REQUIREMENTS FOR MN^{++} AND MG^{++} IN RAT LIVER ACETYL-COA CARBOXYLASE AND FATTY ACID BIOSYNTHESIS ACTIVITIES

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Vagelos (1964) reviewed the evidence that fatty acid biosynthesis in higher organisms proceeds through a series of reactions catalyzed by acetyl-CoA carboxylase and a fatty acid synthetase multi-enzyme complex. Acetyl-CoA carboxylase, discovered by Wakil (1958) appears to be the rate-limiting enzymatic step in the reaction series (Ganguly, 1960; Numa, et al, 1961; Korchak and Masoro, 1962a). The activity of this enzyme is stimulated by citrate and isocitrate (Matsuhashi, et al, 1964; Martin and Vagelos, 1962; Waite and Waikil, 1962; Kallen and Lowenstein, 1962) and is inhibited by long-chain fatty acyl-CoA derivatives (Bortz and Lynen, 1963; Numa, et al, 1965) and by free fatty acids (Korchak and Masoro, 1964). Acetyl-CoA carboxylase can exist in different molecular weight forms with sedimentation coefficients of 20_s and 45_s, i.e. unaggregated, low molecular weight, protomeric forms and aggregated, high molecular weight, polymeric forms respectively (Vagelos, et al, 1963; Gregolin, et al, 1966).

While studying the capacity of particle-free, rat-liver supernatants to convert 1-^{14}C-acetate and 1-^{14}C acetyl-CoA into long-chain fatty acids, Korchak and Masoro (1962a) consistently obtained higher rates of ^{14}C-incorporation than

had previously been reported. In seeking reasons for this difference, we noted that while most workers had used incubation media containing predominantly magnesium (Mg^{++}) as divalent cation, Korchak and Masoro used manganese (Mn^{++}).

To determine how Mn^{++} promotes higher levels of fatty acid biosynthesis than Mg^{++}, we studied the divalent cation requirement 1) for the incorporation of ^{14}C-citrate into long-chain fatty acids by particle-free supernatants of rat-liver; and 2) for the carboxylation of acetyl-CoA with $NaH^{14}CO_3$ by partially purified rat-liver acetyl-CoA carboxylase. Mn^{++} consistently caused higher fatty acid biosynthetic activity and higher specific activities of acetyl-CoA carboxylase than Mg^{++}. Moreover, the conversion of citrate into fatty acids in the presence of optimal Mg^{++} concentrations was enhanced by adding Mn^{++} to the system. Sucrose density gradient sedimentation studies indicate that Mn^{++} + ATP is a specific requirement for activity in the low molecular weight forms of acetyl-CoA carboxylase.

Experimental Procedure

Liver supernatants were prepared from rats (Wistar strain, albino, average wt. 350 grams) by differential centrifugation. The liver was homogenized in either cold 0.25 M sucrose or cold 0.15 M KCl and centrifuged in a Servall (model RC-2) refrigerated centrifuge at 16,000 g for 15 minutes to sediment cell debris, nuclei and mitochondria. The resultant supernatant was further centrifuged in a Spinco (model L) ultracentrifuge at 105,000 g for 60 minutes to sediment microsomes.

The supernatant was harvested by aspiration, care being taken to avoid contamination with either loosely sedimented pellet material or the lipoid overlay. The 105,000 g supernatant was either incubated with 1,5-^{14}C-citrate

and required cofactors for measuring incorporation of label into long-chain fatty acids (see legend of figure 1) or was fractioned with 0-30% ammonium sulfate at pH 7.3.

When the latter procedure was employed, the precipitated protein was sedimented at 25,000 g at 0°C for 25 minutes. The pellet was dispersed in 0.02 M phosphate buffer (potassium salts), pH 7.4, and dialyzed with gentle stirring for 2 hours against 200 volumes of the same buffer. Immediately after dialysis, the enzyme solution was centrifuged at 850 g for 10 minutes. The supernatant is referred to as fraction f_1. This fraction contained fatty acid synthetase, isocitrate dehydrogenase and 7 to 8-fold purified acetyl-CoA carboxylase. All preparative operations were conducted at 0 to 2°C.

Sucrose density gradients were prepared by the method of Martin and Ames (1961). Incorporation of ^{14}C into long-chain fatty acids was determined by the method described by Korchak and Masoro (1962b). Acetyl-CoA carboxylase activity was measured by the recovery of acid-stable material from ^{14}C bicarbonate (Martin and Vagelos, 1962). All radioactivity was assayed by liquid scintillation spectrometry. Protein was determined by the method of Lowry, et al, (1951).

