459. *The Action of Two Antibacterial Agents on the Growth of* Bact. lactis aerogenes.

By B. J. McCarthy and Sir Cyril Hinshelwood.

A study has been made of the effect on the growth of *Bact. lactis aerogenes* of 2 : 4-dinitrophenol and of disodium dihydrogen ethylenediamine-*NNN'N'*-tetra-acetate at sub-lethal concentrations.

Over the wide range of concentrations employed the presence of this sodium salt affects only the rate of multiplication of the cells without inducing an initial lag phase, the growth rate being reduced progressively with increase in concentration. Repeated subculture at a given concentration of the toxic agent reduces the inhibitory effect but does not completely eliminate it.

On the other hand 2 : 4-dinitrophenol evokes a more usual type of response, the lag shown at a first subculture in the drug medium being rapidly eliminated. Various distinct sub-strains are developed by serial subculture at particular concentrations. The lag characteristics of any given sub-strain tested at a series of drug concentrations are quantitatively related to the concentration at which it has been trained.

The training to the drug has no detectable effect on the respiratory properties of the cells but affects their ability to undergo adaptation to new sources of carbon.

The development of drug resistance to toxic agents by a given kind of bacterium may follow very varied patterns. The adaptive response of *Bact. lactis aerogenes* to 2 : 4-dinitrophenol and to ethylenediamine-*NNN'N'*-tetra-acetic acid (E.D.T.A.) illustrates this phenomenon and is here considered in some detail.

Experimental Details and Techniques

The stock strain of *Bact. lactis aerogenes* used was maintained in " Lemco " broth, and acclimatised when required to a standard minimal medium made by mixing the following solutions: 10 ml. of aqueous glucose (50 g./l.); 10 ml. of phosphate buffer ($Na_2HPO_4,12H_2O$ 16 g./l., KH_2PO_4 2·96 g./l.; pH = 7·1); 5 ml. of aqueous ammonium sulphate (5 g./l.); 1 ml. of magnesium sulphate ($MgSO_4,7H_2O$ 1 g./l.; containing 5·2 mg. of ferrous sulphate per l.).

The two drugs used in the experiments were dissolved in the standard phosphate buffer and the pH finally adjusted by the addition of a few drops of concentrated sodium hydroxide. The appropriate amount was then added to the above medium in place of all or part of the 10 ml. of phosphate solution so as to obtain the required final concentration.

The solid agar medium consisted of the same materials with the addition of 1·25% of agar. The following amounts were dissolved in 800 ml. of glass-distilled water: $Na_2HPO_4,12H_2O$ 8·0 g., KH_2PO_4 1·5 g., $(NH_4)_2SO_4$ 0·6 g., $MgSO_4,7H_2O$ 0·05 g., agar 10·0 g.

Population changes in liquid cultures were estimated with the aid of a Hilger " Spekker " light-absorptiometer, calibrated against the microscope count of actual numbers of bacteria made in a hæmocytometer counting chamber. For these purposes samples of an actively growing log-phase glucose culture were used. With the calibration so obtained turbidimetric readings can be expressed as a number equal to the cell count, in 10^6 per ml., of a glucose culture of the same optical density. This was used in plotting growth curves for estimations of lag and mean generation time.

The Action of Disodium Dihydrogen Ethylenediamine-*NNN'N'*-Tetra-acetate

The effect of this drug on the kinetics of growth when the bacteria are subcultured for the first time into a medium containing it is rather unusual. Throughout the wide range of concentrations used, 5 to 10,000 mg./l., the cells were able to multiply without a preliminary phase of adjustment although at a rate decreasing with increasing concentration. In Fig. 1 this is shown by a plot of the mean generation time (m.g.t.) against the logarithm of the concentration. Fig. 2 gives the actual growth curves corresponding to some of these determinations.

For this series of measurements five tubes containing the E.D.T.A. salt at 0, 10, 100, 1000, and 10,000 mg./l. were inoculated in parallel with 0·1 ml. of an actively growing culture.

