RESPIRATORY ACTIVITY OF CELL-FREE EXTRACTS FROM AZOTOBACTER

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It is now clear that in most animal tissues that have been thoroughly investigated the main pathway of terminal respiration takes place via the citric acid cycle as developed by Krebs (1940), Green *et al.* (1948), and others. The mechanism of oxidative reactions of bacteria, however, is still confused because of the lack of sufficient experimental data and the presence of conflicting reports. For example, Karlsson and Barker (1948) reported that cells of *Azotobacter agile* did not metabolize acetic acid via the pathway of the cycle and suggested that some unknown mechanism may be involved. Ochoa (personal communication) has found, however, that extracts of this organism contain the enzyme that condenses acetate to citrate. Ajl (1950) presented evidence of the citric acid cycle in the metabolism of *Escherichia coli* and *Aerobacter aerogenes* but believes that an additional oxidative pathway such as a C_2—C_2 condensation may also function. Recently Foulkes (1951) has shown that although yeast cells did not metabolize added tricarboxylic acids, cell-free extracts can be obtained capable of oxidizing these acids at a rate approaching 40 per cent of the rate for pyruvate oxidation by the intact cells.

The respiration of *Azotobacter* is of particular interest because this organism has the highest rate of oxidation (based on cell dry weight or nitrogen) of any microorganism yet reported and because its oxidative metabolism is related to nitrogen fixation. *Azotobacter vinelandii*, strain "original", was selected for this study as dehydrogenase activity had been demonstrated in cell-free extracts from this organism by Lee, Burris, and Wilson (1942).

METHODS

Preparation and testing of extracts. Cells were obtained by growing *A. vinelandii* in Burk's sucrose-mineral salt medium under strong aeration in large bottles. The cultures were incubated at 34 C for periods of from 24 to 36 hours; then the cells were removed by means of a Sharples centrifuge. The cell paste was washed once by resuspending in a ten times volume of cold 0.2 per cent KCl solution and recentrifuged. The resulting cell paste containing approximately 80 per cent water was used to prepare the bacterial extracts.

Of the various methods used to break the cells, grinding in a bacterial mill, triturating with alumina powder, and treatment with ultrasonic radiation gave the most active preparations. The wet-crushing of cells in a bacterial mill as described by Booth and Green (1938) was a convenient method of preparing

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extract in quantity. The mill[2] consists of a stainless steel roller-bearing tightly fitted in a steel housing so that the rollers are under pressure. The bacterial suspension is pumped through the rotating bearing with the resultant crushing of cells. Approximately 30 g of cell paste were diluted to the consistency of a thick cream with 10 ml of 0.2 per cent KCl and milled for from 30 to 60 minutes at 2 C. The suspension was kept near pH 7.0 during the crushing process by the dropwise addition of 1 N NaOH as indicated by bromthymol blue indicator on a spot plate. Forty minutes of milling resulted in breakage of approximately 75 per cent of the cells as judged by microscopic examination. The mixture was diluted to 125 ml with cold 0.2 per cent KCl and centrifuged for a sufficient period (usually about 20 min at 2,500 G) to remove all intact cells and the larger cell particles. The translucent extract was light brown in color and contained from 2.0 to 3.5 mg N per ml. It was stable for several hours when stored at 0 C and could be kept for several weeks with gradual loss of activity when frozen and stored at −10 C.

Ultrasonic extracts were prepared by diluting 5 g cell paste to 20 or 40 ml with 0.2 per cent KCl solution in plastic centrifuge tubes. The tubes were placed in a water-cooled bath through which a 300 kilocycle ultrasonic wave impinged on the bottom of the tube. The power input of the generator was 1,000 watts. Approximately 20 minutes of exposure resulted in at least 75 per cent cell breakage. During the period of irradiation, the temperature of the cell suspension rose to 30 C, which was likely deleterious to the final respiratory activity. After exposure the extracts were cooled, centrifuged to remove cells, and frozen until used. Nitrogen values ranged from 2.0 to 4.0 mg per ml.

Alumina grinding (McIlwain, 1948) also was used successfully to prepare active extracts. Ten grams of cells were mixed with 10 g of alumina powder (Merck reagent grade) and triturated in a large chilled mortar for about 10 minutes under refrigeration. The cell paste was diluted with cold 0.2 per cent KCl solution or 0.02 M phosphate buffer (pH 7.0) and centrifuged to remove cells and alumina powder. It was necessary to use a force of 10,000 G for 15 minutes to obtain a translucent extract. The extracts contained from 1.0 to 3.0 mg N per ml.

