Differences between Manganese and Magnesium Ions with Regard to Fatty Acid Biosynthesis, Acetyl-Coenzyme A Carboxylase Activity and Malonyl-Coenzyme A Decarboxylation

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Fatty acid-biosynthetic activity in rat liver cytosol fractions is much greater when the bivalent cation in the assay system is Mn^{2+} than when it is Mg^{2+} . This difference between bivalent cations can be abolished if the cytosol fractions are preincubated with isocitrate and the bivalent cation for 30 min before assay of fatty acid-biosynthetic activity. In a search for the biochemical basis of this phenomenon, the following differences between Mg^{2+} and Mn^{2+} were established: (1) Mn^{2+} promotes acetyl-CoA carboxylase activity of the protomeric form of the enzyme under conditions in which Mg^{2+} does not; (2) Mn^{2+} + ATP have little inhibitory effect on the polymerization of acetyl-CoA carboxylase whereas Mg^{2+} + ATP are markedly inhibitory; (3) under conditions in which utilization of malonyl-CoA in condensation reactions is prevented, the steady-state concentration of malonyl-CoA formed by a cytosol fraction is much greater with Mn^{2+} than with Mg^{2+} . The role that each of these specific differences between Mn^{2+} and Mg^{2+} might play in causing liver cytosol preparations to have greater fatty acid-biosynthetic activity in the presence of Mn^{2+} is discussed.

The biosynthesis of fatty acids involves the conversion of acetyl-CoA into malonyl-CoA (Wakil, Porter & Gibson, 1957) in a reaction catalysed by acetyl-CoA carboxylase [acetyl-CoA- CO_2 ligase (ADP), EC 6.4.1.2] (Wakil, 1958). Since the rate-limiting step in fatty acid biosynthesis is believed to be the acetyl-CoA carboxylase-catalysed reaction (Ganguly, 1960; Numa, Matsuhashi & Lynen, 1961; Korchak & Masoro, 1962), this step has been focused upon as a site of regulation. This reaction is stimulated by citrate, isocitrate and a variety of dicarboxylic acids (Martin & Vagelos, 1962; Waite & Wakil, 1962; Matsuhashi, Matsuhashi, Numa & Lynen, 1962), citrate and isocitrate being the most effective stimulators, and is inhibited by long-chain fatty acyl-CoA derivatives (Bortz & Lynen, 1963; Numa, Ringlemann & Lynen, 1965) and by free fatty acids (Korchak & Masoro, 1964). Citrate and isocitrate have been shown to promote the transformation of low-molecular-weight protomeric forms of acetyl-CoA carboxylase into high-molecular-weight polymeric forms, and it has been suggested that this change in quaternary structure markedly increases the activity of the enzyme (Vagelos, Alberts & Martin, 1963; Matsuhashi, Matsuhashi & Lynen, 1964; Gregolin *et al.* 1968).

Although the bivalent metal ions (Mn^{2+} and Mg^{2+}) are known to be involved in acetyl-CoA carboxylase activity, it was not previously certain whether both of these bivalent cations were required or whether either would suffice when present at optimum concentration (Waite & Wakil, 1962; Matsuhashi *et al.* 1964; Gregolin, Ryder & Lane, 1968). Definitions of similarities and differences between Mn^{2+} and Mg^{2+} with regard to fatty acid biosynthesis, the activity of acetyl-CoA carboxylase and malonyl-CoA decarboxylation are the subjects of this paper. A preliminary report of this work has appeared (Scorpio & Masoro, 1968).

EXPERIMENTAL

Materials. Acetyl-CoA, dithiothreitol and CoA were purchased from Calbiochem (Los Angeles, Calif., U.S.A.), NADP⁺, ATP, glucose 6-phosphate, creatine phosphate, creatine phosphokinase, glucose 6-phosphate dehydrogenase and trisodium DL-isocitrate from Sigma Chemical Co. (St Louis, Mo., U.S.A.), MnCl_2 and MgCl_2 from Merck, Sharpe and Dohme Inc. (Rahway, N.J., U.S.A.) and [1,5- ^{14}C]citric acid, [3- ^{14}C]malonic acid and [1- ^{14}C]acetyl-CoA from New England Nuclear Corp. (Boston, Mass., U.S.A.). Rats (Wistar strain, albino, average

weight 350g) were obtained from Charles River Breeding Laboratories (N. Wilmington, Mass., U.S.A.).

