Inhibition by cycloserine of mitochondrial and cytosolic aspartate aminotransferase in isolated rat hepatocytes

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Isolated hepatocytes were incubated with L-cycloserine and then treated with digitonin so that mitochondrial and cytosolic fractions were obtained in 5 s. Mitochondrial and total cellular aspartate aminotransferases (EC 2.6.1.1) were inactivated in parallel. The enzyme was also inhibited in isolated mitochondria incubated with L-cycloserine. These results, in contrast with previous reports, indicate that cycloserine reacts equally with mitochondrial and cytosolic aspartate aminotransferases.

The role of the malate–aspartate shuttle in metabolic processes is frequently examined with transaminase inhibitors such as amino-oxyacetate and cycloserine. Although amino-oxyacetate is effective at much lower concentrations, some workers (e.g. Meijer *et al.*, 1978; Sano & Swick, 1979; Rognstad, 1979; Hensgens *et al.*, 1980) have used cycloserine because of reports that it does not penetrate the mitochondrial membrane, and therefore inhibits only the cytosolic aspartate aminotransferase (EC 2.6.1.1) (Parrilla *et al.*, 1974; Meijer *et al.*, 1975, 1978). A transaminase inhibitor with those properties would be a valuable tool in metabolic research. However, the assignment of those properties to cycloserine is based in part on a cell-fractionation study in which 50% of the total cellular aspartate aminotransferase activity was indicated to be cytosolic (Meijer *et al.*, 1978). We have described modifications (Janski & Cornell, 1980a) of the digitonin fractionation procedure (Zuurendonk & Tager, 1974) which permit separation of cytosolic and mitochondrial fractions from rat hepatocytes in 3 s under conditions that cause more than 90% release of cytosolic enzymes and less than 2% release of mitochondrial enzymes. By using that modified procedure and measuring the rates of release of marker enzymes for cytosol and mitochondria, it was found that only 16% of the cellular aspartate aminotransferase is located in the cytosol (Janski & Cornell, 1980a). Because of this much lower value for the cytosolic fraction of that enzyme, we have conducted experiments with rat hepatocytes and isolated mitochondria to test whether mitochondrial aspartate aminotransferase is inhibited by cycloserine.

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Experimental

Male Wistar rats (170–210 g), obtained from Carworth (Wilmington, MA, U.S.A.), were starved for 2 days and used as the source of hepatocytes or mitochondria. Isolated hepatocytes were prepared by the method of Berry & Friend (1969), modified as described by Cornell *et al.* (1973) and Krebs *et al.* (1974). ATP contents were measured in all cell preparations and ranged from 2.3 to 2.8 $\mu\text{mol/g}$ wet wt. of cells; these values are similar to those found for freeze-clamped liver from starved rats (2.5 $\mu\text{mol/g}$; Krebs *et al.*, 1974). Hepatocytes were suspended in saline medium (Krebs & Henseleit, 1932) containing 2.5% (w/v) bovine serum albumin and were incubated at 38°C in the presence of O_2/CO_2 (19:1). Liver mitochondria were isolated as described by Johnson & Lardy (1967), and the three preparations had respiratory control ratios (O_2 uptake with 0.25 mM-ADP/ O_2 uptake when ADP is depleted) of 8.2, 10.6 and 11.0 when incubated with 5 mM-glutamate + 5 mM-malate and other conditions as described by Estabrook (1967). Where statistics are given, the values are means $\pm$ S.E.M. for experiments with three different hepatocyte or mitochondrial preparations.

L-Cycloserine was a gift from Dr. W. E. Scott (Hoffman–LaRoche, Nutley, NJ, U.S.A.). Digitonin was purchased from ICN Pharmaceuticals (Cleveland, OH, U.S.A.) and was rendered water-soluble as described by Janski & Cornell (1980a). Ultra-pure-grade sucrose was obtained from Schwarz/Mann (Orangeburg, NY, U.S.A.), bromodecane from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and bromododecane from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Analytical enzymes and

cofactors were from Boehringer (Mannheim) Corp. (Indianapolis, IN, U.S.A.) or from Sigma.

