Gel Electrophoretic Analysis of Peanut Proteins and Enzymes. II. Effects of Thiol Reagents and Frozen Storage

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Peanut proteins and enzymes solubilized in the presence of high concentrations of dithiothreitol (DTT) or β-mercaptoethanol (β-ME), then examined by polyacrylamide or starch gel electrophoresis, showed significant changes in their mobilities and activities, respectively, when compared to those of control extracts (without thiol-reducing compounds). Frozen storage altered the solubility characteristics and electrophoretic patterns of these proteins, especially when the samples contained high concentrations of a reducing reagent. These changes were quite noticeable with protein fractions separated from arachin and with enzymes after DEAE-cellulose chromatography. The results indicate that thiol-reducing compounds and/or frozen storage promote many *in vitro* changes (*e.g.*, molecular weight, conformational, structural, steric hindrance, and functional properties) in extracted peanut proteins and enzymes.

Reactive thiol groups have several roles in proteins; *e.g.*, they help to maintain protein conformation and stability through disulfide bridges or through properly located sulfhydryl groups hydrogen-bonded to oxygen or nitrogen of hydroxyamino or imino groups; they provide binding sites for cofactors and acyl groups necessary for some proteins to attain enzymatic activity; or they participate directly in the catalytic reactions of functional proteins, such as sulfhydryl enzymes.

Reagents such as DTT and β-ME have been extensively used in extractions of proteins and enzymes to prevent oxidation of sulfhydryl groups, decreasing the amount of protein-protein interaction (Anderson, 1968; Baldry *et al.*, 1970; Fukushima and Van Buren, 1970; Gelderman and Peacock, 1965; Hashizuma *et al.*, 1971; Jones and Carnegie, 1971; Klotz *et al.*, 1958; Tucker and Fairbrothers, 1970; Zondag, 1963). However, disulfide bridges which maintain conformational and structural integrity of proteins can sometimes be reduced by these compounds (Shiprin *et al.*, 1970; Sondack and Light, 1971; Stankewicz *et al.*, 1971; Thompson and O'Donnell, 1961; Wall, 1971; Wolf *et al.*, 1962).

In the previous paper, peanut extracts were shown to consist of heterogeneous mixtures of proteins and enzymes which could be chromatographically separated, grouped into identifiable classes, and characterized by gel electrophoretic techniques (Cherry *et al.*, 1973). The purpose of the present investigation was to examine the effects of thiol-reducing reagents and frozen storage on the electrophoretic patterns of the proteins and certain enzymes in these groups.

MATERIALS AND METHODS

Peanuts used in preparations of the total extract and individual fractions of proteins were described by Dechary *et al.* (1961) and Cherry *et al.* (1973). Protein and enzyme preparations were dissolved in phosphate buffer, pH 7.9, I = 0.01, with and without 20 m*M*, 0.25 *M* β-ME or 10 m*M*, 0.125 *M* DTT, and examined at three concentrations by polyacrylamide disk and starch gel electrophoretic techniques (Cherry *et al.*, 1973). Dialyses to remove the thiol reagent from treated samples were performed against the same phosphate buffer for 48 hr at 4°. Frozen samples were stored overnight or for 2 to 7 days at −20°.

RESULTS AND DISCUSSION

In many electrophoretic analyses of seed proteins, several treatments have been employed to cleave disulfide or thiol-hydrogen bonds; *e.g.*, urea, detergents, and/or thiol-reducing reagents (Holden *et al.*, 1971; Klotz *et al.*, 1958; Koshiyama, 1970; Warren and Gordon, 1971). Urea and detergents can dissociate many different types of bonds as well as protein–lipid (or carbohydrate) interactions, whereas thiol reagents are more specific for (–SH) associated linkages (Anderson, 1968; Sondack and Light, 1971; Stankewicz, 1971; Wall, 1971). Since the purpose of this investigation was to confine the induced changes as much as possible to sulfur-containing linkages, total protein extracts from peanuts and fractions separated by DEAE-cellulose chromatography were treated with β-ME or DTT only, and/or frozen storage, and then examined for molecular changes by gel electrophoretic techniques (Ornstein, 1964; Peterson, 1971).

