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EFFECT OF PROPIONATE AND PYRUVATE ON CITRULLINE SYNTHESIS AND ATP CONTENT IN RAT LIVER MITOCHONDRIA

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SUMMARY

Propionate inhibits citrullinogenesis when succinate (plus rotenone) or glutamate are the oxidizable substrates used. Propionate decreases the intramitochondrial concentration of carbamylphosphate by decreasing the ATP content. When the energy supply for citrullinogenesis is provided by an influx of exogenous ATP, propionate is no longer an inhibitor. Pyruvate inhibits citrullinogenesis with glutamate but not with succinate (plus rotenone) as oxidizable substrates. Propionate and pyruvate deplete mitochondrial ATP but probably by different mechanisms.

Hyperammonemia is frequently observed in propionyl-CoA carboxylase, methylmalonyl-CoA mutase and racemase deficiencies (1). In cases with neonatal onset, the ammonemia is constantly increased (unpublished results) reaching up to ten times the normal values. An accumulated metabolite has been implicated in the genesis of hyperammonemia : propionate inhibits ureogenesis in rat liver slices (2) and citrullinogenesis in isolated mitochondria from rat liver (3). Our work was aimed to clear how propionate might decrease citrullinogenesis. Two hypotheses were evaluated : (a) inhibition of the enzymes, carbamylphosphate synthetase (CPS) or ornithine carbamyltransferase (OCT) ; (b) decreased concentrations of either a substrate (ATP) or activator (acetyl glutamate) of citrullinogenesis.

MATERIALS AND METHODS

Male Wistar adult rats weighing 200 to 300 g were used in the experiments. Rats had free access to a standard food obtained from the Usine d'Alimentation Rationnelle Villemoisson France (AO_2 diet with 19 % of protein). Non fasting animals were killed by decapitation. Livers were immediately removed to prepare homogenates in 0.1 % cetyltrimethyl ammonium bromide (4), or mitochondria in 0.27 M sucrose (5). Most chemical reagents and the purified ornithine carbamyl transferase were obtained from Sigma Chemical Company. Hexokinase, glucose 6-phosphate dehydrogenase and NADP were obtained from Boehringer-Mannheim. All incubations were at 37°C in air. Protein determinations were made by the biuret method (6).

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Effect of propionate on kinetics of CPS and OCT

Kinetics of CPS (4) and OCT (7) were studied in rat liver homogenates. The final concentration of propionate used was 10 mM. Citrulline produced was determined according to a modified procedure (8) of Marsh's method (9).

Measurement of citrullinogenesis by isolated mitochondria

Freshly isolated mitochondria (about 5 mg of protein per assay) were incubated in 2 ml of reaction mixture containing 35 mM KCl, 50 mM Tris-HCl buffer pH 7.4, 1 mM EDTA, 1 mM $MgCl_2$, 10 mM ornithine, 10 mM NH_4Cl, 16.6 mM $KHCO_3$, 5 mM potassium phosphate, 6.75 mM sucrose. Then pH was adjusted to 7.4 and the mixture was bubbled by 95 % O_2, 5 % CO_2 before use. After 10 minutes, incubations were stopped by 1 ml of 1 M perchloric acid. The citrulline produced was assayed in the supernatant obtained by centrifugation at 11 000 g for 2 minutes.

Assays of mitochondrial substrates

Mitochondria (about 20 mg of protein per assay) were incubated in the same mixture as used for the measurement of citrullinogenesis but without ornithine to avoid any production of citrulline. The incubations were stopped by 0.3 M perchloric acid (final concentration) except for the measurement of the acetylglutamate-like substances when 0.3 M HCl was used to avoid the inhibition of carbamylphosphate synthetase by perchlorate (10). The mixtures were chilled at 4°C for 10 minutes then centrifuged 2 minutes at 11 000 g. The supernatants were neutralized at pH 7.0 with $KHCO_3$, then centrifuged at 4°C and the assays were made from the supernatant. A blank was made for each assay on a nonincubated sample.

1 - Carbamylphosphate assays were performed immediately after the pH of the extract was adjusted. The incubation mixtures contained : 200 mM triethanolamine buffer pH 7.7, 5 mM ornithine, 3 units of OCT, perchloric extract 150 µl in a final volume of 500 µl. After 20 minutes the reaction was stopped by 500 µl of 5 % (w/v) trichloracetic acid and the citrulline produced was assayed in the supernatant obtained by centrifugation at 11 000 g. Carbamylphosphate plots were linear from 1 to 100 nmoles.

