THE NATURE OF THE ADAPTIVE LAG OF PSEUDOMONAS FLUORESCENS TOWARD CITRATE[1]

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During the course of studying the terminal respiration of *Pseudomonas fluorescens*, detailed adaptive patterns were worked out with particular respect to compounds conceivably concerned in the terminal oxidation of carbon compounds. The general techniques of simultaneous adaptation were used in establishing these patterns (Stanier, 1947; Karlsson and Barker, 1948; Suda *et al.*, 1949, 1950), and it was hoped that scrutiny of these data would lead to new information on the mechanisms of terminal respiration. However, early in the study it became obvious that there existed rather profound differences between the enzymatic behavior of the whole cell and the enzymatic content of extracts of these same cells. The following illustrates this point: fumarate grown cells oxidize fumarate rapidly and immediately but oxidize citrate very slowly for the first 70 minutes and then at a rapidly increasing rate until a maximum is reached, at which point the oxidation continues at the newly established level. In other words, the response of fumarate grown cells to citrate is adaptive. Following the dictates of simultaneous adaptation one should expect that prior to becoming adapted to citrate the fumarate grown cells should possess little, if any, of the enzymatic components required for citrate oxidation. As shown in the present study this is not the case—both fumarate grown and fumarate grown citrate adapted cells appear to have nearly equivalent amounts of a citrate oxidizing system, as shown by the disappearance of citrate from extract reaction mixtures. This apparent discrepancy between the enzymatic behavior of resting cellular suspensions and cellular extracts has been noted many times in the past and permeability has been used to explain the phenomenon. In studies on terminal respiration Campbell and Stokes (1951) have shown that *Pseudomonas aeruginosa* grown with acetate as the sole source of carbon oxidizes the following only after a "period of adaptation": citrate, *cis*-aconitate, *iso*-citrate, α-ketoglutarate, succinate, and fumarate. Acetate grown cells oxidized acetate, pyruvate, and malate immediately. Dried preparations of these acetate grown cells had the ability to oxidize *all* the compounds immediately. "Therefore," quoting Campbell and Stokes, "the limiting factor in the immediate utilization of these intermediates by resting cells was the impermeability of the cell membranes." Stone and Wilson (1952) noted a lag period in sucrose grown *Azotobacter vinelandii* cells oxidizing succinate, malate, fumarate, and α-ketoglutarate. Evidence then was presented to show that sucrose grown *A. vinelandii* extracts have a pathway of oxidation involving the tricarboxylic acid cycle. The failure of whole cells to attack certain intermediates without a lag was ascribed to "permeability" with the further proviso that this term implies orientation of substrate at the enzyme surface as well as cell membrane penetration.

So far as we are aware no attempt has been made to investigate the nature of this lag on any basis other than that of permeability. The results reported here indicate that during the oxidation of citrate by fumarate grown cells an adaptive process does occur in addition to the mechanisms of penetration.

METHODS

Pseudomonas fluorescens, strain A 3.12 (Stanier, 1947), was used throughout the study because it is capable of growing on a variety of compounds as a sole source of carbon without ancillary growth factors and possesses the requisite genetic background for a variety of adaptive responses. The medium employed and the method of growing cells were essentially those of Stanier (1947), except that 0.1 per cent of yeast extract was

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added routinely to the medium. The inclusion of yeast extract was shown to have no effect on the adaptive patterns of the cells but did ensure better yields of organisms. After washing, the cells were turbidimetrically standardized to contain 4.0 mg of cells (dry weight) per milliliter. Thus, all results are strictly comparable as to cell substance present.

Extracts of cells were prepared in conventional fashion from heavy suspensions of cells by sonic oscillation and centrifugation. Citrate was quantitated by the method of Speck, Moulder, and Evans (1946). Protein was determined by use of the quantitative biuret (Gornall *et al.*, 1949). The usual type of Warburg equipment was used to obtain manometric data.