Several other methods of preparation of cell-free extracts were attempted including drying at room temperature in air, drying in vacuum, lyophilization, and drying in acetone—all followed by grinding. None of these procedures gave extracts with activity comparable to those described before. Any process which required drying reduced drastically the oxidative activity of the whole cells before any grinding was done.

Freezing the cells and grinding in a mortar in the presence of CO_2 snow and powdered glass gave extracts with some activity but did not cause sufficient cell breakage. Likewise, shaking the cell paste violently in the presence of fine glass beads resulted in fairly active preparations (Heckly, 1949).

It appears that active cell-free extracts can be obtained by any method which breaks the cell under conditions in which it is kept moist and cold. Either dilute KCl or phosphate buffer is satisfactory as a suspending medium provided the pH

[2] Unicam Instruments (Cambridge) Ltd.

is maintained near neutrality. Salt concentrations materially higher than 0.2 per cent KCl were deleterious to the activity of the extracts.

The respiratory activity of the extracts was determined by measuring the rate of oxygen uptake with standard manometric procedures. The system used for most of the experiments with extracts consisted of 0.2 ml phosphate buffer (K^+ salt), 0.2 M, pH 6.85; 0.1 ml $MgSO_4$, 0.02 M; 5 to 20 micromoles substrate, 0.5 ml bacterial extract containing from 1.0 to 1.5 mg N; and water to make a total volume of 3.0 ml in the Warburg flask. The atmosphere was air and temperature 33 C. When whole cells were used, approximately 0.2 ml of a 1:20 cell suspension containing from 0.8 to 1.0 mg N per ml was substituted for the extract.

TABLE 1

Activity of cell-free extracts compared to cell preparations

TREATMENT		OXYGEN UPTAKE AS Q_{O_2} (N)		
		Acetate	Citrate	Succinate
Cell preparations				
2D1	Air dry 25 C	90	80	—
3S	Cell suspension	2350	120	3350
3L	Lyophylized	80	75	300
4A	Acetone dry	30	—	10
4D	Vacuum dry 25 C	50	—	25
5S	Cell suspension	3300	100	4200
16S	Cell suspension	2000		2700
Cell-free extracts†				
3J1	B-G mill	180	455	180
5J1	B-G mill	850*	450	—
5C1	CO_2 grind	—	240	95
6U2	Ultrasonic	550*	370	—
7J1	B-G mill	675*	500	120
11M1	Alumina grind	260*	200	—

* Acetate is "sparked" by 2 μM of fumarate.

† In these designations, the number refers to a particular cell paste and the letter denotes the treatment.

EXPERIMENTAL RESULTS

Comparison of extracts with cell suspensions. The supernatant extracts obtained by breaking the cells possessed respiratory activity for a number of substrates related to the Krebs cycle. A comparison of activity for some typical extracts with the rate of oxidation of untreated cell suspensions is shown in table 1. To compare the preparations on a common basis the figures for oxygen uptake are given in terms of Q_{O_2} (N), calculated as the oxygen uptake in μl O_2 per hour per mg of N contained in the reaction vessel. The rate used for the calculation of the Q_{O_2} values was that of the fastest 20 minute period, generally occurring in the initial 20 minutes with the cell extracts and after varying lag periods with the cell suspensions. As the cell extracts did not attack different substrates at the same

relative rates as the intact cells, Q_{O_2} (N) values are shown for three substrates, acetate, citrate, and succinate. To allow a more equable comparison of the extracts with the cell suspensions, several of the acetate values are given with the addition of 2 μM fumarate as a "sparker" for reasons that will be explained in a subsequent section.

It will be noted that cell suspensions 3S, 5S, and 16S readily attack both acetate and succinate, the Q_{O_2} (N) values ranging from 2,000 to 4,000. The cells showed little or no activity on citrate, with some, hardly more than the endogenous uptake. When the cells were dried in air, vacuum, acetone, or by lyophilization, the activity was almost entirely removed. Likewise, cell-free extracts made from such dried preparations were without oxidative power.

