THE TRICARBOXYLIC ACID CYCLE IN THE OXIDATIVE AND SYNTHETIC METABOLISM OF KLEBSIELLA PNEUMONIAE

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Evidence for and against the operation of a tricarboxylic acid cycle (TCA) in bacteria has been obtained by testing the utilization of the intermediate compounds by resting cells, by using the technique of simultaneous adaptation, by noting the action of specific inhibitors, and by recovery techniques using radioactive tracers with and without added nonisotopic carriers. Karlsson and Barker (1948) using whole cells concluded against the presence of a TCA cycle in *Azotobacter agilis* since growth in each intermediate resulted in adaptation to the more oxidized but not the more reduced compounds in the cycle, since there was no malonate inhibition, and since labeled succinate and oxalacetate were not recovered when labeled acetate was metabolized. Stone and Wilson (1952*a*) working with *Azotobacter vinelandii* showed that while whole cell preparations metabolized the TCA intermediates with a lag period or were inactive, cell-free extracts oxidized all the compounds without demonstrable lag periods. Furthermore they showed (Stone and Wilson, 1952*b*) that carboxyl tagged acetate was incorporated into citrate, α-ketoglutarate, and succinate by cell-free extracts of the same organism. On the basis of their studies using metabolic inhibitors, labeled compounds with unlabeled carriers, and the technique of simultaneous adaptation, Ajl (1950, 1951), Ajl and Kamen (1950), and Ajl *et al.* (1951) concluded that *Micrococcus lysodeikticus* and citrate adapted *Aerobacter aerogenes* metabolized acetate via a TCA cycle. On the other hand, they reported that *Escherichia coli* and acetate grown *A. aerogenes* metabolized acetate via a dicarboxylic acid cycle which was preceded by the Knoop-Thunberg condensation of two molecules of acetate.

Swim and Krampitz (1952) using radioactive tracers showed that the Knoop-Thunberg condensation of two molecules of acetate to form succinate did not occur in *E. coli*; hence, they concluded against the presence of a dicarboxylic acid cycle in this organism. Furthermore, they (Swim and Krampitz, 1954) isolated labeled trias as well as dicarboxylic acids when radioactive acetate was metabolized in the absence of unlabeled carriers. Campbell and Stokes (1951) demonstrated that drying cells of *Pseudomonas aeruginosa* eliminated the lag periods noted when resting cells were tested against the tricarboxylic acids as well as succinate and fumarate. Barrett and Kallio (1953) by using amino acid analogues presented evidence to indicate that the penetration of citrate into cells of *P. fluorescens* was controlled by an adaptively produced enzyme. Krebs *et al.* (1952), on the basis of the very low activity of whole cell and freeze dried preparations of yeast against various citric acid cycle intermediates and of the lack of malonate inhibition, concluded against the presence of a di- or a tricarboxylic acid cycle in this organism. They concluded that two different pathways could exist: one for growth and one for energy.

In the studies to be described the oxidation of tricarboxylic acid cycle intermediates and their ability to serve as sole sources of carbon for the growth of *Klebsiella pneumoniae* were studied. Adaptation to the intermediates was ascertained by observing the growth in and dissimilation of each of the compounds by cells adapted to utilize each of the other intermediates as the sole source of carbon.

MATERIALS AND METHODS

The organism used in these studies was strain SA2 of *K. pneumoniae*. Serologically it is type A (1). Biochemically it is lactose positive, citrate positive, indole negative, methyl red negative, and Voges-Proskauer positive. This strain is well capsulated and pathogenic for mice. Inocula for the growth studies were prepared by growing the cells for 12–18 hours at 37 C in media composed of 1 per cent peptone plus 0.5 per cent glucose or in the following synthetic medium: NH_4NO_3, 0.4 g; glucose, 0.5 g; phosphate solution, 10.0 ml; salt solution, 0.2 ml; $FeSO_4$, 1.0

ml; distilled H_2O, 100.0 ml. The phosphate solution was prepared by mixing 0.1 M solutions of Na_2HPO_4 and KH_2PO_4 in a ratio of 6.9 to 3.1 so as to give a pH of approximately 7.0; the stock iron salt solution contained 100 μg/ml of $FeSO_4 \cdot 7H_2O$; and the salt solution was prepared as follows: $MgSO_4 \cdot 7H_2O$, 22.5 g; $CuSO_4 \cdot 5H_2O$, 50.0 mg; $ZnSO_4 \cdot 7H_2O$, 40.0 mg; $MnCl_2 \cdot 4H_2O$, 15.0 mg; distilled H_2O to 100.00 ml.

