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INHIBITION BY Mn^{2+} OF CITRATE SUPPORTED PROGESTERONE BIOSYNTHESIS IN MITOCHONDRIAL FRACTIONS OF HUMAN TERM PLACENTAE

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SUMMARY

Mitochondrial fraction prepared from human term placentae were incubated with [^{14}C]-cholesterol in the presence of citrate, *cis*-aconitate or isocitrate. A stimulatory effect of these metabolites on cholesterol to progesterone conversion was found. In contrast to the other metabolites tested, citrate supported progesterone biosynthesis was found to be inhibited by manganese ions. This effect occurred in intact mitochondria only and was accompanied by a decreased citrate consumption.

INTRODUCTION

Manganese is known to be important in the reproductive system of mammalians. Animals kept on a diet deprived of manganese lose their libido and fecundity due to impaired function of ovary and testis [1, 2]. The manganese levels in endometrium and plasma of normal women undergo cyclic changes during menstrual cycle, with a rise of manganese concentration during the proliferative phase [3]. However the specific role for manganese in these processes has not yet been elucidated.

Recent observations in our laboratory [4–6] indicate that progesterone biosynthesis in the human placental mitochondrial fraction is stimulated by malate, citrate and isocitrate. The stimulatory effect of malate and its precursors was increased by the presence of Mn^{2+}, due to the activatory role of Mn^{2+} for mitochondrial $NADP^+$-linked malic enzyme. Results presented here show an inhibitory effect of this ion on the citrate-supported cholesterol to progesterone conversion. Although our findings are concerned with progesterone biosynthesis in placental mitochondria, a more general role of this ion in citrate metabolism is discussed.

EXPERIMENTAL

Chemicals

The following were obtained from Boehringer Corp., London: NAD^+, NADH, $NADP^+$, NADPH and citrate lyase. Citrate, *cis*-aconitate, DL-isocitrate, malate and malate dehydrogenase were supplied by Sigma Chem. Co., and pregnenolone and progesterone by Koch–Light Laboratory. [4-^{14}C]-Cholesterol (S.A.: 58 mCi/mmol), [^{3}H]-progesterone (S.A.: 255 mCi/mmol), [^{3}H]-pregnenolone (S.A.: 6·9 Ci/mmol) were obtained from Radiochemical Centre, Amersham. All other chemicals used were analytical grade products of Polish origin.

Preparation of placental mitochondria. Mitochondrial fraction from human term placenta was prepared as described previously [4]. The 105,000 *g* supernatant of sonicated placental mitochondria was prepared by suspending the mitochondrial fraction (30 mg protein per ml) in 2 vol. of cold redistilled water and subjecting it to ultrasonication at 20 KHz (MSE-Ultrasonic Disintegrator) for 4 × 30 s with 1 min intervals for cooling. The sonicated mitochondria were centrifuged at 10,000 *g* for 20 min, and the supernatant obtained was centrifuged at 105,000 *g* for 45 min.

Incubation. Incubation was carried out at 37°C in air with constant shaking for 1 h. Incubation mixture contained: 20 mM potassium phosphate buffer pH 7·4, 10 mM magnesium sulphate, 1 mM $NADP^+$ and 15 mg mitochondrial protein suspended in 0·154 M KCl or 3 mg protein of the 105,000 *g* supernatant of sonicated mitochondria, and appropriate amounts of Krebs cycle metabolites. Total volume of the incubation mixture was 2·5 ml. Incubation was initiated by the addition of 0·15 μCi [^{14}C]-cholesterol and terminated by freezing.

Isolation of steroids. To isolate ^{14}C labelled steroids the incubation mixtures were transferred to conical tubes containing known amounts of [^{3}H]progesterone and/or [^{3}H]pregnenolone. Nonradioactive progesterone (2 mg) and pregnenolone (2 mg) were also added as "cold" carriers. The contents of tubes were extracted twice with 10 ml of ether (to extract progesterone) or once with 10 ml of ether and 10 ml of methylene chloride (to extract pregnenolone). The organic and aqueous phases were separated by centrifugation and the organic phase was evaporated to dryness. The dry residue was subjected to thin-layer

chromatography on Silica gel G (Merck) impregnated with Rhodamine 6 G. On developing the chromatogram in (i) methylene chloride–ether (5:2, v/v), three fractions were obtained: progesterone, pregnenolone and cholesterol. Progesterone and pregnenolone fractions were further purified by thin-layer chromatography in the following systems: (ii) benzene–ethyl acetate (3:2 v/v) (iii) benzene–ethanol (9:1, v/v). Purification was continued to the constant $^{14}C/^{3}H$ ratio in the end products.