The fractions of proteins and enzymes analyzed were described in the previous paper (Cherry *et al.*, 1973). These include the total cotyledonary extract (sodium chloride or phosphate buffer-soluble proteins), Fraction I + II + III (conarachin), Fraction I (albumins), Fraction II + III (conarachin less albumins but including smaller globulins and α-conarachin), Fraction III (α-conarachin), Fraction IV (arachin), and Fraction VI (arachin minus an arachin contaminant). The enzymes selected include esterase, peroxidase, catalase, leucine aminopeptidase, acid phosphatase, alcohol dehydrogenase, and INT-oxidase. Since the effects of β-ME and DTT on peanut proteins and enzymes were similar, both compounds are discussed interchangeably.

Total Protein Extract. Proteins extracted with and without low concentrations of thiol-reducing reagent and then separated electrophoretically before and after frozen storage produced the same banding pattern (Figure 1A). With high concentrations of thiol reagent, however, these proteins showed a collective increase in electrophoretic mobility from region 0–2.5 cm 2.5–4.0 cm (Figure 1B). In addition, the latter samples contained more distinct bands at 4.5 to 7.0 cm than extracts containing little or no sulfhydryl reagent (Figure 1, compare B to A). Whether this is due to high concentrations of thiol reagents releasing subunits from the large molecular weight globulins and/or exposing more negatively charged proteins can not be confirmed by gel electrophoresis alone.

After frozen storage some protein precipitated in the samples containing little or no thiol reagent, whereas in the presence of high concentrations of these compounds large amounts of precipitate were formed. Electrophoresis of precipitates from samples containing either little (Figure 1F) or no (Figure 1D) thiol reagent showed mainly quantitative differences compared to extracts of cryoprecipitated arachin (Figure 1E), prepared as described by Neucere (1969). Precipitates from samples with high con-

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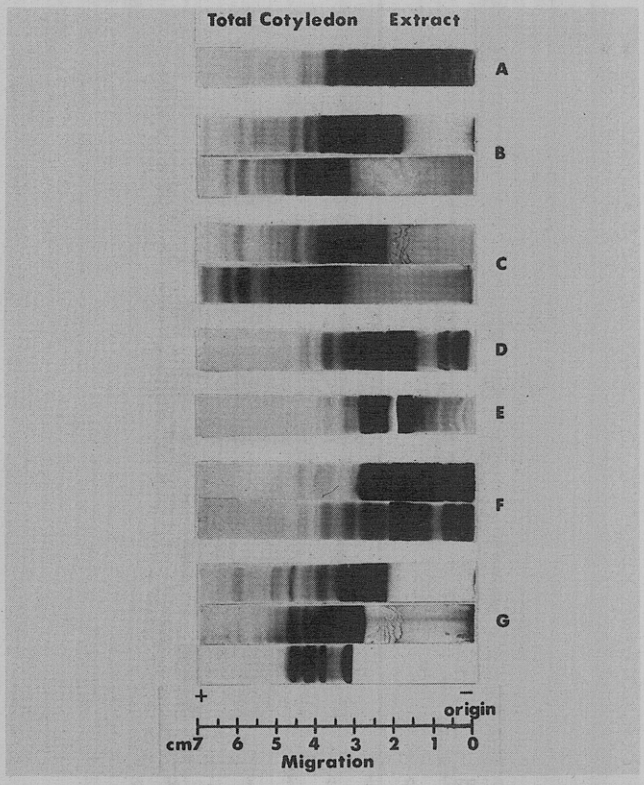

Figure 1. Typical disk gel electrophoretic patterns of samples containing cotyledonary proteins treated (T) and not treated (NT) with high and low concentrations of thiol-reducing reagents (20 m*M*, 0.25 *M* β-ME, or 10 m*M*, 0.125 *M* DTT) and/or frozen storage (FS). A, D, E, F: proteins soluble after NT or T (20 m*M* β-ME, or 10 m*M* DTT) before and after FS, NT proteins of A precipitated after FS, a cryoprecipitate fraction, and T proteins of A precipitated after FS, respectively. B, C, G: proteins soluble after T (0.25 *M* β-ME, or 0.125 *M* DTT), proteins of B soluble after FS, and proteins of B precipitated after FS, respectively.

