THE INFLUENCE OF THE ENVIRONMENT ON ACETATE METABOLISM IN ESCHERICHIA COLI[1]

H. EDWIN UMBARGER

Department of Bacteriology and Immunology, Harvard Medical School, Boston, Massachusetts

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In a previous communication (Umbarger, 1953), the effects of adding glucose to the growth medium on strain K-12 of *Escherichia coli* and a mutant derived from it were compared. It was reported that high levels of glucose in the culture fluids at the time of harvesting the cells had a marked influence on the rate of acetate oxidation exhibited by those cells. It had been observed that, from a casein hydrolyzate medium with either no glucose or a limited amount of glucose, cells were obtained which oxidized acetate rapidly. If, on the other hand, an amount of glucose was added to the medium such that glucose remained at the time of harvest, the cells oxidized acetate at a much slower rate.

The environmental influence on the ability of *E. coli* to oxidize acetate has been observed by others. For example, Oginsky *et al.* (1949) reported that *E. coli* grown without shaking in a complex medium was almost inert toward acetate. Ajl (1950) noted a difference in the acetate oxidizing capacity between cells grown in an acetate medium and cells grown in glucose medium.

In the present communication, the effects of certain environmental factors on acetate and glucose oxidation are reported and correlated, where possible, with the activity of the first enzymatic step in the citric acid cycle, namely, the condensation of oxalacetate and acetate. The observations reported here have a direct bearing on the relationship of the citric acid cycle to the growth of *E. coli*. In addition, the findings should serve to emphasize the apparent precision with which bacteria synthesize the varying kinds of enzymes which their genotypes permit in amounts which adapt them to their immediate environment in the most favorable way conceivable.

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MATERIALS AND METHODS

The organism employed throughout was the K-12 strain of *E. coli*. The basal medium was that of Davis and Mingioli (1950) except that citrate was omitted. Difco casamino acids, technical and Difco yeast extract were each added to the basal medium, where indicated, in a concentration of 0.2 per cent. Glucose, when present, was added after autoclaving to give a concentration of one per cent.

For manometric experiments, cells were grown for 18 hours at 37 C under the conditions indicated and harvested by centrifugation and washed twice in a volume of 4×10^{-5} M phosphate buffer (pH 7.0) equal to one-half or one-quarter of growth medium.[2] The concentrations of substrates were chosen so that both the rate and the extent of oxidation could be observed. Aerobic growth was achieved by agitation of 250 ml Erlenmeyer flasks containing 100 ml of medium at 37 C. For anaerobic growth 250 ml of medium in 250 ml Florence flasks were incubated without shaking. The dry weights of cells were determined by reference to curves relating turbidity to dry weight obtained by drying turbid suspensions over P_2O_5.

Citrate synthesizing activities were determined using cell-free extracts prepared by grinding the twice washed cell paste with an equal weight of ignited aluminum oxide (Baker's analyzed). The ground cell mass was suspended in four parts of 0.02 M $NaHCO_3$. The suspension was cleared by centrifugation at 22,000 $\times$ G for 15 minutes. All operations in preparing the extracts were performed in a 4 C cold room. The extracts thus obtained were stored in the deep freeze.

The activities of the extracts were determined

[2] Whereas the cells were quite difficult to pack when centrifuged in distilled water, this small quantity of buffer served to permit packing with as little centrifugation as six minutes at 7,200 rpm in a Sorvall SS-1 centrifuge.

