THE INFLUENCE OF AGE ON ENZYMATIC ADAPTATION IN MICROORGANISMS[1]

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Hegarty (1939) observed a marked shortening during physiological youth of the lag period required by *Streptococcus lactis* for carbohydrase adaptation, and concluded that the cell is best prepared during physiological youth for enzymatic adaptation. In view of the paucity of data on other microorganisms, Hegarty's results have been accepted as being applicable to many microorganisms (Gunsalus, 1951). However, during investigations on formic hydrogenlyase, we observed that the adaptability of *Escherichia coli* is greatest in cells harvested from the stationary period of growth rather than during physiological youth. Also, results with the nitrate reductase system of *E. coli* and adaptation of *Pseudomonas fluorescens* to the oxidation of benzoic and gluconic acids differ markedly from those of Hegarty and suggest a more general interpretation of the variation in adaptive ability during the growth cycle.

METHODS

Cell suspensions of *E. coli*, strain S, from yeast extract broth cultures of different ages were prepared as described previously (Pinsky and Stokes, 1952). The cells at all ages were devoid of hydrogenlyase activity but contained low preadaptive levels of nitrate reductase. Hydrogenlyase adaptation was carried out and activity determined by the standard manometric procedure outlined previously (Pinsky and Stokes, 1952). Briefly, H_2 production was determined in cell suspensions incubated in formate and the energy and amino acid supplements, glucose and hydrolyzed casein, which are essential for adaptation. Cells from 12 hour cultures develop considerable hydrogenlyase activity after a lag of approximately 30 minutes. There is no detectable cell proliferation during adaptation. For nitrate reductase adaptation the same standard cell suspensions as used for formic hydrogenlyase were incubated with an equal volume of a solution of supplements of the following final concentrations: 0.83 per cent hydrolyzed casein, M/60 glucose, and M/60 $NaNO_3$. The adaptation process was carried out at 28 C in test tubes that could be alternatively stoppered or flushed with N_2. At various intervals preceded and followed by flushing with N_2, 0.1 ml samples were removed and analyzed for the NO_2^- produced from NO_3^- with the Griess-Ilosvay reagent essentially as outlined by Pollock (1946). The same experimental procedure was used for the determination of preadaptive nitrate reductase activities except that the supplements were M/60 $NaNO_3$ and M/60 Na formate. Without the amino acid and energy supplements, the NO_3^- consumption was linear and hence unadaptive.

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The cells originally contain no nitrite reductase but adapt to this enzyme concomitantly with nitrate reductase. This results in a loss of NO_2^- which lowers the nitrate reductase activity values. The error is not very significant since it is fairly constant in all determinations and reduces the true values only by approximately 10 per cent. It can be corrected for by accompanying each nitrate reductase determination with similar determinations on suspensions containing progressively lower concentrations of NO_3^-. Such treatment does not alter the adaptation curves before exhaustion of the substrate. The difference between the theoretical and observed maximal values of NO_2^- at each NO_3^- concentration indicates nitrite reductase activity which may then be added to the uncorrected NO_3^- values. This procedure has been carried out with old and young cells and yields results that are not materially more useful than the uncorrected ones which are reported.

The adaptive behavior of *P. fluorescens* was followed by essentially the same manometric procedure described by Stanier (1947). Two micromoles of benzoate or 5 μM of gluconate per vessel were used. The cells were grown in the same way as *E. coli* with the exception that the inoculum for the cultures was 20 hours rather than 8 hours old.

RESULTS

Effect of age on hydrogenlyase adaptation by E. coli, strain S. The fate of the hydrogenlyase forming system during growth of *E. coli* is illustrated in figure 1, in which the adaptive activities and the growth of the culture are both plotted against age. Beginning with the 0 hour value obtained with the inoculum, an immediate and sharp decrease in adaptive activity occurs as the cells enter and pass through the early part of the exponential period, and this continues until a steady low value is reached. Then as the growth curve begins to level off, the adaptive activity quickly regains a maximal value which is retained even well into the stationary phase. The lag periods of adaptation by the young cells which develop the lowest hydrogenlyase activity are 100 per cent or more longer than those of the old cells which develop maximal activity. It thus appears that formic hydrogenlyase adaptation is favored by aging rather than physiological youth.