The Figure shows that, after division has taken place about twice, the cells begin to grow

FIG. 1. *Plot of mean generation time against log of E.D.T.A. salt concentration.*

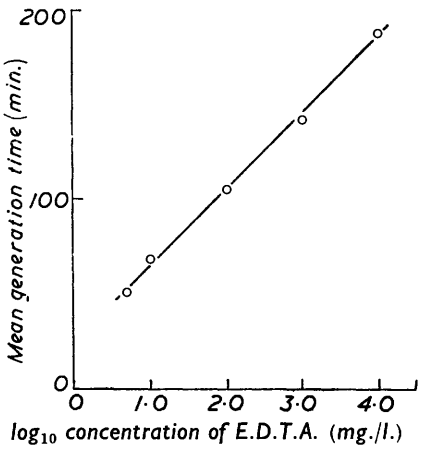
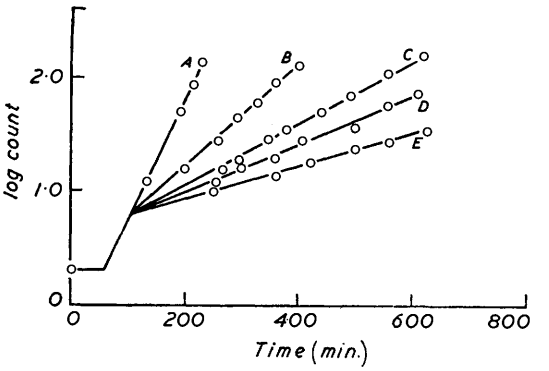


FIG. 2. *Growth curves for cultures containing (A) 0 (B) 10, (C) 100, (D) 1000, and (E) 10,000 mg. 0, E.D.T.A. salt per l.*



at a reduced rate. The dependence of this new rate of multiplication on the concentration is reflected in the fan-wise distribution of the portions of the growth curve corresponding to logarithmic growth.

Measurements were continued throughout a process of training by two series of subcultures

TABLE 1. *Training to E.D.T.A.*

Initial subculture in disodium dihydrogen ethylenediamine-*NNN'N'*-tetra-acetate		Mean generation times of successive subcultures at 10 mg./l.			
Concn. (mg./l.)	Mean generation time (min.)	No. of previous subcultures at 10 mg./l.	Mean generation times (min.) at 10 mg./l.	100 mg./l.	1000 mg./l.
0	31	0	68	105	142
5	51	5	62	—	—
10	68	9	37, 83 *	—	—
100	105	11	37, 57 *	—	—
1000	142	13	46	—	—
10000	187	17	63	—	—
		19	47	—	—
		21	50	—	—
		22	51	67	78
		23	59	—	—
		36	48	—	—
		43	50	—	—
		45	49	—	—
		59	46	—	—
		61	48	—	—
		66	46	46, 68 *	81

Mean generation times of successive subcultures at 100 mg./l.

No. of previous subcultures at 100 mg./l.	Mean generation times (min.) at 10 mg./l.	100 mg./l.	1000 mg./l.
0	68	105	142
5	—	80	—
15	—	68	—
21	51	72	77
23	—	70	—
38	—	50	—
40	47	53	80
45	—	51	—

* Composite growth curve.

carried out at concentrations of 10 and 100 mg./l. When tests were to be made the parent strain was subcultured into two tubes in parallel from one of which samples were taken at 20 min. intervals for the determination of growth rate, the second tube serving as the routine day-to-day subculture. From time to time a complete set of growth curves at drug concentrations of 0, 10, 100, and 1000 mg./l. were determined for the trained strain (Table 1).

The two Tables of mean generation times indicate clearly that training takes place. There is an apparent limit to the training process at m.g.t. values of about 46 and 51 min. for the respective strains. These figures may indeed represent an approach to the same limit, particularly in view of the fact that training at the higher concentration was carried out for a somewhat shorter period.

Prolonged growth by serial subculture was shown to be impossible at concentrations as high as 1 g./l., where the cells would not grow after the third subculture. This result is probably connected with Lodge and Hinshelwood's observation [1] that growth of *Bact. lactis aerogenes* is completely inhibited at concentrations of magnesium sulphate below 2×10^{-6} g./l. The concentration of magnesium sulphate hydrate in the standard medium is 4×10^{-2} g./l. so that at concentrations of the E.D.T.A. salt in the region of 1 g./l. the Mg^{++} concentration is probably reduced below this critical level by complex formation.

The training process does not appear to result in the ability of cells to use the drug as a source of carbon, neither of the trained strains being able to grow in a medium containing the compound as sole source. This is not to say, however, that the drug is not metabolised in some way.

