THE METABOLISM OF SPECIES OF STREPTOMYCES

VI. Tricarboxylic Acid Cycle Reactions in Streptomyces coelicolor

VINCENT W. COCHRANE and HARRY D. PECK, JR.

Wesleyan University, Middletown, Connecticut

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In the preceding paper of this series (Cochrane, 1952) it was shown that bulk formation of succinic acid by *Streptomyces coelicolor* (Müller) Waksman and Henrici is effected via reductive carboxylation of pyruvate or a derivative of it. There was no evidence that bulk formation of succinate results from a block in an oxidative pathway such as the tricarboxylic acid cycle. Nevertheless, it was pointed out that the evidence, being restricted to the problem of the origin of succinate, in no way bore on the operation of such a cycle in the organism.

Ajl (1951) has reviewed recently the problem of the existence of the tricarboxylic cycle in bacteria and other microorganisms, concluding with regard to bacteria that the cycle has been shown conclusively in only a few forms, and concluding further that there are some bacteria in which the full set of reactions does not occur. The same review points out that in the aerobic filamentous fungi some of the distinctive reactions of the cycle, e.g., the oxidative decarboxylation of α-ketoglutaric acid, have never been demonstrated.

In the present work, the over-all respiratory pattern of *S. coelicolor* has been studied with whole cells and extracts. Since simple manometric data alone can never afford critical evidence, some of the key reactions have been investigated chemically, and the incorporation of acetate into α-ketoglutarate has been followed with the aid of carbon-14.

MATERIALS AND METHODS

Streptomyces coelicolor was grown for these experiments in Difco nutrient broth supplemented with glucose (0.04 M), potassium phosphate buffer (0.033 M, pH 7.4), $MgSO_4$ (0.001 M), and minor elements (Cochrane and Dimmick, 1949). Cultures were grown at 25 C on a reciprocating shaker (stroke 9 cm, frequency 95 strokes per minute), except as noted otherwise.

For manometric work with whole cells the pellets of growth were washed 3 times on a coarse sintered glass funnel with salt solution (K_2HPO_4, 0.003 M; $MgSO_4$, 0.001 M), blended 30 seconds in 10 weights of water, and added by pipette to the respiration vessels. Cell-free preparations were made by grinding with 3.3 weights of powdered pyrex glass (40 mesh) in the alkaline isotonic KCl mixture of Potter (1948). Grinding was continued in a chilled mortar until a change in consistency indicated substantially complete destruction—usually about 10 minutes. Glass and cell debris were centrifuged out at 2,500 rpm in a Sorvall model XL centrifuge, and the supernatant, adjusted to contain approximately 1 mg nitrogen per ml, was used in the Warburg or other vessel.

Methods for use and determination of carbon-14 were as previously described (Cochrane, 1952). Citrate was determined by the method of Weil-Malherbe and Bone (1949), pyruvate and α-ketoglutarate by the method of Friedemann and Haugen (1943), and acetate by the chromatographic method of Bueding and Yale (1951), as modified by Kohlmiller and Gest (1951). Conventional manometric methods were used with a bath temperature of 30 C. In experiments with whole cells and acidic substrates, a pH of 5.6 was maintained to facilitate penetration of substrate into the cell. Adenosine triphosphate, diphosphopyridine nucleotide (65 per cent purity), thiamin pyrophosphate, and α-ketoglutaric acid were obtained from commercial sources.

RESULTS

Oxidation of carbon compounds by intact cells. In table 1 are collected data from several experiments on the respiratory capacity of whole cells. The values are reported after subtraction of endogenous respiration.

It is evident first that older cells are more active than younger, the effect of age (days from inoculation to harvest) being especially marked

TABLE 1

Respiration of intact cells of different ages
(Expressed as Q_{O_2} (N)*)

SUBSTRATE†	AGE OF CELLS,‡ DAYS		
	2	3	4
Glucose, 0.01 M	171	274	368
Pyruvate, 0.01 M	42	86	108
Acetate, 0.005 M	−2	41	60
Citrate, 0.01 M	14	0	5
α-Ketoglutarate, 0.01 M	7	1	2
Succinate, 0.01 M	62	78	103
Fumarate, 0.01 M	10	12	74
DL-Malate, 0.02 M	7	11	57
Lactate, 0.01 M	14	38	—

* Endogenous subtracted.