Further experiments were designed to determine whether the low enzymatic activity of the young cells is due to a limited availability of the nutrients required for adaptation, i.e., amino acids, glucose, and formate. It is possible, for instance, that permeability to these essential nutrients is altered during youth. However, the Q_{O_2} values for the oxidation of the important amino acids (glutamic acid, aspartic acid, arginine, cysteine, serine, threonine, and glycine) were 57, 98, 7, 13, 137, 31, and 12, respectively, with 4 hour cells and 27, 116, 5, 31, 95, 30, and 8, respectively, with 12 hour cells. Oxygen uptake always occurred immediately after adding the amino acids. Also, both young and old cells dissimilate glucose anaerobically immediately after tipping. In addition they have high and immediate Q_{O_2} values, greater than 100, for formic dehydrogenase. Therefore, the lowered adaptability of the young cells cannot be attributed to impermeability or lag in permeability to any of the important substrates required for adaptation.

There is also the possibility of a more rapid inactivation of the hydrolyzed casein supplement by the young cells under the anaerobic conditions of adaptation, resulting from destruction of aspartate which is highly critical for hydrogenlyase adaptation (Pinsky and Stokes, 1952). However, the anaerobic decomposition of aspartic acid proceeds at the same rate with both 4 hour and 12 hour cells, as judged by the rate of NH_3 liberation.

On the basis of CO_2 liberation from bicarbonate buffer, the Q_{CO_2} of glucose is 72 for the 4 hour cells and 28 for the 12 hour cells. The possibility thus arises that the young cells may exhaust their critical glucose supply before adaptation has become appreciable. Also, it is possible that they may require more hydrolyzed casein than the old cells. As illustrated in figure 2, neither a 5-fold nor a 10-fold increase in the glucose concentration nor a 2½-fold increase in the hydrolyzed casein concentration stimulates markedly the activity of the young cells. If these

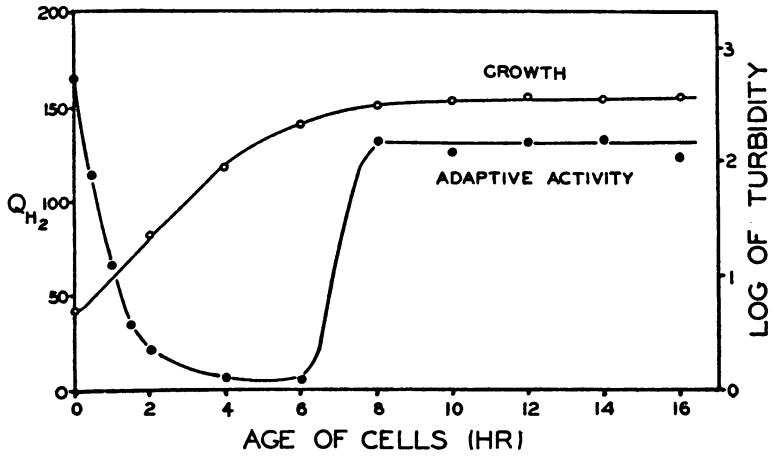

Figure 1. The influence of age on adaptation of *Escherichia coli* to formic hydrogenlyase. The growth curve is superimposed for comparison. Turbidity values are recorded in Klett-Summerson units.

supplements are present together at their highest concentrations, the activity of the young cells becomes appreciable, but the activity of the old cells is also considerably higher under the same conditions. Of greater significance, however, is the persistently longer lag period of the younger cells which cannot be shortened by the use of greater amounts of supplements even though the final activity is raised. Since the length of the lag is characteristic of the autocatalytic rate of enzyme synthesis, the younger cells have apparently less inherent ability to synthesize formic hydrogenlyase. It is possible that a factor critical for adaptation is present in the old cell but lacking in the young one. With this idea in mind attempts were made to improve the low adaptability of the young cells with sonic and boiled extracts of the old cells. These were not as effective as the increased concentration of amino acid supplements in stimulating final adaptive activity and had no effect on the long lag period. It was thought that perhaps the postu-

lated factor might be indicated by a higher rate of endogenous oxidation by the old cells. However, the rates were the same for the young and the old cells. Furthermore, aeration for 8 hours to deplete the endogenous reserves of the old cells reduces their adaptive activity by only 15 per cent.

The drop in the activity curve in figure 1 could be attributed to an enhanced sensitivity of the physiologically young cells to adverse treatment involved in preparing the cell suspensions. This possibility may be conclusively ruled out, however, for several reasons. First, the viability of the young cells with lowest activity is not appreciably impaired since the growth of such cells, as indicated by an increase in turbidity after the initial nonproliferation period, is as great and often considerably greater than that of the old cells with greatest activity.