Hepatocyte suspensions were fractionated with the use of brominated hydrocarbons (Cornell, 1980) and the centrifugation procedure described by Janski & Cornell (1980a). A 0.2 ml portion of an incubated cell suspension was added to 1.0 ml of fractionation medium (0.25 M-sucrose/20 mM-4-morpholinepropanesulphonic acid/3 mM-EDTA, pH 7.0) at 23°C. When digitonin was present, the concentration was 4 mg/ml of fractionation medium. After 5 s in the fractionation medium, cells were sedimented through the brominated-hydrocarbon layer. The fluid remained above the hydrocarbon layer after centrifugation contained the cytosolic fraction; both layers were removed sequentially, and the cell pellets were resuspended in 1.2 ml of fractionation medium containing 0.1% (v/v) Triton X-100. In the present paper 'mitochondrial fraction' is defined as the enzyme activity in the material sedimenting through the hydrocarbon layer after exposure of cells to digitonin, and the 'total cellular activity' refers to enzyme activity in cells exposed to digitonin-free fractionation medium and centrifuged through a hydrocarbon layer.

All enzyme assays were performed at 38°C, and 1 unit of activity is defined as the amount of enzyme catalysing conversion of 1 μ mol of substrate/min. Aspartate aminotransferase was measured during the first 2 min of reaction in the presence of 25 mM-aspartate and otherwise as described by Bergmeyer & Bernt (1974). Lactate dehydrogenase (EC 1.1.1.27) and adenylate kinase (EC 2.7.4.3) were assayed as described by Bergmeyer *et al.* (1974), with modifications (Janski & Cornell, 1980a). Citrate synthase (EC 4.1.3.7) was assayed by the procedure of Shepherd & Garland (1969).

Results and discussion

To measure accurately the accessibility of mitochondrial aspartate aminotransferase to cycloserine, the conditions for fractionating hepatocytes were selected so as to minimize the time of exposure to digitonin and damage to the mitochondria. For all cell-fractionation experiments reported here, the conditions were as described in the Experimental section. With those conditions, digitonin caused release of 87% of the marker enzyme for the cytosol (lactate dehydrogenase), 7.8% of the marker for the mitochondrial intermembrane space (adenylate kinase), but only 0.4% of the marker for the mitochondrial matrix (citrate synthase). The percentage of aspartate aminotransferase released was calculated from the difference between total activity in the cells and that in the mitochondrial fractions, and was 12–13% in either the absence or presence of cycloserine in the hepatocyte incubation. By appli-

cation of the previously described procedure for calculating subcellular distribution of enzymes (Janski & Cornell, 1980a), the results given here indicate that 14% of the cellular aspartate aminotransferase is cytosolic and 86% mitochondrial. These results agree very well with the values of 16 and 84% obtained under a different set of fractionation conditions and with the data showing that the conditions of the digitonin fractionation can be varied without affecting the calculated distributions of enzymes located in the cytosol and the mitochondrial matrix (Janski & Cornell, 1980a,b).

For the experiments described in Table 1, hepatocytes were incubated with L-cycloserine under conditions similar to those used by Meijer *et al.* (1978), who concluded that cycloserine inhibits aspartate aminotransferase predominantly in the cytosol. In contrast with that conclusion, the results in Table 1 show that L-cycloserine is equally inhibitory to mitochondrial and total cellular aspartate aminotransferase. As a test of the possibility that inhibition of the mitochondrial enzyme occurred either during or after cell fractionation, the following experiment was conducted. Hepatocytes were incubated at 38°C for 20 min in the absence of L-cycloserine, and 0.2 ml of cells was added to 1.0 ml of fractionation medium containing L-cycloserine to

Table 1. *Effect of L-cycloserine on aspartate aminotransferase activities in isolated hepatocytes*

Hepatocytes (108, 112 or 114 mg wet wt. in 3.8 ml) were preincubated for 15 min at 38°C with oleate and with or without L-cycloserine. Then lactate and NH_4Cl in 0.2 ml were added; after an additional incubation for 30 or 80 min, 0.2 ml portions were removed and exposed to fractionation medium with or without digitonin as described in the Experimental section. Final concentrations in the 4.0 ml incubation mixtures were: oleate, 1.0 mM; lactate, 10 mM; NH_4Cl , 10 mM; L-cycloserine, 1.0 mM. For the total cellular activity in the three cell preparations, 1.00 represents 549, 520 or 485 units/g wet wt. of cells; for the mitochondrial fraction, 1.00 represents 480, 471 or 424 units/g wet wt. of cells.

