ACETIC ACID OXIDATION BY ESCHERICHIA COLI: EVIDENCE FOR THE OCCURRENCE OF A TRICARBOXYLIC ACID CYCLE[1]

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The tricarboxylic acid cycle has received general acceptance as a mechanism to explain the oxidation of acetic acid by animal tissues. The data obtained in studies with bacteria do not satisfy many of the criteria on which the cycle was based in animal tissues (Krebs, 1948–1949). In the preceding report, Saz and Krampitz (1954) critically evaluated this problem as applied to bacteria. Techniques were described which permit the isolation of intermediates of acetate oxidation in the absence of added carriers. These intermediates were in approximately complete isotopic equilibrium with each other, with the recovered acetate, and with the respiratory carbon dioxide. These data were in contrast with the results obtained with carrier experiments employing *Micrococcus lysodeikticus* (Saz and Krampitz, 1950; Ajl and Kamen, 1950) and *Escherichia coli* (Swim and Krampitz, 1950; Ajl and Kamen, 1950). In the latter case, isotope from acetate-2-C^{14} was not incorporated into α-ketoglutarate to a significant extent. The carrier succinate, on the other hand, was highly radioactive, and the carboxyl carbons were in almost complete isotopic equilibrium with the respiratory carbon dioxide. These data could be interpreted as evidence that the tricarboxylic acid cycle is of no quantitative importance as a mechanism for the oxidation of acetate by *E. coli* and that the major mechanism involves acetate condensation to succinate (Thunberg condensation) and the other reactions of the dicarboxylic acid cycle. It has been shown by Saz and Krampitz (1954) that nonequilibration between metabolic α-ketoglutarate and that added as a carrier can account for the observed results, and, therefore, the evidence for the dicarboxylic acid cycle was unsatisfactory. The results of experiments to be reported in this communication show that acetate is oxidized by *E. coli* via the tricarboxylic acid cycle. This conclusion is based on the isotope distribution found in intermediates which were isolated when acetate-2-C^{14} was employed as substrate in the absence of added carriers.

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MATERIALS AND METHODS

E. coli, strain E-26, was grown for 18 hours with constant aeration at 30 C in a medium containing 0.2 per cent beef extract (Difco), 0.2 per cent yeast extract (Difco), 0.4 per cent Bactopeptone, 0.2 per cent sodium chloride, 0.2 per cent sodium acetate, and 10 per cent tap water. The cells were harvested and washed twice with distilled water. Freshly harvested cells were employed in all experiments.

Isotope techniques. The methods used in the preparation of C^{14}-acetate, in the degradation of acetate, citrate, and α-ketoglutarate, and in the measurement of radioactivity were the same as those described by Saz and Krampitz (1954). The succinate first was converted to propionate by *Micrococcus lactilyticus* (Swim and Krampitz, 1954), and the latter compound was degraded according to Phares (1951).

Chromatographic separation of acids on celite. Ten g of celite[2] and 4 ml of 0.2 N sulfuric acid were mixed thoroughly in a mortar and a fine slurry prepared with chloroform (previously equilibrated with 0.2 N sulfuric acid) which was added in two g portions to an 18 mm chromatographic tube. Each portion was packed uniformly with a glass pestle. Then, 50 to 100 ml of chloroform were forced through the column under 15 lb pressure. The aqueous sample, in a volume not exceeding one ml, was acidified to a pH of less than two with 10 N sulfuric acid, mixed thoroughly with two g of dry celite, and transferred to the column and uniformly packed. The

[2] Johns Manville celite, no. 535, was washed with ether and dried prior to use on the column.

