Re-activation by glutamate or aspartate of amino-oxyacetate-inhibited aspartate aminotransferase *in vitro* and in isolated hepatocytes

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Experiments with isolated rat hepatocytes and with cell extracts indicate, in contrast with previous reports, that pyruvate does not block or reverse the inhibition of aspartate aminotransferase (EC 2.6.1.1) by amino-oxyacetate. That inhibition, however, is partially overcome by glutamate or aspartate either in cell extracts or in whole cells incubated with substrate combinations that cause accumulation of those amino acids.

Amino-oxyacetate, an inhibitor of transaminases, has been widely used in metabolic studies, especially those concerned with the malate-aspartate shuttle. An example of such studies is the report by Rognstad & Katz (1970) showing that, in the rat, gluconeogenesis from lactate is inhibited by amino-oxyacetate, whereas glucose synthesis from pyruvate is not. Those observations supported concepts about the different origins of cytosolic oxaloacetate from these two compounds. That is, when lactate is the substrate for gluconeogenesis, oxaloacetate is transferred from mitochondria to cytosol via the aspartate shuttle and a dual transaminase mechanism. With pyruvate no transamination is involved, but, rather, the outflow of mitochondrial malate provides the immediate precursor of cytosolic oxaloacetate and reducing equivalents for glucose synthesis (Borst, 1963; Lardy *et al.*, 1965; Haynes, 1965; Walter *et al.*, 1966; Krebs *et al.*, 1967).

More recently, it has been reported (Meijer *et al.*, 1974; Meijer & van Dam, 1974) that little or no inhibition of transaminase reactions was observed when pyruvate and amino-oxyacetate were added simultaneously to incubations of isolated hepatocytes, but inhibition was seen when the cells were preincubated with amino-oxyacetate before addition of pyruvate. Besides this suggestion that pyruvate can block the action of amino-oxyacetate, Smith *et al.* (1977) have concluded that the inhibition of aspartate aminotransferase (EC 2.6.1.1) caused by amino-oxyacetate can be reversed by pyruvate, even

when the latter is added to hepatocyte suspensions preincubated with the inhibitor. Those reports raised some questions about the concepts of gluconeogenesis cited above and about the results of other studies where amino-oxyacetate has been used in the presence of pyruvate as a substrate. For this reason, and to understand better the action of amino-oxyacetate, we have conducted experiments to test whether pyruvate or other materials can reverse the inhibitory effect of amino-oxyacetate on aspartate aminotransferase.

Experimental

Isolated hepatocytes were prepared from 48 h-starved male Wistar rats (170–220 g; Carworth, Wilmington, MA, U.S.A.) by the procedure of Berry & Friend (1969) modified as described by Cornell *et al.* (1973) and Krebs *et al.* (1974). As an index of cell quality, ATP content was measured for each cell preparation and in all cases was 2.0–2.5 $\mu\text{mol/g}$ wet wt. of cells. Incubation of hepatocytes and extraction of metabolites were as described by Krebs *et al.* (1974) and Crow *et al.* (1978). Amino-oxyacetate, NAD^+ and NADH were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Enzymes for analytical purposes and 5,5'-dithiobis-(2-nitrobenzoic acid) were from Boehringer Mannheim (Indianapolis, IN, U.S.A.), and other chemicals were reagent-grade commercial products.

For determination of aspartate aminotransferase activity, hepatocyte suspensions or incubation mixtures were centrifuged for 15 s at room temperature

