

The Kinetic Properties of Citrate Synthase from Rat Liver Mitochondria

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1. Citrate synthase (EC 4.1.3.7) was purified 750-fold from rat liver. 2. Measurements of the Michaelis constants for the substrates of citrate synthase gave values of $16\ \mu\text{M}$ for acetyl-CoA and $2\ \mu\text{M}$ for oxaloacetate. Each value is independent of the concentration of the other substrate. 3. The inhibition of citrate synthase by ATP, ADP and AMP is competitive with respect to acetyl-CoA. With respect to oxaloacetate the inhibition by AMP is competitive, but the inhibition by ADP and ATP is mixed, being partially competitive. 4. At low concentrations of both substrates the inhibition by ATP is sigmoidal and a Hill plot exhibits a slope of 2.5. 5. The pH optimum of the enzyme is 8.7, and is not significantly affected by ATP. 6. Mg^{2+} inhibits citrate synthase slightly, but relieves the inhibition caused by ATP in a complex manner. 7. At constant total adenine nucleotide concentration made up of various proportions of ATP, ADP and AMP, the activity of citrate synthase is governed by the concentration of the sum of the energy-rich phosphate bonds of ADP and ATP. 8. The sedimentation coefficient of the enzyme, as measured by activity sedimentation, is 6.3 s, equivalent to molecular weight 95 000.

In studies of the control of citrate synthesis in rat liver mitochondria, it was found that palmitoyl-carnitine oxidation in the presence of L-malate proceeded largely to acetoacetate if oxidative phosphorylation was coupled, but to citrate if an uncoupling agent was added. The effect of the uncoupling agent on citrate synthesis was partially reversed by the addition of ATP and oligomycin (Shepherd, Yates & Garland, 1965). These observations led us to investigate the kinetic properties of citrate synthase (EC 4.1.3.7) from rat liver mitochondria, with regard to the possibility that a direct inhibitory effect of ATP on the enzyme was of importance in controlling the rate of citrate synthesis. Preliminary accounts of part of this work have been published (Shepherd & Garland, 1966; Garland, 1968; Garland, Shepherd, Nicholls & Ontko, 1968). Inhibitory effects of ATP on citrate synthase from a number of other sources have been described [*Saccharomyces cerevisiae* and pig heart (Hathaway & Atkinson, 1965); pig heart (Kosicki & Lee, 1966); *Citrus limon* (Bogin & Wallace, 1966); *Saccharomyces carlsbergensis* (Light, 1969); ox liver and ox heart (Jangaard, Unkeless & Atkinson, 1968)].

MATERIALS AND METHODS

Reagents. CoASH, oxaloacetic acid, NAD^+ , NADH, AMP, ADP, ATP and L-malate dehydrogenase (EC 1.1.1.37) were purchased from Boehringer Corp. (London) Ltd. (London W.5), L-malate was from Koch-Light

Laboratories Ltd. (Colnbrook, Bucks.), 5,5'-dithiobis-(2-nitrobenzoic acid) was from Aldrich Chemical Co. Inc. (Milwaukee, Wis., U.S.A.), tris base was from Sigma (London) Chemical Co. Ltd. (London S.W.6) and Whatman DEAE-cellulose from W. and R. Balston Ltd. (Maidstone, Kent). Calcium phosphate gel was prepared as described by Dixon & Webb (1964, p. 42), and acetyl-CoA by the method of Simon & Shemin (1963). A fluorescent impurity present in commercial AMP was removed by ion-exchange chromatography on Dowex 1 (formate form).

Assays. The following reagent assays were used: acetyl-CoA (Srere, Brazil & Gonen, 1963); AMP and ADP (Adam, 1963); ATP (Lamprecht & Trautschold, 1963); oxaloacetate (Hohorst & Reim, 1963); NAD^+ and NADH (Klingenberg, 1963). Citrate synthase was assayed by either of two methods, according to the sensitivity required. In each case the initial velocity was measured from the recorder trace of extinction or fluorescence versus time.

Spectrophotometric assay with 5,5'-dithiobis-(2-nitrobenzoic acid). The method of Srere *et al.* (1963) was used during the purification procedure. In detail, 5–30 milliunits of citrate synthase were assayed in 2.0 ml. of tris-chloride buffer, pH 8.0 (100 mM), containing (final concentrations) 5,5'-dithiobis-(2-nitrobenzoic acid) (0.1 mM), acetyl-CoA (50 μM) and oxaloacetate (50 μM). The last addition was oxaloacetate, and the change in E_{412} was measured in a cuvette of 1 cm. light-path with a Beckman DB spectrophotometer at 25°.

Fluorimetric assay with malate dehydrogenase. This is a modification of the coupled assay described by Ochoa, Stern & Schneider (1951), and the concentrations of L-malate and NAD^+ were lowered to allow the use of fluorimetric measurements of NADH (Shepherd & Garland, 1966) with a modified Eppendorf fluorimeter (Garland, Shepherd & Yates, 1965). The assay mixture of 2.0 ml. at

25° contained tris-chloride buffer, pH 7.4 (100 mM), except where otherwise stated, L-malate and NAD⁺ at concentrations indicated in the legends to the relevant figures, and 50 μg. (36 units) of malate dehydrogenase. The malate dehydrogenase reaction reached equilibrium almost instantaneously, and the resultant fluorescence signal on the

But, by the stoichiometry of reaction (1):

$$\frac{d[\text{malate}]}{dt} = \frac{d[\text{NAD}^+]}{dt} = -\frac{d[\text{NADH}]}{dt}$$

Therefore:

$$\frac{d[\text{oxaloacetate}]}{dt} [\text{NADH}] = -\frac{d[\text{NADH}]}{dt} \{[\text{oxaloacetate}] + K'([\text{NAD}^+] + [\text{malate}])\}$$

or:

$$\frac{d[\text{oxaloacetate}]}{dt} = -\frac{d[\text{NADH}]}{dt} \left(\frac{[\text{oxaloacetate}] + K'([\text{NAD}^+] + [\text{malate}])}{[\text{NADH}]} \right)$$

recorder was reset to zero if necessary. Citrate synthase (20–30 milliunits) was then added, followed by acetyl-CoA to initiate the reaction. Calibration of the fluorimeter was made with NADH that had been assayed spectrophotometrically. Account was taken of internal quenching by making successive additions of NADH to the fluorimeter cuvette containing 2.0 ml. of tris-chloride buffer, pH 7.4 (100 mM), and then constructing a calibration curve relating fluorescence to NADH concentration over the range of interest (0–10 μM).

Stoichiometry of the coupled assay. Criticism has been levelled at the non-stoichiometry of this assay system, and

Provided that no oxaloacetate is present initially:

$$[\text{NADH}] = [\text{oxaloacetate}] = (K'[\text{NAD}^+][\text{malate}])^{\frac{1}{2}}$$

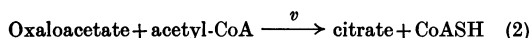
Combining reactions (1) and (2) it can be seen that:

$$v = -\left(\frac{d[\text{oxaloacetate}]}{dt} + \frac{d[\text{malate}]}{dt} \right) \\ = \frac{d[\text{NADH}]}{dt} \frac{d[\text{oxaloacetate}]}{dt}$$

Therefore:

$$v = \frac{d[\text{NADH}]}{dt} \left(1 + \frac{[\text{oxaloacetate}] + K'([\text{malate}] + [\text{NAD}^+])}{[\text{NADH}]} \right) \\ = v_{\text{obs.}} \left(2 + \frac{K'([\text{malate}] + [\text{NAD}^+])}{[\text{NADH}]} \right)$$

Pearson (1965) has derived the factors necessary for correction of acetyl-CoA concentrations assayed by this method. In kinetic studies with citrate synthase it is often necessary to vary the oxaloacetate concentration (i.e. the malate and NAD⁺ concentrations before addition of malate dehydrogenase), and this could alter the stoichiometry between NAD⁺ reduction and citrate synthesis. However, the exact stoichiometry between the initial rate of citrate synthesis and the observed rate, that of NAD⁺ reduction, may be calculated as follows:



Let v be the rate of citrate synthesis, $v_{\text{obs.}}$ be the observed rate, i.e. $d[\text{NADH}]/dt$, and f be the factor of proportionality, $v/v_{\text{obs.}}$. If sufficient malate dehydrogenase is present, then reaction (1) can be taken to be at equilibrium continuously, i.e.:

$$[\text{Oxaloacetate}][\text{NADH}] = K'[\text{malate}][\text{NAD}^+]$$

where K' is the pH-dependent apparent equilibrium constant.