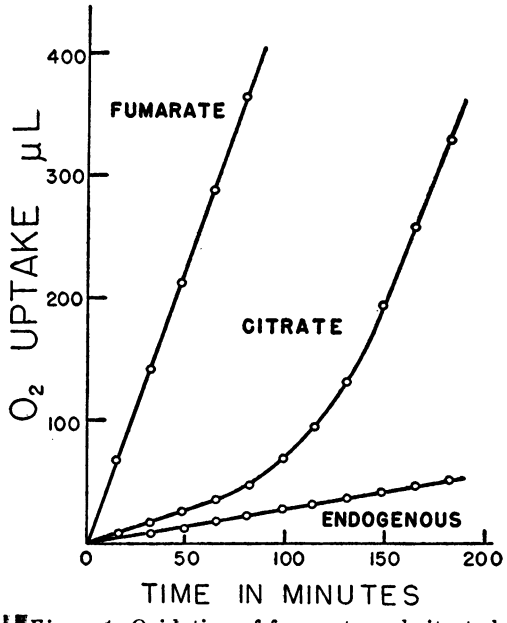

Figure 1. Oxidation of fumarate and citrate by fumarate grown cells. Each vessel contained 1 ml (4 mg) cells, 50 µM substrate as indicated, in 0.025 M phosphate buffer, pH 7.4. Bath temperature 30 C.

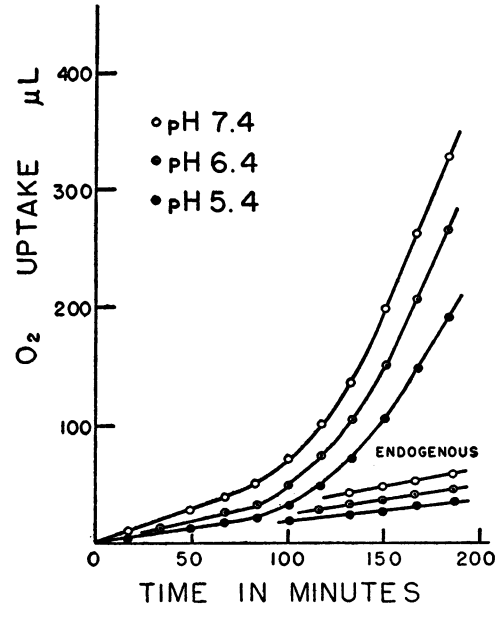

Figure 2. Effect of pH on citrate oxidation by fumarate grown cells. Conditions as in figure 1 except for pH as shown. Substrate pH was adjusted to correspond with buffer prior to experiment.

RESULTS AND DISCUSSION

Oxidative rates of fumarate grown cells oxidizing citrate and fumarate are depicted in figure 1. Clearly, in the case of citrate, there is a slow, steady oxidation at a rate significantly higher than the endogenous oxygen consumption followed by an increasing rate until the maximal is attained at which point the oxidation continues at the newly established rate. This is a typical adaptive type of response and indicates that some type of adaptive enzyme system is being formed during the lag to become operative after about 70 minutes under the conditions of the experiments. The validity of this concept was checked by several methods as indicated in the following sections.

Effect of pH on citrate adaptation. When the experiment outlined in figure 1 is carried out at various pH's, the rates of citrate oxidation are as shown in figure 2. Evidently, pH changes have an effect on the slow, steady oxidative rate (lag) and also affect the final rate of oxidation when the cells are fully adapted. More noteworthy is the observation that the inception of the adaptive part of the rate curve is not abolished at any pH in the range from 5.4 to 7.4. These data probably indicate that factors other than pH are involved in this instance but they do provide an approach to "permeability" different from the classical changing of buffers in the reaction vessels. Thus, if the entrance of citrate into the cell is dependent on two processes, one involving simple passage across an

osmotic barrier and the other involving an adaptive synthesis of some sort, it should be possible to separate the two processes or at least to abolish the adaptive portion of the oxidative rate curves by the use of agents known to prevent adaptive processes.

