clearly mixtures. The mass spectra of authentic ethyldimethylthiazoles and 2,4- and 2,5-dipropylthiazole were reported previously (Buttery *et al.*, 1973).

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Ron G. Buttery*
Louisa C. Ling

Western Regional Research Laboratory
Agricultural Research Service
U. S. Department of Agriculture
Berkeley, California 94710

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Effects of Freezing in the Presence of β-Mercaptoethanol on the Antigenicity of Peanut Proteins

Using immunoelectrophoresis, qualitative effects of freezing in the presence of β-mercaptoethanol (R–SH) on the immunological properties of peanut proteins were determined. When total protein extracts were frozen, only three major proteins remained soluble and antigenic after thawing. Isolated proteins of the arachin and conarachin systems were completely inactivated after freezing in R–SH; the conarachin proteins were more sensitive to reduction than arachin. Slight heterogeneity in migrations was observed for some of the proteins after treatment with R–SH. These results suggested that some determinant groups could be maintained by intra- and/or interchain disulfide bonds.

Thiol compounds are widely used as protective reagents for sulfhydryl groups, enzyme activators, and as reducing agents for disulfides. Depending on the stoichiometry and equilibrium constants for a given system, effects of these reagents vary with concentration and experimental conditions (Wall, 1971). In biological studies of plant and animal tissues, samples are often frozen awaiting experimentation. Changes in electrophoretic migrations and enzyme activities of peanut proteins as effected by thiol reagents and freezing have been reported (Cherry and Ory, 1973). Characterization of the major peanut proteins by immunoelectrophoresis (IEA) was reported previously (Daussant *et al.*, 1969). The purpose of this study was to determine the effects on antigenic properties of peanut globulins (total extracts and isolated fractions) after they were treated with β-mercaptoethanol (R–SH) and then frozen and thawed.

EXPERIMENTAL SECTION

Whole (full-fat) seeds were extracted in phosphate buffer (pH 7.9, ionic strength 0.01) and centrifuged, and the supernatants were made to 0.02 or 0.25 *M* R–SH and frozen for 15 hr (including a control) before analysis. Isolated α-arachin (Neucere, 1969) and the conarachins (Dechary *et al.*, 1961) were made to 0.02 or 0.25 *M* R–SH in phosphate buffer for similar analyses.

Protein contents were determined by the method of Lowry *et al.* (1951). Immunoelectrophoresis was performed in Ionagar No. 2 (Colab Laboratories, Inc., Glenwood, Ill.) according to Grabar and Williams (1953) employing 4 V/cm for 2 hr at room temperature. All sample wells were filled with approximately 0.75 mg of protein and all troughs were filled three times with immune serum *vs.* the fresh total protein extract. After washing out serum proteins, the precipitin lines were stained with 0.1% Amido Black in 7.0% acetic acid and destained with 7.0% acetic acid. The immune serum against the total protein extract was prepared by Antibodies, Incorporated (Davis, Calif.).

RESULTS AND DISCUSSION

Figure 1 shows the IEA of total protein extracts in R–SH after freezing and thawing. No major changes were observed in the total precipitin patterns before freezing (compare the first three samples at the top). A slight cathodic shift of components A and B was observed in the samples containing R–SH, however. After freezing in 0.02 and 0.25 *M* R–SH, only the supernatant of 0.25 *M* R–SH showed a major change in pattern. In the latter, only some subunits of α-arachin (D), one of the so-called α-arachin contaminants (A), and α_2-conarachin (E) retained their immunological properties. The corresponding precipitates shown in the last three samples were very similar except one of the components (F) was not observed in the 0.25 *M* R–SH precipitate.

To test the effect of the reagent on isolated protein, purified α-arachin and the conarachin fraction were treated with R–SH and tested before and after freezing. Since one end of the molecule is an alcohol that could possibly cause denaturation without effecting disulfide bonds, an additional control treated with 0.25 *M* ethanol was used. These results are shown in Figure 2. For the α-arachin preparation (part A) no changes in antigenicity were induced by either 0.02 *M* R–SH or ethanol before and after freezing. After freezing in 0.25 *M* R–SH, no antigenicity for α-arachin was observed in either the supernatant or