Preparation of liver cytosol fractions f_0 and f_1 . The liver cytosol preparation f_0 was obtained in the following manner. Rats were killed by decapitation, and livers were perfused *in situ* with 50 ml of 0.85% NaCl at 37°C, excised and placed in cold 0.25M-sucrose or 0.15M-KCl. The pooled livers were minced, homogenized and centrifuged in a Servall model RC-2 refrigerated centrifuge at 16000g for 15 min to sediment cell debris, nuclei and mitochondria. The resultant supernatant was further centrifuged in a Spinco model L ultracentrifuge at 105000g for 60 min to sediment microsomes. The supernatant was collected by aspiration with a drawn-out pipette, care being taken to avoid contamination with either sedimented pellet material or the lipid overlay. This fraction is called f_0 .

To prepare the liver cytosol fraction f_1 , fraction f_0 was prepared in 0.15M-KCl, adjusted to pH 7.3 with KOH and fractionated with $(\text{NH}_4)_2\text{SO}_4$. The suspension of protein precipitated between 0 and 30% saturation with $(\text{NH}_4)_2\text{SO}_4$ was adjusted to pH 7.3, stirred gently for 30 min and sedimented at 25000g for 25 min. The pellet was washed once in 30%-saturated $(\text{NH}_4)_2\text{SO}_4$ and resedimented before being dissolved in either 20mM-potassium phosphate buffer, pH 7.4, or in 20mM-tris-maleate buffer, pH 7.4, to a final protein concentration of approx. 5 mg/ml. The suspension was then dialysed with slow stirring for 2 h against 200 vol. of the parent buffer, during which a small amount of flocculent precipitate appeared. Immediately after dialysis, the flocculent precipitate was sedimented by centrifugation at 850g for 10 min and the supernatant, called f_1 , was saved. Fraction f_1 contained acetyl-CoA carboxylase, fatty acid synthetase and isocitrate dehydrogenase activities, in agreement with the findings of Matsushashi *et al.* (1964). All preparative operations were carried out at 2°C. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Assay of fatty acid-biosynthetic activity. To measure the fatty acid-biosynthetic activity of fraction f_0 or f_1 it was incubated at 37°C in a 50 ml Pyrex screw-cap centrifuge tube containing $[1,5\text{-}^{14}\text{C}]\text{citrate}$ (potassium salt) as detailed in the legend of Table 1 or $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ as detailed in the legend of Table 2. The reaction was terminated by adding 0.5 ml of 30% (w/v) KOH in 50% (v/v) ethanol containing palmitic acid (0.25%) as carrier. Saponification and extraction of lipids and the determination of ^{14}C incorporation into long-chain fatty acids were conducted by the method described by Masoro, Korchak & Porter (1962).

Assay of acetyl-CoA carboxylase activity. The acetyl-CoA carboxylase activity of fraction f_0 or f_1 was assayed by measuring the recovery of acid-stable material from $[^{14}\text{C}]\text{bicarbonate}$ (Martin & Vagelos, 1962). This material, when hydrolysed in alkali and subjected to paper chromatography (Halenz & Lane, 1960), migrated as a discrete radioactive spot concurrently with authentic $[^{14}\text{C}]\text{-malonic acid}$. The standard assay for acetyl-CoA carboxylase was carried out at 37°C in a glass test tube. The assay system contained (final concentrations) in a total volume of 0.5 ml: 50mM-tris-maleate buffer, pH 7.4; 1mM-dithiothreitol; 10mM-citrate (potassium salt), pH 7.4; MgCl_2 and MnCl_2 at concentrations described in the legends of appropriate tables and figures; 3.3mM-ATP;

