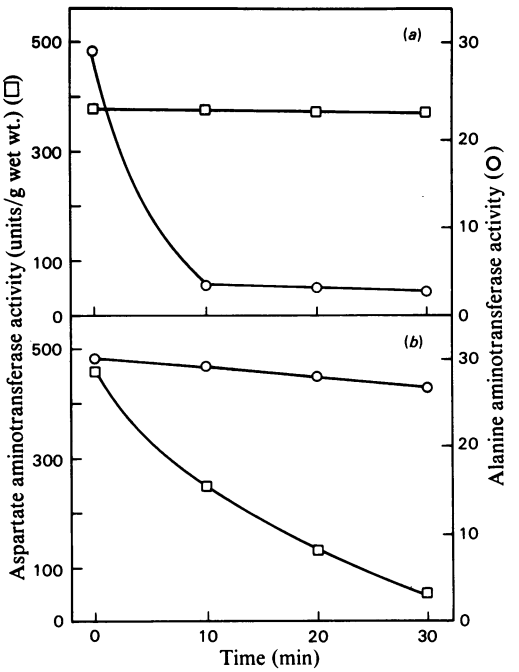

Fig. 1. *Time course of inactivation by cycloserine or AMB of AlaAT (○) and AspAT (□) in hepatocytes*
Hepatocytes were incubated at 38°C for the indicated times with either (*a*) 25 μM-L-cycloserine or (*b*) 250 μM-AMB. Cells were then separated from the medium and extracted as described in the Experimental section to release total enzyme contents. Results are for a representative experiment.

Table 1. *Concentration-dependence for inhibition by L-cycloserine or AMB of AlaAT and AspAT in hepatocytes*
Hepatocytes were incubated for 30 min with inhibitors at the indicated concentrations. Results are means ± S.E.M. for three cell preparations that contained in the absence of inhibitor 29.1 ± 3.0 and 414 ± 17 units of AlaAT and AspAT respectively.

Inhibitor (μM)	Inhibition (%) AlaAT	AspAT
Cycloserine		
25	89.1 ± 0.5	3.1 ± 1.2
50	90.1 ± 0.7	7.9 ± 0.6
100	90.5 ± 0.5	18.4 ± 1.8
500	93.2 ± 0.3	70.1 ± 1.5
AMB		
100	4.7 ± 4.2	62.4 ± 1.7
250	11.2 ± 5.8	85.7 ± 1.2
500	29.5 ± 1.9	93.6 ± 0.6

both AlaAT and AspAT (see below), as well as several other transaminases (Wong *et al.*, 1973; Crabb & Harris, 1978; Tischler *et al.*, 1982). With isolated rat hepatocytes incubated in the absence of substrates we have found (Fig. 1*a*) that 25 μM-L-cycloserine caused about a 90% decrease in the cellular AlaAT activity in 10 min, whereas AspAT was inhibited by only about 5% after 30 min. However, as shown by the data in Table 1, inhibition of AspAT was proportional to the concentration of the inhibitor and reached 70% at 500 μM-L-cycloserine (see also Janski & Cornell, 1981).

AMB

AMB is an irreversible inactivator thought to be relatively specific for AspAT (Rando *et al.*, 1976), and that property has led to recommendations for the use of this aminotransferase inhibitor in metabolic studies (Smith *et al.*, 1977; Smith & Freedland, 1981). The time course of action of 250 μM-AMB in hepatocyte incubations is shown in Fig. 1(*b*). The inactivation of AspAT developed gradually, reaching about 90% after 30 min, and, at that time, AlaAT activity was decreased by only 10–12%. Thus the effectiveness of this inhibitor against AlaAT and AspAT is the converse of that seen with cycloserine (Fig. 1). The relatively long time required for inhibition of AspAT by low concentrations of AMB is inconvenient, but, although we have confirmed the results of Smith & Freedland (1981) showing 90% inactivation of hepatocyte AspAT in 15 min with 1 mM-AMB, we also find 48–50% inactivation of AlaAT with that concentration of inhibitor; with 500 μM, 90% inactivation of AspAT occurred in 20 min, and, as shown in Table 1, AlaAT was decreased by 30% in 30 min incubations. The effect on AlaAT was proportional to AMB concentration between 100 and 500 μM (Table 1). Although it would be desirable to have an inhibitor with less reactivity towards AlaAT, none that we have tested gives the required inactivation of AspAT with as little relative decrease in AlaAT as that obtained with AMB.

