62. THE ENZYMIC DECARBOXYLATION OF OXALOACETATE

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ALTHOUGH oxaloacetate has an important role in cellular metabolism, no enzyme has been described which brings about a rapid decarboxylation of the acid. Breusch [1939] investigated a heat-stable component of mammalian tissue responsible for this decarboxylation but could not identify it with any known physiological agent. The rate of this decarboxylation is comparatively slow. In investigations on the utilization of CO_2 by bacteria, Wood & Werkman [1938; 1940] postulated the reverse reaction, i.e. the carboxylation of pyruvate to form oxaloacetate. This reaction was represented as follows:

Evans & Slotin [1940] have demonstrated the utilization of CO_2 with pigeon liver by the use of radioactive CO_2 . The mechanism suggested by them is essentially the Wood & Werkman reaction. Krebs & Eggleston [1940], also working with pigeon liver, suggested that the reaction occurs in the dissimilation of pyruvate. They postulate that aneurin is required in the fixation. Smyth [1940] came to the same conclusion using vitamin-deficient staphylococci.

It is the purpose of the present communication to report the occurrence of a thermolabile enzyme which brings about the decarboxylation of oxaloacetate to pyruvate and CO_2 . Experimental evidence is presented to show that (1) the magnesium ion and not cocarboxylase is a coenzyme for the enzyme; (2) the products of decarboxylation are pyruvate and CO_2 ; (3) the enzyme is heat-labile and (4) permeability of the cell wall is an important factor to be considered in studies on the enzyme. Furthermore, it is believed that under proper experimental conditions the enzyme described is capable of bringing about the carboxylation involved in CO_2 utilization.

METHODS

Micrococcus lysodeikticus was employed in the investigation. The organisms were grown on a medium containing 1 % glucose, 0.5 % peptone, 0.3 % yeast extract, 10 % tap water and 2 % agar. Inoculations were made on agar slopes in large Roux bottles. After 3 days' incubation at 30°, the cells were washed from the agar surface with distilled water, filtered through glass wool and washed twice in a centrifuge.

Acetone-treated cells. 10 g. of cell paste were mixed with 70 ml. ice-cold acetone and shaken thoroughly for 5 min. The cells were filtered off, suspended in 140 ml. ice-cold acetone and shaken vigorously for 10 min. This process was repeated. The preparation was then placed *in vacuo* to remove traces of acetone. These cells are permeable to oxaloacetate, whereas untreated cells are not.

Lysed cell preparation. To a 10% cell suspension (wet weight basis), one-tenth volume of saliva was added, and the mixture incubated 1 hr. at 36°, whereupon lysis occurred. For control, a 10% cell suspension without saliva was incubated in like manner.

Coccarboxylase- and Mg^{++} -deficient acetone-treated cells. The usual alkaline phosphate washing was employed. 1 g. of acetone-treated cells was washed twice for 10 min. on the shaking machine with 50 ml. phosphate buffer, pH 8.0, and once with 50 ml. distilled water. The cells maintain their deficiency if dried on a porous plate. Coccarboxylase and Mg^{++} cannot be removed by phosphate washing unless the cells are first treated with acetone.

Coccarboxylase- and Mg^{++} -deficient preparation of lysed cells. To 4 ml. of a 20% solution of lysed cells, 6 ml. of saturated $(NH_4)_2SO_4$ were added. The mixture was vigorously shaken for 10 min. and centrifuged. The precipitated particles were made up to a volume of 4 ml. with distilled water and the $(NH_4)_2SO_4$ washing was repeated twice. Finally, the precipitate was washed with distilled water and made up to the original volume of 4 ml.

