the deleterious effects of high temperatures.

Our work has indicated that *Cyanidium* is an obligate acidophile. Earlier reports of growth above *p*H 5 may well be due to the capacity of the organism to acidify the culture medium rapidly, or else the method of inoculation may have introduced acid into the medium (*8, 20*).

The lag periods induced in growth by abrupt temperature changes have also been observed for thermophilic bacteria (*21*), as well as for some mesophilic organisms (*22*). Such lags may be due to adaptive processes involving temperature-sensitive control mechanisms.

From our studies further work with *Cyanidium caldarium* is more feasible. There are indications that this organism offers an opportunity to study the mechanism of accommodation of cells to elevated temperatures, while at the same time serving as a source of heat-stable enzymes.

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23 May 1966

Feedback Inhibition of Glycerol Kinase, a Catabolic Enzyme in Escherichia coli

Abstract. *Fructose-1,6-diphosphate is a feedback inhibitor of the catabolic enzyme, glycerol kinase, in* Escherichia coli. *A mutant was isolated which produced a desensitized enzyme. Glucose was no longer as effective in preventing the utilization of exogenous glycerol by cells which synthesized constitutively such an altered enzyme, even though the usual degree of catabolite repression still operated.*

Feedback inhibition plays a part in the regulation of biosynthetic pathways in bacteria (*1*). A similar type of control has also been discovered in an anaplerotic (or replenishing) pathway (*2*). We now describe a case of feedback inhibition in a catabolic pathway encountered in our study of glycerol dissimilation by *Escherichia coli*.

The utilization of glycerol by cells of *E. coli* is obligatorily dependent upon a specific kinase (*3*). This kinase for glycerol not only mediates the first reaction in the dissimilatory pathway, but it also is responsible for the trapping of the substrate which is freely diffusible across the cell membrane (*4*). Utilization of glycerol can be greatly reduced by the metabolism of glucose, which exerts strong catabolite repression on the glycerol kinase even in cells producing this enzyme constitutively (*5*). During the course of screening for mutants in which the formation of glycerol kinase was resistant to glucose repression, using the ability of a colony to incorporate C^{14}-glycerol in the presence of glucose as a criterion (*6*) we uncovered an anomalous variant (strain 43). This mutant was still as sensitive to the repression as its parent, strain 7, but was able to incorporate glycerol while growing on glucose. This unexpected characteristic suggested that there is another type of control mechanism in addition to catabolite repression and that relief from either of these two controls is sufficient to permit simultaneous utilization of glycerol and glucose.

Results from a study of suspensions of whole cells indicated that the additional control was effected through a kinetic feedback mechanism. Cells of the parental constitutive strain, growing in the logarithmic phase on glucose, were promptly collected and suspended in basal medium. When glycerol was added immediately to such a cell suspension together with phenazine methosulfate and a tetrazolium dye, only a slight stimulation of the rate of reduction of the dye occurred (Fig. 1*A*). However, if the cells were first fasted for 30 minutes at 25°C, there was a rapid reaction when glycerol and the electron