0.4mM-acetyl-CoA; 25mM- $\text{KH}^{14}\text{CO}_3$ (specific radioactivity 4.25×10^5 c.p.m./ μmol); 0.25–0.50 mg of fraction- f_1 or 0.8–1.2 mg of fraction- f_0 protein. The reaction was started when fraction f_0 or f_1 was added to the test tube; the incubation time was 5 min at 37°C unless otherwise indicated. The pH of the system, determined at 37°C in $\text{O}_2 + \text{CO}_2$ (95:5) environment, was found to be 7.25. The reaction was terminated by the addition of 0.5 ml of 0.2M-HCl. To expel residual $^{14}\text{CO}_2$ the contents of each tube were mixed on a vortex rotary mixer, and the air phase was flushed for 30 s with $\text{O}_2 + \text{CO}_2$ (95:5). The denatured protein was sedimented and 0.2 ml of supernatant was transferred to a liquid-scintillation counting vial. The contents of each vial were evaporated just to dryness by simultaneously passing a stream of warm air over the vials maintained on a hot-plate at 60°C and irradiating with an i.r. lamp. The residue was dissolved in 0.2 ml of water and suspended in the liquid-scintillation system described by Malila, DeMartinis & Masoro (1968) for aqueous samples. Radioactivity was determined by liquid-scintillation spectrometry, the channels-ratio method being used to correct for quenching. Under the conditions of this acetyl-CoA carboxylase assay the reaction rate was linear with respect to protein concentration and fraction f_1 had a specific activity eight times that of fraction f_0 .

Sedimentation of fraction f_1 by sucrose-density-gradient centrifugation. Linear sucrose density gradients of 5 ml volume containing (final concentrations) 5–20% (w/v) sucrose, 50mM-tris-maleate buffer, pH 7.4, and 1mM-dithiothreitol were prepared in $\frac{1}{2} \times 2\frac{1}{2}$ in cellulose nitrate centrifuge tubes by the method of Martin & Ames (1961). On each sucrose gradient 0.1 ml of fraction f_1 (approx. 0.5 mg of protein) was carefully layered and the system centrifuged at 125000g for 4 h at 4°C in a Spinco model L ultracentrifuge (SW 50 L rotor). Fractionation was carried out at 2°C by inserting a 27-gauge hypodermic needle through the bottom of the centrifuge tube, sealed against a concave rubber septum to prevent leaks. A continuous series of either 0.14 ml or 0.21 ml fractions was collected dropwise from each centrifuge tube. Each fraction was kept at 2°C until assayed for acetyl-CoA carboxylase activity.

RESULTS

Fatty acid biosynthesis. When the liver cytosol fractions f_0 and f_1 were assayed for fatty acid-biosynthetic activity, the amount of activity found was much greater in incubation systems containing added Mn^{2+} than in systems containing added Mg^{2+} . For example, the incorporation of ^{14}C from $[^{14}\text{C}]\text{citrate}$ into fatty acids by fraction f_0 (liver 105000g supernatant) was much greater with added Mn^{2+} than with added Mg^{2+} (Table 1). It is particularly striking that fraction f_0 prepared from starved rats could incorporate more citrate carbon into fatty acids when the incubation medium contained Mn^{2+} than could fraction f_0 prepared from fed rats when the incubation medium contained Mg^{2+} . The incorporation of $[1\text{-}^{14}\text{C}]\text{acetyl-CoA}$ into fatty acids by fraction f_1 (0–30%-saturation

ammonium sulphate fraction of fraction f_0) was also greater in a Mn²⁺- than in a Mg²⁺-containing incubation medium (Table 2, I and II).

It is known that preincubation of liver cytosol with citrate plus Mg²⁺ enhances its fatty acid-

Table 1. *Effect of Mn²⁺ and Mg²⁺ on incorporation of ¹⁴C from citrate into fatty acids by rat liver fraction f_0*

The incubation mixture contained (final concentrations) in a total volume of 0.5 ml: 50 mM-tris-maleate buffer, pH 7.4; 1 mM-dithiothreitol; 4 mM-ATP; 12 mM-MgCl₂ or 2 mM-MnCl₂; 25 mM-KHCO₃; 5 mM-sodium DL-isocitrate; 0.38 mM-NADP⁺; 20 mM-creatine phosphate; 40 μ g of creatine phosphokinase; 0.16 mM-CoA; 10 mM-potassium [1,5-¹⁴C]citrate (specific radioactivity 4.5×10^4 c.p.m./ μ mol), pH 7.4 and 5.4, mg of fraction- f_0 protein from starved or fed rats as indicated. The incubation was at 37°C. Fed rats were given Purina Chow ad libitum; starved rats were deprived of food overnight.

