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Factors Affecting the Activity of Citrate Synthase of *Acetobacter xylinum* and its Possible Regulatory Role

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The citrate synthase activity of *Acetobacter xylinum* cells grown on glucose was the same as of cells grown on intermediates of the tricarboxylic acid cycle. The activity of citrate synthase in extracts is compatible with the overall rate of acetate oxidation *in vivo*. The enzyme was purified 47-fold from sonic extracts and its molecular weight was determined to be 280000 by gel filtration. It has an optimum activity at pH 8.4. Reaction rates with the purified enzyme were hyperbolic functions of both acetyl-CoA and oxaloacetate. The K_m for acetyl-CoA is 18 μM and that for oxaloacetate 8.7 μM. The enzyme is inhibited by ATP according to classical kinetic patterns. This inhibition is competitive with respect to acetyl-CoA ($K_i = 0.9$ mM) and non-competitive with respect to oxaloacetate. It is not affected by changes in pH and ionic strength and is not relieved by an excess of Mg^{2+} ions. Unlike other Gram-negative bacteria, the *A. xylinum* enzyme is not inhibited by NADH, but is inhibited by high concentrations of NADPH. The activity of the enzyme varies with energy charge in a manner consistent with its role in energy metabolism. It is suggested that the flux through the tricarboxylic acid cycle in *A. xylinum* is regulated by modulation of citrate synthase activity in response to the energy state of the cells.

Organic acids and related substrates are oxidized in *Acetobacter xylinum* by way of the tricarboxylic acid cycle (Gromet *et al.*, 1957; Schramm *et al.*, 1957; Benziman & Burger-Rachamimov, 1962). Oxidative dissimilation of carbohydrates in this organism proceeds largely by way of the pentose phosphate cycle (Schramm *et al.*, 1957). The channelling of sugar carbon into the tricarboxylic acid cycle differs in *A. xylinum* from that in mammalian and yeast systems in that it involves only a segment of the glycolytic pathway, namely the oxidation of triose phosphate formed in the pentose phosphate cycle via the phosphoglyceric acids, pyruvate and acetate (Schramm *et al.*, 1957; Benziman & Abeliovitz, 1964). The net formation of C_4 acids from C_3 precursors, which are essential for the maintenance of the tricarboxylic acid cycle during growth on glucose, is achieved by the action of phosphoenolpyruvate carboxylase (EC 4.1.1.31) (Benziman, 1969*a*). Gluconeogenesis in *A. xylinum*, in the form of cellulose synthesis from tricarboxylic acid-cycle intermediates, differs from that in mammalian, avian and some bacterial systems (Utter, 1969) in that it involves the action of oxaloacetate decarboxylase (EC 4.1.1.3) in conjunction with pyruvate orthophosphate dikinase (EC 2.7.9.1), which catalyses the direct phosphorylation of pyruvate to phosphoenolpyruvate (Benziman & Palgi, 1970; Benziman & Eisen, 1971). Experiments *in vivo* indicate that the oxidative pathways of the tricarboxylic acid cycle with concurrent phosphorylation (Benziman & Levy, 1966) affect the glyconeogenic flux in this organism as well as the flow of sugar carbon into the pentose phosphate cycle (Weinhouse & Benziman, 1972, 1974).

The entry of carbon into the tricarboxylic acid cycle is catalysed by citrate synthase (EC 4.1.3.7). The present paper describes some properties of this enzyme from *A. xylinum*, especially with regard to possible regulatory mechanisms for controlling carbon flow through the various metabolic pathways operative in this organism.

A preliminary report on this work has been published (Swissa & Benziman, 1973).

Materials and Methods

Chemicals

Nucleotides, sugar phosphates, pyruvate, oxaloacetate, 6-phosphogluconate, 2-phosphoglycerate, 2,3-diphosphoglycerate and most of the enzymes were purchased from C. F. Boehringer und Soehne, Mannheim, Germany. Acetyl phosphate, CoA, glyceraldehyde 3-phosphate, phosphoenolpyruvate, *p*-hydroxymercuribenzoate and isocitrate were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Acetyl-CoA was prepared from acetic anhydride and CoA as described by Stadtman (1957).

Cells

The cellulose-synthesizing strain of *A. xylinum* was the same as that used by Schramm & Hestrin

(1954). The culture media, prepared with glass-distilled water, were as follows: substrate (succinate or glucose), 2%; yeast extract (Difco), 0.5%; Bactopeptone (Difco), 0.5%; KH_2PO_4, 0.3%. The succinate medium was adjusted to pH 4.0 with NaOH and the glucose medium to pH 5.0 with HCl. Cells were grown and harvested after 24h as described by Benziman (1969*b*).