Following incubation, the cells were washed twice in saline and resuspended to 90 per cent transmission in standardized 16 mm tubes using a Coleman B spectrophotometer. One-tenth ml amounts were inoculated in triplicate into standardized tubes of synthetic media in which the intermediates replaced glucose. The carbon sources in the synthetic media were filter sterilized and then added to the autoclaved base in concentrations such that the amount of carbon was the same as that in 0.5 per cent glucose. Growth adaptation studies were performed by similarly preparing inocula from tubes showing growth in the various intermediates. Growth was measured turbidimetrically. Cell suspensions for the manometric studies were prepared by inoculating 1 liter Erlenmeyer flasks, each containing 500 ml amounts of the same media used in the growth studies. No differences in the activity of the cell preparations were noted if the cells were aerated during growth by bubbling air into the cultures. The flasks were incubated at 37 C till good growth was obtained. Following incubation, the cells were centrifuged, washed in saline, recentrifuged, and suspended in 0.1 M phosphate buffer, pH 7. The cells were then aerated for three hours, centrifuged, and resuspended in saline to about ten per cent. The manometric studies were done using a Warburg respirometer in an atmosphere of air at 37 C.

RESULTS

No differences in the ability of the cells to utilize the various TCA intermediates as sole carbon sources for growth were noted whether the inocula were grown in the peptone, peptone-glucose, or synthetic glucose media (figure 1). Glucose, malate, oxalacetate, and pyruvate were utilized rapidly. The lag periods in citrate and acetate media were longer; however, all these compounds gave visible growth within 30 hours. Growth appeared next in α-ketoglutarate and *cis*-aconitate media and lastly in succinate and fumarate.

The growth response to glucose, malate, oxalacetate, and pyruvate remained rapid irrespective of the compound to which it was previously

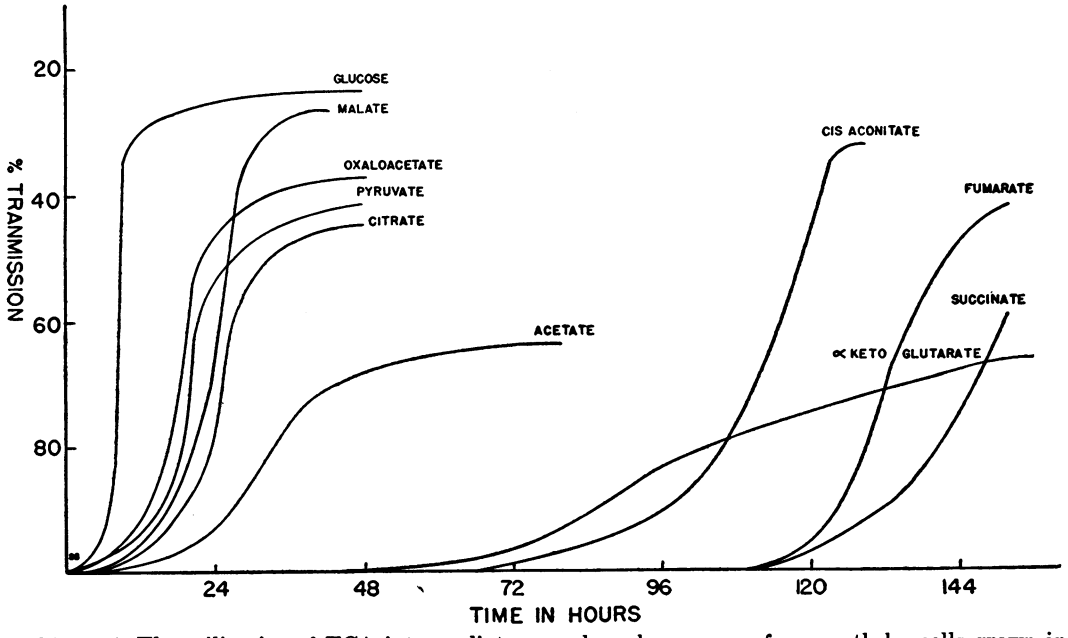

Figure 1. The utilization of TCA intermediates as sole carbon sources for growth by cells grown in glucose synthetic medium.