Nutritional state of rat	Bivalent cation	Incubation time (min)	[1,5- ¹⁴ C]Citrate incorporated (nmol/mg of protein)
Starved	Mg ²⁺	30	1.2
Starved	Mn ²⁺	30	10.9
Fed	Mg ²⁺	30	3.7
Fed	Mn ²⁺	30	25.0
Starved	Mg ²⁺	60	2.5
Starved	Mn ²⁺	60	22.6
Fed	Mg ²⁺	60	9.1
Fed	Mn ²⁺	60	64.3

biosynthetic activity (Fang & Lowenstein, 1967), a phenomenon often called activation. Similar activation follows preincubation of fraction f_1 with isocitrate and either Mg²⁺ or Mn²⁺ (Table 2, III and IV). However, after such preincubation fraction f_1 no longer incorporated ¹⁴C from [¹⁴C]-acetyl-CoA into fatty acids at a rate greater with Mn²⁺ than with Mg²⁺ (Table 2, compare I and II with III and IV). In the particular experiment reported in Table 2, it would appear that the Mg²⁺-containing system had more fatty acid-biosynthetic activity than the Mn²⁺-containing one; but several other experiments, not reported, show this difference not to be significant. When the preincubation system contained ATP as well as isocitrate and Mg²⁺, fraction f_1 incorporated much less [¹⁴C]-acetyl-CoA into fatty acids than when the preincubation took place in the presence of ATP, isocitrate and Mn²⁺ (Table 2, V and VI). The reason for this difference is clarified by the experiments presented below.

Acetyl-CoA carboxylase activity. Since the action of the bivalent cations Mn²⁺ and Mg²⁺ on fatty acid biosynthesis probably involves the acetyl-CoA carboxylase step, the effect of these cations and of ATP on the carboxylase activity of fraction f_1 (without preincubation) was investigated (Fig. 1). At all ATP concentrations tested the maximum carboxylase activity attainable with Mn²⁺ is higher than that attainable with Mg²⁺, and the concentration of Mn²⁺ required to promote this maximum activity is lower than the corresponding concentration of Mg²⁺.

Table 2. *Effect of preincubating fraction f_1 with isocitrate and either Mg²⁺ or Mn²⁺ in the presence and absence of ATP on the incorporation of ¹⁴C from acetyl-CoA into fatty acids*

The procedure for assay of fatty acid-biosynthetic activity was as follows. The incubation mixture for determining incorporation of ¹⁴C from acetyl-CoA into fatty acids contained (final concentrations) in a volume of 0.5 ml: 50 mM-tris-maleate buffer, pH 7.4; 1 mM-dithiothreitol; 80 mM-sucrose; 4 mM-ATP; 20 mM-MgCl₂ or 2 mM-MnCl₂ as indicated; 25 mM-KHCO₃; 10 mM-sodium DL-isocitrate; 0.38 mM-NADP⁺; 20 mM-creatine phosphate; 40 μ g of creatine phosphokinase; 0.4 mM [1-¹⁴C]acetyl-CoA (specific radioactivity 1.4×10^6 c.p.m./ μ mol) and 0.25 mg of fraction- f_1 protein. Each system was incubated at 37°C for 60 min. The general procedure for preincubation was as follows. With the exception of fraction- f_1 , KHCO₃, NADP⁺, acetyl-CoA, ATP and the ATP-regenerating system (creatine phosphate and creatine phosphokinase), all ingredients of the fatty acid-biosynthetic assay system were present at the concentrations noted above. ATP was either totally absent or present at a concentration of 4 mM; sodium DL-isocitrate was present at a concentration of 10 mM and fraction- f_1 protein was present at a concentration of 2 mg per ml of preincubation medium. Preincubation was for 30 min at 38°C.

