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*ENZYMATIC FIXATION OF CARBON DIOXIDE IN α-KETOGLUTARIC ACID**

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During investigations on the heterotrophic assimilation of CO_2 by *Escherichia coli* and *Aerobacter aerogenes*, it was shown that the C_5 compounds, α-ketoglutaric acid and its precursor, glutamic acid, were more effective than the other members of the Krebs cycle or their precursors in replacing CO_2 in the metabolic requirements of the organisms.[1] It was proposed that a fixation over and above the Wood and Werkman reaction takes place and that this reaction may be of great importance to the cell. The present purpose is to report the enzymatic exchange of $C^{13}O_2$ with the carboxyl group of α-ketoglutaric acid and to show a new type of heterotrophic assimilation of CO_2 involving a C_4 and C_1 addition.

A cell-free enzyme preparation from *E. coli* decarboxylates α-ketoglutarate to succinate and CO_2 in the presence of malonic acid as an inhibitor of succinate oxidation. Under proper conditions the same preparation fixes $C^{13}O_2$ in the carboxyl group adjacent to the carbonyl carbon of the C_5 acid.

Any exchange reaction involving CO_2 and resulting in the formation of a carbon-to-carbon linkage is considered to be a fixation reaction. Attempts to demonstrate the carboxylation of succinate have thus far failed, probably because the equilibrium of the reaction is far to the side of decarboxylation and the quantities of α-ketoglutarate formed are too small to detect. A phosphorylated form of succinate is apparently involved since adenosine triphosphate enhances the fixation. The reaction as studied in the presence of malonate is a reversible oxidative decarboxylation involving a half mole of oxygen uptake for each mole of α-ketoglutaric acid utilized for the production of a mole of succinate and a mole of CO_2. Thus,

$$COOHCH_2CH_2COCOOH + O \rightleftharpoons COOHCH_2CH_2COOH + CO_2.$$

Methods.—*E. coli* was grown for 16–18 hrs. at 30°C. in a medium con-

taining 0.8% glucose, 0.4% $(NH_4)_2SO_4$, 0.8% KH_2PO_4, 0.2% yeast extract and 10% tap water at an initial pH of 7.0. The cells were then harvested and ground with powdered glass and subsequently extracted with phosphate buffer solution (Kalnitsky, *et al.*[2]).

The exchange reactions were carried out in 125 ml. Warburg-Barcroft reaction vessels with two side arms. The final concentration of the α-ketoglutarate was 0.01 *M* and that of malonate 0.05 *M*. Sodium salts of both acids were used. Depending on the activity of the juice, a quantity varying between 5 and 10 ml. was added to each flask. The mixture was buffered with 0.2 *M* phosphate at pH 6.6 and 0.07 *M* $NaHC^{13}O_3$. Appropriate concentrations of the two buffers were mixed with the substrate and enzyme after temperature equilibration (30.4°C.) had been established. pH of the resulting mixture was approximately 7.2 and the volume 25 or 30 ml.

The reaction was allowed to continue until approximately half of the substrate was utilized. The residual CO_2 in the mixture was then determined by aeration. Addition of the necessary reagents was made during the course of aeration and heating. The $C^{13}O_2$ was liberated by the addition of 6 *N* H_2SO_4 and collected in 8 ml. of 4 *N* carbonate-free NaOH in specially constructed carbon dioxide absorption tubes.

Since both malonate and α-ketoglutarate are decarboxylated by the same reagents, the former was removed before decarboxylating the keto acid by the addition of 10 ml. of 0.025 *M* sodium bisulfite to the reaction mixture from which the residual CO_2 had been removed. The deproteinated sample was extracted for 72 hrs. with ether. The ether extract contained all of the original malonate as subsequently determined, plus the other dicarboxylic acid (succinic) formed during the reaction.

To the extracted solution was added 0.75 mM. of $NaHC^{12}O_3$, further acidified with H_2SO_4, heated and aerated for 15 minutes. The use of $C^{12}O_2$ rinse precluded residual $C^{13}O_2$ remaining to interfere with the isotope determination of the carboxyl carbon. The $C^{13}O_2$ content of the rinse was always determined.

α-Ketoglutaric acid was degraded with ceric sulfate and $KMnO_4$ to succinate and CO_2. The CO_2 originates from the carboxyl adjacent to the carbonyl group; it was collected and the C^{13} content determined on the mass spectrometer.