The assay system for acetyl-CoA carboxylase contained tris-maleate buffer, pH 7.4, 0.05 M; dithiothreitol, 10^{-3} M; tris-ATP, 3.3×10^{-3} M; potassium citrate, pH 7.4, 0.01 M; $MgCl_2$, 0.012 M or $MnCl_2$, 2×10^{-3} M; $KHCO_3$, 0.025 M; acetyl-CoA, 4×10^{-4} M; $NaH^{14}CO_3$, 1.3×10^6 cpm, sp. act. 5.25×10^5 cpm/μmole; 0.25 to 0.50 mg f_1 protein in a final reaction volume of 0.5 ml, pH 7.3. The system was incubated at 37° for 5 minutes.

Rats were fed standard Purina chow, ad libitum. Fasted rats were deprived of food overnight but had access to water.

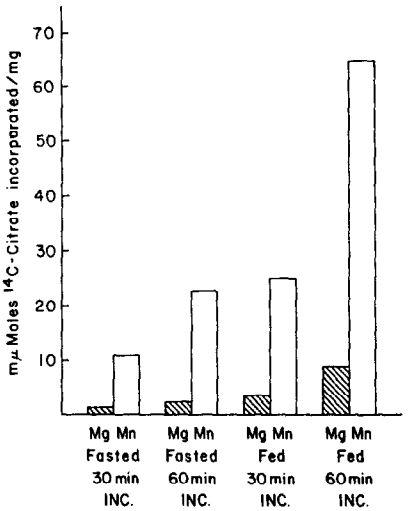

Fig. 1 - Effect of Nature of Divalent Cation on Incorporation of ^{14}C-citrate Into Fatty Acids. The system contained tris-maleate Buffer, pH 7.4, 0.05 M; dithiothreitol, 10^{-3} M; ATP (disodium salt), 4×10^{-3} M; $MgCl_2$, 0.012 M or $MnCl_2$, 2×10^{-3} M; $KHCO_3$, 0.025 M; Na-DL-isocitrate, 5×10^{-3} M; NADP, 3.8×10^{-4} M; creatine phosphate, 0.02 M; creatine phosphokinase, 40 μg; CoA, 1.6×10^{-4} M; 1,5-^{14}C-K-citrate, pH 7.4, 0.01 M (sp. act. 4.5×10^4 cpm/μMole); 5.4 mg rat-liver 105,000 g supernatant protein. The system was incubated at 37°C, pH 7.3, for the times indicated. The final reaction volume was 1.0 ml.

Results and Discussion

Rat-liver supernatants incorporated much more 1,5-^{14}C-citrate into long-chain fatty acids in the presence of Mn^{++} than in the presence of Mg^{++} (Fig. 1). So pronounced was this difference that a 30-minute incubation of "fasted" supernatant in the Mn^{++}-medium converted more citrate into fatty acid than did a 60-minute incubation of "fed" supernatant in the Mg^{++}-medium. The acetyl-CoA carboxylase specific activity of corresponding f_1 fractions (not shown) was also higher in the presence of Mn^{++} than Mg^{++}.

Table I shows the effect of the addition of Mn^{++} on the incorporation of ^{14}C-citrate into long-chain fatty acids by a Mg^{++}-containing supernatant system. The addition of a Mn^{++} concentration of 5×10^{-4} M to a medium

TABLE I

The Effect of Mn^{++} and Mg^{++} on the Incorporation of Citrate into Long Chain Fatty Acids

Divalent Metal ion	mμMoles ^{14}C-Citrate incorporated/mg protein
Mg, 15 mM	2.6
Mg, 15 mM Mn, 0.05 mM	16.6
Mg, 15 mM Mn, 2 mM	15.6
Mn, 2 mM	25.7

The incubation system was the same as described in legend of Figure 1 except 5.2 mg of liver 105,000 g supernatant protein was used and the $[Mg^{++}]$ and $[Mn^{++}]$ were those shown above. The system was incubated for 30 minutes at 37°C.

containing 0.015 M Mg^{++} greatly stimulated ^{14}C incorporation. However, raising the level of Mn^{++} concentration to 2×10^{-3} M did not further increase the incorporation. Citrate incorporation obtained with Mn^{++} in the absence of Mg^{++} was 10-fold greater than incorporation with Mg^{++} as the sole divalent cation (both divalent cations at optimal concentrations, see below). Consistent with these findings is the work of Matsuhashi, et al (1964) who found that maximum activity of highly purified rat-liver acetyl-CoA carboxylase requires Mn^{++} and that this requirement cannot be replaced by increasing concentrations of Mg^{++} alone.

