CITRIDESMOLASE: ITS PROPERTIES AND MODE OF ACTION

by

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Cell-free extracts that dissimilate citrate to oxaloacetate and acetate have been obtained from *Escherichia coli* grown in suitable media[1]. Similar extracts of *Aerobacter aerogenes*[2] and *Streptococcus faecalis*[3] give the same products when freed from oxaloacetic decarboxylase. This cleavage of citrate contrasts with its biosynthesis insofar as coenzyme A is not involved[2,3] and no co-factors in addition to Mg^{++} or Mn^{++} ions have been implicated. We have studied the kinetics of citridesmolase reaction for partially purified extracts of *Esch. coli* and *A. aerogenes* and we suggest a mechanism of citrate cleavage to account for our observations.

EXPERIMENTAL

Methods. The organisms used[1] were *A. aerogenes* NCTC 418 and *Esch. coli* NCTC 5928. *A. aerogenes* was grown in the citrate + mineral salt medium used by DAGLEY AND DAWES[2], and *Esch. coli* in a citrate + peptone + glucose medium replenished with glucose 30 minutes before harvesting[1]. About 10 g of frozen cell paste were crushed without abrasive in a HUGHES bacterial press[4] at −14°, and after taking up in phosphate buffer (27 g KH_2PO_4 per litre, pH 7.0), cell debris was removed by centrifugation. Citridesmolase activity was determined by incubation of extracts at 37°, for periods of time stated, with a solution of pH 7.0 containing per litre 21 g trisodium citrate dihydrate and 5 g KH_2PO_4 to which was added $MgSO_4 \cdot 7H_2O$ (0.4 g) or other salts of divalent metals. This citrate medium will be referred to later as C.M. Protein was then precipitated with an equal volume of cold 10% (w/v) trichloracetic acid and the total keto acid concentration in the clear supernatant was determined according to FRIEDEMANN AND HAUGEN[5]. Paper chromatography of 2:4-dinitrophenylhydrazones, by a modification[6] of the method of CAVALLINI, FRONTALI AND TOSCHI[7], was used to identify pyruvic and oxaloacetic acids. Protein was determined by the method of STICKLAND[8] using crystalline bovine serum albumin as standard.

Crude extracts were partially purified as follows. The protein content was determined and for each milligramme present, 0.01 ml of a solution of protamine sulphate, pH 5.8, containing 17 mg per ml was added. The precipitate containing nucleic acids was discarded, the supernatant saturated with ammonium sulphate and the precipitate taken up in water. When this solution was brought to half-saturation with ammonium sulphate, citridesmolase activity was retained in the precipitate: this was dissolved in phosphate buffer (9 g KH_2PO_4 per litre, pH 7.1) and the solution shaken with calcium phosphate gel[9]. The supernatant from the gel was active and was free from oxaloacetic decarboxylase; Mg^{++} or certain other divalent ions were required for activation. For such preparations from *A. aerogenes* showing initially strong oxaloacetic decarboxylase, 1 mole of citrate usually gave rise to somewhat less than the 1 mole of oxaloacetate required by the proposed mechanism[2]. The oxaloacetate produced was estimated manometrically by decomposition with aniline citrate[10]. A flow-sheet for purification is given in Table I.