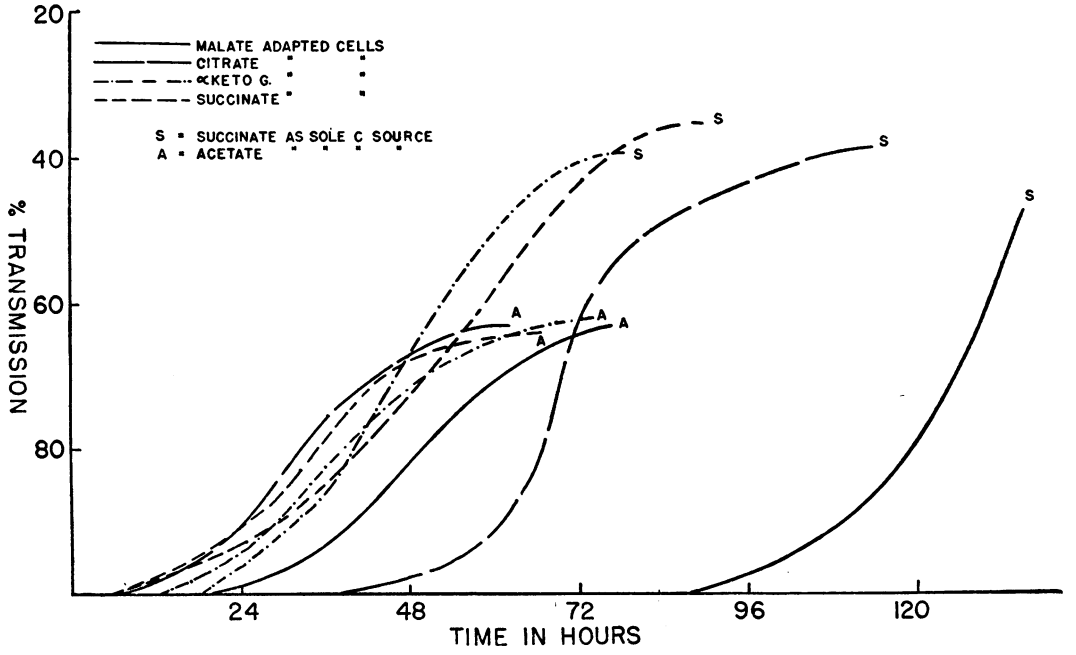

Figure 2. The utilization of succinate and acetate as growth substrates by malate, citrate, α-ketoglutarate, and succinate adapted cells.

