Int. J. Biochem., Vol. 13, pp. 495 to 499, 1981
Printed in Great Britain.

0020-711X/81/040495-05$02.00/0

FACTORS RESPONSIBLE FOR THE INHIBITORY EFFECT OF MALATE ON CITRULLINE FORMATION IN RAT LIVER MITOCHONDRIA

ALEKSANDRA NIEDZWIECKA and JADWIGA BRYŁA*
Institute of Biochemistry, University of Warsaw, Al. Żwirki i Wigury 93, 02-089 Warsaw, Poland

(*Received* 29 *September* 1980)

Abstract—1. The effect of malate, succinate and malonate on citrulline synthesis in rat liver mitochondria was studied.

2. When NH_4Cl was used as the source of ammonia, malate as well as succinate and malonate inhibited the citrulline formation by about 40–50% in mitochondria incubated with glutamate in State 4 as well as in uncoupled (+oligomycin) mitochondria with exogenous ATP as an energy source. The addition of either arginine to mitochondria incubated in State 4 or *N*-acetylglutamate to uncoupled mitochondria only slightly decreased the inhibition of citrulline formation by malate.

3. The inhibitory effect of malate on citrulline formation was not observed in both toluene-treated and sonicated mitochondria as well as on the addition of *n*-butylmalonate into the suspension of mitochondria.

4. When carbamoyl phosphate and ornithine were used as substrates for ornithine carbamoyltransferase the inhibitory effect of malate on citrulline production was observed.

5. Malate decreased significantly the ornithine uptake by mitochondria incubated in the absence and presence of citrulline or *N*-acetylglutamate.

6. The data presented indicate that with glutamate as substrate the inhibition of citrulline formation by malate may be not only due to a decline of *N*-acetylglutamate level as postulated by Meijer & Van Woerkom (1978) but also due to a decrease of mitochondrial ornithine uptake.

INTRODUCTION

It has been shown for some time that the capacity of rat liver mitochondria to synthesize citrulline is higher with glutamate than with succinate as the respiratory substrate (Glasgow & Chase, 1976; Hensgens *et al.*, 1980). According to Meijer & Van Woerkom (1978) and Hensgens *et al.* (1980) the higher rate of citrulline formation in the presence of glutamate is presumably caused by an increased level of intramitochondrial *N*-acetylglutamate, an essential cofactor of the carbamoyl phosphate synthase (EC 2.7.2.5, Grisolia & Cohen, 1953). The inhibitory effect of malate on citrulline formation in mitochondria incubated with glutamate was attributed to a decrease of mitochondrial *N*-acetylglutamate content probably due to removal of acetyl-CoA under these conditions (Meijer & Van Woerkom, 1978). Since it was reported (Glasgow & Chase, 1976) that malonate may also inhibit the rate of citrulline formation in the presence of glutamate we have decided to reinvestigate the effect of dicarboxylates on citrulline synthesis.

The results presented in this communication indicate that the inhibitory effect of malate and presumably of other dicarboxylates (succinate, malonate) on citrulline production might be not only due to a decline of mitochondrial *N*-acetylglutamate level, as postulated by Meijer & Van Woerkom (1978), but also due to a decrease of mitochondrial ornithine uptake.

* To whom all correspondence should be addressed.

MATERIAL AND METHODS

Mitochondrial preparations

Liver mitochondria were isolated from the fed male Wistar rats (200–250 g in weight) as described by Harris *et al.* (1971) but the final wash and suspension of mitochondria were made with 0.3 M mannitol replacing sucrose. Treatment of isolated mitochondria with toluene was done according to Matlib *et al.* (1977). Sonication of mitochondrial suspension (approx 20 mg of protein/ml at 0.1 M Tris–HCl buffer pH 7.4) was carried out for 3 successive periods of 30 sec at 20 kHz using MSE ultrasonic desintegrator.