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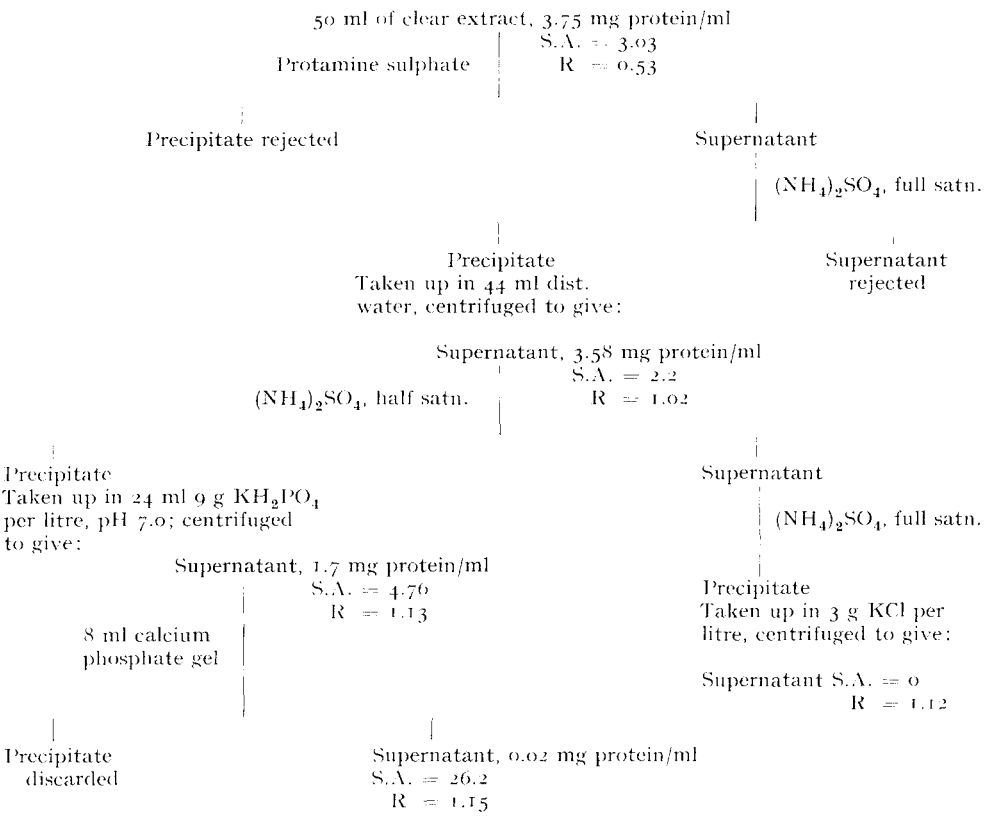
TABLE I

PURIFICATION PROCEDURE

10 g of paste of *A. aerogenes* crushed, extracted with 0.2 *M* KH_2PO_4, pH 7.0, and centrifuged at 25,000 r.p.m. for 45 minutes.



S.A. = specific activity in μmoles/3 ml test/mg N/45 secs.
R = light absorption at 280 mμ/absorption at 260 mμ.

Progress of the reaction: inhibition by oxaloacetate. It was shown for crude preparations of *A. aerogenes*[1] that formation of pyruvate ceased with citrate unattacked, and we have also demonstrated this phenomenon for purified preparations that produce oxaloacetate. Progress curves exhibit a reduction of reaction rate with time, and linear plots of the latter against log $(C_F - C_T)$ are obtained, where C_T and C_F are respectively the concentrations of accumulated keto acids, after time t and after the reaction has ceased. This type of relationship may be readily deduced for a mechanism by which inactivation of enzyme may occur as a result of the reaction taking place: this would be the situation when inhibitory products are formed. That oxaloacetate is the inhibitor responsible was established by the following experiment which makes use of the fact that extracts may be precipitated completely by saturation with ammonium sulphate without loss of activity. An extract of *A. aerogenes*, partially purified to the stage preceding calcium phosphate gel treatment, was precipitated with saturated ammonium sulphate, redissolved in phosphate buffer (9 g KH_2PO_4 per litre, pH 7) and various amounts incubated

