Atti Accademia Nazionale dei Lincei

Classe Scienze Fisiche Matematiche Naturali

Rendiconti

Michela Festa, Alfredo Ruffo

Control by oxalomalate of fatty acids synthesis in rat liver

Atti della Accademia Nazionale dei Lincei. Classe di Scienze Fisiche, Matematiche e Naturali. Rendiconti, Serie 8, Vol. **80** (1986), n.4, p. 238–243.

Accademia Nazionale dei Lincei

<http://www.bdim.eu/item?id=RLINA_1986_8_80_4_238_0>

Biochimica. — *Control by oxalomalate of fatty acids synthesis in rat liver.* Nota di MICHELA FESTA e ALFREDO RUFFO (*), presentata (**) dal Corrisp. A. RUFFO.

RIASSUNTO. — Sono stati determinati gli acidi grassi liberi ed il citrato in omogenati di fegato di ratto dopo 1 ora di incubazione con ossaloacetato 2 mM in presenza ed in assenza di gliossilato o di ossalomalato 1 mM. In presenza di tali composti è stato trovato insieme ad accumulo di citrato, una evidente extra-sintesi di palmitato e stereato. Uguali risultati sono stati ottenuti anche *in vivo* su fegati di ratti iniettati con una soluzione di ossaloacetato e gliossilato noti precursori della sintesi di ossalomalato. La coincidenza dell'accumulo di citrato con la formazione *de novo* di acidi grassi è interpretata come una ulteriore prova del controllo metabolico esercitato dall'ossalomalato sul ciclo citrico.

Oxalomalic acid, a tricarboxylic acid synthesized *in vitro* and *in vivo* by spontaneous condensation of oxaloacetic and glyoxylic acids in the presence of Mg^{2+}, is a powerful competitive inhibitor of aconitase and isocitrate $NADP^+$-dehydrogenase but does not affect the NAD^+-dependent dehydrogenase nor ATP-citrate lyase [1-6]. More recent results [7] showed that at pH 6.8 and at high Mg^{2+} concentrations, NADPH-isocitrate-dehydrogenase reduced oxalomalate to an inactive compound. The chemical reduction of oxalomalate with $NaBH_4$ also produced inactivation. Moreover, an enzyme able to cleave oxalomalate into its primary components, namely glyoxylate and oxaloacetate [8], was found in rat liver mitochondria and in the supernatant fraction.

All these results strongly suggest that oxalomalate may exert a reversible control on the rate of the cycle by inhibiting aconitase and being removed by either NADPH-isocitrate-dehydrogenase or by the cleavage enzyme. If this is correct the amount of citrate accumulated, transferred into cytosol, should furnish sufficient acetyl-CoA equivalents for promoting *de novo* synthesis of fatty acids and activate acetyl-CoA carboxylase, the first enzyme connected with the synthesis.

To investigate this problem we determined the synthesis of citrate and fatty acids in rat livers homogenates incubated in the presence and in the absence of

(*) Laboratorio di Biochimica, Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli, Via L. Rodinò, 22 - 80138 Napoli.

(**) Nella seduta del 12 aprile 1986.

small concentrations of oxalomalate, and, respectively, the mixture oxaloacetate plus glyoxylate. The same determinations were repeated on livers of rats injected with an equimolar solution of oxaloacetate and glyoxylate, known to give origin to oxalomalate *in vivo* [2-6].

MATERIAL AND METHODS. Oxalomalic acid (α-hydroxy-β-oxalosuccinate) Na-salt; cis-oxaloacetic acid 90-95% were Sigma (St. Louis, USA) products. Glyoxylic acid-Na salt was obtained from Fluka A.G. Buks S.G. (Switzerland); silica-gel-60, chloroform, methanol, palmitic and stearic acids were from Merck (Darmstadt, Germany); NADH, grade II, citrate-lyase; lactic and oxaloacetic dehydrogenases were from Boehringer (Mannheim, Germany). Me-8 (Methyl-8-2meq/ml-DMF-Dimethyl-Acetal in Pyridine) was from Pierce Chem.Co. (Rockford, USA). Petroleum ether (40-70 °C), ethanolamine, HCl and all the other reagents were Carlo Erba (Milano) products.