Metabolic effects of cycloserine and AMB

With preincubation. The results presented in Fig. 1 and Table 1 show that it is possible selectively to inactivate AlaAT and AspAT in the absence of substrates. This should lead to selective effects on metabolic pathways that involve either or both of those two enzymes. To test this expectation, we conducted hepatocyte incubations with lactate or alanine as the added substrate and measured rates of glucose and urea synthesis. When lactate is the substrate for gluconeogenesis, its oxidation to pyruvate also yields the required NADH, and, in rat liver, the cytosolic oxaloacetate arises via sequential reactions of mitochondrial and cytosolic aspartate aminotransferases; in contrast, glucose

synthesis from alanine does not involve AspAT, since pyruvate is generated via AlaAT, and, after carboxylation in the mitochondria, the carbon exits to the cytosol as malate, which is then oxidized to yield oxaloacetate as well as the required NADH (Borst, 1963; Lardy *et al.*, 1965; Haynes, 1965; Walter *et al.*, 1966). For the experiments described in Table 2, cells were incubated for 20min without or with an inhibitor before the addition of alanine or lactate. Lysine, which is not glucogenic, was included in the incubations with lactate to accelerate recovery of malate–aspartate-shuttle intermediates and to eliminate any lag in the rate of gluconeogenesis (Cornell *et al.*, 1974).

In hepatocytes preincubated with 25 μM- or 50 μM-L-cycloserine and then incubated with alanine or lactate plus lysine, AlaAT activity was decreased by about 90%, whereas AspAT was decreased by 10% or less. Glucose synthesis from lactate was unaffected, but, when alanine was the added substrate, glucose production was inhibited almost to the rate observed with no added substrate. Likewise, urea synthesis in incubations with alanine and cycloserine was decreased by about 70%, as were alanine utilization and glutamate accumulation, and the amount of aspartate was lowered by 85–90%. These metabolic effects of L-cycloserine are consistent with its selective action on AlaAT when used at low concentrations.

Data in Table 2 also show that AMB has the metabolic effects indicative of a selective inhibitor of AspAT. After preincubation with 250 μM- or 500 μM-AMB, glucose synthesis from lactate was decreased by 90%, whereas with alanine as substrate glucose production was unaffected. Aspartate accumulation was diminished by 50–60%, and, since an intermediate step in urea synthesis requires aspartate in the cytosol, it is not surprising that ureogenesis was decreased by 20–30% by AMB in the incubations with alanine. Although in the incubations with lactate plus lysine 250 μM-AMB had only a small effect on AlaAT activity, alanine accumulation was 80% less than in the absence of inhibitor. This observation is explained by results from other experiments showing that preincubation of hepatocytes with 250 μM- or 400 μM-AMB, by inactivating AspAT, greatly diminishes the capacity for reoxidation of excess cytosolic NADH, and the cumulative conversion of lactate into pyruvate, the substrate for alanine production via AlaAT, is blocked. It should be noted that cessation of glucose synthesis in these experiments occurred with a residual AspAT activity of 39 units/g wet wt. of cells (measured under optimal conditions with hepatocyte extracts). That activity is 15 times the rate of incorporation of lactate into glucose measured in the absence of AMB, and it suggests that a very large excess of cellular AspAT activity is needed for the normal synthesis of glucose from lactate.