Effect of ultraviolet irradiation on the process of citrate adaptation. Ultraviolet light is known to block the formation of adaptive enzymes at levels of irradiation which do not harm preformed enzymes (Swenson and Giese, 1950; Brandt *et al.*, 1951; Entner and Stanier, 1951).

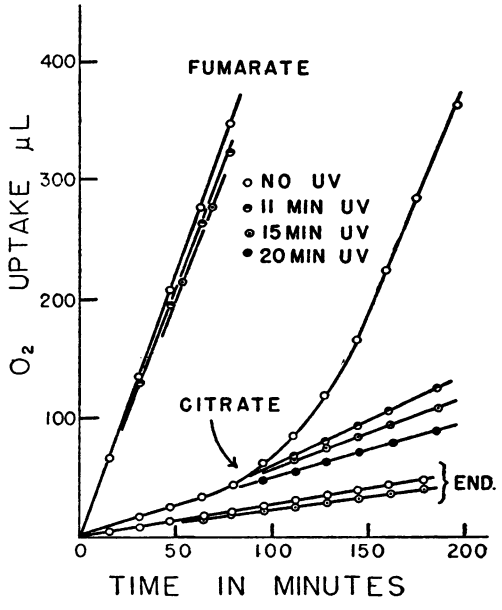

Figure 3. Effect of ultraviolet irradiation on adaptation to citrate by fumarate grown cells. Conditions as in figure 1.

Irradiation was carried out empirically as described by Entner and Stanier (1951), and the effect of such irradiation is illustrated in figure 3. Inspection of the curves leaves little question that the adaptive process has been eliminated at irradiation exposures which have little effect upon fumarate oxidation. That this effect is not specific for some component of the citrate oxidizing system is evidenced by the fact that under the same conditions citrate oxidation by citrate grown cells proceeds undisturbed as does fumarate oxidation by fumarate grown cells. The curves for citrate oxidation by citrate grown cells have been omitted from figure 3 to avoid overcrowding since they are almost identical with the fumarate curves of fumarate grown cells. Interestingly, not until the exposure time is prolonged to the point where large numbers of fumarate grown cells are killed, as demonstrated by a decrease in fumarate oxidation, is the so-called lag rate of citrate oxidation decreased. This suggests that two separate mechanisms exist

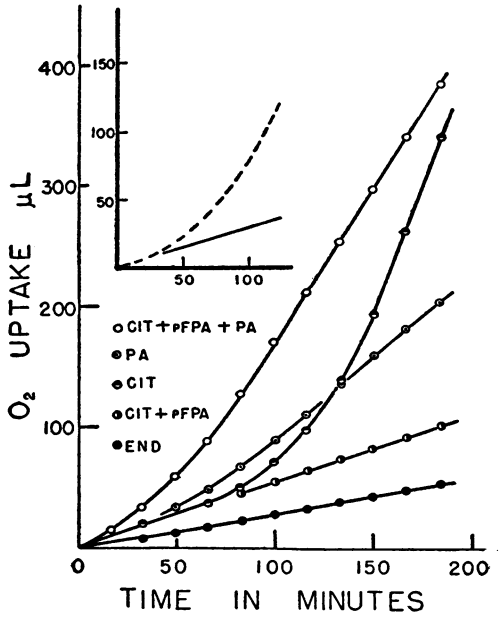

Figure 4. Effect of *p*-fluorophenylalanine on adaptation to citrate by fumarate grown cells. Conditions as in figure 1 except for additions as indicated. The dashed line in the inset represents the values of the system citrate + *p*-fluorophenylalanine + phenylalanine minus the values of phenylalanine alone and the endogenous rate. The solid line in the inset is the result of subtracting endogenous from citrate + *p*-fluorophenylalanine. *p*-Fluorophenylalanine and phenylalanine were present to a final concentration of 0.002 M. CIT = citrate, pFPA = *p*-fluorophenylalanine, PA = phenylalanine, END = endogenous.

either for entrance of citrate into the cell or for its oxidation. One process is abolished by ultraviolet irradiation; the other is not.