Measurement of citrulline synthesis

Mitochondria (about 4 mg protein/ml) were incubated at 30°C under 95% O_2 + 5% CO_2 in a basic medium containing: 15 mM KCl, 2 mM EGTA, 50 mM Tris–HCl pH 7.4, 5 mM phosphate buffer (pH 7.4), 10 mM glutamate, 0.2 mM aminooxyacetate, 30 mM $KHCO_3$, 10 mM NH_4Cl and 10 mM ornithine. The final protein concentration was 4–5 mg/ml. The reactions were started by the addition of mitochondrial suspension to the reaction mixture. At indicated periods of incubation 1 ml samples were withdrawn from the suspension, deproteinized with 1/10 volume of 35% $HClO_4$ (v/v) and centrifuged. Citrulline was determined in deproteinized samples according to Archibald (1944) as modified by Bryła & Harris (1976).

Measurement of mitochondrial ornithine uptake

Uptake of ornithine was measured using the silicone layer technique (Harris & Van Dam, 1968) as described by Bryła & Harris (1976), L-1-[^{14}C]ornithine monochloride was applied.

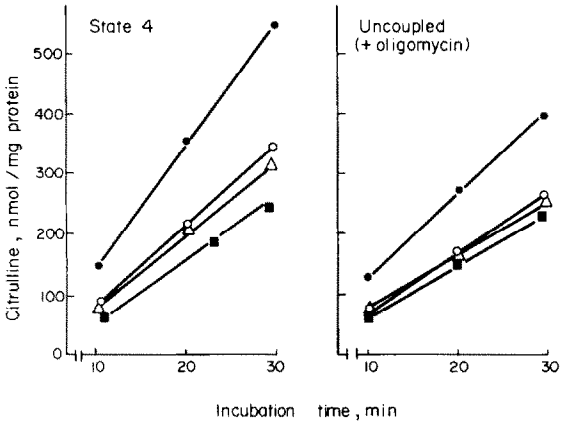

Fig. 1. Effect of dicarboxylic acids on citrulline synthesis in mitochondria incubated in State 4 and uncoupled (+oligomycin) conditions. Mitochondria (4 mg/ml) were incubated in the standard medium in either State 4 or in the presence of 2 μM FCCP, oligomycin (1 μg/mg protein), 4 μM rotenone and 4 mM ATP. Malate (■), malonate (△) and succinate (○) were added at 10 mM concentrations.

Protein

Mitochondrial protein was measured by a biuret reaction as described by Cleland & Slater (1953), with bovine serum albumin as standard.

Chemicals

N-Acetylglutamic acid was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Aminooxyacetate was from Eastman Kodak Co. (Rochester, NY, U.S.A.). L-1-[^{14}C]-Ornithine monochloride (50 mCi/mmol) was from Amersham, England. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was a generous gift of Dr P. Heytler while rotenone was kindly provided by Dr J. L. Howland. All other chemicals were of analytical grade.

RESULTS AND DISCUSSION

Isolated liver mitochondria synthesize citrulline when incubated with ornithine, bicarbonate, NH_4Cl and a source of ATP (Charles *et al.*, 1967). Figure 1 shows that the rate of citrulline synthesis is higher in mitochondria incubated with glutamate in State 4 than in uncoupled (+oligomycin) mitochondria when exogenous ATP is used as an energy source. This observation is in agreement with the results obtained by McGivan *et al.* (1976). According to these authors the rate of citrulline synthesis in the presence of exogenous ATP is lower than when ATP is generated by oxidative phosphorylation. The higher rate of citrulline synthesis with glutamate as respiratory substrate in comparison with that measured in uncoupled mitochondria can also be caused by an increased synthesis of *N*-acetylglutamate in the presence of glutamate (Meijer & Van Woerkom, 1978; Hensgens *et al.*, 1980).

With saturating concentrations of substrates it is in principle possible for the synthesis of citrulline from NH_4Cl to be rate limited either by the activity of carbamoyl phosphate synthase and of ornithine carbamoyltransferase or by the transport of both ornithine and citrulline across the mitochondrial membrane (McGivan *et al.*, 1976). In agreement with Meijer & Van Woerkom (1978) malate results in about 50% decrease of the rate of citrulline formation in mitochondria incubated in State 4 (Fig. 1). The inhibitory effect of malate on this process is also observed in uncoupled (+oligomycin) conditions when exogenous ATP is used as an energy source. Moreover, other dicarboxylates such as malonate or succinate inhibit also the citrulline formation by about 40% under both conditions studied.