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with C.M. for 10 minutes. During this period the reaction ceased, since no increase in keto acid occurred when incubation was prolonged. A linear relation was established between amount of keto acid and amount of enzyme present (Fig. 1). The effect on this relation of first allowing the enzyme to react with citrate was then studied. The extract (5 ml) was incubated with 5 ml of C.M. for 15 minutes, precipitated with ammonium sulphate, redissolved in buffer and various amounts again incubated with C.M. It was found that the preliminary incubation with C.M. had abolished activity. That this effect was not due to citrate itself was shown by omitting Mg^{++} from the C.M. during the 15 minutes incubation. Some reaction occurred since it was impossible to ensure complete freedom from metal activators at such a high enzyme concentration (12 mg protein per 5 ml): the amount of oxaloacetate produced was about one-third of that given when Mg^{++} ions were added to the C.M. For this portion of extract, when precipitated and redissolved in buffer, the plot of amount of enzyme against keto acid formed (line 3, Fig. 1) lay between those for an extract preincubated with phosphate buffer and for inactivated enzyme (lines 1 and 2 respectively). When the enzyme was inactivated during preliminary incubation with C.M., oxaloacetate accumulated to give a concentration of 3.4 m*M*. In three further experiments, 5 ml portions of extract were each incubated for 15 minutes with 5 ml of phosphate buffer containing respectively pyruvate, acetate and oxaloacetate at concentrations of 3.4 m*M*; and after ammonium sulphate precipitation as before, relations were established between amounts of keto acid and recovered enzyme in each case. Incubation with oxaloacetate at 37° resulted in almost complete inactivation; pyruvate and acetate had no effect. These experiments prove that as the reaction proceeds, enzyme is inactivated, and suggest that the agent responsible is oxaloacetate.

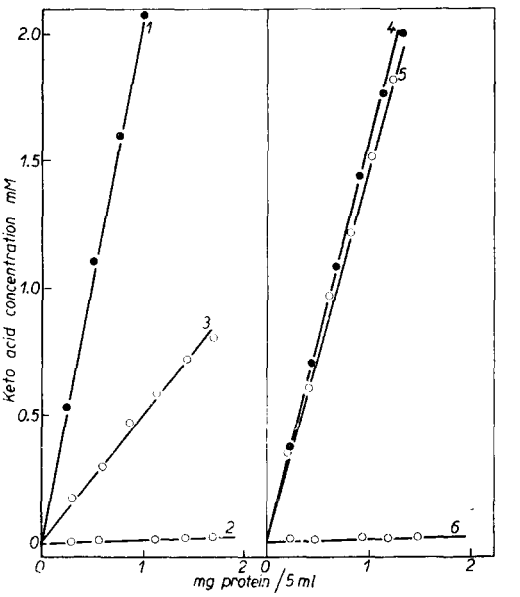

Fig. 1. Relationships between amounts of enzyme protein present and amounts of keto acid produced from citrate in 10 minutes. The enzyme had first been incubated for 15 minutes with (1) phosphate buffer alone (2) citrate medium, with Mg^{++} (3) citrate medium, no added Mg^{++} (4) acetate (5) pyruvate (6) oxaloacetate.

Activation by divalent metal ions. Salts of divalent metals at various concentrations were added to C.M. from which magnesium sulphate had been omitted, and in Fig. 2 logarithms of cation concentrations are plotted against the keto acid concentrations attained after incubation for 10 minutes with purified *A. aerogenes* extract (0.1 mg protein per ml). When soluble, the salts used were sulphates, otherwise they were chlorides; but no difference could be detected when it was possible to use both, as in the case of magnesium. When extracts of *Esch. coli* were used similar results were obtained, namely good activation with Zn^{++}, Mg^{++}, Fe^{++} and Co^{++} and none with Cu^{++}, Hg^{++}, Ba^{++}, Sr^{++} and Ca^{++}; with Mn^{++}, however, activation in the case of *Esch. coli* was markedly greater than for *A. aerogenes* As stated above, citrate decomposition had

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usually ceased at the end of 10 minutes and concentrations of accumulated keto acids at that time measured the extent of the reaction rather than its velocity. To obtain initial reaction rates from which Michaelis constants could be derived, the incubation mixtures were sampled after 30 seconds when the rate of citrate decomposition was approximately linear. In Fig. 4 (a) the reciprocals of these initial velocities are plotted against reciprocals of Mg^{++} ion concentrations for an extract of *A. aerogenes*, and from the slope and ordinate intercept the Michaelis constant is calculated to be $3 \cdot 10^{-3}$ *M*. Using extracts obtained from *Esch. coli* the same value was obtained for Mg^{++} activation but that for Mn^{++} was significantly lower, namely $8 \cdot 10^{-5}$ *M*. Calcium ions inhibited the activation of extracts of both species by other metals. The competitive nature of the inhibition of an extract from *Esch. coli* is shown in Fig. 3. Initial velocities were deter-

