ACETIC ACID OXIDATION BY ESCHERICHIA COLI AND AEROBACTER AEROGENES[1]

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Evidence against the occurrence of the Krebs oxidation cycle in bacterial respiration has been steadily accumulating. *Escherichia coli* and many other bacteria do not readily metabolize the three tricarboxylic acids. *Aerobacter aerogenes* will not readily attack citrate when measured manometrically unless the organism is grown in the presence of this acid as the sole source of carbon. Recently Lenti (1946) was able to show inhibition of succinic acid oxidation in *E. coli* by malonate, but the oxidation of pyruvate was not affected. Karlsson and Barker (1948) obtained evidence against the tricarboxylic acid cycle in *Azotobacter agilis*. There is, therefore, little support for the assumption that the cycle occurs in those bacteria whose intermediary metabolism has been studied in detail. On the other hand, many bacteria, including *E. coli* and *A. aerogenes*, oxidize succinate, fumarate, and malate, and reduce anaerobically oxalacetate to succinate, and this suggests that the Szent-Györgyi system, which constitutes an integral part of the Krebs cycle, is operative in microorganisms. By the use of arsenious oxide and cyclohexanol it has been possible to show that glucose or pyruvate is oxidized aerobically as far as acetic acid without the mediation of the C_4 dicarboxylic acids as catalytic hydrogen carriers. In other words, the possibility of pyruvic acid initially condensing with oxalacetate to form procitric acid or some other C_7 intermediate in its oxidation scheme is ruled out.

By applying the principle of "simultaneous adaptation" (Stanier, 1947), it was observed that acetate that arises from the breakdown of either glucose or pyruvic acid is further oxidized to CO_2 and water without the mediation of *cis*-aconitate or α-ketoglutarate. These results may in turn be used as evidence against the occurrence of the tricarboxylic acid cycle in the organisms under consideration.

METHODS

E. coli or *A. aerogenes* was grown for 16 to 18 hours, with constant aeration, at 30 C in a medium containing 0.8 per cent substrate, 0.4 per cent $(NH_4)_2SO_4$, 0.8 per cent KH_2PO_4, 0.2 per cent yeast extract or 0.07 per cent tryptone, and 20 per cent tap water, at an initial pH of 6.8 to 7.0. The cells were then harvested, washed several times with cold distilled water, and stored in the icebox, or first lyophilized and stored in the dry state. The results vary somewhat, particularly with respect to acetate oxidation by *A. aerogenes*, depending upon the treatment of the cells.

The Barcroft-Warburg respirometer technique was used. The total volume per flask varied from 2.3 to 2.8 ml. Endogenous values were subtracted from those

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obtained in the presence of the substrates. Cells were often suspended in phosphate buffer and aerated for 2 or 3 hours, at room temperature, to reduce endogenous activity to a minimum. If autoxidation is high, no acetate, for example, is utilized in the first hour of the experiment.

Pyruvic acid was determined manometrically by ceric sulfate oxidation. At the conclusion of the experiment, the fermentation liquor was acidified and 0.5 ml of saturated ceric sulfate solution in N H_2SO_4 was added. The reaction was continued for 2 hours, with constant shaking, at 32 C. Acetylmethylcarbinol (AMC) was qualitatively detected by the addition of a few drops of α-naphthol to a solution containing equal quantities of 40 per cent KOH and fermentation liquor. Acetic acid was isolated by steam distillation and estimated by titration.

TABLE 1

Effect of arsenious oxide on glucose oxidation

EXPT. NO.	SUBSTRATE	As_2O_3 CONC.	MANOMETRIC DATA							
			*Escherichia coli**				*Aerobacter aerogenes**			
			O_2 uptake	CO_2 evolved	R.Q.	AMC	O_2 uptake	CO_2 evolved	R.Q.	AMC
			μl	μl			μl	μl		
1	Glucose	0.0005 M†	230	55		−	255	403	1.6	+++
		—	263	366	0.72	−	265	395	1.4	+
	α-Ketoglutarate	0.0005 M	17	14		−	10	15		
		—	154	207	1.5	−	104	157	1.5	
	Pyruvate	0.0005 M	15	10		−	18	210		+++
		—	224	222	1.0	−	169	316	1.86	+

Total volume of reactants, 2.3 ml. Each flask contained 30 mg (dry wt) lyophilized *E. coli* or *A. aerogenes;* 0.5 ml phosphate buffer, 0.2 M, pH 7.0; 0.5 ml 0.025 M substrate (sodium salts in case of acids); 0.3 ml H_2SO_4 or NaOH in cups; water to 2.3 ml. Time, 60 min. Temperature, 32 C.