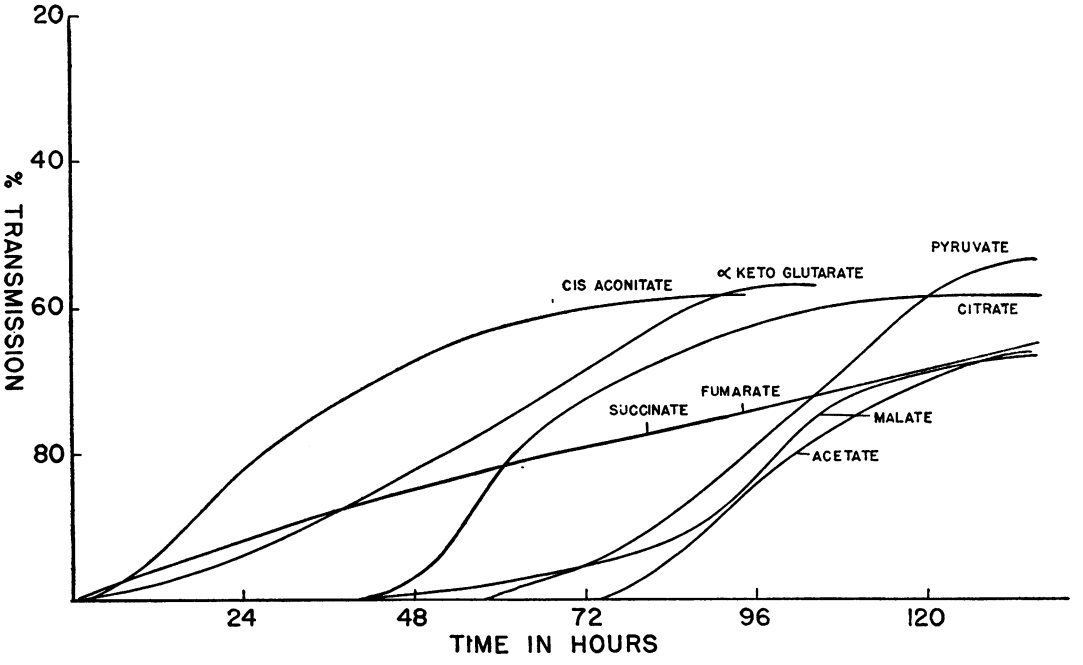

Figure 3. The utilization of α-ketoglutarate for growth by cells adapted to various TCA intermediates. The compound named over each curve denotes the intermediate to which the cells were previously adapted.

adapted. Growth in citrate and acetate lagged behind that in the aforementioned compounds even when the cells were previously grown in citrate and acetate synthetic media. The lag period in succinate and fumarate was not decreased by previous adaptation to malate, oxalacetate, pyruvate, or acetate, was slightly decreased by previous adaptation to citrate, was moderately decreased by previous growth in α-ketoglutarate and *cis*-aconitate, and was greatly decreased by adaptation to succinate and fumarate themselves (figure 2). The lag period in α-ketoglutarate was not affected by previous adaptation to acetate, malate, oxalacetate, and pyruvate, was slightly decreased by growth in citrate, and was almost completely eliminated by previous adaptation in *cis*-aconitate, succinate, fumarate, and α-ketoglutarate itself (figure 3).

The results of the manometric studies were not too definitive. That the slow growth in the succinate and fumarate media was not due to permeability can be seen from the fact that both these compounds as well as malate, oxalacetate, and pyruvate were metabolized rapidly with cells prepared in peptone or peptone-glucose media