Extracts made from the cells by wet crushing with the bacterial mill, grinding with alumina or powdered glass, and breaking by ultrasonic waves all showed significant oxygen uptake with suitable substrates. In the work reported here, the milled extracts appeared to have the most activity probably due in part to the better control of temperature and pH possible by this method. It will be noted that these extracts on sparked acetate and citrate ranged in Q_{O_2} (N) values from 450 to 850 or about 20 per cent of the figures obtained with cell suspensions on acetate and succinate. Therefore, extracts prepared by the Booth-Green mill were used for most of the subsequent work.

Oxidation of acids of the citric acid cycle. The extracts oxidized the acids of the citric acid cycle but appeared to have little activity for fatty acids and most amino acids. Slow but steady rates of oxygen uptake were observed with glucose, sucrose, and some of the phosphorylated hexoses.

An experiment showing the behavior of a cell-free extract on citric, α-ketoglutaric, and succinic acids is illustrated in figure 1. With both citrate and α-ketoglutarate an initial and rapid uptake of oxygen was followed by a slower but constant rate equivalent to that found for succinate. The amount of initial oxygen uptake for citrate approaches one mole of O_2 per mole of citrate which is equivalent to that necessary to oxidize citrate to succinate. Similarly, the oxygen utilized during the initial rapid uptake on α-ketoglutarate is equivalent to 0.5 mole O_2 per mole of α-ketoglutarate, i.e., the quantity necessary to oxidize this compound to succinic acid. Succinic acid was always attacked at a substantially slower rate than other members of the cycle, and the rapid initial uptake observed with these always fell to a rate comparable to that of succinic acid alone. Tests for succinic acid dehydrogenase using methylene blue as a hydrogen acceptor showed that this enzyme was not the limiting factor in the system.

The bacterial extracts effected a rapid initial oxidation of fumaric, L-malic, and oxalacetic acids. In figure 2 the graphs for L-malic and fumaric acids are compared to that of succinate. As with citrate, both L-malate and fumarate follow the first rapid initial rate of oxygen uptake by a decrease to the rate equivalent to succinic acid, and the course of the oxidation of the two compounds appears to be identical. Doubling the amount of fumarate from 5 to 10 μM doubled the amount of oxygen initially taken up and corresponded to about from 1.0 to 1.2 moles O_2 per mole of substrate added. As an extract became older and lost ac-

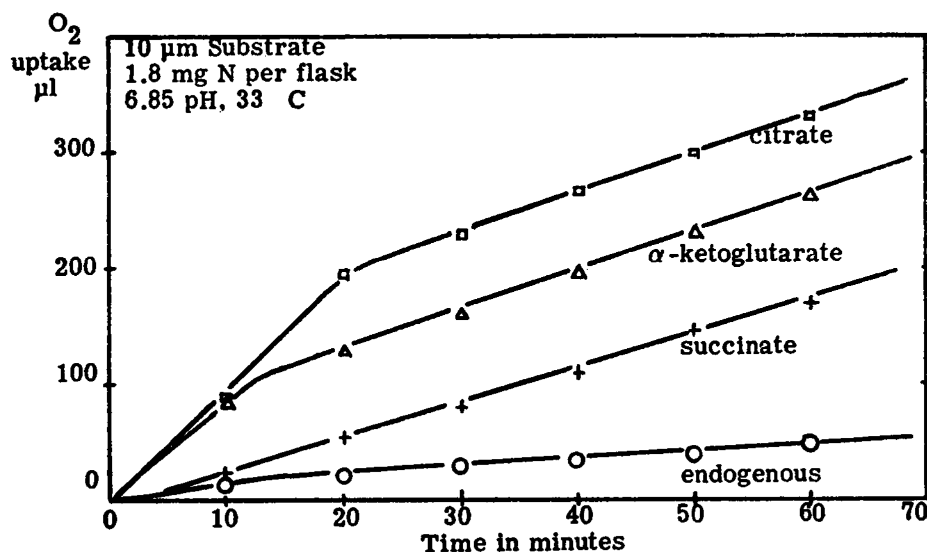

Figure 1. Oxidation of citrate, α-ketoglutarate, and succinate by extract of *Azotobacter vinelandii.*