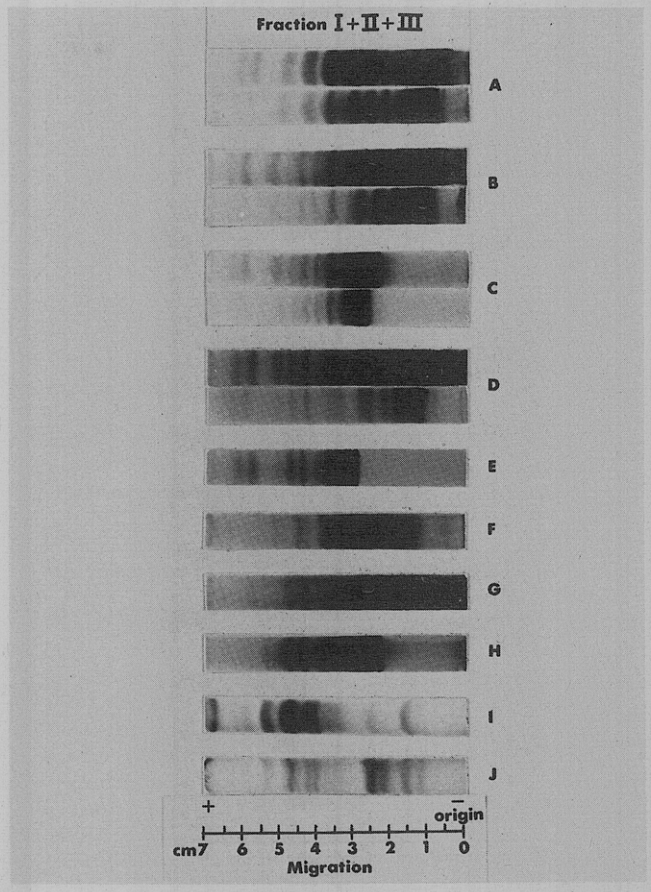

Figure 2. Typical disk gel electrophoretic patterns of fraction I + II + III proteins treated (T) and not treated (NT) with thiol-reducing reagents (20 m*M*, 0.25 *M* β-ME, or 10 m*M*, 0.125 *M* DTT) and/or frozen storage (FS). A, F: proteins soluble after NT before and after FS, and proteins from A precipitated after FS, respectively. B, D, G, I: proteins soluble after T (20 m*M* β-ME, or 10 m*M* DTT), proteins of B soluble after FS, proteins of B precipitated by FS, and proteins of B after dialysis, respectively. C, E, H, J: proteins soluble after T (0.25 *M* β-ME, or 0.125 *M* DTT), proteins of C soluble after FS, proteins of C precipitated by FS, and proteins of C after dialysis, respectively.

centrations of thiol reagents shifted in electrophoretic mobility, as did also the supernatant fractions before and after frozen storage (Figure 1, compare B to C and G). However, gel patterns of the supernatant fractions contained more low molecular weight, negatively-charged proteins in region 5.0–7.0 cm than did the precipitate (compare Figure 1, B, C, and G), especially after frozen storage (Figure 1C). In contrast, the precipitated protein produced distinct bands in region 3.0–5.0 cm (Figure 1G). The use of high concentrations of thiol-reducing reagents and/or frozen storage of proteins in solution induce similar changes in the proteins. The use of these techniques for fractionating specific groups of peanut proteins during the extraction of isolates from a crude mixture has potential, although all changes are not completely reversible, as discussed later. In addition, these "reduced" protein patterns differed from those of the classical fractions obtained by DEAE-cellulose chromatography (Cherry *et al.*, 1973). An examination of the effects of thiol-reducing reagents and frozen storage treatments on DEAE-cellulose separated fractions of peanut proteins was subsequently conducted.