Without preincubation. When substrates and

Table 2. *Aminotransferase activities, metabolic rates and metabolite contents in hepatocytes preincubated with* L-*cycloserine or AMB*

Where L-cycloserine (CS) or AMB was present, each was added to hepatocyte suspensions at zero time; the suspensions were gassed with O_2/CO_2 (19:1), and shaken at 38°C for 20min. Then the indicated substrates were added, and the suspensions gassed again and incubated for 30min at 38°C. Concentrations of substrates were: lactate, 10mM; lysine, 2mM; alanine, 5mM. The values for metabolites are amounts present in the cell suspensions after incubation, except in experiments where alanine was added as substrate; for those instances, the alanine present after incubation was subtracted from that in identical mixtures stopped with acid at zero time to calculate alanine disappearances, and those values are given under the heading of ($\Delta\mu$mol/g). Results are means ± S.E.M. for experiments with three cell preparations.

		Aminotransferase (units/g wet wt.)		Metabolic rates (μmol/min per g)		Metabolites (μmol/g wet wt.)		
Substrates	Inhibitor	AlaAT	AspAT	Urea	Glucose	Alanine	Glutamate	Aspartate
None	–			0.15±0.01	0.07±0.006	0.26±0.01	3.19±0.20	0.15±0.02
Lactate, lysine	–	22.2±3.7	401±22	0.15±0.01	1.31±0.03	8.18±0.59	8.75±0.99	0.59±0.06
	+CS, 25 μM	2.4±0.3	397±20	0.15±0.02	1.31±0.05	5.11±0.24	10.4±0.99	0.70±0.07
	50 μM	2.2±0.2	372±16	0.15±0.01	1.29±0.06	4.69±0.13	10.2±0.58	0.71±0.07
	+AMB, 250 μM	17.4±2.9	39.3±2.6	0.15±0.01	0.13±0.02	1.57±0.25	8.11±0.68	2.93±0.16
	400 μM	15.6±2.6	17.3±1.5	0.15±0.02	0.13±0.02	1.49±0.23	8.68±0.10	1.61±0.12
						($\Delta\mu$mol/g)		
Alanine	–	21.5±3.4	411±8	0.73±0.05	0.45±0.02	70.1±3.4	12.7±1.1	0.90±0.16
	+CS, 25 μM	2.5±0.3	421±11	0.25±0.02	0.16±0.03	21.8±0.87	5.23±1.0	0.14±0.01
	50 μM	2.2±0.1	380±18	0.21±0.02	0.12±0.01	23.3±0.49	3.81±0.46	0.10±0.02
	+AMB, 250 μM	19.7±3.5	62.9±3.6	0.60±0.03	0.41±0.03	58.8±2.9	13.2±1.3	0.44±0.15
	400 μM	18.0±3.3	29.6±3.0	0.50±0.03	0.42±0.02	58.8±2.4	13.4±1.2	0.33±0.08

Table 3. *Aminotransferase activity, metabolic rates and metabolite contents in hepatocytes incubated with simultaneous additions of substrates and cycloserine or AMB*

Hepatocytes were incubated at 38°C for 30min. Substrate concentrations were: lactate, 10mM; lysine, 2mM; alanine, 5mM. Where present, the concentrations of L-cycloserine (CS) or AMB were as indicated. All materials were in the incubation medium when cells were added. For experiments with alanine as the added substrate, the values listed for alanine are for substrate utilization during the 30min incubation (cf. Table 2). Other metabolite values represent contents at the end of the incubations. Results are means ± S.E.M. for three cell preparations, except in the two instances without statistics, which are values obtained with only one of the three preparations.