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and 2500 g. The sedimented cells were resuspended in 50 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 8.4, at a concentration of about 50 mg wet wt. of cells/ml of buffer. The cells were sonically disrupted at 4°C and centrifuged for 45 min at 4°C and 40 000 g. Supernatants were either used immediately or were quick-frozen with liquid N₂ and stored at -80°C; control experiments showed that samples frozen and stored in this manner lost no aspartate aminotransferase activity over a period of 3 months. The standard assay for enzyme activity was a modification of that described by Bergmeyer & Bernt (1974) and contained, in a final volume of 2.0 ml: 88 mM-potassium phosphate, pH 7.4, 0.21 mM-NADH, 11 mM- α -oxoglutarate, 25 mM-aspartate (omitted from the reference cuvette), 7.5 units (1 μ mol/min) of malate dehydrogenase (no. 127892, Boehringer Mannheim) and cell extract. Reaction was initiated by addition of aspartate, and the A_{340} was monitored. In experiments conducted to test whether pyruvate or NH₄Cl can block the inhibitory effect of amino-oxyacetate, the high content of lactate dehydrogenase and glutamate dehydrogenase in hepatocyte extracts made it impossible to use the standard assay procedure. For those instances, aspartate aminotransferase was assayed by coupling it with citrate synthase (EC 4.1.3.7) and using 5,5'-dithiobis-(2-nitrobenzoic acid) (Ellman, 1959) to measure CoA released from acetyl-CoA. A similar procedure was described by Itoh & Srere (1970). The assay mixture contained in a final volume of 2.0 ml: 88 mM-potassium phosphate, pH 7.4, 0.10 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 0.05 mM-acetyl-CoA, 11 units of citrate synthase (Boehringer no. 103381), 11 mM- α -oxoglutarate, 25 mM-aspartate (omitted from the reference cuvette) and cell extract. Reaction was monitored at 409.5 nm, and 14 150 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ was used as the molar absorption coefficient of the anion produced from reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (Riddles *et al.*, 1979). Complete utilization of the acetyl-CoA in this assay would give an absorbance change of 0.71, and the reaction rates observed with hepatocyte extracts or crystalline pig heart aspartate aminotransferase were linear until about 95% of the acetyl-CoA was used. With four hepatocyte extracts, the aspartate aminotransferase activity determined by this procedure was 103 $\pm$ 2% of that found with the standard procedure described above. In this paper, one unit of aspartate aminotransferase activity is the amount causing oxidation of 1 μ mol of NADH or reduction of 1 μ mol of 5,5'-dithiobis-(2-nitrobenzoic acid)/min at 38°C.

Urea was measured by the procedure of Bernt & Bergmeyer (1974), glutamate as described by Cornell *et al.* (1974), and aspartate by the method of Bergmeyer *et al.* (1974) as modified by Crow *et al.*

(1978). All values in the present paper are means $\pm$ S.E.M. for at least three cell preparations or cell extracts.

Results and discussion

When hepatocytes were preincubated for 15 min with amino-oxyacetate and then incubated for 60 min with various substrates (Table 1), urea synthesis during the 60 min period was 94–98% inhibited with all substrate mixtures except pyruvate, NH₄Cl and ornithine. In the latter instance, urea production was 50% of the corresponding amount without amino-oxyacetate. In contrast, with the lactate/NH₄Cl/ornithine substrate mixture, ureogenesis in the 60 min incubation was only 2% of that in the absence of amino-oxyacetate. These results agree well with those of Smith *et al.* (1977), who, on observing the partial restoration of urea synthesis with pyruvate, NH₄Cl and ornithine as substrates, concluded that pyruvate can reverse the amino-oxyacetate inhibition of aspartate aminotransferase. As shown in Table 1, however, in the absence of NH₄Cl and ornithine, 10 mM-pyruvate was ineffective in reversing the amino-oxyacetate inhibition of urea synthesis.

Our results for urea synthesis made it appear unlikely that the restoration seen with pyruvate, NH₄Cl and ornithine could be explained as a re-activation by pyruvate of amino-oxyacetate-inhibited aspartate aminotransferase. However, because of the low rate of ureogenesis in the absence of NH₄Cl and ornithine, it seemed worthwhile to evaluate that possibility directly by measuring the enzyme activity under each of the experimental conditions described in Table 1. It was found that a 15 min exposure of hepatocytes to 0.2 mM-amino-oxyacetate caused a 96% inhibition of aspartate aminotransferase. Since this enzyme is 84% mitochondrial and 16% cytosolic in rat hepatocytes (Janski & Cornell, 1980), our results show, as suggested by others (Parrilla *et al.*, 1974), that amino-oxyacetate readily inactivates aspartate aminotransferase in both cellular compartments. After the subsequent 60 min incubations, the enzyme remained 91–95% inhibited, except where the substrate mixture contained pyruvate, NH₄Cl and ornithine; in that case, enzyme activity was restored to 20% of the control value. In the experiments described in Table 1, aspartate aminotransferase remained 91% inhibited in the incubations with pyruvate as the only added substrate. In separate tests, 10 mM-pyruvate was added to incubations either 15 min before or at the same time as 0.2 mM-amino-oxyacetate, and the enzyme was, respectively, 92 $\pm$ 1% or 93 $\pm$ 2% inhibited at 75 min after addition of amino-oxyacetate. Thus, independent of when it is added relative to amino-oxyacetate, pyruvate, in contrast with previous

Table 1. *Effect of substrates on urea synthesis, glutamate and aspartate content, and aspartate aminotransferase activity in hepatocytes incubated with amino-oxyacetate*