To measure the appearance or utilization of α-keto acids, saturated ceric sulfate in N H_2SO_4 was added and the CO_2 evolved measured. The products of the reactions are acetate and CO_2.

* Glucose-grown cells.

† Values as final concentrations.

EXPERIMENTAL RESULTS

Inhibition experiments. Table 1 is a summary of a typical experiment showing the effect of arsenious oxide on the oxidation and decarboxylation of α-keto acids and glucose by lyophilized preparations of *E. coli* and *A. aerogenes*. The experiments were conducted by incubating the cells with arsenious oxide for 10 minutes in the Warburg vessel before tipping in the substrate. Longer incubation periods gave identical results.

Arsenious oxide completely inhibits the oxidative decarboxylation of α-keto acids. Therefore, if α-ketoglutaric acid is involved in the aerobic breakdown of glucose, as required by the tricarboxylic acid cycle, a considerable reduction in gas exchange should result in the presence of this inhibitor. This was found not to be the case.

In the case of *E. coli*, which does not form acetylmethylcarbinol, a negligible amount of CO_2 is evolved from glucose in the presence of arsenious oxide (table 1). This is to be expected if an initial condensation between pyruvate and some C_4 dicarboxylic acid does not occur during the oxidation of the C_3 keto acid. However, if pyruvic acid is initially condensing to a C_7 intermediate that is in turn decarboxylated, then CO_2 should be given off both from glucose and pyruvate, even in the presence of As_2O_3. The O_2 uptake, on the other hand, is appreciable, e.g., 230 microliters of oxygen taken up in the presence of the inhibitor as compared with 263 microliters in its absence. These results therefore show, first, that the oxygen utilized during the aerobic breakdown of glucose may come from reactions not involving oxidative decarboxylations, and, secondly, that the CO_2 from glucose arises only from the direct breakdown of pyruvate to acetate and carbon dioxide, and not from the decarboxylation of some C_7 intermediate, oxalsuccinate, or α-ketoglutarate. It should also be pointed out that higher O_2 uptake values are consistently obtained from glucose in the absence of As_2O_3 because of the oxidation of pyruvate and to some extent of acetate. The fact that pyruvate oxidation does not proceed via the Krebs cycle will be shown. Pyruvic acid gives rise to acetic acid. Carbon dioxide and water are the only end products of acetate oxidation by both freshly harvested and lyophilized *E. coli*. However, the oxidation of acetic acid is sluggish and proceeds at a rather slow rate unless the organisms are grown with this acid as the chief source of carbon.

The end products of glucose metabolism in the case of *A. aerogenes* are different. This organism is known to form rather large amounts of acetylmethylcarbinol from both glucose and pyruvate, at least under anaerobic conditions (Silverman and Werkman, 1941). Arsenious oxide does not inhibit its formation, and it is, therefore, reasonable to expect CO_2 evolution from glucose or pyruvate even in its presence. Our results confirm this assumption. It is of significance that, in the absence of arsenious oxide, the respiratory quotient for pyruvate varies from 1.86 to 2.1, the average for five different experiments being 1.96. The theoretical R.Q. for acetate and CO_2 to be the compounds formed aerobically from the α-keto acid is 2.0. Acetate was separated and identified. Further, lyophilized cells of *A. aerogenes* do not attack acetate to any appreciable extent (in 60 minutes), and the amounts of acetylmethylcarbinol formed are very small. However, on the addition of As_2O_3, the O_2 uptake with pyruvate drops and the amount of acetylmethylcarbinol increases considerably. It must, therefore, be assumed that the large amount of acetylmethylcarbinol that is formed aerobically in the presence of arsenious oxide is due to the inhibition of the oxidative decarboxylation of pyruvate, thus forcing the reaction chiefly toward acetylmethylcarbinol formation.

As was the case with *E. coli*, somewhat greater values for oxygen uptake are obtained from glucose when As_2O_3 was not added to the reaction vessel. This increase is due presumably to the additional oxygen uptake involved in the oxidative decarboxylation of pyruvate only. Glucose-grown *Aerobacter* cells do not attack acetate to any appreciable extent. It is for this reason that the values for oxygen uptake with and without arsenious oxide are closer for *Aerobacter* than they are for *E. coli*.