TABLE 1

Carbon recovery and isotope distribution after oxidation of acetate-2-C^{14} and nonisotopic succinate

COMPOUND	TIME IN MINUTES					
	0		15		50	
	mM	*cpm per mM*	*mM*	*cpm per mM*	*mM*	*cpm per mM*
Acetate-2-C^{14}	0.49	386,000	0.44	358,000	0.39	346,000
Succinate —COOH	0.62	0	0.59	1,920	0.51	5,060
Succinate —CH_2—		0		18,200		39,800
Respiratory CO_2			0.21	1,440	0.57	3,550

Volume of reactants in 125 ml Warburg vessel 30 ml, containing 7.5 ml 10 per cent suspension *Escherichia coli*, 3.0 mM potassium ferricyanide, 3.0 mM potassium phosphate buffer, pH 6.8, and substrates as indicated; 2.0 ml 3 N sodium hydroxide in center well. Gas phase, nitrogen. Temperature, 30 C. The reactions were terminated by adding 10 mM of sulfuric acid.

last traces of celite were wiped from the sample container with a plug of glass wool which was then pushed down firmly on top of the column.

The column was developed by the successive addition of 100 ml each of chloroform and chloroform plus 5, 10, 15, 20, 25, 30, 35, 40, 50, and 60 per cent (v/v) n-butanol.[3] Each successive solution was added before the last 10 ml of the preceding solution had run into the column. The effluent was collected in 10 ml fractions (20 by 150 mm test tubes). To each tube were added 5 ml of carbon dioxide-free water and one drop of phenol red indicator,[4] and the mixture was titrated by the addition of standard sodium hydroxide. During the titration, intimate contact between the two phases was obtained by bubbling carbon dioxide-free air through the mixture. Heavy emulsions were encountered with butanol concentrations in excess of 35 per cent which made accurate titrations practically impossible. This difficulty was eliminated by adding 5 ml of water-washed chloroform to each tube before titration.

The methods employed in the experiments recorded in tables 1 and 2 differed somewhat from those already described. The acids were removed from the reaction mixture by extraction with ether for 72 hours. The individual acids were recovered from the ether extract as described by Wood *et al.* (1942). Succinate was degraded by pyrolysis of the barium salt (Kushner and Weinhouse, 1949).

[3] Reagent grade chloroform and n-butanol were employed. Each solvent mixture was equilibrated with 0.2 N sulfuric acid.

[4] Phenol red indicator was prepared by triturating 30 mg of phenolsulfonphthalein with 0.85 ml 0.1 N sodium hydroxide and diluting to 100 ml with distilled water.

TABLE 2

Isotope distribution after oxidation of acetate-2-C^{14} in the presence of nonisotopic α-ketoglutarate and succinate

COMPOUND	SPECIFIC ACTIVITY
	cpm per mM
α-Ketoglutarate	1,240
Succinate	60,000
Respiratory CO_2	2,600

Volume of reactants 30 ml, containing 5.0 ml 15 per cent suspension *Escherichia coli*, 3.0 mM phosphate buffer, pH 6.8, 3.0 mM potassium ferricyanide, 0.75 mM acetate-2-C^{14} (386,000 cpm per mM), 0.5 mM α-ketoglutarate, 0.5 mM succinate; 2 ml 3 N sodium hydroxide in center well. Gas phase, nitrogen. Temperature, 30 C. Time, 60 minutes.

RESULTS

Isotope carrier experiments. The initial experiments were conducted under anaerobic conditions employing potassium ferricyanide as a hydrogen acceptor. Under these conditions, resting suspensions of *E. coli* oxidize acetic acid to carbon dioxide and water. The C_4-dicarboxylic acids and α-ketoglutarate also are oxidized, but the C_6-tricarboxylic acids are not utilized. The results obtained from experiments employing acetate-

2-C^{14}, with the addition of members of the tricarboxylic acid cycle as carriers, are presented for comparison with those obtained in the absense of carriers.