Hepatocytes, 70–95 mg wet wt. in a final incubation volume of 4.0 ml, were preincubated at 38°C for 15 min either with no substrate (A) or with 10 mM-NH₄Cl and 5 mM-ornithine (B, C, D). Where indicated, 0.20 mM-amino-oxyacetate was also present during the preincubation period. At the end of that period, lactate (C) or pyruvate (A and D) was added to give a concentration of 10 mM; at this time (0 min incubation) some samples in each set were taken for aspartate aminotransferase assays, some received HClO₄, and others were incubated for an additional 60 min. The results are means $\pm$ S.E.M. for three different cell preparations and are expressed as μ mol/g wet wt. of cells for urea, glutamate and aspartate, and units/g wet wt. for aspartate aminotransferase. The zero-time values in B also apply to C and D.

Incubation time (min)	Substrates	Amino-oxyacetate	Urea		Glutamate		Aspartate		Aspartate aminotransferase	
			0	60	0	60	0	60	0	60
A. Pyruvate	—	—	3.9 $\pm$ 0.4	19.4 $\pm$ 1.7	1.35 $\pm$ 0.18	8.05 $\pm$ 0.73	0.13 $\pm$ 0.02	0.18 $\pm$ 0.05	503 $\pm$ 11	516 $\pm$ 18
	+	—	1.6 $\pm$ 0.1	2.6 $\pm$ 0.1	1.30 $\pm$ 0.31	6.88 $\pm$ 0.76	0.11 $\pm$ 0.02	0.25 $\pm$ 0.09	19 $\pm$ 2	43 $\pm$ 1
B. NH ₄ Cl, ornithine	—	—	5.4 $\pm$ 0.6	32.0 $\pm$ 4.0	1.49 $\pm$ 0.24	1.96 $\pm$ 0.09	0.15 $\pm$ 0.03	0.27 $\pm$ 0.05	497 $\pm$ 16	511 $\pm$ 12
	+	—	1.2 $\pm$ 0.2	2.8 $\pm$ 0.2	1.31 $\pm$ 0.37	1.90 $\pm$ 0.13	0.15 $\pm$ 0.02	0.24 $\pm$ 0.06	18 $\pm$ 1	39 $\pm$ 5
C. Lactate, NH ₄ Cl, ornithine	—	—		169 $\pm$ 8		5.00 $\pm$ 0.30		0.22 $\pm$ 0.03		514 $\pm$ 19
	+	—		4.3 $\pm$ 0.2		2.85 $\pm$ 0.14		0.15 $\pm$ 0.05		26 $\pm$ 3
D. Pyruvate, NH ₄ Cl, ornithine	—	—		148 $\pm$ 9		16.0 $\pm$ 1.5		0.46 $\pm$ 0.08		528 $\pm$ 21
	+	—		73 $\pm$ 5		28.9 $\pm$ 2.2		0.47 $\pm$ 0.11		106 $\pm$ 8

reports (Meijer *et al.*, 1974; Meijer & van Dam, 1974; Smith *et al.*, 1977), has little effect in protecting or restoring aspartate aminotransferase activity. The slight increase in enzyme activity during incubations without amino-oxyacetate (Table 1) was a consistent, but unexplained, observation in these experiments.

In the course of a previous study (Cornell *et al.*, 1974) in which amino-oxyacetate was used as an inhibitor of gluconeogenesis, it was observed that glutamate decreases the rate at which amino-oxyacetate inactivates aspartate aminotransferase *in vitro*. For example, with the crystalline pig heart enzyme, the presence of 0.5 mM-glutamate increased 6-fold the time required for 50% inhibition by 10 μ M-amino-oxyacetate (R. Whitefoot, unpublished work). In addition, Rognstad & Katz (1970) found with kidney-cortex slices that high concentrations of aspartate diminished the inhibition of gluconeogenesis. Because of those observations, glutamate and aspartate were also measured in the experiments described in Table 1, and it was found that hepatocyte incubations with pyruvate, NH₄Cl and ornithine contained 2–3 times as much aspartate and 4–15 times as much glutamate as in incubations with amino-oxyacetate and other substrate mixtures. The large amounts of glutamate produced under those conditions are probably due to increased synthesis via the glutamate dehydrogenase reaction, with pyruvate supplying the α -oxoglutarate. Similar results were obtained in the absence of ornithine, i.e.