The question of oxygen uptake from glucose in the presence of arsenious oxide was further investigated. Since equivalent amounts of pyruvate were obtained, it was assumed that the carbohydrate is broken down by the Meyerhof scheme. In this scheme coenzyme I is involved in the dehydrogenation of glyceraldehyde phosphate to 3-phosphoglyceric acid. Under aerobic conditions, oxygen probably serves as the hydrogen acceptor for the diphosphopyridine nucleotide, since iodoacetate completely inhibits the breakdown of glucose. This last observation can be used to explain the fact that arsenious oxide has little effect on the oxygen uptake in the presence of glucose.

In the experiments of table 2, glucose, pyruvate, acetate, and fumarate were allowed to be oxidized by freshly harvested cells of *A. aerogenes* in the presence

TABLE 2

*Effect of cyclohexanol on the oxidation of glucose, pyruvate, acetate, and fumarate by A. aerogenes**

	MANOMETRIC DATA							
	Substrate							
	Glucose		Pyruvate		Acetate		Fumarate	
	Cyclohexanol							
	Added	Not added	Added	Not added	Added	Not added	Added	Not added
O_2 uptake, μl	356	429	200	265	43	52	0	251
Co_2 evolution, μl	563	585	352	373	24	30	10	461

Total volume of reactants varied from 2.3 to 2.8 ml. Each flask contained 0.5 ml of a 10 per cent suspension of freshly harvested *A. aerogenes*; 0.5 ml phosphate buffer, 0.2 M, pH 7.0; 0.5 ml 0.025 M substrate (sodium salts in the case of acids); 0.3 ml H_2SO_4 or NaOH in cups; 0.5 ml cyclohexanol (1:50 dilution of freshly distilled cyclohexanol); water to desired volume. Time, 60 min. Temperature, 32 C.

* Glucose-grown cells.

and absence of cyclohexanol. We found that cyclohexanol, in addition to preventing the deamination of aspartic and glutamic acids (Ajl and Werkman, 1949), also disturbs the fumarate-malate equilibrium, e.g., in its presence fumarate is not oxidized. The oxidation of glucose and pyruvate, however, remains essentially the same even when relatively high concentrations of cyclohexanol are added. This last observation is of significance since it completely eliminates the possibility of an initial condensation between pyruvate and oxalacetate as a primary step in the oxidation scheme of the C_3 keto acid, at least in the case of *Aerobacter*. If a tricarboxylic acid cycle were involved, the inhibition of any one member should result in a corresponding inhibition of glucose or pyruvate oxidation. This was found not be be the case. Freshly harvested *Aerobacter* breaks down pyruvate chiefly to acetate and CO_2, and some AMC. Acetic acid is further oxidized very

slowly, at least for the first two hours of the experiment. A possible mechanism for its oxidation will be discussed. It may, however, be assumed that unless *Aerobacter* cells are specially grown they will not attack acetate appreciably, and that this volatile acid constitutes the end product of the normal aerobic breakdown of glucose or pyruvic acid.

Adaptation experiments on the mechanism of acetate oxidation. Stanier (1947) was probably the first investigator to utilize the principle of simultaneous adaptation as a technique for the study of metabolic pathways. The method is based upon the hypothesis that if an organism is adapted to a compound, X, it will simul-

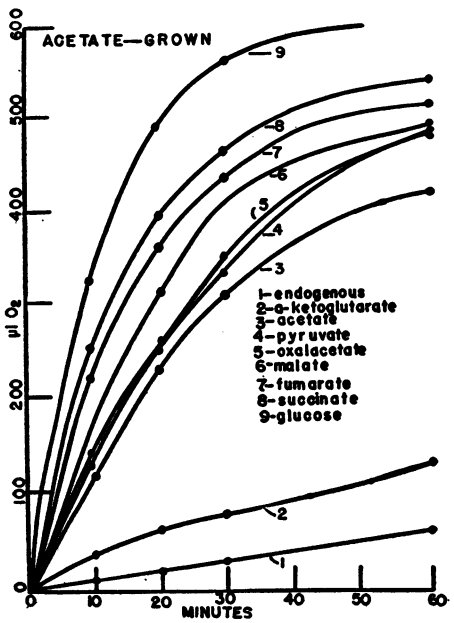


Figure 1. Respiration curves for acetate-grown cells. Total volume 2.3 ml, containing 0.5 ml of a 10 per cent suspension of freshly harvested *E. coli*; 0.5 phosphate buffer, 0.2 M, pH 7.0; 0.5 ml 0.025 M substrate; 0.3 ml NaOH; and water to desired volume. Temperature, 32 C.