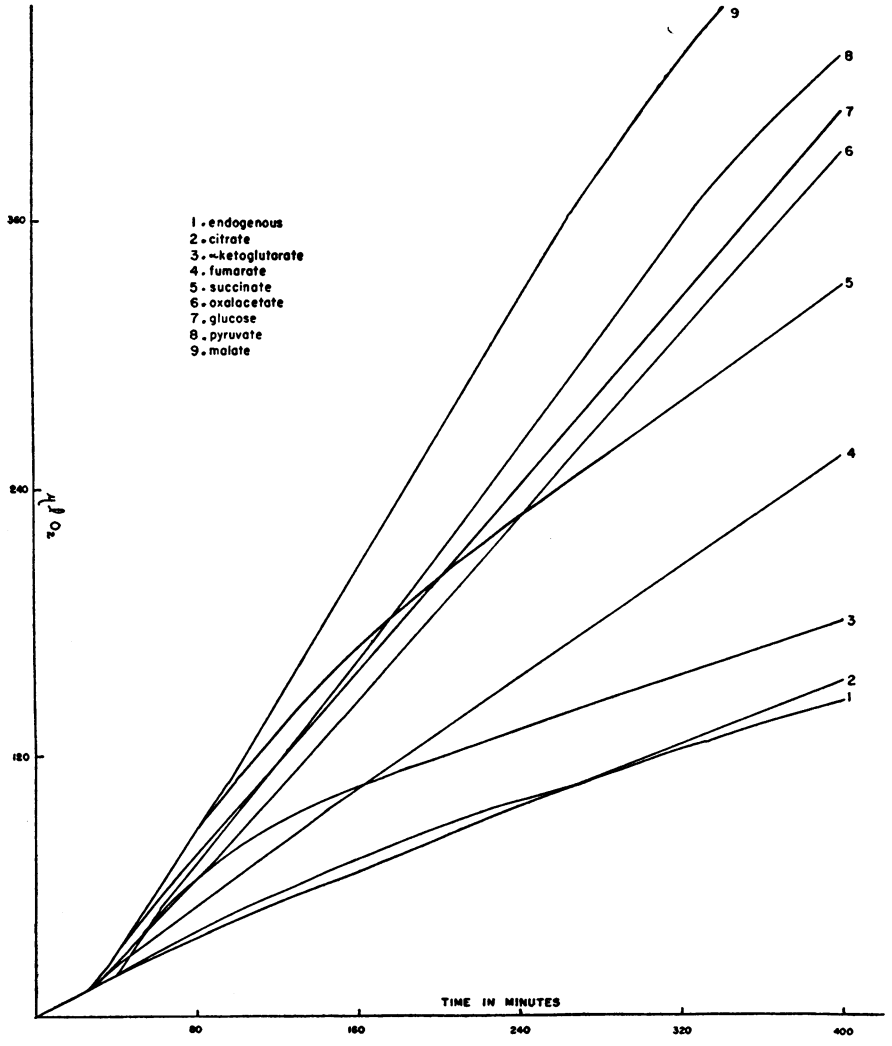

Figure 4. The oxidation of TCA intermediates by peptone grown cells: total volume 3.2 ml, containing 1.0 ml of a 5 per cent suspension of cells (strain SA2); 0.5 ml of phosphate buffer, pH 7.0; 0.5 ml of a 0.015 M of the substrate; 0.2 ml of 10 per cent KOH in the center well; and saline to volume.

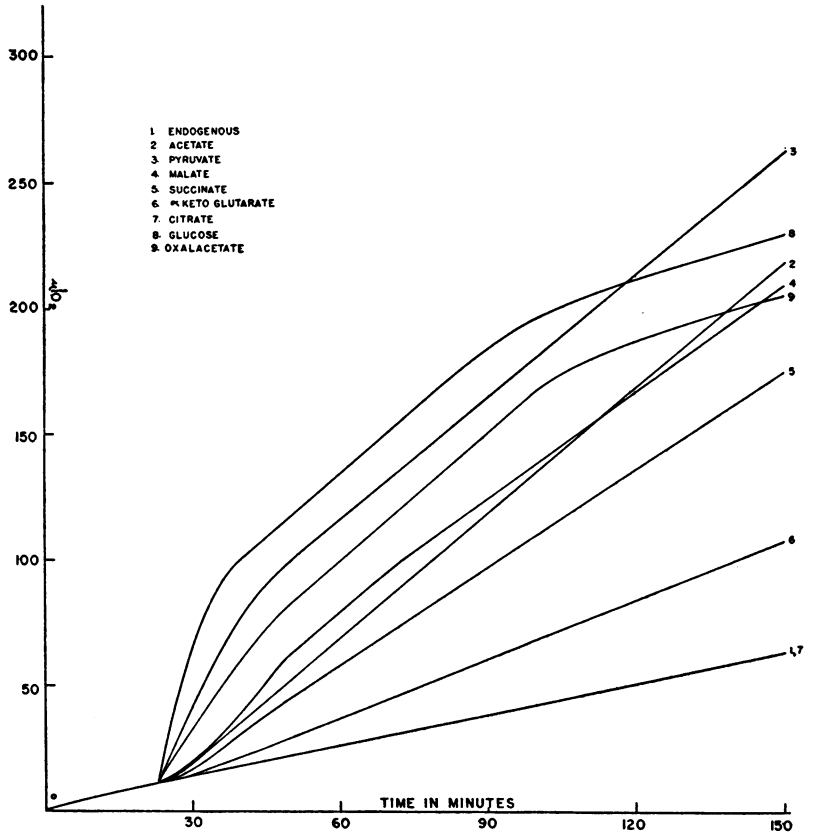

Figure 5. The oxidation of TCA intermediates by oxalacetate adapted cells. The flask contents were the same as those described in figure 4.

(figure 4). Citrate and α-ketoglutarate were oxidized very slowly if at all. Thus, citrate whose very slow oxidation has been attributed by other workers to permeability was a much better growth substrate than succinate which was oxidized rapidly. These two compounds were oxidized slowly even when the cells were grown in synthetic media in which citrate and α-ketoglutarate themselves were the sole sources of carbon. When the cells were grown in malate synthetic medium, the oxidation of both acetate and succinate was inhibited; and, after short initial periods of high activity, the rates of oxidation of malate, oxalacetate, pyruvate, and glucose fell off and then deaccelerated again when the compounds were used up. The same phenomenon was observed with oxalacetate adapted cells (figure 5) with the exception that malate did not show the initial period of high activity but, rather, had a short lag period. No such effects were observed with succinate, α-ketoglutarate, citrate, or acetate adapted cells.