† Complete system: cell suspension, buffer (0.017 M phosphate, pH 5.6), substrate (adjusted to pH 5.6 with KOH), water to 3.2 ml. Gas phase air, temperature 30 C.

‡ Days from inoculation to harvest; cells used immediately after harvest.

with malate and acetate as substrates. Young cells oxidize only glucose, pyruvate, and succinate of the compounds tested; it is significant that the sequence glucose—pyruvate—succinate occurs in this organism and is established early in the culture cycle (Cochrane, 1952; Cochrane and Dimmick, 1949). As cells become older, activity on these substrates increases, and other substrates (malate, lactate, and acetate) are metabolized. In view of the importance of cell permeability (see below), we are inclined at present to believe that the age effect is in fact a permeability effect although admittedly more critical experiments are needed to buttress this belief.

Representative respiratory data are plotted in figure 1, with the purpose of giving a picture of the activity of intact cells. The high endogenous rate is characteristic of the actinomycetes and the fungi (Woodruff and Foster, 1943; Stout and Koffler, 1951) and complicates interpretation of marginal rates of respiration. The cells used for the experiment illustrated were 4 days old and at

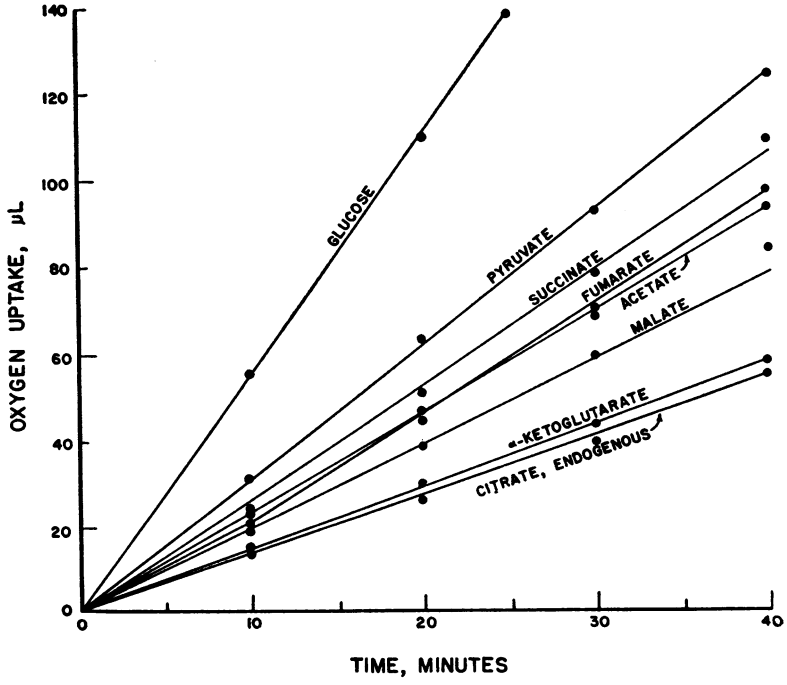

Figure 1. Respiratory metabolism of intact cells of *Streptomyces coelicolor*. All flasks contained phosphate buffer 0.017 M (pH 5.6), Mg^{++} 3.3×10^{-3} M, Mn^{++} 1.0×10^{-5} M, cell suspension 1 ml, water to 3.0 ml. Substrates at 0.01 M, except DL-malate (0.02 M) and acetate (0.005 M), tipped in 20 minutes before start of readings. Acid substrates neutralized with NaOH to pH 5.6. KOH in center well, gas phase air, temperature 30 C.

the peak of their respiratory activity. Even under these favorable conditions, only with glucose is the rate of respiration with substrate more than twice endogenous. Attempts to reduce the endogenous rate by starvation or prolonged aeration failed in that reduction in the endogenous rate was achieved only by such prolonged treatment as to reduce proportionately or more than proportionately the rate of oxidation of glucose and pyruvate.

The data on intact cells, taken by itself, would render very doubtful the applicability to this organism of any cyclic mechanism involving citrate or α-ketoglutarate, especially the latter.

Oxidation of carbon compounds by cell-free extracts. The disadvantages of intact cells as experimental material prompted a shift to cell-free methods. In the actinomycetes and presumably in the filamentous fungi, such methods offer two advantages: elimination of permeability effects and reduction of the endogenous respiration rate.