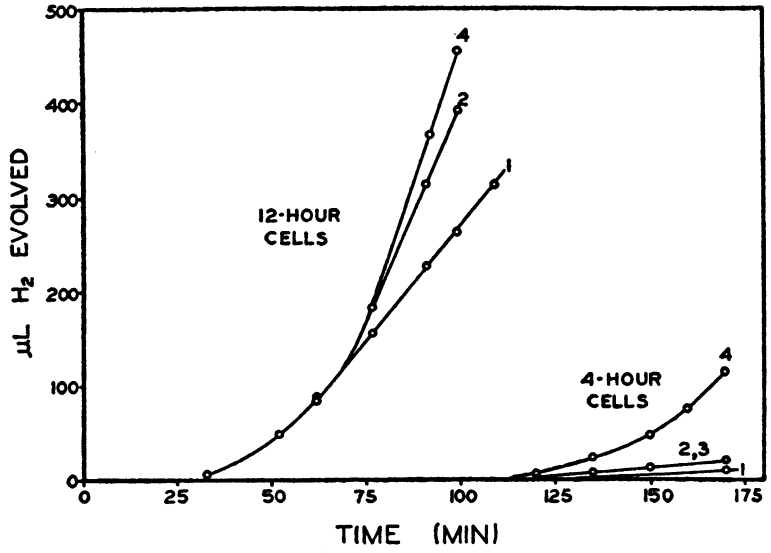

Figure 2. Stimulation of formic hydrogenlyase adaptation by old (12 hour) and young (4 hour) cells of *Escherichia coli* with supplements. (1) Standard concentration of supplements: 0.83 per cent hydrolyzed casein, M/240 Na formate, and M/240 glucose. (2) Supplements as in 1, but with M/48 or M/24 glucose. (3) Supplements as in 1, but with 2.1 per cent hydrolyzed casein. (4) Supplements as in 1, but with 2.1 per cent hydrolyzed casein and M/24 glucose.

Also, many attempts in various ways to minimize the manipulation and treatment of the cells as much as possible failed to decrease the lag periods or materially enhance the final activities of the young cells. This is illustrated by an experiment in which the cultures were both grown and adapted in the same Warburg flasks. A medium containing 1 per cent yeast extract in M/20 phosphate buffer at pH 7.0 received a 5 per cent inoculum from a stationary culture and was incubated in 2.0 ml amounts in Warburg vessels. The cultures were then incubated aerobically with shaking and periodically flushed with air. After various growth intervals KOH was added to the center well, the vessels were flushed with N_2, 2.0 ml of fresh yeast extract-phosphate medium plus glucose and formate were tipped in from the side arms, and the subsequent evolution of H_2 was fol-

lowed. Each determination at a given age was compared with a 0 hour culture which received glucose, formate, and 2.0 ml of "used" yeast extract-phosphate medium recovered from an exact duplicate of the culture being compared. Thus each age could be compared with a 0 hour culture in essentially the same nutritional environment. Also, in this way the entire culture was investigated, and centrifugation, washing, the loss of diffusible factors from the cells, and any radical change in environment were all eliminated. Under such conditions undoubtedly there is some growth since the adaptation curves do not reach steady final values but increase gradually and continuously. Nevertheless, the shapes of the adaptation curves of the cultures which were tested before the stationary growth phase was reached were closely similar to those of the 0 hour controls, especially during the most active periods of adaptation. This indicates that the enzyme

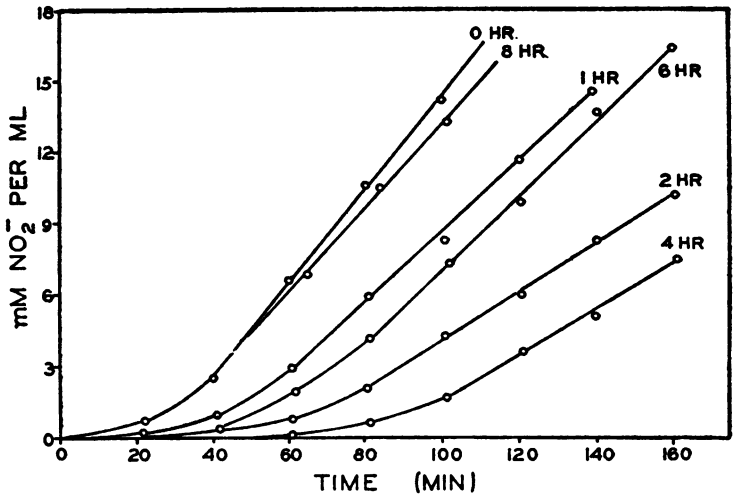

Figure 3. Nitrate reductase adaptation curves of *Escherichia coli* at different ages. The growth curve of the culture is essentially the same as that in figure 1. All suspensions contain 0.75 mg of bacterial cells, as dry weight, per ml.

