Inhibition of Urea Biosynthesis and Conversion of L-Citrulline-6-^{14}C to ^{14}C-Urea by α-Methyl Aspartic Acid and β-Methyl Aspartic Acid (35896)

W. Bruce Rowe and L. L. Miller

Department of Biochemistry, Cornell University Medical College, New York, New York 10021; and Department of Radiation Biology and Biophysics, University of Rochester School of Medicine and Dentistry, Rochester, New York 14620

α-Methyl aspartic acid and *threo-β*-methyl aspartic acid are effective inhibitors of *in vitro* urea biosynthesis in liver homogenates and slices (1–3). However, intraperitoneal injection of α-methyl aspartic acid results in no decrease in urea excretion while *in vitro* urea biosynthesis in liver homogenates prepared from similarly treated animals is strongly inhibited (1). The experiments reported here were undertaken to further investigate urea biosynthesis in the presence of these inhibitors. Urea synthesis and conversion of L-citrulline-6-^{14}C to ^{14}C-urea by liver homogenates have been investigated in the presence of several nitrogen substrates. The effect of these inhibitors on urea synthesis and conversion of L-citrulline-6-^{14}C to ^{14}C-urea by the isolated perfused rat liver has also been investigated.

Materials and Methods. L-Citrulline-6-^{14}C was synthesized from ^{14}C-potassium cyanate and ornithine (4). DL-α-methyl aspartic acid was synthesized as described by Cocker and Lapworth (5), and DL-*threo-β*-methyl aspartic acid as described by Benoiton *et al.* (6). Citrulline and glutamine were purchased from Calbiochem, and aspartic acid from Nutritional Biochemical Co. Fasted male Sprague-Dawley rats were used as liver donors in the perfusion experiments and as donors of livers used in the *in vitro* experiments.

The isolated rat liver perfusion technique of Miller *et al.* (7) was employed in these experiments. Glucose (250 mg) was added to the perfusate at the beginning of the experiments, and amino acid substrates and L-citrulline-6-^{14}C were infused at a constant rate throughout the experiment. Urea nitrogen concentration was determined by the Conway (8) microdiffusion method. Urea specific radioactivity was determined on chromatographically pure urea obtained by Dowex-50 chromatography of a picric acid filtrate of liver or perfusate followed by descending chromatography of the urea fraction in *n*-butanol: methy ethyl ketone:formic acid:H_2O (40:30:15:15). Urea eluted from the latter chromatogram was free of Ninhydrin reactive material and was homogenous in three chromatographic systems. Radioactivity was determined by liquid scintillation counting.

Results. Effect of α-methyl aspartic acid and threo-β-methyl aspartic acid on urea synthesis in rat liver homogenates. Results presented in Table I indicate that the extent of inhibition of *in vitro* urea synthesis by α-methyl aspartic acid and β-methyl aspartic acid is dependent upon the nitrogen substrate employed. Synthesis of urea in the presence of ammonium acetate was strongly inhibited by both α-methyl and β-methyl aspartic acids. Inhibition is less for both analogs of aspartic acid when citrulline was employed as nitrogenous substrate. When aspartic acid as well as citrulline was present, the inhibitory effect of these methyl analogs of aspartic acid is minimal. In the presence of glutamine neither analog exerted significant inhibitory effects on urea synthesis.

In the presence of ammonium acetate, citrulline, or citrulline plus aspartic acid, the inhibition of the conversion of L-citrulline-6-^{14}C to ^{14}C-urea closely parallels inhibition of urea synthesis. However, when glutamine was employed as nitrogenous substrate, the conversion of labeled citrulline to labeled urea was inhibited despite the virtual absence of inhibition of urea synthesis.