Fraction I + II + III Proteins. Electrophoretic alterations of gel patterns for total protein extracts were more prevalent in Fraction I + II + III, classical conarachin, after DEAE-cellulose chromatography (Figure 2). These fractionated proteins showed changes in region 2.5–4.5 cm in the presence of low concentrations of thiols (Figure 2, compare B to A) and most significantly at 0–4.0 cm with higher concentrations (compare A and B to C). Frozen storage induced the precipitation of a large amount of protein in this fraction with or without prior treatment with thiol compounds. Changes in electrophoretic patterns of both the precipitates and supernatants were noted either as diffuse areas at 0–2.5 cm (Figure 2, D and G), as shifts in banding mobility at 0–4.5 cm (Figure 2, D, E, F, and H), or as qualitative and quantitative variations among 4.0–7.0 cm (Figure 2, D to H).

Dialysis of these samples to remove the thiol-reducing reagents did not completely reverse the observed changes, as shown by comparing gels I and J to A, B, and C in Figure 2. Variations in regions 0.5–2.0 and 3.5–5.5 cm were quite evident, indicating that changes induced in proteins by treatment with either high or low concentrations of thiol-reducing reagents are not completely reversible.

Fraction I Proteins. The gel electrophoretic patterns in regions 0–0.5 and 1.0–1.6 cm of Fraction I (albumins) were altered both qualitatively and quantitatively with low concentrations of thiol-reducing reagents (Figure 3, compare A to B). With high concentrations of these reagents, the proteins migrated collectively to region 2.0–4.0 cm (Figure 3C). Frozen storage did not cause any detectable precipitation or further changes in these patterns, suggesting that the effects of either reducing reagents or of freezing on peanut albumins may be quite similar.

Fraction II + III Proteins. Samples of the crude conarachin fraction with or without low concentrations of thiol-reducing reagents or untreated samples subjected to frozen storage all showed similar electrophoretic patterns (Figure 3D). The small amount of protein precipitated