Differentiating with respect to time:

$$\frac{d[\text{oxaloacetate}]}{dt} [\text{NADH}] + \frac{d[\text{NADH}]}{dt} [\text{oxaloacetate}] \\ = K' \frac{d[\text{malate}]}{dt} [\text{NAD}^+] + K' \frac{d[\text{NAD}^+]}{dt} [\text{malate}]$$

Thus:

$$f = 2 + K' \left(\frac{[\text{NAD}^+] + [\text{malate}]}{(K'[\text{NAD}^+][\text{malate}])^{\frac{1}{2}}} \right)$$

or:

$$f = 2 + \left(\frac{K'[\text{NAD}^+]}{[\text{malate}]} \right)^{\frac{1}{2}} + \left(\frac{K'[\text{malate}]}{[\text{NAD}^+]} \right)^{\frac{1}{2}}$$

Hence f will remain constant so long as the $[\text{malate}]/[\text{NAD}^+]$ ratio remains constant. This has been adhered to during the present work, a 20:1 ratio being employed. Since K' is 10^{-5} at pH 7.0, f may be taken to be approx. 2 except when the $[\text{malate}]/[\text{NAD}^+]$ ratio assumes extreme values (10^{-2} or 10^2). K' is pH-dependent and increases tenfold per rise of 1 pH unit. At pH 8.0 f is 2.045 and at pH 9.0 f is 2.14. These errors were not considered worth correcting for in these studies, which were largely carried out at pH 7.4. The error introduced into the pH-dependence curve was not greater than 10% at the highest pH value (9.0). Alternative but less comprehensive corrections have been described by Buckel & Eggerer (1965) and Hardwick (1968).

Purification of citrate synthase from rat liver. Citrate synthase in rat liver is localized intramitochondrially and the first step in the purification of the enzyme was therefore the preparation of mitochondria, since this decreased the amount of starting protein by 50–60% with little loss in activity. Mitochondria were prepared from the livers of 30 rats (Garland *et al.* 1965), except that the two final washings were omitted. The mitochondrial pellet was

resuspended in four times its own volume of potassium phosphate buffer, pH 7.4 (100 mM). Batches (25 ml.) of this 20% suspension were sonicated in stainless-steel centrifuge tubes, cooled by an ice-salt mixture, for 8 min. with a 60 W ultrasonicator (Measuring and Scientific Equipment Ltd., Crawley, Sussex) at 20 kc/sec. The sonicated suspension was centrifuged at 78 000 g for 90 min. at 4° and the precipitate discarded. The supernatants were pooled and brought to 50% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ (31.2 g./100 ml.). The precipitate was removed by centrifugation at 23 000 g for 20 min. at 4° and discarded. The supernatant was brought to 75% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ (17.2 g./100 ml.). The precipitate, after centrifugation as above, was dissolved in 15 ml. of potassium phosphate buffer, pH 7.4 (100 mM), and the solution was dialysed against 5 l. of potassium phosphate buffer, pH 7.4 (20 mM), overnight at 4°. The enzyme solution was placed on a DEAE-cellulose column (40 cm. x 20 cm.) previously equilibrated with potassium phosphate buffer, pH 7.4 (2 mM), and the column was washed with 1 l. of the same buffer. No citrate synthase activity was eluted during this washing, but 150 mg. of protein was removed, including the acetyl-CoA deacylase activity. The column was next washed with 1 l. of potassium phosphate buffer, pH 7.4 (8 mM), and finally with potassium phosphate buffer, pH 7.4 (18 mM). Fractions were collected, and those that contained the bulk of the citrate synthase activity were pooled. To the pooled fractions from the column procedure was added 2 ml. of calcium phosphate gel (18 mg./ml., prepared as described by Dixon & Webb, 1964, p. 42)/100 ml. After mechanical stirring for 1 hr. at 0°, the gel was removed by centrifugation at 25 000 g for 15 min. The enzyme was eluted from the gel with five washings of 10 ml. of potassium phosphate buffer, pH 7.4 (100 mM). The pooled gel eluates were brought to 50% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was centrifuged and discarded. The supernatant was then brought to 75% saturation with solid $(\text{NH}_4)_2\text{SO}_4$ and the precipitate was centrifuged and dissolved in a minimal volume of potassium phosphate buffer, pH 7.4 (100 mM). The enzyme solution was dialysed at 4° overnight against potassium phosphate buffer, pH 7.4 (2 mM), and the precipitate removed by centrifugation.

The purification procedure is summarized in Table 1. The specific activity of citrate synthase prepared as described is 15 units/mg., and by comparison with the specific activity of crystalline citrate synthase from other

sources it would appear to be about 30% pure. It is likely that, with a larger amount of starting material, crystals could be obtained. The main contaminant is malate dehydrogenase (specific activity 33 units/mg.), but this presents no problem since the coupled assay is mainly used in this study. The enzyme has no NADH oxidase or acetyl-CoA deacylase activity, and an adenosine triphosphatase activity of 0.1 unit/mg. occurs only in the presence of Mg^{2+} .

Citrate synthase, prepared as described, is very stable. Dilute solutions in potassium phosphate buffer can be kept at -15° for at least 4 months without any appreciable change in activity, although repeated freezing and thawing leads to loss of activity.

Activity sedimentation. Activity-sedimentation (Cohen, Giraud & Messiah, 1967; Hathaway & Criddle, 1966) experiments were performed with a Spinco model E analytical ultracentrifuge. The cell sector of a synthetic-boundary cell was filled with 0.4 ml. of tris-chloride buffer, pH 7.8 (100 mM), containing (final concentrations) acetyl-CoA (80 μM), oxaloacetate (150 μM) and 5,5'-dithiobis-(2-nitrobenzoic acid) (100 μM); the cap contained up to 2 μg . (30 milliunits) of rat liver citrate synthase, prepared as described, in 0.1 ml. of tris-chloride buffer, pH 7.8 (100 mM). The enzyme was layered over the substrate solution at low speed and then centrifuged at 59 780 rev./min. A 405+436 nm. band filter was used to select the two mercury-arc wavelengths most favourable for the estimation of the thionitrobenzoate anion. Exposures (10 sec.) on Kodak B.10 plates were taken every 8 min. Schlieren optics were not used. Care was taken to exclude any light from the rotor chamber, other than when a photograph was being taken, to minimize the light-sensitive non-enzymic hydrolysis of acetyl-CoA by 5,5'-dithiobis-(2-nitrobenzoic acid) and decay of the thionitrobenzoate anion. A Hilger recording densitometer was used to scan the plates and plot the boundary on a transmission-versus-distance basis. These plots were converted into extinction versus distance and, from the movement of the inflexion point of the front of the colour boundary with time, the sedimentation coefficient for the enzyme, at 21.4°, could be calculated. Since this technique involves the measurement of an active enzyme, care was taken to ensure that substrate was available to the enzyme throughout the experiment. In practice, this was achieved by simulating the conditions of the analytical-ultracentrifuge cell in a spectrophotometer and selecting an enzyme concentration that did not exhaust

Table 1. *Purification of citrate synthase from rat liver*

The specific activity of citrate synthase in this batch of broken mitochondria was about 0.05 unit/mg. and hence the overall purification (at step 7) by this procedure was about 300 from mitochondria or about 750 from whole liver.

Step no.	Purification step	Volume (ml.)	Activity (units)	Protein (mg.)	Specific activity (units/mg.)	Purification	Yield (%)
1	Ultrasonic supernatant	810	635	5780	0.11	1	100
2	50-75%-satd. $(\text{NH}_4)_2\text{SO}_4$	16	143	540	0.27	2.4	22
3	After dialysis	45	280	540	0.5	4.5	44
4	DEAE-cellulose eluate	570	151	35	4.3	39	24
5	$\text{Ca}_3(\text{PO}_4)_2$ -gel eluate	50	97	7.5	13	120	15
6	50-75%-satd. $(\text{NH}_4)_2\text{SO}_4$	2	53	5.5	10	90	8
7	After dialysis	3	40	2.7	15	135	6

the substrates in a typical run time. Lower concentrations of enzyme (15 and 7.5 milliunits) were also used to verify that the sedimentation properties of the enzyme activity did not alter with enzyme dilution.