in experiments with pyruvate and NH₄Cl as substrates, restoration of urea synthesis, re-activation of aspartate aminotransferase, and accumulation of aspartate and glutamate were comparable with those seen with pyruvate, NH₄Cl and ornithine (N. W. Cornell & K. E. Crow, unpublished work). In studies where hepatocytes have been fractionated after incubation under conditions that cause glutamate to accumulate, the mitochondrial concentration of glutamate was calculated to be 10–20 mM and to be 10–35 times higher than the cytosolic concentration (Zuurendonk *et al.*, 1976). If such gradients occur under the conditions used in our experiments, it would mean that the glutamate concentration was greatest in the cellular compartment that contains 84% of the aspartate aminotransferase activity (Janski & Cornell, 1980). These observations suggest that it is the generation of amino acid substrates for aspartate aminotransferase, and not pyruvate itself, which leads to the enzyme re-activation shown in Table 1. In this regard, it should be noted that John *et al.* (1978) used the purified pig heart enzyme and cysteinesulphinat, an aspartate aminotransferase substrate which irreversibly traps the enzyme in the amino form, and were able to measure the dissociation of the enzyme–amino-oxyacetate complex.

As a direct test of the possibility that the amino acid substrates can reverse the effect of amino-oxyacetate on rat liver aspartate aminotransferase, extracts of rat hepatocytes were preincubated for

2.5 min with 10 μ M-amino-oxyacetate and then incubated with test compounds for 1–10 min before initiating the enzyme reaction. The results (Table 2) show that the activity is 76% inhibited after 2.5 min, and the inhibition increases with the time of exposure to amino-oxyacetate, reaching about 90% after 12.5 min. Of the compounds tested, only glutamate and aspartate were effective in reversing the inhibition. Re-activation of aspartate aminotransferase increased with the time of incubation with the amino acid substrate, and, because re-activation continued after the enzyme assay was begun, the results in Table 2 are based on the first 2 min of the assay. NH_4Cl , ornithine, pyruvate or α -oxoglutarate was without effect in these experiments. In addition, it was found that incubating pyruvate with amino-oxyacetate at 38°C in the assay buffer for 10 min before the enzyme was added did not decrease the inhibition (N. Cornell, unpublished work). Although amino-oxyacetate is known to complex with acetaldehyde (Jones *et al.*, 1970), our experiments provide no indication of a reaction between pyruvate and amino-oxyacetate. These results are in agreement with those of Longshaw *et al.* (1972), who detected no effect of pyruvate on inhibition by amino-oxyacetate of purified pig heart

aspartate aminotransferase or that in guinea-pig kidney. Thus there is no evidence, either in cell extracts or in intact cells, of a direct action of pyruvate in preventing or reversing inhibition by amino-oxyacetate. However, the results presented here indicate that substrate combinations that increase the cellular content of glutamate or aspartate can protect or re-activate aspartate aminotransferase. Consequently, in metabolic experiments with amino-oxyacetate and complex substrate mixtures, it would seem advisable to measure the extent of enzyme inactivation.

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Table 2. Effect of substrates, pyruvate, NH_4Cl or ornithine on inhibition by amino-oxyacetate of rat liver aspartate aminotransferase

Extracts of isolated rat hepatocytes were added to aspartate aminotransferase assay mixtures lacking aspartate and α -oxoglutarate, and were preincubated for 2.5 min either without or with 10 μ M-amino-oxyacetate. After the preincubation, either the indicated substance or nothing was added, and the mixtures were incubated for the stated periods. The enzyme assay was then initiated by addition of 25 mM-aspartate and 11 mM- α -oxoglutarate, and the reaction was monitored for 2 min. The temperature was 38°C during the preincubation, incubation and assay periods. Details of the cell extraction and assay procedures are described in the Experimental section. Results are means $\pm$ S.E.M. for three to six hepatocyte extracts. In the absence of amino-oxyacetate, aspartate aminotransferase activity in these extracts was 491 ± 16 units/g wet wt. of cells.

Addition to incubation	Period of incubation (min)	Inhibition (%)	
		Without addition	With addition
2 mM-aspartate	1.0	76 $\pm$ 5	66 $\pm$ 1
	2.0	78 $\pm$ 3	56 $\pm$ 2
	5.0	84 $\pm$ 3	39 $\pm$ 1
2 mM-glutamate	5.0	83 $\pm$ 5	44 $\pm$ 2
10 mM- α -oxoglutarate	10	92 $\pm$ 6	91 $\pm$ 3
10 mM-pyruvate	10	92 $\pm$ 3	92 $\pm$ 2
10 mM- NH_4Cl	10	90 $\pm$ 3	91 $\pm$ 2
5 mM-ornithine	10	91 $\pm$ 4	90 $\pm$ 3

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