The effect of amino acid analogues on the process of citrate adaptation. Halvorson and Spiegelman (1952) have demonstrated the presence of a free amino acid pool in *Saccharomyces cerevisiae* which apparently is concerned in adaptive enzyme synthesis. It was demonstrated that cells

induced to synthesize maltase use considerable quantities of the amino acid pool as compared with nonadapting control cells. The presence of an amino acid analogue prevented utilization of the free amino acid pool and as a consequence prevented the synthesis of an adaptive enzyme in nongrowing cells. This inhibition could be reversed specifically by the corresponding homologous amino acid.

In our experiments the inclusion of amino acid analogues shows a similar effect on the adaptation toward citrate. Two such experiments are graphed in figures 4 and 5. Figure 4 shows the effect of *p*-fluorophenylalanine on the adaptive process. Obviously no adaption occurs. Reversal of the inhibition by inclusion of phenylalanine is somewhat difficult to demonstrate because phenylalanine itself is oxidized. However, if the values for phenylalanine oxidation are subtracted from the values in the system citrate + *p*-fluorophenylalanine + phenylalanine, the result is the dashed curve in the inset of figure 4. Citrate oxidation in the presence of *p*-fluorophenylalanine is given by the solid line in the inset. A much more clear-cut demonstration of the inhibition of substrate induced enzyme synthesis by an amino acid analogue and its reversal is shown by the ethionine-methionine antagonism as graphed in figure 5. Because of the insignificant oxidation of methionine by these cells the adaptive process toward citrate in the presence of ethionine + methionine is very evident. Inhibition and reversal of adaptive enzyme synthesis in *P. fluorescens* by amino acid analogues are especially interesting because all efforts to demonstrate chromatographically a free amino acid pool have been uniformly unsuccessful. Partition chromatograms were carried out (on trichloroacetic acid supernates of sonic lysates of the cells) as described by Feldman and Gunsalus (1950). Identical techniques applied to lysates of a gram positive organism (*Bacillus mycoides*) clearly demonstrated such an amino acid pool. The nature and mechanism of the amino acid analogue depression of substrate induced enzyme synthesis in gram negative organisms are under investigation.

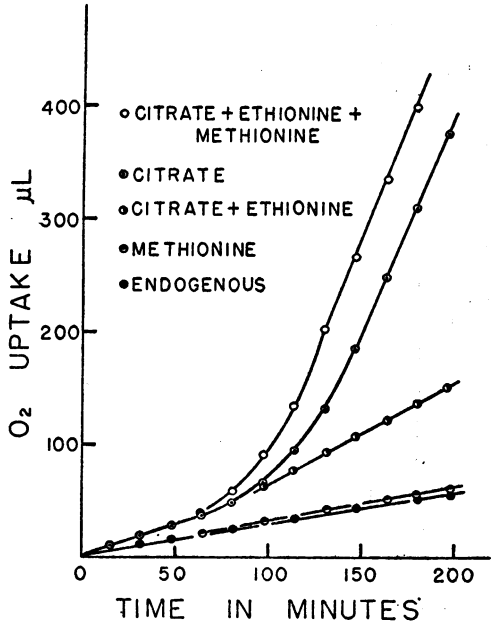

Figure 5. Effect of ethionine on citrate adaptation by fumarate grown cells. Conditions as in figure 1 except for additions as noted. Ethionine was present to a final concentration of 0.0032 M and methionine to a final concentration of 0.0021 M.

Action of streptomycin on the adaptive process. Streptomycin, 30 µg per vessel, prevents the adaptive oxidation of citrate. However, streptomycin appears to have some effect on citrate oxidation itself as shown by an inhibition of citrate oxidation by citrate grown cells. Thus, not too much significance can be attached to the effect of streptomycin on the adaptive process under discussion here.