ANIMALS. Wistar albino rats (160-200 g) were kept fasting for 24 hrs before being injected with 1 ml per 100 g of body weight of a freshly prepared solution containing 50.3 mg of glyoxylate and 92.6 mg of oxaloacetic acid neutralized to pH 7.4. The molar concentration of oxaloacetate was in excess in order to ensure the total transformation of glyoxylate into oxalomalate. The control was injected with 1 ml of 0.9% NaCl. After 1 hr the rats were killed by decapitation and the livers washed and divided in two aliquots. The first was homogenized in 2 volumes of 0.6 N solution of $HClO_4$ neutralized with Na_2CO_3, centrifuged and the supernatant used for citrate determination according to the enzymic method with bacterial citrate-lyase [9]. The second aliquot was homogenized in 20 volumes of a mixture 2 : 1 (v/v) of chloroform and methanol and extracted for total lipids according to Folch (10).

INCUBATION MIXTURES. Rat liver homogenates (1 : 10 w/v) in Krebs phosphate-saline of pH 7.4 free of Ca^{2+} and enriched of Mg^{2+} were incubated in the final volume of 8 ml for 1 hr at 37 °C with 2 mM oxaloacetate in the absence and i nthe presence of either 1 mM glyoxylate or 1 mM oxalomalate. At the end of incubation 2 ml were taken for citrate determination after deproteinization with 0.5 ml of 3 N $HClO_4$ and neutralization with KOH. The remaining aliquot was etxracted with 20 ml of the mixture 2 : 1 (v/v) chloroform-methanol according to Folch [10].

SEPARATION OF FATTY ACIDS. The total lipids were redissolved in chloroform, absorbed on a silica-gel-60 column for removing phospholipids, eluted with chloroform and evaporated to dryness. The residue extracted with petroleum ether was treated with Na_2CO_3 and acidified with H_2SO_4 from which fatty acids were extracted 3 times with petroleum ether, washed twice with water and evaporated at reduced pressure to dryness [11]. Finally they were redissolved in 0.05 ml of Me-8 heated for 20 min at 60 °C and analysed for gas-liquid chromatography Fractovap Carlo Erba, Milano 2900 capillary column in comparison with standards of palmitate and stearate. The analytical details are referred in figs. 1 and 2.

RESULTS

In fig. 1 are reported the profiles of chromatograms of fatty acids extracted from the homogenates, which show an evident increase 1 hr after the incubation in the presence of the inhibitor of aconitase. Besides the increase of the peaks of palmitate (P) and stearate (S), the appearance is to be noticed of some