TABLE I. Inhibition of *in Vitro* Urea Synthesis by Methyl Aspartic Acids.[a]

Substrate:	0.005 *M* Citrulline 0.005 *M* aspartic acid			0.01 *M* Citrulline			0.01 *M* Ammonium acetate			0.01 *M* Glutamine		
Inhibitor:	None	α-Methyl aspartic acid	β-Methyl aspartic acid	None	α-Methyl aspartic acid	β-Methyl aspartic acid	None	α-Methyl aspartic acid	β-Methyl aspartic acid	None	α-Methyl aspartic acid	β-Methyl aspartic acid
Urea synthesis	19.9	16.3	18.9	7.2	3.6	4.5	11.8	0.8	2.6	10.1	10.8	9.8
Inhibition (%)	—	18	5	—	50	38	—	93	78	—	0	0
Citrulline-^{14}C conversion to ^{14}C-urea	13.1	10.8	11.9	3.4	2.2	3.3	32.0	8.0	14.4	27.1	4.9	11.2
Inhibition (%)	—	18	9	—	35	12	—	75	55	—	82	59

[a] Rat liver was homogenized in 3 vol of cold potassium phosphate buffer (0.1 *M*, pH 7.4). Reaction mixtures contained 0.5 ml of homogenate, potassium phosphate buffer (0.05 *M*, pH 7.4), $MgSO_4$ (0.003 *M*), NaATP (0.0013 *M*), Na-fumarate (0.007 *M*), 0.14 μCi L-citrulline-6-^{14}C, methyl aspartate (0.01 *M*) as indicated, and nitrogenous substrate as indicated in a final volume of 3.0 ml. A mixture of oxygen (95%) and carbon dioxide was passed through the samples for 5 min prior to incubation at 37° for 1 hr. Reaction was stopped by adding 9 ml of cold absolute ethanol. After standing at 0° for 1 hr, the samples were centrifuged and the supernatent solutions were evaporated to dryness under a stream of air. The residues were dissolved in a small volume of distilled water. Aliquots of this solution were used for determination of urea concentration and for chromatographic isolation of urea. Urea was isolated by descending chromatography in the solvent: *tert*-butanol:2-butanone:formic acid:water (40:30:15:15). The area of the chromatogram containing urea was cut out and assayed for radioactivity by liquid scintillation counting. Urea synthesis is expressed as micromoles of urea nitrogen per gram of fresh tissue per hour of incubation. The conversion of L-citrulline-6-^{14}C to ^{14}C-urea is expressed as percentage of added radioactivity recovered as radioactivity in urea.

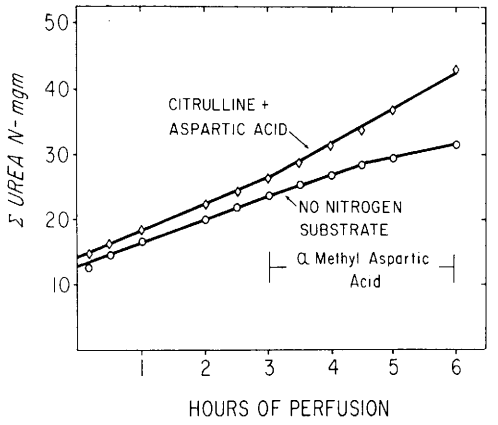

FIG. 1. Effect of α-methyl aspartic acid on urea biosynthesis by the isolated perfused rat liver. In the citrulline + aspartic acid experiment, citrulline (0.25 mmole/hr) and aspartic acid (0.25 mmole/hr) were infused at a constant rate throughout a 6-hr perfusion. In the other experiment no exogenous substrate was infused. After 3 hr of perfusion, 230 mg (1.6 mmoles) of α-methyl aspartic acid were added to the perfusate; and a constant infusion of α-methyl aspartic acid [77 mg (0.5 mmole)/hr] was begun. Cumulative urea nitrogen was calculated from the concentration of urea determined in aliquots of perfusate taken at the times indicated. Each curve represents the results of a single perfusion experiment.