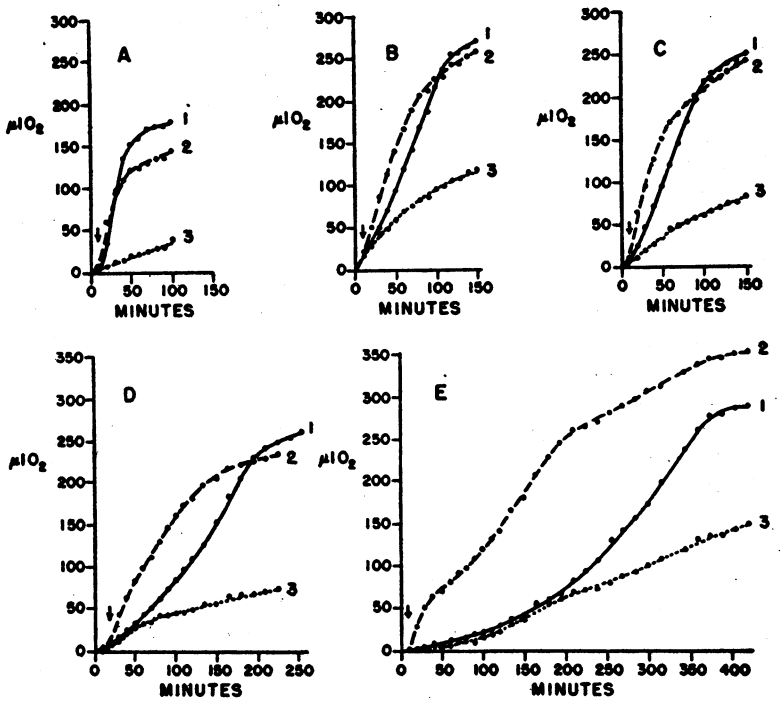

Figure 1. Substrate oxidation by *Escherichia coli* grown under various conditions. Solid line (curve 1), 5 μmoles acetate; broken line (curve 2), 2 μmoles glucose; dotted line (curve 3), endogenous. All vessels contained phosphate buffer 0.067 M (pH 7.0), cells and substrate in a total volume of 2.0 ml. A, 2.7 mgm (dry wt) cells grown aerobically in casein hydrolyzate medium; B, 3.4 mgm cells grown aerobically in casein hydrolyzate and glucose medium; C, 3.0 mgm cells grown aerobically in minimal medium; D, 3.1 mgm cells grown anaerobically in minimal medium; E, 3.4 mgm cells grown anaerobically in casein hydrolyzate, yeast extract, and glucose medium. Arrow indicates tipping of substrate. Temperature 37 C, gas phase air, KOH in center well.

by means of the following assay system modified from that of Novelli and Lipmann (1950): sodium oxalacetate, 25 μmoles; coenzyme A (70 to 75 per cent),[3] 100 μg; L-cysteine, 18.6 μmoles; lithium acetyl phosphate, 6.25 μmoles; $NaHCO_3$, 10 μmoles; $MgSO_4$, 12 μmoles; cell-free extract (containing 1.5 to 2.0 mg N per ml), 0.15 ml; total volume, 0.75 ml. The reaction mixture was flushed with nitrogen (Linde) and incubated for 30 minutes at 30 C. The apparent citrate formed was determined by the method of Ettinger *et al.* (1952) after precipitation of the protein with 9 N H_2SO_4. It was necessary to autoclave the sample at 15 pounds for 30 minutes in order to destroy an unknown material which was formed from oxalacetate during incubation with or without enzyme and which yielded a false positive test in the citrate determination.[4] The values obtained were corrected for blanks obtained in the absence of enzyme.

[3] Obtained from the Nutritional Biochemicals Corporation.

EXPERIMENTAL RESULTS

Effect of environment on glucose and acetate oxidation. The effect of adding glucose to the casein hydrolyzate medium on the oxidation of acetate and glucose by aerobically grown cells can be seen in figure 1A and B. When glucose was added to the medium, the cells exhibited a rate of acetate oxidation considerably lower than

[4] It should be noted that in agreement with the findings of Ettinger *et al.* (1952) freshly dissolved oxalacetate did not react to give a positive reaction when their test was employed. Blakley (1952), employing a different test for citrate, observed a similar interference by a decomposition product of oxalacetate. It is likely that the unknown product was the same in each case.

that of cells grown in a casein hydrolyzate medium without glucose. The Q_{O_2} values for the slow type of acetate oxidation have been found to vary between 25 and 35 in various experiments whereas the Q_{O_2} of rapid acetate oxidation varied from 80 to 120. An additional point observed in examinations of cells grown with both glucose and casein hydrolyzate was that at times an increase in oxygen consumption due to acetate was not seen during the first ten minutes after tipping. Since this effect (observed in the experiment recorded in figure 1B) was not always obtained, it has been attributed to some subtle environmental differences in the growing of the cells even though attempts were made to use identical cultural conditions.

Since the cells grown in the medium with both glucose and casein hydrolyzate presumably employed glucose as their major energy source while using the amino acids as building blocks, it was of interest to examine the oxidations by cells employing glucose not only as an energy source but also as a precursor of cell material. Figure 1C reveals that the cells grown under such conditions oxidized acetate slowly, but without the brief induction period.

