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Studies on the Redox State in Polyacrylamide Gels

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Summary

The oxidation-reduction properties of polyacrylamide gels were examined to test the possibility that persulfate—or other free radical donors—alter proteins during polyacrylamide gel electrophoresis. Polyacrylamide gels formed either by use of persulfate or riboflavin and light as catalysts were shown to oxidize sulfhydryl compounds. Electrophoresis of thioglycolate into the gel in amounts equivalent to persulfate resulted in reducing conditions (titratable SH groups) in the gel.

Acrylamide monomer and, therefore, presumably unreacted monomer in polyacrylamide react with the α-amino group of amino acids: the reaction with glycine proceeded slowly at pH 9 and at acrylamide concentrations comparable to that of amino acid. Acrylamide at high concentrations (0.5 M) also reacted with yeast enolase and, like the thiol groups of reducing agents, prevented the appearance of characteristic changes in the pattern of enolase in polyacrylamide gel electrophoresis, previously attributed to "persulfate damage" and now designated the "enolase band shift."

The enolase band shift was demonstrated in gels photopolymerized by riboflavin in the absence of persulfate. Pattern changes resembling those of

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the enolase band shift were observed after direct incubation of enolase with persulfate at the high level used for polymerization (4 m*M*), although pattern changes indicative of drastic alteration of the protein result from such incubation. The enolase band shift was obtained whether or not gels were purified by pre-electrophoresis which should remove all persulfate. The pattern ascribed previously to persulfate damage only occurred in gels containing urea and was abolished by reaction of the protein with thioglycolate, hydroquinone, dithiothreitol, or acrylamide prior to the migration of the protein into the gel.

Under the conditions of the riboflavin-catalyzed photopolymerization of acrylamide the cyanmethemoglobin molecule was altered; illumination by itself had no such effect.

INTRODUCTION

The high resolving power, economy, and simplicity of polyacrylamide gel electrophoresis (PAGE) has lead to its widespread use (*1*) in spite of a number of problems that are inherent in the use of these gels as media for zone electrophoresis. Several of these problems stem from the fact that polyacrylamide (PA) gels as they are commonly used contain reactants and catalysts of the polymerization reaction. To date, all efforts to remove them from the gels prior to PAGE have introduced additional problems.

When gels are purified by pre-electrophoresis, charged reactants are removed, but the increment in ionic strength over that of the buffer produced by these reactants is not reversed. Also, electrolysis products may enter the gel (*2*, *3*). Residual acrylamide monomer represents a substantial "impurity" since gels can be formed with up to 50% unreacted (extractable) monomer; this is not removed by pre-electrophoresis. Also pre-electrophoresis does not remove a nondialyzable charged compound that resembles polyacrylamide but is given off continuously during electrophoresis (*4*).

Gel purification by diffusion seems most effective (*2*, *5*). However, it is not compatible with apparatus used in PAGE, the operation of which depends on firm wall adherence of polyacrylamide. In addition, purification by diffusion results in a change in the effective gel concentration and pore size (*2*, *5*).

Among the various components of the polymerization reaction of acrylamide which may react with proteins, only persulfate has been studied extensively to date. "Persulfate damage" of proteins was first claimed by Brewer (*6*), then by others (*7–9*). Brewer subjected enolase to PAGE in a modified Tris system of Ornstein and Davis (*10*) in 8 *M*

urea and observed the appearance of a new inactive component in gels formed with persulfate but not in those formed with riboflavin. Therefore, he associated this band shift with persulfate damage. Similar conclusions were reached by others (*7–9*). However, none of these reports of persulfate damage included assessment of pattern changes in PAGE after incubation of the protein in persulfate; none accounted for the fact that persulfate migrates ahead of anionic protein in gels (*11*) nor for the slow kinetics of persulfate as an oxidizing agent (*12*). No persulfate damage has been observed with hemoglobin (*13*) and serum proteins (*14*).

Because of the potential challenge to the safe use of PAGE as a fractionation tool for other proteins, we undertook a systematic study of yeast enolase applying PAGE under the conditions previously used by Brewer (*6*). The enzyme has recently been reported (*26*) to contain 1 SH group/mole.

MATERIALS AND METHODS

Yeast enolase was obtained from Dr. Finn Wold (Dept. of Biochemistry, Univ. of Minnesota, Minneapolis, Minn.). Dithiothreitol was purchased from Calbiochem, thioglycolic acid from Fisher, Ellman's reagent from Gallard-Schlesinger (No. 44137). Urea (9 *M*) was deionized on AG 501D (Biorad) to achieve a specific conductance of 5 μmhos/cm or less. Alternatively, freshly prepared purified urea (Schwarz/Mann No. 909200) of similar conductance was used without deionization.

Preparation of PA Gels

The polymerization of cross-linked PA was carried out as described previously (*15*) except for the conditions stated below. All gels were 6 mm in diameter.

For the experiments on enolase, PA gels (phase GAMMA) (see Nomenclature section) were formed in the buffer system (Rick Prairie, unpublished data) described by Brewer (*6*), designated the "Tris–Brewer" system or in system A (*15*). The properties of this system at 25°C were computed by the program of Jovin (*16*) and are shown in Table 1. The symbols used in the Table are defined in detail elsewhere (*1, 17*) (see also Nomenclature section). Upper gels (stacking gels) for the Tris–Brewer lower gels were made as described previously (*15*) for system A (phase BETA). Separation gels (lower gels) for both buffer

TABLE 1

Physical Parameters Characteristic of the Tris–Brewer Buffer System, Computed by the Program to T. M. Jovin (*16*)[a]

SYSTEM NUMBER

DATE = 09/22/67 COMPUTER SYSTEM NUMBER = CHRAMBACH SYSTEM TRIS-BREWER
POLARITY = - (MIGRATION TOWARD ANODE) TEMPERATURE = 25 DEG. C.

CONSTITUENT 1 = NO. 29 , GLYCINE
CONSTITUENT 2 = NO. 82 , PHOSPHATE-DIBASIC
CONSTITUENT 3 = NO. 99 , CHLORIDE -
CONSTITUENT 6 = NO. 12 , TRIS

	PHASES					
	ALPHA(1)	ZETA(4)	BETA(2)	PI(9)	LAMBDA(8)	GAMMA(3)
C1	0.0384	0.0499		0.0312		
C2			0.0320		0.0188	
C3						0.0400
C6	0.0050	0.0520	0.0590	0.0509	0.0562	0.0597
THETA	0.130	1.043	1.844	1.631	2.989	1.492
PHI(1)	0.044	0.136		0.169		
PHI(2)			0.641		0.893	
PHI(3)						1.000
PHI(6)	0.338	0.130	0.890	0.103	0.633	0.670
RM(1)	-0.032	-0.098		-0.122		
RM(2)			-0.949		-1.090	
RM(3)						-1.552
RM(6)	0.169	0.065	0.445	0.052	0.317	0.335
PH	8.40	8.94	7.20	9.05	7.87	7.80
ION,STR.	0.0017	0.0068	0.0730	0.0053	0.0524	0.0400
SIGMA	0.199	0.796	7.741	0.620	5.564	7.921
KAPPA	100.	388.	3371.	304.	2475.	3580.
NU	-0.159	-0.123	-0.123	-0.196	-0.196	-0.196
BV	0.006	0.027	0.030	0.021	0.034	0.030