forming system, or at least the kinetics of adaptation, was passed on essentially unchanged from the inoculum to the progeny. Thus the lowered adaptability of the younger cells can be attributed to a diluting out effect. When the cells entered the stationary phase of growth, however, their adaptability vastly exceeded that of the 0 hour control with respect to both the magnitude of the final activity and the brevity of the lag period, indicating that the bulk of the enzyme forming potential is developed when the growth levels off.

Influence of age on nitrate reductase adaptation by E. coli, strain S. Nitrate reductase adaptation, like hydrogenlyase adaptation, appears to be favored by aging rather than physiological youth. The adaptation curves in figure 3 for the cells at different ages show a regression of adaptability during early growth, followed by a rapid restoration towards the activity of the original inoculum of old cells when growth slows down. The most striking difference is the progressive

lengthening, as much as 2- to 3-fold, of the time required by the young cells to reach full adaptive activity. In contrast to hydrogenlyase adaptation, the final nitrate reductase activities of cells at different ages do not differ greatly, the least active cells reaching maximal rates only approximately 40 per cent less than

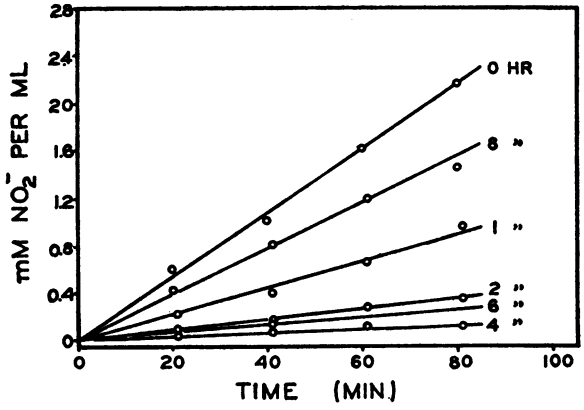

Figure 4. Preadaptive nitrate reductase of *Escherichia coli* at different ages. The suspensions are the same as in figure 3.

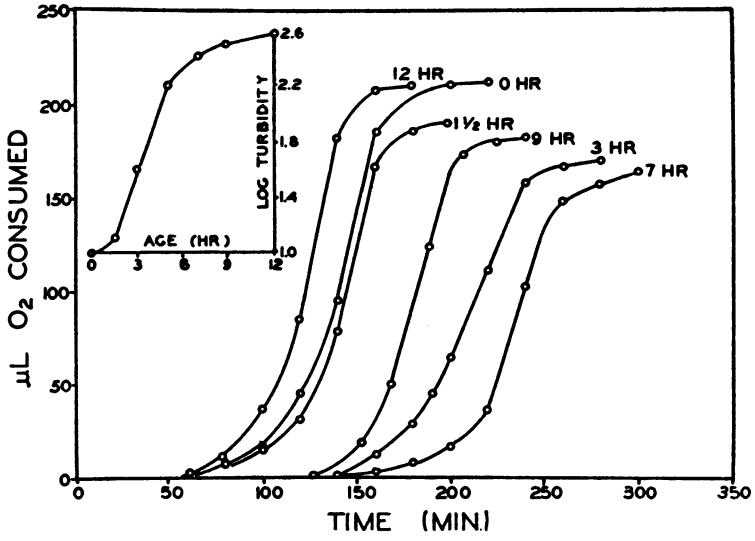

Figure 5. The influence of age on adaptation to benzoate oxidation by *Pseudomonas fluorescens*. Endogenous values have been subtracted. The insert is the growth curve recorded in Klett-Summerson units.

those of the most active cells. The adaptive pattern of nitrate reductase is closely parallelled by the preadaptive activities illustrated in figure 4. The results do not indicate a precise mathematical relationship between the two. Nevertheless, it is clear that as the preadaptive activity decreases during growth, the lag

period is prolonged, and finally both the lag and preadaptive activities are restored to their original values by aging. Because of the interference due to nitrite reduction which is comparatively great during the very early phases of adaptation by the young cells, the shapes of the curves are not strictly comparable as indicators of relative rates of enzyme synthesis. However, it seems justifiable to conclude that the prolonged lag of the younger cells is due to a lower initial rate, rather than a lower autocatalytic rate, of enzyme synthesis.