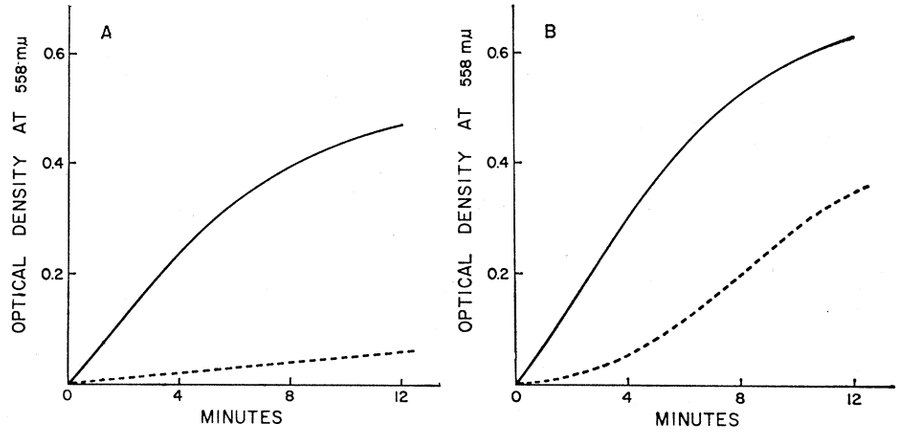

Fig. 1. The cells were grown on 0.2 percent glucose in 25-ml cultures incubated in 300-ml erlenmeyer flasks. When the culture density reached 100 Klett units (420 mμ filter), the cells were collected by centrifugation, washed twice with cold basal inorganic medium, and resuspended in 0.5 ml of the same solution at 0°C. One portion of cells (------) was tested immediately for the glycerol-dependent reduction of 3(4,5-dimethylthiazolyl-1,2)2,5-diphenyltetrazolium bromide in the presence of phenazine methosulfate (*3, 15*). Another portion was first incubated for 30 minutes at 25°C before exposure to glycerol and the hydrogen acceptors (——). The reduction of the tetrazolium was monitored in a Gilford Model 2000 spectrophotometer with automatic recording device. The curves were corrected for the blank in which glycerol was omitted. *A*, Parent strain; *B*, mutant strain.

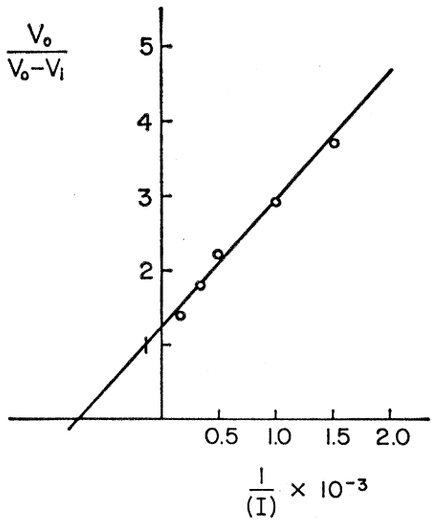

Fig. 2. Inhibition of glycerol kinase as a function of the concentration of FDP. The general condition of assay was the same as that described in Table 1. V_o and V_i are the enzyme activities measured, respectively, in the absence and presence of the inhibitor.

acceptors were added. Inasmuch as glycerol must be phosphorylated before it can be further metabolized, one would expect a priori an opposite result: starvation is likely to deplete the high-energy phosphates and thereby should prolong the lag of glycerol metabolism. In a parallel experiment with mutant cells (Fig. 1*B*), fasting was less essential for lifting the restraint on glycerol consumption. It seemed that cells grown on glucose were replete in an intermediate which inhibited severely the glycerol metabolism of wild-type cells but not that of the mutant cells. That the difference in behavior between the two types of cells reflected the properties of their respective glycerol kinases rather than enzymes further on in the pathway was indicated by the finding that the kinase of the mutant was inactivated at *p*H 9.5, a condition under which the activity of the wild-type enzyme is nearly maximum (*7*).

In an attempt to identify the inhibitor, several glycolytic intermediates were tested at a concentration of 10^{-3} mole/liter, *p*H 7.5, for their effects on the activity of glycerol kinase in extracts freshly prepared from wild-type cells. The compounds included dihydroxyacetone phosphate; glyceraldehyde-3-phosphate; 2,3-phosphoglycerate; 3-phosphoglycerate; 2-phosphoglycerate; fructose-1,6-diphosphate (FDP); fructose-6-phosphate; glucose-6-phosphate; and glucose-1-phosphate. Of these compounds, only FDP was inhibitory. L-α-glycerophosphate was not inhibitory under similar conditions (*7*). This inhibition by FDP was dependent upon *p*H: the maximum effect occurred at *p*H 7.5, and almost no effect was detectable at *p*H 9.5, the usual *p*H for assay of glycerol kinase.

The possibility that the effect observed with FDP was due to an impurity in the sample was excluded by generating the inhibitor enzymatically (Table 1); when $10^{-3}M$ FDP or $2 \times 10^{-3}M$ dihydroxyacetone phosphate was added to an assay system in the presence of both triosephosphate isomerase and aldolase, the wild-type glycerol kinase was inhibited to the same extent. The activity of the kinase is shown as a function of the concentration of FDP from which a value of $10^{-3}M$ was obtained as the inhibition constant (K_i) (Fig. 2). The inhibition of the kinase by FDP was independent of glycerol concentration in the tested range of 10^{-4} to $3 \times 10^{-2}M$. Earlier it had been reported that the enzyme exhibited typical Michaelis-Menten kinetics towards the substrate glycerol with a K_m of $10^{-6}M$ (*4*). Hence, it appears that only the rate of catalysis and not the substrate affinity of the enzyme is regulated by the effector, thus making the kinase a "V" allosteric enzyme (*8*).