2 - ATP assays were made on 100 or 200 µl of the perchloric acid extract by a spectrophotometric method (11).

3 - Acetylglutamate-like substances : the HCl mitochondrial extracts were incubated for 5 hours to destroy the carbamylphosphate produced during the incubation (half life of carbamylphosphate is 50 minutes at 37°C). Under these experimental conditions, the recovery of acetylglutamate was close to 100 %. The acetylglutamate-like substances were assayed by their activation of CPS. Extracts with CPS activity were obtained from sonicated mitochondria dialyzed free of acetylglutamate. Acetylglutamate-like substances were assayed in the following mixture : 0.3 units of CPS, endogenous OCT, 10 mM ATP, 10 mM ornithine, 10 mM $MgCl_2$, 50 mM NH_4HCO_3, 40 mM phosphate buffer pH 7.5 and 450 µl of HCl-mitochondrial extract in a final volume of 1 ml. A blank was prepared by omitting ornithine and was also incubated for 20 minutes A standard curve was obtained with acetylglutamate added in the place of HCl-mitochondrial extract.

RESULTS

Propionate does not modify the Km and Vm of CPS and OCT as determined in homogenates (results not shown). When isolated mitochondria were used, succinate with or without rotenone seems to be the most effective oxidizable substrate and in its presence citrulline production is about five-fold of that obtained without any substrate. Propionate is not effective as

Table I - Effect of propionate on citrullinogenesis

Oxidizable substrates	Number of experiments	With propionate 1 mM	5 mM	10 mM
None	1	80	60	39
Succinate + Rotenone	5	88 ± 6	70 ± 8	38 ± 7 (SD)
Glutamate	2	70 ; 80	53 ; 58	53 ; 54
Pyruvate	1	50	37	30

Results are expressed as a percentage of synthetized citrulline without propionate. Compounds were added to the mixture at the following levels succinate, glutamate, pyruvate, 10 mM ; rotenone 10 µg/ml.

Table II - Effect of propionate on the mitochondrial carbamylphosphate, ATP and acetylglutamate contents.

	Time of incubation (min.) 0	10	30
Without propionate			
Carbamylphosphate	0	26.1 ± 7.0 (5)	-
ATP	1 to 2	8.6 ± 2.5 (5)	-
Acetylglutamate	0.54 ± 0.10 (4)	0.47 ; 0.51 (2)	0.43 ± 0.07 (4)
With 10 mM propionate			
Carbamylphosphate	0	16.1 ± 4.9 (5)++	-
ATP	1 to 2	3.7 ± 1.6 (5)++	-
Acetylglutamate	0.44 ± 0.10 (4)+	0.47 ; 0.51 (2)	0.52 ± 0.14 (4)+

Mitochondria were incubated in an ornithine-free mixture (see Materials and Methods) containing 10 mM succinate plus 10 µg/ml rotenone. The amount of metabolites is expressed in nmoles/mg protein (mean ± SD), + non significant, ++ $p < 0.001$ (paired Student's test) ; number of experiments between brackets.

substrate and moreover propionate inhibits the citrullinogenesis with all oxidizable subtrates (table I). Propionate induces a significant decrease of carbamylphosphate and ATP levels in mitochondria, the acetylglutamate-like substances not being modified (table II). Thus propionate could decrease mitochondrial carbamylphosphate through the decrease of ATP. To test this hypo-

Table III - Effect of exogenous ATP on citrullinogenesis with or without propionate

Compounds added	Without ATP	With ATP
None	0.48 ; 0.65	0.66 ; 1.00
Propionate	0.19 ; 0.30	0.42 ; 0.70
Oligomycin	0.06 ; 0.08	0.37 ; 0.50
2,4-dinitrophenol	0.03 ; 0.05	0.12 ; 0.40
Oligomycin + 2,4-dinitrophenol	0.03 ; 0.05	1.46 ; 2.40
Oligomycin + 2,4-dinitrophenol + propionate	0.03 ; 0.05	1.25 ; 1.92

Compounds were added to the mixture at the following levels : propionate, 10 mM ; oligomycin, 10 µg/ml ; 2,4-dinitrophenol, 0.04 mM ; ATP, 4 mM. Results of 2 experiments are expressed in citrulline produced (µmoles/h/mg protein).