taneously be adapted to all intermediates to which X gives rise in the course of its breakdown. In the study of acetate oxidation by *E. coli* this principle was utilized with some modification. If an organism attacks X only slightly when grown on any medium (not containing X), it is possible to increase the activity upon X by growing the organism in a medium in which the compound in question constitutes the chief source of carbon. The organism grows at a much slower rate, and after a prolonged incubation period cells are obtained with a very high activity upon X. Now, if Y and Z are suspected intermediates in the breakdown of X, then Y and Z should also be attacked at a much faster rate, irrespective of whether the organism is strictly adaptive to these intermediates or not, pro-

vided that the cell also metabolizes *Y* and *Z* at a faster rate, when measured manometrically and when these compounds are added to the growth medium.

E. coli oxidizes acetate, the Szent-Györgyi C_4 dicarboxylic acids, pyruvate, and α-ketoglutarate when grown on a medium containing either glucose or glycerol. When acetic acid constitutes the chief carbon source in the growth medium, the C_2 acid is attacked at a much faster rate per unit time than cells grown in either glucose or glycerol. Simultaneously an increased rate of oxidation is observed with succinate, fumarate, malate, oxalacetate, and pyruvate. Oxidation of α-ketoglutarate and *cis*-aconitate remains low (figures 1 and 2). These results suggest that the C_4 dicarboxylic acids, as well as pyruvate, participate in the

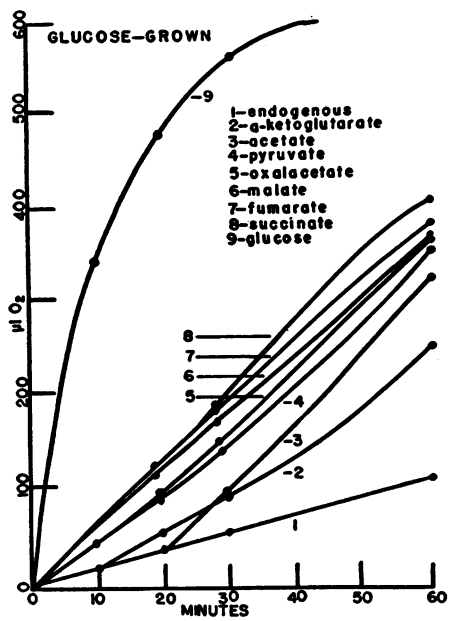
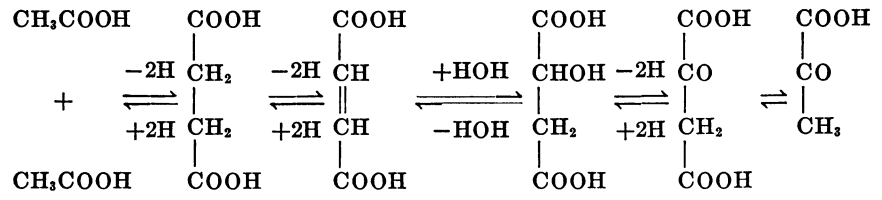


Figure 2. Respiration curves for glucose-grown cells. Total volume 2.3 ml, containing 0.5 ml of a 10 per cent suspension of freshly harvested *E. coli*; 0.5 ml phosphate buffer, 0.2 M, pH 7.0; 0.5 ml 0.025 M substrate; 0.3 ml NaOH; and water to desired volume. Temperature, 32 C.

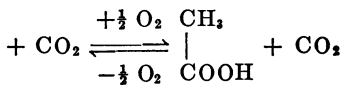
oxidation of acetic acid, at least in the case of *E. coli*. It should be pointed out that figures 1 and 2 are comparable in every respect, that is, cells were grown under identical conditions, they were washed and aerated (to reduce endogenous activity) at the same time, and the experiments were run simultaneously. The values plotted are typical, but they do not represent averages of several different experimental setups. Attention should be called to the graph obtained for oxalacetate. It follows very closely the one obtained for the oxidation of pyruvic acid. This, of course, is to be expected since there is no oxygen involved in the decarboxylation of the labile dicarboxylic acid, and the O_2 uptake is due only to pyruvate, which arises from the initial breakdown of oxalacetate.

DISCUSSION

In view of our findings the following series of reactions may take place when acetate is oxidized by *Escherichia coli*.



Two moles of acetate condensing — Succinate — Fumarate — Malate — Oxalacetate



One mole of acetate remaining

This mechanism for acetate oxidation in muscle was originally proposed by Thunberg and Knoop. Its chief weakness was the complete lack of evidence supporting the assumption of a formation of succinate from acetate.