		Aminotransferase (units/g wet wt.)		Metabolic rates (μmol/min per g)		Metabolites (μmol/g wet wt.)		
Substrates	Inhibitor	AlaAT	AspAT	Urea	Glucose	Alanine	Glutamate	Aspartate
None	–			0.18±0.01	0.11±0.007	0.36±0.01	2.04±0.22	0.07±0.03
Lactate, lysine	–	24.4±3.8	369±2.9	0.18±0.01	1.22±0.02	4.60±0.14	7.88±0.89	0.51±0.03
	+CS, 25μM	3.25±0.84	349±14.2	0.19±0.04	1.23±0.02	3.26±0.27	8.20±0.11	0.60±0.03
	50μM	2.95	341	0.18	1.21	3.01	8.04	0.62
	+AMB, 250μM	20.9±5.4	91.3±8.3	0.17±0.04	1.00±0.14	3.89±0.26	7.71±0.65	1.22±0.08
	400μM	19.6±4.8	50.4±6.3	0.18±0.03	0.69±0.04	3.04±0.29	7.30±0.48	1.46±0.16
						(Δμmol/g)		
Alanine	–	23.8±6.1	371±7.6	0.77±0.10	0.53±0.03	63.5±5.7	9.31±0.56	1.65±0.45
	+CS, 25μM	4.65±1.2	354±14.3	0.60±0.10	0.49±0.05	61.0±2.6	7.43±0.86	0.73±0.09
	50μM	2.98±0.85	336±10.4	0.47±0.07	0.45±0.07	45.6±3.1	5.33±0.61	0.28±0.08
	+AMB, 250μM	23.8±3.9	176±14.8	0.69±0.10	0.52±0.03	66.0±3.5	9.40±0.43	1.34±0.45
	400μM	19.9	95.9	0.63	0.54	63.1	9.37	0.96

inhibitors were added at the same time to hepatocyte incubation mixtures (Table 3), the results were qualitatively similar to those obtained by preincubating the cells with inhibitors (Table 2). However, the inactivation of enzymes, decreases in urea or glucose synthesis, changes in accumulation of alanine, aspartate and glutamate, and the decline in utilization of added alanine, were all smaller when there was no preincubation with cycloserine or AMB. The differences between the results in Tables 3 and 2 are greater for the experiments with AMB than for those with cycloserine, and that contrast reflects the different rates with which these agents inactivate AlaAT or AspAT (Fig. 1). In this regard, it was unexpected that the inactivation of AspAT by AMB would be less with alanine than with lactate plus lysine as added substrate. For example, with 250μM-AMB, in the lactate-containing incubations AspAT was decreased by 75%, whereas in the presence of alanine the decrease was only 53% (Table 3). The same trend occurred with 400μM-AMB and was also apparent, but less pronounced, when cells were preincubated with the inhibitor (Table 2).

Amino-oxyacetate

Amino-oxyacetate is perhaps the most widely used of all aminotransferase inhibitors. In spite of increasing evidence that it is a general reagent reacting with the pyridoxal moiety of many aminotransferases and other enzymes (see the introduction), we tested amino-oxyacetate for its selectivity against hepatocyte AlaAT and AspAT. As seen from the results in Table 4, 50μM-amino-oxyacetate in the absence of added substrates caused 93% inhibition of AspAT within 10min, but, concomitantly, AlaAT was inhibited by 43% at 10min and by 61% at 30min. The same lack of selectivity was observed with lower concentrations of amino-oxyacetate. That is, in 10min incubations with 25μM-amino-oxyacetate, hepatocyte

Table 4. *Inhibition of AlaAT or AspAT in hepatocytes incubated with amino-oxyacetate*

Hepatocytes (0.095–0.115g wet wt. in 4.0ml) were incubated with 50μM-amino-oxyacetate and no added substrates for the indicated times. The cells were then separated from the medium by centrifugation through brominated hydrocarbons as stated in the Experimental section, and the cell pellets were extracted to release total enzyme contents. Percentage inhibition was calculated relative to parallel cell samples incubated without amino-oxyacetate but otherwise treated in the same manner as for incubations containing the inhibitor. Further details of the assays are given in the text. Results are means ± S.E.M. for three cell preparations that contained (in the absence of amino-oxyacetate) 32.7±1.2 and 431±17.5 units/g wet wt. of AlaAT and AspAT respectively.