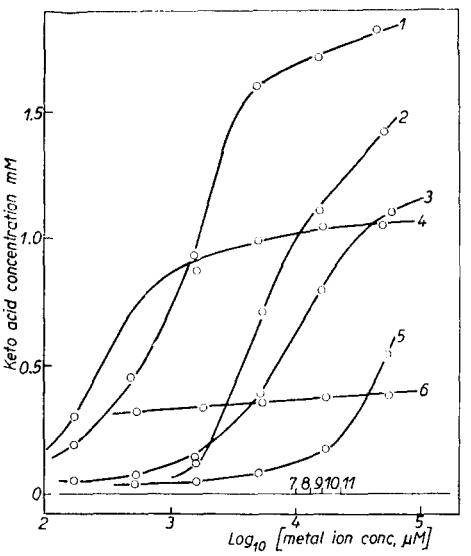

Fig. 2. Activation of enzyme obtained from *A. aerogenes* by various concentrations of divalent metal ions (1) Zn^{++} (2) Fe^{++} (3) Co^{++} (4) Mg^{++} (5) Ni^{++} (6) Mn^{++} (7) Cu^{++} (8) Hg^{++} (9) Ca^{++} (10) Sr^{++} (11) Ba^{++}.

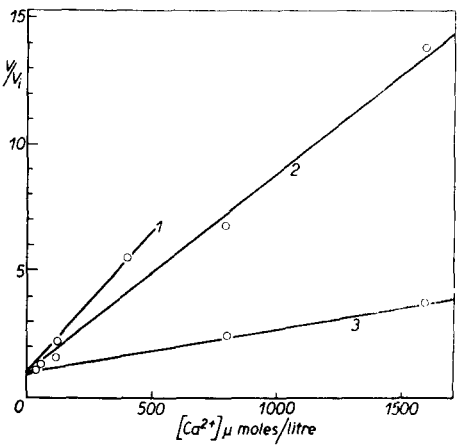

Fig. 3. Competitive inhibition of Mg^{++} ion activation by Ca^{++} ions. v and v_i are respectively reaction velocities in the absence and in the presence of Ca^{++} ions at the concentrations of Mg^{++} ions stated: (1) 0.4 m*M* (2) 1.3 m*M* (3) 4.4 m*M*. The enzyme was obtained from *Esch. coli*.

mined for three series of reaction mixtures containing concentrations of Mg^{++} ions of .434 m*M*, 1.33 m*M* and 4.4 m*M* respectively; various additions of calcium chloride within the range 0–2 m*M* were also made. The ratio v/v_i was then plotted against $[Ca^{++}]$, where v and v_i are respectively initial velocities in the absence and in the presence of the relevant concentration of Ca^{++} ions. The lines so obtained intersect the v/v_i axis at unity and their slopes increase as $[Mg^{++}]$ is reduced; this behaviour is characteristic of competitive inhibition[11].

Other cofactors. The possible dependence of the reaction upon coenzyme A was investigated by treatment of a crude extract with Dowex 1 according to the procedure of CHANTRENNE AND LIPMANN[12]. Our thanks are due to Dr. LAWRENCE L. LACHAT of Armour and Company, Chicago, who kindly supplied us with a liver coenzyme concentrate containing coenzyme A. From Table II it is clear that there is no evidence for

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TABLE II

EFFECT OF ADDITIONS OF COENZYME A TO PREPARATIONS TREATED WITH DOWEX 1

10 ml extract treated with 5 ml solid Dowex 1 for 30 minutes in ice bath. 0.5 ml enzyme incubated with 2.5 ml C.M. for 10 minutes and concentrations of keto acid determined

	Keto acid concentrations (mM)	
	Pyruvate	*Total keto acids*
Treated extract	0.44	0.98
Treated extract + cysteine	0.41	0.88
Treated extract + cysteine + CoA	0.27	0.58
Treated extract + CoA	0.41	0.94

coenzyme A participation: additions of cocarboxylase to dialysed extracts likewise gave no response.

