# *Escherichia coli* Citrate Synthase. Purification and the Effect of Potassium on Some Properties*

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ABSTRACT: Citrate synthase (EC 4.1.3.7) which catalyzes the condensation of acetyl-coenzyme A with oxaloacetate to form citrate, was purified from *Escherichia coli* extracts. The purified enzyme was homogeneous as judged by ultracentrifugation. Potassium ion markedly increased the stability of the enzyme and lowered the apparent $K_m$'s for acetyl-coenzyme A and oxaloacetate. These effects were not specific for potassium, and other monovalent cations were also effective in the decreasing order $NH_4^+ > Na^+ > Li^+ = (CH_3)_4N^+$. The enzyme showed normal Michaelis–Menten kinetics ($K_m$ for acetyl-coenzyme A, $1.1 \times 10^{-4}$ M; $K_m$ for oxaloacetate, $2.1 \times 10^{-5}$ M) at 0.1 M $K^+$; while lower substrate affinities and nonlinear kinetics were observed when $K^+$ was in low concentration or absent from the reaction mixture. The pH optimum of the enzyme was 8.0. The molecular weight, estimated by gel filtration, was 280,000 daltons, and was independent of the concentration of potassium in the eluent. A shift in the ultraviolet absorption spectrum was observed when potassium was added to the salt-free enzyme.

Thus, the enzyme does not aggregate or dissociate in the absence of potassium, but undergoes an apparent conformational change which significantly alters its substrate affinities and sensitivities to various inhibitors.

Several recent reports have appeared on the effect of possible modifiers of *Escherichia coli* citrate synthase activity. Inhibitions by NADH (Weitzman, 1966a) and $\alpha$-ketoglutarate (Wright *et al.*, 1967), two proposed regulators of this enzyme, were reversed by the addition of KCl. The effect of adenine nucleotides was found to be highly dependent upon pH (Jangaard *et al.*, 1968). We had reported earlier that palmityl-CoA is a potent inhibitor (Srere and Whissen, 1967), and its effect is also modulated by KCl. We have also reported that potassium modifies the effect of ATP and NADH on the enzyme (Srere, 1968). Since the properties of *E. coli* citrate synthase seem to be markedly dependent upon the assay conditions, we have studied the effect of KCl and other salts on some of the kinetic, chemical, and physical properties of highly purified *E. coli* citrate synthase.

## Methods and Materials

*E. coli* grown on rich media and harvested at $^3/_4$ log phase were obtained from Grain Processing, Muscatine, Iowa. Acetyl-CoA was prepared by the addition of acetic anhydride to a solution of CoA (Pabst Laboratories) (10 mg/ml) in 0.1 M Tris base (Simon and Shemin, 1953). In some experiments acetyl-CoA (lithium Salt) (Pabst) was used. DTNB[1] was purchased from Aldrich Chemical Co., Inc. DEAE-cellulose (Brown, Co.) was purified as described by Uyeda (1962). Visking dialysis tubing was treated with saturated $Na_2CO_3$ at 100°; and washed with 95% ethanol and then with 0.01 M EDTA before use. Polyacrylamide disc gel electrophoresis was performed according to Davis (1964), using the Canalco Model 12 apparatus. A Spinco Model E was used for analytical ultracentrifugation. Measurements of difference spectra were made with a Cary 15 spectrophotometer. Potassium concentrations were determined with a Perkin-Elmer atomic absorption spectrophotometer, Model 303. Ultrafiltration was performed with a Diaflo apparatus with an XM-50 ultrafilter (Amicon Corp.).

The enzyme was routinely assayed by the DTNB method (Srere *et al.*, 1963). The standard assay mixture contained 150 $\mu$M DTNB, 100 mM Tris-Cl buffer (pH 8.1), 500 $\mu$M acetyl-CoA, 500 $\mu$M oxaloacetate, and 100 mM KCl. The reaction rate was followed at 27° with a Gilford recording spectrophotometer. The increase in absorbance at 412 nm was proportional to enzyme concentration over a wide range and was linear for up to 10 min, at rates less than 0.05 $A$/min. Incubation with or without potassium at 27° with DTNB for short periods of time (<5 min) did not effect the initial velocity. The 233-nm assay (Srere and Kosicki, 1961) was also employed for certain experiments, and the reaction rates were identical with those obtained with the DTNB assay. Protein was estimated from the absorbance at 260 and 280 nm (Warburg and Christian, 1941). Specific activities are expressed as $\mu$moles of CoA formed/1 mg of protein/min.

## Results

*Purification.* Frozen *E. coli* were lyophilized and stored at −40°. All steps were carried out at 0–4° unless otherwise specified.