PHASE ETA(7) X1= 1.361 X2= 0.237 X3= 1.701 X4= 0.296

RECIPES FOR BUFFERS OF PHASES ZETA(4),BETA(2),GAMMA(3),PI(9)

CONSTITUENT		1X PHASE 4	4X PHASE 2	4X PHASE 3	4X PHASE 9
GLYCINE	GM	3.74			0.94
1M PHOSPHORIC ACID	ML		12.80		
1N HCL	ML			16.00	
TRIS	GM	6.30	2.86	2.89	2.47
H2O TO		1 LITER	100 ML	100 ML	100 ML
AT FINAL CONCENTRATION =					
PH(25 DEG.C.)		8.94	7.20	7.80	9.05
KAPPA(25 DEG.C.)		388.	3371.	3580.	304.

[a] For abbreviations, see Nomenclature section.

systems were made in 8 *M* urea, and were polymerized using either 3.6 m*M* ammonium persulfate (AP) or 0.656 m*M* of riboflavin (RN) under illumination for 30 min. Stacking gels (without urea) were always photopolymerized, using 0.656 m*M* RN. *N,N,N',N'*-Tetramethylethylenediamine (TEMED) was added to the polymerization mixture of lower gel and stacking gel to final concentrations of 1.56 and 3.13 m*M*, respectively. Polyacrylamide gels (systems Tris–Brewer and B) used for the measurement of thiol group concentration were polymerized with both AP and RN at concentrations as specified in Table 2.

Polyacrylamide gels (system A, 5%T, 3%C, phase GAMMA; see Nomenclature section) used in the investigation of alteration of hemoglobin by illumination were polymerized using 1.8 m*M* KP and 42.5 m*M* TEMED. Catalyst concentrations for the 5%T gel were designated as 1×, and corresponding multiples as 5× and 10× (see Table 4).

Electrophoresis

Twenty-five micrograms of enolase (1 mg/ml in upper buffer containing 8 *M* urea) was applied to each gel. PAGE was carried out at 25°C as described (*15*) at a constant current of 2 mA/tube.

Pre-electrophoresis of separation gels was carried out for 3 to 5 hr, utilizing lower gel buffer (LGB) in 8 *M* urea in both the upper and lower

TABLE 2

Conditions of Polymerization and of Extraction for the Determination of SH Oxidation by Polyacrylamide Gels

	Gel				Extraction			
Fig. 1	System	KP (m*M*)	RN (m*M*)	pH	Reag.	SH (m*M*)	MeOH (%)	Max. (hr)
A	Tris–Brewer	—	0.665	8.1	CL	60	0	30
		—	0.665	8.1	CL	60	70	30
B	Tris–Brewer	—	0.665	7.3	TG	100	20	50
C	B	2.774	—	3.0	TG	150	0	50
D	B	2.774	—	3.0	TG	150	66	3
E	B	2.774	—	8.1	TG	150	66	3

buffer reservoirs. As a control, both the separation and stacking gels were also subjected to pre-electrophoresis, using upper gel buffer (UGB) in the upper buffer reservoir (cathode) and lower buffer in the lower buffer reservoir (anode).

Various reagents, including hydroquinone, acrylamide, Tris–thioglycolate (thioglycolic acid neutralized by Tris), and dithiothreitol (each in 25% sucrose, 8 *M* urea as an anticonventive medium), were applied in a "reagent layer" above the stacking gel and below the sample. The reagent layer volume was usually 0.5 ml. In some cases, per-electrophoresis was carried out with a reagent layer.

Determination of Thiol Group Activity in Gel Slices

One milliliter of separation gel (system Tris–Brewer or B, nonurea, 10%T, 2%C) was sliced transversely at 1.3-mm intervals (*18*). Slices were suspended in solution (specified in Table 2) containing 150 μmoles or less SH in a tightly sealed vial. This solution was stirred magnetically for the first 2 hr; for longer extraction times, slices were left to diffuse.

The types of gels were also subjected to PAGE with a thioglycolate reagent layer, sliced, and extracted. The SH concentrations of the extracts were determined by the Ellman procedure (*19*), both in the presence and absence of gels.

Determination of Residual Monomer in PA

Gels prepared simultaneously and under identical conditions with those used for the determination of SH activity were sliced and analyzed for methanol-extractable amide nitrogen (residual monomer) as described elsewhere (*3*).

Determination of α-Amino N after Reaction of Glycine with Acrylamide

Glycine and acrylamide in a 1:2 and 1:100 molar ratio were allowed to react in 8 *M* urea in the operative separation gel buffer (PI phase) of the Tris–Brewer system (Table 1). The same reaction was also carried out at pH 11.0 with a glycine: acrylamide molar ratio of 1:200. At various time intervals, fractions were taken, together with controls (without acrylamide) and spotted on filter paper strips. The strips were dried, chromatographed in descent, using acetone–urea–water = 60: 0.5:40, dipped into 0.25% ninhydrin in acetone and dried for 10 min at 60°C.

Altercation of Hemoglobin

A hemolysate (provided by M. Feld, NIH) containing human hemoglobins A and S was added to the polymerization mixture of the stacking gel in 20% sucrose (system A, phase BETA) at a load of 20 μg/gel. The gel was then photopolymerized using 0.131 m*M* RN as previously described (*15*). Control samples were irradiated by nine concentric 20-W fluorescent lights (daylight) for an identical duration in the absence of RN. A further control sample was subjected to loading and PAGE in the dark under otherwise identical conditions.

RESULTS

Experiment A—Titration of Thiol Group Activity in Gels

Figure 1 shows the oxidative reaction of PA gels with sulfhydryl reagents. Polyacrylamide gels (Tris–Brewer and B systems, phase GAMMA, 10%T, 2%C) were polymerized with either 0.656 m*M* RN (cases A and B) and TEMED or KP (2.775 m*M*) and TEMED (cases C, D, and E) (other conditions for these experiments are given under Methods and in Table 2). The extractable monomer in the gels (*3*) was 1.0 m*M* when they were polymerized with RN, and approximately 0.1 m*M* when polymerized with KP. The gels (1 m*l*) were sliced and extracted in either buffer or methanol (as specified in Table 2) containing 150 μmoles or less of SH reagent (dithiothreitol or thioglycolate). Figure 1 shows that the rate of disappearance (presumably oxidation) of SH is increased in the presence of gels as compared with controls whether they were catalyzed by RN or by persulfate.

Experiment B—Establishment of Reducing Conditions in PA Gel by Electrophoretic Introduction of Thioglycolate

PAGE was carried out with a reagent layer of 0.5, 1.0, 2.0, and 5.0 μmoles Tris–thioglycolate (pH 7.20) above Tris–Brewer gels prepared as described in Experiment A. Ellman analysis was carried out on the gel slices after 90 min of stirring in 4-ml, 0.02-*M* Tris–Cl buffer, pH 8.1. Titratable SH found in the gel after electrophoresis of the "stack" (PI-LAMBDA boundary) to approximately one-half of the gel length was 0, 5.0, 4.5, and 38.5% of the amounts applied in the four cases, respectively.