Table 1. Inhibition of crystallized glycerol kinase (*7*) from wild-type *Escherichia coli* K12. Activity was measured by a system in which the adenosine diphosphate formed was followed by the oxidation of reduced nicotinamide-adenine dinucleotide at 340 mμ. The reaction mixture contained: 3 μmole of glycerol, 3 μmole of adenosine triphosphate, 30 μmole of $MgCl_2$, 3 μmole of phosphoenolpyruvate, 0.2 mg of crystallized pyruvate kinase (Boehringer and Sons), 0.1 mg of crystallized lactic dehydrogenase (Boehringer and Sons), 100 μmole of potassium phosphate at *p*H 7.5, and 0.1 μg of glycerol kinase in a final volume of 3 ml. Glycerol kinase was omitted from the blank. Other components were added as indicated. The reaction was carried out at 25°C and was monitored in a Gilford Model 2000 spectrophotometer.

Additions to assay mixture*				Kinase activity (relative units)
$10^{-3}M$ FDP	$2\times 10^{-3}M$ DHA-P	Triose-P isomerase†	Aldolase	
0	0	0	0	1.0
+	0	0	0	0.59
+	0	+	+	.67
0	+	+	+	.67
0	+	0	+	1.0
0	+	+	0	1.0
0	0	+	+	1.0

* FDP, fructose-1,6-diphosphate; DHA-P, dihydroxyacetone phosphate; and Triose-P, triosephosphate. † 50 μg/ml.

The actual role of FDP as the physiological inhibitor of glycerol kinase is evident from the fact that the anomalous mutant, strain 43, produced an enzyme which was refractory to inhibition by the diphosphate. Whereas, at a concentration of $3 \times 10^{-3}M$, FDP caused a fourfold reduction in the activity of the wild-type enzyme (from 2.4 to 0.6 μmole per minute per milligram of protein), virtually no inhibition occurred with the mutant enzyme (from 1.01 to 0.96). Further evidence for this regulatory role of FDP was provided by Böck and Neidhardt in their study of the behavior of an *E. coli* mutant which produced a heat-labile aldolase. At a high temperature, its growth and glycerol could be brought to an abrupt halt by exposure to a trace of glucose. Cells whose growth was thus arrested contained elevated levels of FDP (*9*).

The evolutionary acquisition of a mechanism of feedback inhibition in the case of glycerol kinase may have been causally related to the destiny of this enzyme as an agent for glycerol scaveng-

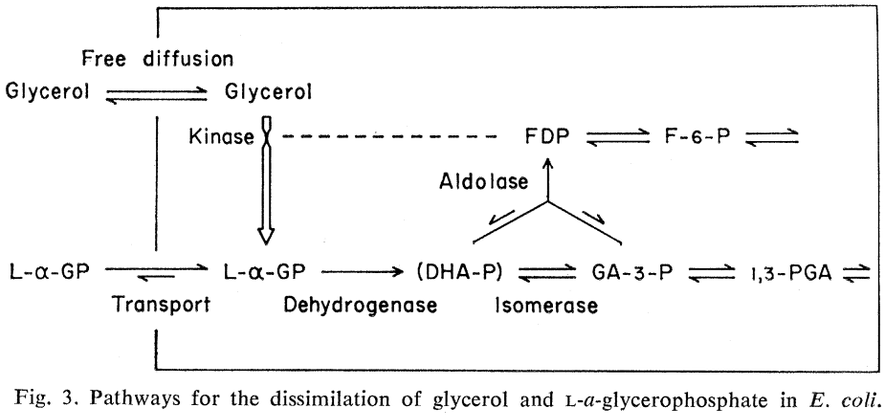

Fig. 3. Pathways for the dissimilation of glycerol and L-*a*-glycerophosphate in *E. coli*.

ing (Fig. 3). Under conditions in which the substrate is scarce, it is advantageous for the cell to increase the production of enzyme to a maximum so that even at partial saturation an adequate input is maintained. However, unless there is a kinetic feedback control, the high level of the kinase may at times cause over-production of L-α-glycerophosphate, thereby retarding growth (*10*). Thus, if the glycerol concentration in the environment suddenly rises, from 10^{-6} to $10^{-5}M$, flooding with the phosphorylated product may occur before a corrective effect by repression can be expressed. The liability of producing derepressed levels of the desensitized kinase in fact could be shown with cells of strain 43 growing on succinate. The addition of glycerol to such a culture severely impeded growth. Cells making the wild-type glycerol kinase did not exhibit this vulnerability.