Determinations of Michaelis constant for citrate. After additions of citrate to tubes containing, per litre, 5 g KH_2PO_4 (pH 7.0) and 0.4 g $MgSO_4 \cdot 7\ H_2O$, the mixtures were incubated at 37° with amounts of purified preparation sufficient for 4 satisfactory determinations of initial reaction rates.

Fig. 4 (b) and (c) shows plots of reciprocals of velocities and citrate concentrations, and Michaelis constants of $2.1 \cdot 10^{-4}$ and $1.6 \cdot 10^{-4}$ *M* are calculated for extracts of *A. aerogenes* and *Esch. coli* respectively. Since saturation of enzyme with substrate occurred at low concentrations it was essential in each case to follow the progress of the reaction to ensure that the keto acid concentration at 30 seconds gave an actual measure of the velocity before all the substrate was decomposed.

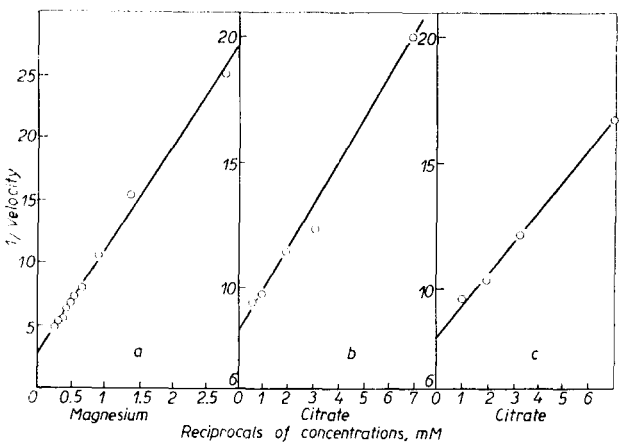

Fig. 4. Reciprocal plots of reaction velocity against substrate concentration. Velocities expressed as concentrations of keto acids (m*M*) attained in the first 30 seconds of reaction. Extracts were obtained from: *A. aerogenes*, (a) and (b); *Esch. coli*, (c).

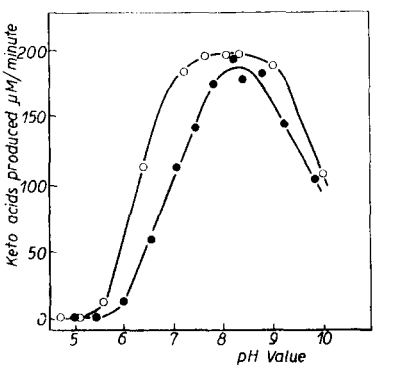

Fig. 5. Effect of pH value upon reaction velocity. Open circles, *A. aerogenes* enzyme; closed circles, *Esch. coli* enzyme.

Effect of pH on activity. Aliquots of citrate medium (21 g trisodium citrate dihydrate, 5 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7\ H_2O$ per litre) were adjusted to give a range of pH values by addition of suitable quantities of sodium hydroxide. At each pH, 2.8 ml of C.M. were incubated at 37° with 0.2 ml of purified enzyme and the accumulated keto acids estimated

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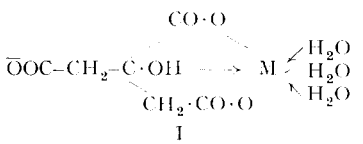
after one minute. The actual pH values after enzyme addition were determined for a set of parallel tubes, using a Muirhead pH meter, and these values are plotted against keto acid production for both *A. aerogenes* and *Esch. coli* in Fig. 5.