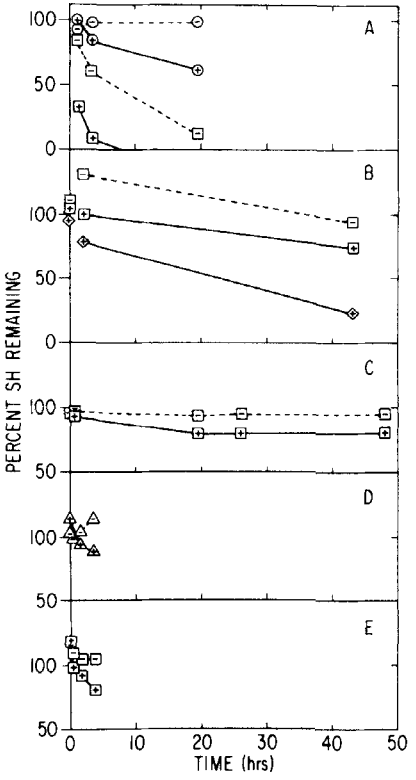


FIG. 1. Percent of free SH (dithiothreitol or thioglycolate) remaining as a function of the time of reaction with polyacrylamide gel slices (+). Polyacrylamide (10.2%T, 2%C) is polymerized by either RN (cases A, B) or KP (cases C, D, and E). The symbol (−) designates controls without polyacrylamide. In A, the circles designate extraction by methanolic buffer. Other conditions are listed in Table 2.

Experiment C—Nonequivalence of Pore Size between AP- and RN-Catalyzed Gels of the Same Concentration

The pore sizes in gels catalyzed by RN and AP were compared on the basis of the R_f's of BSA subjected to PAGE in the two types of gels in system B. Table 3 shows that the R_f values and, therefore, presumably the effective pore sizes in a gel of 10%T, 2%C made with RN are approximately the same as those of an 8%T, 2.4%C gel polymerized by

use of AP. Catalyst concentrations in both cases produced polymerization within 10 min with adequate surfaces although this does not imply equivalent percent polymerization (*3*).

Experiment D—Reaction of Glycine with Acrylamide

Glycine (3.12 m*M*) and acrylamide (70 m*M*) were prepared in the operative (PI phase) buffer, pH 9.05, of the Tris–Brewer system. The solution was permitted to react at room temperature (22°–25°C) for various times and was then spotted on filter paper, chromatographed, and stained with ninhydrin. Ninhydrin-reactive material in the reaction mixture decreased with time and became undetectable after about 600 h, whereas ninhydrin-positive material remained constant in the controls (glycine without acrylamide). When the acrylamide concentration was increased to 1500 m*M*, the ninhydrin-reactive α-amino group of glycine disappeared from the reaction mixture within 24 hr at pH 9.05 or within 30 min at pH 11.0.

Experiment E—The "Enolase Band Shift" in PAGE

Figure 2 depicts the enolase pattern in PAGE (4%T, 5%C) at separation gel buffer concentrations 1×, 2×, and 3× that of the original Tris–Brewer system. Fractionations in gels polymerized with RN and with AP were compared. In gels polymerized with AP, the dominant bands of the enolase pattern exhibit a mobility (R_f) significantly less than in gels polymerized with RN. This effect, designated the "enolase band shift," confirms Brewer's original observation (*6*) and occurs at each of the levels of ionic strength tested. The enolase band shift was demonstrated at several gel concentrations, as shown in Fig. 3 (gel patterns Nos. 1 and 8).

TABLE 3

R_f Values of BSA in System B (*15*) Obtained with Gels at Different Pore Sizes and Initiated by Either AP or RN

	R_f			
	8.2%T, 2.4%C		10.2%T, 2.0%C	
	AP	RN	AP	RN
Monomer	0.524	0.792	—	0.508
Dimer	0.305	0.625	—	0.318

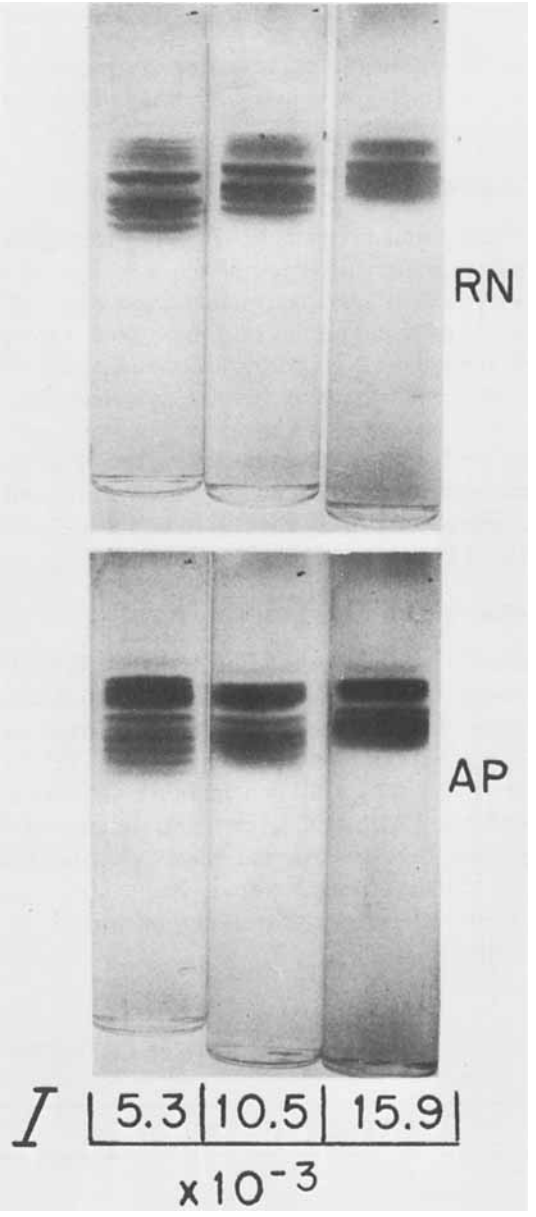


FIG. 2. PAGE (system Tris–Brewer, 4.2%T, 4.8%C) fractionation of enolase in either RN- or persulfate (AP)-initiated gels. The ionic strength of the separation gel buffer is varied from 0.0053 (left) to 0.0105 (center) to 0.159 (right).