Radioactivity measurements and calculations. The radioactivity of isolated steroids was measured using Nuclear Chicago Isocap 300 liquid scintillation spectrometer with an efficiency of 40% for ^{3}H and 60% for ^{14}C. Total incorporation of ^{14}C into [^{14}C]-pregnenolone and [^{14}C]-progesterone was found on the basis of tritium found in the final product of purification procedure.

Citrate determination was performed according to Williamson and Corkey [7].

Aconitase activity was measured in the 105,000 *g* supernatant of sonicated placental mitochondria using *cis*-aconitate as substrate according to the method described by Fansler and Lowenstein [8]. Reduction of $NADP^{+}$ in the presence of citrate in 105,000 *g* supernatant of sonicated mitochondria was measured by scanning continuously the absorbancy at 340 nm.

Oxygen consumption was determined in a Warburg apparatus (20 mg of mitochondrial protein was used).

Protein was determined by the biuret method [9].

RESULTS

The effect of manganese ion on [^{14}C]-progesterone biosynthesis from [^{14}C]-cholesterol in the placental mitochondrial fraction is shown in Table 1. Only radioactive progesterone was formed during the incubation in the presence of every metabolite used whereas no [^{14}C]-pregnenolone was detected. Conversion of cholesterol to progesterone supported by citrate was inhibited by manganese ion by about 70%. This inhibition was not observed when either *cis*-aconitate or DL-isocitrate supported progesterone biosynthesis was measured. When malate was used as source of reductive equivalents, manganese ion caused over 100% stimulation of progesterone biosynthesis. Manganese ion was without effect on the conversion of [^{14}C]-cholesterol to [^{14}C]-progesterone in the presence of NADPH. Addition of Mn^{2+} to the incubation mixture, which contained NADPH and citrate, caused an inhibition which seems to be due to the inhibition of the citrate supported progesterone biosynthesis only.

Table 1. Effect of Mn^{2+} on malate, citrate, *cis*-aconitate, DL-isocitrate and NADPH supported progesterone biosynthesis in placental mitochondria

Additions	Progesterone biosynthesis (d.p.m. ^{14}C per flask)	
	Without Mn^{2+}	With Mn^{2+}
None, control	0	0
Malate, 10 mM	3560 ± 540	7300 ± 780
Citrate, 10 mM	18200 ± 1760	5520 ± 990
cis-Aconitate, 10 mM	19200 ± 1240	18940 ± 1360
DL-Isocitrate, 20 mM	16900 ± 2400	17130 ± 1960
NADH, 1 mM	0	0
NADPH, 1 mM	10350 ± 2040	9060 ± 1980
NADPH, 1 mM + citrate, 10 mM	26470 ± 3010	14140 ± 910

The incubations were carried out with 20 mg mitochondrial protein in 2·5 ml medium pH 7·4 containing 0·15 μCi of [4-^{14}C]-cholesterol, 20 mM potassium phosphate, 10 mM $MgSO_4$, 1 mM $NADP^{+}$ except experiments in which effect of NADPH was investigated, and where indicated 1 mM $MnSO_4$. Incubations were carried out for 1 h at 37°C.

Values are the means from three experiments ±S.D.

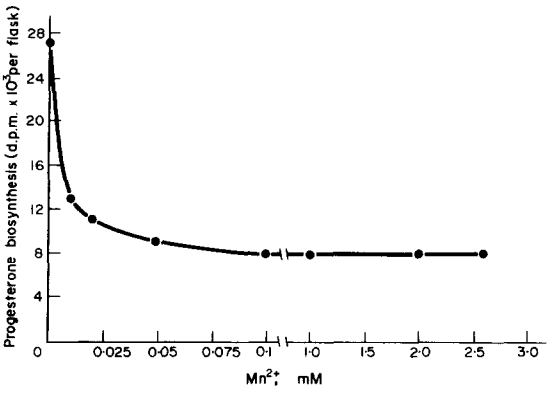

Fig. 1. Inhibitory effect of manganese on citrate supported progesterone biosynthesis in placental mitochondria. Conditions were as in Table 1, except that various concentrations of Mn^{2+} were used.