L-Cycloserine	Incubation time (min)	Relative aspartate aminotransferase activity	
		Total cellular activity	Mitochondrial fraction
—	45	1.00	1.00
+	45	0.43 $\pm$ 0.04	0.41 $\pm$ 0.04
—	95	0.98 $\pm$ 0.02	0.99 $\pm$ 0.01
+	95	0.25 $\pm$ 0.02	0.22 $\pm$ 0.03

give a final concentration of 0.17 mM (the concentration present after cells incubated with 1.0 mM-L-cycloserine were added to fractionation medium as described in Table 1). After exposure for 5 s and centrifugation, the aspartate aminotransferase released into the medium by digitonin was inhibited by $45 \pm 3\%$; however, there was no detectable inhibition of either total cellular activity or that in the mitochondrial fraction. Thus this control experiment indicates that the inhibition of mitochondrial aspartate aminotransferase by L-cycloserine shown in Table 1 occurred during the hepatocyte incubation and not as a consequence of the fractionation.

In other experiments, conducted in a manner similar to those described in Table 1, the concentration of L-cycloserine in the hepatocyte incubation was varied from 0.1 to 5.0 mM, and half-maximal inhibition of total cellular aspartate aminotransferase occurred at 0.70 ± 0.06 mM-L-cycloserine. Meijer *et al.* (1978) reported half-maximal inhibition of total cellular activity as occurring at about 2 mM-DL-cycloserine, which agrees reasonably well with our data, since only the L-isomer of cycloserine is inhibitory to mammalian transaminases (Wong *et al.*, 1973). This agreement between our results and those of Meijer *et al.* (1978) concerning the inhibition of total cellular activity suggests that the difference in results for the mitochondrial aspartate aminotransferase derives from the two different cell-fractionation techniques and not from some subtle difference in hepatocyte preparation or incubation. In this regard, it should be noted that Meijer *et al.* (1978) fractionated hepatocytes by a cavitation method which disrupts cells by mechanical shearing and causes release of as much as 17% of mitochondrial matrix enzymes under conditions giving 86% release of cytosolic enzymes (Tischler *et al.*, 1977).

An experiment with isolated rat liver mitochondria provided additional evidence that aspartate aminotransferase in that compartment can be inhibited by cycloserine. Mitochondria (23 mg of protein/ml) were incubated at 23°C in 0.25 M-sucrose (pH 7.4) with or without 4.0 mM-L-cycloserine. At 20 min and 60 min, a 0.10 ml portion of the incubation mixture was mixed with 1.0 ml of 0.25 M-sucrose (pH 7.4) and layered above 0.15 ml of a brominated-hydrocarbon mixture ($\rho = 1.045$ g/ml; Janski & Cornell, 1980a) in a 1.5 ml Eppendorf centrifuge tube. After 5 s, the mitochondria were centrifuged through the hydrocarbon layer as in the procedure for fractionating hepatocytes, and the sedimented mitochondria were disrupted in 1.0 ml of fractionation medium containing 4 mg of digitonin and 0.1% (v/v) Triton X-100. Aspartate aminotransferase activity was inhibited by $65 \pm 4\%$ and $80 \pm 2\%$ after 20 and 60 min incubation with

L-cycloserine. In a control experiment, mitochondria incubated for 60 min in the absence of cycloserine were transferred to a centrifuge tube, mixed with the sucrose layer containing L-cycloserine at a final concentration of 0.36 mM (the amount present in the sucrose layer after addition of mitochondria which had been incubated with 4.0 mM-L-cycloserine) and then were centrifuged through the hydrocarbon layer. There was no detectable inhibition of aspartate aminotransferase in the sedimented mitochondria, which indicates that the inhibition reported above occurred during incubation of the mitochondria and not during or after the centrifugation step.