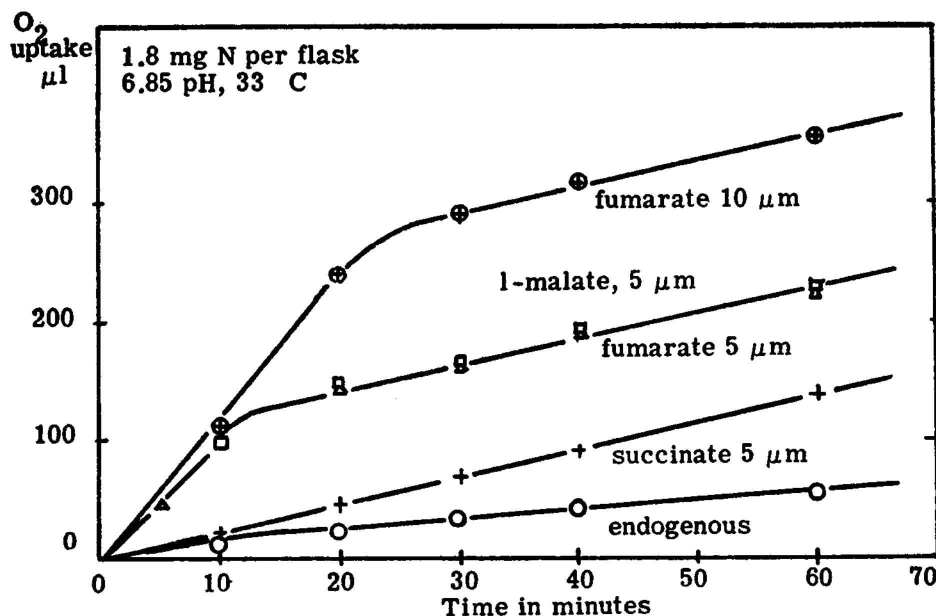

Figure 2. Comparison of the course of oxidation of four carbon dicarboxylic acids by an extract of *Azotobacter vinelandii.*

tivity, the amount of oxygen taken up with L-malate and fumarate fell off to approximately 0.5 mole O_2 per mole of substrate or sufficient to oxidize malate to the level of oxalacetate and pyruvate. When DL-malate was added, only one

half the amount of oxygen was initially taken up, indicating that D-malic acid was not attacked.

It is significant that intact cells of *A. vinelandii* grown on a sucrose-mineral salt medium usually were not able to oxidize citric acid and had lag periods of varying length when added to succinic, fumaric, malic, and α-ketoglutaric acids. It will be noted in figure 3 that although acetate and pyruvate were oxidized immediately, malate and succinate gave rise to a long lag period and citrate was not attacked. The lag period found for malate, succinate, and α-ketoglutarate varied considerably with the different cell suspensions used; however, in every case examined, extracts from these cells attacked these compounds and citrate imme-

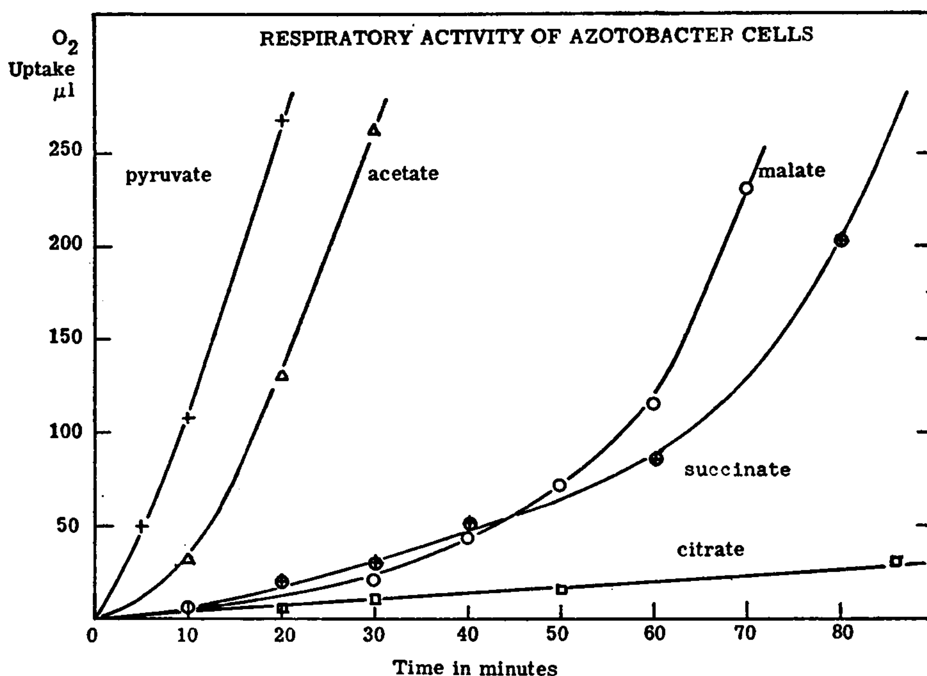

Figure 3. Oxidation of acids of the citric acid cycle by cells of *Azotobacter vinelandii*. Note differences in activity between the cells and the extracts illustrated in figures 1 and 2.

diately with no demonstrable lag period. It appears that cells grown on a sucrose medium are provided with the enzymes necessary to oxidize the acids of the citric acid cycle and that the delay or lack of activity on malate, succinate, or citrate is explicable on the basis of cell permeability.