Time of incubation (min)	Inhibition (%) AlaAT	AspAT
10	43.4±10.4	93.5±0.6
20	49.2±7.9	93.3±0.4
30	61.2±6.4	94.2±0.6

AspAT was inhibited by 92% and AlaAT by 44%; with 10 μM, cellular AspAT was decreased by 72% and AlaAT activity by 38%. These results indicate that it is not possible with amino-oxyacetate to inhibit hepatocyte AspAT to an extent that is useful without also causing an unacceptable decrease in AlaAT activity. Amino-oxypropionate, an analogue of amino-oxyacetate, has previously been shown to have almost the same potency in blocking glucose synthesis from lactate, but without some troublesome side effects of amino-oxyacetate (Harris *et al.*, 1982). We have found, however, that amino-oxypropionate causes similar inhibition of AspAT and AlaAT and has the same low selectivity as those described above for amino-oxyacetate (results not shown).

With regard to the values given here for the inhibition of aminotransferases by amino-oxyacetate, it should be noted that, when hepatocytes are incubated with this inhibitor and enzymes are extracted and assayed *in vitro*, aminotransferases show a slow re-activation on addition of the amino acid substrate. This has been described in detail for AspAT (Cornell *et al.*, 1981), and it also applies to AlaAT. For example, with extracts of cells incubated with 50 μM-amino-oxyacetate, AlaAT activity was inhibited by 47% when calculated from the reaction in the first 4 min after addition of alanine; if the calculation is based on the rate in the 4–8 min interval after initiating the reaction with alanine, the apparent inhibition falls to 38%. In the present work, all values for inhibition by amino-oxyacetate are calculated for the first 4 min interval after the assay reactions were begun with aspartate for AspAT or alanine for AlaAT.

Although it was expected that amino-oxyacetate would inhibit both aminotransferases examined in this study, it was surprising that, in hepatocyte incubations, AspAT was more rapidly and more strongly inhibited than AlaAT. With rat liver extracts prepared by standard homogenization techniques, both Hopper & Segal (1964) and Rofe & Edwards (1978) have reported that AlaAT is much more sensitive to inhibition by amino-oxyacetate than is AspAT. We observed the same order of sensitivity (Table 5) with hepatocyte extracts prepared as described in the Experimental section. Thus, when amino-oxyacetate is used with intact cells, its relative reactivity towards AlaAT and AspAT is reversed from that seen when this inhibitor is used *in vitro*. It is possible that AlaAT is, as *in vitro*, more strongly affected in cells, but is re-activated by cell disruption and extraction to a greater extent than is amino-oxyacetate-inhibited AspAT.

Propargylglycine

Marcotte & Walsh (1975) reported that AlaAT *in vitro* was 50% inactivated in about 5 min by 10 mM-DL-propargylglycine, but no loss of AspAT activity occurred during a 10 min treatment with 100 mM-propargylglycine. Subsequently (Tanase & Morino, 1976) it was found that purified pig heart AspAT was very slowly inactivated when the enzyme was incubated with pyruvate or 2-oxoglutarate as well as 100 mM-L-propargylglycine; the times for half-inactivation in the presence of pyruvate were 2–4 h, but were much decreased when the incubations were conducted in 3 M-potassium formate. These observations *in vitro* suggested that propargylglycine might be useful as a selective inactivator of AlaAT in whole cells. Results of tests with isolated hepatocytes (Table 6) show that 4 mM-DL-propargylglycine is required to achieve 90% inactivation of AlaAT in 20 min. It is likely that the L-isomer is the active component of the racemic propargylglycine used here, but, on that assumption, the concentration of propargylglycine required for 90% inactivation of rat liver AlaAT would still be at least 40 times the concentration of L-cycloserine required to achieve that effect (cf. Table 6 with Table 1 and Fig. 1). In contrast with our expectation based on the enzyme studies *in vitro*, hepatocyte AspAT was partially