The establishment of FDP as the kinetic regulator of glycerol kinase allows momentary carbon surplus derived from glycerol to be deposited in the hexose diphosphate pool, since both triosephosphate isomerase and aldolase are either constitutive or internally induced (*11*), and the equilibrium is much in favor of the synthesis of FDP from the triosephates (*12*).

The inhibition of the kinase by a glycolytic intermediate provides an additional means of excluding glycerol utilization during glucose metabolism. The efficiency of this exclusion is made all the more powerful by the fact that the product of the kinase reaction is the inducer of the enzyme (*13*).

The finding that remote-product inhibition of the first enzyme in a dissimilatory pathway illustrates further the similarity of regulatory mechanisms of anabolic, amphibolic, and catabolic systems in which feedback control can be exercised during enzyme action as well as enzyme synthesis (*14*).

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Dieldrin: Interaction with Nerve Components of Cockroaches

Abstract. *We have evidence that the nerve components of the dieldrin-resistant German cockroach have less binding capacity for dieldrin than those of the susceptible cockroach; the highest interstrain difference was in the crude-nucleus fraction. The dieldrin-nerve complexes are not extracted by many organic solvents.*

Since Busvine (*1*) discussed resistance to dieldrin, as distinct from other resistance phenomena such as DDT resistance, on the basis of symptomology and cross resistance in the housefly, the problem of dieldrin-resistance has been constantly investigated (*2*). The problem has been a delight for geneticists, who discovered a simple, straightforward pattern of inheritance (*3*), and a nightmare for biochemists, who found no significant interstrain differences in the insect's defense mechanisms such as biochemical detoxication, penetration through the cuticle and the nerve sheath, storage, and excretion (*4*). Dieldrin is generally very stable and not readily detoxified by insects, though one exception was recently reported (*5*).

The only direct relation of the action of dieldrin to the central nervous system was observed by Yamasaki and Narashashi (*6*), who discovered that dieldrin-poisoned nerves of the American cockroach (*Periplaneta americana*) showed spontaneous bursts of action potential; they also noticed that the nervous systems of resistant houseflies showed much longer latent periods between the application of dieldrin and the appearance of discharge than the systems of susceptible flies (*7*).

A recent hypothesis attempted to explain the mode of action of DDT on the basis of formation of charge-transfer complexes by nerve components and DDT (*8*). The first step of this charge-transfer process was exemplified by formation of bound DDT, which apparently could be clearly distinguished from free DDT by means of molecular filtration (Sephadex colmun chromatography). This approach to the problem of an insecticide-binding mechanism may be generally applicable throughout the whole group of chlorinated hydrocarbon insecticides, including dieldrin and DDT. The implication is simple: if such a mechanism is directly implicated in dieldrin poisoning, nerve components of dieldrin-resistant individuals should clearly show a different pattern of binding with dieldrin.

The head parts from three strains of the German cockroach (*Blattella germanica* L.) were homogenized, with small Teflon Potter-Elvehjem homogenizers, in 0.25*M* sucrose solution at 0°C at a concentration of three heads per milliliter. The head samples from each strain were carefully weighed to insure equality of the homogenate concentrations among the strains; London, Fort Rucker (both resistant), and CSMA (susceptible) strains averaged 2.517, 2.508, and 2.458 mg, respectively.

In terms of LT_{50} (time to 50-percent mortality; *9*) (at 1 mg of dieldrin per jar having an inner surface of 200 cm^2), Fort Rucker (15 hours) and London strains (45 hours) were four and eleven times more resistant, respectively, than the CSMA strain (4 hours). The C^{14}-dieldrin in either absolute ethanol or acetone was added to the brain homogenate to make the final concentration $1 \times 10^{-5}M$ (final ethanol or acetone concentration, 1 percent) in a 20-ml vial with a screw cap; the system was maintained at 24°C for 1 hour. The reaction was stopped by transferring the vial to an ice bath. The resulting solution was poured into a Sephadex G-50 (medium) column of 1 by 10 cm, and each component was eluted carefully with distilled water;