Experiment F—Effect of Acrylamide Monomer on the Enolase Band Shift

The enolase band shift was observed originally in relatively efficiently polymerized gels (initiated by AP) but not in gels (initiated by RN and light) exhibiting a relatively open pore size as demonstrated in our ex-

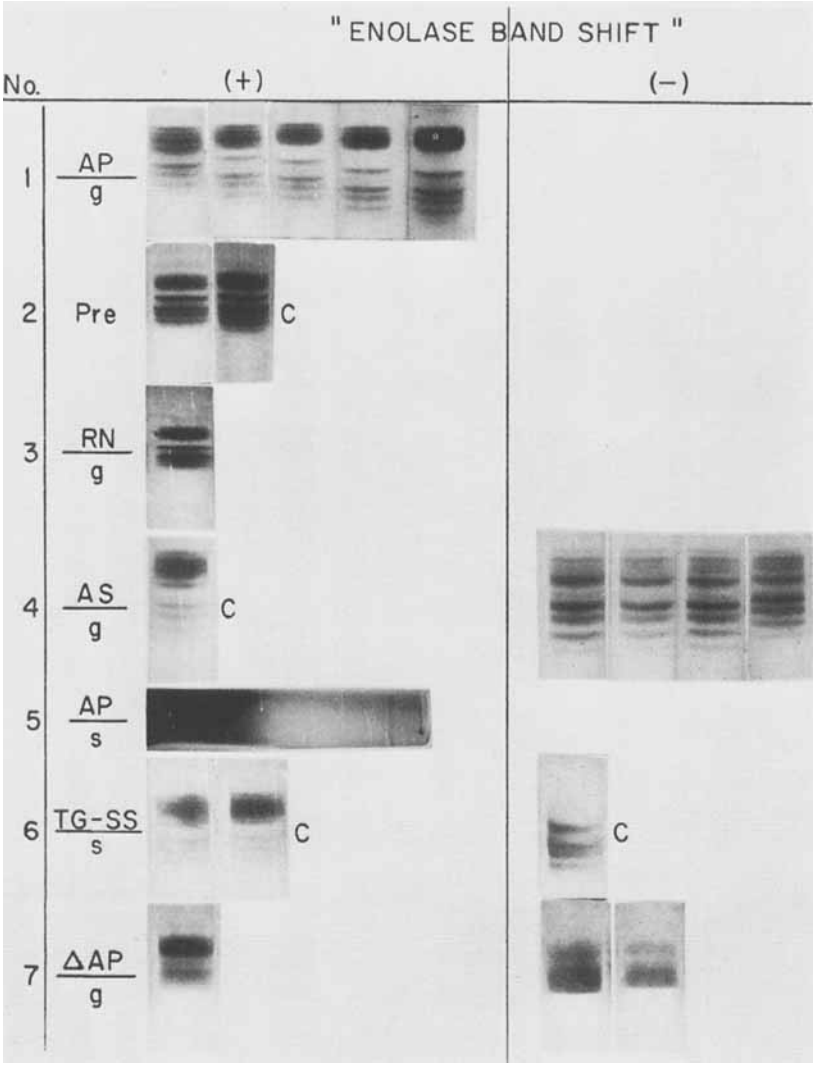
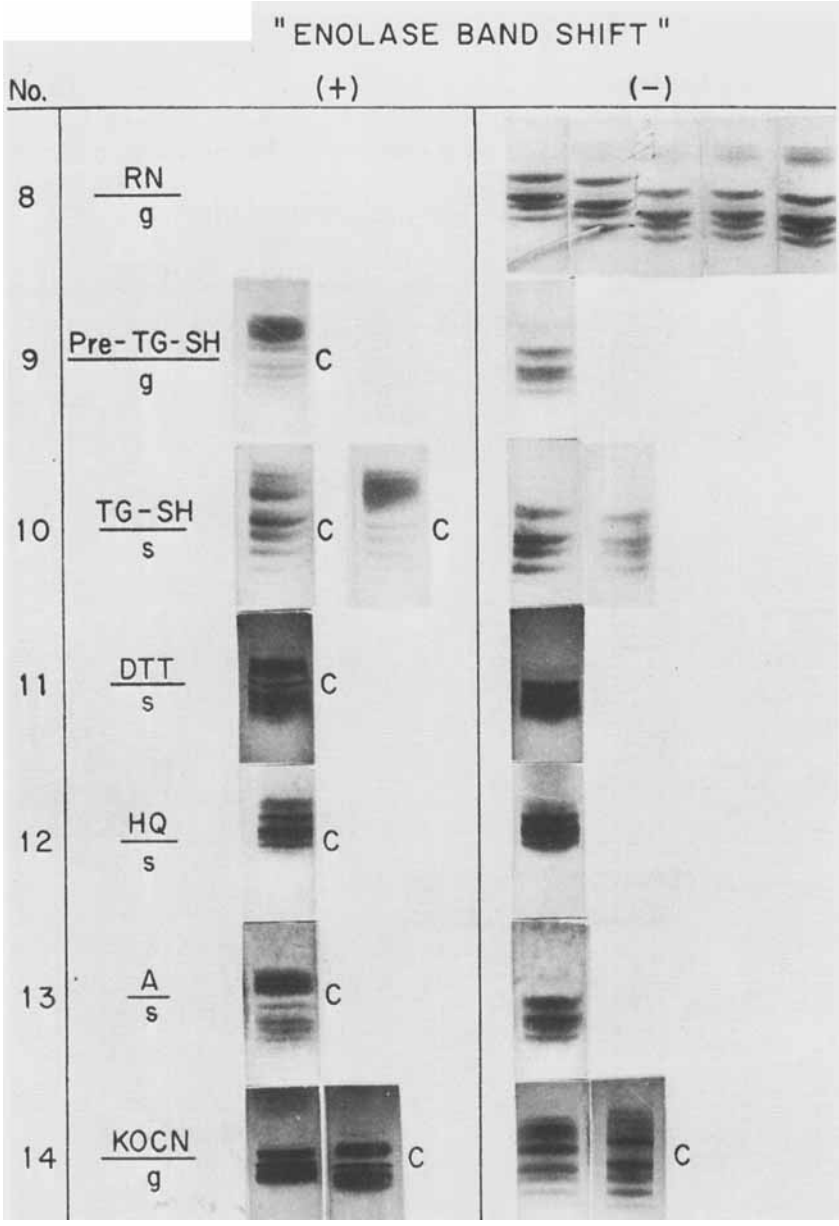


FIG. 3. See page 759 for the legend.



periments (Experiment C and Table 3). Thus, the RN-polymerized gels presumably contain relatively high concentrations of residual acrylamide monomer (*3*). Accordingly, we tested the hypothesis that acrylamide monomer might prevent the enolase band shift. Enolase was subjected to PAGE (Tris–Brewer system, 4.2%T, 4.75%C) within minutes after being mixed with 500 m*M* acrylamide and 8 *M* urea. Figure 3, No. 13, clearly shows that addition of acrylamide to enolase prior to PAGE abolished the enolase band shift. Controls with sucrose and 8 *M* urea alone had no effect on the enolase pattern.