Preparation of oxaloacetic acid. To the Na salt of diethyloxaloacetate (100 g. in 250 ml. water) conc. H_2SO_4 , equivalent to the Na, was added. The free ester was removed as an oily layer by means of a separating funnel and dissolved in 80 ml. ether. The ether solution was washed twice with 50 ml. distilled water, and the ether evaporated off. The ester was hydrolysed by treatment with 4 vol. conc. HCl at room temperature and vigorously shaking the acid solution mechanically for at least an hour. The solution was then placed at -26° for 24–48 hr. The precipitated acid was removed by filtration, dissolved in the requisite amount of warm acetone and recrystallized by adding chloroform. The purity of such a sample as determined by the aniline citrate method [Ostern, 1933] is greater than 98%.

Pyruvic acid was determined by the carboxylase method [Krebs *et al.* 1940] and also by the salicylaldehyde method of Straub [1936]. CO_2 was determined by adding 0.3 ml. of 1:1 H_2SO_4 to the reactants in the manometer vessel.

The Barcroft-Warburg respirometer was used in this study. The total volume of reactants in each cup was 2 ml. Experiments conducted with untreated cells showed an endogenous activity which was deducted from values obtained with the substrates present. Endogenous activity of acetone-treated cells was negligible.

EXPERIMENTAL

Permeability. The untreated cells of *M. lysodeikticus* aerobically oxidized pyruvate, oxaloacetate, malate, fumarate and succinate with almost equal rapidity (Table 1). Anaerobically there was no evolution of CO_2 from any of these salts. The acetone-treated cells behaved similarly to the untreated cells excepting that they oxidized succinate very slowly and decarboxylated oxaloacetate very rapidly anaerobically. Since there was no increase in CO_2 evolution in the presence of the untreated cells and oxaloacetate, it may be assumed that the oxaloacetate did not reach the active enzyme centres. Apparently, the acetone extraction of the cells makes the cell permeable to oxaloacetate. Objections to this explanation may be made, inasmuch as the untreated cells showed an O_2 -uptake with oxaloacetate. It is probable, however, that the oxaloacetate did not penetrate the untreated cell even in the aerobic experiments, and that the O_2 -uptake was due to the oxidation of pyruvate formed by the spontaneous decarboxylation of oxaloacetate. This explanation is supported by the fact that anaerobically with the acetone-treated cells, the quantity of

Table 1. *Anaerobic and aerobic behaviours of Micrococcus lysodeikticus on the C₄ dicarboxylic acids and pyruvate*

Substrate	Untreated cells		Acetone-treated cells		
	Aerobic $\mu\text{l. O}_2$ uptake	Anaerobic $\mu\text{l. CO}_2$ evolved	Aerobic $\mu\text{l. O}_2$ uptake	Aerobic $\mu\text{l. CO}_2$ evolved	Anaerobic $\mu\text{l. CO}_2$ evolved
Pyruvate	248	6	262	320	12
*Oxaloacetate	225	4	255	1220	915
Malate	250	2	298	—	3
Fumarate	228	3	314	—	2
Succinate	240	1	12	—	0

Total volume of reactants = 2 ml. Conc. of substrates 0.025 *M*, 20 mg. cells per cup (dry weight), 0.125 *M* PO_4 buffer pH 6.8; aerobic exp. contained air in gas space, anaerobic contained N_2 . Temp. 30°. Time 1 hr.

* All values corrected for spontaneous decarboxylation of oxaloacetate. Endogenous values deducted in experiments containing substrates.

CO_2 from oxaloacetate (915 $\mu\text{l.}$) is substantially equivalent to the CO_2 from the corresponding aerobic experiment (1220 $\mu\text{l.}$) minus the CO_2 from the aerobic pyruvate experiment (320 $\mu\text{l.}$). Apparently the excess CO_2 in the aerobic experiment is the result of pyruvate dissimilation. Pyruvate is not dissimilated anaerobically; consequently the CO_2 evolved from oxaloacetate under these conditions must have arisen by its decarboxylation, shown by the 915 $\mu\text{l.}$ of CO_2 obtained anaerobically from 928 $\mu\text{l.}$ of oxaloacetate.

