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The Influence of Magnesium Ions and other Bivalent Metal Ions on the Aconitase Equilibrium and its Bearing on the Binding of Magnesium Ions by Citrate in Rat Heart

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The equilibrium citrate/isocitrate concentration ratio for the aconitase reaction (aconitate hydratase, EC 4.1.2.3) is reported to be between 13·5:1 and 15:1 (Eggleston & Krebs, 1949; Krebs, 1953). When hearts from normal rats are perfused with medium containing glucose (5·5mM) and insulin (0·05i.u./ml.) the citrate/isocitrate concentration ratio is reported to be between 7·8:1 and 11:1. The ratio is increased in hearts from alloxan-diabetic rats or in hearts from normal rats perfused with ketone bodies or fatty acids to 15:1 (Garland & Randle, 1964; Bowman, 1966; Randle, Denton & England, 1967). Thus there is a discrepancy between the published equilibrium citrate/isocitrate concentration ratio for aconitase and for hearts from normal rats perfused with glucose and insulin in that the ratio measured in the heart under these conditions is lower. This low ratio is unlikely to be due to displacement of the aconitase reaction from equilibrium because it represents an apparent excess of isocitrate. Moreover the low ratio is unlikely to be due to 'compartmentation' of isocitrate because aconitase has been detected in both mitochondrial and cytoplasmic fractions (P. J. England, R. M. Denton & P. J. Randle, unpublished work). Citrate and isocitrate are known to show different affinities for bivalent metal ions (Sillen & Martell, 1964) and this has led us to investigate the effect of Mg^{2+}, Ca^{2+} and Fe^{2+} on the aconitase equilibrium.

A crude aconitase fraction was prepared from rat heart as follows, all operations being conducted at 0–4°. Rat ventricular muscle (approx. 1g.) was homogenized for 1min. in 10ml. of 0·1M-triethanolamine–chloride buffer, pH7·3, in a Sorvall Omnimixer. The homogenate was centrifuged for 20min. at 40000*g* and the supernatant containing approx. 2 units of aconitase/ml. used to determine equilibrium citrate/isocitrate concentration ratios as follows. Incubation mixtures were made in a volume of 2ml. at 37° with shaking in 15ml. conical centrifuge tubes with 0·1M-triethanolamine–chloride buffer, pH7·3 (unless otherwise stated), and concentrations of sodium citrate, $MgSO_4$, $CaCl_2$ and $FeSO_4$ given in Fig. 1 or the text. The reaction was initiated by addition of aconitase (0·4unit in 0·2ml.) and continued until the concentrations of citrate and isocitrate were constant. For assay of citrate and isocitrate deproteinization was achieved with 0·1ml. of 70% (w/v) $HClO_4$ and perchlorate was removed by neutralization with saturated $KHCO_3$ at 0°. Citrate was assayed spectrophotometrically with citrate lyase, malate dehydrogenase and NADH (Moellring & Gruber, 1966); isocitrate was assayed with isocitrate dehydrogenase and $NADP^+$ (Siebert, 1963). Enzymes and coenzymes were obtained from Boehringer Corporation (London) Ltd., London, W. 5. Under the conditions described the sum of citrate + isocitrate at the end of incubation was the same as the initial citrate. Equilibrium was reached within 10min. of incubation with initial citrate concentrations below 1·5mM and within 15min. with initial citrate concentrations between 1·5 and 3mM. Control experiments showed that aconitase was still active when apparent equilibrium was attained. The crude aconitase fraction was unstable, all activity being lost after 18hr. at 0°; the preparations were therefore used within 2hr.

The effects of Mg^{2+} and Ca^{2+} on the equilibrium citrate/isocitrate concentration ratio for aconitase at pH7·3 are shown in Fig. 1. In the absence of added bivalent metal ion the concentration ratio was 7·8:1, both in the presence and in the absence of mM-EDTA. The concentration ratio measured with an initial citrate concentration of 1mM increased with the addition of Mg^{2+} to a maximum of 33:1 at 2mM-Mg^{2+}. Further increases in Mg^{2+} concentration between 2 and 50mM led to some diminution in the ratio. Qualitatively similar effects were seen with Ca^{2+} (see Fig. 1) and with Fe^{2+} (up to 1mM; not shown). The effects of Mg^{2+} were also investigated at pH8·0 and pH6·5 (0·1M-triethanolamine–chloride buffer). At pH8·0 the concentration ratio in the absence of Mg^{2+} and the effects of Mg^{2+} on the ratio were similar to those seen at pH7·3. At pH6·5 the concentration ratio in the absence of Mg^{2+} was unchanged but the effects of Mg^{2+} were less pronounced. Thus the ratio increased to 19:1 with 2mM-Mg^{2+} at pH6·5 compared with 33:1 at pH7·3. The effects of Mg^{2+} at concentrations below 5mM were to some extent dependent on the initial citrate concentration. For example, with mM-Mg^{2+} the citrate/isocitrate con-