Preparation of extracts

Cells suspended in 5mM-Tris/H_2SO_4 buffer, pH 7.4, were treated for 15min in a Raytheon model DF1 magnetorestriction oscillator at 200W and 10kHz. The sonic extract was then centrifuged in the cold at 10000*g* for 15min and the precipitate was discarded. The resulting supernatant fluid is termed 'crude extract'.

Enzyme assays

Enzyme assays involving the measurement of absorbance changes were performed at room temperature (about 22°C) in a Zeiss spectrophotometer or a Gilford model 240 recording spectrophotometer with either 10mm- or 5mm-light-path quartz cells.

Citrate synthase was assayed by following the appearance of thiol groups as the thioester bond of acetyl-CoA is cleaved, either by the increase in E_{412} by the method of Srere *et al.* (1963), which uses 5,5′-dithiobis-(2-nitrobenzoic acid) (method I), or by the decrease in E_{233} (Ochoa, 1957) (method II). In some experiments enzyme activity was assayed by the continuous polarographic measurement of CoA formation as described by Weitzman (1969). In each case, the reaction rate was proportional to enzyme concentration.

In both methods concentrations in the reaction mixtures (1.0ml) were as follows: glycine buffer, pH 8.4, 100mM; freshly prepared oxaloacetate, 0.25mM; acetyl-CoA, 0.16mM. In method I the reaction mixture also contained 0.1mM-5,5′-dithiobis-(2-nitrobenzoic acid). The reaction was started by the addition of enzyme. A unit of enzyme activity is defined as the amount of enzyme that catalyses the formation of 1 μmol of CoA in 1min under the conditions described. The specific activity is defined as the number of units per mg of protein. Other enzymes tested were assayed by the following procedures: ATPase (adenosine triphosphatase) (EC 3.6.1.3), adenylate kinase (EC 2.7.4.3) and oxaloacetate decarboxylase (EC 4.1.1.3), as described by Benziman & Palgi (1970); isocitrate dehydrogenase (NAD^+ and $NADP^+$) (EC 1.1.1.41 and EC 1.1.1.42) as described by Cox (1969) and Cleland *et al.* (1969); citrate lyase (EC 4.1.3.6) as described by Moellering & Gruber (1966); citrate cleavage enzyme (EC 4.1.3.8) by the method of Takeda *et al.* (1969) and phosphotransacetylase (EC 2.3.1.8) by the method of Klotzsch (1969).

Analytical methods

Protein was determined by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as standard. Citrate was determined by the method of Moellering & Gruber (1966), acetyl-CoA by the method of Tubbs & Garland (1969), and oxaloacetate, ATP and ADP as described by Benziman & Palgi (1970).

Molecular-weight determination

The apparent molecular weight of the enzyme was determined by gel filtration (Determann, 1968) with a column (5cm × 100cm) of Biogel A-0.5, previously calibrated with the following protein standards: horseradish peroxidase (EC 1.11.1.7) (mol.wt. 40000); bovine serum albumin (mol.wt. 68000); yeast hexokinase (EC 2.7.1.1) (mol.wt. 105000); rabbit muscle lactate dehydrogenase (EC 1.1.1.27) (mol.wt. 150000); rabbit muscle pyruvate kinase (EC 2.7.1.40) (mol.wt. 237000); *Escherichia coli* β-galactosidase (EC 3.2.1.23) (mol.wt. 515000).

Energy charge

The effects of energy charge $\{([ATP]+\frac{1}{2}[ADP])/([ATP]+[ADP]+[AMP])\}$ (Atkinson, 1968) on enzyme activity were studied by incubating appropriate mixtures of ATP and AMP in a system containing 0.1M-Tris/HCl buffer (pH 8.0) and 10mM-$MgCl_2$ with 10 units of adenylate kinase at 30°C for 10min to reach equilibrium. Portions of these mixtures were then incorporated into the assay mixture to produce the required total adenine nucleotide concentration.