Sparking reaction. Although the extracts attacked the members of the citric acid cycle at satisfactory rates, with the possible exception of succinate, the oxygen uptake on acetate was slow and varied with different extracts. Pyruvate and lactate were subject to a rapid initial oxidation but appeared to go to the approximate oxidation level of acetate at which point they were likewise markedly retarded. Since cell suspensions of *Azotobacter* attacked acetate rapidly with

little or no lag period, it was necessary to find some reason for the slow acetate oxidation in cell-free extracts.

A probable explanation of the difficulty becomes evident on examination of the citric acid cycle. In figure 4 a schematic diagram of the main reactions of the cycle proposed by Krebs is shown. Essentially a 2-carbon unit represented by acetate is condensed with a 4-carbon unit represented by oxalacetate to form citrate. One revolution of the cycle utilizes 4 atoms of oxygen, generates 2 molecules of CO_2, and is, therefore, equivalent to the complete oxidation of acetic acid. It becomes clear that in an enzyme system representing the reactions of this cycle, a small amount of oxalacetic acid or one of its precursors will be necessary to combine with the acetate, or its 2-carbon equivalent, and thus "spark" the cycle. This sparking phenomenon has been described in studies on animal tissue such as in the cyclophorase preparations of Green *et al.* (1948).

When a small amount of fumarate was added to acetate in the presence of *Azotobacter* extract, oxidation of the acetate was immediate and rapid as shown in figure 5. A low rate of oxygen uptake was observed when 5 μM of acetate or

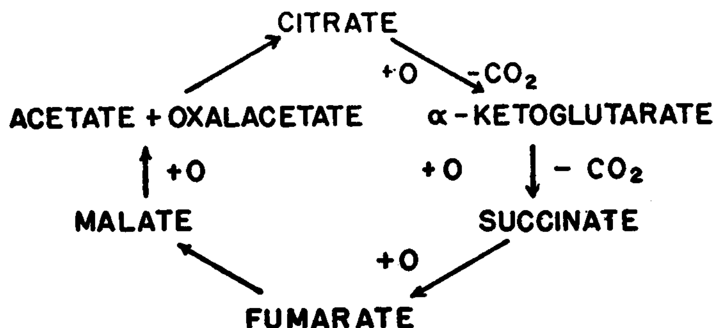

Figure 4. Condensed diagram of the tricarboxylic acid cycle of respiration.

2 μM of fumarate were added separately. When the two compounds were added in conjunction, however, a rapid uptake of oxygen occurred sufficient to account for the quantitative oxidation of 5 μM of acetate to CO_2. All the acids of the citric acid cycle, including succinate, accomplished this sparking action with either acetate or pyruvate.

Data for two experiments showing the sparking reaction in terms of oxygen uptake at intervals of 20, 40, and 60 minutes are given in table 2. In the first experiment the sparking action of 2 μM of fumarate is shown on both pyruvate and acetate. It is evident that pyruvate alone caused more oxygen uptake than acetate since likely it was oxidized to the acetate level before entering the cycle. However, the increased uptake due to the addition of fumarate is more than the sum of either compound added separately. In this experiment the oxidation of both acetate and pyruvate fell off after 40 minutes. In the second experiment the oxygen utilized by acetate and fumarate added separately is less than half that obtained when both were added together. When the acetate concentration was increased to 10 μM, the oxidation proceeded faster, and after 40 minutes, 430 μl of