Table IV - Effect of pyruvate on mitochondrial carbamylphosphate and ATP

		Succinate plus Rotenone	Pyruvate
Carbamylphosphate	1	35.3	21.0
	2	25.9	13.3
	3	26.0	16.8
ATP	1	11.2	6.3
	2	8.2	5.2
	3	7.4	4.9

Mitochondria were incubated for 10 minutes in ornithine-free medium (see Materials and Methods). Compounds were added at the following levels : succinate, pyruvate, 10 mM ; rotenone, 10 µg/ml.

thesis, mitochondrial citrullinogenesis was studied when a mitochondrial influx of ATP is obtained by addition of oligomycin and 2,4-dinitrophenol (12). When mitochondria are incubated without ATP and without any oxidizable substrate, the citrullinogenesis is small and fully suppressed by addition of 10 µg/ml oligomycin plus 0.04 mM 2,4-dinitrophenol. Then the addition of 4 mM ATP induces a strong increase of the citrullinogenesis, this latter being exclusively linked with the ATP influx in mitochondria. Under these conditions propionate inhibits slightly : only 15 to 20 % of inhibition in the place of 70 % obtained with oxidizable substrates (table III).

The citrullinogenesis obtained with pyruvate is less than with succinate or glutamate (results not shown). Pyruvate inhibits the citrullinogenesis of mitochondria incubated with glutamate (40 % at 5 mM and 60 % at 10 mM) but it has no inhibitory effect when succinate plus rotenone take place of glutamate. Mitochondria incubated with 10 mM pyruvate for 10 minutes contain less carbamylphosphate and less ATP than those incubated with succinate plus rotenone (table IV).

DISCUSSION

In agreement with Glasgow and Chase (3) the present study demonstrates an inhibition of citrulline synthesis by propionate, thought an obvious inhibition is only obtained with high propionate concentrations (above 5 mM). Citrulline synthesis requires the production of ATP in mitochondria (13). Without added oxidizable substrate, citrullinogenesis is slight, amounts only 20 % of the maximum rate obtained with succinate and disappears when rotenone is added. It seems therefore to be linked with the NAD-linked oxidation of endogenous substrates. Propionate inhibits citrulline synthesis with or without exogenous substrates. This inhibition is related neither to a direct effect on CPS or OCT activities nor to a decrease of acetylglutamate-like substances. Propionate promotes a decrease of mitochondrial ATP resulting in a decrease of carbamylphosphate synthesis. In addition the lack of an inhibitory effect of propionate when there is an induced uptake of exogenous ATP also indicates that propionate inhibits citrullinogenesis through a depletion of ATP content in mitochondria. This effect of propionate on the ATP content reflects an interference with the energy metabolism. Propionate is not an oxidizable substrate used for the citrulline synthesis. Thus, the synthesis of succinate from propionate seems to be a slow mechanism probably because propionyl-CoA carboxylase and methylmalonyl-CoA mutase and racemase are not very active enzymes. However propionate may yield propionyl-CoA via acetyl-CoA synthetase using ATP and coenzyme A (14) and the ATP formed by the oxidation of reduced substrates would be, therefore, used for the formation of propionyl-CoA and would be not available for citrulline formation. Propionyl-CoA might also condense with oxaloacetate to form methylcitrate (15), a metabolite that would have his own effect on the mitochondrial ATP content.

Pyruvate decreases the ATP content of mitochondria probably by another mechanism, that is acetoacetate formation via the acetylCoA pathway, which is known to decrease both ureogenesis and ATP in isolated hepatocytes (16). Thus glutamate an inhibitor of pyruvate carboxylase (17-18) increases the pyruvate-induced inhibition of citrullinogenesis while this inhibition

does not occurs in presence of rotenone an inhibitor of pyruvate dehydrogenase.

A propionate-induced decrease of the ATP content in mitochondria is perhaps not the sole mechanism responsible of the hyperammonemia observed in human propionyl-CoA carboxylase deficiencies. In fact such severe hyperammonemia occur in the newborn only. This particular sensibility to propionate may be is due to an immature oxidative phosphorylation pathway as shown in neonatal rat liver mitochondria (19).

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