Results

Purification of citrate synthase

The steps reported below were carried out at 0–4°C. A crude extract of succinate-grown cells was centrifuged in a Beckman model L-2 ultracentrifuge at 150000*g* for 60min and the sediment was discarded. The supernatant was applied to a column (1.0cm × 25cm) of DEAE-cellulose (Whatman-DE 52), which was pre-equilibrated with 5mM-Tris/H_2SO_4 buffer, pH 7.4. The column was eluted with a 200ml linear gradient of 0.025–0.15M-KCl in the same buffer. Fractions (6ml) were collected at a flow rate of 60ml/h and assayed for enzyme activity and protein. Citrate synthase was eluted at approx. 0.1M-KCl. Fractions 18–22, containing 50% of the original activity, were combined. Solid $(NH_4)_2SO_4$ was added to 50% saturation (31.3g/100ml) to the combined fractions.

Table 1. *Purification of citrate synthase from Acetobacter xylinum*

Activity was determined by assay method I.

Fraction	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)
Crude extract	14	518.0	352.8	0.68	100
High-speed supernatant	14	288.0	310.8	1.08	88
DEAE-cellulose combined fractions (18–22)	30	12.9	168.0	13.1	48
$(NH_4)_2SO_4$ precipitate (50–70% saturation)	2	4.1	133.4	32.5	38

The salt was added slowly with stirring, after which 15min more was allowed for precipitation. The precipitate was removed by centrifugation at 15000*g* for 15min and discarded. The supernatant solution was then adjusted to 70% satd. $(NH_4)_2SO_4$ (13.7g/100ml), stirred and centrifuged as before. The precipitate was dissolved in a small volume of 0.05M-Tris/H_2SO_4 buffer, pH7.4. This fraction had a specific activity of 32.5, representing a 48-fold purification over the crude extract with 38% recovery of the total activity. The purification procedure is summarized in Table 1.

The purified enzyme preparation was free of malate dehydrogenase, citrate lyase, NADH oxidase, oxaloacetate decarboxylase, acetyl-CoA deacetylase, phosphotransacetylase and isocitrate dehydrogenase activities. When stored in the frozen state the purified enzyme preparation retained most of its activity over a period of 6 months.

Apparent molecular weight

The apparent molecular weight of the enzyme was determined by gel filtration on Biogel A-0.5. It was found to be 280000 by comparing its elution volume with that of known protein standards (see the Materials and Methods section).

Effect of growth medium on enzyme activity

The specific activity of citrate synthase was virtually the same in extracts obtained from cells harvested after 24h growth on either glucose or succinate as sole carbon source.

Stoicheiometry

The stoicheiometry expected for the citrate synthase reaction was observed (0.115μmol of oxaloacetate and 0.112μmol of acetyl-CoA were utilized and 0.110μmol of citrate and CoA were formed by 0.84μg of protein of the purified enzyme in 10min at 23°C).

Effect of pH

Maximum enzyme activity was obtained at pH8.4–8.7. Activity decreased gradually at lower pH values to 50% of maximum activity at pH6.0. No effect of the various buffers tested (sodium acetate, potassium phosphate, glycine/NaOH, piperazine/glycylglycine) was observed at several overlapping pH values. The shape of the pH–activity curve in the pH region 7.5–9.0 was not affected by buffer concentrations up to 200mM.

Effect of uni- and bi-valent cations

Unlike the citrate synthase from some bacteria and higher organisms (Flechtner & Hanson, 1970; Srere, 1971; Johnson & Hanson, 1974), the enzyme of *A. xylinum* was not affected by K^+ and NH_4^+ ions. Addition of these ions (0.02–0.15M) did not increase the activity of an extensively dialysed enzyme preparation, even when assayed at low concentrations of acetyl-CoA (0.02mM) or oxaloacetate (0.01mM). The enzyme was similarly not affected by $MgCl_2$ (1–20mM), $MnCl_2$ (1–10mM), EDTA (1–5mM) or dithiothreitol (1–3mM).

Kinetic constants for substrates

Double-reciprocal plots of velocity against acetyl-CoA concentrations at different concentrations of oxaloacetate are shown in Fig. 1. The apparent K_m values calculated from these plots are 18μM for acetyl-CoA and 8.7μM for oxaloacetate. The K_m of either substrate is independent of the concentration of the other. The apparent K_m values for oxaloacetate and acetyl-CoA did not differ significantly at pH6.0, 8.4 and 9.5. The slopes of the lines obtained in Hill plots (Monod *et al.*, 1963), calculated from the rate-concentration data, indicate that the order of the reaction for both substrates is 1.0.