STEP 1. SONIC TREATMENT. Lyophilized cells (130 g) were rapidly dispersed in five volumes of 0.1 M potassium phosphate buffer (pH 7.0) in a Waring Blendor. The suspended cells were placed in a beaker surrounded by an ice bath and with constant stirring were disrupted by sonication with a Bronwill Biosonik set at maximum output. A few drops of

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[1] Abbreviation used is: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

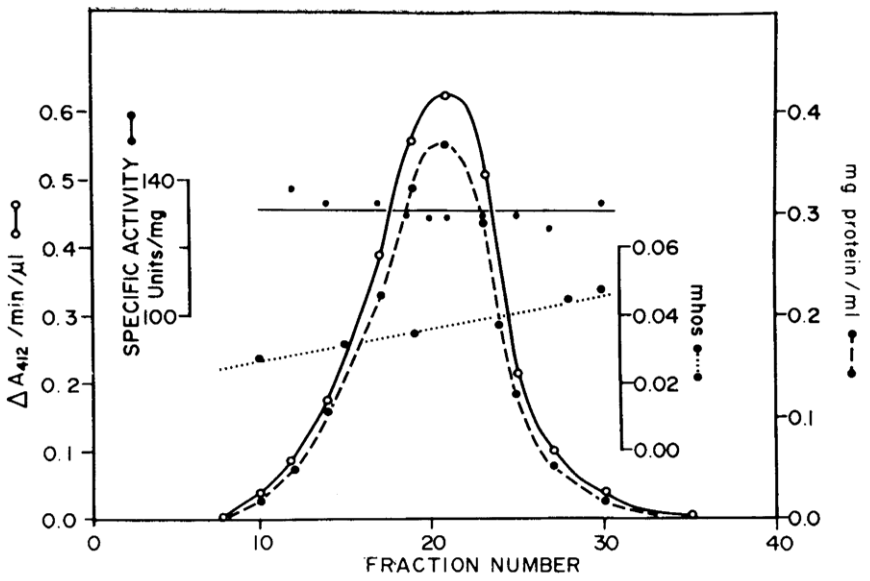



FIGURE 1: Gradient elution from DEAE at constant specific activity. The purified enzyme was added to a 0.9 × 10 cm DEAE-cellulose column equilibrated at 0.01 M $KPO_4$ (pH 6.9)–0.5 mM EDTA. After washing with the same buffer at 0.1 M KCl, a linear gradient was established between 0.1 and 0.25 M KCl. Fractions (4 ml) were collected.

antifoam 60 silicone emulsion were added to the suspension to prevent foaming during sonication. The probe was set a few centimeters below the surface of the suspension and $H_2O$ at 4° was circulated through a coil surrounding the probe. Cell breakage, measured by soluble protein and citrate synthase activity, was complete after 120–150 min. The sonicated suspension was centrifuged at 8000 rpm (14,000*g*) for 2 hr and the supernatant fluid was decanted and retained.

STEP 2. PROTAMINE SULFATE. Protamine sulfate (16 g) as a 2% solution were added slowly with constant stirring. After 10 min of additional stirring the precipitate was separated by centrifugation and discarded.

STEP 3. AMMONIUM SULFATE. Solid ammonium sulfate was added (31.4 g/100 ml) and the mixture was stirred for 30 min. The precipitate was removed by centrifugation at 14,000*g* for 1 hr. Ammonium sulfate (13.5 g) was then added to each 100 ml of supernatant solution and the precipitate was separated by centrifugation, and dissolved in a minimal amount of 0.025 M Tris-Cl (pH 8.1), 0.05 M KCl, and 0.5 mM EDTA solution. The enzyme solution was dialyzed against the same buffer at 4° for 48 hr during which the buffer was changed several times.

STEP 4. DEAE-CELLULOSE CHROMATOGRAPHY. After centrifugation to remove any precipitated protein, the dialysate was added to a 2 × 40 cm DEAE-cellulose column previously equilibrated to identical pH and conductivity with the same buffer used for dialysis. After loading, the column was washed with about 500 ml of a solution of 0.1 M KCl, 0.05 M Tris-Cl (pH 8.1), and 1 mM EDTA. A linear gradient was then established between 0.1 and 0.2 M KCl both at 0.05 M Tris-Cl (pH 8.1). The total volume of eluted gradient was 1 l. The citrate synthase activity eluted between 0.12 and 0.14 M potassium. The fractions containing the activity were pooled and the protein concentrated by ultrafiltration.