While investigating the effects of different divalent cation concentrations on the acetyl-CoA carboxylase specific activity of fraction f_1, it was found that the specific activity obtained with Mn^{++} was consistently higher

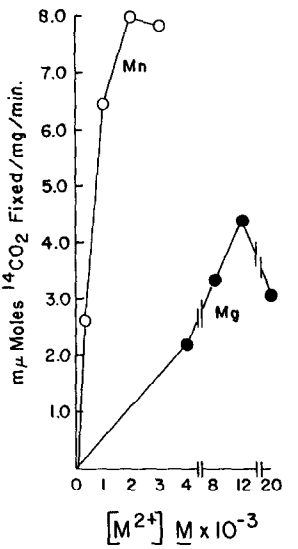

Fig. 2 - Effect of Divalent Cation Species and Concentrations on the Acetyl-CoA Carboxylase Specific Activity of Fraction f_1. The assay is described in text. The X axis refers to the concentration of Mg^{++} or Mn^{++}; open circles on graph refer to Mn^{++} and closed circles to Mg^{++}.

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than the specific activity obtained with Mg^{++} at all divalent cation concentrations tested (Fig. 2).

Using the optimal concentrations of Mn^{++} and Mg^{++} determined by these experiments, we tested the effects of preincubating fraction f_1 with divalent cation and citrate for 30 minutes and ATP for varying lengths of time on its acetyl-CoA carboxylase specific activity (Fig. 3). In contrast to the system not activated by preincubation, also reported in Fig. 3, the citrate + Mg^{++}-activated system had the same activity as the citrate + Mn^{++}-activated system if ATP was omitted during preincubation. However, the

inclusion of ATP in the preincubation inhibited the citrate + Mg^{++}-activated system drastically and the citrate + Mn^{++}-activated system much less. These results supplement the findings of Greenspan and Lowenstein (1967) that Mg^{++} + ATP inhibits rat-liver acetyl-CoA carboxylase activation and those of Ryder, et al (1967) and Numa, et al (1967) that Mg^{++} + ATP inhibits the decarboxylation of the chicken-liver acetyl-CoA carboxylase-biotin-CO_2 complex.

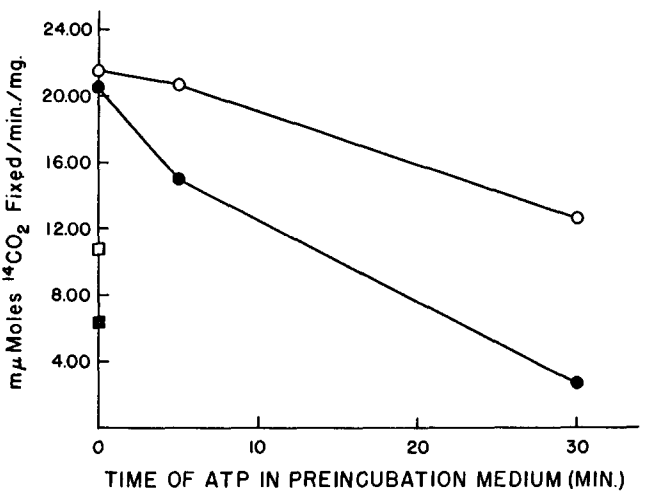

Fig. 3 - Effect of Preincubation With Citrate and Divalent Cation (Activation) on Acetyl-CoA Carboxylase Specific Activity of Fraction f_1 in Relation to Nature of Cation Species and Presences of ATP. The preincubation medium (pH 7.3) contained tris-maleate buffer, pH 7.4, 0.05 M; dithiothreitol, 10^{-3} M; sucrose 0.08 M and potassium citrate, 0.01 M, tris-ATP, 3.3×10^{-3} M. The preincubation was of a 30 minute duration and ATP was present for the length of time indicated on the X axis. The open circles refer to a $[Mn^{++}]$ in the preincubation medium of 2×10^{-3} M and the closed circles to a $[Mg^{++}]$ of 0.012 M. The open squares refer to Mn^{++} containing systems not preincubated and the closed squares similarly to Mg^{++}. The assay for carboxylase activity is described in text.