Preincubation conditions	Bivalent cation in fatty acid-biosynthetic assay and preincubation system	[1- ¹⁴ C]Acetyl-CoA incorporation (nmol/mg of protein)
I (none)	Mg ²⁺	11.2
II (none)	Mn ²⁺	56.3
III (Preincubated without ATP)	Mg ²⁺	176.2
IV (Preincubated without ATP)	Mn ²⁺	130.0
V (Preincubated with ATP)	Mg ²⁺	16.0
VI (Preincubated with ATP)	Mn ²⁺	117.5

Fig. 1. Effect of combining different concentrations of bivalent cations and ATP on acetyl-CoA carboxylase activity of fraction f_1 . All incubation mixtures contained 0.4 mg of fraction- f_1 protein and the standard assay medium described in the Experimental section with concentrations of $MnCl_2$, $MgCl_2$ and ATP varied as indicated. All samples were assayed at 37°C for 5 min. O, ●, 1.6 mM-ATP; Δ, ▲, 3.3 mM-ATP; □, ■, 4.1 mM-ATP; ◇, ◆, 4.9 mM-ATP; X, 6.6 mM-ATP.

Table 3. *Effects of preincubation with citrate and bivalent cations on the acetyl-CoA carboxylase activity of fraction f_1*

All preincubation mixtures contained (final concentrations) in a total volume of 0.25 ml: 50 mM-tris-maleate buffer, pH 7.4; 1 mM-dithiothreitol; 80 mM-sucrose; 0.25 mg of fraction- f_1 protein; and potassium citrate, $MgCl_2$ or $MnCl_2$ in the concentrations indicated. Preincubation was for 30 min at 37°C. After preincubation, an appropriate mixture of components was added to provide the standard acetyl-CoA carboxylase assay system described in the Experimental section; the assay so initiated was carried out at 37°C in a final volume of 0.5 ml. With unpreincubated systems the reaction was initiated by adding 0.25 mg of fraction- f_1 protein.

Components of preincubation medium	Bivalent cation in assay medium	$^{14}CO_2$ fixed (nmol/min per mg of protein)
No preincubation	2 mM- Mn^{2+}	9.6
No preincubation	12 mM- Mg^{2+}	5.2
10 mM-Citrate	2 mM- Mn^{2+}	15.4
10 mM-Citrate	12 mM- Mg^{2+}	12.3
10 mM-Citrate + 2 mM- Mn^{2+}	2 mM- Mn^{2+}	20.0
10 mM-Citrate + 12 mM- Mg^{2+}	12 mM- Mg^{2+}	20.5

The effects of preincubation with citrate and Mn^{2+} or Mg^{2+} on acetyl-CoA carboxylase activity of fraction f_1 were also explored (Table 3). Although in systems not preincubated Mn^{2+} promotes acetyl-CoA carboxylase more effectively than Mg^{2+} , preincubation abolishes this difference.

If the acetyl-CoA carboxylase assay is allowed to occur for 30 min rather than the 5 min used for routine assays of this enzyme a steady-state amount of ^{14}C from $^{14}CO_2$ incorporated in malonyl-CoA is reached; the rate at which steady state is approached is more rapid with Mg^{2+} but the steady-state amount is much greater with Mn^{2+} . Data for the first 20 min of such an experiment are reported in Fig. 2.

The activation of acetyl-CoA carboxylase during preincubation with citrate and Mg^{2+} is inhibited by ATP (Fig. 3), in confirmation of the results reported by Greenspan & Lowenstein (1968). However, if Mg^{2+} is replaced by Mn^{2+} , ATP is less inhibitory. This effect of ATP on the activation of acetyl-CoA carboxylase is consistent with the results relating to fatty acid biosynthesis.

Sucrose-density-gradient-centrifugation studies of acetyl-CoA carboxylase activity. The differences between Mn^{2+} and Mg^{2+} with regard to acetyl-CoA carboxylase were further defined by experiments utilizing sucrose-density-gradient centrifugation. The sedimentation pattern of fraction- f_1 protein in 5–20% sucrose density gradients revealed the presence of slow- and fast-sedimenting peaks of acetyl-CoA carboxylase activity (Fig. 4). The slow-sedimenting peak of activity was observed only when the assay was carried out with Mn^{2+} , but the fast-sedimenting peak of activity was observed with either Mn^{2+} or Mg^{2+} . In these experiments the phosphate concentration was less than 1 mM.

