polymorphism) and mean proportion of loci heterozygous per individual, $\overline{H}$, for Dalton and Hadera populations respectively were: $\overline{A}$ = 1.06, 1.06; $\overline{P}$ = 0.062, 0.062; $\overline{H}$ = 0.021, 0.028. Mean $\overline{H}$ for *P. syriacus* was 0.024. Heterozygosity in *P. cultripes*, based on the 3 specimens analyzed, was 0.011.

The pattern of genetic variation in *Pelobates* is best explained by the environmental variability model. Selection for homozygosity as an adaptive strategy seems to operate in the relatively narrow and constant subterranean niche[5,8,9]. LEVINS'[10] theory of fitness suggests that the amount of genetic variation is adapted to environmental heterogeneity and uncertainty. Therefore, homozygous patterns are expected to be adaptively selected in species occupying relatively narrow and constant niches, as is also postulated by the niche-variation model[3]. The subterranean niche is ecologically narrower, more constant and predictable than surface environments in terms of annual and daily fluctuations of temperature and relative humidity[11].

The evolutionary history of the pelobatids is very long, dating back to Cretaceous times[12], and that of the modern species of *Pelobates* to the Miocene[13]. Throughout its long history *Pelobates* evolved burrowing habits that made it a narrow habitat specialist living in a relatively constant and highly predictable underground environment, and thus insulated from short term environmental perturbations. In Israel, adult spadefoots are mainly seen above ground at night during the short winter breeding season, spending the greater part of their existence, during the dry and hot summer, underground (NEVO, unpublished). Significantly lower genetic variation was found in *Pelobates* when compared to the habitat intermediate species *Rana ridibunda* and *Hyla arborea*, and the habitat generalist *Bufo viridis*, when all 4 species were sampled at the same sites[9]. It therefore appears plausible that the relative narrowness and constancy of the subterranean niche are the major selective determinants of high homozygosity in spadefoots in accord with the prediction of the niche-variation model[3] and the theory of adaptive strategies[10].

Alternative explanations to the extreme homozygous patterns of *Pelobates* can be ruled out. First, genetic drift seems an unlikely agent of homozygosity since both the Dalton and Hadera populations of *P. syriacus* are quite large, involving hundreds, if not thousands, of breeding adults. Second, neutrality also appears unlikely. The evolutionary history of the modern species of *Pelobates* is certainly long enough to result in total fixation if neutrality indeed operated[14]. Nor can migration explain the esterase polymorphism, since the Hadera and Dalton populations are probably geographically disjunct and the two *Pelobates* species are certainly geographically and reproductively isolated.

Relatively low heterozygosity characterizes most subterranean and fossorial mammals yet studied[5], and also subterranean mole crickets, genus *Gryllotalpa*[15]. Similar reduced genetic variation associated with increasingly homogeneous environments has been demonstrated in marine bivalves[16], in bees[17], and was experimentally generated in *Drosophila* by POWELL[18] and MCDONALD and AYALA[19]. Despite some evidence to the contrary[20], a growing body of evidence indicates an overall positive correlation between protein polymorphisms and environmental heterogeneity[9,21,22]. It therefore appears plausible that allelic variation of protein polymorphisms is at least partly adaptive and is regulated in natural populations by selection in accord with an index of environmental diversity.

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Reactivation by Glycerol and Ethylene Glycol of Inactivated δ-Aminolevulinic Acid Synthetase of *Rhodopseudomonas spheroides*

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Summary. Reactivation effects by glycerol and ethylene glycol of inactivated ALA synthetase of *R. spheroides* were observed. Accompanying the reactivation of the inactivated enzyme, K_m value for PLP decreased to levels similar to those of the freshly prepared enzyme.

2-Mercaptoethanol is the most popularly used and potent stabilizer of δ-aminolevulinic acid (ALA)synthetase (E.C. 2.3.1.37), a regulatory enzyme of the tetrapyrrole biosynthetic pathway, of some bacteria and of animal tissues, but the effect becomes less remarkable as the purification of the enzyme proceeds and finally it is inhibitory on the activity of the highly purified ALA synthetase[1,2]. As another stabilizer of this enzyme, some workers recently applied glycerol which is well-known as a cryoprotectant; they reported that 10%[3,4] and 30%[5]

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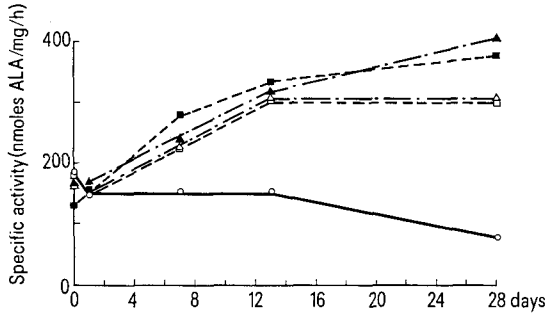
glycerol stabilized ALA synthetase of *Rhodopseudomonas spheroides*. During examination of glycerol, and at the same time of ethylene glycol, on ALA synthetase of *R. spheroides*, we found that both glycerol and ethylene glycol not only protect the enzyme from inactivation, but also reactivate the inactivated enzyme. This interesting finding was further kinetically analyzed and it was found that accompanying the reactivation of the inactivated enzyme *Km* value for pyridoxal-5′-phosphate (PLP), but not for glycine, decreased to similar levels of those of the freshly prepared enzyme.