Cycloserine is a 'quasi-substrate' inhibitor (Braunstein, 1973) of transaminases, and the presence of a substrate, glutamate, will partially block or reverse the inhibition of L-cycloserine of aspartate aminotransferase in rat liver extracts (N. W. Cornell, unpublished work). Because of this, it seemed possible that the effect of cycloserine in hepatocytes could be influenced by other substances in the cell incubation mixtures. Freshly prepared hepatocytes have low amounts of amino acids (Cornell *et al.*, 1974), but when cells are incubated with substrates such as lactate, NH_4Cl and oleate, i.e. those used in the experiments reported in Table 1 and by Meijer *et al.* (1978), the contents of aspartate and glutamate are increased rapidly (Stubbs & Krebs, 1975; Meijer *et al.*, 1978). As shown in Table 2, addition of

Table 2. *Effect of substrates on inhibition of aspartate aminotransferase by L-cycloserine*

Hepatocytes (76, 104 or 113 mg wet wt. in 4.0 ml) were incubated at 38°C with 1.0 mM-L-cycloserine and with or without substrates (1.0 mM-oleate, 10 mM-lactate and 10 mM- NH_4Cl) added at zero time. At the indicated incubation times, 0.2 ml samples were removed and exposed to fractionation medium with or without digitonin as described in the Experimental section. For total cellular activity in the three cell preparations, 1.00 represents 555, 540 or 480 units/g wet wt.; for the mitochondrial fraction, 1.00 represents 486, 481 or 416 units/g wet wt. of cells.

Substrates	Incubation time (min)	Relative aspartate aminotransferase activity	
		Total cellular activity	Mitochondrial fraction
—	6	0.48 ± 0.05	0.45 ± 0.06
—	20	0.22 ± 0.03	0.18 ± 0.03
—	40	0.17 ± 0.01	0.16 ± 0.01
—	60	0.16 ± 0.003	0.14 ± 0.01
+	12	0.86 ± 0.01	0.86 ± 0.03
+	20	0.79 ± 0.04	0.81 ± 0.04
+	40	0.75 ± 0.02	0.77 ± 0.04
+	60	0.71 ± 0.02	0.73 ± 0.01

hepatocytes to incubation mixtures that contained both L-cycloserine and the lactate/ NH_4Cl /oleate substrate combination decreased the rate and the extent of inhibition of aspartate aminotransferase. However, there were no differences in either rate or extent of inhibition of the mitochondrial and total cellular activities. These results suggest that, when cycloserine is used as a metabolic inhibitor with isolated cells, some care must be taken to assure that the effects observed are not influenced by the substrates added to the incubation. In addition, it is worth noting that the maximum effect of 5 mM-L-cycloserine (the equivalent of 10 mM of the DL mixture) seen in our experiments was about 85% inhibition of total cellular aspartate aminotransferase. As the content of that enzyme is about 500 units/g wet wt. of cells (Tables 1 and 2), the residual activity would still be about 75 units/g. In contrast, with only 0.2 mM-amino-oxyacetate, aspartate aminotransferase is 96–98% inhibited (N. W. Cornell, K. E. Crow & R. Whitefoot, unpublished work), and such differences in completeness of inhibition might be significant in comparing and analysing results obtained with cycloserine and amino-oxyacetate.

Because the data presented here indicate that the mitochondrial and cytosolic aspartate aminotransferases of isolated hepatocytes are equally accessible to cycloserine, it would seem preferable to use the more potent inhibitor, amino-oxyacetate, in metabolic experiments concerned with aspartate aminotransferase and the malate–aspartate shuttle. It should be noted, however, that both cycloserine (Wong *et al.*, 1973) and amino-oxyacetate (John *et al.*, 1978) react with transaminases other than aspartate aminotransferase, and, at concentrations above 1 mM, amino-oxyacetate affects ethanol metabolism in a manner that is not explained by inhibition of transaminases (Rognstad & Clark, 1974; Krebs & Stubbs, 1975). Attention should be given to such non-specific effects when cycloserine or amino-oxyacetate is used with whole cells or other complex systems.

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