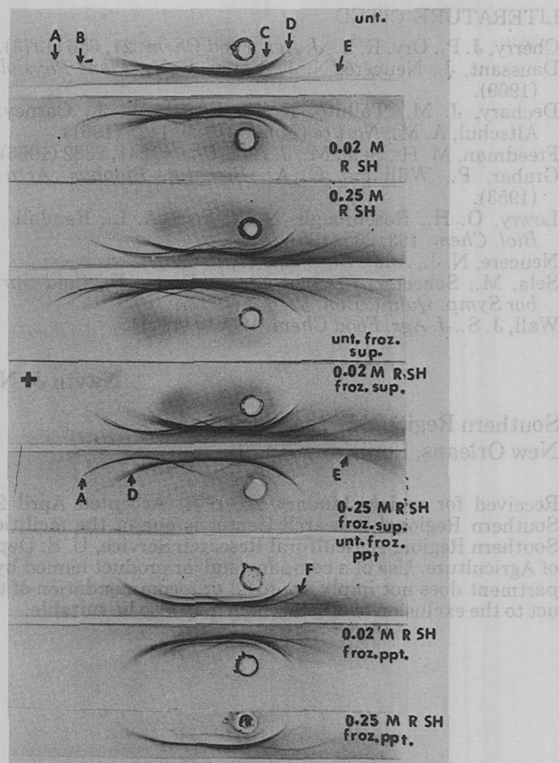

Figure 1. IEA of total protein extracts treated with β-mercaptoethanol (R–SH) followed by freezing and thawing. Description of samples from top to bottom: untreated fresh extract, fresh extract in 0.02 *M* R–SH, fresh extract in 0.25 *M* R–SH, supernatant of untreated extract after freezing and thawing; supernatant of extract in 0.02 *M* R–SH after freezing and thawing, supernatant of extract in 0.25 *M* R–SH after freezing and thawing, precipitate of untreated sample after freezing and thawing, precipitate of extract in 0.02 *M* R–SH after freezing and thawing, precipitate of extract of 0.25 *M* R–SH after freezing and thawing; (A and C) contaminants of α-arachin; (B) α_1-conarachin; (D) α-arachin; (E) α_2-conarachin.

the precipitate (last two samples). All other precipitates (not shown) showed only a trace reaction for α-arachin. Low concentrations of R–SH did not seem to affect the antigenicity of α-arachin after freezing.

For the conarachin proteins (part B) all antigenicities were destroyed after freezing in either low or high concentrations of R–SH (see last two samples in figure). Note also the slight heterogeneity in protein migration at the low concentration of R–SH (arrows pointing to leading edges in first two samples).

Because the exact structures (*i.e.*, primary, secondary, etc.) of all the proteins in the present study are not known, only qualitative interpretation is possible. It is evident that some of the peanut globulins are less subject to reduction when present in a "gross" mixture than as separate entities after freezing and thawing. Perhaps protein–protein (or protein–carbohydrate) interactions in whole extracts inhibited the reduction of buried disulfide bonds during freezing. Since the conarachin proteins are most sensitive to reduction, their antigenic sites are apparently more integrated with disulfide bonds than those in α-arachin.

The slight shifts in electrophoretic mobilities observed in both whole extracts and isolated fractions for some of the proteins suggested possible conformational changes or subunit modifications that altered net charge of the molecules. However, not all determinant groups for certain proteins are necessarily disrupted even though mobilities changed.

The lack of cross-reaction between denatured proteins and the antibodies to the same proteins in their native form could result from either conformational changes of the molecules or from cleavage of peptide bonds (Sela *et al.*, 1967). For serum proteins of rabbit, a study of the role of disulfide bridges in maintaining antigenic specificity concluded that practically all the determinants of one of the globulins were conformation dependent because only negligible cross-reaction was observed after disulfide reduction (Freedman and Sela, 1966). Whether antigenicity

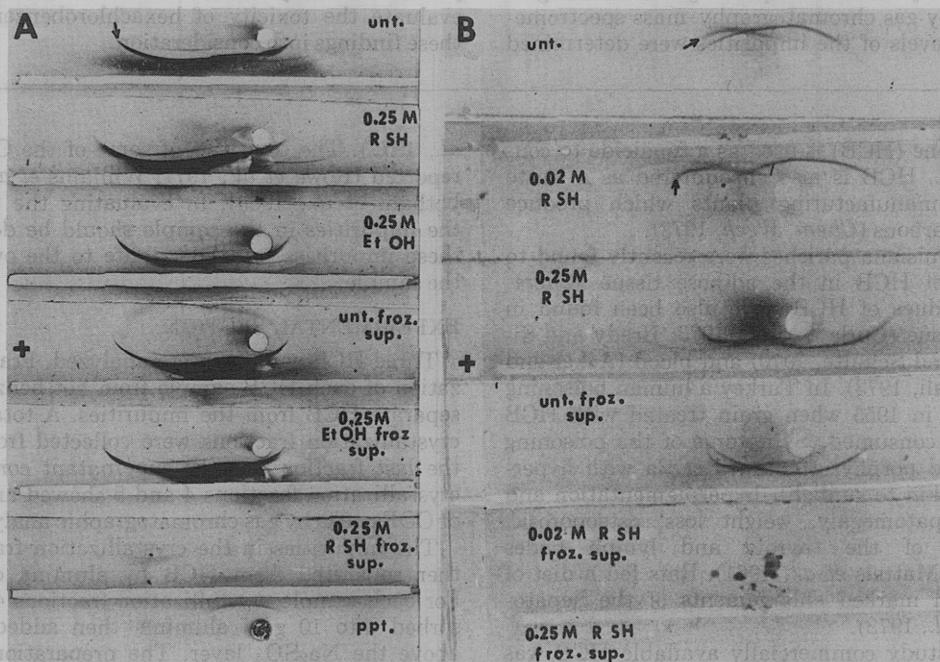

Figure 2. IEA of purified α-arachin (A) and the conarachin proteins (B) dissolved in buffer, treated with R–SH, then frozen, and thawed. Description of samples from top to bottom: (A) fresh untreated sample, fresh sample treated with 0.25 *M* R–SH, fresh sample in 0.25 *M* ethanol, supernatant of untreated sample after freezing and thawing, supernatant of a sample treated with 0.25 *M* ethanol after freezing and thawing, supernatant of sample treated with 0.25 *M* R–SH after freezing and thawing, precipitate of preceding sample; (B) fresh untreated sample, fresh sample treated with 0.02 *M* R–SH, fresh sample treated with 0.25 *M* R–SH supernatant of untreated sample after freezing and thawing, supernatant of sample treated with 0.02 *M* R–SH after freezing and thawing, supernatant of sample treated with 0.25 *M* R–SH after freezing and thawing.