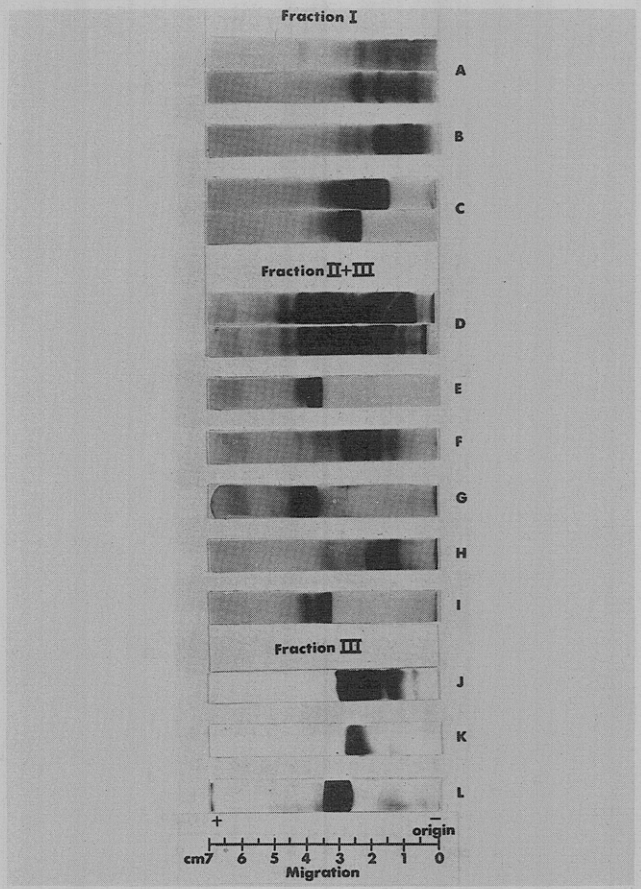

Figure 3. Typical disk gel electrophoretic patterns of Fraction I, Fraction II + III, and Fraction III proteins treated (T) and not treated (NT) with thiol-reducing reagents (20 m*M*, 0.25 *M* β-ME, or 10 m*M*, 0.125 *M* DTT) and/or frozen storage (FS). Fraction I (A to C): proteins soluble after NT before and after FS, proteins soluble after T (20 m*M* β-ME, or 10 m*M* DTT) before and after FS, and proteins soluble after T (0.25 *M* β-ME, or 0.125 *M* DTT) before and after FS, respectively. Fraction II + III (D to I), D, F, H: proteins soluble after NT or T (20 m*M* β-ME, or 10 m*M* DTT) before and after FS, NT or T proteins of D soluble after FS, and NT or T proteins of D insoluble after FS, respectively. E, G, I: proteins soluble after T (0.25 *M* β-ME, or 0.125 *M* DTT), proteins of E soluble after FS, and proteins of E precipitated after FS, respectively. Fraction III (J to L): proteins soluble after NT before and after FS and T (20 m*M* β-ME, or 10 m*M* DTT) before FS, T proteins in J after FS, and proteins soluble after T (0.25 *M* β-ME, or 0.125 *M* DTT) before and after FS, respectively.

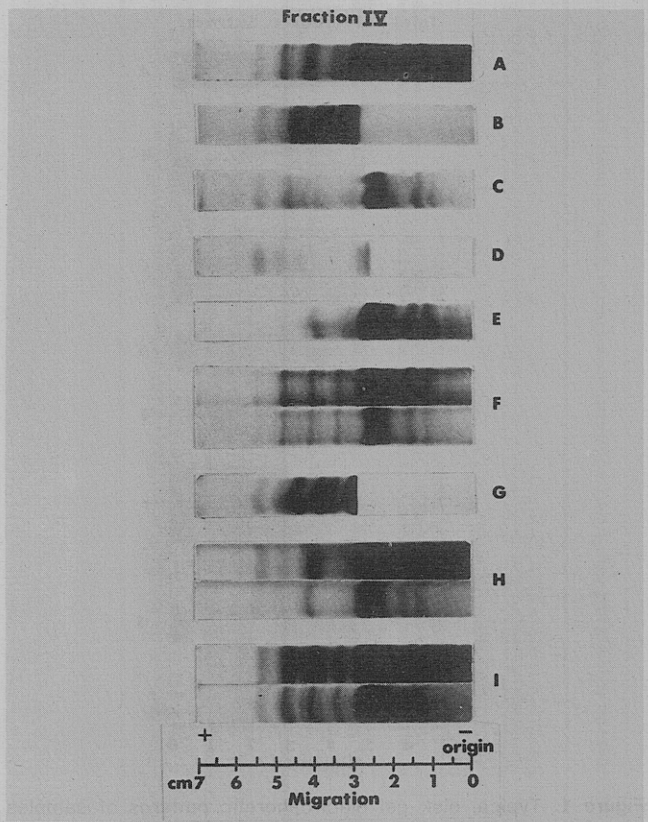

Figure 4. Typical disk gel electrophoretic patterns of fraction IV proteins treated (T) and not treated (NT) with thiol-reducing reagents (20 m*M*, 0.25 *M* β-ME, or 10 m*M*, 0.125 M DTT) and/or frozen storage (FS). A, C, E, F: proteins soluble after NT or T (20 m*M* β-ME, or 10 m*M* DTT) before FS, proteins in A soluble after FS, NT proteins in A precipitated after FS, and T proteins in A precipitated after FS, respectively. B, D, G: proteins soluble after T (0.25 *M* β-ME, or 0.125 *M* DTT), proteins of B soluble after FS, and proteins of B precipitated after FS, respectively. H, I: NT, T proteins of A and B after dialysis.

under these conditions was mostly at 1.0–4.0 cm (Figure 3, compare F to H), the region in which arachin is located. As noted with the other protein fractions, high concentrations of thiol compounds again caused major shifts in the gel patterns (Figure 3, E, G, and I). Protein precipitation from Fraction II + III after frozen storage with high concentrations of thiol reagent was not as great as with Fraction I + II + III, probably because of the virtual absence of arachin from the former (Cherry *et al.*, 1973).

Fraction III Proteins. Major protein changes were observed in the gel pattern for α-conarachin after treatment with high concentrations of this reagent (Figure 3L). At low concentrations, the single band in region 1.3 cm separated as two proteins when compared to that of the untreated samples (Figure 3, compare K to J).