The exclusion of air during growth resulted in cells capable of oxidizing glucose but initially unable to attack acetate. Figure 1D shows that cells grown anaerobically in the glucose-mineral salts medium became adapted to acetate oxidation after a relatively short lag period in agreement with the observation of Oginsky *et al.* (1951) with a methionineless mutant of *E. coli*. This lag period has been observed to vary between 20 and 90 minutes. After the lag period, acetate was observed to be oxidized at the slow rate. For comparison, cells also were grown anaerobically in a medium designed to contain an ample supply of preformed metabolites. For this purpose yeast extract as well as casein hydrolyzate was added to the medium. Figure 1E shows the prolonged lag period which preceded the increase in the rate of oxygen uptake due to acetate. Even after the lag period these cells oxidized acetate more slowly than any of the other types of cells examined. It is perhaps of interest that, occasionally, glucose oxidation was observed to stop after the uptake of two moles of oxygen per mole of substrate (theory for acetate formation) until the time when the rate of oxygen uptake increased in the flask containing acetate, after which glucose oxidation again proceeded.

Effect of environment on citrogenase activity. Of the several questions that might arise from the manometric experiments reported above, the one which seemed of greatest interest was whether the enzyme activities of the citric acid cycle reflected the same environmental influences noted for acetate oxidation. The same cultural conditions employed in the growth of the cells for the manometric experiments were suited particularly for an examination of the role of the citric acid cycle in the growth process since it was possible to separate the two functions of the cycle, namely, the formation of carbon residues for synthetic purposes such as α-ketoglutarate and fumarate and the pathway of terminal respiration. Thus, cells grown anaerobically in the glucose-mineral salts medium would employ the citric acid cycle exclusively for synthesis, whereas cells grown anaerobically in the presence of preformed metabolites would conceivably be able to dispense with both the synthetic function and the energy yielding function. On the other hand, cells grown aerobically in the casein hydrolyzate medium without glucose, although supplied with many preformed metabolites, would have derived their energy almost exclusively from the citric acid cycle.

Accordingly, the citrate synthesizing (citrogenase) activities of the various types of cells were examined. The assumption was made that citrogenase activity would serve as an index of the degree with which the various growth conditions employed influenced the steps in the citric acid cycle although there is no evidence that citrogenase would actually be a valid index of the other enzyme activities.

Table 1 contains the results of these experiments. The figures in the first column represent the apparent citrate present in the cell-free extracts plus any formed from endogenous acetyl donors. The second column shows the increased amount of citrate formed when an acetyl donor was added, in this case, acetyl phosphate. Similar results always were obtained using acetate and adenosine triphosphate as the acetyl donor except in one of three extracts prepared from cells grown in the casein hydrolyzate medium without glucose. In the case of this one extract, acetate plus adenosine triphosphate was only about one-third as effective as acetyl phosphate. The final column in the table gives the range of Q_{O_2} values for acetate oxidation by the various types of cells.

The relative amounts of citrogenase activity

of cells grown in the casein hydrolyzate medium with and without glucose were about the same as the relative Q_{O_2} values for acetate oxidation. Likewise, the extremely low citrogenase activity shown by cells grown anaerobically in the highly enriched medium was compatible with their inability to oxidize acetate previously observed. Most unexpected, however, were the high citrogenase activities exhibited by cells grown either aerobically or anaerobically in the glucose mineral salts medium. This finding suggests that the role of the citric acid cycle as a mechanism for synthesis of carbon chains used in amino acid formation exerts a greater influence on the metabolic pattern in *E. coli* than does its role as a mechanism for terminal respiration.

Adaptation to acetate oxidation. Since cells grown anaerobically in the yeast extract-casein hydrolyzate medium were almost totally devoid of citrogenase activity, it seemed important to determine whether such cells could synthesize citrate after becoming adapted to acetate oxidation. Certainly, this is one criterion that would be fulfilled if acetate is oxidized via the citric acid cycle.

For the experiment,[5] cells were grown as described previously. The washed cells were divided into two parts. The first part was used to prepare a cell-free extract for determining citrogenase activity before adaptation. The second part was suspended in water in the cell concentration usually employed in the manometric experiments. Ordinary Warburg vessels were prepared for the determination of the lag period. The bulk of the suspension was shaken in the incubator at 37 C with acetate and phosphate buffer in the same proportions as employed in the small vessels. In the small vessel, acetate oxidation began 230 minutes after tipping. The cells in the large flask were harvested in the usual way after 335 minutes' exposure to acetate, and a cell-free extract was prepared. At this time, the Q_{O_2} on acetate was about 14.