As shown in Table 1 a significant inhibitory effect of malate on citrulline synthesis was observed at as low as 1 mM malate concentration. This phenomenon may have a physiological significance since the cytosolic malate level may vary from 0.03 to 2.59 mM depending upon metabolic conditions (Siess *et al.*, 1976).

Since according to Meijer & Van Woerkom (1978) the inhibition of citrulline production by malate is due to removal of acetyl-CoA required for *N*-acetylglutamate synthesis, we have checked the effect of malate on citrulline formation in the presence of *N*-acetylglutamate known to penetrate into uncoupled mitochondria (Meijer & Van Woerkom, 1979). As is shown in Table 2, *N*-acetylglutamate stimulates the citrulline synthesis by about 30%. However, on the addition of either malate or malonate into the reaction medium the rate of citrulline formation is by about 30% lower than that measured with *N*-acetylglutamate alone. Furthermore, when mitochondria are incubated in State 4 in the presence of arginine, an activator of *N*-acetylglutamate synthase (EC 2.3.1.1, Shigesada & Tatibana, 1971; Shigesada & Tatibana,

Table 1. Effect of malate concentrations on citrulline synthesis in rat liver mitochondria

Malate concentration (mM)	Citrulline synthesis nmol/mg protein	%
0	213	100
1	164	77
5	122	57
10	65	31

Mitochondria (3.4 mg protein/ml) were incubated in State 4 for 15 min in a basic medium containing 2.5 mM phosphate.

Table 2. Effect of malate and malonate on the citrulline formation in the absence and presence of *N*-acetylglutamate or arginine

Conditions	Additions	Citrulline synthesis (nmol/mg protein) Control	+ Malate	+ Malonate
Uncoupled	None	250 ± 20 (4)	158 ± 18 (4)*	162 ± 20 (4)*
(+ oligomycin)	10 mM *N*-acetylglutamate	364 ± 20 (3)	281 ± 21 (3)†	234 ± 21 (3)*
State 4	None	338 ± 29 (7)	142 ± 17 (6)*	160 ± 9 (4)*
	1 mM arginine	441 ± 22 (3)	264 ± 22 (3)*	—

Mitochondria (about 4 mg/ml) were incubated for 20 min in a basic medium in either State 4 or in the presence of 2 μM FCCP, oligomycin (1 μg/mg protein), 4 μM rotenone and 4 mM ATP. Malate and malonate were added at 10 mM concentrations, where indicated. Data shown are means ± SEM for number of experiments indicated in parentheses. *P* (against corresponding controls): * $P < 0.01$, † $P < 0.05$.

1978), the addition of malate results in an inhibition of the rate of citrulline synthesis by about 40%, i.e. for about 10–20% less than in the absence of arginine. On the basis of these observations it seems that a decrease of the intramitochondrial *N*-acetylglutamate concentration, is not the only factor limiting the rate of citrulline synthesis on the addition of dicarboxylic acids into the reaction medium. The following observations throw some light on the nature of the inhibitory effect of malate on citrulline formation: (i) As shown in Fig. 2 the addition of malate results in a decrease of citrulline production, when carbamoyl phosphate and ornithine are used as substrates for ornithine carbamoyl-transferase (EC 2.1.3.3, Natale & Tremblay, 1974; McGivan *et al.*, 1976); (ii) An inhibition of citrulline synthesis by either malate or malonate is abolished by *n*-butylmalonate (Table 3), an inhibitor of mitochondrial dicarboxylate carrier (Robinson & Chappell, 1967) and (iii) In both sonicated- and toluene-treated mitochondria an inhibition of citrulline formation by malate does not occur (Table 4). In view of these data it seems likely that malate might affect the transport of ornithine into mitochondria. In order to check this possibility we have investigated the effect of malate on ornithine uptake by mitochondria. Studies were carried out under conditions ensuring neither ornithine conversion to citrulline (Bryła & Harris, 1976) nor its transamination (McGivan *et al.*, 1977). The data presented in Table 5 show that malate lowers the mitochondrial ornithine accumulation (Experiment 1). Although in agreement with Bryła & Harris (1976) citrulline increases the mitochondria ornithine uptake, the inhibitory effect of malate on [^{14}C]ornithine accumulation is still observed (Experiment 2). Similarly, an enhanced ornithine uptake on the addition of *N*-acetylglutamate is abolished in the presence of malate (Experiment 3).