The carbon recovery and the isotope distribution obtained after partial oxidation of acetate-2-C^{14} in the presence of unlabeled succinate are presented in table 1. The results of an experiment, in which both succinate and α-ketoglutarate were employed as carriers, are summarized in table 2. The specific activity of the residual succinate (table 1) increased with time, whereas, the ratio of C^{14} in the methylene:carboxyl carbons decreased slightly (9.5 at 15 minutes and 7.9 at 50 minutes). The specific activity of the carboxyl carbons of succinate was somewhat greater than that of the respiratory carbon dioxide which indicated that the latter was derived from the carboxyl carbon of succinate. It is evident that the carrier succinate was in isotopic equilibrium with metabolic succinate because, if this were not the case, the specific activity of the respiratory carbon dioxide would have been greater than that of the carboxyl carbons of succinate. In contrast with the results obtained with succinate, when α-ketoglutarate was employed as carrier, the latter was not labeled to a significant extent (table 2). The relative quantity of C^{14} incorporated into the succinate and respiratory carbon dioxide was essentially the same as when α-ketoglutarate was not added (compare with table 1). These data could be interpreted as evidence against the occurrence of α-ketoglutarate as an intermediate in acetate oxidation. Experiments of this type, however, do not provide any information concerning the degree of isotopic equilibration between carrier α-ketoglutarate and metabolic α-ketoglutarate. In the absence of such data, it was not considered justifiable to exclude α-ketoglutarate as an intermediate.

Succinate and α-ketoglutarate do not accumulate in significant quantities during the oxidation of acetate by *E. coli*. It appeared possible, however, that minute quantities of these compounds are present in the cells, either free or in a combined form, and that by employing a large quantity of cells and micromethods for the isolation and separation of acids it would be possible to isolate succinic and α-ketoglutaric acids. Experiments of this type were performed in order to obtain more definitive information regarding the pathway of acetate oxidation in this organism.

TABLE 3

Quantity and specific activity of acids isolated from Escherichia coli after oxidation of acetate-2-C^{14}

COMPOUND	CELLS		SUPERNATANT	
	Quantity	Specific activity	Quantity	Specific activity
	μM	*cpm per* μM	μM	*cpm per* μM
Citrate	4.2	11,000	2.1	12,800
α-Ketoglutarate	3.4	9,600	0.5	10,400
Succinate	14.7	8,400	121	8,500
Fumarate	1.4	6,800	8.8	8,100
Malate	1.2	8,600	8.7	9,300
Respiratory CO_2	—	—	1,765	860
Acetate	440	4,200	2,850	4,430

Total volume of reactants 500 ml, containing 80 g wet weight *Escherichia coli*, 50 mM phosphate buffer, pH 6.8, 4.0 mM sodium acetate-2-C^{14} (5,080 cpm per μM). Gas phase, air. Temperature, 30 C. Time of incubation, 15 minutes. The reactions terminated by adjusting the pH to 4.5 with hydrochloric acid and reducing the temperature to 0 C.

The acetone extracted cell residue contained 52,800 cpm total. The lipid (acetone soluble, water insoluble) fraction contained 153,000 cpm total (4 cpm per μM carbon). Recovery of C^{14} was 90 per cent. Recovery of carbon was 111 per cent.

This seemed especially important since *E. coli* is one of the organisms that has been stated to employ the dicarboxylic acid cycle (Ajl, 1951*a*).

Isolation of intermediates without added carriers. The reaction vessel consisted of a 2.8 L Fernbach flask and stopper. The stopper was fitted with gas inlet and outlet tubes and a dropping funnel, each containing a stopcock. The gas outlet tube was connected to a series of two bead towers containing 3 N carbonate-free sodium hydroxide. The respiratory carbon dioxide was collected continuously by drawing carbon dioxide-free air through the apparatus. The technique employed was similar to the usual Warburg methods, and the protocol appears beneath table 3. A short incubation period (15 min) was employed in order to minimize incorporation of isotope into cellular components which are not on the direct pathway of acetate oxidation. The cells were removed from the reaction mixture by centrifugation for 5 minutes at 20,000 $\times$ G and then washed once with 250 ml of water. The cells and the combined supernatants were analyzed separately for

their acid content. All operations were performed at 0 to 5 C unless otherwise specified.