The Action of Dinitrophenol

In general, phenols affect bacterial metabolism by the precipitation of proteins, which causes a general reduction in the rate of enzyme reactions within the cell,[2] but in addition to this common property some phenols have more selective actions.[3]

In the adaptation of *Bact. lactis aerogenes* subcultured in sub-lethal concentrations of phenols [4] there are two types of behaviour illustrated by the cases of phenol and thymol. In a medium containing phenol there is simply an extremely slow increase in growth rate whereas with thymol there is an elimination, after a few subcultures, of an initial very long lag. A series of alkyl phenols show intermediate behaviour. Strains resistant to increased concentrations of thymol have been obtained by continued cultivation in its presence, but, in contrast, attempts to produce strains appreciably resistant to higher concentrations of phenol proved unsuccessful.

2 : 4-Dinitrophenol has been widely used in biochemical studies since it has a specific action on certain internal cell processes. Experiments with animal-cell extracts have shown that it inhibits the assimilatory processes through which new structures are built up.[5]

The present study is concerned with the training of *Bact. lactis aerogenes* to resist increased concentrations of dinitrophenol, the mechanisms by which such resistance is acquired, and the effect of the training upon some biochemical properties of the organism. The methods used are similar to those employed in the study of the resistance of *Bact. lactis aerogenes* to proflavine and of *Saccharomyces cerevisiae* to dinitrophenol.[6]

The resistance of the cells in liquid cultures was assessed by means of lag–concentration curves. A series of tubes were prepared containing the standard growth medium and the drug at about ten appropriate concentrations. These were inoculated with 0·1 ml. volumes of the culture in or immediately after the logarithmic phase. The lags in the various media were then plotted against the drug concentration.

This procedure was repeated with various trained strains developed by continued subculture at one given drug concentration. Cells trained at one concentration were inoculated into a higher concentration to start a more highly trained strain. Usually about ten subcultures at a given level were sufficient to bring the training process to a limit but a larger number was given before the strain was tested. Each subculture represents 8—10 generations. In this

[1] Lodge and Hinshelwood, *J.*, 1939, 1692.
[2] Fogg and Lodge, *Trans. Faraday Soc.*, 1945, **41**, 359.
[3] Spray and Lodge, *ibid.*, 1943, **39**, 1.
[4] Phillips and Hinshelwood, *J.*, 1953, 3679.
[5] Lehninger, " Harvey Lectures," Academic Press, New York, 1954, Vol. 49, p. 176.
[6] Wild and Hinshelwood, *Proc. Roy. Soc.*, 1955, *B*, **144**, 287.

way the series of strains was developed for which the lag–concentration curves are plotted in Fig. 3.

The results show that a strain trained to a concentration m is resistant to all concentrations up to approximately $3m$. The resistance of a trained strain, as measured by the drug concentration required to produce a standard lag, is proportional to the " training concentration " (Fig. 4). The lag–concentration curves are shown for only four trained strains, corresponding to 300, 400, 500, and 700 mg./l. although two other strains trained to 1500 and 3000 mg./l. were maintained. Both these were found to be fully resistant, in the sense of showing no lag, to concentrations up to the solubility limit of about 5·6 g./l.

The resistance of some of the above strains was also tested by determining the fraction of the inoculum surviving when spread on a drug agar plate. Results showing the increased survival of the two trained strains are given in Table 2.

Measurements were also made of the times of appearance and the rates of development of colonies on drug plates.[7] A number of cells was plated such that between 50 and 200 of them finally gave rise to colonies. The fraction of cells of the untrained strain surviving to form colonies (α_∞) was about 10^{-5} at the concentration of drug used. For the trained strain it was unity, corresponding to 100% colony formation.

FIG. 3. *Lag–concentration curves for* Bact. lactis aerogenes *trained at various concentrations* (m) *of dinitrophenol. The values of m are shown above each curve* (mg./l.).