STEP 5. HEAT STEP. This solution was adjusted to final

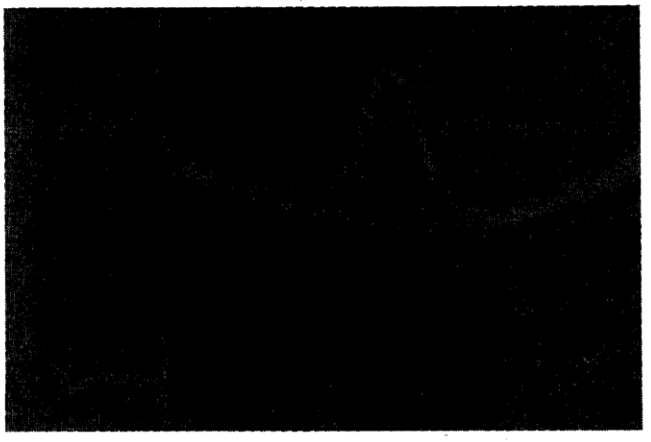


FIGURE 2: Sedimentation velocity profile of *E. coli* citrate synthase. The photograph was taken 82 min after reaching 42,040 rpm at bar angle of 50°. The double-sector cell contained 3.5 mg/ml of enzyme in 1 M KCl and 0.1 M Tris-Cl (pH 8.1). $s_{app}$ = 8.0 S.

TABLE 1: Purification Summary for *E. coli* Citrate Synthase.

| | Total Units | Sp Act. | % Yield |
|---|---|---|---|
| Sonicate supernatant | 22,000 | 0.4 | 100 |
| Protamine $SO_4$ | 20,000 | 0.6 | 91 |
| 50–70% $(NH_4)_2SO_4$ | 18,000 | 2.2 | 82 |
| DEAE-cellulose chromatography | 12,000 | 21 | 54 |
| 65°, 5 min and $(NH_4)_2SO_4$ | 10,000 | 68 | 45 |
| Gel filtration G-200 Sephadex | 6,000 | 150 | 27 |

TABLE II: Protection Against Urea Denaturation.[a]

| Effector | Concn (M) | % Act. at 60 min | % Act. at 20 hr |
|---|---|---|---|
| None | | 0 | 0 |
| KCl | 0.08 | 2 | 0 |
| KCl | 1.0 | 85 | 35 |
| KCl | 2.0 | 100 | 90 |
| $(NH_4)_2SO_4$ | 0.08 | 5 | 0 |
| $(NH_4)_2SO_4$ | 1.0 | 77 | 30 |
| Oxaloacetate | $4 \times 10^{-4}$ | 1 | 0 |
| Oxaloacetate | $5 \times 10^{-3}$ | 60 | 0 |
| Acetyl-CoA | $3 \times 10^{-3}$ | 1 | 0 |
| α-Ketoglutarate | $2 \times 10^{-2}$ | 1 | 0 |
| Citrate | $10^{-2}$ | 2 | 0 |
| NADH | $1.5 \times 10^{-3}$ | 3 | 0 |
| ATP | $10^{-3}$ | 1 | 0 |

[a] Citrate synthase (0.30 unit) was incubated at 25° in 0.05 M Tris-Cl (pH 8.1)–4 M urea, and the given concentration of effector. Values are expressed as per cent of original activity remaining; control samples without urea did not lose activity under these conditions.

concentrations of 0.1 M Tris-Cl (pH 8.1), 2 M KCl, 1 mM EDTA, and about 30 mg of protein/ml.

This solution was brought to 65° in a boiling-water bath and then transferred to a 65° $H_2O$ bath for exactly 5 min with constant stirring, then submerged in an ice bath for 30 min, and the denatured protein then was removed by centrifugation. The enzyme was precipitated from the supernatant by the addition of two volumes of saturated ammonium sulfate solution. The precipitate was collected by centrifugation and resuspended in 45% saturated $(NH_4)_2SO_4$. After stirring for 10 min the insoluble protein was removed by centrifugation and about 90% of the enzyme activity present before the heat treatment was recovered in the supernatant.

STEP 6. GEL FILTRATION. This solution was concentrated by precipitation at 80% saturated ammonium sulfate or by ultrafiltration and was added to a (2.5 × 80 cm) G-200 Sephadex column, previously equilibrated with the same Tris–KCl–EDTA buffer used in step 3. Fractions (10 ml) were collected at a flow rate of about 10 ml/hr. The enzyme activity was eluted with the first protein peak at nearly constant specific activity. These fractions were pooled and concentrated by precipitation with ammonium sulfate or by ultrafiltration. The specific activity of the final preparation was about 150 units/mg with an over-all purification of about 375-fold. A typical purification scheme starting with 135 g (dry weight) of *E. coli* is summarized in Table I.

In some preparations the protamine sulfate step was omitted and the heat step was performed immediately after the ammonium sulfate fractionation. This procedure resulted in a specific activity after heat treatment of about 12, and DEAE chromatography and gel filtration gave a final specific activity of 125–140 with a slightly lower yield.

*Homogeneity*. Attempts to purify the enzyme further were not successful. The enzyme eluted from DEAE-cellulose at pH 6.9 as a single component at constant specific activity (Figure 1).