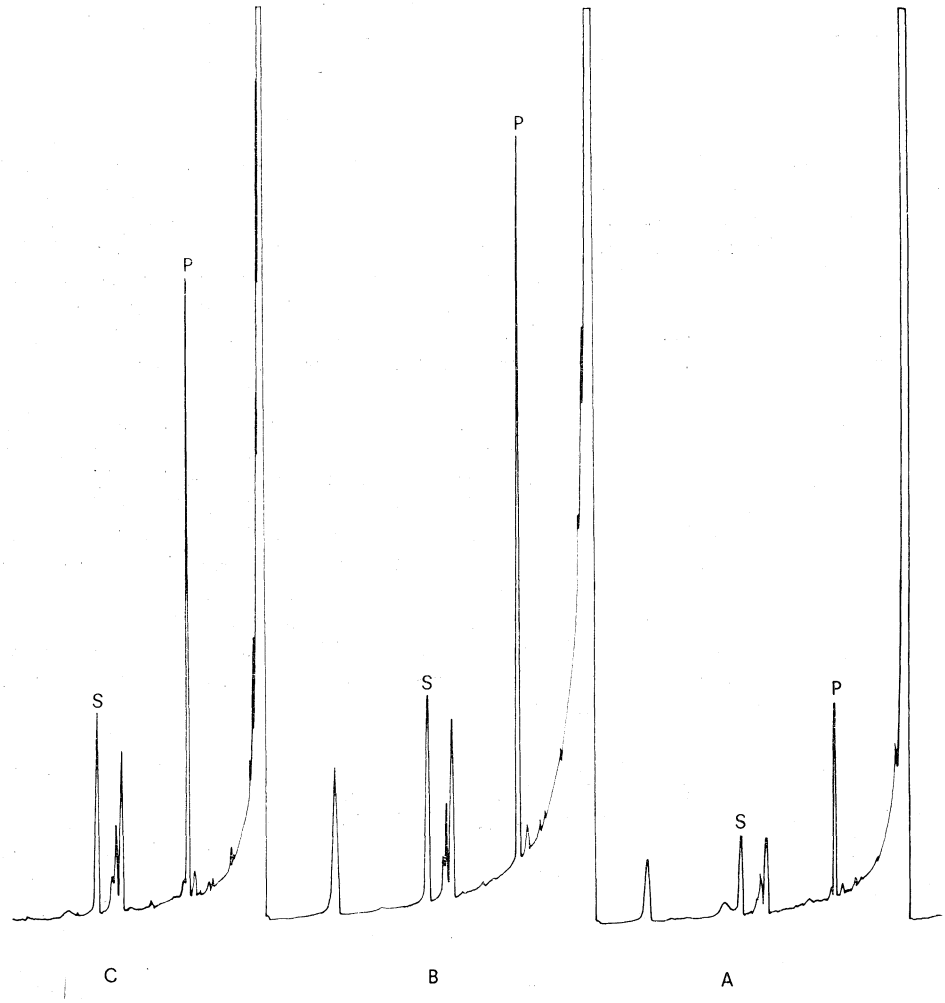

Fig. 1. – Gas chromatogram of liver homogenates incubated and extracted as described in Methods. Aliquots of 0.2 μl of methylated samples were injected in a HACHEREY–NAGEL OV-101 glass capillary columns 25 m × 0.32 mm. Test temp. 185 °C. The heights of palmitate (P) and stearate (S) peaks were compared with those of standards containing in 0.2 μl of Me-8, 0.8 nanomoles of each acid. A = 2 mM oxaloacetate; B = 2 mM oxaloacetate plus 1 mM glyoxylate; C = 2 mM oxaloacetate plus 1 mM oxalomalate.

other peaks related to acids at C-atoms lower and higher than those employed as reference standards. By comparing the standards areas with those of fig. 1 it was possible to quantify the rate of extra-synthesis as reported in Table I. Citrate determined in the same experiment was found to increase between 8 to 10-fold in the samples containing oxalomalate and respectively glyoxylate, confirming our previous results [2-6].

TABLE I.

Synthesis of fatty acids in 1 : 10 rat liver homogenate 1 hour after the incubation at 37 °C, pH *7.4 with 2* mM *oxaloacetate.*

Additions	μ g/g fresh weight	
	Palmitate	Stearate
Nothing	51.2	47.3
1 mM glyoxylate	195.3	130.2
1 mM oxalomalate	169.8	125.5

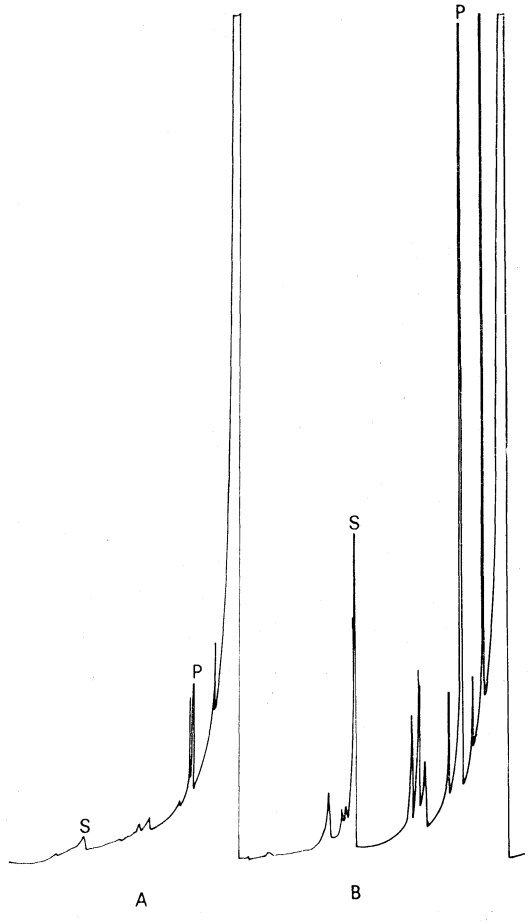

Fig. 2. – Gas chromatogram of fatty acids in rat livers extracted as described in Methods. An aliquot of 2 μl of not-methylated sample was injected into a J. and W. Fused Silica capillary column 15 m × 0.252 mm. Test temp. 200 °C. The heights of palmitate (P) and stearate (S) peaks were compared with those of standards containing in 2 μl of chloroform 5 nanomoles of each acid. A = Control; B = Livers of rats injected with glyoxylate and oxaloacetate.