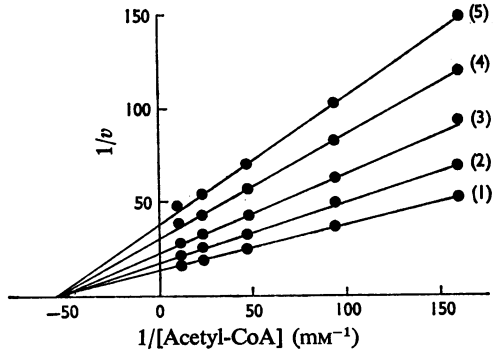

Fig. 1. *Double-reciprocal plot of initial velocity versus acetyl-CoA concentration at various fixed concentrations of oxaloacetate*

The concentrations of oxaloacetate used were: (1), 0.52mM; (2), 0.038mM; (3), 0.019mM; (4), 0.01mM; (5), 0.007mM. The amount of enzyme used per assay was equivalent to 6munits. v is expressed as ΔE_{412}/min (method I).

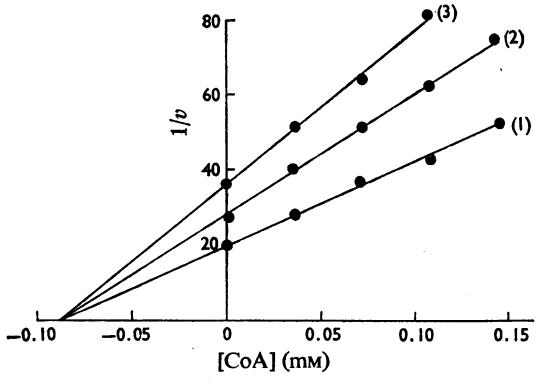

Fig. 2. *Inhibition of citrate synthase by CoA*

Plots of the reciprocal initial velocity against CoA concentration at acetyl-CoA concentrations of 0.133mM (1), 0.03mM (2), and 0.017mM (3), in the presence of 0.25mM-oxaloacetate. The amount of enzyme used per assay was equivalent to 5munits. v is expressed as ΔE_{233}/min (method II).

Effect of reaction products

CoA markedly inhibits the enzyme. The inhibition is non-competitive with respect to acetyl-CoA, with K_i 0.09mM (Fig. 2). The inhibition by CoA is not connected with reversibility of the overall reaction through a mass-action effect, since the inhibition clearly affects the initial velocity of the reaction. Moreover, the extent of CoA inhibition was not affected by varying the concentration of oxaloacetate between 0.05 and 5mM. Citrate at relatively high concentrations also inhibited the enzyme. Thus 10mM-citrate produced 50% inhibition in the presence of an excess of acetyl-CoA and a K_m concentration of oxaloacetate, and 25% inhibition with excess of oxaloacetate and a limiting concentration of acetyl-CoA.

Effect of ATP

The enzyme is inhibited by ATP. Inhibition is linear competitive with respect to acetyl-CoA, with K_i 0.9mM. Hill plots relating velocity and ATP concentration give a slope of 1.0, which is independent of acetyl-CoA concentration. Similarly the slope of the Hill plot for acetyl-CoA is not altered in the presence of ATP. On the other hand, at a fixed concentration of acetyl-CoA, ATP inhibition is non-competitive for oxaloacetate.

In contrast with ATP inhibition of *E. coli* citrate synthase (Srere, 1968; Jangaard *et al.*, 1968), ATP inhibition of the *A. xylinum* enzyme did not vary with pH within the range pH6.0–9.0 with either the concentration of buffer (20–100mM), KCl (20–200mM) or Mg^{2+} (10mM) (cf. Kosicki & Lee, 1966; Flechtner & Hanson, 1969). The extent of ATP inhibition was similarly not affected by 10mM-P_i, 3mM-AMP, 3mM-α-oxoglutarate and 5mM-dithiothreitol.

Other nucleotides were tested for their effect on citrate synthase. In standard reaction mixtures, and in the presence of 18μM-acetyl-CoA, 3mM-ATP produces 65% inhibition and 3mM-ADP produces 35% inhibition, whereas 5mM-AMP, -GTP, -UTP or -CTP do not affect activity. The kinetics of ADP inhibition are similar to those obtained for ATP. Both nucleotides increase the K_m value for acetyl-CoA but do not affect $V_{max.}$. In the presence of an excess of oxaloacetate, the K_m value for acetyl-CoA is increased 3.5-fold by 3mM-ATP and 1.8-fold by 3mM-ADP.