RESULTS

K_m values for acetyl-CoA and oxaloacetate. The method employed in determining the Michaelis constants was that suggested by Florini & Vestling (1957) for two-substrate enzyme systems. Initial reaction velocities were measured for each of five different concentrations of both substrates, making a total of 25 observations altogether. The effect of varying the second substrate concentration on double-reciprocal plots (Lineweaver & Burk, 1934) for acetyl-CoA and oxaloacetate is shown in Figs. 1 and 2. Lines were generally fitted to the experimental points of double-reciprocal plots by the method of least squares (Wilkinson, 1961). The calculations were performed by computer by using a programme written for the purpose (after Cleland, 1963a) that gave values of V_{\max} and K_m together with their standard deviations for each set of

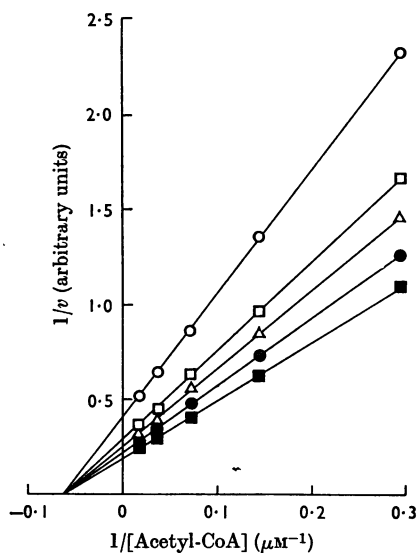


Fig. 1. Double-reciprocal plots of initial reaction velocity against acetyl-CoA concentration for various concentrations of oxaloacetate. The velocity, v , is expressed in arbitrary units, where 1 unit is defined as the production of $1.62 \mu\text{M-NADH/min}$. The concentrations of oxaloacetate were $1.3 \mu\text{M}$ (\circ), $2.5 \mu\text{M}$ (\square), $3.5 \mu\text{M}$ (\triangle), $5.6 \mu\text{M}$ (\bullet) and $10.6 \mu\text{M}$ (\blacksquare), giving K_m values for acetyl-CoA of $16.2 \pm 0.4 \mu\text{M}$, $16.9 \pm 0.7 \mu\text{M}$, $14.9 \pm 0.9 \mu\text{M}$, $15.7 \pm 1.3 \mu\text{M}$ and $16.6 \pm 0.5 \mu\text{M}$ respectively and V_{\max} values of 2.41 ± 0.03 , 3.52 ± 0.06 , 3.91 ± 0.07 , 4.47 ± 0.14 and 5.23 ± 0.06 respectively.

points. It is apparent from Figs. 1 and 2 that the K_m values for acetyl-CoA and oxaloacetate are independent of the concentration of the other substrate. To obtain the best K_m values, the V_{\max} values from Figs. 1 and 2 were taken and replotted

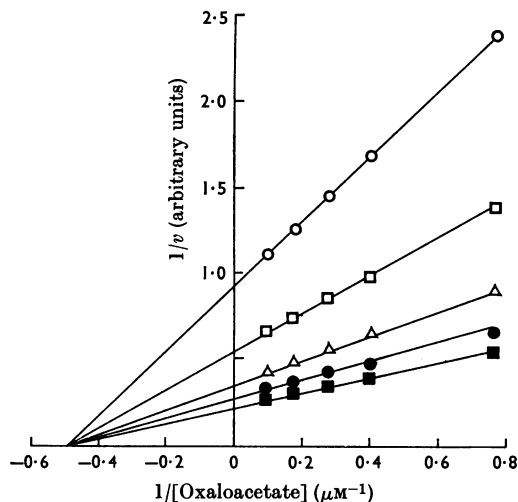


Fig. 2. Double-reciprocal plots of initial reaction velocity against oxaloacetate concentration for various concentrations of acetyl-CoA. The velocity, v , is expressed in arbitrary units, defined in Fig. 1. The concentrations of acetyl-CoA were $3.5 \mu\text{M}$ (\circ), $7.0 \mu\text{M}$ (\square), $14.0 \mu\text{M}$ (\triangle), $27.5 \mu\text{M}$ (\bullet) and $55 \mu\text{M}$ (\blacksquare), giving K_m values for oxaloacetate of $2.03 \pm 0.04 \mu\text{M}$, $1.87 \pm 0.26 \mu\text{M}$, $1.90 \pm 0.17 \mu\text{M}$, $1.96 \pm 0.09 \mu\text{M}$ and $1.96 \pm 0.12 \mu\text{M}$ respectively and V_{\max} values of 1.08 ± 0.01 , 1.83 ± 0.08 , 2.81 ± 0.08 , 3.95 ± 0.06 and 4.71 ± 0.09 respectively.

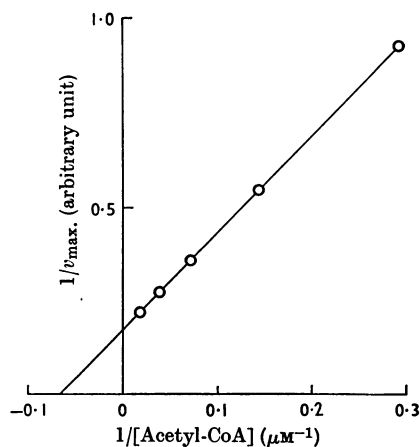


Fig. 3. Double-reciprocal plot of the values of V_{\max} obtained in Fig. 2 against acetyl-CoA concentration. The velocity, V_{\max} , is expressed in arbitrary units, defined in Fig. 1. The value of the K_m for acetyl-CoA was $16.3 \pm 0.8 \mu\text{M}$ and that of the true V_{\max} was 6.16 ± 0.12 .

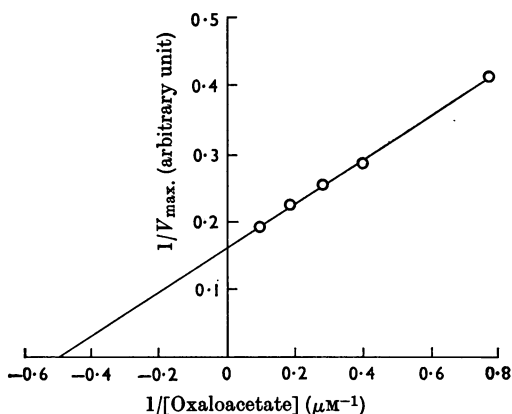


Fig. 4. Double-reciprocal plot of the values of V_{max} in Fig. 1 against oxaloacetate concentration. The velocity, V_{max} , is expressed in arbitrary units, defined in Fig. 1. The value of the K_m for oxaloacetate was $1.99 \pm 0.13 \mu\text{M}$ and that of the true V_{max} was 6.16 ± 0.13 .

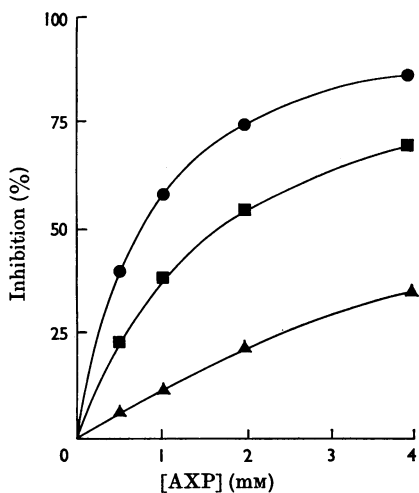


Fig. 5. Inhibition of citrate synthase by ATP (●), ADP (■) and AMP (▲). The concentration of acetyl-CoA was $11 \mu\text{M}$ and that of oxaloacetate was $1.9 \mu\text{M}$.

as a double-reciprocal plot against the appropriate substrate concentration. Hence the V_{max} values from Fig. 2 were used to obtain the K_m for acetyl-CoA as shown in Fig. 3, and those from Fig. 1 were used to obtain the K_m for oxaloacetate as shown in Fig. 4.