Adaptation of P. fluorescens to benzoate and gluconate oxidation. The adaptability of *P. fluorescens* is also favored by aging. As illustrated in figure 5, the cells, when harvested at successive growth stages, require progressively longer periods for the occurrence of O_2 consumption in excess of the high endogenous rates. However, the maximal rates are not appreciably or consistently affected. Cells well into the stationary phase regain the rapid adaptability of the old inoculum. The total O_2 consumption is progressively lowered as the adaptive lag is lengthened. This may be due to a greater oxidative assimilation of the substrate or it may be an artifact produced by the corrections for the endogenous oxidation. Results similar to those with benzoate also were obtained with gluconate.

DISCUSSION

The influence of age on adaptive activity can best be determined if two experimental conditions are fulfilled: (1) the organism should be repeatedly subcultured in the same medium before determining adaptability; (2) the cells should be grown in the absence of added substrate and adapted at all ages under identical conditions in the absence of cell proliferation. In the absence of these conditions interpretation of the data may be complicated by such factors as the effect of changes in the growth medium on adaptability, training, and variations in environment due to growth during adaptation. In our experiments these conditions have been satisfied. Nonproliferation of the cells during adaptation has been established for hydrogenlyase (Pinsky and Stokes, 1952) and in the other cases the extent of cell proliferation, if it occurred at all, must have been small since steady linear rates of final adaptive activity were obtained.

Billen and Lichstein (1951) determined the ability of an essential amino acid supplement to induce the formation of hydrogenlyase when added at various ages to cultures of *E. coli* growing in a glucose, NH_3, salts medium. They found that earlier additions of the amino acids resulted in higher final hydrogenlyase activity when the cells were tested after 24 hours of growth. However, it cannot be concluded from these results that the young cells had the greatest adaptive activity since such data are subject to the complicating effects of cell proliferation and also to secondary changes which could occur during the long period between the addition of the amino acid supplement and the determination of adaptability. With our experimental procedure which employs resting suspensions of *E. coli* and includes the immediate determination of the effect of supplements on adaptation, we find on the contrary that cells grown either aerobically or anaerobically in the medium of Billen and Lichstein (1951) have the greatest adaptive activity during the stationary phase. The same is true for nitrate reductase adaptation.

These results indicate that the enhanced adaptability of the old cells cannot be attributed to the medium or to the presence or absence of O_2.

On the basis of our results of those of Hegarty (1939), enzymatic adaptation may be favored (a) by physiological youth, as in the case of the adaptive carbohydrases of *Streptococcus lactis*, or (b) by aging, as for formic hydrogenlyase and nitrate reductase of *E. coli* and benzoate and gluconate oxidation by *P. fluorescens*. Thus there is no constant relation between the ages of cells and enzymatic adaptability. This is strikingly similar to the variations that occur in enzyme activity with respect to age (Gale, 1943).

The relatively poor adaptability of young cells in our experiments can perhaps be attributed to unsuccessful competition for essential structural units, e.g., amino acids, between the systems which synthesize these enzymes and the other synthetic processes in the cell. But as the competing synthetic processes slow down during aging, the necessary building blocks may become available for the production of the adaptive enzymes. However, this does not hold for the adaptive carbohydrases of *S. lactis*. It may be expected that, in general, the relation of age to adaptive enzyme formation will vary with the particular organism and substrate.

The term adaptability, which has been used somewhat loosely in this discussion, can apply to the final adaptive activity or to the length of the lag before maximal activity is reached. Depending on which criterion is used, age favors adaptation in our experiments to different degrees.

The correlation between adaptability and preadaptive activity of nitrate reductase in *E. coli* is strikingly similar to the findings of De Ley and Vandamme (1951). They noted in yeast cultures grown in beer wort that the preadaptive activity and the adaptability of saccharase gradually and concomitantly increase with age. If in these cases the enzyme forming system is in equilibrium with its products, the preadaptive enzyme level should indicate the level of enzyme forming system and hence the ability of the enzyme forming system and the enzyme to increase.

SUMMARY

The influence of age on enzymatic adaptation has been investigated with respect to the nitrate reductase and formic hydrogenlyase systems in *Escherichia coli* and the gluconate and benzoate oxidizing systems in *Pseudomonas fluorescens*. In all cases, adaptability steadily decreases during the period of active growth from the high level of the original inoculum and is restored as the cells enter the stationary phase of growth. Thus adaptation is favored by aging rather than physiological youth.

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