The cells were mixed in a mortar with 6 ml of 3 M hydrochloric acid and thoroughly triturated with 100 g of powdered glass. This cell-glass paste was mixed with 500 ml of acetone and allowed to stand for 5 hours with occasional shaking and then filtered. The precipitate was resuspended in 250 ml of acetone and allowed to stand overnight. After a second extraction with 250 ml of acetone for 5 hours, all the filtrates were combined and the acetone distilled *in vacuo*. The aqueous residue was filtered and the precipitate (lipids) exhaustively washed with water. The combined filtrate and wash water was distilled *in vacuo*. The residue which contains the nonvolatile acids was chromatographed on celite. The distillate was neutralized, evaporated to dryness, and the residue was chromatographed for acetic acid content.

The volume of the original supernatant (pH 4.5) was reduced to 80 ml by direct distillation. The residue was acidified to congo red with sulfuric acid, and 15 volumes were steam distilled. Acetic acid was isolated from the combined distillates as already described. The volume of the residual fluid (nonvolatile acid fraction) was reduced to 15 to 20 ml, mixed with 30 g of celite, packed into a column (25 mm inside diameter), and extracted with 1,500 ml of ether. The acids were separated from the residue remaining after evaporation of the ether by chromatography on celite.

Lactic and succinic acids are not well separated under the conditions employed. This fraction was treated with acid permanganate to destroy the lactic acid. The succinic acid was recovered from the resulting mixture by extraction with ether and then rechromatographed. The titration values for α-ketoglutarate and citrate were checked by colorimetric methods (Friedemann and Haugen, 1943; Natelson *et al.*, 1948). The individual acids were diluted with a known quantity of the corresponding nonisotopic compound and degraded. The specific activity of each acid before dilution was calculated by applying the appropriate dilution factor.

Figure 1 compares the chromatogram of the acids isolated from the cells with that of a known mixture of acids. The citrate, α-ketoglutarate, and succinate fractions did not contain interfering isotopic material since, upon the application of specific degradation procedures, the sum of the specific activities of each carbon in the respective compounds was essentially equal to that obtained when an aliquot was oxidized to carbon dioxide (*cf* tables 3 and 4). The identity of fumarate, malate, and pyruvate is assumed from their respective peak effluent volumes. The contents of the blank tubes (figure 1), in between the various peaks, were pooled in groups of 2 to 5 tubes and assayed for radioactivity. Tubes 95 to 100 contained a total of 1,500 cpm, and the average activity in the remaining groups of tubes was 80 cpm per tube. These data indicate the efficiency with which the acids are separated by this procedure and also that significant quantities of other radioactive compounds were not present in the mixture. The pyruvate peak contained a total of 880 cpm and was not investigated further.

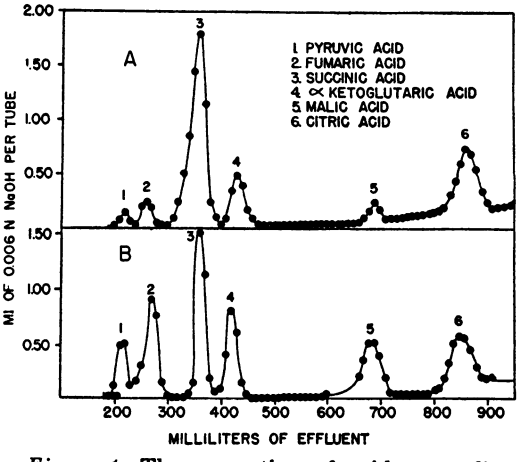
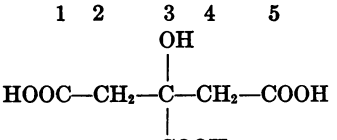
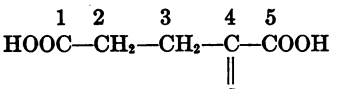
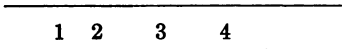


Figure 1. The separation of acids on celite: A—separation of a known mixture of acids; B—acids obtained from the cell fraction.