The data in Table I show a consistent extra-synthesis of palmitate and stearate. The increase is higher in the presence of glyoxylate than in the sample where oxalomalate was added. This small difference was expected because of the tendency of oxalomalate to decarboxylize spontaneously into hydroxyoxoglutarate which inhibits aconitase less than oxalomalate [4] and the presence in homogenates of the enzyme which approximately cleaves 20% of oxalomalate in 1 hr at 37 °C [8]. These results have been confirmed by several experiments employing similar concentrations of either oxalomalate or oxaloacetate plus glyoxylate and by the results reported in the chromatogram of fig. 2 related to the liver of a rat 1 hr after the injection of glyoxylate plus oxaloacetate. The analysis was done on a capillary column different from that of fig. 1 on samples not-methylated, but the results show an extra-synthesis in the livers of the treated rats as evident as that observed in the homogenates (see fig. 1). We have also observed that total lipids diminished about 20% in the livers of the treated rats and did not change appreciably during the incubation of the homogenates.

Discussion

The results reported above show a significant extra-synthesis of fatty acids in rat livers when the citric acid cycle was interrupted at the level of aconitase either by the addition of oxalomalate or by the precursors independent if incubated *in vitro* or injected *in vivo*. In both cases it was already known [2-6] that citrate accumulated, as it has been confirmed in the present experiments. Therefore it is easy to expect that its reflux into cytosol may furnish sufficient substrate to ATP-citrate-lyase for being transformed into oxaloacetate and acetyl-CoA. Since citrate is also a necessary effector of acetyl-CoA-carboxylase, the first enzyme connected with the synthesis of fatty acids, another favourable stimulus that evokes the synthesis was realized. On these bases our results are well explained and suggest a prominent role either of oxalomalate or its precursors in modulating the rate of the whole cycle through the reversible inhibition on aconitase. With regard to the mechanism of the inhibition these new results showing higher values of fatty acids in the homogenates incubated with the precursors, confirm the preceeding ones [4-5] according to which only one of the four stereoisomers of oxalomalate formed during the incubation in the presence of the enzyme would fit together with Mg^{2+} the active site. Thus the chemically synthesized oxalomalate being a raceme resulted less active in producing inhibition.

However when oxalomalate blocks aconitase the observed *de novo* synthesis of fatty acids represents a convenient demonstration of the diversion of the catabolic route of the cycle into ancillary reactions yielding anabolic sequences. Since the synthesis of oxalomalate depends on the presence of two cellular metabolites, one of which, glyoxylate, is less contained in animal tissues, the exceptional reactivity of its aldheyde group with the acidic methylene carbon of oxa-

loacetate suggests that, whenever they meet, they will react quickly in the presence of Mg^{2+} and form oxalomalate in order to control the metabolic rate of the cycle if the physiological request of cells needs fatty acids for lipogenesis.

Acknowledgements. This work was supported by grants from the Ministry of Education, Rome and by the University of Naples. Thanks are due to Miss A. Angiolillo and to Mr. G. Laudadio and Mr. A. Senatore for technical assistance.

REFERENCES

[1] RUFFO A., TESTA E., ADINOLFI A. and PELIZZA G. (1962) – « Biochem. J. », *85*, 588-593.

[2] RUFFO A., ADINOLFI A., BUDILLON G. and CAPOBIANCO G. (1962) – « Biochem. J. », *85*, 593-600.

[3] RUFFO A., ADINOLFI A. and MALCOVATI M. (1966) – « Biochim. and Biophys. Acta Library. Regulation of Metabolic processes in Mitochondria », *7*, 518-525. Ed. by J.M. Tager *et al.*, Elsevier Publish Co., Amsterdam.

[4] RUFFO A., TESTA E., ADINOLFI A., PELIZZA G. and MORATTI R. (1967) – « Biochem. J. », *103*, 19-23.

[5] ADINOLFI A., GUARRIERA-BOBYLEVA V., OLEZZA S. and RUFFO A. (1971) – « Biochem. J. », *125*, 557-562.

[6] ADINOLFI A., SPERANZA M.L., GUARRIERA-BOBYLEVA V. and RUFFO A. (1973) – « Ital. J. Biochem. », *22*, 92-103.

[7] ADINOLFI A., MENNA G. and RUFFO A. (1981) – « Rend. Accad. Naz. Lincei », *59*, 265-270.

[8] FESTA M., LAUDADIO G. and RUFFO A. – Abstract Commun. 30° Congress of Biochemistry, Ischia 1984, 379.

[9] DAGLEY S. (1974) – « Methods of Enzym. Analysis », vol. 3, p. 1562-1565, Ed. by H.U. Bergmeyer; Acad. Press N.Y. and London.

[10] FOLCH L., LEES M. and SLOAM-STANLEY P.A. (1957) – « J. Biol. Chem. », *226*, 497.

[11] J.C. DITMER and M.A. WELLS (1969) – *Methods in Enzymology*, *14*, 482-530, Ed. by J.M. Lowenstein, Acad. Press, N.Y. and London.