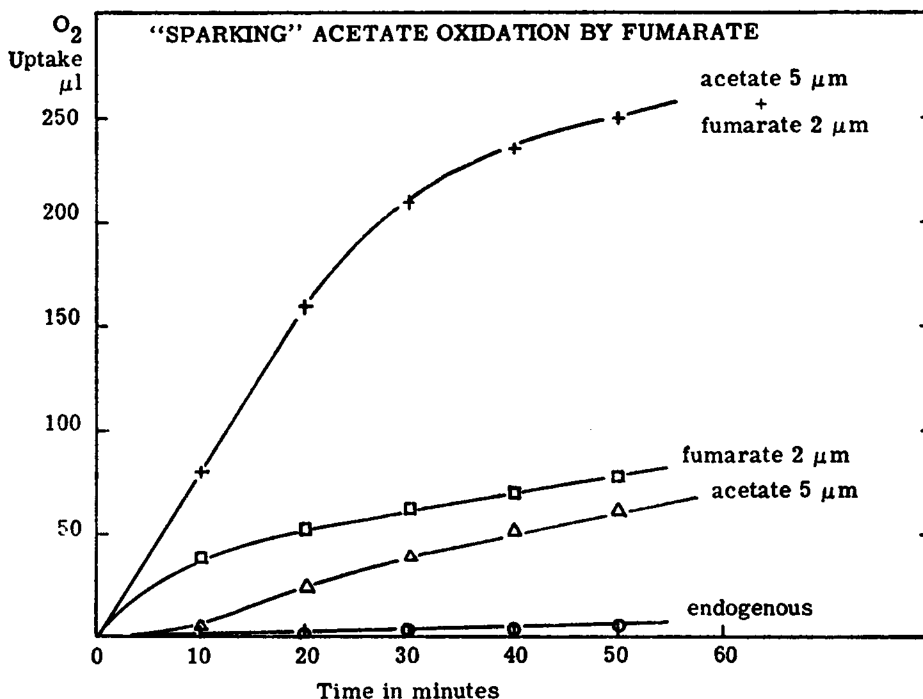

Figure 5. Oxidation of acetate and fumarate singly and together by an extract of *Azotobacter vinelandii.*

TABLE 2

"Sparking" reaction with cell-free extracts of Azotobacter vinelandii

TREATMENT	O_2 UPTAKE IN μL		
	20 min	40 min	60 min
Extract 8J1			
Endogenous	4	8	11
Acetate 10 μM	27	49	61
Pyruvate 10 μM	60	100	130
Fumarate 2 μM	43	50	57
Acetate 10 μM + fumarate 2 μM	145	275	350
Pyruvate 10 μM + fumarate 2 μM	165	270	300
Extract 10J1			
Endogenous	−5	0	—
Acetate 5 μM	38	68	—
Fumarate 2 μM	35	50	—
Acetate 5 μM + fumarate 2 μM	180	216	—
Acetate 10 μM + fumarate 2 μM	254	430	—

8J1—1.1 mg N/flask.
10J1—1.2 mg N/flask.

O_2 were taken up—a figure which approaches the theoretical 448 μl O_2 required for complete oxidation of 10 μM of acetate.

Effect of phosphate and magnesium ions. Most of the milled extracts from *A. vinelandii* could carry out the entire series of oxidations from substrate to oxygen with a quite simple system, indicating that the coenzymes that participate in the process are usually present in the bound state. Two mineral constituents appear to be necessary or at least stimulatory to the system, phosphate and magnesium ions. When a portion of extract 8J1 was dialyzed for 4 hours at 0 C against distilled water, approximately 80 per cent of the activity remained for the oxidation of citrate and sparked acetate. When magnesium ion was omitted from the system, a further decrease in activity of about 30 per cent occurred, while when the phosphate was omitted, a sharp decrease to about 20 per cent of the dialyzed control was observed. No other ion tested substituted satisfactorily for

TABLE 3

Effect of Coenzyme A (CoA) on sparking reaction

EXTRACT	TREATMENT	Q_{O_2} (N)
10J1	Acetate 5 μM + sparker*	470
10J1	Acetate 5 μM + sparker + CoA†	630
12J1	Acetate 20 μM + sparker	162
12J1	Acetate 20 μM + sparker + CoA	660
8M1	Acetate 20 μM + sparker	85
8M1	Acetate 20 μM + sparker + CoA	450
8M1	Fumarate 20 μM	800
8M1	Fumarate + CoA	650

* 2 μM of fumarate or malate used as sparker.

† 25 μg of CoA added per 3.0 ml.

Mg^{++} in the oxidation of acetate, although Mn^{++} functioned as an adequate replacement in the oxidation of fumarate and L-malate.