When fraction f_1 was assayed in the presence of increasing concentrations of potassium phosphate,

Fig. 2. Time-course of acetyl-CoA carboxylation in fraction f_1 and the effect of bivalent metal ion species on unpreincubated and citrate-preincubated enzyme. Preincubations and acetyl-CoA carboxylase assays were carried out as described in Table 3 and the Experimental section except for the assay times indicated on the abscissa. $\circ$, Enzyme assayed with 2 mM-MnCl₂; $\bullet$, enzyme assayed with 12 mM-MgCl₂.

Fig. 3. Effect of ATP on the activation of acetyl-CoA carboxylase by citrate plus Mn²⁺ or Mg²⁺. Preincubations were carried out as described in Table 3 with the exception that 3.3 mM-ATP was added for the elapsed time indicated on the abscissa. After preincubation, acetyl-CoA carboxylase activity was assayed as described in Table 3 and the Experimental section. Percentage activation was calculated from the arbitrary value of 100 assigned to the increase in ¹⁴CO₂ fixed after preincubation of fraction f_1 with citrate and either 2 mM-MnCl₂ ($\circ$) or 12 mM-MgCl₂ ($\bullet$) in the absence of ATP.

Fig. 4. Sedimentation of fraction- f_1 acetyl-CoA carboxylase activity by sucrose-density-gradient centrifugation. In trace *a* enzyme was assayed with 2 mM-MnCl₂; in trace *b* enzyme was assayed with 12 mM-MgCl₂. Sucrose density gradients contained no added phosphate. Protein fraction f_1 was prepared by suspension in and dialysis against 20 mM-tris-maleate buffer, pH 7.4, instead of 20 mM-phosphate. Additional experimental details are given in the Experimental section.

acetyl-CoA carboxylase activity in the Mg²⁺-containing medium increased almost linearly with respect to phosphate concentration (Fig. 5); however, in the Mn²⁺-containing medium increasing the phosphate concentration did not stimulate. After sedimentation of fraction f_1 by sucrose-density gradient centrifugation in the system containing

20 mM-phosphate, slow- and fast-sedimenting peaks of acetyl-CoA carboxylase activities were observed when the assay was carried out with either Mn²⁺ or Mg²⁺ (Fig. 6).

The acetyl-CoA carboxylase activity promoted by

Fig. 5. Effect of potassium phosphate and bivalent metal ions on acetyl-CoA carboxylase activity of fraction f_1 . All incubation mixtures contained 0.4 mg of fraction- f_1 protein and the standard assay medium described in the Experimental section. Potassium phosphate ($\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$, pH 7.4) was added at increasing concentrations as indicated. O, Enzyme assayed with 2 mM- MnCl_2 ; ●, enzyme assayed with 12 mM- MgCl_2 . Protein fraction f_1 was prepared by suspension in and dialysis against 20 mM-tris-maleate buffer, pH 7.4, instead of 20 mM-phosphate. Acetyl-CoA carboxylase activity was assayed by the standard procedure described in the Experimental section.

Fig. 6. Effects of phosphate on the sedimentation of fraction- f_1 acetyl-CoA carboxylase activity by sucrose density-gradient centrifugation. In trace a enzyme was assayed with 2 mM- MnCl_2 ; in trace b enzyme was assayed with 12 mM- MgCl_2 . Sucrose density gradients contained 20 mM-potassium phosphate ($\text{KH}_2\text{PO}_4 + \text{K}_2\text{HPO}_4$, pH 7.4). The assay medium contained 10 mM-phosphate. Protein fraction f_1 was prepared by suspension in and dialysis against 20 mM-potassium phosphate. Additional details are given in the Experimental section.

Mn^{2+} in the medium containing less than 1 mM-phosphate decreased when fraction f_1 was aged at 2°C for 20 h (Fig. 7). This decrease related to a loss in the slow-sedimenting peak of acetyl-CoA carboxylase activity (Fig. 8) and was probably due to the instability of protomeric forms of the enzyme.