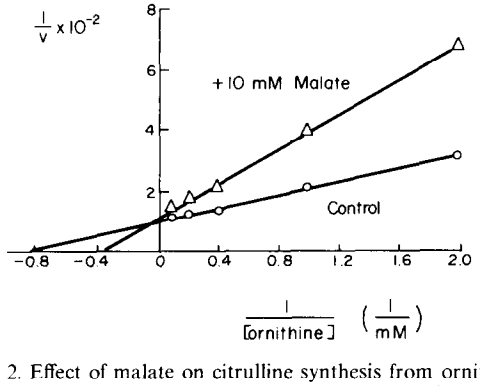

Fig. 2. Effect of malate on citrulline synthesis from ornithine and carbamoyl phosphate. Lineweaver–Burk plot. Mitochondria (5 mg/ml) were incubated in ammonia-, glutamate- and bicarbonate-free basic medium containing corresponding amounts of Tris–HCl, 20 μM rotenone, 10 mM carbamoyl phosphate and various concentrations of ornithine. The rate of citrulline synthesis was obtained from analyses of samples withdrawn after 1, 2 and 3 min of incubation. In the presence of 10 mM ornithine the rate of citrulline formation (v) was equal to 86 nmol × min^{-1} × mg^{-1} of protein.

Table 3. Effect of malate and malonate on citrulline formation in the absence and presence of *n*-butylmalonate

	Additions	Citrulline synthesis (nmol/mg protein) −*n*Butylmalonate	+*n*-Butylmalonate
Experiment 1	None	408	438
	5 mM Malate	222	470
	5 mM Malonate	270	448
Experiment 2	None	433	458
	5 mM Malate	268	443
	5 mM Malonate	246	—

Mitochondria (4 mg protein/ml) were incubated for 20 min in the basic medium in State 4 in either the absence or presence of 25 mM *n*-butylmalonate.

Table 4. Effect of malate on citrulline synthesis in intact, sonicated and toluene-treated mitochondria

	Mitochondria	Citrulline synthesis (nmol/mg protein) −Malate	+Malate
Experiment 1	Intact	257	150
	Sonicated	386	398
	Toluene-treated	458	430
Experiment 2	Intact	280	144
	Sonicated	388	360
Experiment 3	Intact	229	104
	Toluene-treated	485	462

Intact, sonicated and toluene-treated mitochondria were incubated for 20 min in the basic medium. Intact mitochondria (about 3 mg protein/ml) were incubated in State 4, while both sonicated and toluene-treated mitochondria (about 2 mg protein/ml) were incubated in the presence of 10 μg oligomycin, 0.45 mM NAD^+, 4 mM ATP and 10 mM *N*-acetylglutamate. Malate was added at 10 mM concentration, where indicated.

Table 5. Effect of malate on the uptake of [^{14}C]ornithine by rat liver mitochondria in the presence of various phosphate concentrations

	Additions	[^{14}C]Ornithine uptake (nmol/mg protein)
Experiment 1	None	0.39
	+10 mM Malate	0.31
Experiment 2	None	0.40
	+1 mM Citrulline	0.63
	+10 mM Malate	0.51
Experiment 3	None	0.30
	+10 mM *N*-acetylglutamate	0.45
	+10 mM Malate	0.29

The system for Experiments 1 and 2 consisted of mitochondria (3 mg protein/ml) incubated in the ammonia-, glutamate- and bicarbonate-free basic medium containing 3% dextran, oligomycin (1 μg/mg protein), 10 mM glucose, 0.1 mM ADP and 2 units of hexokinase. 0.2 mM [^{14}C]-ornithine was added after 1 min of preincubation. In experiment 3 mitochondria were preincubated for 1 min in the presence of 20 μM rotenone and oligomycin (1 μg/mg protein) followed by the addition of 1.5 μM FCCP. Time of incubation was 2 min. Other additions to the incubation medium as indicated.