Table 5. *Inhibition of AlaAT or AspAT in vitro with or without preincubation with amino-oxyacetate*

Hepatocytes were extracted as described in the Experimental section for release of total cellular enzyme activities. Samples of the cell extracts were added to enzyme assay buffer at 38°C, followed by amino-oxyacetate at the indicated concentrations. In some instances, ('−preincubation'), reaction was initiated immediately; in others ('+preincubation'), reaction was initiated after 15 min at 38°C. Inhibitions were calculated relative to assays without amino-oxyacetate but run in parallel with those containing the inhibitor. Results are from a representative set of assays with an extract containing 439 and 29.3 units/g wet wt. of AspAT and AlaAT respectively.

		Inhibition (%)			
		AlaAT		AspAT	
[Amino-oxyacetate] (μM)	Preincubation ...	−	+	−	+
1.0		89.8	92.4	28.9	60.4
5.0		96.3	98.3	76.1	93.6

Table 6. *Inactivation by* DL-*propargylglycine of AlaAT or AspAT in hepatocytes*
Hepatocytes were incubated for 20min at 38°C, separated from the medium as described in the Experimental section, and the cell pellets extracted for enzyme measurements. Results are means ± S.E.M. for experiments with three hepatocyte preparations.

[DL-Propargylglycine] (mM)	AlaAT		AspAT	
	(units/g wet wt.)	(% inhibition)	(units/g wet wt.)	(% inhibition)
0	29.1 ± 1.6		422 ± 14	
2	5.42 ± 0.74	80.8 ± 3.8	385 ± 15	8.8 ± 3.7
4	2.36 ± 0.18	91.7 ± 1.2	355 ± 13	15.9 ± 0.6
6	1.74 ± 0.11	94.0 ± 0.5	345 ± 12	18.3 ± 0.3
8	1.91 ± 0.36	93.8 ± 1.1	332 ± 14	21.5 ± 2.1
10	1.56 ± 0.14	94.7 ± 0.2	313 ± 11	25.9 ± 0.7

inactivated by propargylglycine, and the concentration causing a 90% decrease in AlaAT also lowered AspAT activity by 16% (Table 6). Thus, compared with cycloserine, propargylglycine is slightly less selective, as well as requiring much higher concentrations for its actions against aminotransferases in intact cells.

Vinylglycine

Vinylglycine (2-aminobut-3-enoic acid), the parent compound for AMB, is an irreversible inactivator of pig heart AspAT *in vitro* (Rando, 1974*a*), but it is described as having no effect on AlaAT (Marcotte & Walsh, 1976). However, when tested with extracts of rat hepatocytes, DL-vinylglycine inhibited both aminotransferases, and the inhibition of AlaAT was slightly greater than that of AspAT. It is not known whether this discrepancy is due to species differences, to differences in states of enzyme purity or to some other cause. We also tested vinylglycine in incubations of intact hepatocytes and found its action to be weaker than with cell extracts. That is, 4mM-DL-vinylglycine caused 57% and 69% inhibition, respectively, of AspAT and AlaAT *in vitro* (Table 7), but, in 30min incubations of three cell preparations with 4mM-DL-vinylglycine, the observed percentage decreases (means ± S.E.M.) were only 22.4 ± 1.9 for AlaAT and 5.7 ± 0.9 for AspAT. Thus, both in hepatocytes and *in vitro*, vinylglycine was more active against AlaAT. This compound, however, is the weakest aminotransferase inhibitor of any that we have tested with rat hepatocytes, and it seems unlikely that vinylglycine would be useful as an aminotransferase inhibitor in studies of cellular metabolism.