FIG. 3. PAGE (system Tris–Brewer) fractionation of enolase under fourteen different conditions. (The fractionations for conditions 1–7 are on p. 757; for 8–14 on p. 758.) Reagents added to the enzyme sample (s) or to the separation gel (g) or pre-electrophoresis of the separation gel (Pre) are indicated in the figure. Controls are designated C. All gels contain 8 *M* urea and residual acrylamide monomer. Symbols "plus" and "minus" designate an enolase band shift from faster to slower migrating species (+) or the absence of such a shift (−). (No. 1) AP initiated gels (3.6 μmoles AP/gel); from left to right—3.2, 4.2, 5.2, 6.2, 7.2%T, 0.2% Bis. (No. 2) Pre-electrophoresis of gels containing 3.6 μmoles AP/gel, 5.2%T, 3.8%C. Right—not pre-electrophoresed gel of the same composition. (No. 3) RN-initiated gels, 2.5 μmoles RN/gel, 6.2%T, 3.2%C. (No. 4) Ammonium sulfate—gels containing 0, 1, 5, and 10 μmoles of sulfate are shown from left to right in the (−) column (3.2%T, 6.25%C, RN-initiated gels.) Left (+) column—same gel, except initiated with 3.6 μmoles AP/gel. (No. 5) Enolase (25 μg) reacted with 61 μmoles AP prior to PAGE (5.2%T, 3.8%C gel, 3.6 μmoles AP/gel). (No. 6) Reagent layer of oxidized thioglycolate. Left—gel containing 3.6 μmoles AP; center—control without oxidized thioglycolate; right—control with reduced thioglycolate. (No. 7) 3.6, 0.72, and 0.36 μmoles AP/gel (left to right). (No. 8) RN-initiated gel; 2.5 μmoles RN/gel. From left to right— 3.2, 4.2, 5.2, 6.2, 7.2%T, 0.2% Bis. (No. 9) Pre-electrophoresis with Tris–thioglycolate; 3.6 μmoles AP/gel. Left—control containing no thioglycolate. (No. 10) Thioglycolate; 0.7 μmoles Tris–thioglycolate reacted with enolase (25 μg) prior to PAGE. Gels initiated by RN or AP, 3.2%T, 6.25%C. From left to right—control of RN gel without thioglycolate; control of AP gel without thioglycolate; RN gel (2.5 μmoles RN/gel) with thioglycolate; AP gel (3.6 μmoles AP/gel) with thioglycolate. (No. 11) Dithiothreitol; 0.2 μmoles dithiothreitol reacted with enolase (25 μg) prior to PAGE; gels of 5.2%T, 3.8%C. Left—control without dithiothreitol. (No. 12) Hydroquinone; reagent layer of 2 μmoles hydroquinone/gel (enolase load 25 μg); AP-initiated gel of 5.2%T, 3.8%C. (No. 13) Enolase (25 μg) in 0.5 *M* acrylamide, AP-initiated gel, 4.2%T, 4.8%C. Left—control without acrylamide. (No. 14) From left to right—gel 0.1 *M* in KOCN, 6.2%T, 3.2%C, RN-initiated; same gel without KOCN; gel 0.01 *M* in KOCN, 3.2%T, 6.2%C, RN-initiated; same gel without KOCN.

Experiment G—Effect of RN on the Enolase Band Shift

Although the enolase band shift originally observed with AP-polymerized gels was not seen in all RN-polymerized gels in the present study, e.g., Fig. 3, No. 8, this effect can occur in RN-initiated gels (Fig. 3, No. 3) in the absence of persulfate. The enolase band shift in RN-polymerized gels was observed most frequently, but not exclusively, when the urea in the gels was freshly deionized.

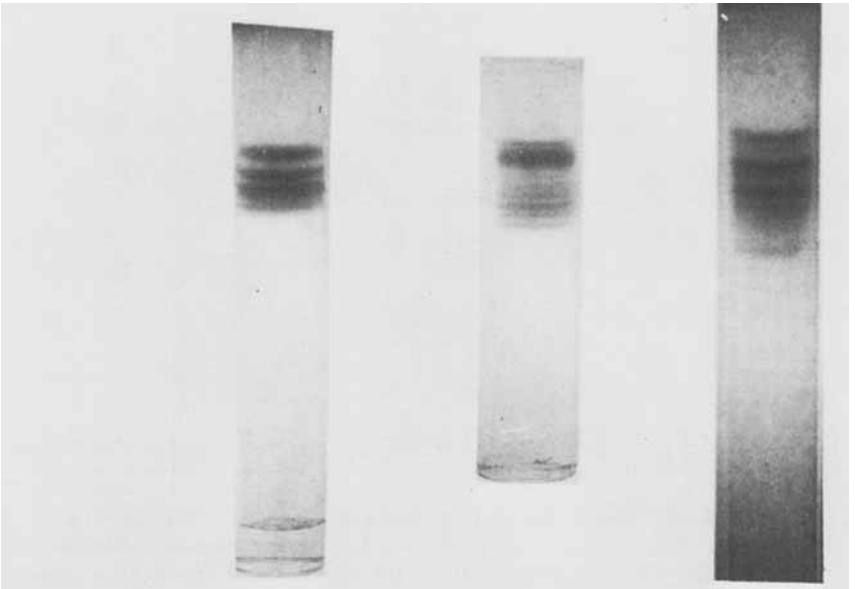
Experiment H—Effect of Urea on the Enolase Band Shift

Figure 4 (center and right) shows that a nonurea Tris-Brewer system does not show the enolase band shift even when the gels have been polymerized with persulfate. The difference in pattern between urea- and nonurea Tris–Brewer systems suggests that urea is involved in the genesis of the enolase band shift. Since urea seemed necessary for the enolase band shift the question arose whether cyanate binding (*20*, *21*) to the protein was involved. Figure 5 and Fig. 3, No. 14, show that with increasing cyanate concentration in the gel, the bands of the enolase pattern appear increasingly condensed. At 0.05 *M* KOCN, this concentration of bands produces a pattern with a predominant slow band such as seen in the enolase band shift. However, a shift of intensity from faster to slower migrating components as associated with the enolase band shift cannot be observed as a function of KOCN concentration.

Experiment I—Effect of pH, Stacking Limits, and Ionic Strength on the Enolase Band Shift

a. Urea gels in system A do not show the enolase band shift, in contrast to those in the Tris–Brewer system (Fig. 4, center and left). The difference in pattern (Fig. 4, center and left) between the urea gels in these two systems suggests an effect of pH (pH (GAMMA) 7.80 vs 8.84; pH (PI) 9.05 vs 9.45], ionic strength (0.0053 vs 0.0158), or the fact that the Tris–Brewer system has a smaller unstacking limit [RM (1,9)] than system A (0.122 vs 0.244, respectively). If the enolase band shift is analogous to the interferon alteration at low pH (*7*), it could conceivably depend on the lowering of pH (GAMMA) by 1 pH unit.

Both the low ionic strength and the low RM(1,9) might contribute to high R_f values and, therefore, to the resolution of the faster (active) from slower (inactive) enolase components.