Figure 1 shows the effect of different Mn^{2+} concentrations in the presence of 10 mM citrate on progesterone biosynthesis in placental mitochondria. The inhibitory effect increases with the rise of manganese ion concentration up to 0·05 mM. Higher con-

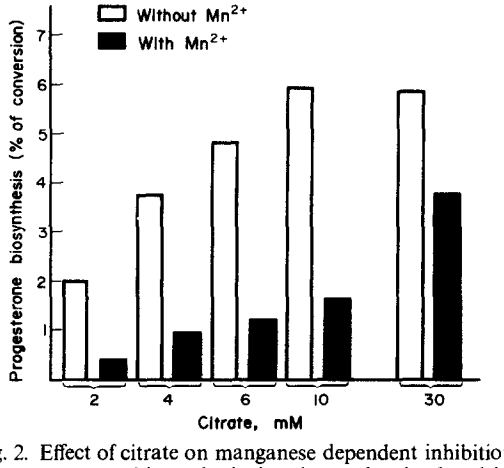

Fig. 2. Effect of citrate on manganese dependent inhibition of progesterone biosynthesis in placental mitochondria. Conditions were as in Table 1, except that various concentrations of citrate were used.

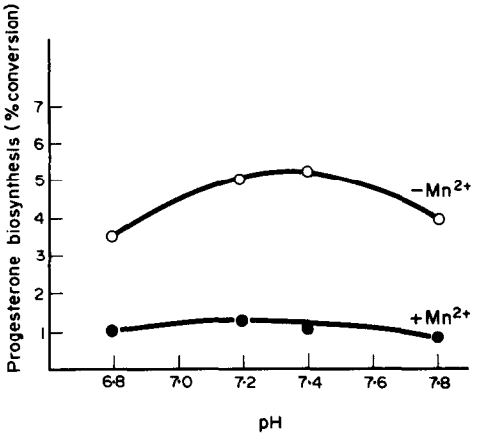

Fig. 3. Effect of $[H^+]$ concentration on citrate supported progesterone biosynthesis in placental mitochondria. Conditions were as in Table 1, except that phosphate buffer of various pH was used.

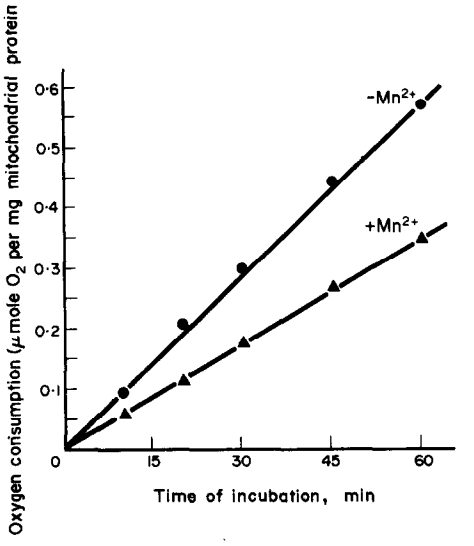

Fig. 4. Effect of Mn^{2+} on citrate oxidation in placental mitochondria. Conditions were as in Table 2.

centrations did not essentially alter the degree of the inhibition. No complete inhibition was observed with any of the concentrations used. The increase of citrate concentration (Fig. 2) did not reverse the degree of inhibition, a partial diminution of the inhibitory effect being noted when 30 mM citrate was used. There were no significant changes of degree of inhibition by manganese ions in experiments carried out at different pH (Fig. 3). Addition of 0·5 mM malate to the incubation medium did not alter the degree of inhibition and was without effect on citrate-supported progesterone biosynthesis.