Materials and methods. R. spheroides (NCIB 8253) cells were cultured as previously[2]. ALA synthetase was purified from crude extracts of *R. spheroides* as previously[2] by the treatment with calcium phosophate gel and the fractionation with ammonium sulfate. Precipitates collected between 30% and 50% saturation of ammonium sulfate were dissolved in 50 m*M* phosphate buffer (pH 7.4) containing 10 m*M* 2-mercaptoethanol and



Reactivation by glycerol and ethylene glycol of the partially inactivated ALA synthetase. The partially inactivated enzyme by repeated freezing and thawing was preincubated with 30 and 50% glycerol or ethylene glycol (7–7.6 mg protein per ml), respectively, at −20 °C.
○, control; □, 30% glycerol; △, 30% ethylene glycol; ■, 50% glycerol; ▲, 50% ethylene glycol.

Michaelis constants for PLP and glycine[a]

Enzymes	Specific Activity (nmoles ALA formed per mg protein/60 min)	K_m	
		PLP (μM)	Glycine (mM)
Active	622.7	9.5	40
(freshly prepared)	411.2	10	50
	630.9	7	—
Inactivated	192.5	17.4	46.5
	210.3	30	46.5
	225.7	24.4	38
Reactivated by			
Glycerol	339.2	6	41.6
	537.7	8.5	45.5
Ethylene glycol	332.5	7.7	41.7

[a] Michaelis constants of the freshly prepared, inactivated, and reactivated enzyme were determined for PLP and glycine. The partially inactivated enzymes were prepared from the freshly prepared one by repeated freezing and thawing or by aging at room temperature. The reactivated enzymes were prepared from the partially inactivated enzyme by preincubation with 50% glycerol or ethylene glycol at −20 °C.

and dialyzed against the same buffer. ALA synthetase activity was assayed as described by TUBOI et al.[6] with slight modifications.

Results and Discussion. After storage of the partially purified ALA synthetase in a medium of 50 m*M* phosphate buffer (pH 7.4) containing 10 m*M* 2-mercaptoethanol at 4 °C for 3 days, about 50% loss of the original activity was observed. By the addition of 30–50% glycerol or ethylene glycol to the enzyme solution, however, the enzyme activity was completely stabilized. Lower concentrations of glycerol or ethylene glycol than 30% was not, or less, effective on the enzyme. Stability of the enzyme also depends on the storage temperature. As shown in the Figure, the loss of the enzyme activity was well prevented even in the absence of either alcohol when the enzyme was stored at −20 °C (control curve), but finally a gradual loss of the enzyme activity occurred after prolonged storage. In sharp contrast to the control experiment, 30% and 50% glycerol or ethylene glycol not only stabilized the enzyme, but also reactivated the enzyme. The original activity of the enzyme used in this experiments was about 300 nmoles/mg/60 min and the partially inactivated enzyme (at about the half of the original enzyme activity) was reactivated to the full original activity after 2 weeks storage at −20 °C. The reactivation effect by both the alcohols on the inactivated enzyme, however, was not observed when the preincubation was carried out at higher temperature than 0 °C or after prolonged storage of the inactivated enzyme so far tested. Failure to observe the reactivation at higher temperature than 0 °C is probably due to the faster rate of inactivation by some inhibitor or by proteinase contained in the enzyme preparation than that of the reactivation by the alcohols. Indeed, reactivation of the inactivated enzyme at higher temperature than 0° could be observed when the highly purified enzyme was preincubated with either alcohol. The Table shows the kinetic analysis of the freshly prepared, partially inactivated, and reactivated enzymes. Michaelis' constants for glycine of the 3 enzymes showed similar values, while K_m values for PLP of the inactivated enzyme were larger than those of the freshly prepared enzyme. Accompanying the reactivation, however, K_m value for PLP decreased to levels similar to those observed with the freshly prepared enzyme. Activations of other enzymes by glycerol which resulted from lowering K_m for substrates were reported by other workers[7,8], and in such cases the activations occurred instantaneously in the assay mixture. The effect of both alcohols on ALA synthetase, however, was observed only when the enzyme was preincubated with either alcohol, and the addition of either alcohol to the assay mixture had no effect or was rather inhibitory on the enzyme activity. These results suggest that the lability of protein conformation around the PLP-binding site of ALA synthetasde is responsible for the partial inactivation. Ethylene glycol showed an identical effect as glycerol described above, and the advantage of the former reagent for practical use is its lower viscosity in comparison to glycerol.

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