Artifact Produced in Disc Electrophoresis by Ammonium Persulfate

Abstract. *Ammonium persulfate, a common polymerizing agent for acrylamide gels, can inactivate yeast enolase and produce increased electrophoretic heterogeneity during disc electrophoresis in gels containing 8M urea. The use of riboflavin and light for polymerization or thioglycolate for removal of the persulfate are feasible alternatives.*

The widespread use of acrylamide gels for electrophoretic work and chromatography has been accompanied by the equally widespread use of ammonium persulfate, a free radical generator and strong oxidant, to polymerize the acrylamide.

I have found that under some conditions the persulfate can inactivate yeast enolase and produce additional electrophoretically distinguishable protein. The latter finding is shown in Fig. 1. A preparation of yeast enolase which gave a single major band on disc electrophoresis in the absence of urea was electrophoresed in gels containing 8*M* urea and various concentrations of persulfate. The composition of the upper and lower gels is as described by Jovin *et al.* (*1*), except that the upper gels contained no urea, and the buffer in the lower gels was $\Gamma/2 = 0.04$ tris-HCl, *p*H 7.8. The lower gels were polymerized by 0.4 μmole of persulfate per milliliter in tubes 1 and 2; 1.2 μmole/ml in tubes 3, 4, and 8; and 4 μmole/ml in tubes 5 and 6. Tube 7 was polymerized with riboflavin and light. Tubes 7 and 8 are from a separate experiment. Tris-thioglycolate (0.7 μmole in 50 percent sucrose) was layered onto the gels in tubes 2, 4, and 6 before the addition of 25 μg of yeast enolase in upper buffer containing 10 percent sucrose. Fifty micrograms were electrophoresed in tubes 7 and 8. All tubes were run at 1.5 ma per tube for 1.5 hours before staining.

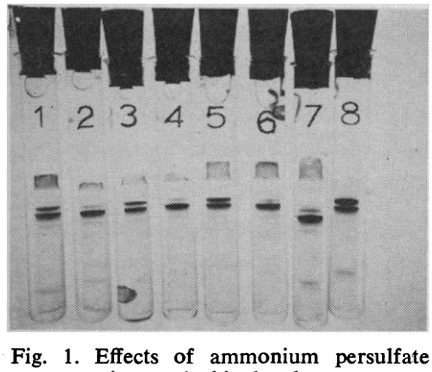

Fig. 1. Effects of ammonium persulfate concentration and thioglycolate on yeast enolase electrophoresis patterns in gels containing 8*M* urea. The lower gels were polymerized by 0.4 mole of persulfate per milliliter of gel in tubes 1 and 2; 1.2 mole/ml in tubes 3, 4, and 8; and 4 mole/ml in tubes 5 and 6. The lower gel in tube 7 was polymerized with riboflavin and light. Thioglycolate was used in tubes 2, 4, and 6.

In the presence of increasing concentrations of persulfate, an increasing fraction of the total protein migrates in a band with a lower R_F (tubes 1, 3 and 5). With thioglycolate (tubes 2, 4, and 6), or on lower gels polymerized with riboflavin and light (tube 7), only one major band is observed. The use of thioglycolate with riboflavin-polymerized gels reduces, but does not eliminate, the small amount of slower-migrating component seen in tube 7 (not shown).

Thioglycolate is a reducing agent with an electrophoretic mobility equal to that of the tracking dye. It migrates ahead of the enzyme and should remove persulfate before the latter can come into contact with the enolase. The total amount of persulfate is in excess, but most of it will migrate in the same direction as the protein. Yeast enolase contains no cysteine or cystine (*2*), and is unaffected by thioglycolate.