The data in table 2 show that, as would be expected, citrogenase activity was virtually absent in the unadapted cells, but after adaptation considerable activity could be demonstrated. Thus, citrogenase can be considered as an addition to the growing list of enzymes known to be adaptive.

[5] This experiment was suggested by Dr. Clarke T. Gray of this department.

TABLE 1

Citrogenase activity in cell-free extracts

GROWTH CONDITIONS	CITRATE FORMED (μMOLES/MG N/30 MINUTES)		
	Acetyl phosphate omitted	Complete system	Acetate oxidation Q_{O_2}
Casein hydrolyzate, aerobic	0.40	5.53	80–120
Casein hydrolyzate, glucose, aerobic	0.47	1.89	25–35
Glucose-mineral salts, aerobic	1.37	11.10	30–40
Glucose-mineral salts, anaerobic	0.25	7.42	0*
Casein hydrolyzate, yeast extract, glucose, anaerobic	0.42	0.75	0†

* After brief lag period, the Q_{O_2} on acetate was between 25 and 35.

† After prolonged lag period, the Q_{O_2} on acetate was about 18.

TABLE 2

The adaptive formation of citrogenase activity

EXTRACT FROM	Q_{O_2} ACETATE	CITRATE FORMED (μMOLES/MG N/30 MINUTES)	
		Acetyl phosphate omitted	Complete system
Unadapted cells	0	0.32	0.22
Adapted cells	14	0	6.61

The nature of the process of adaptation to acetate oxidation in cells grown anaerobically in the minimal medium appeared to be more of a mystery. Since cells of this type exhibited more than enough citrogenase activity to permit even rapid acetate oxidation (see table 1) and exhibited good oxygen uptake with glucose, it seemed reasonable that some specific link between one of the dehydrogenases of the citric acid cycle and the cytochrome system might have been absent. However, as more cell preparations were examined, it became obvious that the length of the adaptive period (ranging from 20 to 90 minutes) was related intimately to the endogenous respiration of the cells. This relationship can be seen in figure 2 containing plots of oxygen uptake with and without acetate. Each curve has been displaced by 50 minutes for clarity.

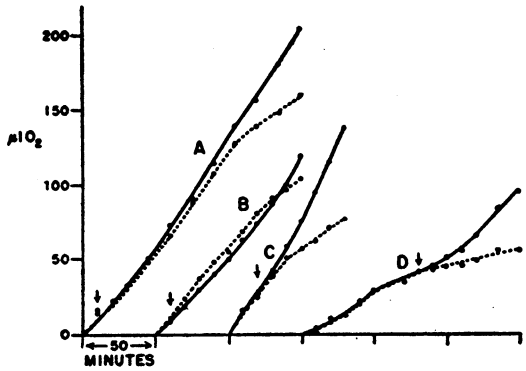

Figure 2. Lag in acetate oxidation and endogenous respiration in *Escherichia coli* grown anaerobically in minimal medium. Solid line, 5 μmoles acetate; dotted line, endogenous. A, 3.2 mgm (dry wt); B, 2.6 mgm cells; C, 3.0 mgm cells; D, 3.1 mgm cells, substrate tipped after endogenous respiration decreased. For other conditions see figure 1.

Invariably, it has been observed that at almost the time that the endogenous respiration decreases in rate, a net increase in oxygen uptake due to acetate can be demonstrated. Furthermore, if acetate is tipped after the endogenous rate has decreased, much less or no "lag" in acetate oxidation is observed. It would seem, therefore, that this phenomenon is not an adaptation in the usual sense of the term.

DISCUSSION

The data presented here should serve in a general way as a demonstration of the importance of the environment on the enzymatic equipment and consequent metabolic behavior of the bacterial cell. From the few properties of *E. coli* that have been reported here to be so different when the cells were harvested from a relatively few kinds of media, it seems justified to conclude that any interpretation of the significance of a given enzyme reaction or series of reactions to the growth of an organism must take into consideration the type of medium employed to grow the cells. When an enzymatic reaction has been demonstrated, the following questions should be asked: under what conditions is the enzyme formed or functioning and, equally important, to what extent does it contribute to the economy of the cell during growth?