is lost after general rearrangement of cleaved disulfide bonds through interchain or intrachain mechanisms in this system remains to be determined. The conclusion is that freezing in the presence of R-SH discriminately disrupted the determinant groups of peanut proteins; the conarachin system was more sensitive to reduction than α-arachin, and some of the antigenic determinants were protected in whole extracts.

The thiol study on the major peanut proteins reported by Cherry and Ory (1973) showed drastic electrophoretic modifications on disk gels after adding R-SH that were not as readily discernible in the present study. It should be pointed out, however, that dissociation of variable sized subunits (possibly having equal net charges) can be detected through the "sieve" effect on acrylamide gels; this is not necessarily true in agar electrophoresis. Hence, in view of the disk study, perhaps some determinant groups are maintained by interchain disulfide bonds.

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Navin J. Neucere

Southern Regional Research Center
New Orleans, Louisiana 70179

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Evidence of Chlorodibenzo-*p*-dioxin and Chlorodibenzofuran in Hexachlorobenzene

Three commercial hexachlorobenzene preparations were analyzed for toxic impurities. The impurities were separated from the hexachlorobenzene by fractional crystallization followed by alumina chromatography. The contaminants were identified by electron capture gas chromatography and by gas chromatography-mass spectrometry. The levels of the impurities were determined when the standards were available. Pentachlorobenzene was the major contaminant in all preparations. A number of other compounds were found including hepta- and octachlorodibenzofuran and octachlorodibenzo-*p*-dioxin. It is important that any studies which are conducted to evaluate the toxicity of hexachlorobenzene take these findings into consideration.

Hexachlorobenzene (HCB) is used as a fungicide to control bunt of wheat. HCB is also encountered as a waste by-product from manufacturing plants which produce chlorinated hydrocarbons (*Chem. Week*, 1973).

Cattle in two Louisiana parishes were recently found to have high levels of HCB in the adipose tissue (*Chem. Week*, 1973). Residues of HCB have also been found in human adipose tissue (Curley, *et al.*, 1973; Brady and Siyali, 1972; Acker and Schulte, 1970) and blood (Acker and Schulte, 1970; Siyali, 1973). In Turkey a human poisoning outbreak occurred in 1955 when grain treated with HCB was inadvertently consumed. Symptoms of the poisoning in Turkey included porphyria cutanea tarda with hypersensitivity of the skin to sunlight, hyperpigmentation and hypertrichosis, hepatomegaly, weight loss, osteoporosis, and enlargement of the thyroid and lymph nodes (Schmid, 1960; DeMatteis *et al.*, 1961). Rats fed a diet of 0.2% HCB showed marked enlargements of the hepatocytes (Medline *et al.*, 1973).

In the present study commercially available HCB was analyzed for toxic impurities which may have been formed during the manufacturing process. The chlorodibenzo-*p*-dioxin (CDD's) and the chlorodibenzofurans (CDF's) were of particular interest because these compounds have previously been found as contaminants in pentachlorophenol (Firestone *et al.*, 1972; Jensen and Renberg, 1972; Villanueva *et al.*, 1973), 2,4,5-T, Silvex, and 2,4-D (Woolson *et al.*, 1972). The toxicities of some of the CDD's have been reported (Rowe *et al.*, 1971; Williams *et al.*, 1972; Higginbotham *et al.*, 1968). In evaluating the toxicity of HCB the impurities in the sample should be determined, since these impurities may contribute to the overall toxicity of the sample.

EXPERIMENTAL SECTION

Three HCB samples were analyzed. Fractional crystallization of each HCB sample from hot benzene was used to separate HCB from the impurities. A total of four or five crystallization fractions were collected from each sample; the last fraction was the supernatant concentrated. Only crystallization fractions 4 and 5 showed any possible CDD or CDF peaks by gas chromatographic analysis.

The impurities in the crystallization fractions were further separated from HCB by alumina chromatography. For each sample crystallization fractions 4 and 5 were adsorbed into 10 g of alumina, then added to the column above the Na_2SO_4 layer. The preparation and elution of the columns are otherwise previously described (Firestone *et al.*, 1972). The alumina used was Fisher No. A-540. Since recovery studies have shown that the CDD's and CDF's elute primarily in fraction 3 and a small amount in fraction 4, these two alumina fractions from each column were concentrated.

The alumina fractions were analyzed by electron cap-