Effect of coenzymes. Several coenzymes were added to the cell-free system to determine whether any additional coenzyme or other factor was stimulatory. A small increase was observed in the oxidation of acetate when boiled yeast or *Azotobacter* extract was added. Cytochrome c, cocarboxylase, and adenosine triphosphate had no significant effect on any of the systems tested. Coenzyme A (CoA), which has been shown by Stern and Ochoa (1949) to be instrumental in the condensation of acetate with oxalacetate to form citrate, stimulated the sparking reaction, and in some of the less active extracts, diphosphopyridine nucleotide increased the rate of oxidation. Flavin adenine dinucleotide gave a small boost to the rate of succinate oxidation.

A summary of several experiments with coenzyme A is shown in table 3, in which data for 3 experiments with coenzyme A are given in terms of Q_{O_2} (N)

values. A 34 per cent increase in the rate of oxidation was observed when 25 μg of a crude preparation of coenzyme A (6 per cent purity) were added to extract 10J1 in the presence of acetate plus sparker. This increase is of the order usually observed with milled extracts. However, with extract 12J1, which had a low initial activity, additional coenzyme A increased the rate of oxidation by a factor of 4.

The extracts made by grinding with alumina were generally low in activity for acetate without coenzyme A as shown by an increase of from 85 to 450 μl of O_2 per hour when 25 μg coenzyme A were added to the 3.0 ml mixture in the reaction flask. That the stimulation by coenzyme A was not necessarily obtained with other steps in the tricarboxylic acid cycle is shown by the fact that the initial rate of oxygen uptake with fumarate was not helped by the addition of the coenzyme A preparation. The slight inhibition, however, was likely due to impurities in the crude preparation rather than to coenzyme A itself.

Diphosphopyridine nucleotide stimulated oxidation with some of the extracts, especially those that were of low activity originally or in older preparations that had diminished oxidative power. Flavin dinucleotide helped somewhat in the

TABLE 4

Effect of diphosphopyridine nucleotide (DPN) and flavin adenine dinucleotide (FAD) on succinate oxidation

(Extract 16J1—1.2 mg per flask)

TREATMENT	O_2 UPTAKE; μL IN 40 MIN		
	Trial 1	2	3
Succinate alone	67	68	60
Succinate + DPN	73	—	89
Succinate + FAD	—	80	—
Succinate + DPN + FAD	115	108	120

DPN 100 μg and FAD 6 μg per flask in experiments given in tables 4 and 5.

oxidation of succinate and α-ketoglutarate but not with acetate. A summary of the effect of diphosphopyridine nucleotide and flavin adenine dinucleotide on succinate oxidation is shown in table 4. The data are given in μl O_2 uptake after 40 minutes time; 100 μg of diphosphopyridine nucleotide (Schwarz Laboratories) and 6 μg of flavin adenine dinucleotide were added per 30 ml of reaction mixture. Both diphosphopyridine nucleotide and flavin adenine dinucleotide produced some increase in oxygen uptake on succinate when added separately; together the two nucleotides brought about a 60 to 100 per cent increase.

Further experiments on the effect of diphosphopyridine nucleotide and flavin adenine dinucleotide are shown in table 5. In the presence of α-ketoglutarate, 100 μg of diphosphopyridine nucleotide per reaction vessel produced some increase as did flavin adenine dinucleotide. The stimulation by flavin adenine dinucleotide of the oxidation of α-ketoglutarate may well be due to its effect on succinate oxidation, as succinate formed by the initial oxidation of α-ketoglutarate might add to the oxygen utilizing capacity of the system. Flavin adenine dinucleotide was not observed to have any beneficial effect on acetate oxidation,

but in some extracts diphosphopyridine nucleotide showed a significant stimulation as is evident by comparing the uptake with extract 16J1 after 20 minutes oxidation. However, with extract 17J1 the effect of both flavin adenine dinucleotide and diphosphopyridine was negligible.

DISCUSSION

The relative ease with which cells of *A. vinelandii* may be broken to release an apparently soluble enzyme solution provides a valuable system for the study of the oxidative metabolism of these organisms. Further interest is attached to the respiration of this system because of the high efficiency of the *Azotobacter* cells in carrying out the oxidation of simple substrates such as glucose, pyruvate, or acetate. It would be expected that the enzyme systems released from these organisms would also possess a high activity compared to enzymes of other tissues.