DISCUSSION

The results of the present study show the following differences between Mn^{2+} and Mg^{2+} with regard to the acetyl-CoA carboxylase activity and the net formation of malonyl-CoA by a 0–30% saturation ammonium sulphate fraction of rat liver cytosol (fraction f_1). First, Mn^{2+} promoted acetyl-CoA carboxylase activity of the protomeric form of the enzyme under conditions in which Mg^{2+} did not. Secondly, Mn^{2+} +ATP had little inhibitory effect on the polymerization of acetyl-CoA carboxylase whereas Mg^{2+} +ATP were markedly inhibitory. Thirdly, under conditions in which utilization of malonyl-CoA in condensation reactions was prevented, the steady-state concentration of malonyl-CoA was much greater with Mn^{2+} than with Mg^{2+} .

Evidence supporting the first point was obtained by the sucrose-density-gradient-centrifugation studies of the acetyl-CoA carboxylase activity of fraction f_1 . Mn^{2+} but not Mg^{2+} was found to promote the catalytic activity of slow-sedimenting material in the assay systems containing less than 1 mM-phosphate. Further sucrose-density-gradient analysis showed that this difference did not result from a polymerization of acetyl-CoA carboxylase by Mn^{2+} and citrate during the assay for enzymic activity (R. M. Scorpio & E. J. Masoro, unpublished work). It is striking that, by increasing the phosphate concentration to 20 mM, the slow-sedimenting forms of acetyl-CoA carboxylase were equally active with either Mn^{2+} or Mg^{2+} . Apparently the activity of the protomeric forms of the enzyme were sensitive to the ionic environment and under certain conditions only Mn^{2+} was capable of bringing about enzymic activity. Further purification of acetyl-CoA carboxylase by adsorption on calcium phosphate gel (Matsushashi *et al.* 1964) resulted in the disappearance of the difference between Mg^{2+} and Mn^{2+} relative to the enzymic activity of slow-sedimenting material (R. M. Scorpio & E. J. Masoro, unpublished work); this could result from a loss of labile proto-

Fig. 7. Effects of aging on fraction-*f*₁ acetyl-CoA carboxylase activity. All incubation mixtures contained the standard assay medium described in the Experimental section. ○, Fraction *f*₁, not aged, enzyme assayed with MnCl₂; ●, fraction *f*₁, not aged, enzyme assayed with MgCl₂; △, fraction *f*₁, aged, enzyme assayed with MnCl₂; ▲, fraction *f*₁, aged, enzyme assayed with MgCl₂. Immediately after suspension in and dialysis against 20 mM-tris-maleate buffer, pH 7.4, fraction *f*₁ was divided into two fractions of equal volume. One fraction (not aged) was centrifuged (see the Experimental section) and the supernatant was assayed immediately for acetyl-CoA carboxylase activity by the standard procedure described in the Experimental section. The other fraction (aged) was allowed to stand at 2°C for 20 h before centrifugation and assay of supernatant.

meric forms or from their conversion into polymeric forms during exposure to the high concentrations of phosphate required in the purification procedure. The former seems more likely, since aging caused a disappearance in the activity of the slow-sedimenting forms (Fig. 8). However, it should also be noted that Gregolin *et al.* (1968) have found that phosphate can promote the polymerization of highly purified chicken liver acetyl-CoA carboxylase. The large amount of purified enzyme necessary to determine which of these possibilities is the case could not be readily obtained; therefore, since it was not a major point of our study, such experiments were not carried out.

The second point, i.e. that Mn²⁺ + ATP have much less inhibitory effect on citrate-induced polymerization of acetyl-CoA carboxylase than Mg²⁺ + ATP, is established by the data reported in Table 2 and Fig. 3. That Mg²⁺ + ATP inhibit acetyl-CoA carboxylase polymerization has been previously shown by Fang & Lowenstein (1967) and by Greenspan & Lowenstein (1968). The latter authors also proved that this inhibition was not the result of metal-ion chelation by ATP (Greenspan & Lowenstein, 1967). The possible difference in free ATP concentration between the Mg²⁺ + ATP and the Mn²⁺ + ATP systems cannot be of sufficient magnitude to account for the much lower inhibitory

effect of the Mn²⁺ + ATP system on the polymerization of acetyl-CoA carboxylase (Walaas, 1958).