Oxidation of putative intermediates by cell-free preparations (figure 2) suggests immediately a more consistent pattern of metabolism than could be inferred from data on intact cells. Both citrate and α-ketoglutarate are oxidized, the former very rapidly. In the case of fumarate, data are included to show the stimulatory effect of diphosphopyridine nucleotide; the same stimulation has been observed with malate or succinate as substrate, suggesting that succinate and fumarate are metabolized at least in part via malate. Rates of oxidation of the 4-carbon dicarboxylic acids are approximately equal. These findings and the demonstration of enzymatic

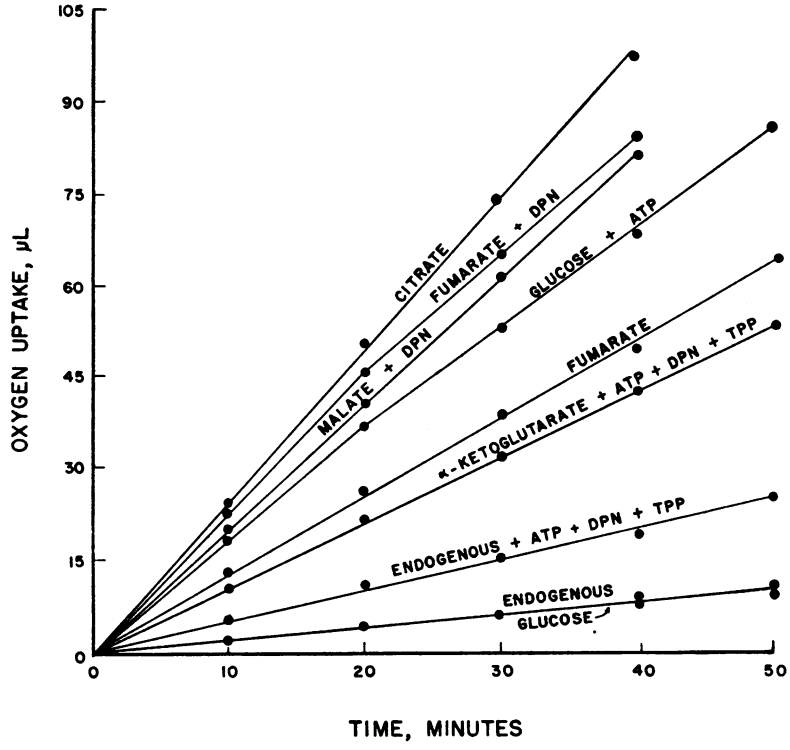

Figure 2. Respiratory metabolism of a cell-free extract of *Streptomyces coelicolor*. All flasks contained phosphate buffer 0.008 M (pH 7.3), Mg^{++} 3.3×10^{-3} M, Mn^{++} 1.0×10^{-5} M, methylene blue 1.0×10^{-3} M, extract 1.0 ml, water to 3.0 ml. Substrates at 0.01 M, except DL-malate (0.02 M), tipped in 10 minutes before start of readings. Supplements as shown: DPN (diphosphopyridine nucleotide) 5.9×10^{-3} M, ATP (adenosine triphosphate) 1.0×10^{-3} M, TPP (cocarboxylase) 8.5×10^{-5} M. KOH in center well, gas phase air, temperature 30 C.

decarboxylation of oxalacetate (table 2) are all consistent with the operation of a tricarboxylic acid cycle.

In no case was it possible to obtain a cell-free preparation capable of oxidizing pyruvate or acetate at manometrically detectable rates although, as shown earlier, intact cells oxidize

TABLE 2

*Decarboxylation of oxalacetate by cell-free extracts**

SYSTEM	CO_2, μL†
Extract 1 ml, oxalacetate 30 μM	319
No extract, oxalacetate 30 μM	170
Extract 1 ml, no substrate	2
Decarboxylase activity [319 − (170 + 2)]	147

* All flasks contained phosphate buffer 0.005 M (pH 7.3), Mg^{++} 3.3×10^{-3} M, Mn^{++} 1.0×10^{-4} M, water to 3.2 ml. Gas phase air, temperature 25 C.

† Average of duplicate flasks. Time 30 minutes.

TABLE 3

*The incorporation of C^{14} from acetate-2-C^{14} into α-ketoglutarate by intact resting cells**

	INITIAL†	FINAL
Acetate, μM	158	116
α-Ketoglutarate, μM	406	360
Acetate specific activity, mμc/mg C	434	387
Acetate total activity, mμc	1645	1076
α-Ketoglutarate specific activity, mμc/mg C	0.00	1.06
α-Ketoglutarate total activity, mμc	0.00	22.9

* Cells grown 72 hours on rotary shaker in buffered (pH 6.0) nutrient broth (Difco) plus glucose (0.04 M) and $MgSO_4$ (0.001 M).