By the use of a preparative disc electrophoresis apparatus (*1*), these protein fractions were isolated by electrophoresis with and without thioglycolate (Fig. 2). Samples containing 20 to 25 mg of yeast enolase were electrophoresed in gels polymerized by 1.2 μmole of persulfate per milliliter. Where indicated, 20 μmole of tris-thioglycolate were used. Other conditions are given above or in Jovin *et al.* (*1*). In this apparatus, the protein is collected as it is electrophoresed off the lower gel. Figure 2 gives the 280 mμ absorption versus water of the fractions collected as a function of time of electrophoresis after the appearance of the tracking dye (and thioglycolate). The eluting buffer provides a background absorbance of about 0.09. The shoulder at 1 to 2 hours is not protein, but some ultraviolet-absorbing contaminant from the gel.

The enzyme electrophoresed with thioglycolate migrated as a single species (Fig. 2, open circles) and had a specific activity of about half that of the original enzyme. This is the normal recovery of activity from urea-treated enzyme. The specific activity was constant across the peak. The faster of the two peaks obtained by electrophoresis without thioglycolate (closed circles) had about 10 percent of the original specific activity; the slower component, representing about half of the eluted protein, had no activity.

The enzyme under some circumstances dissociates into subunits (*3*). However, combining the two fractions did not increase the activity, nor did neutralization of combined fractions after acid-urea treatment (*4*). The latter process has been shown to restore 50 percent of the original activity to yeast enolase which has been aggregated by boiling or precipitated with trichloroacetic acid. Treatment of the individual or combined fractions with thioglycolate also had no effect. I conclude that the loss of activity occurring upon electrophoresis in the absence of thioglycolate is due to a chemical modification, probably an oxidation, of the enzyme by persulfate.

It is easy to show that, of all the gel ingredients, only the persulfate inactivates the enzyme. The persulfate does this only in the presence of urea, suggesting that it operates by attacking residues which in the native enzyme are inaccessible. The difference spectrum of persulfate-inactivated enzyme in 8*M* urea versus untreated enolase in the same solvent shows that tyrosines or tryptophans are affected by the persulfate. It is obvious, however, that any accessible oxidizable group, such as

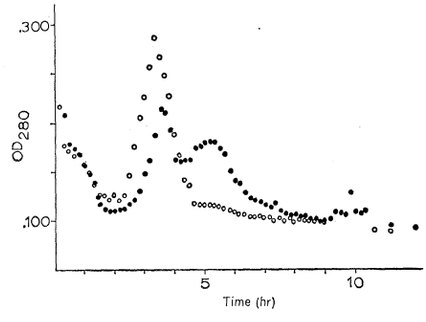

Fig. 2. Effect of thioglycolate on the elution pattern obtained in preparative disc electrophoresis of yeast enolase. The solid circles show the pattern obtained by electrophoresis without reducing agent. The profile represented by the open circles was obtained with thioglycolate.

sulfhydryl groups, can be oxidized even without urea during disc electrophoresis in persulfate-polymerized gels.

I recommend the use of riboflavin and light for polymerizing lower gels, rather than persulfate. The use of substances such as thioglycolate is of course restricted to proteins unaffected by these compounds.

JOHN M. BREWER
Department of Biochemistry, University of Georgia, Athens 30601

References and Notes

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2. B. G. Malmstrom, in *The Enzymes*, P. Boyer, H. Lardy, K. Myrbäck, Eds. (Academic Press, New York, 1961), vol. 5, p. 471.
3. J. M. Brewer and G. Weber, in preparation.
4. E. W. Westhead, *Biochemistry* **3**, 1062 (1964).
5. I thank Gene Michaels for preparing the photograph used as Fig. 1. Supported by NSF grant GB 5918.

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Temperature Tolerance of Some Antarctic Fishes

Abstract. *Three species of Antarctic fishes which live in constantly near-freezing waters have a markedly low upper-lethal temperature of 6°C ; this is the lowest upper-lethal temperature reported for any organism. The fishes survive supercooling to −2.5°C. Data on brain metabolism in vitro support the hypothesis that the central nervous system is a primary site of thermal injury.*

The temperature-tolerance limits of fishes and the physiological and biochemical factors setting these limits have been the subject of much study. Brett (*1*) reviewed the temperature-tolerance limits of a large number of fish species; Fry discussed the factors generally involved in creating these limits (*2*). Fry indicated that there is a lack of information about the temperature-tolerance limits of polar marine fishes which may spend their entire lives in near-freezing waters. There is still considerable uncertainty about the thermally sensitive site (or sites) which imposes thermal-tolerance limits on a species. This is particularly true in the case of the upper-lethal temperature which is often far lower than the temperatures at which enzyme denaturation is expected to occur.