The quantity and specific activity of each acid which was isolated are recorded in table 3. The isotope distribution found in the individual carbons of citrate, α-ketoglutarate, succinate, and the residual acetate is presented in table 4. The average specific activity of the carboxyl carbons of citrate, α-ketoglutarate, and succinate was 980 cpm per μM as compared with 860 cpm per μM for the respiratory carbon dioxide. If these compounds were not intermediates, the respiratory carbon dioxide would have a higher specific activity than these carboxyl carbons. According to the mechanism of the tricarboxylic acid cycle, the isotope distribution in succinate and acetate

TABLE 4

Isotope distribution in acids isolated from Escherichia coli

COMPOUND	SOURCE	SPECIFIC ACTIVITY,* CPM PER μM CARBON					
		1	2	3	4	5	6
	Cells	800	3,600	3,000	3,600	800	1,090
	Supernatant	720	3,200	2,700	3,200	940	980
	Cells	1,180	3,050	3,050	1,180	1,025	
	Supernatant†	—	—	—	—	—	
	Cells	940	3,400	3,400	940		
	Supernatant	1,150	3,150	3,130	1,150		
	Cells	50	4,200				
	Supernatant†	—	—	—	—		

* The 1,5 and 2,4 carbons of citric acid and the 1,4 and 2,3 carbons of α-ketoglutaric acid were not separated by the degradation procedures employed, and, therefore, these figures are average values for each of the respective pairs of carbon atoms.

† These acids were not degraded.

would reflect the distribution expected to be found in citrate. Similarly, the type of citrate would determine the isotope distribution in α-ketoglutarate and succinate. The data (table 4) are in good agreement with these requirements. There are, however, two minor deviations from this basic pattern. The carbon atoms of citrate are numbered arbitrarily as follows: 1 and 2 represent the acetate moiety and 3 through 6 indicate the oxalacetate (C_4-dicarboxylic acid) portion of the molecule. Carbons 3 and 6 are obtained separately by the degradation procedures, and, therefore, the values for 4 and 5 should be 3,000 and 1,090 cpm per μM since they correspond to the other two carbons of the C_4-acid. By difference, the specific activity of carbon 1 would be 510, there being 1,600 cpm in carbons 1 plus 5 and 1,090 in carbon 5. Similarly, carbon 2 would contain 4,200 cpm per μM. Carbon 1 has a higher specific activity than the anticipated value because the carboxyl carbon of the residual acetate contained only 50 cpm per μM. This difference may result from the conversion of a small quantity of oxalacetate to a C_2 which is utilized more readily than acetate *per se*. The second anomaly is that the average specific activity of the 1, 4 carbons of α-ketoglutarate, based on citrate, should be $1{,}755\left(\frac{510 + 3{,}000}{2}\right)$ as compared with the observed value of 1,180 cpm per μM. The latter value, on the other hand, is in agreement with the labeling found in the succinate. The full explanation of these differences is not apparent. Three possibilities are suggested to be involved: (1) the α-ketoglutarate may be diluted by equilibration with the glutamate of the cells, (2) the carboxyl carbons of succinate may be diluted by exchange with the respiratory carbon dioxide, and (3) analytical errors.

It is apparent that components of the tricarboxylic acid cycle were produced by endogenous reactions during the oxidation of acetate-2-C^{14}. For example, the specific activity of the acetate decreased from 5,080 cpm per μM to 4,250 cpm per μM, the specific activity of the respiratory carbon dioxide was less than that of the carboxyl carbons of the various acids, and the carbon recovery was 111 per cent. In addition, 0.88 mM of acetate was oxidized, and the specific activity of the individual acids would be expected to be

somewhat higher than the observed values unless considerable dilution had occurred.