DISCUSSION

Any mechanism for citridesmolase action must take account of three salient properties established above, namely: the progressive inactivation of enzyme by oxaloacetate that occurs as the reaction proceeds, the ability of certain divalent metal ions to activate the system and the competitive inhibition of this process by Ca^{++} ions. SMITH and collaborators have produced abundant evidence that the influence of metal ions upon enzyme action is related to their capacity for chelation[13]. In the present system a mechanism involving chelation appears to be essential because citrate chelates so strongly that, in our experiments, chelated metal ions must greatly exceed in number those that are free. Consequently, although it remains possible that participation of cofactors in addition to divalent ions may be discovered in the future, and that our present proposals would need to be modified accordingly, it is improbable that any satisfactory mechanism will be devised that ignores the powerful tendency of citrate to chelate with metals.

From physicochemical studies, BOBTELSKY AND JORDAN[14] proposed formula I for chelate compounds of citrate with divalent metals and established that the hydrogen of the hydroxyl group is labilized as a result of coordination with the metal. According to the mechanism we propose (II), it is this hydrogen which migrates when $-CH_2.COOH$ is split off and acetate is produced. If there is a tendency for oxaloacetate to be retained on the active site as shown, the enzyme will become progressively inactivated.



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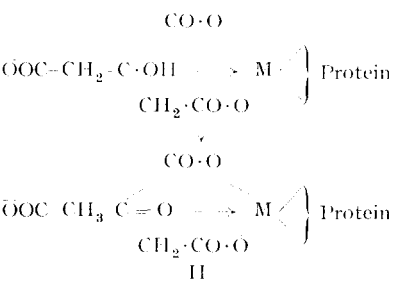

II

It is also evident that a metal which is able to chelate with citrate but unable to activate the enzyme may inhibit competitively those metals which can take part in the reaction: this is the case for calcium. All the active metals have ionic radii, for coordination number six[15], within the range 0.72 ± 0.08 A; Ca^{++} ions (0.99 A) and other ions that do not activate, have radii outside this range. It might be argued that Ca^{++} could inhibit simply by removing free citrate ions from the system. That this is not the case follows from a consideration of our quantitative results. When Ca^{++} ions inhibited at concentrations up to 2 m*M* (Fig. 3) citrate was present, in C.M., at 71 m*M* and Mg^{++} at 0.4 to 4.4 m*M*. In our determinations of the Michaelis constant for citrate we showed that its concentration must be reduced to about 0.2 m*M* before the velocity was reduced to one-half of its maximum, and it is therefore clear that inhibition by Ca^{++} ions cannot be due to reduction in available citrate. BOBTELSKY AND JORDAN[14] showed a sharp

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reduction in the stability of citrate chelate compounds as pH decreased and it is suggestive that we found activity largely abolished below pH 6 in the range at which the compounds dissociate. KORNBERG, OCHOA AND MEHLER[16] have shown that oxaloacetic acid, and other β-ketocarboxylic acids, are decarboxylated catalytically by metal ions alone with the formation of spectroscopically observable intermediate metal-substrate complexes. Formulae for the latter were not proposed, and we do not suggest that the oxaloacetate necessarily remains bound to the enzyme in the precise manner shown in II; clearly a subsequent rearrangement of bonds might occur. It may be observed that according to our mechanism the unsymmetrical dissimilation of the citrate molecule is a consequence of its three-point attachment to the enzyme *via* the metal.

It is a pleasure to acknowledge the assistance given by Mr. J. SYKES and Mr. R. G. STICKLAND in certain experiments, to Mr. J. SMILLIE for skilful technical assistance throughout and to the Carnegie Trust for the Universities of Scotland for a personal grant for apparatus to one of us (E.A.D.).