Fig. 1. Effect of lysis on the decarboxylation of oxaloacetate. Total volume of reactants 2.0 ml. Anaerobic; 0.125 *M* PO_4 buffer pH 6.8, temp. 30°. — 2.5 % untreated cells + 920 $\mu\text{l.}$ oxaloacetate. ---- 2.5 % lysed cells + 920 $\mu\text{l.}$ oxaloacetate. -.-.- 2.5 % untreated cells + 0.1 volume saliva + 920 $\mu\text{l.}$ oxaloacetate.

Objections may also be raised that if the other C_4 acids reach their enzyme centres, oxaloacetic acid should likewise do so. Apparently other compounds having the grouping $-\text{CO}.\text{CH}_2.\text{CO}-$ do not penetrate the cell wall, e.g. malonic acid, generally recognized as an inhibitor of succinate oxidation, shows no

inhibition with *M. lysodeikticus*, even after long incubation before the succinate addition. Apparently, the malonate does not penetrate the cell to the enzyme centre. This hypothesis cannot be tested with our acetone preparation, inasmuch as it does not oxidize succinate (Table 1).

Fig. 1 further substantiates the cell permeability hypothesis. *M. lysodeikticus*, as shown by Fleming [1924], is lysed by lysozyme, an enzyme present in both egg albumin and saliva. In the figure the decarboxylations of oxaloacetate by untreated cells, by previously lysed cells and by cells that were lysed during the course of the experiment are compared. If cell permeability is limiting the decarboxylation of oxaloacetate, it is to be expected that as lysis proceeds the enzyme will become liberated and be available for decarboxylation. Decarboxylation by the untreated cell was identical with the spontaneous decarboxylation; therefore the two curves are superimposable. The lysed preparation showed a remarkable ability to decarboxylate oxaloacetate, and the experiment in which lysis proceeded in the presence of oxaloacetate showed an increasing rate of decarboxylation. Saliva alone does not increase the spontaneous decarboxylation. The results of these experiments clearly establish the importance of cell permeability.

Products of the decarboxylation of oxaloacetate. Pyruvic acid and CO_2 are formed by the anaerobic breakdown of oxaloacetate (Table 2). For each oxaloacetic acid molecule decarboxylated, one molecule of pyruvate and one of CO_2

Table 2. *Products of anaerobic decarboxylation of oxaloacetate by an acetone preparation*

Material determined	Method of determination	Quantity obtained ($\mu\text{l.}$)	Recovery %
Oxaloacetate dissimilated	Aniline citrate	998	—
Pyruvate	Carboxylase	958	97
Pyruvate	Salicylaldehyde	970	98
CO_2	Acidification	947	96

were found. The pyruvate determinations by both the carboxylase and the salicylaldehyde methods agreed, and there was an equivalent recovery of CO_2 on acidification. The oxaloacetate was determined by the aniline citrate method.

Effects of Mg^{++} and cocarboxylase on the enzyme. The question next arises as to the relationship between oxaloacetate carboxylase and pyruvate carboxylase. Krebs & Eggleston [1940] and Smyth [1940] are of the opinion that the two are either identical or very similar. It is well known that yeast carboxylase not only decarboxylates pyruvate but also oxaloacetate. The products of decarboxylation of the latter are acetaldehyde and two molecules of CO_2 . There are two possible mechanisms by which yeast carboxylase may decarboxylate oxaloacetate: initial β -decarboxylation or initial α -decarboxylation.

β -decarboxylation

α -decarboxylation

To obtain information relative to this question, the Mg^{++} and the cocarboxylase requirements of the two reactions have been determined. It is well known that

in both the aerobic and the anaerobic decarboxylations of pyruvic acid, cocarboxylase and Mg^{++} are required. With *M. lysodeikticus* it was found that the organism likewise requires cocarboxylase and Mg^{++} for the α -decarboxylation. On the contrary, for the β -decarboxylation of oxaloacetate only Mg^{++} is required. The difference in the coenzyme requirements indicates that the two enzymes are not alike. Whether decarboxylation of oxaloacetate by yeast involves two enzymes or occurs solely by α -decarboxylation must await further investigation. The present investigation shows that with bacteria the former is the case.