DISCUSSION

These observations are in accord with those of Baskett and Hinshelwood (1950) who found that the addition of succinate to a glucose synthetic medium shortened the lag phase from that obtained with glucose alone. This was so in spite of the fact that the lag phase in succinate was forty-five times as long as that in glucose. Baumstark (1953) in our laboratory noted a similar shortening of the lag phase when succinate was added to acetate synthetic medium.

The slow growth of glucose grown cells on succinate, fumarate, α-ketoglutarate and, to a lesser extent, citrate and acetate media confirms the results of Ravin (1952); and the strain of *K. pneumoniae* used apparently corresponds to the "C* or slow growing class" of mutants of *A. aerogenes* which he described.

On the basis of the described results it would appear (1) that fumarase is absent and the TCA cycle as such is not present in this organism and

that the intermediate and end products resulting from the oxidation of fumarate cannot be assimilated to form all the compounds necessary for the growth of the organism or (2) that different pathways exist for the growth and energy needs of the organisms and that compounds at this level do not pass back and forth between the pathways. The long growth lag periods in succinate and fumarate as opposed to the short ones in malate, oxalacetate, and pyruvate would argue against the presence of the cycle. Even more convincing was the inability of cells grown in the compounds below fumarate in the cycle, malate, oxalacetate, pyruvate, and acetate, to adapt to rapid growth in succinate, fumarate, or α-ketoglutarate while cells grown in α-ketoglutarate, *cis*-aconitate, and citrate adapted only to a degree correlated with their proximity to these compounds in the cycle.

A low permeability to succinate and fumarate as an explanation for all the data is not compatible with the rapid oxidation of these two compounds in manometric experiments. Thus citrate, *cis*-aconitate, and α-ketoglutarate, which were oxidized at very low rates, served as more rapidly utilized substrates for growth than succinate or fumarate. Furthermore, the very fact that simultaneous adaptation did occur in the growth studies would argue against permeability as an explanation. A second explanation for the slow utilization of succinate and fumarate as growth substrates might be that derivatives of the free compounds as they arise in the cycle but not the free compounds themselves are active. However, both succinate and fumarate were oxidized rapidly in the manometric experiments; this could have been due to the larger number of cells and the absence of a nitrogen source in the manometric experiments.

Assuming the TCA cycle as such is absent, on the basis of the data presented one might postulate several alternate pathways for the utilization of the various intermediates in the cycle. However, this will have to await the confirmation of these results by the isolation of the intermediates and by tracer studies. Such experiments are now in progress.

SUMMARY

The oxidation of tricarboxylic acid cycle intermediates and their ability to serve as sole sources of carbon for the growth of *Klebsiella pneumoniae* were studied. Adaptation to the intermediates was ascertained by observing the growth in and dissimilation of each of the compounds by cells adapted to utilize each of the other intermediates as the sole source of carbon. Glucose, malate, oxalacetate, and pyruvate were utilized rapidly. The lag period in citrate and acetate media was longer. Growth appeared next in α-ketoglutarate and *cis*-aconitate and after 5–6 days in succinate and fumarate media. The growth response to glucose, malate, oxalacetate, and pyruvate remained rapid irrespective of the compound to which it was previously adapted. The lag period in succinate and fumarate was not decreased by previous adaptation to malate, oxalacetate, pyruvate, or acetate, was slightly decreased by previous adaptation to citrate, was moderately decreased by previous growth in α-ketoglutarate and *cis*-aconitate, and was greatly decreased by adaptation to succinate and fumarate themselves.

Succinate, fumarate, oxalacetate, malate, pyruvate, and acetate were oxidized rapidly; citrate and *cis*-aconitate were not oxidized at all; and α-ketoglutarate was oxidized only slightly if at all by peptone and peptone-glucose grown cells. There was little if any oxidation of citrate by citrate grown cells. In view of the evidence presented, it is suggested in *K. pneumoniae* strain SA2 that fumarate does not go to malate and that the tricarboxylic acid cycle as such is not present.

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