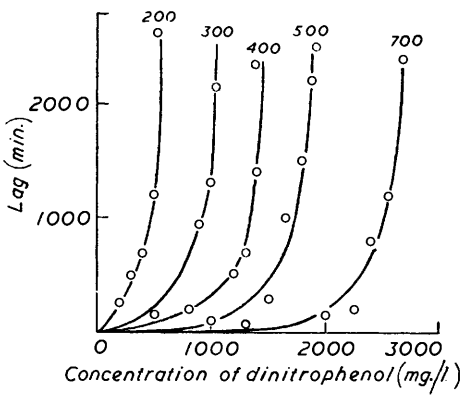


FIG. 4. *Concentrations of dinitrophenol giving lags of* 1200 *minutes for* Bact. lactis aerogenes *trained at various concentrations* (m) *of dinitrophenol.*

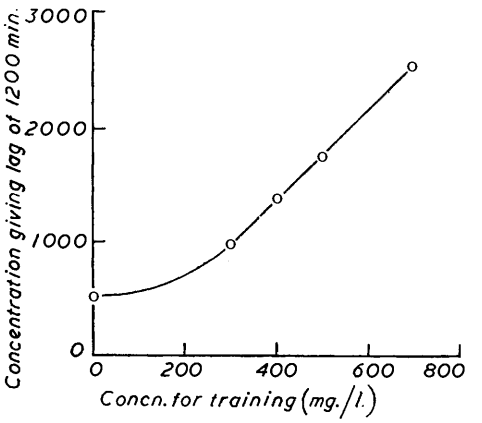


TABLE 2. *Survival on dinitrophenol–agar plates.*

Concn. of dinitrophenol in the agar (mg./l.)	Fraction surviving: Untrained strain	Strain trained at 400 mg./l.	Strain trained at 1500 mg./l.	Concn. of dinitrophenol in the agar (mg./l.)	Fraction surviving: Untrained strain	Strain trained at 400 mg./l.	Strain trained at 1500 mg./l.
0	1	1	1	750	3×10^{-8}	—	—
125	6×10^{-1}	1	1	1000	1×10^{-8}	6×10^{-1}	7×10^{-1}
250	4×10^{-2}	8×10^{-1}	1	2000	—	4×10^{-2}	3×10^{-1}
375	6×10^{-4}	—	—	3000	—	6×10^{-4}	2×10^{-1}
500	1×10^{-5}	9×10^{-1}	9×10^{-1}				

The time at which each colony on the plate became visible to the naked eye was recorded, and the sizes were then measured at intervals of 4—8 hours. The results were expressed as the fraction (α_t) of the inoculum which had appeared and the fraction which, by a time t from inoculation, had reached a standard size designated " large." Fig. 5 shows the increase with time both in the total number, T, and the number of " large " colonies, L, by plotting α_t/α_∞ against t.

The delay in the appearance of colonies from the untrained strain as compared with those from the trained strain is not due to the presence of a large excess of sensitive cells. Experiments in which a small number of trained cells were mixed with a large excess of sensitive cells

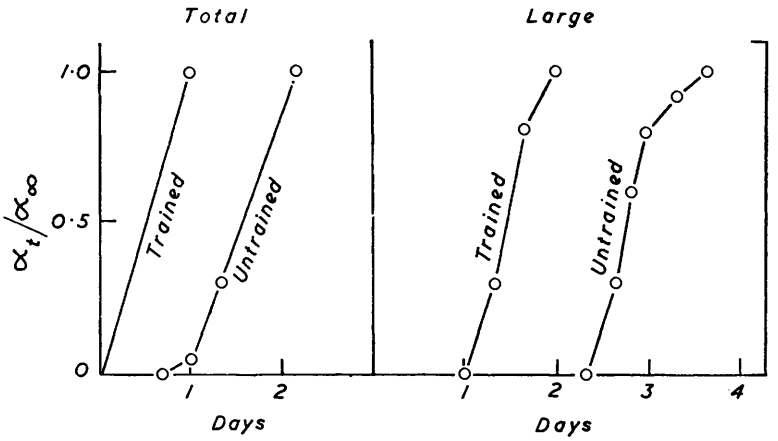
[7] Dean and Hinshelwood, *Proc. Roy. Soc.*, 1955, *B*, **144**, 297.

showed that the former developed into colonies at the same rate as when plated alone. In this experiment the fully trained cells were easily differentiated from the small fraction surviving from the untrained culture. They all formed colonies after one day, while the untrained cells did not do so until two or three days after inoculation. Large colonies were formed from trained cells in two days and from untrained in three or four days. These time relationships are in accordance with the results of the previous experiment in which the two types of cell were plated separately.