Fraction IV Proteins. Low concentrations of thiol-reducing agents had no effect on the electrophoretic pattern of untreated samples of arachin (Figure 4A), but high concentrations caused a striking change (Figure 4B). As expected, frozen storage of Fraction IV, with or without low concentrations of thiol reagents, induced heavy cryoprecipitation of arachin. This precipitation was even greater in samples containing high concentrations of thiol reagent. After removal by centrifugation of the precipitates from treated and untreated preparations, the remaining supernatants showed significant decreases in protein bands compared to samples that had not been frozen and thawed (Figure 4, compare C to A and D to B). Proteins in the precipitates differed considerably from those in the supernatant fraction (Figure 4, compare E and F to C, and G to D). Both gels E and F contain similar patterns in the upper half of the gels (region 0–3.0 cm) but a new group of bands appears in region 3.0–5.5 cm for proteins containing low concentrations of thiol reagent (Figure 4F). As expected from the cryoprecipitation of arachin by Neucere (1969), frozen storage of Fraction IV can differentially precipitate proteins, but it also dissociates many of these molecules into smaller proteins, with or without reducing agents. Tombs (1965) and Tombs and Lowe (1967) reported that the two major protein bands of arachin are composed of a number of subunits. Cherry *et al.* (1973) showed that the number of low molecular weight proteins in the lower half of electrophoretic gels is increased when arachin is purified extensively by DEAE–cellulose chromatography. These studies using thiol-reducing agents to dissociate arachin produce results similar to those obtained by DEAE–cellulose chromatography.

Dialysis of Fraction IV after treating with a high concentration of thiol reagent (Figure 4, gels I) reversed the large shift in mobility of proteins (gels B and A), but not all of the smaller protein bands in the lower half of the

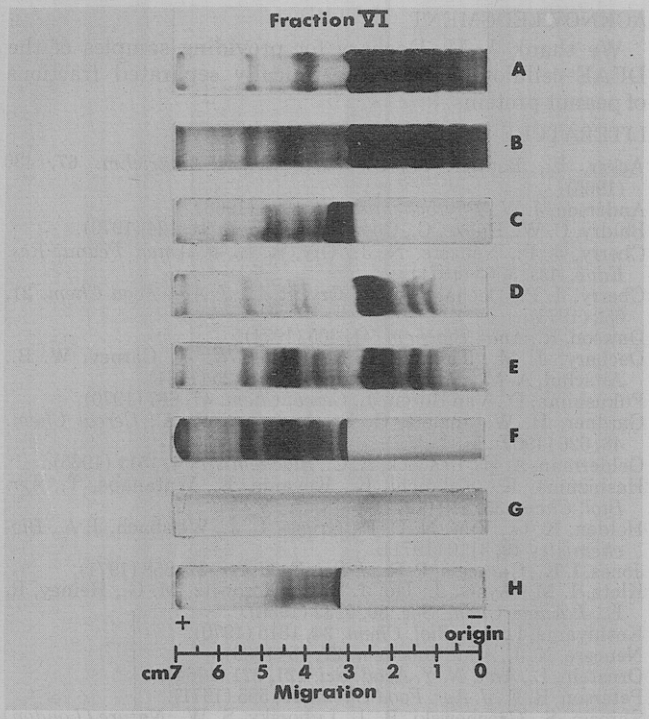

Figure 5. Typical disk gel electrophoretic patterns of Fraction VI proteins treated (T) and not treated (NT) with thiol-reducing reagents (20 m*M*, 0.25 *M* β-ME, or 10 m*M*, 0.125 *M* DTT) and/or frozen storage (FS). A, D: proteins soluble after NT, and proteins of A soluble after FS, respectively. B, E: proteins soluble after T (20 m*M* β-ME, or 10 m*M* DTT), and proteins of B soluble after FS, respectively. G: proteins of A and B precipitated after FS. C, F, H: proteins soluble after T (0.25 *M* β-ME, or 0.125 *M* DTT), proteins of C soluble after FS, and proteins of C precipitated after FS, respectively.