Gel filtration on Bio-Gel P-300 also gave a single symmetrical elution pattern at constant specific activity. A single sym-

TABLE III: The Effect of Various Salts on the Catalytic Activity of *E. coli* Citrate Synthase.[a]

| Salt | $A_{412}$/min |
|---|---|
| None | 0.012 |
| KCl | 0.12 |
| $K_2SO_4$ | 0.12 |
| $(NH_4)_2SO_4$ | 0.10 |
| NaCl | 0.09 |
| LiCl | 0.045 |
| Tris-Cl | 0.045 |
| $(CH_3)_4NCl$ | 0.044 |

[a] Each reaction cuvet contained 0.1 mM DTNB, 20 mM Tris-Cl (pH 8.1), 0.25 mM acetyl-CoA, 0.5 mM oxaloacetate, 100 mM cationic equivalent of the above salts, and 80 ng of freshly diluted enzyme.

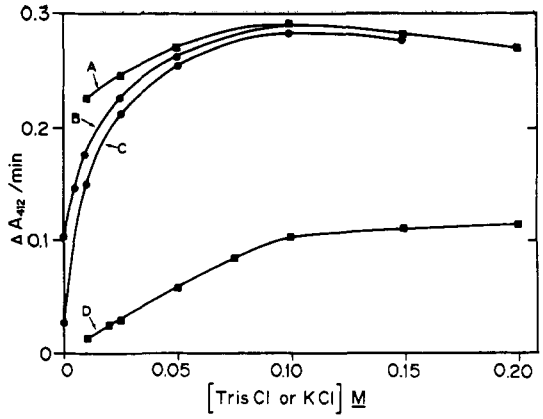



FIGURE 3: The effect of KCl and Tris-Cl concentration on the velocity of the reaction catalyzed by *E. coli* citrate synthase. Acetyl-CoA (0.25 mM), oxaloacetate (0.50 mM), DTNB (0.1 mM), and identical amounts of purified enzyme were included in each reaction cuvet. Tris-Cl (pH 8.1) and KCl concentrations as shown. (A) Tris-Cl at 0.1 M KCl, (B) KCl at 0.1 M Tris-Cl, (C) KCl at 0.02 M Tris-Cl, and (D) Tris-Cl without KCl.

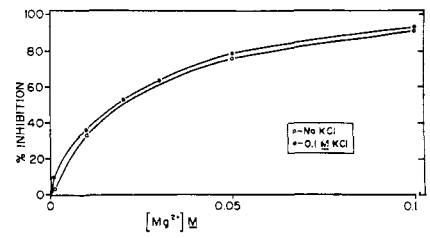
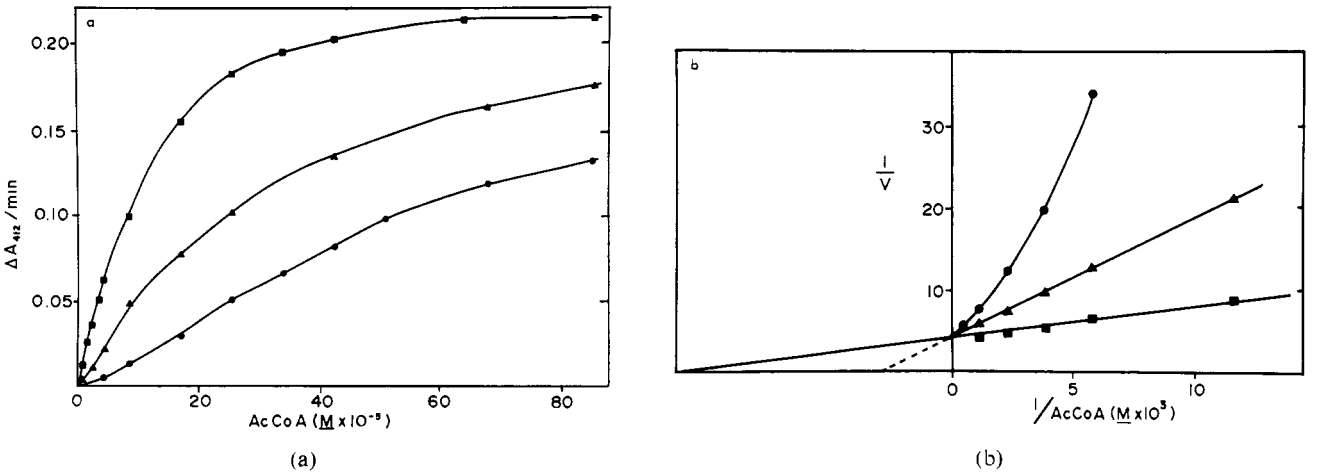



FIGURE 4: The effect of $Mg^{2+}$ on the reaction velocity. Each reaction cuvet contained 0.25 mM acetyl-CoA, 0.50 mM oxaloacetate, 0.1 mM DTNB, identical amounts of enzyme, and $MgCl_2$ as indicated. (A) ●——●, 0.1 M KCl; (B) ○——○, without KCl.