To determine whether manganese exerts its inhibitory effect on the citrate metabolism or on some other part of the citrate dependent progesterone biosynthesis, the effect of Mn^{2+} on citrate levels and oxygen consumption by placental mitochondria was measured. In Table 2 data from three experiments are presented. One mM Mn^{2+} caused an inhibition of citrate and oxygen utilization by placental mitochondria and a more pronounced effect on progesterone biosynthesis in every case. Inhibition of citrate oxidation by placental mitochondria is presented in Fig. 4. The inhibitory effect of Mn^{2+} occurred from the first minutes of incubation and did not change with the time.

In contrast Mn^{2+} had no inhibitory effect on citrate-supported pregnenolone and progesterone biosynthesis in the supernatant of sonicated mitochondria. The decrease of citrate concentration (data not shown) and the increase of manganese ion concentration (Table 3), did not change the effect of manganese ion on steroid biosynthesis.

Figure 5 shows the effect of different Mn^{2+} concentrations on the aconitase activity in 105,000 *g* supernatant of sonicated mitochondria. Non-competitive inhibition by manganese using *cis*-aconitate as substrate was observed. A K_i value of about 4 mM was found. No inhibitory effect of Mn^{2+} was detected in $NADP^+$ reduction in the presence of 10 mM citrate with the supernatant of sonicated mitochondria (Table 4).

DISCUSSION

Mitochondria from human term placentae possess an electron transporting chain involved in cholesterol

Table 2. Effect of Mn^{2+} on oxygen and citrate utilization accompanying progesterone biosynthesis in the placental mitochondrial fraction

Expt	Mn^{2+} (1 mM)	Uptake (μmol)		Progesterone biosynthesis (d.p.m. ^{14}C)		
		Oxygen	Citrate	Per flask	Per μmol O_2	Per μmol of citrate
1.	–	7·5	15	18220	2430	1210
	+	6·3	10	5480	870	550
2.	–	10·0	16	26100	2610	1630
	+	6·4	11	14980	2340	1360
3.	–	7·2	8	18500	2570	2310
	+	3·6	4	6010	1670	1500

Conditions were as in Table 1. 10 mM citrate was used, citrate and oxygen utilization was measured as described in the Experimental.

Table 3. Citrate (10 mM) supported pregnenolone and progesterone biosynthesis in the supernatant (105,000 *g*) of sonicated placental mitochondria in the absence and in the presence of manganese ion. Effect of manganese concentrations

Manganese (mM)	Pregnenolone biosynthesis (d.p.m. ^{14}C per flask)	Progesterone biosynthesis (d.p.m. ^{14}C per flask)
0	11540 ± 1200	3280 ± 420
2	17760 ± 2230	4580 ± 380
4	14080 ± 970	3210 ± 350
8	13320 ± 1120	4730 ± 430
10	15850 ± 1430	5340 ± 490

Experimental conditions as in Table 1, except that 3 mg of supernatant (105,000 *g*) of sonicated mitochondria instead mitochondrial fraction was used. Manganese concentration was as indicated in the Table.

Values are the means from three experiments ±S.D.

side-chain cleavage. NADPH is a highly specific electron donor in this system [10, 11]. The mitochondrial fraction used in the present experiments is not able to utilize NADH as electron donor in the conversion of cholesterol to progesterone (Table 1). Stimulatory effect of Krebs cycle metabolites on the side-chain cleavage reaction results from mitochondrial $NADP^+$-malic enzyme activity in the case of malate, and mitochondrial $NADP^+$-isocitric dehydrogenase in the case of isocitrate, *cis*-aconitate and citrate [6].

Stimulation of progesterone biosynthesis from cholesterol by citrate, shown in the present experiments, requires several steps in citrate to isocitrate metabolism (i) translocation of citrate across mitochondrial membrane barrier, (ii) citrate conversion into *cis*-aconitate, (iii) *cis*-aconitate conversion into isocitrate and (iv) citrate conversion into isocitrate, without *cis*-aconitate being an intermediate. Steps ii–iv are based on the scheme of aconitase action described by Villafranca [12].