System	A	Tris-Brewer	
pH (PI)	9.45	9.05	
RM (1,9)	−0.244	−0.122	
Initiator	AP	AP	
% T	5.2	5.2	10.2
Urea (M)	8	8	0

FIG. 4. PAGE [system Tris–Brewer and system A (*15*)] fractionation of enolase in urea- and nonurea gels. The operative pH of the separation gels is designated as pH (PI) (*1, 16*). The RM(1,9) is the constituent mobility of the trailing ion, relative to the mobility of sodium, in the operative separation phase: it is also designated as the "unstacking limit" (*1, 16*) since ions with constituent mobilities less than the value of RM(1,9) are unstacked. Gel concentration is designated as %T.

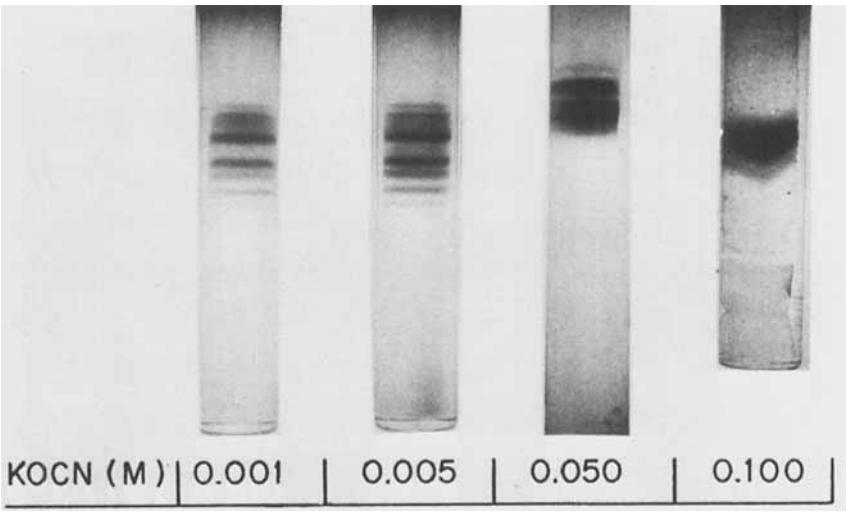


FIG. 5. PAGE (system Tris–Brewer, RN-initiated, 3.2%T, 6.25%C) fractionation of enolase in separation gels containing various amounts of KOCN. An additional concentration is depicted in Fig. 2, No. 14.

b. Figure 6 shows that enolase is stacked in nonurea upper gels (Tris–Brewer, 3.125%T, 20%C) but not in upper gels containing 8 *M* urea. Figure 6 also shows that the operational stacking phase (ZETA phase) resolves in the region of the "AP band" two slowly migrating bands that are not seen in the operative separation phase (phase PI). The density of bands in the region of the AP band is less in the RN-polymerized upper gel (ZETA phase) than in AP-polymerized separation gels (PI phase).

Unstacking in urea gels may be caused by unfolding of the enolase molecule in urea and lowering of its constituent mobility; or, it may be due to the same molecular alteration in urea gels that is responsible for the lowering of the migration velocity of enolase in the enolase band shift.

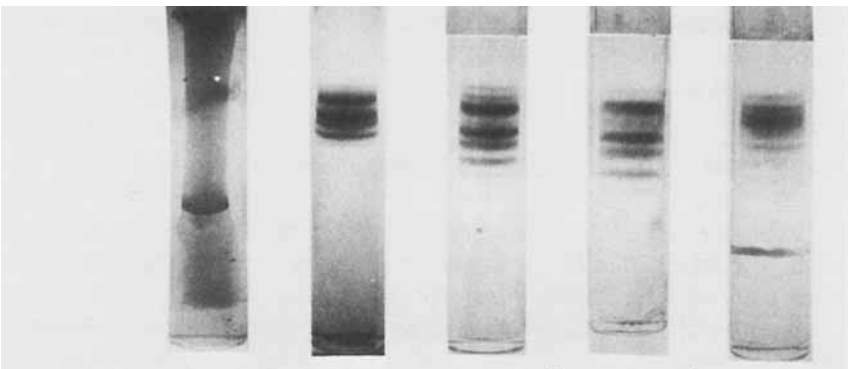
Experiment J—Absence of an Effect of Pre-electrophoresis on the Enolase Band Shift

Figure 3, No. 2, shows that pre-electrophoresis of the separation gel (Tris–Brewer system, 3.6 μmoles AP/gel, 5.2%T, 3.9%C) for 2 hr at

4 mA/tube, does not prevent the enolase band shift. (*Note.* In this experiment, all persulfate should have been removed from the gel before entry of the protein.)

Experiment K—Effect of Concentration of Persulfate and of Sulfate on the Enolase Band Shift

Persulfate may exert its effect on enolase as a free radical donor, in common with photosensitized RN. It may also alter the ionic strength in two different ways: first, through covalent binding of sulfate to the chain termini of polyacrylamide (*22*) and second, by increase of sulfate and/or ammonium ion concentration. These two possible mechanisms were studied with the results shown in Fig. 3, Nos. 4 and 7.



% T	3.125	3.125	2.2	2.2	2.2
% C	20	20	9	9	9
8M urea	−	+	+	+	+
Initiator	RN	RN	RN	RN	AP
Phase	ZETA	ZETA	ZETA	PI	PI

FIG. 6. PAGE (Tris–Brewer system) fractionation of enolase in stacking gels (phase ZETA) (*1*, *16*) or separation gels (phase PI) (*1*, *16*) as a function of the choice of initiator (RN or AP) and the presence of 8 *M* urea.

Figure 3, No. 7, shows that gels (Tris–Brewer, 5.2%T, 3.9%C) polymerized with 3.6, 0.72, and 0.36 μmoles AP/ml (and 2, 1, 1 μl TEMED/ml gel, respectively) exhibit the enolase band shift in proportion to AP concentration. This confirms Fig. 1 of Ref. *6*.

Figure 3, No. 4, shows the fractionation of enolase (Tris–Brewer system, separation gel only, 3.2%T, 6.3%C) in RN-polymerized gels containing 0.001, 0.005, and 0.01 *M* ammonium sulfate. The presence of ammonium sulfate at these concentrations (which are one to ten-fold higher than the AP concentration used as a control) does not give rise to a significant increase in band intensity in the region of AP bands. This rules out the possibility that AP may affect the enolase pattern by simple alteration of ionic strength. In view of the high molar ratio of persulfate to protein at all three AP concentrations used, it is unlikely that a direct free radical reaction of persulfate with the protein would be dependent on a tenfold variation of AP concentration. But if the reaction is mediated by acrylamide monomer or urea, which are present in very high concentrations, a tenfold change in AP concentration could be rate-limiting in producing chain sulfonylation or a free radical product of urea.

Experiment L—Direct Incubation of Persulfate with Enolase

Enolase (1.06 mg) was incubated at 25°C with 13.9 mg AP in 0.5 ml 8 *M* urea; then 50-μl fractions were withdrawn at various time intervals and subjected to PAGE (Tris–Brewer, 5.2%T, 3.9%C). The enolase pattern resulting from incubation in this concentration of persulfate does not simulate the enolase band shift although a drastic change in pattern suggestive of destruction is observed (Fig. 3, No. 5). For all time intervals investigated, the patterns were identical to the one shown.