Data in Table 3 show the results with acetone-treated cells made deficient in cocarboxylase and Mg^{++} . Deficiencies were determined by observing the lack of ability of the preparation to oxidize pyruvate and oxaloacetate aerobically.

Table 3. *Pyruvate and oxaloacetate metabolism by Mg^{++} and cocarboxylase deficient and non-deficient acetone-treated cells*

Substrate	Deficient cells plus					Mg^{++} and thiamin	Non-deficient acetone-treated cells
	No addition	Mg^{++}	Cocarboxylase	Mg^{++} and cocarboxylase	Mn^{++}		
Pyruvate, μ l. O_2 uptake	14	16	17	212	18	18	250
*Oxaloacetate, aerobic, μ l. O_2 uptake	18	20	19	219	22	22	255
*Oxaloacetate, aerobic μ l. CO_2 formed	41	927	38	1206	932	926	1228
*Oxaloacetate, anaerobic, μ l. CO_2 formed	32	930	30	930	934	926	928

Total volume of reactants 2 ml. 0.025 *M* oxaloacetate, 0.006 *M* $MgCl_2$, 0.006 *M* $MnSO_4$, 25 μ g. cocarboxylase or thiamin, 20 mg. cells per cup (dry weight), 0.125 *M* PO_4 buffer pH 6.8; aerobic exp. contained air in gas space, anaerobic contained N_2 . Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation values of oxaloacetate deducted from substrate values. With Mg^{++} 178 μ l. Without Mg^{++} 94 μ l.

Upon addition of Mg^{++} and cocarboxylase to the deficient cells, the oxidation of pyruvate took place. 212 μ l. O_2 were taken up with cocarboxylase and Mg^{++} addition, and only 14 μ l. with no addition. The non-deficient acetone-treated cells took up 250 μ l. O_2 . When either Mg^{++} or cocarboxylase alone was added, no oxidation occurred. Therefore, it is apparent that both Mg^{++} and cocarboxylase are essential for pyruvic acid oxidation. The addition of aneurin and Mg^{++} had no effect, indicating that the mechanism for phosphorylating the vitamin had been destroyed by the acetone treatment.

The difference between pyruvate and oxaloacetate decarboxylations is apparent if the effects of Mg^{++} and cocarboxylase on oxaloacetate dissimilation are considered. Considering O_2 uptake alone, the difference is not evident, since both Mg^{++} and cocarboxylase are required for O_2 utilization. This oxidation does not involve oxaloacetate but pyruvate arising from the decarboxylation of the oxaloacetate. The picture, however, is different with respect to CO_2 evolution. The deficient cells were unable to decarboxylate oxaloacetate, e.g. only 41 μ l. CO_2 were liberated. The addition of Mg^{++} restored both the anaerobic decarboxylation (930 μ l.) and the aerobic decarboxylation (927 μ l.) to the original values, which were comparable with those found when both Mg^{++} and cocarboxylase were added (930 μ l. anaerobically and 1206 μ l. anaerobically). Obviously cocarboxylase is not required for the decarboxylation of oxaloacetate. Mg^{++} increased the spontaneous decarboxylation of oxaloacetate and proper corrections were made.

In Table 4 data are given which were obtained with a lysed deficient preparation. With this preparation it should be stated that the dehydrogenase activity had been almost destroyed by lysing; consequently there was little O_2 uptake with pyruvate. This is in accordance with the findings of Penrose & Quastel [1930]. Values representing this O_2 uptake, therefore, have been omitted from the Table. The oxaloacetate decarboxylation values, however, show substantially the same results as those obtained with the acetone-treated preparation.