Experimental.—Succinic acid and carbon dioxide are the products formed by the aerobic dissimilation of α-ketoglutaric acid in the presence of malonate by the cell-free enzyme preparation of *E. coli* (table 1). The reaction is similar to that reported by Ochoa[3] using cat heart as the source of α-ketoglutaric dehydrogenase. When the action of the bacterial succinic dehydrogenase is blocked by sodium malonate, one molecule of succinate and one molecule of CO_2 are formed for each atom of oxygen taken

up in the oxidative decarboxylation of the keto acid. Succinate determinations by the silver salt method or by the oxidation with succinic dehydrogenase obtained from beef heart agreed well with the values for oxygen uptake and CO_2 evolved. No attempts were made to determine the products when malonate was omitted since the respiratory quotient of 1.2 to 1.3 obtained (theoretical 1.25) indicated a complete oxidation of α-ketoglutarate to CO_2 and water.

TABLE 1

OXIDATIVE DECARBOXYLATION OF α-KETOGLUTARATE BY A CELL-FREE ENZYME PREPARATION OF *E. coli*

MATERIAL DETERMINED	WITH MALONATE*	R.Q. THEORY	R.Q. EXPTL.	WITHOUT MALONATE†	R.Q. THEORY	R.Q. EXPTL.
α-Ketoglutarate utilized; mM.	0.02	...	...	0.002	..	..
Succinic acid formed; mM.	0.017	...	...	0.0	..	..
O_2 uptake; μl.	109	2.0	2.1	103	1.25	1.30
CO_2 liberated; μl.	239			135		

Total volume of reactants 2.8 ml. 0.0035 *M* α-ketoglutarate, 0.05 *M* PO_4 buffer, pH 6.8, 1 ml. bacterial juice per cup. Temp., 30.4°C.

All values are corrected for enzyme blanks.

* 0.031 *M* sodium malonate.

† Time, 2 hrs.

The cell-free enzyme preparations are generally not highly active on α-ketoglutarate or succinate but were used because malonic acid does not block the oxidation of succinate when the intact bacterial cell is employed (table 2). It was assumed that malonate did not penetrate the cell, therefore the bacteria were treated with various solvents to increase the permeability of the cell wall to the inhibitor. However, no satisfactory results were obtained. Quastel and Wooldridge[4] have shown that exposure of *E. coli* to toluene brings about a number of inactivations. The enzymes for lactic, succinic and formic acids were found intact. Our results are in agreement. Toluene and acetone treated cells remain active on succinate and become permeable to malonate; however, the same cells show no activity on α-ketoglutarate. *n*-Propyl alcohol destroys both enzymes.

Recently, Klotz and Tietze[5] reported on the inhibition of succinic acid oxidation by structurally related sulfonic acids using rat liver homogenates. No such inhibition was noted with bacterial cells (table 3). However, in the case of bacterial juices, high concentrations are effective. The endogenous activity of the juice remains essentially unchanged even when a concentration as 0.08 *M* of inhibitor is used. Malonate not only inhibits succinic dehydrogenase but reduces the endogenous activity as well. Since toluene-treated cells remain active on succinic acid, this treatment does

TABLE 2

EFFECT OF MALONIC ACID ON SUCCINIC DEHYDROGENASE

	METHOD OF INVESTIGATION							
	MANOMETRIC; OXYGEN UPTAKE, μL. SUBSTRATES					THUNBERG; REDUCTION OF M.B. % REDUCTION SUBSTRATES		
ENZYME PREPARATION	SUCCINATE	SUCCINATE + MALONATE*	α-KETOGLUTARATE	α-KETOGLUTARATE + MALONATE	MALONATE	TIME, HRS.	SUCCINATE	SUCCINATE + MALONATE
Suspension of washed *E. coli*	446	516	186	193	30	2	90	90
Toluene-treated suspension of *E. coli*	103	3	18	−10	0	2	80	0
Acetone-treated suspension of *E. coli*	201	63	62	33	...	...	..	..
n-Propyl alcohol-treated suspension of *E. coli*	−6	−2	23	6	0	...	..	..
Cell-free enzyme preparation of *E. coli*	93	65	257	153	−6	0.4	90	0

Manometric data: Total volume of reactants 2.3 ml. 0.0043 *M* α-ketoglutarate and succinate, respectively, 0.05 *M* PO_4 buffer, pH 7.0. Bacterial suspensions were added at the rate of 30 mg. per cup, dry weight, bacterial juice, 1 ml. per cup. Temp., 30.4°C.