Table 7. *Inhibition by vinylglycine of rat liver AlaAT and AspAT in vitro*
Total cell extracts from three hepatocyte preparations were obtained as described in the Experimental section. Extract, the appropriate assay buffer and DL-vinylglycine at the indicated concentrations were mixed and incubated at 38°C for 15min. Then other assay components (the appropriate keto acid, coupling enzyme and NADH) were added as quickly as possible and reactions initiated with alanine or aspartate. Results are means ± S.E.M. for measurements with three cell extracts that contained, in the absence of vinylglycine, 34.0 ± 1.1 and 417 ± 26 units/g wet wt. of cells, respectively, of AlaAT and AspAT.

[DL-Vinylglycine] (mM)	Inhibition (%)	
	AlaAT	AspAT
1	30.4 ± 0.5	23.9 ± 1.4
2	47.8 ± 0.8	39.8 ± 1.5
4	68.6 ± 2.9	56.9 ± 2.4

Ethyl hydrazinoacetate

The use of ethyl hydrazinoacetate in metabolic experiments was first reported by Rognstad (1980), who found that this inhibitor acted with many of the characteristics of amino-oxyacetate. That is, with isolated rat hepatocytes, both compounds decreased the rates of gluconeogenesis from lactate, but not from pyruvate or fructose. Ethyl hydrazinoacetate is a derivative of hydrazine, and amino-oxyacetate is a substituted hydroxylamine. Since both hydrazine and hydroxylamine are carbonyl reagents with general reactivity towards aldehydes and, more slowly, towards ketones, it might be expected that ethyl hydrazinoacetate would inhibit a variety of enzymes similar to that described for amino-oxyacetate (see the introduction). In the present study, we have examined only AlaAT and AspAT, and in hepatocyte incubations we observed a pattern of inhibition resembling that shown for amino-oxyacetate in Table 4. Ethyl hydrazinoacetate was, however, less inhibitory; at a concentration of 50 μM in three experiments with hepatocytes, this compound decreased AspAT activity by 52.3 ± 5.8% and AlaAT by 33.7 ± 3.4% in 30min incubations. Thus, with ethyl hydrazino-

acetate, as with amino-oxyacetate, it appears that inhibition of AspAT, which is adequate for metabolic studies, would be accompanied by a large inhibition of AlaAT.

Discussion

In this paper we have described aminotransferase inhibitors in terms of their selective action against AlaAT and AspAT. Generally inhibitors are discussed in terms of their specificity, but the description of an inhibitor as specific often is taken to mean that it acts at a unique site or with one class of enzymes. We have chosen to refer to selectivity in order to emphasize the probability than all metabolic inhibitors, when used with intact cells or more complex systems, will have multiple sites of action. For example, pyrazole and its 4-substituted derivatives are well-known inhibitors of alcohol dehydrogenase, and occasionally they are spoken of as being specific for that action (see, e.g., Blomstrand *et al.*, 1979). However, 4-hydroxypyrazole, a liver metabolite of pyrazole, has been shown to be a potent inactivator of catalase *in vivo* (MacDonald & Pispa, 1980) and in isolated hepatocytes (Harris *et al.*, 1982). Furthermore, each of the inhibitors used in the present study acts against enzymes other than aminotransferases. Of those, AMB, which probably has the fewest reported additional sites of action, has been shown to be a substrate for deamination by threonine dehydratase (Kapke & Davis, 1975), to cause inactivation of tryptophan synthase (Miles, 1978), to antagonize methionine uptake and utilization and inhibit the growth of cultured tumour cells (Tisdale, 1980), and to inhibit enzymes of *S*-adenosylmethionine synthesis (Sufrin *et al.*, 1982).