On the basis of data presented the inhibition of citrulline formation by malate may be explained not only by a decline of mitochondrial *N*-acetylglutamate content resulting in a decrease of carbamoyl phosphate synthase activity (Meijer & Van Woerkom, 1978) but also by a diminished ornithine accumulation which may limit the ornithine carbamoyltransferase activity. The latter suggestion is likely in view of the facts that: (i) Ornithine carbamoyltransferase has a high K_m for ornithine—about 1.4 mM according to Reijchard (1957) and 1.8 mM according to Raijman (1974); (ii) Under conditions of urea formation the ornithine content in rat hepatocytes is below 0.3 mM (Meijer *et al.*, 1978) and (iii) The activity of ornithine aminotransferase, which in liver mitochondria may compete for ornithine with ornithine carbamoyltransferase under physiological ornithine concentrations is rate-limited by the transport of ornithine across the mitochondrial membrane (McGivan *et al.*, 1977).

According to Meijer *et al.* (1978) an increase of urea formation from 25 to 193 μmol/g dry wt per hr on the addition of NH_4Cl to the suspension of rat hepatocytes incubated with lactate and oleate is accompanied by a marked decrease of the cytosolic malate concentration (from 2.07 to 0.29 mM). Thus, the inhibitory effect of malate on the mitochondrial ornithine accumulation may have physiological implications.

SUMMARY

The effect of malate, malonate and succinate on citrulline synthesis was studied in rat liver mitochondria incubated under different metabolic conditions. The addition of these dicarboxylic acids to mitochondria incubated in the presence of glutamate in both State 4 as well as in uncoupled (+oligomycin) mitochondria with exogenous ATP as an energy source inhibited the citrulline formation by about 50%. On the addition of either *N*-acetylglutamate or arginine the inhibitory effect of malate and malonate was only slightly decreased, indicating that a decrease of the intramitochondrial *N*-acetylglutamate concentration is not the only factor limiting the rate of citrulline synthesis in the presence of dicarboxylic acids. When carbamoyl phosphate and ornithine were used as substrates for mitochondrial citrulline synthesis a decrease of citrulline production by malate was still observed. The inhibitory effect of malate on citrulline formation did not appear in both sonicated- and toluene-treated mitochondria as well as after addition of *n*-butylmalonate into the reaction medium. These observations together with a diminished ornithine accumulation in mitochondria incubated in the presence of malate suggest that the inhibition of citrulline formation by malate may be due to not only a decreased *N*-acetylglutamate level in mitochondria, as postulated by Meijer & Van Woerkom (1978), but also due to a diminished ornithine uptake which may limit the activity of ornithine carbamoyltransferase.

Acknowledgements—This investigation was supported in part by the grant of the Polish Academy of Sciences (MR II.1.1.4). The participation of Mrs T. Surala in some experiments is acknowledged.

REFERENCES

ARCHIBALD R. M. (1944) Determination of citrulline and allantoin and demonstration of citrulline in blood plasma. *J. biol. Chem.* **156**, 121–142.

BRYŁA J. & HARRIS E. J. (1976) Accumulation of ornithine and citrulline in rat liver mitochondria in relation to citrulline formation. *FEBS Lett.* **72**, 331–336.

CHARLES R., TAGER J. M. & SLATER E. C. (1967) Citrulline synthesis in rat liver mitochondria. *Biochim. biophys. Acta* **131**, 29–41.

CLELAND K. W. & SLATER E. C. (1953) Respiratory granules of heart muscle. *Biochem. J.* **53**, 547–556.