† Washed cells suspended in buffer (0.017 M, pH 6.0) plus addenda as shown plus water to 40 ml, incubated 6 hours on rotary shaker at 25 C.

both. The rapidity of oxidation of citrate in the absence of added cofactors suggests that sufficient triphosphopyridine nucleotide is present in the preparation, as contrasted with the deficiency of diphosphopyridine nucleotide evidenced by the data on fumarate oxidation.

The manometric results establish at best only a presumption as to mechanism. It should be noted that with *S. coelicolor*, as with most other cell-free bacterial systems, it was necessary in aerobic experiments to use an artificial hydrogen carrier, methylene blue, to replace the still unknown terminal electron-transfer system. The remaining experiments to be reported were designed to demonstrate as unequivocally as possible the existence of specific reactions. Certain of the classical methods for studying metabolic systems proved inapplicable to *S. coelicolor*; with intact cells, neither sparking experiments nor malonate inhibition experiments gave clear-cut results, and the inability of extracts to oxidize pyruvate and acetate further restricted the possible experimental designs.

The incorporation of acetate-2-C^{14} into α-ketoglutarate. It has been shown earlier (Cochrane, 1952) that the methyl carbon of acetate can be trapped in succinate although at a low level only. In the present work (table 3), exogenous α-ketoglutarate was added to a cell suspension metabolizing labeled acetate; the rationale of this method is outlined by Ajl (1951).

Utilization of α-ketoglutarate was very slow although measurable over the time period used (6 hours). Nevertheless, detectable activity was trapped in the keto acid, while in the control sample, made up exactly as the experimental but harvested immediately and purified in parallel with it, there was none. Of a total of 569 mμc lost by acetate in the experimental flask, 23 mμc, or 4 per cent, was recovered in the α-ketoglutarate.

Obviously, extracellular α-ketoglutarate is not in equilibrium with the metabolic pool if it is assumed that acetate is oxidized over free α-ketoglutarate. In view of the demonstration earlier in this paper of the effect of grinding on α-ketoglutarate oxidation, it is believed that the explanation lies in the relative impermeability of the cells to the substrate. We interpret the experiment as a qualitative indication of the involvement of α-ketoglutarate or a derivative of it in acetate oxidation.

The negligible dilution of acetate activity presumably means that free acetate is not formed from α-ketoglutarate. This is in contrast to the results with glucose (Cochrane, 1952), during the oxidation of which acetate formation can be detected both by isotope dilution and by analysis.

The oxidation of α-ketoglutarate. In the tricarboxylic acid cycle in animal tissues, α-ketoglutarate is oxidatively decarboxylated to succinate,

and the further oxidation of succinate can be blocked by malonate (Stumpf *et al.*, 1947). In the presence of malonate the respiratory quotient should approximate 2 and there should be a yield of one mol of carbon dioxide per mol of substrate disappearing.

Preliminary experiments with cell-free systems metabolizing succinate in the presence of malonate indicated that the inhibitor is effective, but only at higher concentrations than usually recommended (Pardee and Potter, 1949); a concentration of 0.03 M malonate proved necessary to cause 85 per cent inhibition of the oxidation of 0.01 M succinate.

Using malonate at the level indicated, its effect on the oxidation of α-ketoglutarate (table 4) is found to be as expected. The respiratory quotient is raised from about 1.4 to about 2.0, and there is reasonable agreement between carbon dioxide evolved and substrate utilized in the presence of malonate.

TABLE 4

*The oxidation of α-ketoglutarate by cell-free extracts**

	NO MALONATE	MALONATE 0.03 M
Oxygen uptake, μM†	5.31	3.12
Carbon dioxide output, μM†	7.28	6.34
Respiratory quotient	1.37	2.03
α-Ketoglutarate utilized, μM	6.4	7.0

* System: Na α-ketoglutarate 0.01 M, phosphate buffer 0.008 M (pH 7.3), Mg^{++} 3.3×10^{-3} M, Mn^{++} 1.0×10^{-4} M, DPN (diphosphopyridine nucleotide) 5.9×10^{-5} M, ATP (adenosine triphosphate) 1.0×10^{-3} M, cocarboxylase 8.5×10^{-5} M. KOH in center well, time 155 min, temperature 30 C, gas phase air.