The observation that the K_m values are independent of the concentration of the second substrate suggests that the enzyme has a random Bi Bi mechanism (Cleland, 1963b) and is similar to that

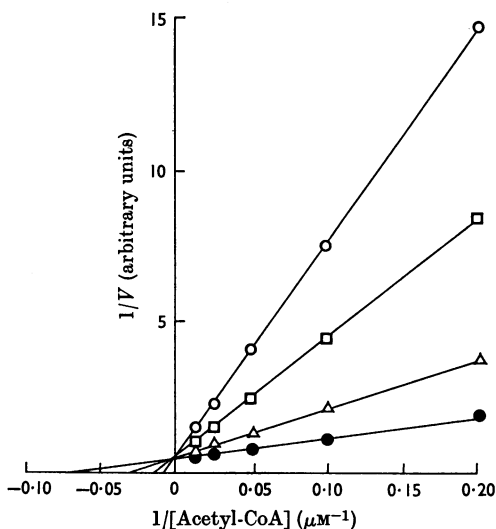


Fig. 6. Double-reciprocal plots of initial reaction velocity against acetyl-CoA concentration to show the effects of 4mM-AMP (Δ), 4mM-ADP (\square) and 4mM-ATP (\circ) on the K_m for acetyl-CoA; \bullet , control with no added adenine nucleotide. The velocity, v , is expressed in arbitrary units, where 1 unit is defined as the production of $0.8 \mu\text{M-NADH/min}$. The concentration of oxaloacetate was $34 \mu\text{M}$. The K_m for acetyl-CoA in the absence of adenine nucleotides was $17.2 \pm 1.2 \mu\text{M}$ and the K_m values in the presence of AMP, ADP and ATP were $27.5 \pm 2.2 \mu\text{M}$, $65.6 \pm 3.8 \mu\text{M}$ and $143 \pm 5 \mu\text{M}$ respectively. The values of V_{max} were not significantly different.

for the enzyme from pigeon breast muscle (Sreere *et al.* 1963). With the enzyme from moth flight muscle the K_m values decrease with increasing concentration of the second substrate (Sreere *et al.* 1963), whereas for pig heart citrate synthase, which appears to have very unusual kinetics, the K_m values increase with increasing concentration of the second substrate. The K_m values for acetyl-CoA and oxaloacetate, $16 \mu\text{M}$ and $2 \mu\text{M}$ respectively, are at the low end of the range for citrate synthases (see the Discussion section and Table 3).

Inhibition of citrate synthase by adenine nucleotides. Citrate synthase from rat liver is inhibited by adenine nucleotides (Fig. 5). The concentrations of acetyl-CoA and oxaloacetate were about the K_m values. The order of inhibition is: ATP > ADP > AMP.

Double-reciprocal plots of the initial reaction velocity against acetyl-CoA concentration in the absence and presence of ATP, ADP or AMP (Fig. 6) demonstrated that the inhibition was competitive with respect to acetyl-CoA, the K_m value $17 \mu\text{M}$ being increased to apparent values of $143 \mu\text{M}$, $66 \mu\text{M}$ and $27 \mu\text{M}$ respectively. From the results of Fig. 6

and at an oxaloacetate concentration of $34\mu\text{M}$ the K_i values for ATP, ADP and AMP were calculated to be 0.55mM , 1.4mM and 6.7mM respectively. These results are in agreement with those for citrate synthase from yeast (Hathaway & Atkinson, 1965), pig heart (Kosicki & Lee, 1966), ox heart and liver (Jangaard *et al.* 1968) and the lemon (Bogin & Wallace, 1966) on the basis of competitive inhibition with respect to acetyl-CoA. However, similar double-reciprocal plots of the initial reaction velocity against oxaloacetate concentration demonstrated the existence of a more complex situation (Fig. 7). AMP appeared to inhibit in a strictly competitive manner, increasing the K_m from $2.0\mu\text{M}$ to an apparent value of $3.5\mu\text{M}$, while the V_{max} was not significantly altered. On the other hand, both ATP and ADP altered the K_m and V_{max} significantly, explicable in terms of a mixed inhibition. There are few references in the literature to K_m values for oxaloacetate (see Table 3) and fewer still to the effect of ATP on them. Indeed, only Hathaway & Atkinson (1965) have reported that 'the apparent affinity of the enzyme (yeast) for oxaloacetate does not seem to be affected by ATP'. The reason for this situation is undoubtedly the difficulty in measuring, by conventional techniques, small apparent differences in a K_m value as low as $2\mu\text{M}$. The results reported here, obtained with a sensitive fluorimeter, are sufficiently accurate to permit unequivocal interpretation.

The inhibition of citrate synthase by ATP at two concentrations of acetyl-CoA is shown in Fig. 8. Inhibition for corresponding concentrations of ATP was noticeably greater at the lower acetyl-CoA concentration. A reciprocal plot of initial reaction velocity against inhibitor concentration, for two or more substrate concentrations, provides a more reliable estimate of K_i values, and indicates additionally whether the inhibition is competitive or non-competitive.

Such a plot for the actual velocities of Fig. 1 is shown in Fig. 9. The K_i for ATP (given by $-[I]$ at the point of intersection of the two plots) was 0.25mM and, since the intersection did not coincide with the abscissa, the inhibition was not competitive with respect to acetyl-CoA, contrary to the results shown in Fig. 6. It may be that the estimate of 0.25mM for the K_i for ATP is a more reliable value than that of 0.55mM obtained from Fig. 6, or, more probably, that the K_i diminishes as the oxaloacetate concentration is decreased, since the oxaloacetate concentration for Fig. 9 was $3.5\mu\text{M}$, whereas that for Fig. 6 was $34\mu\text{M}$.

Similar experiments, but with various oxaloacetate concentrations, are shown in Figs. 10 and 11. It is apparent from Fig. 10 that the variations in oxaloacetate concentration had a smaller effect on the inhibition by ATP than did corresponding

variations in acetyl-CoA concentration, and that at higher oxaloacetate concentrations there was no effect at all. These results are reflected in Fig. 11. The value of the K_i from the intersection of the plots for the high and middle concentrations of

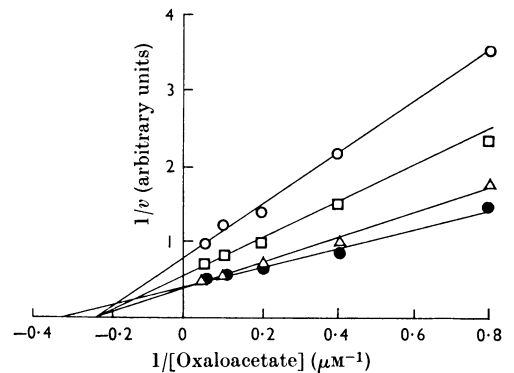


Fig. 7. Double-reciprocal plots of initial reaction velocity against oxaloacetate concentration to show the effects of 4mM -AMP (Δ), 4mM -ADP (\square) and 4mM -ATP (\circ) on the K_m for oxaloacetate; \bullet , control with no added adenine nucleotide. The velocity, v , is expressed in arbitrary units, where 1 unit is defined as the production of $2.1\mu\text{M}$ -NADH/min. The concentration of acetyl-CoA was $84\mu\text{M}$. The K_m for oxaloacetate in the absence of adenine nucleotides was $2.0 \pm 0.3\mu\text{M}$ and the K_m values in the presence of AMP, ADP and ATP were $3.5 \pm 0.6\mu\text{M}$, $4.8 \pm 0.4\mu\text{M}$ and $4.8 \pm 0.7\mu\text{M}$ respectively. The value for V_{max} in the absence of adenine nucleotides was 2.2 ± 0.1 and the V_{max} values in the presence of AMP, ADP and ATP were 2.3 ± 0.1 , 1.9 ± 0.1 and 1.3 ± 0.1 respectively.

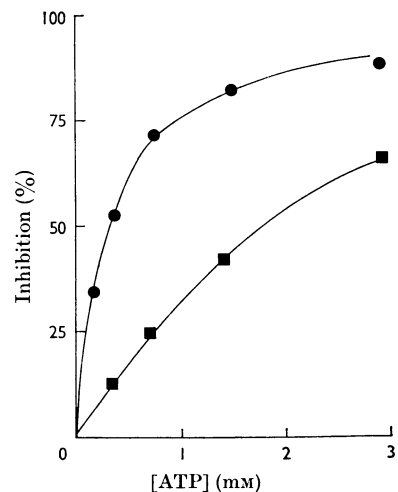


Fig. 8. Inhibition of citrate synthase by ATP at high and low concentrations of acetyl-CoA. The concentration of oxaloacetate was $3.5\mu\text{M}$ and the two concentrations of acetyl-CoA were $2\mu\text{M}$ (\bullet) and $20\mu\text{M}$ (\blacksquare).