Thunberg data: Total volume 6.0 ml. 0.0033 *M* succinate, 0.0066 *M* malonate, 0.066 *M* PO_4 buffer, pH 7.2. Bacterial suspensions, 0.5 ml. 10% suspension wet weight. Dilution of methylene blue 1/5000.

* 0.0086 *M* sodium malonate.

TABLE 3

OXIDATION OF SUCCINIC ACID IN THE PRESENCE OF 1,2-ETHANE DISULFONIC ACID

ENZYME PREPARATION	SUBSTRATE	OXYGEN UPTAKE, μL.	METHYLENE BLUE REDUCTION TIME, HRS.	% REDUCTION
Suspension of washed *E. coli*	Succinate	312	0.6	75
			0.4	95
	Succinate + inhibitor	352	0.3	95
Toluene-treated *E. coli*	Succinate	107	2	75
	Succinate + inhibitor	103	2	80
Cell-free enzyme preparation of *E. coli*	Succinate	224	...	..
	Succinate + inhibitor	60	...	..

Manometric data: Total volume 2.3 ml. 0.0043 *M* succinate, 0.05 *M* PO_4, buffer, pH 7.2, 0.08 *M* 1,2-ethane disulfonic acid. Bacterial suspensions were added at the rate of 30 mg. per cup dry weight, bacterial juice—1 cc. per cup. Temp., 30.4°C.

Thunberg data: Total volume 6.0 ml. 0.0033 *M* succinate, 0.16 *M* inhibitor, 0.066 *M* PO_4 buffer, pH 7.0. Bacterial suspensions, 0.5 cc. of a 10% suspension wet weight. Dilution of methylene blue 1/5000.

not alter permeability to 1,2-ethanedisulfonic acid. Type of the inhibition is yet to be determined. Apparently the sulfonate ion forms an enzyme complex of almost the same strength as that with malonate.

Because of the difficulties with intact cells, it was necessary to employ a cell-free preparation which not only oxidatively decarboxylates α-ketoglutarate to succinate and CO_2 but under the proper experimental conditions carboxylates succinate to form the C_5 keto acid (table 4).

TABLE 4

EXCHANGE OF HEAVY CARBON DIOXIDE IN THE CARBOXYL GROUP OF α-KETOGLUTARIC ACID DURING ENZYMATIC DECARBOXYLATION

			α-COOH OF α-KETOGLUTARATE			RESIDUAL $NaHC^{13}O_3$ —RINSE—			MALONATE		COOH GROUPS
EXPT. NO.	ATP ADDED, mg.	α-KETOGLUTARATE ADDED, mM.	RECOVERED, mM.	EXCESS C^{13} %	C^{13} FIXED, mM. × 10^4	EXCESS C^{13}, %	RECOVERED,* mM.	EXCESS C^{13}, %	ADDED, mM.	RECOVERED, mM.	EXCESS C^{13}, %
1	..	0.25	0.16	0.171	2.7	2.79	0.75	0.00	1.25	1.24	0.00
2	..	0.25	0.15	0.00	0.0	..	..	..	1.25	..	..
3	..	0.25	0.09	0.06	0.5	..	..	..	Not added	..	..
4a†	..	0.25	0.18	0.102	1.8	..	..	..	1.25	1.23	..
4b	40	0.25	0.19	0.372	7.0	..	..	..	1.25	1.25	..

Reactions were carried on in 125-ml. Erlenmeyer flasks with two side arms which were attached to the Warburg-Barcroft manometers. The final concentration of the α-ketoglutarate was 0.01 *M* and that of malonate 0.05 *M*. Depending on the activity of the juice, a quantity varying between 5 and 10 ml. was added to each flask. The mixture was buffered with 0.2 *M* phosphate, pH 6.6 and 0.07 *M* $NaHC^{13}O_3$ (3.113% excess). Appropriate concentrations of the two buffers were mixed with the substrate and enzyme after temperature equilibrium (30.4°C.) had been reached. The pH of the resulting mixture was approximately 7.2 and the volume 25 or 30 ml.