Various effects of prolonged cultivation of *Bact. lactis aerogenes* in the presence of high concentrations of dinitrophenol were also investigated. The strain used for these tests had been given more than 50 subcultures at 1500 mg./l. of the drug, and then showed no lag at concentrations up to the solubility limit.

Preliminary experiments showed that the simple biochemical tests normally used to define the organism were not affected by the training process. The trained strain was still able to ferment sucrose, lactose, and inositol, but not dulcitol, and it produced acetylmethylcarbinol (3-hydroxybutan-2-one) when grown in glucose.

FIG. 5. *Effect of training on growth of colonies on plates.* (*For explanation of symbols see text.*)



In view of the well-known specific action of dinitrophenol on the metabolism of cell extracts experiments were made to assess the respiratory and synthetic ability of dinitrophenol-trained cells. To test the respiratory activity of these cells they were spread on agar plates containing 2 : 3 : 5-triphenyltetrazolium chloride at 30 mg./l., this compound being applicable for the estimation of dehydrogenases, acting as hydrogen acceptor in place of oxygen and being reduced to a carmine-red formazan.[8] The colonies from the trained cells became coloured in the same time, and to the same intensity, as the colonies of control plates prepared with cells unexposed to the action of dinitrophenol. Moreover, when mixtures of the two types of cell were used as inocula the resulting colonies were not distinguishable. Cells having a permanent respiratory deficiency induced by exposure to the drug should have been detectable by such tests.

Another simple means of assessing the efficiency of the respiratory enzyme system consists in measuring the maximum population attainable in a medium containing a limiting amount of glucose, so that multiplication ceases on account of exhaustion of the food supply and the maximum population is linearly related to the concentration of the glucose. (The medium actually used contained one-fiftieth of the usual concentration.) Aerobic processes of oxidation make available much more energy for each unit of dissimilated carbohydrate than anaerobic mechanisms. Thus if normal aerobic mechanisms are impaired the glucose will be much less efficiently used and a much smaller population will be reached. The maximum populations, as measured by turbidity 24 hours after inoculation, are given in Table 3, which shows close

[8] Brodie and Gots, *Science*, 1951, **114**, 40; 1952, **116**, 588; Smith, *Science*, 1951, **113**, 753; Kun and Abood, *ibid.*, 1949, **109**, 144; Kun, *Proc. Soc. Expt. Biol. Med.*, 1951, **78**, 195; Lindemann, *Schweiz. Z. Allg. Pathol. Bakteriol.*, 1954, **17**, 311.

agreement between the results for trained and untrained cells. Even with the drug actually present in the limiting medium the population reached is not significantly less than normal.

In another series of experiments the rate of adaptation to new sources of carbon was examined. Measurements were made of the lags shown when the highly trained strain was given a first subculture in a medium containing various new carbon sources. The drug-trained

TABLE 3. *Glucose utilisation (total cell mass per ml. after exhaustion of glucose) by normal and trained strains.*

Strain	Drug concn. (mg./l.)	Turbidity reading	Equiv. population in 10^6/ml.	Drug concn. (mg./l.)	Turbidity reading	Equiv. population in 10^6/ml.
Trained to 1500 mg./l.	0	1·33	92	500	1·35	88
		1·32	94		1·36	86
					1·34	90
Control	0	1·32	94	0	1·32	94

strain and a normal strain as control were grown in a limiting glucose medium until all the glucose was exhausted and then inoculated into the new medium so as to give an initial count of about 1×10^6/ml.

A strain of *Bact. coli mutabile* was also used in these experiments. The dinitrophenol-trained strain was given 20 subcultures at 1500 mg./l. and was resistant to the solubility limit.

The lags of the trained strains and the controls are given in Table 4. In each case training to high concentrations of dinitrophenol lengthened the lag shown in the new carbon substrate by about 50%. A parallel series of tubes were inoculated in which the medium contained 500 mg./l. of dinitrophenol in addition to the new substrate. Although the trained strains were completely resistant to the dinitrophenol at this concentration in the sense of showing no lag in its presence in a glucose medium, they were unable to undergo the adaptation necessary to grow in the new carbon sources.