Effect of energy charge

The effect of varying energy charge, at a constant total adenine nucleotide concentration, on the K_m for acetyl-CoA is presented in Fig. 3. The enzyme appears to have a high affinity for acetyl-CoA at low energy-charge values. The K_m value for this substrate is not significantly affected by energy charge in the region 0–0.5, but a sharp decline in affinity occurs in the region of 0.6 charge.

Effect of reduced nicotinamide nucleotides

Unlike the citrate synthase from *E. coli* and other Gram-negative bacteria (Weitzman & Jones, 1968; Flechtner & Hanson, 1970), the enzyme from

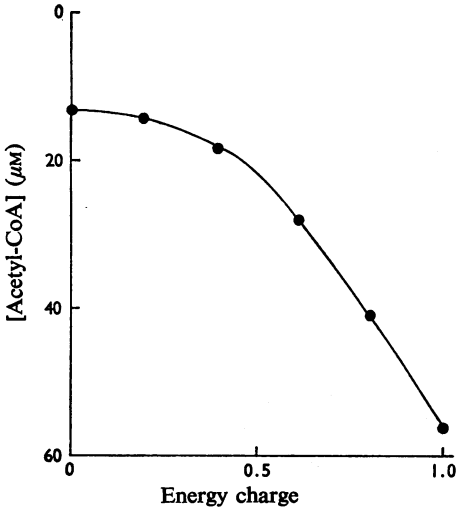

Fig. 3. *Apparent affinity of citrate synthase for acetyl-CoA as a function of energy charge*

Activity was measured by method I. Values plotted are concentrations of acetyl-CoA required for half-maximum velocity in the presence of 0.25mM-oxaloacetate as determined from Lineweaver–Burk plots at each energy-charge value. Total adenylate concentration was 3mM. Desired values of the energy charge were obtained as described in the Materials and Methods section.

A. xylinum is not inhibited by NADH. Activity was not influenced by 2mM-NADH over a wide pH range (7.0–9.5) even when tested at a low acetyl-CoA concentration (15μM). In these experiments enzyme activity was measured both by assay methods I and II and also by the procedure involving the continuous polarographic monitoring of CoASH formation (Weitzman, 1969).

The enzyme is inhibited by NADPH. However, relatively high concentrations of NADPH were required to produce substantial inhibition, 2mM-NADPH being required to produce 50% inhibition. The inhibition by NADPH was dependent on the concentration of acetyl-CoA present but independent of that of oxaloacetate. Thus 1mM-NADPH produced 35% inhibition at 18μM-acetyl-CoA, but no inhibition at 160μM-acetyl-CoA. The degree of NADPH inhibition was not affected by varying the pH between 6.0 and 9.0, nor by the presence of either 100mM-KCl, 100mM-NaCl, 3mM-AMP or 3mM-α-oxoglutarate.

Effect of thiol reagents

Pretreatment of the purified enzyme with *p*-hydroxymercuribenzoate at 25°C for 5min inhibited its activity; 50% inhibition of the enzyme was obtained at 3μM-*p*-hydroxymercuribenzoate. The percentage inhibition was independent of the concentrations of either acetyl-CoA or oxaloacetate. The inhibition by the mercurial compound was immediately reversed by dithiothreitol. Thus an enzyme completely inactivated with 10μM-*p*-hydroxymercuribenzoate regained 80% of its original activity on the addition of 1mM-dithiothreitol.

Unlike the action of thiol reagents on *E. coli* citrate synthase (Weitzman, 1966), the inhibition by *p*-hydroxymercuribenzoate of the *A. xylinum* enzyme could not be prevented nor reversed by the addition of 0.2M-KCl or -NaCl to the enzyme. An enzyme partially inactivated by *p*-hydroxymercuribenzoate retained its sensitivity to inhibition by ATP. The kinetic pattern of ATP inhibition of such an enzyme did not differ from that of untreated enzyme.

Effect of other metabolites

A number of compounds related to carbohydrate metabolism were also tested as effectors. Assays were made at substrate concentrations close to their respective K_m values. Glucose, α-oxoglutarate, malate, succinate, phosphoenolpyruvate, fumarate, isocitrate, acetate, acetyl phosphate, glyceraldehyde 3-phosphate, pyruvate, fructose 6-phosphate, glucose 6-phosphate, fructose diphosphate and 3-phosphoglycerate tested at 1–5mM did not appreciably affect the rate of the reaction.