SUMMARY

Cell free extracts of *A. aerogenes* and *Esch. coli* showing citridesmolase activity were partially purified by removal of nucleic acids, precipitation with half saturated ammonium sulphate and treatment with calcium phosphate gel. Oxaloacetic decarboxylase activity was thereby eliminated and additions of suitable amounts of one of the following ions were needed for activity: Mg^{++}, Zn^{++}, Fe^{++}, Co^{++}, Ni^{++} or, for *Esch. coli* extracts, Mn^{++}. No requirements for other co-factors, including coenzyme A, could be demonstrated. Additions of Ca^{++} inhibited competitively activation by other metals. Extracts became inactive during the course of the reaction with excess citrate present: this was apparently due to inhibition by the oxaloacetate that accumulated. Michaelis constants for extracts from both species were determined for citrate and Mg^{++} and for Mn^{++} in the case of *Esch. coli*. Activities were optimal at pH 8.2 and rapidly decreased in the range pH 7 to 6. These observations may be explained by a mechanism involving complexes of the enzyme protein with chelate compounds of citrate and the activating metals.

RÉSUMÉ

Des extraits d'*A. aerogenes* et d'*Esch. coli* sans cellules montrant une activité citridesmolase furent en partie purifiés en enlevant les acides nucléiques, en les précipitant avec du sulphate d'ammoniaque à demi saturé, et en les traitant avec du gel de phosphate de calcium. L'activité décarboxylase oxaloacétique fut ainsi éliminée et il fallut ajouter des quantités convenables d'un des ions suivants pour produire de l'activité: Mg^{++}, Zn^{++}, Fe^{++}, Co^{++}, Ni^{++} ou, pour les extraits d'*Esch. coli*, Mn^{++}. La nécessité d'aucun autre co-facteur, y compris la coenzyme A, n'a pu être démontrée. Des additions de Ca^{++} produisirent une inhibition compétitive de l'activation d'autres métaux. Les extraits devinrent inactifs au cours de la réaction en présence d'un excès de citrate: ce qui semble dû à l'inhibition provoquée par l'accumulation d'oxaloacétate. Les constantes de Michaelis pour des extraits tirés des deux espèces furent déterminées pour le citrate et le Mg^{++} et pour le Mn^{++} dans le cas d'*Esch. coli*. Les activités furent optima à pH 8.2 et diminuèrent rapidement entre les valeurs pH 7 et 6. Ces observations pourraient s'expliquer par un mécanisme comprenant des complexes de la protéine de l'enzyme avec des composés chelates de citrate et avec les métaux activants.

ZUSAMMENFASSUNG

Zellfreie Extrakte von *A. aerogenes* und *Esch. coli*, die eine citratspaltende Aktivität zeigten (Citratdesmolase), wurden teilweise gereinigt durch Entfernung der Nucleinsäuren, Fällung mit halb-gesättigter Ammoniumsulfatlösung und Behandlung mit Calciumphosphat-Gel. Oxalacetat-Decarboxylase wird hierbei ebenfalls entfernt. Um die Citratdesmolase-Aktivität wieder anzuregen werden den Zellextrakten entsprechende Konzentrationen von Mg^{++}, Zn^{++}, Fe^{++}, Co^{++}, oder Ni^{++} zugesetzt. Ausser den obigen Ionen hat sich Mn^{++}, im *Esch. coli*-Extrakt wirksam gezeigt. Ein Bedarf an anderen Cofaktoren wurde nicht erwiesen, einschliesslich des Coenzyms A. Ca^{++}-Zugabe behinderte

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die Aktivierung durch Metallionen vollständig. Citrat-Überschuss wirkte allmählich hindernd auf die Extraktaktivität, wahrscheinlich wegen der Anreicherung von Oxalacetat. Die Michaelis-Konstante wurde für Citrat und Mg^{++} in beiden Extrakten und für Mn^{++} im *Esch. coli*-Extrakt bestimmt. Die Aktivitäten waren optimal bei pH 8.2 und nahmen rasch ab im Bereich von pH 7-6. Die experimentellen Beobachtungen können mit einem Mechanismus, der Komplexverbindungen zwischen dem Enzymprotein, Citrat und den Aktivierungs-Metallen einschliesst, erklärt werden.

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