(a) (b)

FIGURE 5: The effect of acetyl-CoA concentration on the reaction velocity at various concentrations of KCl. (a) The reaction velocity is plotted against the concentration of lithium acetyl-CoA in the reaction mixture. (■——■) 0.1 M KCl, (▲——▲) 5 × $10^{-3}$ M KCl, (●——●) 5 × $10^{-5}$ M KCl. (b) Lineweaver–Burk plots of the same data.

metrical peak was observed with analytical ultracentrifugation (Figure 2). The apparent $s_{20}$ was 8.0 S.

Polyacrylamide disc electrophoresis revealed at least one minor component. Some preparations showed two or three bands all of which were enzymatically active when eluted from duplicate gel slices.

*Stability and Protection against Denaturation.* The enzyme was routinely stored in 2 M KCl and 0.1 M Tris-Cl (pH 8.1)–1 mM EDTA at −10° and no appreciable loss of activity was observed, even after several months. The enzyme is resistant to heat denaturation in high concentrations of salt, and this property was used in the purification procedure.

Potassium ion also protected the enzyme against urea denaturation (Table II). Other monovalent cations provided partial protection. OAA was effective in providing protection against urea denaturation, but other substrates, products, or possible effectors of the enzyme (acetyl-CoA, citrate, α-ketoglutarate, NADH, and ATP) offered little or no protection under these conditions (Table II).

*Cationic effect on Enzyme Activity.* The salt composition of a citrate synthase solution markedly influences the catalytic activity. At the usual substrate concentrations the activity is promoted by monovalent cations and inhibited by divalent cations. The relative effect of potassium and other cations on the reaction velocity is shown in Table III. An absolute requirement for potassium could not be demonstrated and catalytic activity was still observed in dilute Tris (0.005 M) when other cations were rigorously removed below detectable levels by treatment with Chelex-100 (Bio-Rad).

The activity is apparently not a function of ionic strength, as the nature of the anion seems to be without effect. The effect of $K^+$ and Tris concentration on the catalytic activity is shown in Figure 3.

$Mg^{2+}$ inhibits the enzyme activity (Figure 4) and the extent of inhibition is only slightly affected by 0.1 M potassium. The inhibition was greater at low concentrations of acetyl-CoA, while the apparent $K_m$ for oxaloacetate was only slightly affected by $10^{-2}$ M $Mg^{2+}$. The extent of inhibition by $Ca^{2+}$ was similar.

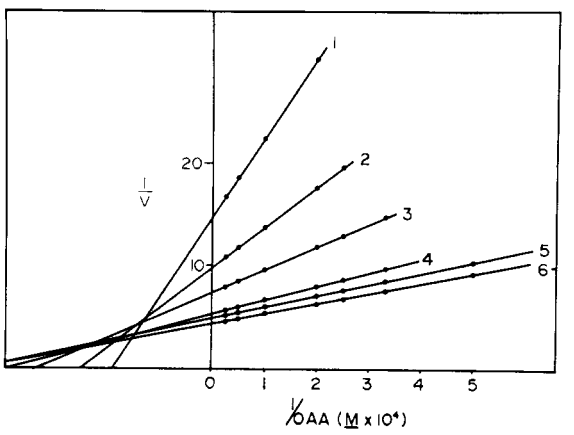



FIGURE 6: Lineweaver–Burk plot for oxaloacetate. 1, 2, 3, and 4, $10^{-4}$ M KCl; 5 and 6, 0.1 M KCl. Acetyl-CoA was 0.25, 0.50, 1.0, 3.0, 0.25, and 0.50 mM for lines 1–6, respectively.

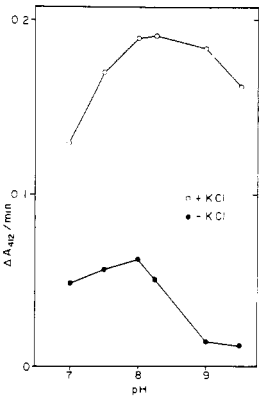



FIGURE 7: pH optimum. Each reaction mixture contained 0.1 M Tris-Cl, 400 μM acetyl-CoA, and 500 μM oxaloacetate. The pH was measured before and after the reaction. Identical amounts of enzyme were added to each reaction cuvet. (●——●) Without KCl and (○——○) 0.1 M KCl.