Citrate oxidation by extracts of fumarate grown and fumarate grown citrate adapted cells. If cellular extracts are prepared from fumarate grown cells, it is found that citrate is utilized rapidly by these extracts as evidenced by oxygen uptake and citrate disappearance. Oxygen uptake data are not very satisfactory because of inordinately high endogenous values particularly in concentrated extracts. For this reason citrate disappearance was used throughout these studies. Here, the question arises of the relative amounts of the citrate oxidizing system in fumarate grown cells and in cells which have been grown on fumarate and subsequently adapted to citrate. In other words, the adaptive nature of citrate oxidation by whole cells may manifest itself by an *increase* in a system which already exists in fumarate grown cells. Therefore, comparison of the two extracts was carried out. Fumarate grown cells were incubated 12 to 15 hours in 10 liter carboys

containing the medium described under Methods. The medium was aerated vigorously during the incubation period. After harvesting and washing, the cell crop was divided into two equal portions, one of which was lysed immediately by sonic vibration. The second fraction of cells was adapted to citrate by suspending in 10 liters of phosphate buffer (0.02 M) and adding 3 g of neutralized citrate. After two hours under aeration the cells in the suspension were adapted to citrate as demonstrated by an immediate, rapid uptake of oxygen on citrate in the Warburg

TABLE 1

Comparative rates of citrate oxidation by extracts of Pseudomonas fluorescens

SOURCE OF EXTRACT	SOURCE OF KOCHSAFT	CITRATE DISAPPEARING IN 30 MINUTES (μM/MG EXTRACT PROTEIN)
Fumarate grown cells	None	2.46
Citrate grown cells	None	2.87
Fumarate grown cells	Fumarate grown cells	2.75
Fumarate grown cells	Citrate grown cells	2.77
Citrate grown cells	Fumarate grown cells	2.80
Citrate grown cells	Citrate grown cells	2.90

Each reaction vessel contained 62.5 μM citrate, phosphate buffer pH 7.4 (0.025 M), Kochsaft where indicated, and extract equivalent to 6 to 9 mg protein.

apparatus by an aliquot of the suspension, and these cells then were reharvested, washed, and disrupted in the sonic vibrator. Data from experiments measuring extract oxidation of citrate are summarized in table 1. There are several possible interpretations of these data. Obviously, there is no dramatic difference between citrate disappearance in the two types of cells. It can be argued that the methods are not delicate enough to detect subtle differences in enzymatic constitution and the small differences pictured in table 1 are the actual differences which result in the marked increase of citrate oxidizing capacity of citrate adapted fumarate grown cells. It must be admitted, however, that a citrate oxidizing system is present in fumarate grown cells and the difference in adaptation is an increase of this system. If, on the other hand, it is agreed that no marked difference obtains in the citrate oxidizing systems of the two types of cells, what is the nature of the adaptive type of response shown? It may be that some type of transport or carrier system is being synthesized by fumarate grown cells and its completion would enable citrate to be "carried" across the osmotic barrier and perhaps oriented on the enzyme surface. This synthesis would be carried out enzymatically and would result in the type of adaptive curve obtained. The carrier concept has been discussed by Doudoroff (1951) and more recently by Osterhout (1952). The data presented here are consistent with the carrier concept as an explanation for the adaptive lag in the oxidation of citrate by cells grown on fumarate, i.e., the lag represents the time required for the induced enzymatic synthesis of carrier molecules for citrate. The nature of these carriers has not yet been suggested, but it may be pointed out that carrier and oxidative function need not be synonymous.

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SUMMARY

The oxidation of citrate by fumarate grown cells of *Pseudomonas fluorescens*, strain A 3.12, is characterized by what appear to be two separate processes. One manifests itself as a slow, steady rate of oxidation, and the second shows the rapid increase in oxidative rate characteristic of adaptive responses. The latter process is abolished by agents specific for inhibiting induced enzymatic syntheses. Fumarate grown and fumarate grown citrate adapted cells appear to have equivalent amounts of a citrate oxidizing system present. The inconsistencies of whole cell enzymatic behavior and enzymatic content are discussed briefly.

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