The results presented provide evidence for a strong

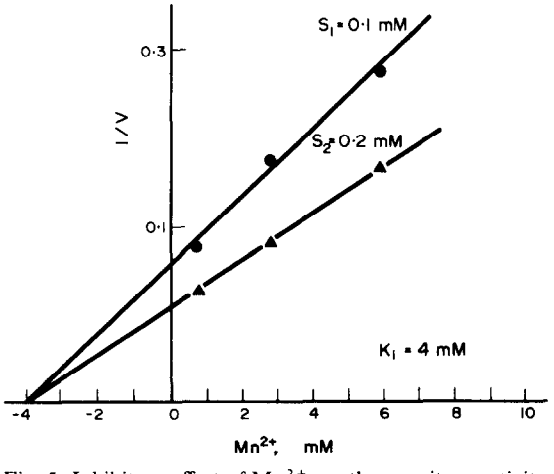

Fig. 5. Inhibitory effect of Mn^{2+} on the aconitase activity in the supernatant (105,000 *g*) of sonicated placental mitochondria. Dixon plot. The reaction mixture contained: 20 mM Tris–HCl buffer, pH 7·4, 100 mM NaCl, 0·1 mM or 0·2 mM *cis*-aconitate, supernatant (105,000 *g*) of sonicated mitochondria 0·8 mg protein and water to give a final volume of 3·0 ml. Decrease in absorbance at 240 nm followed. Unicam SP-800 spectrophotometer was used. Concentrations of Mn^{2+} were as indicated in the Figure.

inhibitory effect of manganese ion on citrate-supported progesterone biosynthesis from cholesterol in the placental mitochondrial fraction. A lack of inhibitory effect on the *cis*-aconitate and isocitrate supported progesterone biosynthesis, as well as the stimulatory effect of Mn^{2+} on malate supported progesterone biosynthesis, indicate that the inhibition is connected with the presence of citrate and that Mn^{2+} does not directly inhibit the reactions of cholesterol to progesterone metabolism. In accordance with this supposition is the lack of manganese inhibitory effect on NADPH supported progesterone biosynthesis. The results of experiments in which the effect of Mn^{2+} on NADPH plus citrate-supported progesterone synthesis were examined, showed a partial inhibition which could be accounted for by the citrate presence. These results indicate that there is no inhibition of cholesterol to progesterone conversion by citrate-Mn^{2+} complex. In agreement with this conclusion are the results of experiments performed on the 105,000 *g* supernatant of sonicated mitochondria. These results confirmed the suggestion that citrate plus manganese ion do not exhibit an inhibitory effect neither on the cholesterol side-chain cleavage or on 3β-hydroxysteroid dehydrogenase, 4 → 5, ene isomerase system.

On the basis of the experiments described we propose that Mn^{2+} inhibits in intact mitochondria some steps in citrate to isocitrate metabolism which affect

Table 4. Effect of manganese on $NADP^+$ reduction in the presence of citrate and isocitrate by supernatant of sonicated placental mitochondria

Addition	$NADP^+$ reduced (nmol per min/mg of protein)
DL-Isocitrate, 20 mM	770 ± 40
DL-Isocitrate, 20 mM + Mn^{2+}, 1mM	1000 ± 90
Citrate, 10 mM	6 ± 0·8
Citrate, 10 mM + Mn^{2+}, 1 mM	9 ± 0·3

Reduction of $NADP^+$ was measured spectrophotometrically at 340 nm in 2·5 ml medium pH 7·4 containing 20 mM potassium phosphate, 10 mM $MgSO_4$ and 1 mM $NADP^+$.

Values are the means from five experiments ± S.D.

indirectly cholesterol to progesterone conversion by decreasing NADPH generation.

Results in which the effect of Mn^{2+} on citrate and oxygen consumption were measured (Table 2) confirmed the suggestion that the inhibition of progesterone biosynthesis in intact mitochondria is a result of a decreased citrate utilization. However, there is no inhibition of citrate supported progesterone biosynthesis in the 105,000 *g* supernatant of sonicated mitochondria (Table 3). Additionally a small stimulatory effect of Mn^{2+} on pregnenolone and progesterone biosynthesis, as well as on NADPH formation from isocitrate and citrate was observed (Table 4).