Table 4. *Metabolism of deficient and non-deficient lysed cells with oxaloacetate*

Substrate	Deficient lysed cells plus						Non-deficient lysed cells
	No addition	Mg ⁺⁺	Coccarboxylase	Mg ⁺⁺ and cocarboxylase	Mn ⁺⁺	Mg ⁺⁺ and aneurin	
*Oxaloacetate, anaerobic, μ l. CO_2	52	928	48	934	927	922	917
*Oxaloacetate, aerobic, μ l. CO_2	54	925	49	916	922	918	920

Total volume of reactants 2 ml. 0.025 *M* oxaloacetate, 0.006 *M* $MgCl_2$, 0.006 *M* $MnSO_4$, 25 μ g. cocarboxylase or aneurin, 20 mg. cells per cup (dry weight), 0.125 *M* PO_4 buffer pH 6.8; aerobic exp. contained air in gas space, anaerobic contained N_2 . Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation of oxaloacetate values deducted. With Mg^{++} 178 μ l. Without Mg^{++} 94 μ l.

Effect of heat on the enzyme. It is well known that there is a heat-stable component present in mammalian tissue that activates the decarboxylation of oxaloacetate [Breusch, 1939]. The heat stability of our enzyme therefore has been studied.

That our preparation is not the heat-stable component is shown by Table 5. These results indicate that the enzyme is heat-labile and is almost completely destroyed at 100° in 5 min. The small value obtained under these conditions may be due to the same component discussed by Breusch.

Table 5. *Effect of heat on enzyme*

Substrate	Lysed preparation				Acetone preparation	
	No heat	60° 10 min.	80° 10 min.	100° 5 min.	No heat	100° 5 min.
*Oxaloacetate, anaerobic, μ l. CO_2	944	498	40	15	948	30

0.025 *M* oxaloacetate, 0.125 *M* PO_4 buffer pH 6.8, 2.5% lysed preparation per cup; 20 mg. acetone preparation per cup. Gas space N_2 . Total volume of reactants 2.0 ml. Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation values deducted.

DISCUSSION

The results reported here appear to differ from those reported by Smyth [1940] and Krebs & Eggleston [1940]. These investigators believe that aneurin is necessary for the synthesis of oxaloacetate from pyruvate and CO_2 . Smyth, working with staphylococci, showed that oxaloacetate could replace aneurin in the dismutation of pyruvate. He concluded that aneurin is required for the synthesis of oxaloacetate, which acts as a hydrogen acceptor in the dismutation

of pyruvate to lactate, acetate and CO_2 . Therefore in the presence of oxaloacetate the vitamin is not necessary for the dismutation. If this were the case, our vitamin-deficient acetone preparation should oxidize pyruvate in the presence of oxaloacetate. This was not true in our experiments, since O_2 -uptake and decarboxylation of pyruvate are obtained only on addition of Mg^{++} and cocarboxylase to the oxaloacetate experiments. It must be borne in mind that with oxaloacetate as the substrate, there is a sufficient supply of pyruvate at all times, owing to spontaneous decarboxylation of oxaloacetate. It should be pointed out that the results of Smyth are by no means conclusive proof of the claimed function of the vitamin. Although the anaerobic dissimilation of pyruvate has not been studied as thoroughly as the aerobic oxidation, it is probable that the component parts of the system are very similar. In the aerobic dissimilation of pyruvate [Lipmann, 1939; Banga *et al.* 1939], cocarboxylase, a C_4 dicarboxylic acid, inorganic phosphate, adenine nucleotide and Mg^{++} are components of the system. The oxidation thus requires cocarboxylase even in the presence of C_4 acids. Certainly cocarboxylase, in this case, has a function other than in the synthesis of C_4 acids. The system investigated by Smyth is very complex and there is a probability of a multiple deficiency. The removal of any one of these deficiencies might stimulate the reaction. The speed of the reaction may be limited by an additional factor when either aneurin or C_4 acids or both are added. Particularly in view of our findings, it seems that there may be another explanation of Smyth's results.