* mM. represent amounts recovered after treatment with $NaHC^{12}O_3$.

† Experiments 4*a* and 4*b* were carried out simultaneously.

It is apparent that the decarboxylation of α-ketoglutaric acid is reversible. The per cent excess of C^{13} in the α-carboxyl of the keto acid was much higher when the reaction was conducted in the presence of malonate and adenosine triphosphate. In the absence of malonate the products of the reaction were water and CO_2. As soon as succinate was formed, it was oxidized. No detectable carboxylation of succinate occurred, consequently the reaction is not recognized as reversible. Since the error of the mass spectrometer is ±0.02, a per cent excess of 0.06% C^{13} is questionable.

In the presence of malonate succinic acid accumulates to enhance the fixation of CO_2. At approximately half time of the reaction carboxylation is readily detectable.

In common with other carboxylations, this one is endergonic as is borne out by its enhancement by adenosine triphosphate (table 4).

It should be pointed out that at times reversibility could not be demonstrated. This may have been due to faulty procedure in the preparation of the juice. For example, when the juice is not centrifuged long enough to remove all of the unground cells, enough of them will be present to oxidize much of the succinate formed since their succinic dehydrogenase is not blocked by malonate, and consequently no CO_2 can be shown to be fixed. Partial denaturation of the enzyme or some component of the enzyme system may also account for experiment 2 in table 4. However, the discrepancies in this experiment are not too serious when compared with the overwhelming evidence for the reversibility (Expts. 1, 3, 4*a* and 4*b*). Any significant failure of malonate to block succinate dehydrogenation may result in fixation of carbon dioxide in the α-ketoglutaric acid. Our previous experience would lead us to believe that this has not occurred under the conditions.

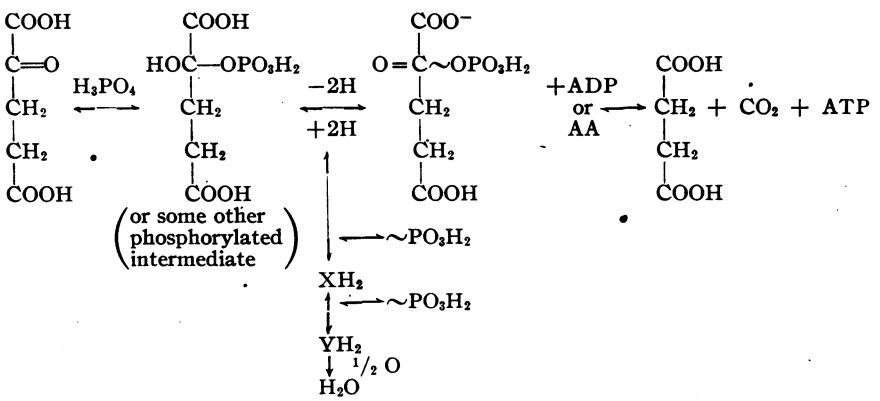
Discussion.—Although the present status of the Krebs cycle in bacterial metabolism is still uncertain, the evidence thus far obtained favors the occurrence of such a cycle in certain bacteria at least in principle. The results of Ajl, *et al.*,[1] on the replacement of CO_2 are of particular significance in this connection since metabolites normally occurring in that cycle are able to replace CO_2 thus showing not only a common function of these compounds in replacing CO_2, but potential interconversions among these compounds as well.

Every reaction of the Krebs cycle except the one studied has been shown to be reversible. According to the present findings, a complete reversal of the oxidative degradation of foodstuff would now be possible. By carboxylation and reduction, α-ketoglutaric acid would be converted to citric acid and the latter would split into acetic and oxalacetic acids as shown by Brewer and Werkman.[6] Further, oxalacetic acid would be reduced to succinic acid by way of malic and fumaric acids, and the succinate thus formed would be converted to α-ketoglutaric acid by reductive carboxylation. In this way, α-ketoglutaric acid would be constantly regenerated. Therefore, CO_2 and H_2 entering the cycle at various points during metabolism would emerge as pyruvate which could then be used for the synthesis of carbohydrates.