Because of the problems outlined above, six aminotransferase inhibitors were tested in hepatocyte incubations to determine which inhibitors are capable of causing at least a 90% decrease in either AlaAT or AspAT activity with an acceptably small effect on the other and to determine the lowest concentrations required to achieve that degree of selective inhibition. For AlaAT, 90% inhibition was obtained with 50 μM-L-cycloserine or 4 mM-DL-propargylglycine, and the concomitant decreases in AspAT were about 10% for cycloserine and 16% for propargylglycine (Tables 1 and 6). The difference in effects of these two inhibitors on AspAT is probably of no metabolic significance, since isolated rat hepatocytes contain 400–500 units of AspAT/g wet wt., and a decrease as large as 16% would still leave a cellular activity of more than 300 units/g. However, the high concentration of propargylglycine required for inactivation of AlaAT might increase the possibility of spurious effects due either to actions on other classes of enzymes or to metabolism of the inhibitor itself. The latter is known to be a problem with millimolar concentrations of amino-oxyacetate (Harris *et al.*, 1982). Thus L-cycloserine seems to be the best material for selective inactivation of AlaAT. For AspAT, selective inactivation can be achieved with AMB, although more slowly than with cycloserine acting on AlaAT (Fig. 1). Inactivation of AspAT was accelerated by increasing the concentration of AMB from 250 μM to 1 mM, but that was accompanied by greater inactivation of AlaAT (see Table 1 and the text). Therefore, for metabolic experiments, it was decided to keep the inhibitor concentration below 500 μM and to preincubate for 20 min to allow for inactivation of AspAT.

When hepatocytes were preincubated with AMB or cycloserine (Table 2), we observed large metabolic effects indicative of selective inactivation of AlaAT or AspAT. However, all of those effects were diminished when the inhibitors and substrates were added simultaneously (Table 3). Although AlaAT was decreased by cycloserine, other measurements show that this occurred largely during the second half of the 30 min incubation period; that is, the time course for AlaAT shown in Fig. 1 was displaced to the right. In agreement with that observation, with no preincubation alanine utilization was not inhibited by 25 μM-L-cycloserine, and the decrease caused by 50 μM (28%) was less than half of the inhibition (67%) when cells were preincubated with 50 μM-cycloserine (Tables 2 and 3). It was previously reported (Janski & Cornell, 1981) that the inactivation of hepatocyte AspAT caused by 1 mM-L-cycloserine was greatly diminished when the inhibitor and a lactate/oleate/NH_4Cl substrate mixture were simultaneously added to incubations. Likewise, with AMB, aspartate has been shown to prevent the inactivation of AspAT *in vitro* (Rando, 1974*b*), and, in hepatocyte experiments, preincubation with AMB was required for full inactivation of AspAT when the incubations also included ornithine, NH_4Cl and either lactate or pyruvate (Smith & Freedland, 1981). It was unexpected, however, that, as seen in Table 3 and also evident in Table 2, AspAT would be partially protected from inactivation by AMB when alanine was the metabolic substrate for hepatocytes. That observation may be partly explained by the large accumulations of glutamate (a reactant for AspAT) that occur when hepatocytes metabolize alanine.

The results presented here show that selective inhibition of AlaAT by L-cycloserine and of AspAT by AMB can be achieved in isolated hepatocytes by using low concentrations of those inhibitors. The results also indicate several other

conclusions. First, data on aminotransferase inhibition *in vitro* should not be assumed to give reliable guides as to which enzymes will be affected or the relative extents to which they will be inhibited in whole cells. Secondly, since all aminotransferase inhibitors interact with pyridoxal at catalytic sites of enzymes, metabolic substrates that cause large cellular accumulations of amino acids may prevent or diminish the effects of inhibitors. In those instances, especially, cells should be preincubated with inhibitors before metabolic substrates are introduced. Finally, since aminotransferase inhibitors as well as other types may be expected to have multiple cellular sites of action, the approach of determining the lowest inhibitor concentration required to block the intended enzyme should decrease the possibility that observed metabolic effects might be caused by other actions of the inhibitor.

We are grateful to Dr. Raymond S. Ochs for helpful discussions of this work.

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