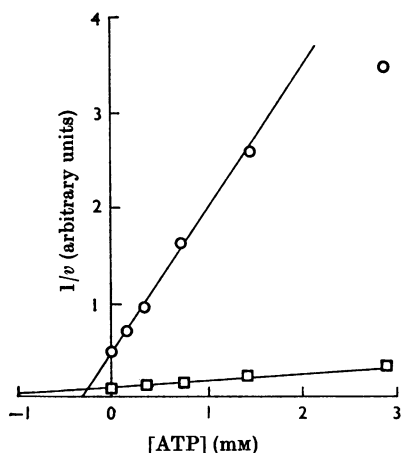


Fig. 9. Reciprocal plot of initial reaction velocity against the concentration of ATP for the data of Fig. 8. The velocity, v , is expressed in arbitrary units, where 1 unit is defined as the production of $10\mu\text{M}\cdot\text{NADH}/\text{min}$. The two concentrations of acetyl-CoA were $2\mu\text{M}$ (\square) and $20\mu\text{M}$ (\circ).

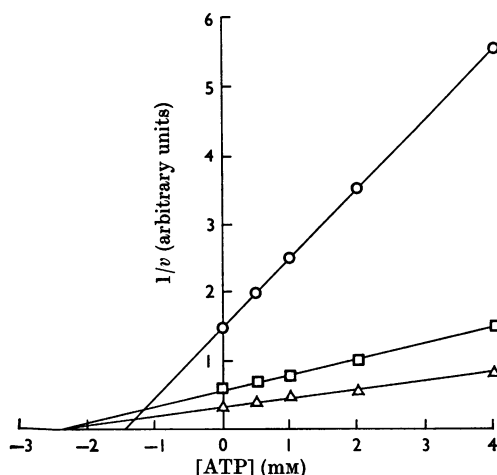


Fig. 11. Reciprocal plot of initial reaction velocity against the concentration of ATP for the data of Fig. 10. The velocity, v , is expressed in arbitrary units, where 1 unit is defined as the production of $0.8\mu\text{M}\cdot\text{NADH}/\text{min}$. The concentrations of oxaloacetate were $0.7\mu\text{M}$ (\circ), $2.8\mu\text{M}$ (\square) and $10.3\mu\text{M}$ (\triangle).

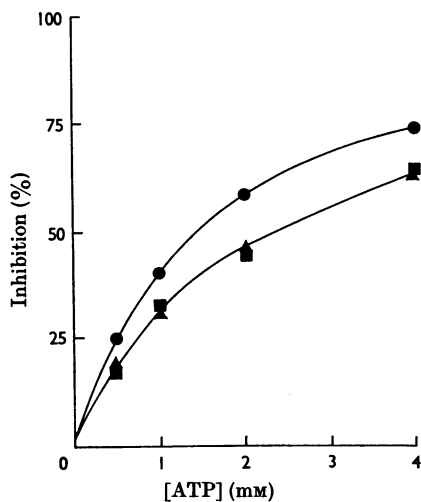


Fig. 10. Inhibition of citrate synthase by ATP at various concentrations of oxaloacetate. The concentration of acetyl-CoA was $60\mu\text{M}$ and the concentrations of oxaloacetate were $0.7\mu\text{M}$ (\bullet), $2.8\mu\text{M}$ (\blacktriangle) and $10.3\mu\text{M}$ (\blacksquare).

oxaloacetate was 2.3mM and the inhibition was strictly non-competitive, since the intersection lies on the abscissa, whereas that for the middle and low oxaloacetate concentrations was 1.2mM and the inhibition had become competitive with respect to oxaloacetate. These results corroborate the conclusion drawn from the results of Fig. 7, that the inhibition by ATP is partially competitive and partially non-competitive with respect to oxaloacetate.

Co-operative effect of ATP inhibition at low substrate concentrations. The inhibition by ATP was investigated at acetyl-CoA and oxaloacetate concentrations of $8\mu\text{M}$ and $1\mu\text{M}$ respectively, i.e. about half of the K_m value for each substrate. The relationship between the extent of inhibition and the concentration of ATP was sigmoidal, implying that this inhibitor is acting co-operatively (Shepherd & Garland, 1966). From these findings the fractional binding of ATP (as the fractional inhibition) was used to form a Hill plot (Hill, 1910; Changeux, 1961), as shown in Fig. 12. The slope of the central section of this plot is 2.5, suggesting the involvement of several binding sites for ATP (Monod, Wyman & Changeux, 1965). Co-operativity has been reported for the inhibition of NADH of citrate synthase from *Azotobacter lwoffii* (Weitzman, 1967), but has not previously been shown for inhibition of citrate synthase by ATP.

Effect of pH on the activity of citrate synthase. The pH optimum for citrate synthase, as measured by the coupled malate dehydrogenase assay in the forward direction, has been reported to be 8.5 (Kosicki & Srere, 1961), and a similar result of 8.7 was obtained for the enzyme from rat liver (Fig. 13). A study of the effect of $2.15\text{mM}\cdot\text{ATP}$ on citrate synthase over the pH range 7.0–9.0 under conditions identical with those described in Fig. 13 showed that percentage inhibition of the enzyme by ATP was constant. Kosicki & Lee (1966) made a similar observation with the pig heart enzyme.

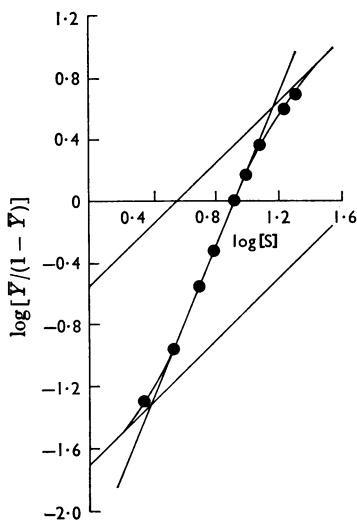


Fig. 12. Hill plot for inhibition of citrate synthase at low substrate concentrations ($8\mu\text{M}$ -acetyl-CoA and $1\mu\text{M}$ -oxaloacetate). Y is the fractional inhibition of the enzyme, and $[S]$ is $10^4 \times$ ATP concentration (M).

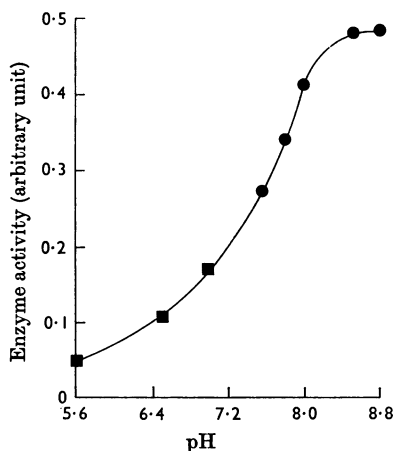


Fig. 13. Effect of varying the pH on the activity of citrate synthase. Substrate concentrations were $21\mu\text{M}$ -acetyl-CoA and $8.6\mu\text{M}$ -oxaloacetate, which was maintained at different pH values by using varied concentrations of L-malate and NAD^+ (although the ratio of these two substrates was constant, as discussed in the Materials and Methods section). The buffers used were 0.1M -tris-chloride from pH 7.6 to pH 8.8 (●) and 0.1M -potassium phosphate from pH 5.6 to pH 7.0 (■). The rates, v , are expressed as $\text{nmoles/min./}1.8\mu\text{g.}$ of protein.