Experiment M—Effect of Reducing Agents on the Enolase Band Shift

Thioglycolate, hydroquinone, and dithiothreitol reverse or abolish the enolase band shift when they are allowed to react with enolase prior to electrophoretic fractionation. Figure 3, No. 9, shows that pre-electrophoresis of the gel with thioglycolate prior to application of the sample has the same effect. Thioglycolate is equally effective when admixed to the sample (Fig. 3, No. 10) or when applied as a reagent

layer under the sample (*6*). However, to verify that this represented a direct effect of the reducing agent on the protein, rather than on the gel, thioglycolate was replaced by a reducing agent that was un-ionized at the operative pH of 9.05. Figure 3, Nos. 11 and 12, show that the characteristic enolase band shift in AP gels is abolished by reaction of enolase with either dithiothreitol or hydroquinone prior to PAGE. Since these agents do not move electrophoretically into the gel, this suggests a direct effect of the reducing agents on the protein.

Experiment N—Molecular Size of Shifted Enolase Components

Figure 7 shows a Ferguson plot (*15*) of log R_f vs gel concentration for the enolase bands in the Tris–Brewer system vs gel concentration (constant 0.2% Bis; 3, 4, 5, 6% acrylamide). The relatively slowly moving bands associated with the enolase band shift exhibited by RN as well as by persulfate-initiated gels appear indistinguishable. Their retardation coefficient (K_R) (*15*) is essentially the same as that for the more rapidly moving (presumably "native") enolase components. Thus, the enolase band shift is not associated with an appreciable change in the effective size of the molecule—aggregation, dimerization, and/or fragmentation can be excluded as explanations for this effect.

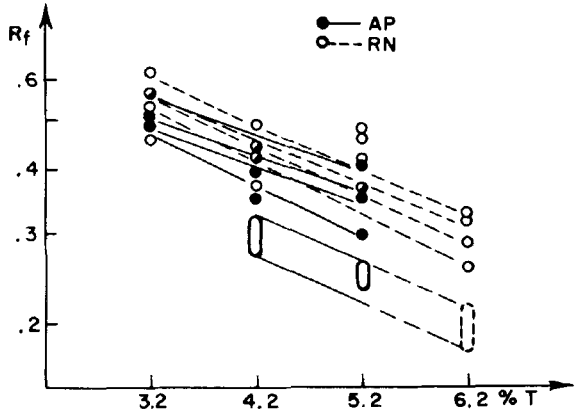


FIG. 7. R_f values of enolase in gels of various %T initiated by either RN (○) or AP (●) or both (◑), plotted vs %T. The degree of cross-linking at 3.2, 4.2, 5.2, and 6.2%T was 6.25, 4.75, 3.84, and 3.22%C, respectively.

Experiment O—Effect of Photosensitized RN and of Illumination on Hemoglobin

The demonstration of damage to enolase by direct incubation with persulfate (Fig. 3, No. 5, and Experiment L) is analogous to the finding of damage to hemoglobin by RN under illumination reported by Pastewka et al. (*13*). These authors also claimed a relatively minor effect by light even in the absence of RN. This prompted us to repeat the experiment. Hemoglobin was irradiated under the routine conditions of the photopolymerization of acrylamide (*15*) in the presence and absence of RN in sucrose solution. Electrophoresis of these two samples was carried out either under illumination or in the dark. Table 4 shows that the characteristic R_f values of hemoglobins A and S are not changed under any of these conditions except when the sample was irradiated in the presence of RN. Increasing the persulfate and TEMED concentrations is also without effect on R_f values under these conditions (Table 4). Thus, in the absence of RN, illumination does not damage the hemoglobin molecule under the conditions used here.

DISCUSSION

Since Brewer's claim that residual persulfate in PA gels damages enolase (*6*) and produces artifactual bands by an oxidative mechanism, the use of PAGE as a fractionation tool for proteins has been held sus-

TABLE 4

R_f Values of Hemoglobins A and S (System A, 5%T, 3%C) Illuminated in the Presence and Absence of RN

RN present during illumination prior to PAGE	PAGE		Relative concentration		R_f	
	Light	Dark	KP	TEMED	Hb (A)	Hb (S)
−	+		1	1	0.622	0.517
		+			0.616	0.515
	+		1	10	0.603	0.500
		+			0.603	0.506
	+		5	1	0.623	0.516
		+			0.633	0.515
+	+		1	1	Smear	
		+			Smear	

pect. However, the hypothesis that proteins are directly oxidized by persulfate invited challenge, since persulfate has a much higher mobility than proteins, and accordingly should be swept out of the gel in front of proteins, as confirmed by benzidine staining of gels (*11*). Also, a direct effect of persulfate on protein seemed unlikely since despite its high thermodynamic oxidation potential, persulfate is "inert" due to the slow kinetics of its oxidative reactions (*12*). This left an alternative hypothesis that persulfate could exert an indirect oxidizing effect on proteins through secondary reactants produced by its reaction with other components of the polymerization mixture (acrylamide monomer, polymer, buffer constituents, or solvents). In fact, a stable free radical has been implicated in the mechanism of action of persulfate and considered responsible for the hydrolysis of proteins (*9*).

We have systematically reinvestigated the enolase band shift as a function of the presence of acrylamide monomer, of gel buffer (pH, ionic strength, stacking limits), choice and concentration of initiator (persulfate, riboflavin), solvent (urea, water), ionic components (cyanate, ammonium sulfate), and reducing agents (dithiothreitol, thioglycolate, hydroquinone) with and without pre-electrophoresis. This has led us to perform preliminary studies designed to determine, at least semiquantitatively, the REDOX conditions within polyacrylamide gels. Although neither the genesis of the enolase band shift nor the REDOX conditions within PA gels have been fully elucidated, several findings are relevant to protein fractionation by PAGE.

1. Initiation of the polymerization of acrylamide with either RN under illumination or persulfate results in gels that can oxidize thiol groups (and presumably most proteins). This was demonstrated by the back-titration of an excess of Cleland's reagent in which gel slices were suspended and equilibrated (Experiment A). Brewer (*6*) was, therefore, correct in postulating that persulfate-initiated gels are oxidative (see Experiment G). However, Brewer's inference that RN-initiated gels are less oxidative or nonoxidative appears overly optimistic—it appears that all gels made from vinyl monomers have to be considered potentially capable of producing artifact bands by free radical reactions with protein. Nonetheless, no unequivocal demonstration of oxidative damage of protein during PAGE has been made in the sense that the oxidizing agent reacting with the protein and the nature of the alteration on the protein remain unknown. This conclusion applies to PAGE in persulfate or illuminated RN-catalyzed gels alike. In contrast to alterations of

protein during PAGE, the free radical damage of protein during photopolymerization of a "sample gel" (*10*) can be avoided simply by use of procedures (e.g., Ref. *15*) that omit such a gel.

2. A practical remedy for the oxidative nature of polyacrylamide gel is available. Reducing conditions may be established within polyacrylamide gels by pre-electrophoresis with a charged reducing agent (e.g., thioglycolate), as evidenced by the appearance of titratable SH in the diffusate of gel slices after such pre-electrophoresis. This confirms a previous suggestion by Brewer (*6*).