The results reported in Figures 2 and 3 reflect a clear distinction between Mn^{++} and Mg^{++}-supported acetyl-CoA carboxylase activity. Since the results in Figure 3 show that this distinction between cations is manifested by the non-activated and divalent cation-citrate-ATP-activated enzyme

preparations, it would seem that it was the unaggregated protomeric form of the enzyme which responded to the nature of the divalent cation. To test this possibility further, 0.1 ml aliquots of fraction f_1 (0.4 mg protein) were layered on 5% to 20% continuous sucrose gradients in centrifuge tubes and sedimented at 125,000 g for 4 hours. From each tube, 26 sub-fractions, decreasing in sucrose density from 1 to 26, were collected and assayed for acetyl-CoA carboxylase activity (without preincubation). In the lower density region of the gradient (Fig. 4) the enzyme solution possessed noticeably

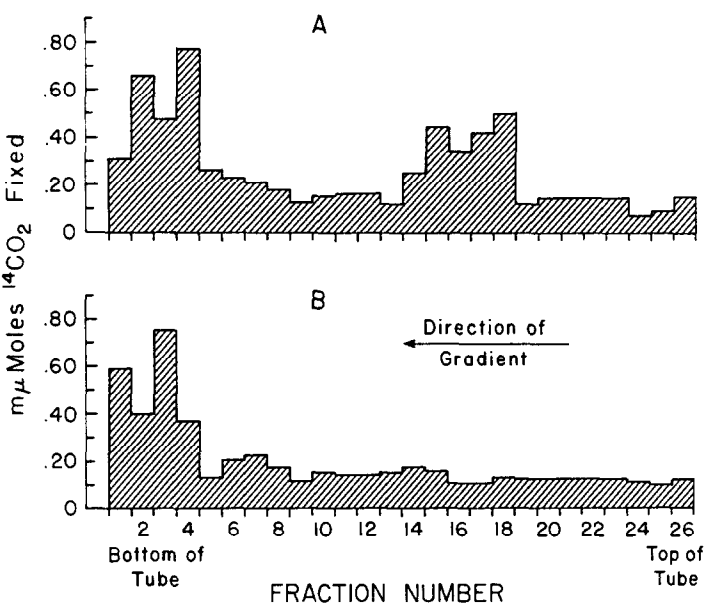

Fig. 4 - The Profile of Acetyl-CoA Carboxylase Activity in Sucrose Density Gradient Sedimentation of Fraction f_1. Each gradient system contained tris-maleate buffer, pH 7.4, 0.05 M; dithiothreitol 10^{-3} M; sucrose, 5 to 20% in a final volume of 4.55 ml. Fraction f_1 (0.1 ml containing 0.35 mg protein) was layered on top of 5% sucrose and the system centrifuged at 125,000 g for 4 hours. Serial fractions (0.16 ml each) were collected with a syringe needle (No. 22) from the bottom of each centrifuge tube. Each fraction was assayed by adding an aliquot of assay medium (see text) to a final volume of 0.27 ml at 37°C pH 7.4 for 5 minutes. A refers to assay with Mn^{++} and B to assay with Mg^{++}.

higher activity when assayed with Mn^{++} than with Mg^{++}. No significant activity differences relative to either divalent cation appeared in the higher density region of the gradients. Possibly related to these findings are the recent observations of Alberts and Vagelos (1968) who found that acetyl-CoA carboxylase of E. coli contains two fractions, one catalyzing carboxylation of the biotin-enzyme and the other catalyzing transfer of CO_2 from the carboxy-biotin-enzyme complex to acetyl-CoA; both fractions appear to be monomeric forms of enzyme protein, the carboxylating fraction exhibiting a strong requirement for Mn^{++} and biotin.

Our observations strongly indicate that rat-liver acetyl-CoA carboxylase contains active monomeric forms. According to sedimentation studies conducted with unpreincubated enzyme, the activity of the monomeric forms specifically require Mn^{++} + ATP. Replacement of Mn^{++} with Mg^{++} results in much less activity. However, preincubation of the enzyme with Mg^{++} and citrate, under conditions which cause polymerization and full activity of the enzyme (Greenspan and Lowenstein, 1967), completely abolishes the requirement for Mn^{++}. Moreover, the similarity in the magnitude of Mn^{++} and Mg^{++}-supported activity of polymeric forms indicates that these forms of rat-liver acetyl-CoA carboxylase show little specificity for Mn^{++} in preference to Mg^{++}. Further characterization of the active monomeric forms are in progress.

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