Effect of bivalent metal ions on the inhibition of citrate synthase by ATP. Evidence has been presented (Kosicki & Lee, 1966; Lee & Kosicki, 1967) to show that bivalent metal ions, in addition to

causing a slight inhibition of citrate synthase from pig heart, also decrease the inhibition caused by nucleotides. Rat liver citrate synthase is also affected by bivalent metal ions. The effect due to Mg^{2+} is shown in Fig. 14. When ATP was absent Mg^{2+} caused an inhibition, but this was small, amounting to only 13% at a Mg^{2+} concentration of 4mM . At concentrations of ATP greater than 2mM the effect of Mg^{2+} was to decrease the inhibition caused by ATP in a fairly straightforward manner: the higher the Mg^{2+} concentration, the greater the decrease in ATP-induced inhibition. However, the exact relationship between the Mg^{2+} concentration and the decrease in ATP-induced inhibition at ATP concentrations greater than 2mM is revealing. By comparison of the same enzyme activity obtained for two differing concentrations each of Mg^{2+} and ATP, it would seem that concentrations of 0.5mM -, 1mM -, 2mM - and 4mM - Mg^{2+} appear to bind approx. 0.5mM -, 1mM -, 1.5mM - and 2.5mM -ATP respectively. The stability constant of the MgATP^{2-} complex is 73000M^{-1} (O'Sullivan & Perrin, 1964), and a reasonable explanation of the Mg^{2+} -induced decrease of ATP inhibition might be the formation of this very stable complex and the postulate that this is non-inhibitory. At low concentrations of Mg^{2+} (up to 1mM) this could be the case, since the Mg^{2+} concentration and the apparent decrease in ATP concentration tally. But to explain the results at higher Mg^{2+} concentrations either the MgATP^{2-} complex is an inhibitor or some other Mg^{2+} -ATP inhibitor complex, perhaps enzyme-bound, exists. For example, with starting concentrations of 4mM - Mg^{2+} and 4mM -ATP, the equilibrium concentrations would be 0.23mM - Mg^{2+} -, 0.23mM -ATP and 3.77mM - MgATP^{2-} . Yet the apparent concentration of ATP, to judge from the enzyme activity, was 1.75mM . It should be stated that, with an acetyl-CoA concentration of $12\mu\text{M}$, little Mg^{2+} would be bound to this substrate. The complexity of the situation is emphasized by the results for ATP concentrations less than 2mM . The curves exhibit sigmoidicity and cross each other, as indeed they must, since Mg^{2+} itself is an inhibitor. This was most pronounced at an Mg^{2+} concentration of 2mM , when reactivation followed by severe inhibition occurred as the ATP concentration was increased.

The effect of Ca^{2+} on the ATP inhibition of citrate synthase is fairly similar to that of Mg^{2+} . As shown in Fig. 15, Ca^{2+} itself does not inhibit the enzyme, but does decrease the ATP inhibition, although not to the same extent as Mg^{2+} . In fact a Ca^{2+} concentration of 1mM appears to bind approx. 0.5mM -ATP. Kosicki & Lee (1966) have noted that Ca^{2+} is less efficient than Mg^{2+} at decreasing the ATP inhibition of citrate synthase from pig heart, and have attributed this to the

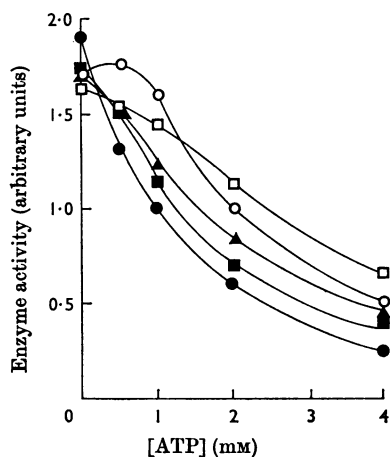


Fig. 14. Effect of Mg^{2+} on the ATP inhibition of citrate synthase. Substrate concentrations were $12\mu M$ -acetyl-CoA and $2.6\mu M$ -oxaloacetate. Activities are expressed as nmol/min./ $1.8\mu g.$ of protein. The concentrations of Mg^{2+} (as $MgCl_2$) were 4 mM (\square), 2 mM (\circ), 1 mM (\blacktriangle), 0.5 mM (\blacksquare) and none (\bullet).

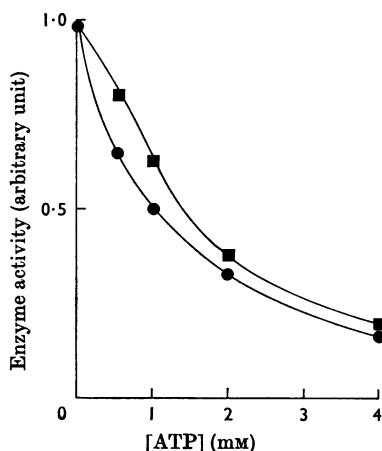


Fig. 15. Effect of Ca^{2+} on the ATP inhibition of citrate synthase. Assay conditions were as for Fig. 14. The concentrations of Ca^{2+} (as $CaCl_2$) were 1 mM (\blacksquare) and none (\bullet).

lower stability constant for the $CaATP^{2-}$ complex of $30000M^{-1}$ (O'Sullivan & Perrin, 1964). But, as with Mg^{2+} , the apparent residual ATP concentrations are too high if a simple non-inhibitory metal ion-adenine nucleotide complex is the only way in which the free ATP concentration is lowered. The effect of very low concentrations of Ca^{2+} (10 – $0.001\mu M$) on the activity of citrate synthase was investigated with the Ca^{2+} -ethylenedioxybis(ethyleneamino)tetra-acetate buffers of Hansford & Chappell (1967), but no effect was detectable.

Three-way plots for the adenine nucleotide inhibition of citrate synthase. The adenine nucleotide concentration of mitochondria is about 10 – 12 nmol/mg. of mitochondrial protein distributed in an aqueous volume of 1 – $2\mu l.$ /mg. of mitochondrial protein (Klingenberg & Pfaff, 1966). As a result of the impermeability of the mitochondrial membrane to adenine nucleotides except by an exchange process, this content remains constant unless specific steps are taken to cause depletion (Meisner, 1967). Since citrate synthase is an intramitochondrial enzyme, and is affected by all three adenine nucleotides, experiments were designed to investigate the behaviour of citrate synthase in a system where the total nucleotide concentration, $[AXP]$, remained constant, but individual nucleotide concentrations were varied.

The results can be presented conveniently in the form of a three-way plot, since there are three variables ($[ATP]$, $[ADP]$ and $[AMP]$) and one restraint ($[AXP]$ is constant). An explanation of the contributory adenine nucleotide concentrations within the three-way plot is given in Fig. 16(a). Points within the triangle correspond to the presence of all three adenine nucleotides at concentrations that may be read off the appropriate scale, points along the sides of the triangle correspond to the situation where only two nucleotides are present and each apex corresponds to ATP, ADP or AMP alone. Although any point within the triangle has a unique combination of adenine nucleotide concentrations, there are lines where the sum of the energy-rich bonds is constant ($ATP \equiv 2 \times \sim P$; $ADP \equiv 1 \times \sim P$; $AMP \equiv 0 \times \sim P$). The direction of these constant $[\sim P]$ lines is shown in Fig. 16(b) and they are calibrated in terms of the 'energy charge' associated with them (after Atkinson, 1967; Atkinson & Walton, 1967), where the energy charge is defined as the fractional phosphorylation of the adenine nucleotide system, i.e. $[\sim P]/2[AXP]$.