Of specific interest, it has been shown here that citrogenase in *E. coli* is an adaptive enzyme. Its synthesis has been shown to be induced by its substrate, acetate. Because citrogenase is of importance in terminal respiration as well as in the synthesis of the key metabolite, α-ketoglutarate, it is of considerable survival value to the organism that its activity is greater when the need for it is greater. When its activity might be of little use either for energy or for biosynthesis as, for example, under anaerobic conditions in a medium enriched with a seemingly ample supply of preformed metabolites, its activity is virtually nonexistent. Conversely, its activity is greatest when the cell is growing aerobically in a medium in which the entire spectrum of cellular components is synthesized from a single carbon source, glucose.

Since it has been shown only that the induction of citrogenase activity accompanies adaptation to acetate oxidation in the cells grown anaerobically in the rich medium, one can conclude that the lack of citrogenase activity is one of the metabolic blocks in cells of this type. It seems quite likely that adaptation to acetate oxidation would be accompanied by the induced biosynthesis of several other enzymes.

It is not possible at this time to interpret satisfactorily the apparent adaptation to acetate oxidation in cells grown anaerobically in the minimal medium. With such cells, a net uptake of oxygen due to the presence of acetate cannot be demonstrated until the endogenous respiration has decreased from its initial rate. A possibility that suggests itself is that these cells which have a relatively high endogenous respiration are employing initially the enzymes of the citric acid cycle to their maximum capacity so that no increase in the rate of oxygen can be shown when acetate is present. Experiments are in progress which will aid in deciding whether it is the oxidation of acetate *per se* or the endogenous respiration that is suppressed during the period of apparent adaptation to acetate oxidation.

It is of interest that Grunberg-Manago and Gunsalus (1953) have shown that aconitase activity in cells grown anaerobically in minimal medium is only about one-third that of aerobically grown cells. While the suppression due to anaerobiosis is considerably greater than was found here in the case of citrogenase, the type of environmental effect is the same in both cases.

Another point that should be stressed concerning this work is that so far no observations have been made which are not compatible with the hypothesis that the citric acid cycle is the

sole pathway for terminal respiration in *E. coli*. While the dicarboxylic acid pathway may exist in some degree, all recent evidence, particularly that of Swim and Krampitz (1952), suggests that it functions, at most, in only a minor role.

Finally, it might be appropriate to consider the significance of the conditions which have been demonstrated to be required for the production of maximal citrogenase activity. These are: oxygen, a source of acetate (an inducer of its production), and the *need* for certain metabolites (e.g., α-ketoglutarate) that are synthesized via a pathway involving that enzyme. The last condition is probably a fairly universal requirement of many, if not all, biosynthetic enzymes. Other cases analogous to this have been reported previously. For example, the addition of methionine to the medium of *E. coli* specifically suppresses, in *E. coli*, the ability to form methionine from its precursor, homocysteine (Cohn *et al.*, 1953). Similarly, the activity of the valine-alanine transaminase is less in cells grown in the presence of valine than in its absence (Adelberg and Umbarger, 1953). The elucidation of the mechanism behind such a suppression in enzyme synthesis by the presence of the reaction product in the medium would be an important step forward in our goal of understanding the connecting links between the environment and genotype, on the one hand, and the final enzymatic constitution of the bacterial cell on the other.

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SUMMARY

Glucose and acetate oxidation by cells of *Escherichia coli* grown under several kinds of environment has been observed. It was found that acetate was oxidized most rapidly by those cells which derived their energy for growth from the aerobic dissimilation of amino acids, presumably involving active acetate and the steps of the citric acid cycle as intermediates. A net uptake of oxygen due to acetate could not be demonstrated with anaerobically grown cells without a lag period. Cells which were grown aerobically in the presence of glucose oxidized acetate at about one-third the rate exhibited by cells grown on amino acids alone.

Citrogenase activity was greatest in cells employing the citric acid cycle for both metabolite synthesis and energy. Citrogenase activity was virtually absent from cells grown anaerobically in a medium containing casein hydrolyzate, yeast extract, and glucose. Such cells developed citrogenase activity, however, after being exposed to acetate oxidation.

Cells grown anaerobically in a glucose-mineral salts medium did not exhibit an increased oxygen uptake due to acetate until the rate of endogenous respiration decreased. The lag period before acetate oxidation in this case probably does not involve enzymatic adaptation.

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