GLASGOW A. M. & CHASE H. P. (1976) Effect of pent-4-enoic acid, propionic acid and other short-chain fatty acids on citrulline synthesis in rat liver mitochondria. *Biochem. J.* **156**, 301–307.

GRISOLIA S. & COHEN P. P. (1953) Catalytic role of glutamate derivatives in citrulline biosynthesis. *J. biol. Chem.* **204**, 753–757.

HARRIS E. J., TATE C., MANGER J. R. & BANGHAM J. A. (1971) The effect of colloids on the appearance and substrate permeability of rat liver mitochondria. *J. Bioenergetics* **2**, 221–232.

HARRIS E. J. & VAN DAM K. (1968) Changes in total water and sucrose space accompanying induced ion uptake or phosphate swelling of rat liver mitochondria. *Biochem. J.* **106**, 759–766.

HENSGENS H. E. S. J., VERHOEVEN A. J. & MEIJER A. J. (1980) The relationship between intramitochondrial *N*-acetylglutamate and activity of carbamoyl-phosphate synthetase (ammonia). *Eur. J. Biochem.* **107**, 197–205.

MATLIB M. A., SHANNON W. A. & SRERE P. A. (1977) Measurement of matrix enzyme activity in isolated mitochondria made permeable with toluene. *Archs Biochem. Biophys.* **178**, 396–407.

MCGIVAN J. D., BRADFORD N. M. & MENDES-MOURAO J. (1976) The regulation of carbamoyl phosphate synthase activity in rat liver mitochondria. *Biochem. J.* **154**, 415–421.

McGivan J. D., Bradford N. M. & Beavis A. D. (1977) Factors influencing the activity of ornithine aminotransferase in isolated rat liver mitochondria. *Biochem. J.* **162**, 147–156.

Meijer A. J., Gimpel J. A., Deleeuw G., Tischler M. E., Tager J. M. & Williamson J. R. (1978) Interrelationship between gluconeogenesis and ureogenesis in isolated hepatocytes *J. biol. Chem.* **253**, 2308–2320.

Meijer A. J. & Van Woerkom G. M. (1978) Control of the rate of citrulline synthesis by short-term changes in *N*-acetylglutamate levels in isolated rat-liver mitochondria. *FEBS Lett.* **86**, 117–121.

Meijer A. J. & Van Woerkom G. M. (1979) Transport of *N*-acetylglutamate across the liver mitochondrial membrane. In *Function and Molecular Aspects of Biomembrane Transport* (Edited by Quagliariello E., Palmieri F., Papa S. & Klingenberg M.), pp. 365–368. North-Holland/Biomedical Press, Amsterdam.

Natale J. P. & Tremblay G. C. (1974) Studies on the availability of intramitochondrial carbamoyl phosphate for utilization in extramitochondrial reactions in rat liver. *Archs Biochem. Biophys.* **162**, 357–368.

Raijman L. (1974) Citrulline synthesis in rat tissues and liver content of carbamoyl phosphate and ornithine. *Biochem. J.* **138**, 225–232.

Reichard P. (1957) Ornithine carbamyl transferase from rat liver. *Acta chem. scand.* **11**, 523–527.

Robinson B. H. & Chappell J. B. (1967) The inhibition of malate, tricarboxylate and oxoglutarate entry into mitochondria by 2-*n*-butylmalonate. *Biochem. biophys. Res. Commun.* **28**, 249–255.

Shigesada K. & Tatibana M. (1971) Enzymatic synthesis of *N*-acetylglutamate by mammalian liver preparations and its stimulation by arginine. *Biochem. biophys. Res. Commun.* **44**, 1117–1124.

Shigesada K. & Tatibana M. (1978) *N*-Acetylglutamate synthase from rat liver mitochondria. Partial purification and catalytic properties. *Eur. J. Biochem.* **84**, 285–291.

Siess E. A., Brocks D. G. & Wieland O. H. (1976) Subcellular distribution of key metabolites in isolated liver cells from fasted rats. *FEBS Lett.* **69**, 265–271.