The quantity of succinate which was isolated exceeded that of the other intermediates (table 3). This result was not surprising since, even under highly aerobic conditions, appreciable quantities of succinate are formed when fumarate or malate are employed as substrates. It is not known whether the acids isolated from the supernatant were liberated from the cells during the incubation period or upon subsequent treatment of the reaction mixture.

DISCUSSION

An attempt has been made in these studies to clarify some of the controversial points concerning the mechanism of acetate oxidation by *E. coli*. The results obtained from isotope carrier experiments employing acetate-2-C^{14} show that the relative quantities of isotope incorporated into these carrier compounds vary greatly. The succinate carrier was not only highly radioactive but also achieved a high degree of isotopic equilibration with metabolic succinate since the specific activity of the carboxyl carbons was somewhat greater than that of the respiratory carbon dioxide. The α-ketoglutarate carrier, on the other hand, was not labeled to a significant extent. The results obtained when acetate-2-C^{14} was oxidized in the absence of added carriers were not in accord with those of the carrier experiments. For example, the α-ketoglutarate was not only highly radioactive but was in isotopic equilibrium with other members of the tricarboxylic acid cycle. It is clear that α-ketoglutarate, when employed as a carrier, does not equilibrate to a significant extent with metabolic α-ketoglutarate. The significance of lack of equilibration has been discussed by Saz and Krampitz (1954), who showed that a similar situation exists in *M. lysodeikticus*. The results of studies with both *E. coli* and *M. lysodeikticus* demonstrate quite conclusively that, unless a carrier compound attains approximately complete isotopic equilibrium with that produced metabolically, it is impossible to evaluate the status of this compound as an intermediate in the metabolic pathway under investigation. Ajl (1950, 1951*a*, *b*), and Ajl and Kamen (1951) concluded that α-ketoglutarate is not an intermediate in acetate oxidation by *E. coli* and that the major pathway is via the dicarboxylic acid cycle. These conclusions were based primarily on data obtained from isotope carrier experiments and are not in accord with the present findings. Similarly, the results of isotope carrier experiments reported for *Azotobacter agile* (Karlsson and Barker, 1948) and *Aerobacter aerogenes* (Ajl, 1951*c*) require further clarification before the mechanism of acetate oxidation by these organisms can be established.

The citrate, α-ketoglutarate, succinate, fumarate, and malate which were obtained by direct isolation in the absence of added carriers were in isotopic equilibrium with each other and in reasonable equilibrium with the residual acetate. The conclusion that these compounds are intermediates in acetate oxidation is supported by the fact that the specific activity of the carboxyl carbons of citrate, α-ketoglutarate, and succinate was somewhat greater than that of the respiratory carbon dioxide. The isotope distribution found in these intermediates indicates that the tricarboxylic acid cycle is of quantitative importance in the oxidation of acetate by *E. coli*. The occurrence of acetate condensation (dicarboxylic acid cycle), as an additional mechanism for acetate oxidation, is in no way excluded by the data which are presented. Citrate, α-ketoglutarate, and succinate would have the observed isotope distribution even if the dicarboxylic and tricarboxylic acid cycles were operating simultaneously.

SUMMARY

Acetate-2-C^{14} was oxidized simultaneously with unlabeled α-ketoglutarate and succinate by *Escherichia coli*. A negligible amount of isotope was incorporated into the α-ketoglutarate carrier, whereas the succinate was highly radioactive. An experiment is described employing acetate-2-C^{14} as substrate in the absence of added carriers. Citrate, α-ketoglutarate, succinate, fumarate, and malate were isolated and found to be in isotopic equilibrium with each other and with the respiratory carbon dioxide and in approximate equilibrium with the residual acetate. The isotope distribution found in these intermediates is presented as evidence for the occurrence of the tricarboxylic acid cycle in this organism. It is concluded that the lack of incorporation of isotope into an added carrier cannot be considered conclusive evidence against the occurrence of this compound as an intermediate.

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