Recently, however, a few other reports appeared in the literature that might be used as presumptive evidence to support this mechanism for acetate oxidation in bacteria. Slade and Werkman (1943), using C^{13}-labeled acetate, demonstrated the formation of succinate from acetate in the presence of glucose and under anaerobic conditions. Randles and Birkeland (1947) reported that if *E. coli* was grown under aerobic conditions in the presence of acetate, the rate of methylene blue reduction with a series of organic acids, including acetate, succinate, malate, and fumarate, was greatly increased, thus indicating that these mediate in the oxidation of acetate by this organism. However, at the present writing the situation is such that clear proof of the exact mechanism of acetate oxidation is completely lacking.

Assuming that the modified technique of "simultaneous adaptation" we employed in this study is correct, it becomes apparent that α-ketoglutaric acid does not participate in the oxidation of acetate (figures 1 and 2), at least so far as *E. coli* is concerned. Additional data are, of course, desired to prove the scheme conclusively, and experiments in this direction are now in progress, using methyl-labeled acetate as the tracer.[2] *Cis*-aconitate is also a very unlikely intermediate since *E. coli* will not attack this compound readily, regardless of what constitutes the carbon source in the growth medium. The organism can, however, be grown on the C_6 acid as a carbon source, and these cells can attack *cis*-aconitate. Data of this nature exclude postulations of permeability phenomena being involved or modified compounds being the true intermediates in certain metabolic pathways. The same is true in the case of α-ketoglutarate, e.g., when the organism is grown

[2] Samuel J. Ajl and Martin D. Kamen, to be published.

in its presence, it is immediately attacked. In addition, the biological purity of α-ketoglutarate was checked by allowing the sodium salt of this keto acid to be oxidized by other organisms (*Micrococcus lysodeikticus*, for example) known to metabolize α-ketoglutaric acid readily. When the same concentrations of α-ketoglutarate were used, 240 μl of oxygen were taken up by *M. lysodeikticus*, whereas only 34 μl of oxygen were utilized by our suspensions of *E. coli* for the same period of time. The eliminations, therefore, of α-ketoglutarate and *cis*-aconitate from the *dicarboxylic acid cycle* for acetate oxidation appear to be valid.

A word must be said in connection with our "modified" technique of simultaneous adaptation that was employed in this study. We measured throughout the course of this work an *increased* enzymatic response upon certain metabolites suspected to be involved in the breakdown of acetate, because none of the metabolites in question are strictly adaptive. Of course, the problem is simpler and the results more spectacular if the compounds to be tested are completely adaptive, as in the case of *Azotobacter agilis* (Karlsson and Barker, 1948), but such cases are relatively few and ours was an attempt to utilize the technique of simultaneous adaptation by studying increased enzymatic responses rather than responses alone. If proper precautions are taken so that simultaneous experiments are completely analogous, an increased enzymatic response upon a certain suspected intermediate is as valid as a response alone upon the same intermediate, which might have resulted from a previous adaptation of the organism upon the precursor of this intermediate. In one case the initial activity upon a given compound is low to begin with and the increased response is measured, and in the second case the initial activity is zero and again the response is measured; but the principle is the same in both instances provided proper precautions are taken in the former. Now the method becomes a very broad one and utilizable in the study of a great many metabolic pathways.

By the use of cyclohexanol and arsenious oxide it was possible to demonstrate that an initial condensation of pyruvate with some C_4 acid is not involved in the oxidation of pyruvic acid in the organisms studied. Aerobically the keto acid is initially oxidatively decarboxylated to acetate and CO_2, the former being further oxidized to CO_2 and water by the scheme outlined above. The scheme represents a series of cyclic reactions involving the utilization of 4 atoms of oxygen and the liberation of 2 moles of CO_2 for each molecule of acetic acid.

SUMMARY

The oxidation of carbohydrate via the tricarboxylic acid cycle is well established in animal tissue, but its status in bacterial metabolism is uncertain. Data have been obtained that indicate that the cycle as such does not occur in the respiration of *Escherichia coli* and *Aerobacter aerogenes*. Pyruvate is oxidatively decarboxylated to CO_2 and acetate, and the latter undergoes a series of cyclic reactions without the participation of either *cis*-aconitate or α-ketoglutarate. These results, together with the lack of evidence for the successful isolation of either the C_5 keto acid or citric acid during the oxidation of pyruvate, appear to eliminate the possibility that the tricarboxylic acid cycle is operative in bacterial metabolism.

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