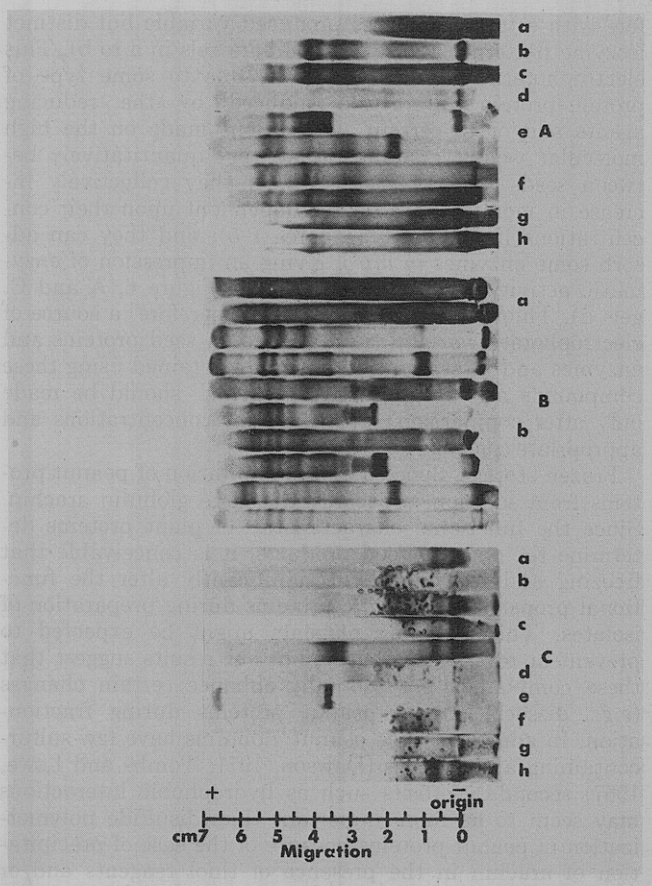

Figure 6. Typical disk gel electrophoretic zymograms of enzymes (A, B: esterases; C: peroxidases) treated (T) and not treated (NT) with thiol-reducing reagents (20 m*M*, 0.25 *M* β-ME, or 10 m*M*, 0.125 *M* DTT) and/or frozen storage (FS). A (a to d): esterase zymograms of soluble proteins from Fraction I, Fraction II + III, Fraction I + II + III and total cotyledonary extracts, and Fraction IV, respectively, before and after FS. (e, f): esterase zymograms of b and c after T (0.25 *M* β-ME, or 0.125 *M* DTT) and T (20 m*M* β-ME, or 10 m*M* DTT), respectively. (g, h): esterase zymograms of samples from e and f after dialysis. B (a to b): esterase zymograms of samples NT and T (0.25 *M* β-ME, or 0.125 *M* DTT), respectively. C (a to d): peroxidase zymograms of soluble proteins from Fraction I, Fraction II + III, Fraction I + II + III and total cotyledonary extract, and Fraction IV, respectively, before and after FS. (e, f): peroxidase zymograms of c after T (0.25 *M* β-ME, or 0.125 *M* DTT) and T (20 m*M* β-ME, or 10 m*M* DTT) respectively. (g, h): peroxidase zymograms of samples from e and f after dialysis.

gels reassociated into their original pattern. The number of bands at 3.0 to 5.5 cm was increased after dialysis, whether samples contained low or high concentrations of thiol reagents (gels I). These patterns were quite similar to those of the precipitated fraction after treatment with low concentrations and frozen storage (Figure 4, compare I to F). Untreated samples prior to dialysis showed no significant electrophoretic changes (Figure 4, compare H to A), indicating that alterations induced in arachin by high concentrations of thiol-reducing reagents are only partially reversed by dialysis, and that some of the subunits reorganize into new configurations and some remain unchanged.