Krebs & Eggleston [1940] investigated the dissimilation of pyruvate by pigeon liver and found an increased pyruvate disappearance in the presence of NaHCO_3 and CO_2 . They concluded that the pyruvate was used in CO_2 fixation, forming oxaloacetate which then undergoes reactions of the citric acid cycle. Aneurin was concluded to be concerned with the fixation reaction because the vitamin stimulated pyruvate dissimilation by liver from an avitaminous pigeon. This stimulation was not obtained by employing breast muscle tissue from the same bird. They concluded that the vitamin acts in a reaction occurring in the dissimilation by liver but not in that by breast muscle. Furthermore, since oxaloacetate synthesis was considered to occur in liver but not in breast muscle, the vitamin was thought to act in this synthesis. The suggested function of the CO_2 is in accord with the observations made with bacteria and is attractive particularly in view of the results of Evans & Slotin [1940]. The evidence for the function of the vitamin in this reaction is, however, not conclusive. Krebs & Eggleston presented no proof that the breast muscle was deficient in aneurin. It cannot be assumed that in an animal suffering from vitamin deficiency all of the organs are equally deficient [cf. Ochoa & Peters, 1938]. Thus the liver of an avitaminous pigeon may be limited in its metabolism of pyruvate, whereas the breast muscle may still contain sufficient cocarboxylase for an active reaction. Therefore, the evidence offered by Krebs & Eggleston that aneurin does not function in pyruvate metabolism of breast muscle needs confirmation, since the vitamin activity will not be apparent if the muscle is not deficient, and therefore their conclusion concerning the function of the vitamin in oxaloacetate synthesis may have another explanation.

We have been unable by use of the enzyme from *M. lysodeikticus* to obtain carboxylation of pyruvate. Inasmuch as the ΔF of the fixation reaction is positive, and therefore requires energy from a coupled reaction, the vitamin may be required in this second reaction. The possibility exists that in our preparation conditions were such that the enzyme catalysing the energy-yielding reaction has been destroyed, and consequently we obtain only the decarboxylation

reaction. We are at present attempting to reverse the reaction to obtain carboxylation. It is to be remembered that our preparation was inactive with aneurin but active with the phosphorylated vitamin, indicating that the phosphorylating mechanism has been destroyed. It is probable that the energy-yielding reaction necessary for carboxylation may be coupled with a phosphate exchange which did not occur in our preparation.

SUMMARY AND CONCLUSIONS

An enzyme preparation obtained from *Micrococcus lysodeikticus* is described which catalyses the reaction: $\text{HOOC} \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{COOH} \rightarrow \text{CH}_3 \cdot \text{CO} \cdot \text{COOH} + \text{CO}_2$. Magnesium ions are apparently required for the reaction, whereas cocarboxylase and aneurin have no effect. The enzyme is heat-labile, and thus is not the heat-stable compound occurring in mammalian tissue which stimulates the decarboxylation of oxaloacetate. Indications are that the enzyme is required in the carboxylation reaction involved in the CO_2 utilization of Wood & Werkman.

Permeability relationships must be considered in working with oxaloacetate and whole cells of *Micrococcus lysodeikticus*.

REFERENCES

- Banga, Ochoa & Peters (1939). *Biochem. J.* **33**, 1980.
Breusch (1939). *Biochem. J.* **33**, 1757.
Evans & Slotin (1940). *J. biol. Chem.* **136**, 301.
Fleming (1924). *Lancet*, **206**, 1303.
Krebs & Eggleston (1940). *Biochem. J.* **34**, 1383.
— Eggleston, Kleinzeller & Smyth (1940). *Biochem. J.* **34**, 1234.
Lipmann (1939). *Symposia on Quantitative Biology*, **7**, 248.
Ochoa & Peters (1938). *Biochem. J.* **32**, 1501.
Ostern (1933). *Hoppe-Seyl. Z.* **218**, 160.
Penrose & Quastel (1930). *Proc. roy. Soc. B*, **107**, 168.
Smyth (1940). *Biochem. J.* **34**, 1598.
Straub (1936). *Hoppe-Seyl. Z.* **244**, 117.
Wood & Werkman (1938). *Biochem. J.* **32**, 1262.
— — (1940). *Biochem. J.* **34**, 7.