We studied the temperature tolerance of several Antarctic fishes during an investigation of the cold-adaptation mechanisms of these organisms. The fishes used were three notothenioid species from McMurdo Sound (77°51′S, 166°38′E): *Trematomus bernacchii, Trematomus borchgrevinki,* and *Trematomus hansoni.* The family Nototheniidae occurs almost entirely in the Antarctic; the genus *Trematomus* is not known to occur in waters with temperatures higher than 1° to 2°C (*3*). The populations we sampled probably resided all year long in McMurdo Sound. Only *T. borchgrevinki* could not be captured throughout the year; its apparent disappearance during the three midwinter months may have been a consequence of our sampling procedures.

Table 1. Median resistance times for three Antarctic fishes adapted to -1.9°C. Time is given in minutes (1 week = 10,080 minutes). Numbers in parentheses represent numbers of specimens.

Species	Median time of death									
	15°C		10°C		8°C		7°C		5°C	
T. bernacchii	6	(6)	140	(12)	430	(8)			12,960	(6)
T. hansoni	8	(18)	60	(10)					15,840	(6)
T. borchgrevinki	7	(12)	81	(14)			1,905	(16)		

The temperature of the environment of these fishes is extremely cold and remarkably stable. The average temperature was −1.9°C; temperature variation, either with depth or through the year, was of the order of 0.1°C (*4*).

All specimens were captured through holes in the sea ice, with either wire traps or conventional hook-and-line fishing techniques (*5*). The fish were transported from the collecting sites to the biology laboratory at McMurdo Station in insulated containers; mortality during transit was negligible. Specimens were held, until use, in 190- or 760-liter aquariums which were continually aerated and filtered; the sea water was changed every 3 to 4 days. All species survived well under these holding conditions, and several specimens were held for periods in excess of 9 months with no apparent ill effects.

All experimental fish were held for 1 week or less at a temperature of −1.9° ± 0.1°C. There were not enough specimens and holding facilities available to permit the acclimation of groups of fish to different temperatures. We were therefore unable to establish a complete zone of tolerance (*6*) for the species. Specimen weights ranged from 34 to 177 g for each species. In view of the fact that most individuals arrived in the laboratory with full guts, no attempt was made to feed the specimens during this brief holding period. The photoperiod was regulated to match that of the environment, and, because most of the experiments were conducted during the austral winter, most of the specimens were held under conditions approximating total darkness.

In each experiment on lethal temperature, a group of four to ten fish of one species was placed in a 190-liter aquarium containing fresh, well-aerated sea water previously heated to the desired experimental temperature. Temperature variation was no greater than 0.1°C. The time of death was taken as the time at which opercular movement ceased and could not be reactivated by mechanically stimulating the specimen. Fish which had attained this state could not be revived if placed into cooler water. The highest temperature at which 50 percent of the fish survived for 1 week or longer is considered to be the upper incipient lethal temperature (*1*).

Determination of a lower incipient lethal temperature was not possible, due to our inability to supercool sea water to temperatures lower than −2.5°C. The longest period that we succeeded in keeping sea water this cold in an ice-free state was 3 days. Fish held in this supercooled water during this period showed no signs of distress; however, the appearance of ice crystals in the water led to rapid mortality, a phenomenon also observed with Arctic fishes by Scholander *et al* (*7*).

The lethal-temperature data (Table 1) indicate that all three species have a markedly low upper-lethal temperature. The upper incipient lethal temperature based on all data in Table 1 is approximately 6°C. We believe that this is the lowest upper-lethal temperature reported for any organism. The narrow thermal-tolerance range of these Antarctic fishes is consistent with the tendency (*1*) for the thermal-tolerance range to decrease as the adaptation temperature decreases.

The data on brain metabolism in