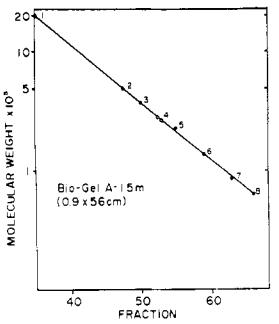



FIGURE 8: Estimation of molecular size of *E. coli* citrate synthase by gel filtration. Blue Dextran and catalytic amounts of several enzymes were added to a Bio-Gel A 1.5 M column (0.9 × 56 cm) and eluted with 0.05 M Tris-Cl (pH 8.1), 0.1 M KCl, and 0.1 M mercaptoethanol. The method of assay and molecular weights (in daltons) of the markers were: (1) Blue Dextran (absorbance at 625 nm), $2 \times 10^6$ (Pharmacia); (2) rat liver citrate cleavage enzyme (Srere, 1959), $5 \times 10^5$ (Inoue *et al.*, 1966); (3) rabbit muscle phosphofructokinase (Uyeda and Racker, 1965), $3.8 \times 10^5$ (Paetkau and Lardy, 1967); (4) *E. coli* citrate synthase; (5) rabbit muscle pyruvate kinase (Cottam *et al.*, 1968), $2.37 \times 10^5$ (Warner, 1958); (6) rabbit muscle aldolase (Racker, 1947), $1.58 \times 10^5$ (Kawahara and Tanford, 1966); (7) pig heart citrate synthase (Ochoa *et al.*, 1951), $8.7 \times 10^4$ (P. A. Srere, unpublished data); (8) pig heart malate dehydrogenase, $6.5 \times 10^4$ (Kun, 1963). Anti pig heart citrate synthetase rabbit serum (0.1 ml) was added to 0.1 ml of fractions 49–56 and assayed by the malate dehydrogenase method 30 min later, in order to accurately determine the *E. coli* citrate synthase elution pattern in the presence of pig heart citrate synthase. Several of the above markers were also run individually and eluted at nearly identical fractions.

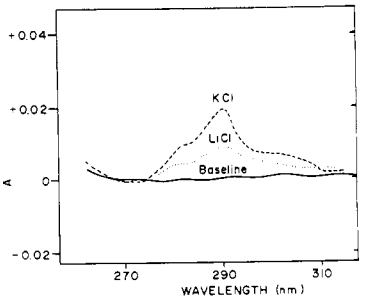



FIGURE 9: Difference spectra observed with addition of 0.1 M salt. Base line from two identical solutions of enzyme (2 mg/ml in 0.01 M Tris-Cl, pH 8.1). Concentrated salt solution was added to one cuvet to a final concentration of 0.1 M, distilled water was added to blank. Addition of KCl to buffer solution alone gave no discernable spectral change.

*Kinetics.* When 0.1 M KCl is included in the reaction mixture, normal Michaelis–Menten kinetics are observed. The $K_m$ values calculated from double-reciprocal plots are $1.1 \times 10^{-4}$ M for acetyl-CoA and $2.1 \times 10^{-5}$ M for oxaloacetate. The latter is not dependent upon acetyl-CoA concentration.

However, marked changes occur in the kinetic behavior when potassium is in low concentration or absent from the reaction mixture. The apparent $K_m$ for acetyl-CoA increases severalfold and nonlinear kinetics are observed but the maximum velocity is unchanged (Figure 5). On the other hand, the reciprocal plot for oxaloacetate at low potassium is linear except at very low concentrations of oxaloacetate and the apparent $K_m$ increases and becomes dependent upon the concentration of acetyl-CoA (Figure 6).

*pH.* The optimal pH is 8.0 (Figure 7). The change in enzyme activity with changes in pH is dependent upon the potassium ion concentration. The maximum potassium effect at these substrate concentrations is seen at pH 9.0 where a 15-fold stimulation of activity occurs by assaying in the presence of 0.1 M KCl, while at pH 7.0, KCl increases the catalytic rate only 2–3-fold. Nearly identical results were obtained with the 233-nm assay. Thus, the salt effect on pH dependence is not due to any limitation of the DTNB assay.

*Molecular Size.* The molecular size of *E. coli* citrate synthase was estimated by gel filtration on precalibrated columns of Bio-Gel A 1.5 M (Figure 8) as approximately 280,000 daltons. It was found that the most reliable calibration was achieved when several enzyme markers were employed and run simultaneously with the citrate synthase. The removal of potassium does not result in dissociation or aggregation of the enzyme, since the $V_e/V_0$ ratio and recovery were not significantly altered when dilute Tris buffer was used as the eluent in the absence of other monovalent cations (Table IV).

*Cationic Effects on Ultraviolet Difference Spectra.* Since monovalent cations, particularly potassium, caused pronounced changes in the substrate affinities, and the extent of inhibition by possible physiological effectors, some type of conformational change would be anticipated. Evidence for a change in the solvating or electrostatic environment of enzyme chromophores was found by observing shifts in the ultraviolet absorption spectrum when the enzyme in 0.01 M Tris-Cl (pH 8.1) was adjusted to 0.1 M salt. $Na^+$ caused an effect nearly equivalent to that of $K^+$, and $Li^+$ caused qualitatively similar but less extensive changes (Figure 9). The ultraviolet spectrum of pig heart citrate synthase is completely unaffected by the addition of potassium, nor are kinetic constants of the pig heart enzyme influenced by KCl.