† Endogenous subtracted.

TABLE 5

Metabolic activities of cell-free extracts

SUBSTRATE(S)	ADDENDA*	OXYGEN UPTAKE μL	PRODUCT			
			Compound	Calc† μM	Found μM	Ratio 420/520‡
A. The oxidation of citrate to α-ketoglutarate (KGA)§						
None	Arsenite	2	KGA	—	0.0	—
Citrate, 30 μM	Arsenite	80	KGA	6.96	7.26	1.94
B. The oxidation of malate to pyruvate (PA)§						
None	DPN	14	PA	—	0.0	—
DL-Malate, 75 μM	DPN	86	PA	6.43	5.27	1.37
C. The formation of citrate from malate and acetate‖						
None	YE, ATP, TPP	—	Citrate	—	0.41	—
DL-Malate, 60 μM + acetate, 15 μM	YE, ATP, TPP	—	Citrate	—	2.02	—

* Na arsenite 6.7×10^{-3} M; DPN (diphosphopyridine nucleotide) 5.9×10^{-5} M; YE (yeast extract) 7.5 mg/ml; ATP (adenosine triphosphate) 7.5×10^{-4} M; TPP (thiamin pyrophosphate) 6.5×10^{-5} M.

† From oxygen uptake.

‡ Ratio of optical densities at 420 and 520 mμ (for authentic α-ketoglutarate 1.95, for authentic pyruvate 1.23).

§ System: phosphate buffer 0.008 M (pH 7.3); Mg^{++} 3.3×10^{-3} M; Mn^{++} 1×10^{-4} M; methylene blue 1×10^{-3} M; extract 1 ml. Fluid volume 3.2 ml, in Warburg flasks; KOH in center well, gas phase air, temperature 30 C. Time 45 min.

‖ System: phosphate buffer 0.006 M (pH 7.3); Mg^{++} 2.5×10^{-3} M; Mn^{++} 1×10^{-4} M; methylene blue 7.5×10^{-4} M. Volume 4 ml, in test tubes. Temperature 25 C, time 60 min.

While complete proof of the reaction in question would involve isolation of the product, succinic acid, the results presented, particularly the

effect of malonate on the respiratory quotient, seem sufficiently convincing for the present.

The metabolism of citrate. Conversion of citrate to α-ketoglutarate by a cell-free preparation in the presence of arsenite and methylene blue is shown in table 5 (A). The agreement between oxygen uptake and α-ketoglutarate formation is as close as can be expected. The rate of the reaction, if some allowance is made for the effect of arsenite, is reasonably close to the rate of oxidation of citrate by the same preparation (figure 2), indicating that in these preparations at least the entire metabolism of citrate occurs over α-ketoglutarate.

The metabolism of malate. In the tricarboxylic acid cycle in animal tissues malate is oxidized to pyruvate (Wood, 1946). While the sequence is not of course distinctive, any organism possessing the cycle should convert malate to pyruvate. Since cell-free preparations of *S. coelicolor* oxidize pyruvate only slowly, it is possible to isolate pyruvate as a product of malate oxidation. The data of table 5 (B) indicate that about 83 per cent of the oxygen uptake with malate as substrate can be accounted for as pyruvate. No attempt was made to discriminate between pathways of malate oxidation which involve or do not involve oxalacetate as intermediate.

In view of the results described in the next section, it is probable that the deficiency of pyruvate not accounted for represents condensation to citrate, the occurrence of which would increase simultaneously oxygen uptake and decrease pyruvate recovery.

The formation of citrate. The same cell-free preparation is shown, in table 5 (C), to carry out the synthesis of citrate from acetate and malate. Malate was used for convenience; in other respects the system used draws upon the demonstration (Stern and Ochoa, 1949) of a soluble condensing enzyme in animal tissues. Pyruvate can be substituted for acetate in this system. The need for yeast extract and adenosine triphosphate was not determined; inclusion of diphosphopyridine nucleotide was based on its stimulatory effect on malate oxidation. Activity of different preparations varied widely, from virtually nil to over five times the activity of that shown; in view of the number of factors involved in this system and the crude extraction methods used, perhaps this variability is not surprising.