Effect of α-methyl aspartic acid and threo-β-methyl aspartic acid on urea synthesis in the isolated perfused rat liver. The results presented in Fig. 1 indicate that the rate of urea synthesis by the isolated perfused rat liver was essentially the same in the presence of α-methyl aspartic acid (3 to 6 hr) as it was in the control period (0 to 3 hr), before addition of α-methyl aspartic acid. This lack of inhibitory effect was observed in the absence of exogenous nitrogen substrate and in the presence of substrate concentrations of citrulline and aspartic acid.

The rate of urea synthesis in the presence of endogenous substrate was not affected by the presence of β-methyl aspartic acid (Fig. 2). In the experiment using citrulline and aspartic acid as nitrogen substrates, the rate of urea synthesis was significantly increased following addition of β-methyl aspartic acid (Fig. 2).

The rate of conversion of L-citrulline-6-^{14}C to ^{14}C-urea was investigated in similar experiments, the results of which are reported in Fig. 3. The rate of incorporation of radioactivity in perfusate urea was not altered by the addition of α-methyl aspartic acid to the perfusion system. However, addition of β-methyl aspartic acid caused an increase in the rate of appearance of citrulline radioactivity in perfusate urea. Thus, β-methyl aspartic acid causes both an increase in the rate of urea synthesis and in the rate of conversion of L-citrulline-6-^{14}C to ^{14}C-urea.

Discussion. It is evident from results presented above, as well as from results of the studies of Cedrangolo *et al.* (1, 2) and Severina (3), that both α-methyl aspartic acid and *threo-β*-methyl aspartic acid are effective inhibitors of *in vitro* urea synthesis. Our experiments have demonstrated this inhi-

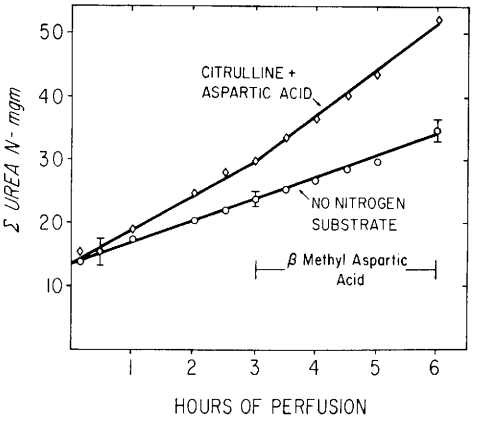

FIG. 2. Effect of β-methyl aspartic acid on urea biosynthesis by the isolated perfused rat liver. In the citrulline + aspartic acid experiment, citrulline (0.25 mmole/hr) and aspartic acid (0.25 mmole/hr) were infused at a constant rate throughout a 6-hr perfusion. In the other experiment no exogenous substrate was infused. After 3 hr of perfusion, 230 mg (1.6 mmoles) of β-methyl aspartic acid were added to the perfusate and a constant infusion of 77 mg (0.5 mmole)/hr was begun. Cumulative urea nitrogen was calculated from the concentration of urea determined in aliquots of perfusate taken at the times indicated. (citrulline + aspartic acid curve) represents the results of a single perfusion experiment; (no nitrogen substrate curve) represents the average results of two perfusion experiments. The range of values obtained at 30 min, 3 and 6 hr is indicated by vertical bars.

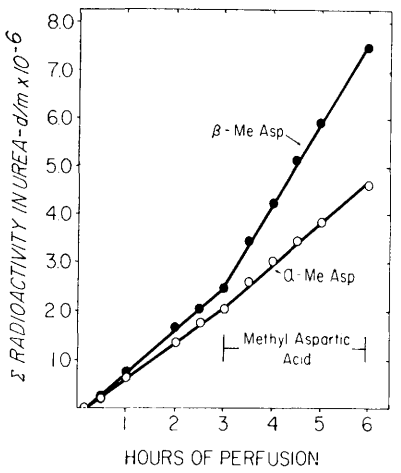

FIG. 3. Effect of DL-α-methyl aspartic acid and DL-*threo*-β-methyl aspartic acid on conversion of L-citrulline-6-^{14}C to urea by the isolated perfused rat liver. L-Citrulline-6-^{14}C was infused at a constant rate of 1.72 μCi/hr throughout a 6-hr perfusion. No exogenous substrate was infused. After 3 hr of perfusion, 230 mg (1.6 mmoles) of methyl aspartic acid were added to the perfusate and a constant infusion of 77 mg (0.5 mmole)/hr was begun. Incorporation of radioactivity into urea was calculated from the specific radioactivity of urea isolated from the perfusate (see Methods). The concentration of urea was determined in aliquots of perfusate. Each curve represents the results of a single perfused experiment.