TABLE 4. *Adaptation to new carbon sources (time lag).*

Substrate	Trained strain	Control	Substrate	Trained strain	Control
		Bact. lactis aerogenes			
Acetate	700 min.	480 min.	D-Arabinose	230 hr.	160 hr.
	750 ,,	560 ,,		254 ,,	162 ,,
				246 ,,	169 ,,
				220 ,,	142 ,,
		Bact. coli mutabile			
Succinate	480 min.	310 min.	Lactose	144 hr.	96 hr.
	500 ,,	340 ,,		150 ,,	98 ,,

DISCUSSION

The actions of the two inhibitors studied present a sharp contrast with one another. Dinitrophenol shows a familiar pattern of behaviour such as is shown with proflavine and other compounds, namely, the production of a lag when the cells are first subcultured into the drug medium and its rapid elimination in successive subcultures. Striking differences in the action of disodium dihydrogen ethylenediamine-*NNN'N'*-tetra-acetate are its failure, at any concentration, to give rise to a lag, and the wide range of concentrations over which it may be used without completely inhibiting growth.

Quantitative interpretation of the results obtained for the latter inhibitor is difficult, first because the presence in the medium of Mg^{++} ions with which the inhibitor forms a complex makes the concentration of both uncertain and, secondly, because the results obtained relate to the mean generation time which is a more complex property than the lag and is determined by all the interdependent processes connected with metabolism and synthesis.

There are, however, two mechanisms which would account naturally for the training processes outlined above: (1) Certain enzyme systems require specific trace cations as

activators. By complex formation with such traces the drug could reduce the activity of the enzyme. (2) The drug is specifically adsorbed on one or more enzymes and reduces their activity. In either of these eventualities the cells might develop, to a limited extent, alternative pathways of metabolism in which the inhibited enzyme is unnecessary. The adoption of the new pathway and its development to a state of maximum efficiency would be manifested as a gradually increasing growth rate, the limiting mean generation time indicating the attainment of the state of maximum efficiency for the new pathway. Alternatively, adaptation might take place by expansion of the enzyme or enzymes affected so as to compensate the reduced activity. Again a gradual increase in growth rate would be expected.

The training to dinitrophenol may be considered in the light of two usual alternatives: selection within an initially heterogeneous population, or induced changes in individual cells. The lag–concentration curves show that a number of stable strains can be obtained differing in degree of resistance, this degree being simply related to the training concentration. There is the usual difficulty in constructing a plausible hypothesis attributing these separate strains, each with definite limits of resistance, to the selection of initially resistant cells. Unless a surprising range of mutations is postulated and thus an indefinite number of distinct types of pre-existing resistant cells, growth in any inhibitory concentration should lead to a strain immune to all concentrations. It is difficult to imagine that training in 300 mg./l. should select out pre-existing types resistant to 1100 mg./l. whilst training in 400 mg./l. selects a different type resistant up to 1900 mg./l.

The experimental results are more in accordance with the mechanism in which exposure of the culture to the drug causes a response in the majority rather than in a very small proportion of the cells.

The difference in the time of appearance of colonies on drug plates when trained and untrained strains are plated is very significant. The extra 28 hours taken for the formation of 100% of colonies from the untrained strain must, it would seem, be due to the gradual adaptation taking place on the plate, the retardation having been shown not to be due to the presence of a large excess of sensitive cells. Thus even the small proportion surviving from the untrained inoculum are quantitatively not of the type in the trained inoculum.

The lags observed during adaptation to new carbon sources of the dinitrophenol-trained strain show two interesting features. First, they are increased by about 50% as compared with those of a normal strain, and this fact suggests a modification of some of the biochemical properties of the cells by the training. If the adaptive processes involve a change in the balance of the various enzyme systems it is perhaps to be expected that changes also will occur in the adaptive potentialities of the cells. Secondly, in no case would the trained strain become adapted to the new carbon source in presence of the drug even at a concentration well below that to which the cells were resistant. Dinitrophenol inhibits enzymic adaptation in normal unadapted bacteria through the prevention of synthesis, but the trained strain fails to become adapted although, as was shown by various simple tests, the normal synthetic processes appear to function even in the presence of the drug. Particularly in cases where the lag period is a long one, as in the adaptation to D-arabinose, it may be the long-term protein-precipitating properties which are operative rather than the specific action to which the cells are apparently resistant. During a prolonged lag considerable slow degeneration of the cell could take place, though it must remain a possibility that the drug inhibits the formation of adaptive enzymes even while normal enzymes still function.

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