DISCUSSION

The experiments presented are designed to demonstrate the existence in *S. coelicolor* of the reactions of the tricarboxylic acid cycle. The particular systems used have been dictated in part by the nature of the material, particularly the stability of separated enzymes.

The involvement of α-ketoglutarate in acetate oxidation is indicated by experiments in which C^{14} from acetate-2-C^{14} is found to be trapped in exogenous α-ketoglutarate. These experiments, by themselves, are not critical because of the very low level of activity trapped, but the involvement of α-ketoglutarate is proved by two other lines of evidence.

In the first place, citrate is oxidized stoichiometrically to α-ketoglutarate by cell-free preparations, and the rate is high enough so that it is reasonable to believe that all oxidation of citrate occurs over α-ketoglutarate. Direct chemical evidence for the conversion by yeast of citrate to α-ketoglutarate has been reported by Foulkes (1951); Kornberg and Pricer (1951) have shown also that yeast can carry out the reaction *iso*-citrate to α-ketoglutarate. In the bacteria, Altenbern and Housewright (1951) have determined by indirect means that *Brucella abortus* converts citrate and *cis*-aconitate to α-ketoglutarate, the product being determined as glutamate after transamination or as alanine after a postulated sequence of reactions involving transamination.

Secondly, the effect of malonate on the oxidation of α-ketoglutarate suggests that succinate is its oxidation product. Here the evidence is indirect, consisting of the demonstration that malonate changes the respiratory quotient with α-ketoglutarate as substrate from about 1.4 to about 2.0. This is taken to mean that the poison, shown experimentally to block the oxidation of succinate at the concentration used, has in effect isolated the oxidative decarboxylation of α-ketoglutarate. The same approach has been used earlier, e.g., by Stumpf *et al.* (1947).

Earlier results (Cochrane, 1952) made probable the existence of an oxidative pathway from succinate to pyruvate via fumarate and malate although the reductive reactions were the ones demonstrated. In the present work, we have assumed the conversion of succinate to malate on the basis of the earlier findings, with the additional evidence that diphosphopyridine nucleotide

stimulates succinate and fumarate oxidation by crude cell-free preparations. The conversion of malate to pyruvate by cell-free extracts is shown to occur with good agreement between oxygen uptake and pyruvate recovery.

Finally, the condensation of acetate (or pyruvate) and a product of malate oxidation, presumably oxalacetate, to citrate is effected by cell-free extracts of *S. coelicolor*.

While it has been shown that the organisms can decarboxylate oxalacetate, no specific effort has been made to test for the obligatory involvement of oxalacetate in the conversion of malate to pyruvate. However, since malate can supply the 4-carbon moiety for citrate synthesis, it is likely on grounds of comparative biochemistry that oxalacetate is in fact an intermediate. The same conclusion is suggested by the observation that either cyanide or glutamate accelerates malate oxidation (*unpublished data*).

The entire sequence of reactions postulated for the tricarboxylic acid cycle in animal cells is covered by these experiments although several individual reactions, e.g., the conversion of fumarate to malate and the demonstration of aconitase, have not been attempted or thought worth attempting. While there is of course no evidence as to the quantitative importance of the tricarboxylic acid cycle, it seems that the organism is capable of carrying out all of the reactions. As in most other microbial systems, aerobic respiration by cell-free extracts required an artificial hydrogen carrier, and to that extent the reactions described are all model systems. It is to be hoped that further investigations of terminal respiration properly so-called, i.e., the transfer of hydrogen to its ultimate acceptor, may in time make more natural systems available for study.

It may be noted that there is some evidence in the taxonomically related *Mycobacterium* that at least some of the crucial reactions of the cycle occur (Edson, 1951; Ochoa *et al.*, 1951).

Methodologically, the use of cell-free extracts has proved to be essential, primarily because of the low permeability of the cells to certain key substrates. It seems likely that work with other actinomycetes and with the filamentous fungi will be facilitated by the use of these methods and for the same reasons. While there is some danger that too great concentration on isolated enzyme systems will lead to neglect of the organisms as they exist in nature, the relative simplicity and abstractness of these methods should allow more rapid progress in the study of filamentous organisms than has been possible with more complex systems of growing or resting cells.

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It is a pleasure to acknowledge the hospitality of the Brookhaven National Laboratory and of Dr. Martin Gibbs, in whose laboratory the experiments with carbon-14 were performed.