The third difference between Mg²⁺ and Mn²⁺ relates to the much higher concentration of malonyl-CoA formed in the presence of Mn²⁺ when acetyl-CoA, bicarbonate, ATP and fraction *f*₁ are incubated under conditions that should prevent further utilization of malonyl-CoA in condensation reactions. Although the steady-state concentration of malonyl-CoA is much greater with Mn²⁺, the initial rate of malonyl-CoA formation and the speed of reaching the steady-state concentration is faster with Mg²⁺. The kinds of reaction that can be involved in reaching these steady-state concentrations are only two: carboxylation of acetyl-CoA to form malonyl-CoA and decarboxylation of malonyl-CoA. Malonyl-CoA is a potent inhibitor of acetyl-CoA carboxylase in the presence of Mg²⁺ (Gregolin, Ryder, Warner, Kleinschmidt & Lane, 1966) and Mn²⁺ (R. M. Scorpio & E. J. Masoro, unpublished work). Therefore the most likely explanation for the higher steady-state concentration of malonyl-CoA in the presence of Mn²⁺ is that the decarboxylation of malonyl-CoA is faster in the presence of Mg²⁺ (from our results and others it is unlikely that this decarboxylation can primarily involve a simple reversal of the acetyl-CoA carboxylase

Fig. 8. Sedimentation of acetyl-CoA carboxylase activity of aged and not-aged fraction f_1 by sucrose-density-gradient centrifugation. In trace *a* fraction f_1 was not aged and enzyme was assayed with 2mM-MnCl₂; in trace *b* fraction f_1 was not aged and enzyme was assayed with 12mM-MgCl₂; in trace *c* fraction f_1 was aged and enzyme was assayed with 2mM-MnCl₂. Sucrose density gradients contained no added phosphate. Protein fraction f_1 was prepared by suspension in and dialysis against 20mM-tris-maleate buffer, pH 7.4, instead of 20mM-phosphate. Additional experimental details are given in the Experimental section.

reaction). Since malonyl-CoA decarboxylation, a separate activity from simple mass-action reversal of the carboxylation reaction, does not require bivalent metal ions (Gregolin *et al.* 1968), it is possible that Mn²⁺ acts to facilitate transfer of malonyl-CoA from a site that promotes decarboxylation on the complex of enzymes involved in fatty acid synthesis to a site where condensation reactions involving malonyl-CoA would occur on the fatty acid synthetase if the necessary cofactors were present. The results in Table 2 are consistent with this suggestion.

The two differences noted with regard to the effects of Mg²⁺ and Mn²⁺ on acetyl-CoA carboxylase activity explain, at least in part, our finding that fatty acid biosynthesis occurs at a much higher rate with Mn²⁺ than with Mg²⁺ in liver cytosol preparations assayed in the routine fashion in a medium containing ATP, citrate or isocitrate and low concentrations of inorganic phosphate. Under these conditions the protomeric forms of acetyl-CoA carboxy-

lase will be active from the very start of the fatty acid-biosynthetic assay when Mn²⁺ is present but not when Mg²⁺ and no Mn²⁺ is present. Also, during the assay citrate or isocitrate activation of acetyl-CoA carboxylase will occur if Mn²⁺ is the bivalent cation but not if Mg²⁺ is. Since acetyl-CoA carboxylase is usually the rate-limiting enzyme in fatty acid biosynthesis, the increase in its activity should cause an increase in the rate of fatty acid biosynthesis, as indeed is the case. That Mg²⁺ causes a higher rate of decarboxylation of malonyl-CoA than Mn²⁺ may also partly account for the higher rates of fatty acid biosynthesis with Mn²⁺. Also, if the hypothesis that Mn²⁺ facilitates the transfer of malonyl groups to the condensation site of fatty acid synthetase is the case, this also would serve as part of the mechanism for enhancement of fatty acid biosynthesis by Mn²⁺.

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