In a system devoid of CO_2, α-ketoglutaric acid may function in one or two ways. First, it may be oxidatively decarboxylated to yield succinic acid and CO_2, the former being utilized by the organism in place of CO_2 and secondly, the keto acid may function as a substrate for transamination, a mechanism responsible for the interconversion of proteins and carbohydrates. Since growth depends on the presence of carbon dioxide, which is replaced by α-ketoglutarate to a greater extent than any of the C_4 acids,

it is entirely possible that during the normal metabolic processes of the bacterial cell the C_1 to C_4 addition may be of greater importance to the cell than some of the other fixation reactions known to occur.

The mechanism of the reversibility may be represented:



where X and Y are intermediary hydrogen acceptors.

Of the 50 energy-rich phosphate bonds that are generated when one molecule of glucose is completely oxidized, not more than two are formed during the initial anaerobic stages that lead to the formation of lactic acid. The residual 48 bonds therefore must arise during the subsequent oxidative stages. Twenty-four (~) must be generated from each molecule of lactate. Lactate is dehydrogenated to pyruvate and the keto acid oxidatively degraded through the Krebs cycle. But of all the reactions involved in this cycle only two are known to undergo oxidative decarboxylations yielding energy-rich phosphate bonds, i.e., the oxidative decarboxylation of pyruvate and of α-ketoglutarate. It follows that some 22 additional energy-rich phosphate bonds are formed as a result of other reactions. It is postulated that the additional bonds are formed during the transfer of electrons from the primary acceptor to oxygen. If such bonds are generated, their energy could conceivably be used to reverse the intermediary steps of the reaction studied. That this may actually be the case is borne out by the fact that ATP enhances the reversibility (table 4) by increasing the concentration of energy-rich phosphate bonds.

Summary.—Reversibility of the following reaction has been demonstrated with a cell-free enzyme preparation of *E. coli*.

$$COOHCH_2CH_2COCOOH + O \longleftrightarrow COOHCH_2CH_2COOH + CO_2.$$

Adenosine triphosphate enhances the reversibility.

Occurrence of this reaction explains the function of α-ketoglutaric acid in replacing CO_2.

* Journal Paper No. J-1578 of the Iowa Agricultural Experiment Station. Project No. 746.

[1] Ajl, S. J., White, A. G. C., and Werkman, C. H., *J. Bact.*, **54**, 23 (1947).
[2] Kalnitsky, G., Utter, M. F., and Werkman, C. H., *Ibid.*, **49**, 595–602 (1947).
[3] Ochoa, S., *J. Biol. Chem.*, **155**, 87–100 (1944).
[4] Quastel, J. H., and Wooldridge, W. R., *Biochem. J.*, **22**, 689–702 (1922).
[5] Klotz, I. M., and Tietze, F., *J. Biol. Chem.*, **168**, 399–400 (1947).
[6] Brewer, C. R., and Werkman, C. H., *Enzymologia*, **6**, 273–281 (1939).

*ANTIBIOTIC SUBSTANCES FROM BASIDIOMYCETES III. COPRINUS SIMILIS AND LENTINUS DEGENER**

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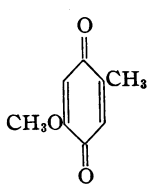
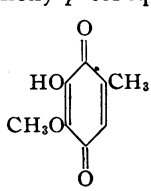
In the course of our investigations of the production of antibiotic substances by Basidiomycetes, two fungi, *Coprinus similis* and *Lentinus degener*,[1] were found to form the same antibacterial substance. This has been determined to be 5-methoxy-*p*-toluquinone (I).



I



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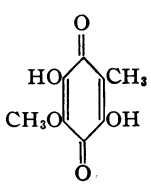

III

The identification of this compound as a mold product is of particular interest because two related compounds, fumigatin (II) and spinulosin (III), have previously been obtained from the culture liquids of fungi. Fumigatin is produced by *Aspergillus fumigatus*[2] spinulosin by *Penicillium spinulosin*[3] and by *A. fumigatus*.[4]

Antibacterial Activity of Culture Liquids.—Each of the two fungi was grown at 25°C. in 2800-ml. Fernbach flasks containing 1 liter of modified Czapek-Doz medium with dextrose and corn steep solids on coils of beechwood shavings[5] which furnished mechanical support for the mycelium.

Cultivated under these conditions, *C. similis* produced in about one month culture liquids with an activity of 128 or 256 dilution units per milliliter when tested against *Staphylococcus aureus* by serial dilution. The antibacterial activity remained essentially unchanged in such cultures for two months or more. When the activity of the culture liquids reached