3. Polyacrylamide gels may contain 0.5–40% unpolymerized acrylamide monomer, depending on polymerization conditions (*3*). This monomer can react with α-amino, sulfhydryl, and phenolic hydroxyl groups at elevated pH (*23*, *24*). In this study it was found to react slowly at pH 9 with the α-amino group of glycine. The effect of acrylamide in preventing the enolase band shift also suggests a reactivity with enolase and possibly proteins in general. Since PAGE is most commonly carried out at pH 9 or above (*10*, *25*), a reaction of proteins with acrylamide might be of general importance as a source of artifacts in PAGE.

4. The shift in the enolase band pattern which was associated with inactivation of the enzyme (*6*) previously attributed to persulfate damage (*6*) is also seen in some RN-catalyzed gels, in the complete absence of persulfate. At the relatively high concentration of persulfate tested, the specific band pattern shift cannot be discerned after direct incubation of enolase with persulfate, although under these conditions a drastic pattern alteration indicative of destruction occurs. This evidence alone is still compatible with the idea that the enolase band shift is due to oxidation by some free radical reagent or reaction product generated in the gel–urea system. An oxidative mechanism for the shift is also supported by its reversibility with thioglycolate, dithiothreitol, hydroquinone (but not oxidized thioglycolate) as originally pointed out by Brewer (*6*).

The hypothesis of free radical damage to enolase is also compatible with the fact that acrylamide, a free radical receptor, is able to protect enolase from alteration. However, if the enolase band shift is simply due to free radical donors, why is it not seen in the absence of urea, and why does the degree of purity of the urea seem to affect the frequency with which the shift is observed in RN-polymerized gels? Possibly, a free radical species derived from urea could be involved.

Brewer suggested that the enolase band shift was due to a gel compo-

nent, persulfate, and implied that the reversal of this effect was mediated by the gel, after the gel had been reduced by thioglycolate. However, the finding that un-ionized acrylamide, dithiothreitol, and hydroquinone (which is also essentially un-ionized at pH 9.05), can prevent the shift would imply that enolase reacts before it enters the gel. One apparent (though unlikely) counterhypothesis would be to assume a reaction of the protein, during the 1 to 2 min prior to initiation of electrophoresis, with photosensitized RN or a secondary reactant at the upper surface of the nonurea stacking gel. But this leaves the urea requirement for the band shift unexplained. Another hypothesis would be to assume that the reactive free radical donor (derived from urea ?) is positively charged under the conditions used and, therefore, brought in contact with enolase during electrophoresis.

In summary, we conclude that the enolase band shift is due, at least in part, to a reaction of enolase occurring prior to its electrophoretic entry into the gel. This conclusion is also consistent with the facts that (a) direct incubation with a high concentration of persulfate does not generate a typical enolase band shift, (b) pre-electrophoresis is ineffectual, and (c) persulfate migrates ahead of all proteins in all anionic buffer systems in gels. Although the principal species that reacts with enolase to produce the shift cannot be identified it appears to involve urea. Carbamylation (*20, 21*) appears to be ruled out since the enolase pattern after addition of KOCN to the gel (Fig. 3, No. 14) does not simulate the patterns obtained in the presence of persulfate (Fig. 3, No. 1).

Since the slow (inactive) enolase bands and the fast (active) bands exhibit parallel Ferguson plots (this is an approximation since these data are limited to a narrow range of gel concentration and %C is not held constant), it is very unlikely that the active and inactive enolase species could vary markedly in molecular size or conformation. Rather, these two species most likely differ in net charge. Conceivably, a free radical reactant could contribute a positive charge to the inactive, more slowly migrating enolase species. The protective effect exerted by uncharged acrylamide or reducing agents (both free radical acceptors) prior to the entrance of enolase into the gel may be due to reaction with a cationic free radical donor (other than TEMED) that has migrated into the sample phase. It is conceivable that the urea molecule in the presence of persulfate or RN (with illumination), TEMED, and acrylamide monomer provides a free radical donor species that can react with protein, possibly at the tyrosyl groups. This hypothesis is presently under investigation.

NOMENCLATURE

ABS	absolute value of
ALPHA	upper buffer phase prior to electrophoresis
BETA	stacking phase prior to electrophoresis
BV	buffer value
C1	constitient concentration of trailing ion of the stack
C2	constituent concentration of leading ion of the stack
C3	constituent concentration of ion in the separation phase prior to electrophoresis
C6	constituent concentration of common ion of the system
C7	constituent concentration of ion in the restacking phase
CONSTITUENT 1	trailing ion of the stack
CONSTITUENT 2	leading ion of the stack
CONSTITUENT 3	ion of the separation phase prior to electrophoresis
CONSTITUENT 4	ion of elution buffer phase
CONSTITUENT 5	ion of lower buffer phase
CONSTITUENT 6	counterion common to all phases
CT7	ion in the upper buffer for restacking
GAMMA	separation phase prior to electrophoresis
IS, I, or ION STR.	ionic strength
KAPPA	specific conductance (μmhos/cm)
LAMBDA (8)	separation phase after migration of the leading ion (C2) of the stack into the GAMMA phase
MAX	maximal value of
NO.	number
NU	boundary displacement (cm^3/Fd)
PHASE 2	stacking phase prior to electrophoresis
PHASE 3	separation phase prior to electrophoresis
PHASE 4	operative stacking phase; also the arbitrary composition of the upper buffer
PHASE 9	operative separation phase
Phase BETA (2)	stacking phase prior to electrophoresis
Phase GAMMA (3)	separation phase prior to electrophoresis

Phase LAMBDA (8)	separation phase after migration of the leading ion of the stack into phase (3)
Phase PI (9)	operative separation phase
Phase PSI (5)	operative restacking phase
Phase TAU (6)	restacking phase prior to electrophoresis
Phase ZETA (4)	operative stacking phase
PHI (1)	ratio of ionized to un-ionized CONSTITUENT 1
PHI (2)	ratio of ionized to un-ionized CONSTITUENT 2
PHI (3)	ratio of ionized to un-ionized CONSTITUENT 3
PHI (6)	ratio of ionized to un-ionized CONSTITUENT 6
PI(9)	operative separation phase
RM (1)	mobility of constituent 1 relative to Na^+
RM (2)	mobility of constituent 2 relative to Na^+
RM (3)	mobility of constituent 3 relative to Na^+
RM (6)	mobility of constituent 6 relative to Na^+
RM (1,4)	relative mobility of constituent 1 in phase (4) (Lower Stacking Limit)
RM (2,2)	relative mobility of constituent 2 in phase (2) (Upper Stacking Limit)
RM (1,9)	relative mobility of constituent 1 in phase (9) (Unstacking Limit)
Separation gel (lower gel)	gel made in PHASE 3, operative in PHASE 9
Stacking gel (upper gel)	gel made in PHASE 2, operative in PHASE 4
SIGMA	relative conductance (C/cm^3)
THETA	ratio of constituents within one phase
ZETA (4)	operative stacking phase

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