SUMMARY

Whole cells oxidize some but not all of the compounds of the tricarboxylic acid cycle, failing in particular to metabolize citrate and α-ketoglutarate. Cell-free preparations oxidize glucose (with a requirement for adenosine triphosphate), citrate, α-ketoglutarate, succinate, fumarate, and malate and decarboxylate oxalacetate. Selected reactions or groups of reactions found to be catalyzed by cell-free extracts include the oxidation of citrate to α-ketoglutarate, the conversion of malate to pyruvate, and the condensation of malate and acetate (or pyruvate) to citrate. Whole cells incorporate, although at a slow rate, carbon-14 from acetate-2-C^{14} into α-ketoglutarate. The effects of diphosphopyridine nucleotide on malate and fumarate oxidation and of malonate on the oxidation of α-ketoglutarate are also consistent with the operation of a tricarboxylic acid cycle.

It is concluded that *Streptomyces coelicolor* is able to carry out the reactions of the tricarboxylic acid cycle although no data are as yet available as to the quantitative importance of this pathway to the organism.

REFERENCES

AJL, S. J. 1951 Terminal respiratory patterns in microorganisms. Bact. Revs., **15**, 211–244.

ALTENBERN, R. A., AND HOUSEWRIGHT, R. D. 1951 Alanine synthesis and carbohydrate oxidation by smooth *Brucella abortus*. J. Bact., **62**, 97–105.

BUEDING, E., AND YALE, HELEN W. 1951 Production of α-methylbutyric acid by bacteria-free *Ascaris lumbricoides*. J. Biol. Chem., **193**, 411–423.

COCHRANE, V. W. 1952 The metabolism of species of *Streptomyces*. V. The role and the

pathway of synthesis of organic acids in *Streptomyces coelicolor*. J. Bact., **63**, 459–471.

COCHRANE, V. W., AND DIMMICK, ISABEL 1949 The metabolism of species of *Streptomyces*. I. The formation of succinic and other acids. J. Bact., **58**, 723–730.

EDSON, N. L. 1951 The intermediary metabolism of the Mycobacteria. Bact. Revs., **15**, 147–182.

FOULKES, E. C. 1951 The occurrence of the tricarboxylic acid cycle in yeast. Biochem. J., **48**, 378–383.

FRIEDEMANN, T. E., AND HAUGEN, GLADYS E. 1943 Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem., **147**, 415–441.

KOHLMILLER, E. F., AND GEST, H. 1951 A comparative study of the light and dark fermentations of organic acids by *Rhodospirillum rubrum*. J. Bact., **61**, 269–282.

KORNBERG, A., AND PRICER, W. E., JR. 1951 Di- and triphosphopyridine nucleotide isocitric dehydrogenases in yeast. J. Biol. Chem., **189**, 123–136.

OCHOA, S., STERN, J. R., AND SCHNEIDER, M. C. 1951 Enzymatic synthesis of citric acid. II. Crystalline condensing enzyme. J. Biol. Chem., **193**, 691–702.

PARDEE, A. B., AND POTTER, V. R. 1949 Malonate inhibitions of oxidations in the Krebs tricarboxylic acid cycle. J. Biol. Chem., **178**, 241–250.

POTTER, V. R. 1948 The homogenate technique. Methods in Medical Research, **1**, 317–336.

STERN, J. R., AND OCHOA, S. 1949 Enzymatic synthesis of citric acid by condensation of acetate and oxaloacetate. J. Biol. Chem., **179**, 491–492.

STOUT, HELEN A., AND KOFFLER, H. 1951 Biochemistry of filamentous fungi. I. Oxidative metabolism of glucose by *Penicillium chrysogenum*. J. Bact., **62**, 253–268.

STUMPF, P. K., ZARUDNAYA, K., AND GREEN, D. E. 1947 Pyruvic and α-ketoglutaric oxidase of animal tissue. J. Biol. Chem., **167**, 817–825.

WEIL-MALHERBE, H., AND BONE, A. D. 1949 The microestimation of citric acid. Biochem. J., **45**, 377–381.

WOOD, H. G. 1946 The fixation of carbon dioxide and the inter-relationships of the tricarboxylic acid cycle. Physiol. Revs., **26**, 198–246.

WOODRUFF, H. B., AND FOSTER, J. W. 1943 Microbiological aspects of streptothricin. I. Metabolism and streptothricin formation in stationary and submerged cultures of *Actinomyces lavendulae*. Arch. Biochem., **2**, 301–315.