bition in terms both of net urea synthesis and of conversion of L-citrulline-6-^{14}C to ^{14}C-urea. The observation that urea synthesis from glutamine is not inhibited while the conversion of labeled citrulline to urea is inhibited warrants confirmation and further investigation.

At a concentration of 20 m*M* in the perfusate, neither compound inhibits urea synthesis in the isolated perfused rat liver. This concentration is twice that required for inhibition in the homogenate experiments. α-Methyl aspartic acid has no effect on the rate of urea synthesis and no effect on the rate of conversion of citrulline to urea. β-Methyl aspartic acid causes an *increase* in the rate of urea synthesis which is paralleled by an increase in the rate of conversion of citrulline to urea.

These results indicating that the methyl aspartates do not inhibit urea synthesis in the perfused liver are in accord with the experiments of Cedrangolo *et al.* (1). In their experiments rats were given intraperitoneal injections of α-methyl aspartate at 8-hr intervals. No decrease in urea excretion over a 72-hr period was found, although *in vitro* urea synthesis by liver homogenates (and slices) prepared from these rats was strongly inhibited. It has been suggested that the failure of methyl aspartates to inhibit *in vivo* urea synthesis is due to a failure of these compounds to penetrate the liver cell (9). The results reported here are not inconsistent with this interpretation. The failure of the methyl aspartates to inhibit urea synthesis may be a result of a failure to obtain sufficiently high intracellular concentrations of inhibitors to affect urea synthesis. However, it is clear that β-methyl aspartate does penetrate the liver cell since it stimulates urea synthesis.

Summary. DL-α-Methyl aspartic acid and DL-*threo*-β-methyl aspartic acid have been shown to inhibit, *in vitro*, both urea synthesis and the conversion of L-citrulline-6-^{14}C to ^{14}C-urea. In the presence of glutamine as nitrogenous substrate, the conversion of citrulline to urea is inhibited while urea synthesis is not inhibited by these methyl aspartates. No inhibition of urea biosynthesis or of conversion of L-citrulline-6-^{14}C to ^{14}C-urea could be demonstrated in the isolated perfused rat liver using these inhibitors.

1. Cedrangolo, F., Della Pietra, G., De Lorenzo, F., Papa, S., and Cittadini, D., Enzymologia **25**, 308 (1962).

2. Cedrangolo, F., Della Pietra, G., Cittadini, D., Papa, S., and De Lorenzo, F., Nature (London) **195**, 708 (1962).

3. Severina, I. S., Biokhimiya **27**, 943 (1962).

4. Smith, L. H., J. Amer. Chem. Soc. **77**, 6691 (1955).

5. Cocker, W., and Lapworth, A., J. Chem. Soc., London **1931**, 1391.

6. Benoiton, L., Winitz, M., Colman, R. F., Birnbaum, S. M., and Greenstein, J. P., J. Amer. Chem. Soc. **81**, 1726 (1959).

7. Miller, L. L., Bly, C. G., Watson, M. L., and Bale, W. F., J. Exp. Med. **94**, 431 (1951).

8. Conway, E. J., "Microdiffusion Analysis and Volumetric Error," p. 162. MacMillan Co., New York (1958).

9. Sakami, W., and Harrington, H., Annu. Rev. Biochem. **32**, 355 (1963).

Received May 21, 1971. P.S.E.B.M., 1971, Vol. 138.