Fraction VI Proteins. As noted with Fraction IV, the purified arachin (Fraction VI) showed an increased number of protein bands in region 3.5–7.0 cm after treatment with either high or low concentrations of thiols, before and after frozen storage (Figure 5). Little or no precipitate was produced in either treated or untreated samples after frozen storage (Figure 5, G and H). It appeared that the arachin in these precipitates had been dissociated into lower molecular weight subunits which were now resistant to any further changes. When Figures 1 and 5 are compared, it is evident that changes in the total protein extracts were less than those for partially purified protein fractions. While these results indicate a significant role of –SS– and –SH groups in association of these proteins (or in maintenance of their natural conformations) in total protein extracts, they can not foretell the extent of this role nor the effects of the induced changes on their functional properties.

Selected Enzymes. Thiol-reducing reagents had no effects on the catalytic properties of leucine aminopeptidase and INT-oxidase, but did reversibly inhibit most of the alcohol dehydrogenase, acid phosphatase, and catalase activities. A small amount of bubbling activity of catalase is visible in gels in Figure 6C.

Figure 6 contains zymograms of esterase (gels in A and B) and peroxidase (gels in C) which were altered by thiols. Enzyme activities in Fraction I albumins (gels a), Fraction II + III crude conarachin (gels b), total cotyledonary extract (gels c) and Fraction IV, with enzyme activity adsorbed to it (gels d), are compared to samples treated with high (gels e) and low (gels f) concentrations of thiol reagent, followed by dialysis to remove these compounds (gels g and h, respectively). Low thiol concentrations (gels f) caused a decrease in both esterase and peroxidase activities but high concentrations altered the isozyme patterns (gels e) both quantitatively and qualitatively. Removal of the reducing reagents by dialysis reversed some of these effects (gels g and h), but some changes remained for peroxidase and esterase activities in regions 0–2.0 cm and 5.0–7.0 cm, respectively (compare in A and C, gels g and h to c).

Further studies on the use of thiol reagents and/or frozen storage to remove large proteins which seem to inter-

fere with esterase analyses produced variable but distinct isozyme patterns (Figure 6B; compare gels in a to b). This electrophoretic variability may be due to some type of protein–protein interaction induced by the reducing agents and/or to certain observations made on the high molecular weight globulins: they vary quantitatively between seeds (Cherry *et al.*, 1971); they collectively increase in mobility to a degree dependent upon their concentrations in the seeds (Figures 1–5); and they can adsorb some enzymes *in vitro*, giving an impression of enzymatic activity associated with them (Figure 6, A and C, gels d). Thiol-reducing reagents are, therefore, a source of electrophoretic variability in studies on seed proteins and enzymes and interpretation of results obtained using these compounds as "SH-protective reagents" should be made only after comparisons with different concentrations and appropriate controls.

Frozen storage alone caused precipitation of peanut proteins from solution, especially the large globulin arachin. Since the functional characteristics of plant proteins determine their use in food products, it is conceivable that freezing and thawing could significantly alter the functional properties of specific proteins during preparation of isolates. Thiol-reducing reagents might be expected to prevent or reverse this effect, but our results suggest that these compounds may actually enhance certain changes (*e.g.*, dissociation) in peanut proteins during fractionation. In addition, since peanut globulins have few sulfur-containing amino acids (Dawson, 1971; Tombs and Lowe, 1967) secondary effects such as hydrophobic interactions may seem to be more important than disulfide polymerization in peanut proteins because of the ease of precipitation of arachin in the presence of thiol reagents and/or frozen storage.

Certain enzymes such as peroxidase (Gardner *et al.*, 1969), phenoloxidase (Anderson, 1968), lipoxygenase (Wagenknecht, 1959), and lipase (Acker and Beutler, 1965) have been shown to influence shelf-life or flavors of foods. If one could remove or reduce these enzyme activities by adding acceptable thiol-reducing reagents during the preparation of protein isolates from oilseed meals (*e.g.*, as with phenoloxidase activity by Anderson, 1968; and the peroxidase activities in Figure 6), the stability and possibly the functional properties of the protein isolates might be improved.

ACKNOWLEDGMENT

We thank J. M. Dechary for providing samples of the DEAE-cellulose chromatographically separated fractions of peanut proteins.

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Received for review October 30, 1972. Accepted March 8, 1973. J. P. Cherry was a National Research Council Postdoctoral Research Associate. One of the facilities of the Southern Region, Agricultural Research Service, U. S. Department of Agriculture.