*SH Titration and the Effect of DTNB.* About 30 SH groups/mole react with DTNB when the enzyme is incubated in 5 M urea; five to seven SH groups per mole will react with DTNB in the absence of denaturing agents. Potassium affects both the initial rate and the extent of the reaction of enzyme with DTNB (Figure 10). But in both the presence and absence of $K^+$ several SH groups can react with DTNB with little or no loss of enzyme activity. In these experiments the enzyme was incubated with excess DTNB and aliquots were taken for determining enzyme activity at various times under the

TABLE IV: Lack of Effect of KCl on Elution Volume from Bio-Gel P-200.

| KCl (M) | $V_e/V_0$ | % Recovery |
|---|---|---|
| $10^{-1}$ | 1.19 | 95 |
| $10^{-3}$ | 1.21 | 90 |
| $10^{-6}$ | 1.18 | 84 |

[a] *E. coli* citrate synthase (10–20 units) was eluted from a Bio-Gel P-200 (0.9 × 32 cm) column. Blue Dextran was run concomitantly to measure $V_0$.

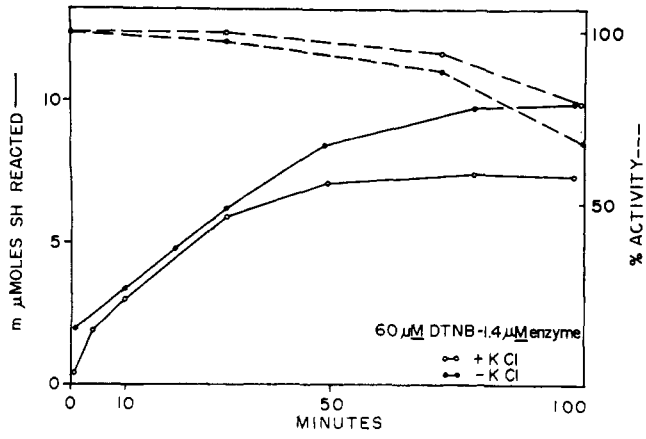



FIGURE 10: Reaction of *E. coli* citrate synthase with DTNB. Enzyme (385 μg) was incubated with 60 mμmoles of DTNB in 0.1 M Tris-Cl (pH 8.1) with and without 0.1 M KCl. The change in absorbance at 412 nm calculated as millimicromoles of SH reacted is shown for each mixture along with the per cent of initial activity as measured by aliquots of each solution in the standard assay.

usual assay conditions. When the enzyme is incubated in DTNB without $K^+$ at 25° the reaction with SH is essentially complete after 2 hr, with only a 20–40% loss of activity. The activity decreases slowly for several hours with no further DTNB–SH reaction; when $K^+$ is added to such an incubation it continues to lose activity but at a slower rate.

## Discussion

The preparation of *E. coli* citrate synthase in a homogeneous form as judged by ultracentrifugation is reported here for the first time. It is not certain if the variable heterogeneity observed with disc electrophoresis is due to preexisting isoenzymes or is the result of the electrophoretic process on the pure enzyme, or alteration of the enzyme during the purification procedure. Samples taken from the ascending and descending slopes of symmetrical elution patterns from gel filtration gave essentially the same electrophoretic pattern.

Weitzman (1966a) reported that incubation of *E. coli* citrate synthase with $10^{-4}$ M DTNB without potassium for 5 min resulted in complete loss of activity. Our data do not support this observation and we have found that the DTNB assay can be employed with the *E. coli* enzyme, even in the absence of potassium. Although the enzyme was certainly less stable without potassium and more vulnerable to reaction with DTNB, the activity was stable in the reaction cuvet for the times necessary to make kinetic measurements. Since identical potassium ion stimulation was observed with the 233-nm assay, which requires only enzyme, substrate, and buffer, we can eliminate the possibility that DTNB is the cause of the limited activity without $K^+$.

In studying the reaction of DTNB with the purified enzyme we found that about five SH groups are available for slow reaction with DTNB at 0.1 M KCl. Two additional SH groups react if potassium is not present. The maintenance of full activity during the initial reaction with DTNB and the finding that the eventual loss of activity was not directly related to reaction with SH groups, makes it highly unlikely that SH groups are involved in the active site. Despite the fact that the loss of activity is minimal, one must consider the possibility that the properties of the enzyme (such as inhibition by effectors) may be altered by the DTNB reaction.

Although potassium ion is necessary for optimal activity, it is not specific in that many cations significantly influence the enzyme activity. We do not know how tightly $K^+$ can be bound to the enzyme. Extensive dialysis against distilled water resulted in precipitation and complete loss of enzyme activity. While dialysis against dilute Tris buffer could remove potassium below detectable amounts, such a preparation was active and addition of KCl could give nearly 100% of the original activity.