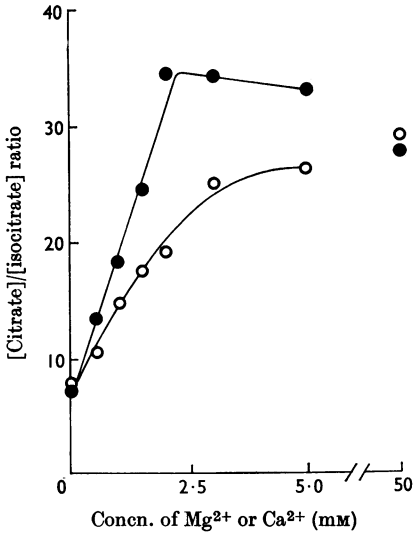

Fig. 1. Effect of Mg^{2+} and Ca^{2+} on the equilibrium citrate/isocitrate concentration ratio with rat heart aconitase. Incubations were made at 37° in 0·1M-triethanolamine–chloride buffer, pH 7·3, with an initial sodium citrate concentration of 1mM. The concentrations of $MgSO_4$ or $CaCl_2$ were as shown. Citrate and isocitrate were assayed spectrophotometrically after attainment of equilibrium. For further details see the text. The values in the absence of Mg^{2+} were means of five determinations; the remainder were single or duplicate determinations. ●, Mg^{2+}; ○, Ca^{2+}.

centration ratio varied linearly with the initial citrate concentration being 18·6:1 at 0·5mM-citrate and 16:1 at 2·8mM-citrate.

In hearts from normal rats perfused with glucose and insulin the citrate/isocitrate concentration ratio in the heart was 7·6 ± 0·7 (means ± S.D.). The mean value suggests that the free Mg^{2+} in the heart under these conditions may be near zero and that little Mg^{2+} is available to citrate from complexes formed between Mg^{2+} and other anions (the total magnesium in rat heart is approx. 10 μmoles/g. wet wt.; Watchorn & McCance, 1937). When hearts from normal rats are perfused with substrates such as acetate in addition to glucose and insulin the concentration ratio increases to 15:1 and the concentration of citrate increases from approx. 0·5mM to 3mM. If citrate and isocitrate are in equilibrium through the aconitase reaction during perfusion with substrates such as acetate, then the increased ratio may indicate that Mg^{2+} is bound by citrate under these conditions. Calculations based on the apparent stability constant of magnesium citrate and the influence of Mg^{2+} concentration on the aconitase equilibrium with 3mM-citrate indicate that approx. 0·7 μmole of Mg^{2+} would be bound to citrate/ml. of intracellular water. It seems probable that there is an equilibrium in rat heart between complexes of Mg^{2+} with various anions, and the rise in citrate concentration with substrates such as acetate may be one factor leading to the formation of magnesium citrate. Other bivalent ions such as Ca^{2+} could have similar effects though their concentration in rat heart is apparently low in relation to that of Mg^{2+} (Watchorn & McCance, 1937).

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Bowman, R. H. (1966). *J. biol. Chem.* **241**, 4092.
Eggleston, L. V. & Krebs, H. A. (1949). *Biochem. J.* **45**, 578.
Garland, P. B. & Randle, P. J. (1964). *Biochem. J.* **93**, 673.
Krebs, H. A. (1953). *Biochem. J.* **54**, 78.
Moellring, H. & Gruber, W. (1966). *Analyt. Biochem.* **17**, 369.
Randle, P. J., Denton, R. M. & England, P. J. (1967). *Symp. biochem. Soc.* (in the Press).
Siebert, G. (1963). In *Methods of Enzymatic Analysis*, p. 318. Ed. by Bergmeyer, H. U. Berlin: Verlag Chemie.
Sillen, L. B. & Martell, A. E. (1964). *Stability Constants of Metal Ion Complexes*. London: The Chemical Society.
Watchorn, E. & McCance, R. A. (1937). *Biochem. J.* **31**, 1379.