In three-way plots of experimental results the adenine nucleotide concentration contours are omitted, and the value of the enzyme activity at a point corresponding to a particular combination of adenine nucleotide concentrations is printed at that point. Such a plot is shown in Fig. 17. The total adenine nucleotide concentration was 5 mM and measurements of enzyme activity were made at intervals of 1 mM for all three nucleotides, there being 21 measurements in all. Continuous contours were drawn through points of equal activity, interpolation being used where necessary. The greater the gradient between contours, the greater the rate of change of activity of citrate synthase with respect to the variables ($[ATP]$, $[ADP]$ and $[AMP]$). It is apparent that the activity contours are almost parallel to the lines of constant $[\sim P]$ and that citrate synthase activity can be controlled more

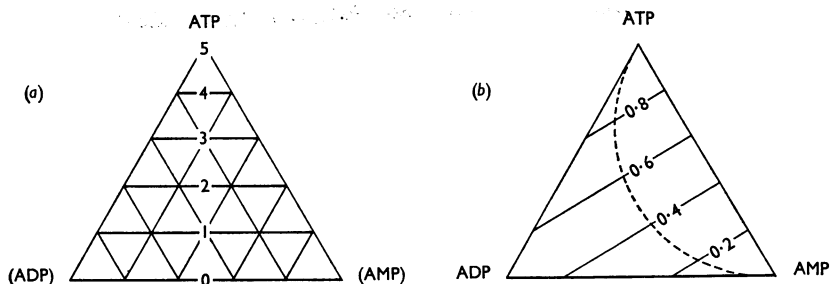


Fig. 16. Key to three-way plots for adenine nucleotide inhibition of citrate synthase. The scale of nucleotide concentrations for the three-way plots of Fig. 17 is shown in (a). The concentration contours, in units of $\mu\text{moles/ml.}$, are shown for ATP as thick lines. The thinner lines for ADP and AMP are left unnumbered for clarity. The total concentration of adenine nucleotides, [AXP], is 5 mM. In (b) is indicated the direction of the lines linking points where the concentration of the sum of the energy-rich bonds is constant (i.e. lines of constant $[\sim P]$). They are numbered in terms of the energy charge associated with them, after Atkinson (1967). The broken line represents the equilibrium of adenylate kinase in the present adenine nucleotide system, assuming a value of 0.8 for the equilibrium constant.

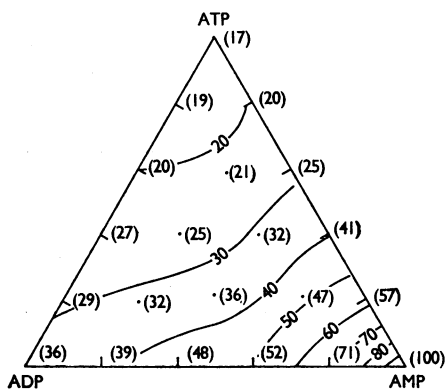


Fig. 17. Three-way plot of the adenine nucleotide inhibition of citrate synthase in the absence of Mg^{2+} . Substrate concentrations were $5\mu\text{M}$ -acetyl-CoA and $5\mu\text{M}$ -oxaloacetate. The total adenine nucleotide concentration, [AXP], was 5 mM. The values in parentheses are the actual enzyme activities at that particular combination of concentrations of ATP, ADP and AMP expressed as percentages of the rate with 5 mM-AMP, which was equivalent to 32 nmoles/min./mg. of mitochondrial protein. Activity contours are plotted through points with the same activity, interpolation being used where necessary.

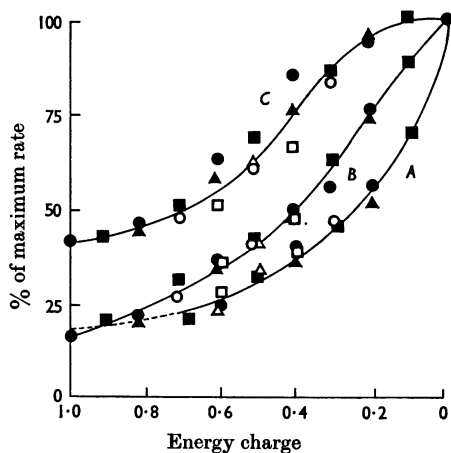


Fig. 18. Relationship between the activity of citrate synthase and the energy charge of 5 mM-adenine nucleotide. Activity is expressed as a percentage of the rate observed at 5 mM-AMP (i.e. the energy charge is zero). The concentrations of Mg^{2+} , acetyl-CoA and oxaloacetate were: curve A, zero, $5\mu\text{M}$ and $5\mu\text{M}$; curve B, 2.0 mM, 6.0 μM and 0.6 μM ; curve C, 2.0 mM, $5\mu\text{M}$ and $5\mu\text{M}$. The 100% rates expressed as nmoles/min./mg. of mitochondrial protein were: curve A, 32; curve B, 12; curve C, 30. In each curve the concentration of ADP was 5 mM (Δ), 4 mM (\square), 3 mM (\circ), 2 mM (\blacktriangle), 1 mM (\blacksquare) and zero (\bullet). Each symbol belongs to the nearest curve.

closely by changes in $[\sim P]$ rather than in one adenine nucleotide in particular.

Accordingly, the enzyme activities from Fig. 17 were replotted against the energy charge of the corresponding combination of adenine nucleotide concentrations as shown in Fig. 18 (curve A). From the grouping of the points it is apparent that enzyme activity is proportional to energy charge under the conditions of assay.

As shown in Fig. 14, Mg^{2+} , particularly at a concentration of 2 mM, imparts a sigmoidicity to the relationship between ATP concentration and the inhibition of citrate synthase, and it might be expected that the presence of Mg^{2+} in a three-way plot experiment would, by lessening the inhibition

at lower concentrations of ATP, alter the activity contour pattern. The experiment of Fig. 17 was therefore repeated in the presence of 2mm-magnesium chloride and the activity contours were shifted in the direction of ATP, but remained parallel to the lines of constant $[\sim P]$. The results are shown as plots of enzyme activity versus energy charge in Fig. 18 (curve *C*). In this experiment the greatest inhibition was only 60%, and since the concentration of oxaloacetate at $5\mu\text{M}$ was considerably above the likely intramitochondrial concentration (see the Discussion section) the experiment was repeated, again in the presence of Mg^{2+} , but at an oxaloacetate concentration of $0.6\mu\text{M}$. In the plot against energy charge (Fig. 18, curve *B*) the maximum inhibition is now increased to 85% and the curve again shows sigmoidicity, if a little less pronounced than in curve *C*.

DISCUSSION

Regulatory role of citrate synthase. Our interest in the kinetic properties of this enzyme arose from the well-documented observation that citrate synthesis in rat liver mitochondria can be inhibited during fatty acid oxidation. It has usually been assumed (e.g. Wieland, Weiss & Eger-Neufeldt, 1964) that the increased flow of acetyl-CoA to acetoacetate in this condition is a result rather than a cause of the diminished rate of citrate synthesis. Attempts to provide an enzymic basis for the control of acetoacetate synthesis in rat liver mitochondria have therefore been directed largely at citrate synthase rather than the less well-characterized enzymes of acetoacetate synthesis. The development of techniques capable of measuring steady-state concentrations of intramitochondrial acetyl-CoA during fatty acid oxidation (Garland *et al.* 1965) provided the first clear evidence that citrate synthesis was inhibited by a mechanism other than an activation of an alternative route of acetyl-CoA utilization (i.e. acetoacetate synthesis).

Although a regulatory role seems established for citrate synthase in rat liver mitochondria, the same cannot readily be said for the enzyme in a number of other situations. This much is apparent from a comparison of the maximal demand on the enzyme in the intact tissue with the maximum activity of the enzyme. Such a comparison for three sources is made in Table 2, and it is apparent that, whereas in rat liver the actual flow through citrate synthase is not greatly in excess of the maximal activity of the enzyme, in rat heart and *Saccharomyces carlsbergensis* the flow rate is considerably less (20–40-fold) than the maximal enzyme activity. A final decision as to whether citrate synthase is regulatory or not in any given situation can be made only on the basis of experiments with intact preparations. No evidence of this sort has been presented, to our knowledge, for any source other than mammalian liver, and it may be that citrate synthase is regulatory only in this tissue, which is unique in its capacity to convert acetyl-CoA into acetoacetate rather than citrate. If this is so, it can be anticipated that the kinetic properties of citrate synthase of liver would be different from that of other sources, and any attempt (Williamson, Browning & Olson, 1968) to explain the regulation of citrate synthesis in liver on the basis of the properties of the enzyme from other sources (e.g. pig heart; Kosicki & Lee, 1966) is potentially misleading.

Comparison with citrate synthase from other sources. Table 3 summarizes the findings of workers in several laboratories on citrate synthase from other sources and compares them with those described here for the rat liver enzyme. Clearly there is a wide variety of properties, and no general conclusions can be drawn.

Kinetic studies at low concentrations of oxaloacetate. Technical difficulties arise in measuring the enzyme activity at the K_m and lower values of oxaloacetate concentration if the K_m value itself is low (e.g. $2\mu\text{M}$). These difficulties are overcome by using a fluorimeter of suitable sensitivity, or, for

Table 2. *Characteristics of citrate synthesis and citrate synthase from several sources*

References: ¹ Srere & Kosicki (1961); ² Scholz & Bücher (1965); ³ Shepherd (1968); ⁴ Garland, Newsholme & Randle (1962); ⁵ Pette (1966); ⁶ Schollmeyer & Klingenberg (1961); ⁷ D. Shepherd & P. B. Garland (unpublished work); ⁸ P. A. Light (unpublished work). Further details are given in the text.