The inhibition by $Mg^{2+}$ was for the most part independent of the presence or absence of $K^+$. It should be noted that Weitzman used $10^{-2}$ M $Mg^{2+}$ in his assay system (Weitzman, 1966a,b) which under our conditions gives about 50% inhibition, apparently due to an increase in the $K_m$ for acetyl-CoA.

Most of the differences between our results and those of others can be explained by their use of assay conditions in which the salt composition was not rigidly controlled. At the low concentrations of acetyl-CoA employed (Weitzman, 1966a,b; Wright *et al.*, 1967; Jangaard *et al.*, 1966) the activity is highly dependent on the salt composition.

Potassium ions cause several concurrent changes in the properties of the enzyme, including an increase in substrate affinities, a decrease in the effectiveness of several inhibitors, an increase in the resistance to heat and urea denaturation, and a shift in the ultraviolet spectra. These changes are apparently the result of a conformational change in the enzyme structure. The substrate velocity curves for acetyl-CoA when potassium was in low concentration or excluded from the reaction were sigmoidal in shape, suggesting cooperativity among reactive sites under these conditions. However, it seems unlikely that these phenomenon are of physiological consequence since whatever the enzyme environment in the cell it is doubtlessly not lacking for $K^+$, which has been reported to be about 0.1 M in *E. coli* (Lubin and Kessel, 1960).

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# Nature of the Active Site of a Subunit of the First Component of Human Complement*

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ABSTRACT: A highly purified subunit of human complement ($C1\bar{s}$) was found to hydrolyze the amino acid ester, *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester. The $K_m$ of *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester for the purified enzyme was $5.6 \times 10^{-5}$ M, the $V_{max}$ was $2.19 \times 10^{-6}$ mmole/min, and the $k_{cat}$ was 1.22 $sec^{-1}$. The reaction was inhibited competitively by a variety of guanidine, amidine, and aromatic compounds of low molecular weight. The results indicated that the active center of human $C1\bar{s}$ consists of an anionic binding site in conjunction with a hydrophobic binding site.

The serum complement system of proteins has been well documented as a group of interacting proteins some of which may exhibit enzymatic properties (Müller-Eberhard, 1967). This conclusion was based on studies of the interaction of purified components of complement as well as the behavior of the intermediate complement complexes with antibody-sensitized sheep erythrocytes (Müller-Eberhard, 1967; Nelson, 1965; Mayer, 1961).

The detailed enzymatic nature of the purified complement proteins, however, has only begun to be investigated. For example, it has been known for some time that the first component of human and guinea pig complement contains an esteratic activity for synthetic amino acid esters which is inseparable from its activity in hemolysis of EA[1] (Becker, 1956; Lepow *et al.*, 1956), and that this esterase enzyme, termed $C\bar{1}$ for guinea pig complement and $C1\bar{s}$ for the human complement, probably has an anionic binding site not unlike that of trypsin (Becker, 1965).

It has also been shown that $C\bar{1}$ exists in both human and guinea pig serum in the form of a proenzyme (Lepow *et al.*, 1965; Borsos and Rapp, 1963). In the human system C1 is made up of three components, C1q, C1r, C1s; C1r converts C1s into the active enzyme $C1\bar{s}$ (Naff and Ratnoff, 1968). Recently, Westfall *et al.* (1969) have shown that butanol, methyl Cellosolve, methanol, and ethanol can noncompetitively inhibit $C1\bar{s}$. On the other hand, little is understood about the manner in which C1s generates a specificity for C2 and C4, or the exact chemical nature of the reaction of C1s with these components.

As a first step toward defining on a chemical basis the reaction of $C1\bar{s}$ with other complement components, it was decided first to examine more carefully some of the parameters involved in the binding of synthetic amino acid ester substrates to this enzyme. Analysis of the inhibition kinetics of trypsin and chymotrypsin with various types of charged and noncharged low molecular weight competitive inhibitors has led to the development of a concept of the chemical nature of the active site of these proteases, thus explaining how these enzymes generate a specificity for given amino acids in a polypeptide chain (Niemann, 1964; Mares-Guia and Shaw, 1965; Baker, 1967). This approach was used in this study to obtain similar data on human $C1\bar{s}$, namely (a) the development of a rapid spectrophotometric assay for $C1\bar{s}$ using the substrate *N*-Z-L-Tyr-*p*-Np, and (b) direct evidence for an anionic binding site in human $C1\bar{s}$.

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[1] Terminology for the complement system is that suggested in the *Bull. World Health Organ. 39*, 935 (1968). Thus $C1^-$ is the enzymatically active form of C1s, the third subunit of the first component (C1) of human complement. The other subunits of C1 are C1q and C1r. C2 and C4 are the second and fourth components of complement. EA, sheep erythrocytes treated with antisheep erythrocyte antiserum; $C\bar{1}$ inactivator is the naturally occurring serum inhibitor of $C1\bar{s}$.