If we assume that the properties of aconitase in intact mitochondria are the same as in the 105,000 *g* supernatant of sonicated mitochondria, the inhibitory effect of Mn^{2+} on citrate-supported progesterone biosynthesis in placental mitochondria is a result of limited citrate penetration across the mitochondrial membrane. To reconcile the results, the following assumptions have to be made: (i) Mn^{2+} is an inhibitor of the citrate transport, but not of other tricarboxylates, (ii) increased citrate concentrations, up to 30 mM and a long time of incubation do not abolish this "selective" inhibition and (iii) citrate penetration which is inhibited by Mn^{2+} is not malate dependent.

Results of several published experiments indicate that the transport of tricarboxylic acids through the mitochondrial membrane is mediated by a common specific carrier which catalyses an exchange diffusion of a tricarboxylate for a tricarboxylate or a dicarboxylate [13–15]. These findings are in contrast to the results of our experiments in which malate was without effect on citrate supported progesterone biosynthesis, and did not reverse the inhibitory effect of Mn^{2+} on progesterone synthesis in the presence of citrate.

The effect of manganese presented here is similar to that of fluorocitrate on isocitrate formation in intact but not disrupted rat liver mitochondria, described by Eanes *et al.* [16]. Explanation of these results as an inhibition of tricarboxylate carrier has been not confirmed by Brand *et al.* [17], who found that fluorocitrate inhibits neither isocitrate oxidation nor the exchange of internal citrate or L-malate in rat liver mitochondria. These authors suggested mitochondrial aconitase inhibition by fluorocitrate as the most probable explanation.

Results presented in this paper argue with the assumption that inhibition of aconitase activity is responsible for the effect of manganese ion on the citrate utilization and in consequence on the inhibition of progesterone biosynthesis by intact mitochondria. In intact placental mitochondria low concentrations of Mn^{2+}, even in the presence of high concentrations of citrate, exerted an inhibitory effect on progesterone biosynthesis, which could not be observed with supernatant of sonicated mitochondria. From these results we may assume that an active aconitase-metal–citrate complex is present in the 105,000 *g* supernatant of sonicated mitochondria, and an inactive aconitase-Mn^{2+}–citrate complex is formed in intact mitochondria.

It cannot be excluded that in our experiments mitochondrial membrane disruption lowered the sensitivity of aconitase to manganese ions in the supernatant of sonicated mitochondria. It is well documented that in liver, isocitrate formation from citrate does not involve *cis*-aconitate as an obligatory intermediate [12]. Also the incomplete inhibition, even at high concentrations of Mn^{2+} (Fig. 1) suggest two pathways for citrate to isocitrate conversion in intact placental mitochondria. The citrate to *cis*-aconitate pathway (on the step of *cis*-aconitate from citrate formation) may be inhibited by Mn^{2+}, whereas the conversion of citrate into isocitrate, without *cis*-aconitate as an intermediate, is unaffected by the presence of this ion. We assume that the first is connected with the existence of intact mitochondrial membranes. Inhibition by Mn^{2+} of *cis*-aconitate utilization in supernatant of sonicated mitochondria may indicate that a small part of "membrane dependent" aconitase activity is present in the supernatant of sonicated mitochondria. Non-competitive inhibitory effect of Mn^{2+} is similar to those obtained by Eanes and Kun [18] in purified aconitase preparation from rat liver mitochondria. High (4 mM) K_i value, for supernatant of sonicated placental mitochondria disagree with the fact that low concentrations of Mn^{2+} caused inhibitory effect in intact mitochondria. These findings may be explained by a structural change of released "membrane dependent" form of aconitase.

It should be stressed that the proposed hypothesis is not proven for human placental mitochondria.

The physiological significance of the inhibitory effect of manganese is also of interest. Conversion of citrate to isocitrate in placental mitochondria is a limiting step for isocitrate supply—a substrate of isocitric dehydrogenase. The K_m values of isocitric dehydrogenase for isocitrate and of aconitase for citrate were found to be 0·016 mM and 1·3 mM, respectively. Thus aconitase is the enzyme requiring a higher substrate concentration for its efficient activity. This enzyme may be additionally regulated by endogenous manganese in intact mitochondria. Although the concentration of Mn^{2+} within placental mitochondria is unknown, the concentration of Mn^{2+} in rat liver mitochondria was found to be 0·03 mM [19]. At this concentration manganese caused a significant inhibitory effect on progesterone biosynthesis in intact placental mitochondria.

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