Source	Whole tissue ($\mu\text{moles/min./g. fresh wt.}$)		Isolated mitochondria ($\text{nmoles/min./mg. of protein}$)	
	Extractable activity	Flow rate in intact tissue	Extractable activity	Maximal flow rate in intact mitochondria
Rat liver	2.8 ¹	2.1–2.4 ²	100 ³	50 ³
Rat heart	39 ¹	2.2 ⁴	1600 ^{5,6}	70 ⁷
<i>Saccharomyces carlsbergensis</i>	—	—	3500 ⁸	85 ⁸

Table 3. *Kinetic properties of citrate synthase from various sources*

References: ¹ Kosicki & Srere (1961); ² Kosicki & Lee (1966); ³ Lee & Kosicki (1967); ⁴ Bogin & Wallace (1966); ⁵ Srere *et al.* (1963); ⁶ Hathaway & Atkinson (1965); ⁷ Weitzman (1966); ⁸ this paper; ⁹ Jangaard *et al.* (1968). The values of K_i are with respect to acetyl-CoA. — means no data available. Comp. means competitive.

Source	K_m (μM)		Interdependence of K_m values	Inhibition by ATP	Form of inhibition by ATP	K_i for ATP (mM)
	Acetyl-CoA	Oxaloacetate				
Pig heart	11* ¹	16* ¹	Yes ¹	Yes ^{2,3}	Comp. with acetyl-CoA ²	0.52 ² , 0.67 ³
Lemon ⁴	—	—	—	Yes	Comp. with acetyl-CoA, non-comp. with oxaloacetate	—
Moth flight muscle ⁵	200*	~10	Yes	—	—	—
Pigeon breast muscle ⁵	180*	~1	No	—	—	—
<i>Saccharomyces cerevisiae</i> ⁶	~2	—	—	Yes	Comp. with acetyl-CoA, non-comp. with oxaloacetate	0.11†
<i>Escherichia coli</i> ⁷	500	—	No	—	—	—
Rat liver ⁸	16*	2*	No	Yes	Comp. with acetyl-CoA, mixed with oxaloacetate	0.25
Ox liver ⁹	5.8	6	Yes	Yes	Comp. with acetyl-CoA only	—

* The true value at infinite concentration of the second substrate is given. Other values are apparent.

† Calculated from the published data.

the spectrophotometric assay, a sensitive apparatus such as a dual-wavelength spectrophotometer. A strong reason for studying citrate synthase at low concentrations of oxaloacetate arises from the results in Fig. 10, which demonstrate that the manner in which ATP inhibits citrate synthase depends on the concentration of oxaloacetate. At an oxaloacetate concentration of 0.7 μM the inhibition by ATP was partially competitive with respect to oxaloacetate, whereas at 2.8 μM - and 10.3 μM -oxaloacetate the inhibition was non-competitive. It has been calculated that the intramitochondrial concentration of oxaloacetate is between 0.1 and 0.5 μM (Williamson, Lund & Krebs, 1967), and the kinetic properties of citrate synthase at these liver concentrations of oxaloacetate are to be preferred when considering the properties of the enzyme *in vivo*. Using an alternative approach to that of Williamson *et al.* (1967), Garland *et al.* (1968) also concluded that the intramitochondrial concentration of oxaloacetate was at or below its K_m value with citrate synthase.

K_m value for acetyl-CoA. Shepherd *et al.* (1965) showed that, in mitochondria converting palmitoyl-carnitine into citrate at a variety of rates, not more than half the maximal rate of citrate synthesis was obtained at an intramitochondrial acetyl-CoA content of 0.25 nmole of acetyl-CoA/mg. of mitochondrial protein. Calculated on the basis that all the acetyl-CoA was in the intramitochondrial water

content of 1 $\mu\text{l.}/\text{mg.}$ of protein, it follows that not more than half the maximal rate of citrate synthesis was obtained at an intramitochondrial acetyl-CoA concentration of 0.25 mM. This value contrasts markedly with the K_m value of 16 μM obtained with purified citrate synthase (Fig. 3), and it is not currently clear whether this discrepancy is due to modification of the enzyme properties or our failure to make the correct assumptions about the intramitochondrial distribution and intramitochondrial activity coefficient of acetyl-CoA (see also Garland *et al.* 1968).

Activity-sedimentation studies. In the absence of ATP the sedimentation coefficient of citrate synthase was 6.3 s at 21.4°. This value was the same at all three enzyme concentrations used. Assuming a frictional ratio of 1.15, the molecular weight was estimated to be 95 000. The sedimentation coefficient was unaltered by the presence of 4 mM-ATP, but the profile of the boundary flattened more rapidly. This suggests that ATP had increased the diffusion coefficient of the enzyme. Although these observations are preliminary and incomplete, they are reported because they illustrate the convenience with which a CoASH-releasing enzyme can be studied by a combination of activity sedimentation (Cohen *et al.* 1967) and the 5,5'-dithiobis-(2-nitrobenzoic acid) assay for thiols (Ellman, 1959).

Intramitochondrial concentration of citrate synthase. If it is assumed that the specific activity of

pure citrate synthase is 45 units/mg., then it may be calculated that a mitochondrial activity of 0.1 unit/mg. of mitochondrial protein corresponds to 2.2 μ g. of citrate synthase/mg. of mitochondrial protein. Taking an intramitochondrial water content of 1 μ l./mg. of protein and a molecular weight for citrate synthase of 95000, it follows that uniform distribution of the enzyme in the available water would give an enzyme concentration of 23 μ M (see also Srere, 1968). This concentration is two orders of magnitude greater than the calculated intramitochondrial concentration of oxaloacetate, and three to four orders of magnitude greater than the citrate synthase concentration used in both the kinetic and activity-sedimentation experiments.

Inhibition of citrate synthase by adenine nucleotides. The inhibition of citrate synthase by adenine nucleotides increases with their degree of phosphorylation, AMP being the least effective inhibitor and ATP the most. The structural similarities between adenine nucleotides and acetyl-CoA may explain their competitive behaviour. However, no such similarity is apparent between ATP and oxaloacetate, and hence the competitive inhibition by ATP with respect to oxaloacetate is allosteric in the sense originally used by Monod & Jacob (1961) and Changeux (1961). Liver is the only tissue where strong evidence is available in support of the idea that the activity of citrate synthase is rapidly and directly modified ('fine control'; Ashworth & Kornberg, 1963) in response to metabolic changes, and it may therefore be relevant to such a regulatory role that the maximal activity of citrate synthase in liver is close to the operating activity, and the inhibition by ATP is competitive not only with acetyl-CoA but also with oxaloacetate. These two features have not been observed with citrate synthase from other sources.

There are two possible ways in which the inhibition of citrate synthase by ATP could be of regulatory significance. In the first, the intramitochondrial concentration of each adenine nucleotide would be constant and cause a constant increase in the K_m values for acetyl-CoA and oxaloacetate. Although such a mechanism would not in itself cause changes in the rate of citrate synthesis, it would require to be taken account of in making a quantitative assessment of the effects of any other regulatory mechanism. It is conceivable that the apparently high K_m value of intramitochondrial citrate synthase for acetyl-CoA is due to the effects of intramitochondrial adenine nucleotides, which have a calculated concentration of 12mM for the sum of ATP, ADP and AMP (Klingenberg & Pfaff, 1966). The second way in which the inhibition of citrate synthase by adenine nucleotides could be of regulatory significance would involve changes in their phosphorylation such that the enzyme was

inhibited (increased phosphorylation of adenine nucleotides) or activated (decreased phosphorylation). Whether or not such a regulatory mechanism exists can be indicated only on the basis of experiment with more complex systems such as isolated mitochondria or the perfused liver. The evidence on this point is conflicting (Garland *et al.* 1968; Williamson *et al.* 1968), and a decisive answer will probably have to await experimental techniques that permit measurements of the true concentrations of metabolites at intramitochondrial and intracellular enzyme sites.

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