TABLE 5

Effect of diphosphopyridine nucleotide (DPN) and flavin adenine dinucleotide (FAD) on acetate and α-ketoglutarate oxidation

EXTRACT	TREATMENT	O_2 UPTAKE; μL IN MIN	
		20	40
17J1	α-Ketoglutarate	48	66
	α-Ketoglutarate + DPN	63	82
	α-Ketoglutarate + DPN + FAD	67	96
16J1	α-Ketoglutarate + DPN	32	62
	α-Ketoglutarate + FAD	54	106
16J1	Acetate*	106	—
	Acetate + DPN	180	—
17J1	Acetate*	166	312
	Acetate + DPN	172	318
	Acetate + DPN + FAD	171	317

* Acetate 10 μM sparked by 2 μM malate.

The fact that Q_{O_2} (N) values in the neighborhood of 600 or more were obtained which are roughly equivalent to 20 per cent of the oxidative activity of the intact cell bears out this expectation and establishes the *Azotobacter* extract as one of the most active oxidizing enzyme systems yet reported.

The ability of the cell-free systems to oxidize acetate rapidly in the presence of a small concentration of any of the members of the citric acid cycle provides evidence that the pathway of oxidation is via this cycle. All the members of the cycle tested were oxidized with satisfactory speed with the possible exception of succinate, and all the members of the cycle, including succinate, were able to effect the "sparking" of acetate and pyruvate in a manner analogous to the cyclophorase preparations from animal tissue. The stimulatory effect of coenzyme A on the oxidation of acetate is further evidence that the citric acid cycle is concerned. This cofactor has been shown by Ochoa (personal communication) to function in the coupling of acetyl phosphate and oxalacetic acid to form citrate

and thus initiate the reactions of the cycle. The stimulatory action of diphosphopyridine nucleotide, although not as clear cut as the effect of coenzyme A, offers a further analogy to the systems of animal tissue carrying out the reactions of the citric acid cycle. Similarly the effect of flavin dinucleotide on succinate oxidation would be predicted from animal tissue studies.

The oxidative behavior of the intact cells on the acids of the citric acid cycle compared to the action of cell extracts is also worthy of note. The work with *A. vinelandii* presents a clear cut example in which the cell suspensions often will not attack an important acid of the cycle (for example, citrate), and yet the extracts show that the respiratory system is present to initiate immediate oxidation. Furthermore the oxidation of all the other members of the cycle tested, such as succinate, malate, and α-ketoglutarate, showed a definite lag period in contrast to acetate and pyruvate which were attacked immediately. In view of the recent popularity of the method of "simultaneous adaptation" in testing for intermediates of a metabolic pathway by analysis of the lag periods when grown on different substrates (Stanier, 1947), it is perhaps worthwhile to emphasize once more that results from all such experiments in which cell suspensions are used must be interpreted with considerable reservation unless the factor of permeability can be definitely eliminated. Unfortunately, this is seldom possible since "permeability" here implies not only ability to penetrate the cell wall but also to get to the enzyme surface at which the reaction occurs.

The fact that the cell is not immediately permeable to a given compound obviously does not rule out its formation and function as an intermediary arising inside the cell. In their work with *A. agile* Karlsson and Barker (1948) interpret the result that cells grown on acetate require time to adapt to succinate, malate, and α-ketoglutarate as evidence that these compounds could not function in the oxidation of acetate and hence that *A. agile* does not utilize the citric acid cycle as a main pathway. In the present work cells of *A. vinelandii* showing a similar behavior were found to produce extracts giving an entirely different result.

The isotopic carbon data of Karlsson and Barker are somewhat more difficult to explain if the Krebs cycle functions in *A. agile*. Their results, however, could be due to the lack of equilibration between the intracellular intermediates formed during active metabolism and a relatively large and inactive pool of succinate or oxalacetate existing outside the cell.

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SUMMARY

Cell-free extracts of *Azotobacter vinelandii* having the ability to oxidize acetate, pyruvate, and the acids of the citric acid cycle have been prepared by wet crush-

ing with a bacterial mill, grinding with alumina, and irradiation by ultrasonic waves. The oxidation of acids of the citric acid cycle proceeded without any demonstrable lag period although the parent cell suspensions required varying lag periods or were inactive on these substrates.

The clear cut demonstration of the "sparking" effect in the oxidation of acetate and pyruvate by these extracts and the stimulation found with coenzyme A suggest that the primary pathway of oxidation of *A. vinelandii* is via the tricarboxylic acid cycle.

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