Discussion

The citrate synthase of *A. xylinum* appears to be constitutive, it being found at equivalent concentrations in cells grown on succinate or glucose. This conclusion is consistent with previous reports on the participation of the tricarboxylic acid cycle in the oxidative metabolism of organic acids and sugars in *A. xylinum* (Gromet *et al.*, 1957; Schramm *et al.*, 1957; Benziman & Burger-Rachamimov, 1962).

The irreversibility of the citrate synthase reaction makes it a susceptible point for metabolic control. As proposed by Garland (1968), citrate synthase may have a regulatory role only in systems where its $V_{max.}$ activity is not in considerable excess of the maximum rate of acetyl-CoA production or oxidation. The *A. xylinum* enzyme appears to fulfil this requirement: the $V_{max.}$ of the enzyme released by sonic disintegration is 0.38μmol/min per mg of cells, whereas whole cells oxidize acetate, in the presence of small amounts of succinate, at a rate of 0.15μmol/min per mg of cells (Weinhouse & Benziman, 1972).

A possible mechanism for controlling the activity of citrate synthase is suggested by its sensitivity to inhibition by ATP. The K_i value (0.9mM) with respect to acetyl-CoA is within the physiological concentration range of ATP. Since ATP is the ultimate end pro-

duct of the tricarboxylic acid cycle, the ATP-linked control of citrate synthase, acting as a negative feedback, may serve as a physiological mechanism by which the entry of acetate into the cycle is kept in step with the energy needs of the cell. Such a mechanism is compatible with the response of the enzyme to the adenylate energy charge (Fig. 3), which is similar to that predicted by Atkinson (1968) for enzymes involved in ATP-regenerating sequences.

Variations in citrate synthase activity in response to changes in the cellular concentration of the different adenine nucleotides may have additional physiological significance when one considers the effect of similar changes on the activities of the key enzymes responsible for the channelling in these cells, of pyruvate into the gluconeogenic route (Benziman & Palgi, 1970) and of phosphoenolpyruvate into either the anaplerotic pathway of oxaloacetate synthesis (Benziman, 1969*a*) or the catabolic route of pyruvate formation (Benziman, 1969*c*).

Citrate synthases from various sources have been shown to differ in their response to ATP, ADP, NADH, α-oxoglutarate, K^+, Mg^{2+} and pH. The pattern of inhibition by various effectors has led Weitzman and his co-workers (Weitzman & Jones, 1968; Weitzman & Dunmore, 1969*a*) to propose that citrate synthases from diverse organisms fall into two or three distinct categories which correlate with established taxonomic divisions. It was further proposed that differences in regulatory behaviour are accompanied by large differences in the molecular sizes of the enzymes (Weitzman & Dunmore, 1969*b*). According to this proposal citrate synthases from Gram-negative bacteria are all sensitive to inhibition by NADH, whereas inhibition by ATP is characteristic of enzymes from Gram-positive bacteria and higher, eukaryotic organisms. The properties of the enzyme from *A. xylinum*, a Gram-negative bacterium, are not consistent with this classification. Thus the enzyme is insensitive to inhibition by NADH under all conditions tested, and on the other hand, it is strongly inhibited by ATP. Further, whereas according to Weitzman & Dunmore (1969*b*) the NADH-insensitive citrate synthases are considerably smaller proteins than the NADH-sensitive synthases, the *A. xylinum* enzyme has a molecular weight of 280000 and is therefore as large a protein as the NADH-sensitive synthases. The non-competitive inhibition of *A. xylinum* citrate synthase by CoA, with respect to acetyl-CoA, is worthy of notice. This is in contrast with the competitive inhibition by CoA of the enzymes from *Azotobacter vinelandii*, *Acinetobacter anitratum* and *Bacillus subtilis* (Johnson & Hanson, 1974).

The citrate synthase from *A. xylinum* is susceptible to inhibition by NADPH. The inhibition appears to be competitive with respect to acetyl-CoA, thus resembling NADH inhibition of citrate synthases from Gram-negative bacteria (Weitzman & Jones, 1968; Flechtner & Hanson, 1970). However, unlike the effect of NADH on these enzymes, the NADPH inhibition was not reversed by AMP, nor was it dependent on pH and ionic strength (cf. Weitzman, 1966; Flechtner & Hanson, 1970). The physiological significance of this inhibition is, however, questionable, considering the relatively high concentration of NADPH required to obtain substantial inhibition.

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